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Validation of a Non-Invasive Blood-Sampling Technique for Doubly-Labelled Water Experiments

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ABSTRACT Two techniques for bleeding small mammals have been used in doubly-labeled water (DLW) studies, including vena puncture and the use of starved nymphal stages of hematophagous reduviid bugs (Reduviidae, Hemiptera). In this study, we tested the validity of using reduviid bugs in doubly-labeled water experiments. We found that the isotope enrichment in initial blood samples collected with bugs was significantly lower compared to isotope enrichment in blood samples obtained using vena puncture. We therefore used the desiccation method for estimating total body water (TBW) in DLW experiments because TBW calculated using the isotope dilution method was overestimated when blood samples were collected using reduviid bugs. In our validation experiment with nectar-feeding bats (*Glossophaga soricina*), we compared estimates of daily energy expenditure (DEE) using DLW with those derived from the energy balance method. We considered Speakman's equation (controlling for 25% fractionated water loss) as the most appropriate for our study animal and calculated DEE accordingly. On average, DEE estimated with DLW was not significantly different from the mean value obtained with the energy balance method (mean deviation 1.2%). We conclude that although bug hemolymph or intestinal liquids most likely contaminate the samples, estimates of DEE are still valid because the DLW method does not depend on absolute isotope enrichments but on the rate of isotope decrease over time. However, dilution of blood with intestinal liquids or hemolymph from a bug may lead to larger variation in DEE estimates. We also tested how the relative error of DLW estimates changed with varying assumptions about fractionation. We used three additional equations for calculating DEE in DLW experiments. The basic equation for DLW experiments published by Lifson and McClintock (LM-6) assumes no fractionation, resulted in an overestimate of DEE by 10%. Nagy's equation (N-2) controls for changes in body mass but not for fractionation. Using Nagy's equation, DEE was overestimated by 8%. Under the assumption that 50% of total water flux fractionates, the alternative equation by Lifson and McClintock (LM-35) DEE was underestimated by 5%. The best fit between estimates of DEE based on DLW and energy balance measurements was derived by assuming that 32% of total water flux (TWF) is fractionated. We conclude that the outcome of DLW experiments is sensitive to assumptions regarding evaporative water loss, and thus recommend Speakman's equation 7.17 for use with bats. *J. Exp. Zool. 296A:87–97, 2003.*

INTRODUCTION

The doubly-labeled water (DLW) method ($^2HH^{18}O$ or $^3HH^{18}O$) established by Lifson and colleagues (Lifson et al., '49, '55; Lifson and McClintock, '66) provides a unique basis for estimating daily energy expenditure (DEE) and daily water flux (DWF) in free-ranging animals. This method relies on the fact that the turnover of the oxygen pool in an animal depends on loss of carbon dioxide and water over time, whereas the turnover of the hydrogen pool depends only on loss of water. By measuring the washout rates of hydrogen and oxygen with heavy isotopes, it is possible to estimate rates of carbon dioxide production (Lifson and McClintock, '66). The accuracy of this method has been tested in a variety of animals, and these validation experiments revealed an average difference of approximately

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3% when results of DLW studies were compared with other methods (Speakman, '97). In part, the accuracy of the DLW method is based on the fact that the underlying assumptions of the method are not be violated by the experimental protocols (Lifson and McClintock, '66; Nagy, '80; Nagy and Costa, '80). Previous validation studies have shown that the extent of isotopic fractionation during pulmocutaneous evaporation greatly affects the outcome of validation trials (summarised in Speakman, '97). In their initial paper, Lifson and McClintock ('66) published equations to adjust for fractionation. These equations are based on the assumption that 50% of the water flux evaporates, which may be high for many animals (Haggarty et al., '88; Tiebout and Nagy, '91; Speakman, '97). Tiebout and Nagy ('91) showed that the DLW method ($^3HH^{18}O$) provides accurate estimates of daily energy expenditure and water flux in hummingbirds when the lower percentage of fractionated water loss and specific fractionation factors for the body temperature of the study animal are incorporated into the equation. Similarly, Speakman ('97) proposed a modified equation for estimating the rate of carbon dioxide production in mammals based on physical fractionation at 37°C and a ratio of 25% evaporative water loss to total water flux. His equation has been validated recently for growing precocial chicks (Visser and Schekkerman, '99; Visser et al., 2000), and the results generally support the view that fractionation can be precisely controlled for in DLW experiments.

Acquiring sufficient quantities of blood from small vertebrates for DLW studies can be challenging (Kunz and Nagy, '88). Vena puncture has been used successfully in numerous DLW studies on birds and bats (reviewed in Nagy et al., '99; Speakman, '97), although if blood is shunted away from the periphery, it may be difficult to obtain sufficient quantities for analysis. Sometimes, vena pucture may lead to excessive blood loss. To avoid these two problems, von Helversen and Reyer ('84) and von Helversen et al. ('86) used starved reduviid bugs to obtain blood from bats. Many reduviid bugs feed exclusively on blood of mammals or birds and are well adapted for accessing and acquiring blood quickly. This method also has several other advantages: 1) the bugs by themselves find an optimal place for inserting their proboscis, 2) the bugs heparinize the ingested blood, and 3) the animals from which blood is taken are less stressed by this procedure compared to other blood sampling techniques (von Helversen and Reyer, '86; C. Voigt, unpublished observation).

The principal goals of this study were to validate the use of reduviid bugs for blood sampling in DLW experiments. To do so, we used estimates of DEE using the DLW method to evaluate this bleeding procedure on captive, nectar-feeding bats, 10 g *Glossophaga soricina*, and compared these estimates with estimates based on an energy balance method. Although the direct measurement of CO_2-production with a gas analyzer is the method of choice when validating the DLW method, we found it justified to use the energy balance method for the following reasons. First, bats spend most of the night foraging on the wing and flight requires a certain amount of space. A large flight enclosure, however, would preclude a correct application of respirometry. Secondly, the study of energy budgets is facilitated in nectar-feeding animals, because digestive efficiency is high and experimental monitoring of nectar intake is relatively easy (e.g., Winter, '98; Winter and von Helversen, '98; Voigt, 2000, 2003).

An additional goal of this study was to compare inter- and intra-individual variation in DLW estimates. Variation in DLW estimates in experiments based on sequential captures of animals can be as a high in an individual as it is between individuals (Speakman et al., '94; Berteaux et al., '96). This observation is important for studies in which DLW estimates are compared between individuals, because a high intra-individual variation of DLW estimates may obscure significant differences in DEE between individuals or between populations. We conducted repeated measurements in three individuals of *G. soricina* to test whether the DLW estimates or error in estimates depends on the number of trials, and whether the error in the method is similar within an individual and between individuals. Finally, we evaluated three commonly used equations for estimating DEE in DLW experiments (all single-pool models) to evaluate the effect of assumptions concerning the ratio of fractionated water loss.

MATERIAL AND METHODS

Equilibration of isotopes in total body water following subcutaneous injection

We conducted an experiment with 20 *Glossophaga soricina* (10 females and 10 males) to determine the timing of the plateau during equilibration of isotopes with the body water. In each bat, we injected 100 µl of sterile water labeled

with deuterium (4.65 atom%). At intervals of 15 to 60 minutes, we took a single blood sample from each bat by puncturing the propatagial vein with a 25-gauge needle. At the time of blood-sampling, the body masses of the bats were measured (Mettler PM-100, accuracy 1 mg). Blood samples were collected in heparinized microcapillary tubes, flame sealed and stored in a freezer below 0°C. Sample preparation and analysis followed the procedure described below.

Potential dilution of blood

To evaluate whether the hemolymph or intestinal liquids from reduviid bugs would dilute the blood samples and thus affect the isotope enrichment, we compared the enrichment of deuterium in blood samples taken with a standard method (vena puncture) and with bugs within a short time interval from the same bats. We subcutaneously injected 100 µl of the isotopically labeled water solution into 7 *Leptonycteris curasoae* (4 females and 3 males) (Glossophaginae). For this experiment we choose this 20 to 25 g bat, because we required maxium volume of blood for two samples. From each bat, we collected a total of ca. 200 µl after an equilibration time of 1.5 hours. We first took a blood sample using a reduviid bug and immediately thereafter took a blood sample from the propatagial vein using vena puncture. On average, only two minutes elapsed between the two sampling events and thus we expected any observed differences in isotope ratios between the two samples to be caused by the contamination of blood with the bug hemolymph. Blood samples were collected in heparinized microcapillary tubes, flame sealed and stored in a freezer below 0°C. We calculated the amount of total body water (TBW; g) with the following equation (1):

$$TBW = 18^{*}Q^{*}(c_i - c_d)/(c_b - c_i) \qquad (1)$$

With Q representing the dose of the injection water (mol), c_b the basal isotope enrichment of 18-O (atom%), c_i the initial isotope enrichment of 18-O (atom%) and c_d the isotope enrichment of 18-O (atom%) in the labeled water. The factor 18 converts moles of H_2O into gram. The percentage TBW was calculated by dividing TBW by the body mass (g).

In other experiments, we measured the TBW of starved *Triatoma infestans* L4. We sacrificed 15 bugs and weighed them immediately to the nearest 1 mg (Mettler, PM-100). Afterwards, the insects were put in a drying oven and dried until constant mass. The body mass of the dried insects was measured to the nearest 1 mg (Mettler, PM-100). We calculated the amount of body water for each insect by subtracting the body mass before drying with the body mass after drying.

Doubly-labeled water experiments

We randomly selected 7 *Glossophaga soricina* (4 males and 3 females) from a captive colony of ca. 50 individuals maintained in greenhouse facilities at the University of Erlangen-Nürnberg. The animals were introduced into an air-conditioned room (25°C), where they were maintained under a light:dark regime of 12 h:12 h, and ad libitum food (ca. 17% mass/mass honey water, banana and pollen). Relative humidity was measured with a conventional hygrometer calibrated at regular intervals with a ceramic hygrometer (Festo Inc., Esslingen, Germany) to an accuracy of 5%. Relative humidity ranged between 60 and 80% (mean 70%) but was constant throughout the experiments. For the validation experiment, a single bat was introduced into a flight cage (7 m long, 1 m wide and 2 m high). The cage was constructed with polyurethane sheeting (Winter and von Helversen, '98) which prevented the bat from landing anywhere other than on a piece of cork suspended from an electronic balance (Mettler PM-100, accuracy 1 mg). Body mass and the duration of flight and resting intervals were recorded from the balance that was interfaced with a personal computer (see Winter and von Helversen, '98). Because we never observed bats landing on the ground, we assumed that they were on the wing when they were absent from the roost (suspended cork). A ventilator was used to draw fresh air into the flight cage. Thirty-seven µl of sugar solution was dispensed into a feeder by a computer-controlled pump when bats inserted their head into the feeder and interrupted a light beam. The feeder was covered with smooth plastic to prevent the bat from clinging to it. We controlled for loss of sugar water at the feeder by placing paper sheets on the floor and periodically checking it for droplets of water. We found no evidence for loss of sugar water and thus assumed that all sugar water pumped into the feeder was ingested. One bat (M4) escaped from the flight cage during one experiment. Thus, time budget measurements are missing for this individual.

Prior to each experiment, a single bat was captured at approximately 15:30 h, weighed, and injected subcutaneously with 100 µl water that was enriched with isotopes of oxygen-18 (30 atom

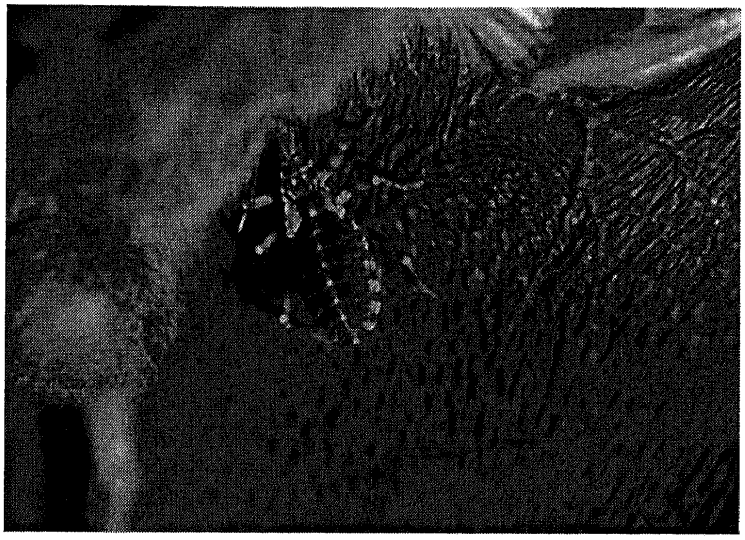

Fig. 1. Blood-sucking bug (*Triatoma infestans*, nymphal stage 4, Reduviidae) ingesting blood from the abdomen of a nectar-feeding bat (view on the ventral side of the bat). The left wing of the bat was extended to its full length (forearm visible in the upper right corner of the picture and left leg and one testes in the left part of the picture).

%) and deuterium (10 atom %). The mean duration of equilibration was 1.5 hours$\pm$13 min (SD). During this time, bats lost an average of 0.13$\pm$0.09 g (SD) body mass. Each initial blood sample was obtained from the bat at 17:00 h, one hour before the onset of darkness. We followed von Helversen et al. ('86) in using starved hematophagous reduviid bugs (Reduviidae: *Dipetalogaster maxima*, nymphal stage 3 and *Triatoma infestans*, nymphal stage 4) for collecting blood. For collecting blood we positioned the bat on its back, opened one wing, and then released a bug onto the wing membrane in proximity to the forearm (Fig. 1). The bug penetrated the forearm or abdomen with its proboscis after a few minutes or even only a few seconds of searching. After the bug had ingested approximately 50 to 80 µl of blood, we removed and decapitated it, and collected the blood with a microcapillary tube. All tubes were immediately flame-sealed and stored below 0°C until analysis. After taking the initial blood sample, the bat was returned to the flight cage (ca. 17:10 h). The following afternoon we repeated the procedure at 17:00 h. Each time, bats were weighed to the nearest 1 mg and thus differences in the two measurements that we recorded reflected daily changes in body mass.

The total body water content of five individuals at 17:00 h was determined by desiccation. After weighing and sacrificing the bats, we dried the carcasses for three days at 90°C and repeatedly weighed each bat until constant mass was achieved. We used the mean body water content of these animals as an estimate of the total body water pool during DLW experiments, because we assumed that the reduviid bugs diluted the blood samples with hemolymph when used in DLW experiments (see results and discussion below). Samples for the analysis of background concentration of oxygen-18 and deuterium were taken from 8 *G. soricina* at 17:00 h following the procedure described above. Body mass at the time of initial blood sample (ca. 17:00 h) was multiplied by the mean percent total body water (66.8%; mean value for 5 desiccated specimens).

We distilled the blood as described in Nagy ('83), and analyzed oxygen isotopes in the water samples using the guanidine-hydrochloride method (Wong and Klein, '86, Wong et al., '87a, '87b). We employed the zinc-reduction method for the analysis of hydrogen isotopes in the water samples (Kendall and Coplen, '85; Coleman et al., '89; "Hayes" Zinc, Indiana University). All samples were analyzed at least in duplicate or triplicate using a Finnigan Delta-S isotope ratio mass-spectrometer at Boston University's Stable Isotope Laboratory. A laboratory standard was analyzed in duplicate after every sixth sample. Triplicate values for oxygen-18 (ppm) showed a standard deviation of 0.41% and those for deuterium (ppm) of 0.05%.

Webb et al. ('92) found that the evaporate water loss of a similar sized insectivorous bat was equal to ca. 30% of the total water loss. Thus, we calculated the rate of carbon dioxide production (rco_2; mol h^{-1}) with equation (2) recommended by

Speakman (=equation 7.17 in Speakman, '97; henceforth abbreviated as S-7.17), controlling for 25% evaporative water loss to total water flux at 37°C body temperature.

$$rco_2 = (N/2.078) * (k_O - k_D) - 0.0062 * N * k_D \quad (2)$$

with N representing the size of the body water pool (mol), k_O and k_D representing the fractional turnover rates (k) of oxygen-18 and deuterium. In general, the fractional turnover rates were calculated using equation (3):

$$k = [\ln(c_i - c_b) - \ln(c_f - c_b)]/t \quad (3)$$

with c_b representing the basal isotope enrichment (atom%), c_i the initial isotope enrichment (atom%), c_f the final isotope enrichment (atom%) and t the time that elapsed between the initial and final sample. The rate of carbon dioxide production (rco_2; mol h^{-1}) was converted to hourly energy expenditure (E; kcal h^{-1}), following Lusk ('76):

$$E = rco_2 * 22.4 * (3.185 + 1.232 * RQ) \quad (4)$$

with RQ equaling the respiratory quotient (=ratio between carbon dioxide produced and oxygen consumed). Using indirect calorimetry, Voigt and Winter ('99) measured an RQ=1 for two species of nectar-feeding bats, indicating complete combustion of sugar during nightly feeding activity. We converted E into DEE (kJ d^{-1}), by multiplying E with 4.184 (J cal^{-1}), and the duration of the experiment (h d^{-1}). Values are expressed in means for individuals $\pm$ 1 SD.

To quantify the variability in DEE that was caused by the inaccuracy of isotope analysis, we calculated an estimate of error for each experiment. As stated, each sample was analyzed either in duplicate or triplicate depending on the amount of water available. For each DLW experiment, we calculated the range of DEE that resulted from combining the multiple measurements of initial and final samples of both isotopes. For example, if all samples of a DLW trial were analyzed in triplet, we received a total number of 36 possible combinations for DEE and if all samples were analyzed in duplicates the total number of combinations equaled 16. The error of each estimate of DEE was then calculated as the percent standard deviation of estimates. The median of the percent standard deviation for all trials was 3.8%, which was less than that reported in other validation studies (e.g., Berteaux et al., '96).

Energy balance method

During validation experiments, each bat was maintained on a diet of 17% sugar solution (mass/mass, Atego refractometer, accuracy 0.2%), which is a common sugar concentration in nectar of bat-pollinated plants (von Helversen, '93). Dry mass of sugar consisted of 26% sucrose, 37% glucose and 37% fructose (heat of sugar combustion equals 15.91 kJ g^{-1}; Wieser, '86). Conversion of the sugar solution into a corresponding mass of dry sugar and water, respectively, followed Wolf et al. ('83), with one litre of 17% sugar solution equaling 1.069 kg of mass and consisting of 182 g sugar and 887 g H_2O. We assumed that nectar-feeding bats absorbed 100% of the ingested sugar solution. Winter ('98) demonstrated by enzymatic analysis that the urine of glossophaginae bats contains no significant amounts of sugar. A digestive efficiency of almost 100% is commonly found in other nectar-feeding animals, such as hummingbirds (Karasov et al., '86). Daily energy expenditure was calculated as the sum of the daily energy intake and the amount of daily metabolized fat, assuming a metabolic heat equivalent of 31.17 kJ per gram change in body mass (mean value from six nectar-feeding bat species; Winter and von Helversen, '98). We estimated an average RQ for each experiment by converting the DEE that was due to sugar and fat catabolism, into the corresponding oxygen and carbon dioxide volume using standard conversion factors (Schmidt-Nielsen, '90), and by dividing total Vco_2 by total Vo_2.

Comparison of different equations

We compared three other equations for estimating DEE in DLW experiments using measurements from the energy balance method. The original equations for estimating the rate of CO_2 production (Lifson and McClintock, '66) assume either no fractionation (equation 6 in Lifson and McClintock, '66, henceforth abbreviated as LM-6) or fractionation due to evaporative water loss that amounts to 50% of total water flux (equation 35 in Lifson and McClintock, '66, henceforth abbreviated as LM-35). Nagy's equation 2 (Nagy, '80, henceforth abbreviated as N-2) does not control for fractionation but instead controls for linear changes in body mass.

For all calculations we used Microsoft Excel (Version 97) and for statistical analysis SPSS (SPSS Inc. 1998). The treatment of animals during the experiments complied with national

guidelines and Boston University's Animal Care and Use Committee.

RESULTS

Equilibration of isotopes in body water after subcutaneous injection

After injection of labeled water into bats, the enrichment of deuterium reached a plateau after approximately 1.5 hours (Fig. 2). We, therefore, assumed that the isotopes were equally distributed in the body water pool at 1.5 hours after injection and chose this as the equilibration time for the following experiments.

Potential contamination of blood samples with bug hemolymph

The enrichment in deuterium was significantly lower in the blood samples taken with bugs compared to those samples taken without bugs (paired t-test, $t_6=4.1$, $P<0.05$). The difference in isotope abundance averaged 0.00449 atom% (0.00266 SD), which was equal to 10% of the overall enrichment in deuterium. Estimates of TBW based on the enrichment of deuterium in the blood samples taken with bugs averaged 81.6 % (±6.3 % SD). This was 13.5 % higher than TBW estimated on the basis of the enrichment of deuterium in the samples taken *without* bugs 68.1% (±5.9% SD), or TBW measured with the desiccation method (66.8 %±3.2 % SD). Because of the good agreement between the latter two methods, we chose the desiccation value of TBW for the DLW experiments in *Glossophaga soricina*. In starved *Triatoma infestans* L4, the amount of body water averaged 97 µl (±27 µl SD, n=15).

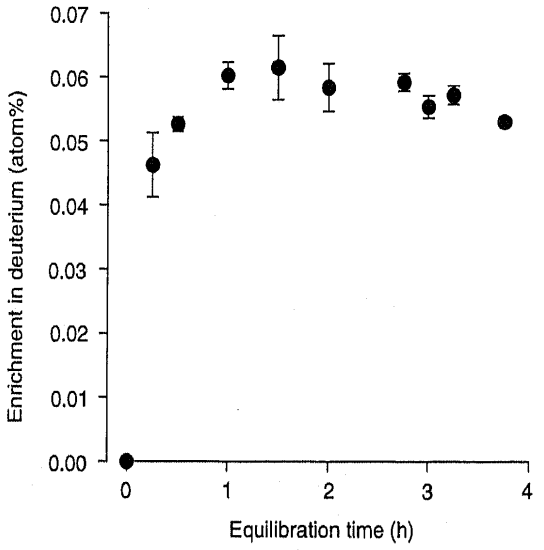

Fig. 2. Enrichment of deuterium (atom%) (± SD) in blood samples taken from 10 g *Glossophaga soricina* in relation to the time elapsed after injection (hour). The concentration of deuterium reaches a maximum (=plateau) at approximately 1.5 hours following subcutaneous injection.

Doubly-labeled water method

Background concentrations for oxygen-18 expressed in atom% were 0.19944 (±0.00003 SD, n=3) for 1994, 0.19997 (±0.00054 SD, n=3) for 1995, and 0.19990 (±0.00054 SD, n=2) for 1998. Background concentrations for deuterium expressed in atom% were 0.01481 (±0.00013 SD, n=3) for 1994, 0.01535 (±0.00015 SD, n=3) for 1995 and 0.01497 (±0.00046 SD, n=2) for 1998. All estimates of TBW based on isotope dilution were significantly higher than the mean value of total body water measured by desiccation (two-tailed t-test on arcsine-transformed data, all $t_6>4.9$, $P<0.05$). The average ratio between isotope washout rates (k_O/k_D) was 1.32±0.03. Speakman's equation (S-7.17; Speakman, '97) resulted in a mean rate of CO_2 production of 0.00455±0.00118 mol h^{-1}. Conversion of the rate of carbon dioxide production into the corresponding rate of energy turnover yielded 51.4±13.3 kJ d^{-1}.

The range of deviations for repeated measurements among individuals was high (Table 1). The variance of error in DEE estimates from M1 was not significantly different from the variance of errors for all other individuals (mean value for M1 excluded) (arcsine-transformed data, ANOVA: $F_{6,6}=0.68$, $P>0.05$). Thus, errors in the DLW estimates were not consistent within an individual. The number of the trial did not significantly explain the deviation of DEE estimates ($F_{1,6}=0.63$, $P > 0.05$).

Energy balance method

The mean body mass of the seven *Glossophaga soricina* was 9.50±0.88 g. On average, the

TABLE 1. Intra-individual variation in precision of estimates of daily energy expenditure (DEE) based on doubly-labeled water experiments in Glossophaga soricina[1]

Individual	Daily energy expenditure[2] Mean±1 SD	Min.	Max.
M1 (n=6)	5.2±11.6%	−16%	15%
M2 (n=2)		−5%	15%
W2 (n=2)		−5%	2%

[1](DLW estimate–balance result)/ balance result.
[2]All values were calculated with the equation controlling for 25% evaporative water loss.

bats lost 0.29±0.31 g during the validation experiments, which was significant based on a paired t-test (t_6=3.8, P<0.05). The mean water intake equaled 14.7±3.0 ml d^{-1} (M4 excluded) and mean sugar intake 2.6±1.2 g d^{-1}. This corresponds to an average energy intake of 41.7±19.0 kJ d^{-1} during the experiments. At the onset of the night, bats gained mass after ingesting sugar water at the feeder (Fig. 3). Bats increased their body mass during foraging by as much as 0.5 g. During the last half of the night, bats maintained nearly constant body mass. The mean body mass during the night equaled 10.00±0.96 g. Hence, body mass at 17:00 h—the time of blood sampling—and the night differed, on average, by 0.5 g, equalling ca. 5%. Each foraging bout averaged less than one minute (0.62±0.5 min), and bats visited the feeder an average of 450 times per night.

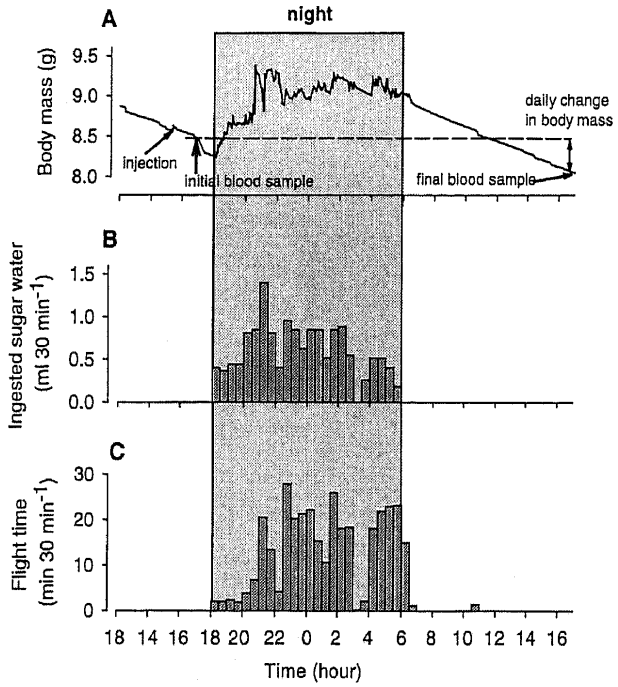

Fig. 3. Typical experimental day for a *Glossophaga soricina*: (A) pattern of body mass change over the course of a day; (B) amount of ingested sugar water (ml) per half hour; (C) flight time (min) per half hour. We measured the rate of evaporative water loss for each bat prior to a doubly-labelled water experiment by dividing the daily loss of body mass over time. Total evaporative water loss was estimated for each experiment on the basis of the time budget and the assumption that rate of evaporative water loss during flight was 11 times higher compared to rest. An animal was injected at 15:30 h with doubly-labelled water and a first blood sample was drawn from the bat at 17:00 h. The night started at 18:00 h and lasted 12 hours. A second (final) blood sample was obtained at 17:00 h after 24 hours. We refer to daily change in body mass as the difference in body mass between measurements taken at 17:00 h after 24 hours.

Based on the energy balance method, the estimated daily energy expenditure equaled 50.9±11.6 kJ d^{-1}. Average daily RQ of all bats was 0.93±0.08, and was not statistically different from unity (one-tailed t-test, t_6=0.3, P >0.05).

Comparison of the two methods of choice

A multiple regression analysis, with the log-transformed DEE as the dependent variable and daily change in body mass and log-transformed body mass as the independent variables, failed to reach a significant level (r^2=0.08; body mass: t_6=0.55, p>0.05, difference in body mass: t_6=0.46, p>0.05). Thus, we did not control for body mass when comparing DEE between individuals. We found good agreement between the DLW estimates of DEE and the expected values based on the energy balance method (mean deviation 1.2% see Table 2, Fig. 4). The slopes of linear regressions that were calculated with the least-squares method did not differ significantly from one (t-test, t_6=0.53, P>0.05), and the intercepts of these regressions did not deviate significantly from zero (t-test, t_6=0.28, P>0.05). Hence, we found no significant deviation from unity.

Comparison of different equations

Using Lifson and McClintock's equations yielded the following results. Calculation without compensation for fractionation (LM-6) resulted in an average hourly CO_2 production of 0.00493±0.00129 mol h^{-1}. When compensating for fractionation (LM-35), the average hourly CO_2 production was 0.00427±0.00109 mol h^{-1}. Thus, mean DEE was 55.9±14.6 kJ d^{-1} when calculated without correcting for fractionation and 48.5± 12.3 kJ d^{-1} when correcting for fractionation. Equation N-2 (Nagy, '80) yielded an average mass-specific rate of CO_2 production of 11.72± 2.22 ml g^{-1} h^{-1}, which equaled an average daily energy turnover of 55.2±14.4 kJ d^{-1}.

In using Speakman's equation, DEE deviated from measured values of the energy balance method by only 1.2%, indicating that his equation was more accurate for the DEE compared to a range of errors from −5 and 10% when using previously published equations (Fig. 5, Table 2). When fractionation was disregarded, estimates of DEE deviated significantly (LM-6) or were marginal significantly different (N-2) from results of the energy balance method (Table 2). By contrast, equations that controlled for fractionation yielded

TABLE 2. Comparison of mean errors (including sign) for estimating daily energy expenditure in doubly-labeled water studies

Equation	Factors controlled	Mean error[1]	SD	Max.	Min.	Mean values (n=7)[2]		All trials (n=14)[2]	
LM-6	No	9.5%	9.6%	23%	−7%	t=2.61	$P<0.05$	t=2.49	$P<0.05$
N-2	Daily change in body mass	8.1%	9.5%	21%	−8%	t=2.29	$P=0.06$	t=2.04	$P=0.06$
S-7.17	Fractionation at 37°C with EWL=25%	1.2%	9.0%	13%	−14%	t=0.45	$P=0.67$	t=0.36	$P=0.73$
LM-35	Fractionation at 25°C with EWL=50%	−4.8%	8.8%	5%	−20%	t=1.49	$P=0.19$	t=2.54	$P=0.05$

[1](DLW estimate–balance result)/ balance result.
[2]Two-tailed paired t-test.

estimates that did not differ significantly from results of the energy balance method (Table 2).

DISCUSSION

The effect of blood sampling using reduviid bugs on DLW estimates

Using reduviid bugs for blood sampling potentially offers a practicable method in doubly labeled water studies. However, this method bears a fundamental problem, i.e., hemolymph or intestinal liquids of the bugs may dilute the blood sample and, thus, bias the isotope enrichment. In the experiment with *Leptonycteris*, we demonstrated that the enrichment of deuterium in blood samples taken with bugs were significantly lower compared to the enrichment in samples taken with vena puncture. Thus, it is likely that the hemolymph from the bug mixed with and diluted the bat blood. Calculation of TBW based on the enrichment of deuterium in blood samples taken *with* bugs yielded significantly higher values. In contrast, calculation of total body water (TBW) based on the enrichment of deuterium in blood samples taken by vena puncture (=without bugs) yielded values that were almost identical to those obtained with desiccation.

On average, starved *Triatoma infestans* had 97 µl of body water. To study the effect of this amount of "bug water" on the estimate of percent TBW, we ran the following simulation. Under the

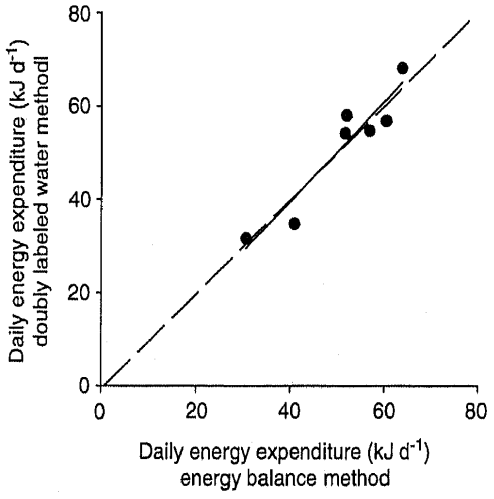

Fig. 4. Comparison of estimated daily energy expenditure (kJ d^{-1}) from the doubly-labeled water experiment with the measurements of the energy balance method for *Glossophaga soricina*. The dashed lines represent equivalence and the solid line is a fitted least-squares regressions over mean values for individuals.

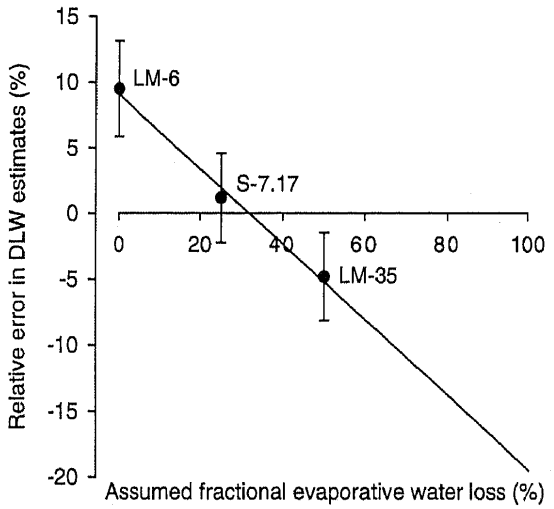

Fig. 5. Average error in DLW estimates in relation to the assumed ratio of fractionated water loss to total water flux (mean±SE). Three different equations were plotted to compare different assumptions with regard to fractionated water loss on the precision of the DLW estimates (the deviation of N-2 is similar to that of LM-6 (see Table 2) and for the reason of clarity results of N-2 were disregarded on this graph). The basic equation for calculation of CO_2 production rate that was published by Lifson and McClintock ('66, LM-6) yielded an overestimate of almost 10%. On the other hand, using Lifson and McClintock equation LM-35 that controls for 50% fractionated water loss resulted in an underestimate of −5%. Speakman's equation (1997, S-7.17) that controls for 25% fractionation water loss yielded the most accurate results. A linear regression (solid line) that was calculated after the least squares method showed that the assumption of 32% fractionated water loss to total water flux yielded the best fit between DLW and energy balance method (regression equation: %error = 9.117–0.286 *percent fractionated water loss).

assumption that the "bug water" had an isotope enrichment identical to the average background level of the body water of bats, the contamination of bat blood with 97 µl "bug water" would reduce the initial isotope enrichment of 100 µl bat blood by about 30%. If this biased initial enrichment were incorporated into equation 1, the estimated TBW of the bat would be 130% instead of 68%. In the experiment on *Leptonycteris*, TBW was overestimated by ca. 14% when calculated on the basis of isotope enrichment from blood collected with reduviid bugs. An overestimate of 14% would be equivalent to dilution of 100 µl bat blood with approximately 20 µl body water from the bug.

In general, we suggest that desiccation values be taken as estimates of TBW if reduviid bugs are used for blood sampling in DLW experiments. In experiments with *Glossophaga soricina*, we overestimated TBW using the plateau method, which is consistent with the findings of the experiment on *Leptonycteris*. Because estimates of daily water flux (DWF) and DEE in DLW experiments are based on the decrease of isotope abundance over time and not on absolute values, the dilution effect of bug hemolymph should not be a problem with the DLW technique. This is supported by our results that show a good fit of the DEE estimate between the doubly labeled water method and the energy balance method (Fig. 4, Table 2). However, it is possible that varying amounts of hemolymph and intestinal liquid from the bug mix with the blood of the initial and final sample and thus contribute to an increase in variation of DLW estimates.

Variation of the doubly-labeled water estimates

The imprecision of DEE estimates may be partly explained by false assumptions regarding, for example, the RQ. Based on the nutritional energy intake and daily change in body mass, we estimated the amount of catabolized fat and sugar and derived an RQ for each trial. If this respiratory quotient is incorporated into equation (4) (see Lusk, '76), the error of estimate was $-0.5 \pm 9.7\%$ SD. Thus, the incorporation of the RQ failed to improve the precision of the DLW estimates, as revealed by the same magnitude of error.

Unaccounted factors such as variation in background isotope concentration, differences in fractionated water loss, or changes in TBW may have affected our estimates of DEE. Observed decreases in body mass over the course of an experiment could influence the decline of isotopes for two reasons. First, mobilization of body fat produces metabolic water that potentially has a different isotope ratio when compared to nutritional water (violation of assumption 5 in Lifson and McClintock, '66: all substances entering the body water pool of an animal are comparable to background level). Second, daily changes in body mass could reflect a change in the TBW over the course of an experiment (violation of assumption 3 in Lifson and McClintock, '66: TBW is constant throughout the measurement period). In general, a decrease in pool size during the course of an experiment would result in an underestimate of the CO_2 production rate.

Relative humidity also may have affected our experiment owing to the intake of unlabeled isotopes via skin and lung. When condensed, unlabeled water mixes with the labeled water molecules of the body, the washout rates of heavy isotopes should increase, and hence DLW estimates should be overestimated. Lifson and McClintock ('66) and Nagy and Costa ('80) emphasized that an uptake of unlabelled isotopes would only affect the estimate of water flux but not of CO_2 production, since the latter is based on the relative difference in the washout rates of two elements. Gettinger ('83) found a high accuracy of DLW estimates in small rodents living at 75% relative humidity when equations did not compensate for fractionation, and concluded that the uptake of unlabelled isotopes from water vapor compensated for fractionation effects. By contrast Tiebout and Nagy ('91) found that a correction for fractionation was superior for estimating water flux in tropical hummingbirds, which contradicts a compensatory effect of the pulmocutaneous uptake of unlabelled water.

By comparing repeated measurements of individuals, we found no consistency in the deviation of DLW estimates among individuals or a correlation between the deviation and the sequential number of the trial. This suggests that the frequently observed imprecision of the DLW water method (see review in Speakman, '97) is probably not caused by physiological differences among individuals, at least in our study.

Comparison of different equations

By using equations LM-6 and LM-32 (Lifson and McClintock, '66), the estimate of DEE was 9.5% higher and 4.8% lower than estimates using the energy balance method (Table 2). Nagy's equation

N-2 (Nagy, '80) improved the estimates of LM-6 by controlling for the loss of body mass in our study animals. However, the deviation was still 8.1% compared to the energy balance method. Speakman's equation S-7.17 (Speakman, '97) provides an estimate of DEE that deviated by 1.2% from results derived from the energy balance method. The statistical analysis of this comparison reveals that estimates derived from LM-6 were significantly different from results of the energy balance method (Table 2). If we consider all trials, both equations by Lifson and McClintock deviated significantly from the results of the energy balance method (Table 2). By contrast, results derived from Speakman's equation S-7.17 did not differ significantly from those derived from the energy balance method (Table 2). These results as well as those reported by others (Tiebout and Nagy, '91; Visser and Schekkerman, '99, Visser et al., 2000) suggest that fractionation processes may affect DLW experiments and that appropriate equations should be used to control accordingly. In the absence of knowledge about the correct ratio of evaporative water loss to daily water flux, we suggest that Speakman's equation 7.17 (Speakman '97) be used for estimating the rate of CO_2 production in DLW experiments.

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