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Validation of the doubly labeled water method in Japanese Quail *Coturnix c. japonica* chicks: is there an effect of growth rate?

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Abstract The Doubly Labeled Water (DLW) method was validated against respiration gas analysis in growing Japanese Quail chicks (between 1 week and 3 weeks of age) as well as in birds after having achieved sexual maturity (7 weeks of age). A comparison was made between a strain selected for high growth rates (P-strain, $n = 18$), and a non-selected strain (C-strain, $n = 18$). Relative growth rates of individual chicks during the measurement ranged from -13.8% day^{-1} to 23.1% day^{-1}. When employing a single-pool model (eq. 34, Lifson and McClintock 1966), it was found that the relative error of the DLW method was sensitive to assumptions concerning fractional evaporative water loss. The best fit was obtained after taking a fractional evaporative water loss value of 0.33. When applying this value for all chicks, it was found that neither strain, relative growth rate of the chick during measurement, nor age significantly contributed to the explained variance. When employing two-pool models, it was found that the DLW method significantly underestimated the true rates of CO_2 production at all assumed levels of fractional evaporative water loss. Based on an evaluation of DLW validation studies in growing shorebirds, terns, and quail we recommend Speakman's Eq. 7.17 (Speakman 1997) for general use in young birds.

Key words Doubly labeled water · Energy expenditure · Japanese Quail *Coturnix c. japonica* · Growth · Fractional evaporative water loss

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Abbreviations C_b isotope concentration of background · C_d isotope concentration of dose · C_i isotope concentration at start · C_f isotope concentration at end · DLW doubly labeled water · f_1 fractionation factor · k fractional turnover rate · k_d fractional turnover rate ^2H · k_o fractional turnover rate ^{18}O · M average body mass · M_f body mass at end · M_i body mass at start · N amount of body water · Q dose of isotopes administered · r_{CO_2} rate of CO_2 production · $r_{CO_2\text{-}DLW}$ rate of CO_2 production measured with doubly labeled water · $r_{CO_2\text{-}IR}$ rate of CO_2 production measured with respiration gas analysis · $R_{dilspace}$ ratio ^2H and ^{18}O body water dilution spaces · r_G fractional evaporative water loss · RGR relative growth rate · r_{H_2O} water efflux rate · $r_{H_2O\text{-}uncorr}$ water efflux rate uncorrected for fractionation effects · t time · TBW total amount of body water

Introduction

Since the publication of the seminal paper of Lifson and McClintock (1966), the Doubly Labeled Water (DLW) method has frequently been used to measure the rate of carbon dioxide production in free-living adult birds and mammals (Speakman 1997). The application of this method is based on the assumption that, following administration of a pulse-dose of ^2H and ^{18}O, ^2H atoms leave the body water pool exclusively as water, and ^{18}O only as water and carbon dioxide gas (Lifson and McClintock 1966). The DLW method has been validated in adult birds of about 15 species over a size range spanning three orders of magnitude (for the most recent review see Speakman 1997).

In growing birds the application of the DLW method has been hampered by uncertainties concerning the routes of disappearance of both labels from the body water pool. For growing birds, it has been questioned as to whether ^2H and ^{18}O atoms not only leave the body water pool as water and carbon dioxide gas, but also via

incorporation into growing tissues. If incorporation does occur, and if the rates of incorporation differ between ^{2}H and ^{18}O, the error in the estimated rate of carbon dioxide production could be up to 25% (Williams and Nagy 1985; Weathers and Sullivan 1991). The first validation study of the DLW method in growing birds was performed on the Arctic Tern (*Sterna paradisaea*), a semi-precocial species (Klaassen et al. 1989). It was found that the DLW method underestimated the true rate of carbon dioxide production by 10.3% on average, which may suggest that differential incorporation of ^{2}H and ^{18}O does occur in this species during growth. These authors employed eq. 35 of Lifson and McClintock (1966) to calculate the rate of carbon dioxide production from DLW measurements to correct for fractionation of the heavy isotopes. In this equation it is assumed that a fraction of 0.5 of the water efflux is lost through evaporative pathways. Later on it was demonstrated that the fit of the Arctic Tern validation study could be substantially improved by assuming a fractional evaporative water loss of 0.2 instead of 0.5 (Visser and Schekkerman 1999). An identical result was obtained in growing precocial shorebird chicks (Black-tailed Godwit, *Limosa limosa*, and Northern Lapwing, *Vanellus vanellus*; Visser and Schekkerman 1999). After employing eq. 35 of Lifson and McClintock (1966; assuming a fractional evaporative water loss value of 0.5), it was found in these birds that the DLW method underestimated the true rate of carbon dioxide production by an average of 11.6%. However, the best fit was obtained at a fractional evaporative water loss of 0.13. When applying this value, no correlation could be detected between the relative growth rate of the chick during the measurement and the relative error of the DLW method (Visser and Schekkerman 1999).

In spite of these observations, differential incorporation of ^{2}H and ^{18}O in growing tissues in young birds cannot be ruled out. The effect of this process can be obscured in a mathematical sense, for instance by the policy of fitting values for the fractional evaporative water loss, instead of relying on direct measurements. To further investigate the effect of growth rate on the application of the DLW method, a validation study was performed in growing chicks of two different strains of the Japanese Quail (*Coturnix c. japonica*): one strain that had been selected for high postnatal growth rate (P-strain) and another non-selected strain (C-strain). Measurements were performed in young chicks (exhibiting high relative growth rates) as well as in adults after achievement of sexual maturity. If differential incorporation of ^{2}H and ^{18}O does play a role during the application of the DLW method, and if this effect is related to growth rate, we would expect that in young fast growing chicks the DLW method would have a strong tendency to underestimate the 'true' rate of CO_2 production ('true' r_{CO_2}). As far as we are aware, this is the first study in which the DLW method has been validated in an animal species over such a wide range of developmental stages.

Materials and methods

Animals and housing

Japanese Quail neonates of a fast growing (P-strain) and normal growing (C-strain) strain were obtained from a commercial farm (N.V. Nouwen, Lommel, Belgium). Until the age of 6 days, birds were kept in wooden cages ($l \times b \times h$: $67 \times 39 \times 44$ cm^3) with sawdust bedding in continuous light, and ad libitum access to quail starter food and water, to ensure maximum possible body mass gain. A 40-W heating lamp was placed in each cage to provide a temperature gradient sufficient for selection of the preferred temperature by the chicks. At 6-days-old, the light regime was switched to L:D 18:6 (0900 hours lights on MET). From this age onwards birds had ad libitum access to water and food pellets (Institute for Animal Science and Health, ID-DLO, The Netherlands) containing 27.7% (w/w) crude protein and 17 kJ wet g^{-1} (gross energy content as determined by bomb calorimetry, own measurement).

Experimental procedure

Chicks of at least 1 week of age were considered to be sufficiently independent to remain unattended in a respiration chamber for 24 h. Validation experiments were performed on growing chicks 1-week-old (P-strain: $n = 6$, C-strain: $n = 7$), 2-weeks-old (P-strain: $n = 6$, C-strain: $n = 5$), and 3-weeks-old (P-strain: $n = 6$, C-strain: $n = 6$). In addition, measurements were performed at sexual maturity (7 weeks of age) on five birds of each strain. Each bird was used once, except for one chick of the C-strain that was used at 1 week and 7 weeks of age.

After measuring body mass (to the nearest 0.1 g, Sartorius QT 6100 balance), the chicks were injected with a DLW mixture (enrichment 62.3% ^{18}O and 31.9% ^{2}H). The amount of mixture injected was determined by weighing the syringe before and after injection to the nearest 0.1 mg on an analytical balance (Mettler H54), and ranged between 1.5 mg g^{-1} (adults) and 3 mg g^{-1} (young chicks). To avoid injection of the DLW into one of the air sacs, the skin was gently lifted manually, and the needle was carefully inserted into the intraperitoneal cavity. If leakage of the DLW occurred, no validation experiment was performed for that chick. This strict protocol enabled us to calculate the individual-specific size of the body water pool on the basis of the principle of isotope dilution (see below). After administration, the birds were kept individually in cardboard boxes during a period of 1 h for complete equilibration of the injected isotopes with body water (Speakman 1997; Visser and Schekkerman 1999). During this interval, the birds had no access to water or food. Exactly 1 h after administration, the bird was reweighed to determine its mass at the start of the validation experiment (M_i; g). The brachial vein was then punctured with a sterile needle for blood collection in 4–5 15-µl capillary tubes (initial sample) which were immediately flame-sealed with a propane torch; samples were stored at 5 °C till isotope analysis. The bird was then immediately placed in an airtight metabolic chamber with ad libitum water and food from the same source as in the holding cages. The metabolic chamber was placed in a light- and temperature-regulated cabinet. The light schedule was identical to conditions in the home cages, and ambient temperature in the respiration chamber was regulated at 35 ± 1 °C in chicks of 1 and 2 weeks of age, and at 32 ± 1 °C in older chicks (i.e., at thermoneutrality; G.H., Visser, unpublished data). With this setup a maximum of five birds could be measured simultaneously, each in a separate metabolic chamber (see below). Exactly 24 h after having taken the initial blood sample, the metabolic chamber was opened. The bird was reweighed (body mass at end, M_f; g) and immediately thereafter another blood sample was taken (final sample) following the same procedure as described earlier. The relative growth rate of the chick (RGR, % day^{-1}) during the validation experiment was calculated as:

$$RGR = 100 \cdot (M_f - M_i)/M \quad (1)$$

where M represents the average body mass (g) during the experiment.

In each age category, three birds were used to determine the age-specific background levels for ^2H and ^{18}O. These birds were not used for validation experiments. We refrained from taking a background sample from each animal prior to the measurement, because pilot experiments revealed that such a frequent sampling procedure could interfere with the normal growth performance of the chick during the measurement in the respiration chamber. To circumvent the lack of information on the individual-specific background levels, we applied a relatively high dose of DLW (see Nagy 1980 for a discussion on the relevance of the background levels), making the DLW calculations almost insensitive to minor differences in the background levels. The enrichments of the final blood samples for ^2H and ^{18}O were at least 670 ppm (i.e., 520 ppm excess) and 2960 ppm (i.e., 960 ppm excess), respectively.

Measurement of carbon dioxide production with infrared gas analysis

r_{CO_2} was measured over 24-h periods in an open air flow system (indirect calorimetry). Dry air was pumped through the chambers at rates varying with age (from ca. 25 l h^{-1} at 1 week to ca. 120 l h^{-1} at 7 weeks of age), to obtain a difference in the in- and out-flowing air of about 0.5% CO_2 (i.e., at about the level of our gas standard for calibration of the CO_2 analyzer). The flow rate of each chamber was measured on the inlet air with a mass-flow controller (type 5850E Brooks with maximum capacities of 60 l h^{-1} and 300 l h^{-1}), that was calibrated against a soap foam flow meter (Bubble-O-Meter, La Verne, Calif., USA). The excurrent air was dried over molecular sieves (3 Å, Merck). The carbon dioxide concentration was measured with an infrared gas analyzer (Leybold Heraeus BINOS-IR), and the oxygen concentration with a zirconium oxide analyzer (Applied Electrochemistry S-3A/II), both to an accuracy of 0.01%. We calibrated both analyzers daily with certified gas standards (AGA, Amsterdam). To verify the O_2 and CO_2 concentrations of the certified gas standard, an inter-laboratory comparison was performed with the Respiration Unit of the Wageningen University, The Netherlands. We employed six channels simultaneously, using valves to switch between the channels once per minute (washout time 45 s), so that for each channel the values were recorded automatically at 6-min intervals. The reading for the CO_2 concentration of the excurrent air for each chamber was made during the last 10 s of the flushing period. Five channels were used for measurements of respiration gasses, and the sixth channel was used for the measurement of the inlet air. r_{CO_2} was calculated as the difference between the CO_2 concentrations of the outlet and inlet air times the flow rate, taking into account minor changes in the volumes of outlet and inlet air at RQ's below 1 (Nolet et al. 1992).

Isotope analysis

Isotopic enrichments of the blood samples were determined in triplicate at the Centre for Isotope Research as previously described by Visser and Schekkerman (1999). In brief, each capillary was first distilled in a vacuum line, and after complete cryogenic transfer of the distilled water sample into a quartz vial (placed in liquid air), 2 ml CO_2 gas was added (quantity determined from accurate pressure readings). The vial was then placed in a thermostatically controlled water bath (Tamson TC 45) at 25.0 °C during 48 h for equilibration of the CO_2 gas with the distilled water sample. Thereafter, the vial was placed in a dry-ice alcohol mixture, and CO_2 gas was cryogenically trapped in another quartz vial placed in liquid air. Finally, the water sample was cryogenically transferred over a uranium oven, and the H_2 gas was trapped in a quartz vial with active charcoal. $^2H/^1H$ and $^{18}O/^{16}O$ isotope ratios were determined from H_2 and CO_2 samples, respectively, on a SIRA 9 isotope ratio mass spectrometer (IRMS) with dual inlet for reference and sample gas. For each isotope, we applied internal gas standards at the background level and at high enrichment daily to estimate the level of cross-contamination between reference and sample channels (Meijer et al. 2000). In addition, for each batch we applied at least three differently enriched internal water standards that have been calibrated against IAEA standards. These were taken such that they covered the entire enrichment range of the test samples. Each internal standard was measured in quadruplicate. To verify the isotope enrichment of the original DLW mixture, a dilution was made with tap water (with known isotope concentrations), which was analyzed in the same batches as the blood samples.

Calculation of the size of the body water pool

For each bird, the amount of body water (TBW; g) was calculated using the principle of isotope dilution for ^{18}O:

$$TBW = 18 \cdot Q \cdot (C_d - C_i)/(C_i - C_b) \quad (2)$$

where Q is the size of the DLW dose (moles), C_d the ^{18}O concentration of the injectate (atom percent), C_i the individual specific ^{18}O enrichment of the initial blood sample (atom percent), and C_b the average ^{18}O concentration of the background (atom percent). The factor 18 was used for the conversion of moles to grams. Equation 2 was also used to calculate the amount of body water on the basis of ^2H dilution by taking for C_d the ^2H concentration of the injectate (atom percent), for C_i the individual specific ^2H enrichment of the initial blood sample (atom percent), and for C_b the average ^2H concentration of the background (atom percent). For each bird, the dilution space ratio ($R_{dilspace}$, dimensionless) was calculated by dividing the TBW value obtained from ^2H dilution by the value obtained from ^{18}O dilution (Speakman 1997).

Calculation of the fractional turnover rates of ^2H and ^{18}O

Fractional turnover rates were calculated for ^2H (k_d; day^{-1}) and ^{18}O (k_o; day^{-1}) with the general equation:

$$k = [\ln(C_i - C_b) - \ln(C_f - C_b)]/t \quad (3)$$

where C_f represents the isotope enrichment of the final blood sample (atom percent), and t the time (day) elapsed between taking the initial and final blood sample.

Calculation of water efflux rates

First, for each bird, rate of water efflux ($r_{H_2O\text{-uncorr}}$, g day^{-1}) was calculated using Eq. 4 of Nagy and Costa (1980), which takes into account the changes in the size of the body water pool during the measurement, but not the fractionation effects of evaporative water loss. It was assumed that for M_i and M_f the percentage of body water did not change. Second, water efflux rates (r_{H_2O} g day^{-1}) were calculated correcting for isotope fractionation effects due to evaporative water loss (Speakman 1997, Eq. 7.6):

$$r_{H_2O} = r_{H_2O\text{-uncorr}}/(r_G \cdot f_1 + 1 - r_G) \quad (4)$$

where r_G represents the proportion of water flux lost through evaporative pathways (here taken as 0.33, see Results), and f_1 the fractionation factor (taken as 0.94, Speakman 1997).

Calculation of r_{CO_2}

The r_{CO_2} was calculated with the following general equation:

$$r_{CO_2} = 22.4 \cdot [N/2.078(k_o - k_d) - r_G \cdot 0.0249 \cdot Nk_d] \quad (5)$$

where N represents the size of the body water pool as assessed from ^{18}O dilution (mol). The factor 22.4 of the equation is applied for the conversion of moles to l (STPD). This is basically Eq. 34 of Lifson and McClintock (1966), but with the most recent estimates for the

different fractionation processes after assuming a ratio of equilibrium to kinetic fractionation of 3:1 for evaporative water loss (Speakman 1997). Unfortunately, it is virtually impossible to measure rates of evaporative water loss in animals that have free access to water and food. Therefore, to investigate the relative importance of the assumptions concerning fractional evaporative water loss for each bird, r_{CO_2} values were calculated at r_G values of 0 (i.e., no evaporative water loss, as proposed by Nagy 1980), 0.25 (as proposed by Speakman 1997), 0.50 (as proposed by Lifson and McClintock 1966), 0.75, and 1 (all water is lost through evaporative pathways, i.e., no defecation).

In addition, r_{CO_2} values were calculated with a two-pool model (Eq. 7.43, Speakman 1997) by taking the average $R_{dilspace}$ obtained for our entire data set (see Results). For this model we also investigated the effect of the assumed fractional evaporative water loss on the relative error of the DLW method.

Statistics

The method of respiration gas analysis to measure $r_{CO_2}(r_{CO_2\text{-IR}})$ was considered as the 'golden standard'. Thus, relative errors in r_{CO_2} values obtained from DLW measurements ($r_{CO_2\text{-DLW}}$) were calculated as:

$$\text{Error} = 100(r_{CO_2\text{-DLW}} - r_{CO_2\text{-IR}})/r_{CO_2\text{-IR}} \quad (6)$$

Relative errors of the DLW method for the two strains were compared with a Student *t*-test. Analysis of covariance was used to investigate the effects of body mass, age, strain, relative growth rate of the chick on the relative error of the DLW method (MANOVA procedure in SPSS/PC V4.0). In all cases $P < 0.05$ was used to determine statistical significance.

Results

Growth rate measurements

Table 1 lists the various characteristics of the two strains at the ages measured. For each strain, 18 experiments were performed on chicks, and 5 on birds that had achieved sexual maturity (Table 1). In the birds of the C-strain, two females produced an egg during the measurement. Until 3 weeks of age, the average absolute growth rates were highest in chicks of the P-strain, ranging from 7.9 g day^{-1} to 9.4 g day^{-1}. For chicks of the C-strain these average values ranged from 6.4 g day^{-1} to 8.0 g day^{-1}. In P-strain chicks, average relative growth rates decreased from 18.7% day^{-1} at 1 week of age to 4.5% day^{-1} at 3 weeks of age. Corresponding values for birds of the C-strain were 16.0% day^{-1} and 6.1% day^{-1}, respectively. In adult animals, P-strain birds showed a slight increase in body mass, and C-strain birds a slight decrease.

r_{CO_2}: single-pool models

As a first step in the analysis, we calculated the r_{CO_2} at each of the assumed levels of fractional evaporative water loss (from 0 to 1, see Eq. 5) for each DLW measurement. Next, we determined for each value the error in the r_{CO2} relative to respiration gas analysis (Eq. 6). We then calculated for birds of both strains, for all ages, the mean relative error values for each level of fractional evaporative water loss (Fig. 1). It was found that after assuming that fractional evaporative water loss did not occur ($r_G = 0$), the DLW method significantly overestimated the true r_{CO_2} by 5.2% (SE = 1.33, $t_{45} = 3.92$, $P < 0.001$). At a fractional evaporative water loss level of 0.25 the DLW method overestimated the true r_{CO_2} by only 1.3% (SE = 1.26), which was not significantly different from 0 ($t_{45} = 1.00$, $P = 0.323$). Assuming fractional evaporative water losses of 0.5, 0.75 or 1, the DLW method significantly underestimated the true r_{CO_2} by 2.7% (SE = 1.20, $t_{45} = 2.26$, $P < 0.029$), 6.7% (SE = 1.16, $t_{45} = 5.76$, $P < 0.001$), and 10.6% (SE = 1.14, $t_{45} = 9.34$, $P < 0.001$), respectively. For these mean values, the relationship between the relative error of the DLW method (error %), and the assumed fractional evaporative water loss (r_G, dimensionless) can be approximated by linear regression as:

$$\text{Error} = 5.2 - 15.8 \cdot r_G \quad (7)$$

Table 1 Average body mass (g), absolute rate of body mass gain (g day^{-1}), relative rate of body mass gain (% day^{-1}), total body water volume (*TBW*, %; based on the dilution space of ^{18}O), rate of water efflux (r_{H_2O}, g), rate of CO_2 production measured by respiration gas analysis ($r_{CO_2\text{-IR}}$, l day^{-1}) and by the doubly labeled water (DLW) method ($r_{CO_2\text{-DLW}}$, l day^{-1}; Eq. 8), and the relative error of the DLW method (%; Eq. 6) in relation to age (weeks) in two strains of Japanese Quail. $n = 5$ for P-strain birds of 7 weeks of age (adult), and C-strain birds of 2 weeks and 7 weeks of age (adult); $n = 6$ for P-strain birds of 1, 2, and 3 weeks and C-strain birds of 3 weeks of age; $n = 7$ for C-strain birds of 1 week of age. Values are means ± SE

Age (weeks)	Body mass (g)	Body mass gain		*TBW* (%)	r_{H_2O} (g day^{-1})	$r_{CO_2\text{-IR}}$ (l day^{-1})	$r_{CO_2\text{-DLW}}$ (l day^{-1})	Error (%)
		(g day^{-1})	(% day^{-1})					
P-strain								
1	46.7 ± 6.5	8.7 ± 2.2	18.7 ± 4.4	78.6 ± 1.5	21.0 ± 3.4	4.2 ± 0.6	4.3 ± 0.6	1.5 ± 10
2	101.7 ± 8.6	9.4 ± 5.6	9.0 ± 4.9	77.3 ± 0.8	30.7 ± 3.5	7.2 ± 0.8	7.5 ± 0.7	4.5 ± 7.4
3	155.0 ± 26	7.9 ± 8.4	4.5 ± 5.7	76.2 ± 1.3	27.1 ± 7.3	8.5 ± 1.7	8.3 ± 1.7	−1.7 ± 5.5
7	294.4 ± 41	8.7 ± 6.8	3.2 ± 2.5	66.7 ± 6.2	62.1 ± 9.7	12.4 ± 1.2	12.2 ± 1.3	−2.2 ± 10
C-strain								
1	40.1 ± 6.6	6.4 ± 0.8	16.0 ± 1.7	79.5 ± 1.9	18.0 ± 2.2	3.7 ± 0.5	3.8 ± 0.3	4.2 ± 6.2
2	71.8 ± 8.9	8.0 ± 0.5	11.3 ± 1.6	78.4 ± 0.6	23.7 ± 3.2	6.1 ± 0.6	5.8 ± 0.9	−6.2 ± 6.7
3	122.0 ± 17	7.3 ± 2.6	6.1 ± 2.3	75.7 ± 1.2	29.7 ± 6.6	7.4 ± 1.0	7.0 ± 0.5	−4.7 ± 11
7	183.9 ± 13	−6.8 ± 12	−3.5 ± 6.0	62.4 ± 3.4	40.9 ± 19	8.2 ± 1.8	8.4 ± 1.9	2.9 ± 4.4

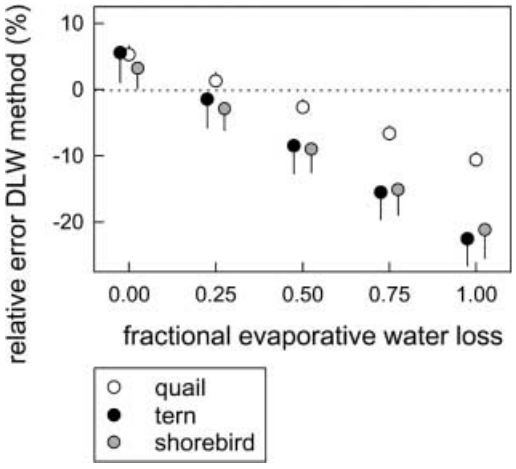

Fig. 1 Relationship between assumed fractional evaporative water loss and the mean relative error of the DLW method in growing chicks of the Japanese Quail (*open symbols*; this study), shorebirds (*grey symbols*; Visser and Schekkerman 1999), and Arctic Tern (*solid symbols*; Visser and Schekkerman 1999). Values are means, and *bars* indicate 1 SE

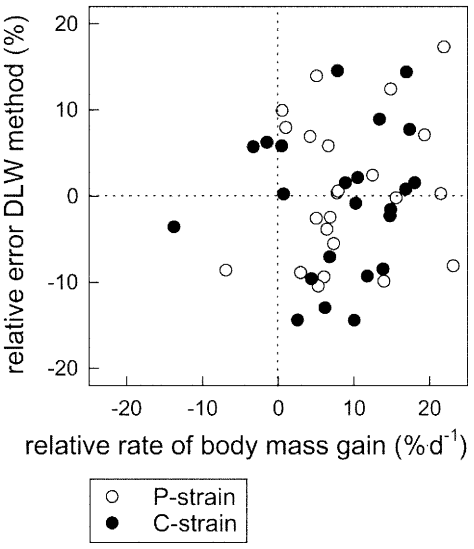

Fig. 2 Relationship between the relative growth rate of the chick during the measurement and the relative error of the DLW method in Japanese Quail chicks of the P- and C-strain (indicated by *open* and *solid* symbols, respectively). Equation 8 was used to calculate the rate of CO_2 production measured with doubly labeled water ($r_{CO_2\text{-DLW}}$), and Equation 6 was used to calculate the relative error of $r_{CO_2\text{-DLW}}$ relative to the rate of CO_2 production measured with respiration gas analysis ($r_{CO_2\text{-IR}}$)

The best fit (average relative error = 0) was obtained by assuming a fractional evaporative water loss of 0.33. This value was entered in Eq. 5 to yield:

$$r_{CO_2} = 22.4 \cdot [N/2.078(k_o - k_d) - 0.00822 \cdot Nk_d] \quad (8)$$

We used this equation to calculate the r_{CO_2} for each DLW measurement, and Eq. 6 to calculate the relative error of each DLW estimate to the value obtained with respiration gas analysis. For all individual values the relative errors ranged between −14.4% and 17.3%. The age-specific average errors ranged between −6.2% and 4.5% (Table 1). The relative errors of r_{CO_2} in the two females that produced an egg during the measurement were −3.6% and +5.8%, which suggests that Eq. 8 also holds during the egg-laying phase.

Analysis of covariance was used for both strains to investigate the relationship between the relative error of the DLW method and the relative growth rate of the chick during the measurement (observed range from −13.8% day^{-1} to 23.1% day^{-1}; Fig. 2). The analysis revealed that the interaction term (strain × relative growth rate) did not significantly contribute to the explained variance ($F_{1,42} = 0.04$, $P = 0.846$). After deleting this term from the model, it was found that there was neither a significant effect of strain ($P = 0.646$) nor of the relative growth rate of the chick during the measurement ($P = 0.292$). We performed a similar analysis for the relationship between the relative error of the DLW method and the age of the chick. After deleting the insignificant interaction term (strain × age, $F_{1,42} = 0.92$, $P = 0.343$) it was found that there was neither an effect of strain ($P = 0.602$) nor of age ($P = 0.638$). Therefore, we can conclude that Eq. 8 is applicable in birds of both strains over the range of relative growth rates observed and ages employed.

r_{CO_2}: two-pool models

For all birds, the average value for $R_{dilspace}$ was 1.035 (SE = 0.0017). Analysis of covariance was used for both strains to investigate the relationship between $R_{dilspace}$ and the body mass at the time of injection. The analysis revealed that the interaction term (strain × body mass) did not significantly contribute to the explained variance ($F_{1,42} = 1.68$, $P = 0.201$). After deleting this term from the model, neither strain ($P = 0.156$) nor body mass ($P = 0.273$) significantly contributed to the explained variance. Thus, it can be concluded that one value for $R_{dilspace}$ is applicable in chicks of both strains over the range of body masses observed.

The value of 1.035 was then applied to calculate r_{CO_2} from the two-pool model at fractional evaporative water loss levels of 0, 0.25, and 0.5. At these levels of fractional evaporative water losses, two-pool models significantly underestimated the true r_{CO_2} by 5.7% (SE = 1.17, $t_{45} = 4.82$, $P < 0.001$), 9.8% (SE = 1.15, $t_{45} = 8.50$, $P < 0.001$), and 13.9% (SE = 1.15, $t_{45} = 12.11$, $P < 0.001$), respectively. Thus, at each level of fractional evaporative water loss the two-pool models significantly underestimated the true r_{CO_2}.

Discussion

Application of the DLW method in Japanese Quail in relation to growth rate

The experiment was designed to test the applicability of the DLW method in two different strains of the Japanese

Quail, in an attempt to evaluate the occurrence of differential rates of ^{2}H and ^{18}O incorporation in growing tissues. We demonstrated that in both strains from the age of 7 days onwards until the achievement of sexual maturity one single-pool model could be adequately employed to calculate the r_{CO_2} from DLW measurements (Eq. 8). This equation is a refinement of Eq. 34 of Lifson and McClintock (1966), after making the assumption that a fixed fraction (here estimated as 0.33) of the entire water efflux is lost through evaporative pathways, instead of the value of 0.5 assumed by the authors. The fact that one equation could be used over a wide developmental range, and over a wide range of relative growth rates, suggests that differential incorporation rates between ^{2}H and ^{18}O are unlikely to occur in the Japanese Quail, at least not at such a level that the application of the DLW method becomes problematic. In fact, relative errors of the DLW method obtained in chicks are not systematically different from those obtained in adults (including laying females). Given the finding that the $R_{dilspace}$ value did not change with body mass, it can be assumed that the ratio of protein to fat accretion did not change during the postnatal period (Coward 1990).

Towards a general understanding of DLW application in growing chicks

Up till now, three studies have been performed to validate the DLW method in growing birds: the semi-precocial Arctic Tern (Klaassen et al. 1989; recalculated by Visser and Schekkerman 1999), precocial Northern Lapwing and Black-tailed godwit (Visser and Schekkerman 1999), and in two strains of the Japanese Quail (this study). In these studies the same general model has been used (Eq. 34, Lifson and McClintock 1966). All three studies showed that the DLW method was shown to be applicable in growing birds. In none of the studies could a significant relationship be detected between the relative growth rate of the chick during the measurement and the relative error of the DLW method. The studies also demonstrated that the error of the DLW method relative to measurements of the respiration gas (the 'golden standard') was highly sensitive to the assumptions concerning fractional evaporative water loss. In all three studies, employment of a fractional evaporative water loss value of 0.50 (as assumed by Lifson and McClintock 1966) resulted in a statistically significant underestimation of the true r_{CO_2} (Fig. 1). The largest error was made in chicks of the shorebirds (average relative error of −9.0%) and Arctic Tern (−8.5%), and the smallest in Japanese Quail chicks (−2.7%). Much better fits were obtained using Speakman's (1997) Eq. 7.17 that employs fractional evaporative water loss value of 0.25: mean relative errors range from −2.9% in shorebirds to +1.3% in the Japanese Quail (Fig. 1). In fact, in none of these studies did the average relative error differ significantly from zero. On the other hand, neglecting fractionation effects due to evaporative water loss resulted in a systematic overestimation of the r_{CO_2} by 3–5% (Fig. 1). Therefore, we can be tentatively conclude that using Speakman's (1997) Eq. 7.17 in growing precocial or semi-precocial birds gives the best estimates if no possibility exists for performing a validation study. It cannot be excluded that fractional evaporative water losses are higher in naked altricial nestlings that lack plumage for insulation.

Sensitivity of the DLW method concerning assumptions on fractional evaporative water losses

In the three groups of species mentioned, the slope of the relationship between the assumed fractional evaporative water loss and the relative error of the DLW method was highest in Arctic Tern (−28.1), and lowest in the Japanese Quail (−15.8; Fig. 1). Thus, the sensitivity of the DLW method differs considerably with respect to assumptions concerning fractional evaporative water loss. It has been argued on the basis of theoretical modeling that this sensitivity strongly depends on the rates of water efflux relative to r_{CO_2} (Roberts 1989). Therefore, in an attempt to explain differences observed between the three groups of species, we examined in more detail the r_{CO_2} (measured with respiration gas analysis), rate of water efflux, and the ratio of water efflux rate to r_{CO_2} (Fig. 3; water economy index; Nagy and Petersen 1988).

First, we used analysis of covariance to compare the separate allometric relationship between r_{CO_2} and body mass for the three different groups of species (Fig. 3A). ANCOVA revealed that the interaction term species × body mass contributed significantly to the explained variance ($F_{2.47} = 25.7$, $P < 0.001$). The lowest value for the allometric scaling exponent was found in the Japanese Quail (0.62) and the highest in shorebirds (1.10; Fig. 3a).

Second, a similar analysis was performed to compare the allometric relationships between water efflux rates and body mass. After deleting the insignificant interaction term (species × body mass), it was found that species ($F_{2.49} = 37.8$, $P < 0.001$), and body mass ($F_{1.49} = 62.8$, $P < 0.001$) contributed significantly to the explained variance (Fig. 3B). The common allometric scaling exponent was 0.45 (SE = 0.057), and the intercept values for Japanese Quail, shorebirds and terns were 3.34, 4.96, and 6.91, respectively (Fig. 3B). Thus, at a given body mass, water fluxes were lowest in the Japanese Quail, and values were higher in shorebirds and terns by 48% and 107%, respectively. In nearly all cases observed levels were well above levels predicted on the basis of an allometric relationship for adult birds in captivity (Nagy and Peterson 1988). Interestingly, both shorebird and Quail chicks were fed a pellet diet, while water fluxes were considerably higher in shorebird chicks; tern chicks were fed a fish diet. In all cases, water efflux rates observed in growing chicks were higher than

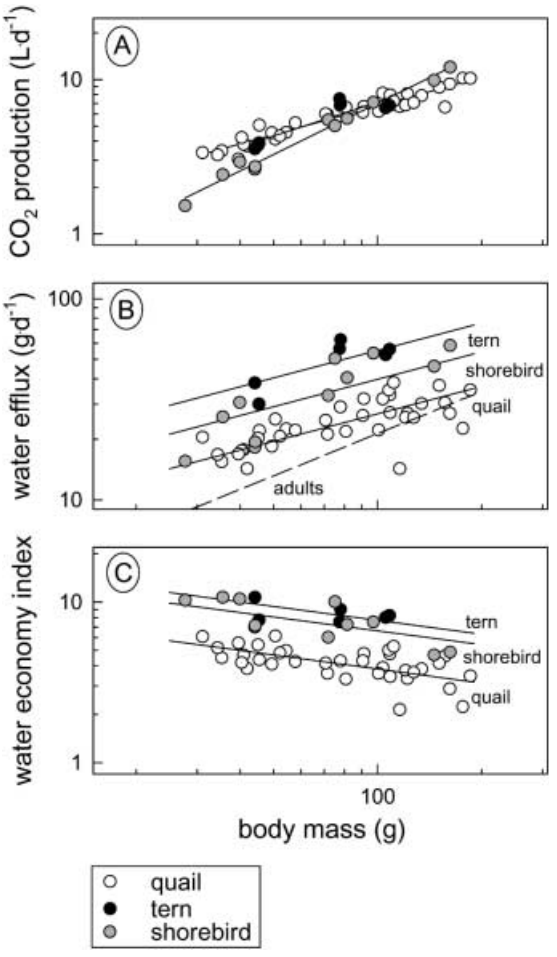

Fig. 3 Allometric relationships between body mass and rates of CO_2 production (**A**), rates of water efflux (**B**), and water economy index (**C**) in growing chicks of the Japanese Quail (*open symbols*; this study), shorebirds (*grey symbols*; Visser and Schekkerman 1999), and Arctic Tern (*solid symbols*; Visser and Schekkerman 1999). *Drawn lines* refer to the fitted equations for each species. The allometric relationship between body mass and water efflux rates in captive adult birds (*broken line*; Nagy and Peterson 1988) is shown in **B**

predicted on the basis of an allometric relationship for adult birds in captivity.

Third, we performed a similar analysis to compare the separate allometric relationships between body mass and the index of water economy (rate of water flux relative to the rate of carbon dioxide production, as proposed by Nagy and Peterson 1988). After deleting the insignificant interaction term, and after assuming a common allometric scaling exponent which had a value of −0.29 (SE = 0.051, $F_{1,49} = 32.6$, $P < 0.001$), it was found that the intercepts differed significantly ($F_{2,49} = 58.2$, $P < 0.001$). At a given body mass, water economy index was lowest in chicks of the Japanese Quail, and values were higher for shorebirds and terns by 72% and 101%, respectively (Fig. 3c).

There is a striking relationship between the level of water economy index and the sensitivity of the DLW method to assumptions concerning evaporative water loss. On the one hand, water economy index was low in Japanese Quail chicks, and the DLW method appeared to be relatively insensitive to assumptions concerning evaporative water loss. On the other hand, water economy index was high in tern chicks, and the DLW method appeared to be more sensitive with respect to assumptions concerning fractional evaporative water loss. As indicated in Fig. 3a and b, the chicks of these groups especially differ with respect to their levels of water efflux.

Application of the DLW method during growth and adulthood

The Japanese Quail is the first animal species in which the DLW method has been validated over a wide developmental range, and no information is available on other species to make a comparison. In humans, validation studies have been performed over an even broader range of developmental stages, from pre-term and term babies to adults at normal and high working rates (for the most recent review see Speakman 1997). The results on humans also showed that one general model (Eq. A6 derived by Schoeller et al. 1986) is applicable at all stages. In contrast to the model employed by us on the Japanese Quail, Schoeller et al.'s (1986) Eq. 6 is based on the assumption that the rate of breath water loss (which is subject to fractionation) is directly related to the r_{CO_2}, and that the rate of transcutaneous water loss through the skin (also subject to fractionation) is directly related to the surface area of the naked skin. Due to the fundamental differences in the respiratory systems and the levels of skin protection between birds and mammals, it is difficult to make a direct comparison between both models (see also the discussion on this issue in Speakman 1997, p. 110). Yet it is encouraging that in both humans and animals the application of the DLW method to juveniles during growth appears not to be invalidated by the partial incorporation of ^{18}O and ^{2}H in growing tissues.

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