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Validation of the doubly labeled water method under low and high humidity to estimate metabolic rate and water flux in a tropical snake (*Boiga irregularis*)

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Anderson, Nancy L., Thomas E. Hetherington, and Joseph B. Williams. Validation of the doubly labeled water method under low and high humidity to estimate metabolic rate and water flux in a tropical snake (*Boiga irregularis*). *J Appl Physiol* 95: 184–191, 2003. First published March 28, 2003; 10.1152/japplphysiol.00692.2002.—This study uses indirect calorimetry to assess the effects of humidity on the accuracy of the doubly labeled water (DLW) technique to predict metabolic rate and water flux in brown treesnakes (*Boiga irregularis*). The DLW technique accurately predicted total water efflux in brown treesnakes under low-humidity conditions and found that the total number of water molecules exchanged with the environment under humid conditions was not significantly different than maximum net total evaporative water loss under low humidity conditions plus fecal water loss. Because of changes of total body water of >12%, the DLW technique overestimated metabolic rate by a factor of 2.2 under low-humidity conditions. Under high-humidity conditions, the DLW technique overestimated metabolic rate in brown treesnakes by a factor of 4.6. Researchers using the DLW technique in humid or moist environments should be cautious because this study indicates that DLW estimates of metabolic rate may be inflated when large amounts of water vapor are exchanged through the skin or respiratory passages.

total evaporative water loss; carbon dioxide production

THE DOUBLY LABELED WATER (DLW) method has been used to estimate energy expenditure and water turnover in free-ranging mammals, birds, reptiles (lizards, snakes, tortoises), and arthropods (19, 20, 30). The technique relies on injection of isotopes of hydrogen [deuterium (D) or tritium (^{3}H)] and oxygen (^{18}O) into a study animal. As the animal consumes oxygen (O_2) and produces carbon dioxide (CO_2), concentrations of isotopes in the total body water (TBW) pool decrease. Fluid samples (i.e., blood, saliva, tears, etc.) taken after isotope equilibration and at the end of the experimental period are analyzed for isotope concentrations from which calculations of isotope turnover can be made (14, 16, 21, 32). Appropriate equations use turnover rates to calculate rates of water flux and CO_2 production ($\dot{V}_{CO_2}$).

$\dot{V}_{CO_2}$, as estimated by the DLW technique, has been compared with standard laboratory measurements of expired CO_2 in species of mammals, birds, lizards, and the desert tortoise (*Gopherus agassizii*). Mean errors in these studies are generally ±11% (19), but individual deviations can vary by ±30% (32). However, the method has not been validated on snakes, i.e., ectotherms that have larger surface to volume ratios than most other animals (9, 17, 18, 22, 32, 35). Despite this lack of assurance that the DLW technique can provide reliable estimates of metabolic rate for snakes, it has been used on them in the wild in a number of studies (2, 4, 23, 25, 30). In addition, although snake diversity is greatest in tropical regions, most DLW studies on snakes have been conducted on species living in xeric and temperate habitats. This study validates the DLW technique for use in a tropical snake species.

The assumptions of the DLW method have been previously detailed (14). Violation of any of these assumptions can lead to errors in estimates of $\dot{V}_{CO_2}$, but the one that is most likely to be violated for studies on tropical forest snakes is that water or CO_2 does not enter the body with inspired air or through the skin. In tropical environments, water vapor content of air is high, and its influx through the skin or mucous membranes and consequent dilution of the hydrogen and oxygen isotopes can potentially generate a significant source of error in DLW calculations. Working on kangaroo rats, Nagy and Costa (21) reported that errors in DLW estimates increased from 3 to 44% as relative humidity (RH) increased from 4 to 20%. Whereas the DLW technique was within ±10% when validated on small desert lizards in low-humidity conditions (9, 18), the technique overestimated energy expenditure by a factor of ~4 in small tropical lizards tested under high-humidity conditions (Ref. 17; Nagy KA, personal communication). In contrast, Van Marken Lichtenbelt (35) found only a small increase in errors (−1.5–7.4%) in DLW estimates of $\dot{V}_{CO_2}$ measured in adult green iguanas [air temperature (T_a): 30–35°C] as RH increased from 55 to 65%. Green iguanas are large, thick-skinned animals with a lower surface-to-volume

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ratio than the smaller lizards studied by Nagy and Costa (21), and the test range of RH was also well below saturation (35).

As part of a study of the biology of the introduced brown treesnake (*Boiga irregularis*) on the island of Guam, we conducted a validation study to assess the accuracy of the DLW technique in this tropical snake. We asked whether the DLW method could accurately predict $\dot{V}CO_2$ and water flux in brown treesnakes, an arboreal ectotherm with a high surface-to-volume ratio that lives in an environment where the water vapor density is high. We found that in high humidity, DLW estimates of $\dot{V}CO_2$ were several times greater than those resulting from indirect calorimetry. Given that DLW estimates can overestimate metabolism of snakes in humid environments, we recommend caution in interpretation of results from studies on this group that do not have validated methods. In addition, we found that it is difficult to validate the DLW technique for estimating $\dot{V}CO_2$ in a tropical forest reptile under low-humidity conditions because large evaporative water losses result in physiological dehydration before the reptile produces enough CO_2 to result in measurable drops in the oxygen isotope due to metabolism. In this case, it is necessary to design the validation study to maintain either a stable TBW pool or constant $\dot{V}CO_2$.

MATERIALS AND METHODS

Terminology

In this paper, total water efflux (TWE) represents total water molecules leaving the body. TWE does not infer a net loss and is used to describe DLW estimates of the total number of water moles exchanged between the body and the environment. Under low-humidity conditions, TWE approaches equality with net TWE because no water is available in the environment, so exchange of water with the environment is unidirectional, a net loss. Net total evaporative water loss (TEWL) represents the net loss of water molecules from the body through evaporation and is used to describe indirect calorimetry measurements of net water vapor loss.

The research reported here complies with The Principles of Animal Care, publication no. 86-23 (revised 1985) of the National Institutes of Health and with current laws of the state of Ohio.

Biology of Brown Treesnakes

Colubrid snakes native to Papua New Guinea, the Solomon Islands, Indonesia, and coastal Australia, i.e., brown treesnakes (*B. irregularis*), were introduced to the island of Guam in the late 1940s (27). Here, they inhabit the moist tropical forests, are chiefly arboreal, and are nocturnally active (27). T_a and RH within the rainforest ranges from 23 to 32°C (mean 27°C) and 40 to 100% (mean 72%), respectively (1). Average rainfall ranges from 8 to 40 cm/mo (27).

Validation of DLW Method in Brown Treesnakes in Low and High Humidity

We used adult brown treesnakes, captured on Guam, to compare DLW estimates of water flux and $\dot{V}CO_2$ with values determined by indirect calorimetry and hygrometry techniques (11, 12, 15) under high-humidity conditions ($n = 8$; mean body mass = 130 ± 7 g) and under low-humidity conditions ($n = 6$; mean body mass = 134 ± 8 g). Snakes were housed at The Ohio State University for 3–6 mo before experiments (T_a: 24–29°C; 12:12-h light-dark cycle) and fed one mouse every 2–3 wk. Water was provided ad libitum. Snakes were fasted for 1 wk before each study. Because brown treesnakes on Guam feed approximately once every 4 days (1), we fed each snake one 3- to 5-g skink (*Carlia fusca*), their most common prey species, every 4 days of the experiment (27).

Before each trial, we weighed each snake (±0.1 g) and obtained three 15-μl blood samples (flame sealed in glass capillary tubes) via the tail vein or by cardiocentesis for measurement of background levels of D and ^{18}O. All isotope concentrations were measured by Henk Visser by using mass spectroscopy at The Centre for Isotope Research, Gronigen, The Netherlands. We administered 0.006 g of DLW/g snake intraperitoneally (2.5 ml of 99.9% D_2O in 25.0 ml of 10.0% $H_2{}^{18}O$). After injection, we placed each snake in a cage for 2 h to allow the DLW to equilibrate with the TBW pool and then drew blood to determine the initial concentration of isotopes.

After feeding the snakes, we placed each snake in a wire mesh cage that we lowered into a 1.25-liter metabolic chamber and then sealed the chamber with a metal lid. Below a wire platform, we placed mineral oil on the bottom of the chamber to collect feces and urine. Chamber T_a was maintained at 26°C, the approximate mean body temperature of free-ranging brown treesnakes on Guam (1), by circulating water through copper coils surrounding the chamber. Water temperature was controlled (±0.1°C) by a Neslab circulating water bath (Neslab, Portsmouth, NH).

Air from a cylinder flowed through columns of Drierite (W. A. Hammond Drierite, Xenia, Ohio), soda lime, and Drierite (dew point temperature: less than −37°C, RH of <0.1%) at a rate of 198.7 ml/min STPD for the high-humidity trials and 102 ml/min in the low-humidity trials. For the low-humidity trials, air directly entered the chamber, but for the high-humidity trial, the air stream was humidified to a dew point temperature of 24.9°C (RH = 93.7%) by a dew point generator (Li-cor, Lincoln, NE) before entrance to the metabolism chamber. Water vapor was trapped downstream of the metabolic chamber in a column of Drierite that we changed and weighed (±0.1 g) daily. We measured the parts/million (ppm) of CO_2 in air exiting the chamber with an infrared CO_2 analyzer (Li-cor) calibrated with a primary CO_2 standard (1,879.8 ppm). Exiting air also passed through Ascarite, as an additional quantification of CO_2 (Mallinckrodt and Baker, Phillipsburg, NJ), then through another tube of Drierite. Both columns were weighed at the beginning and end of each trial (±0.0001 g), and the differences in masses were added to give a gravimetric measure of CO_2. The linear regression coefficient between the analyzer estimates and gravimetric measurements of the mass of CO_2 was not significantly different from 1.0 ($r^2 = 99.9\%$, $P < 0.0001$). Voltages were averaged over 5-min intervals by a Campbell data logger using PC208W software (Campbell Scientific, Logan, UT). Data from the logger was downloaded into Excel (Microsoft, Redmond, WA) spreadsheets for conversion of voltage values to data parameters (APPENDIX A) and for further data analysis.

After 4 days, we removed each snake from the chamber, weighed and fed it, and took a blood sample (<0.25 ml) for determination of isotope concentrations; the entire process required <30 min. If snakes lost >2 g of body mass (1–2% body mass loss), we injected them intraperitoneally with enough 0.9% saline (±0.0005 g via Mettler balance) to balance mass loss. We scored excrement in the chamber as not present (0 g), scant (0.1–1.9 g), moderate (2.0–3.9 g), or large (4.0–5.9 g). Wet mass estimates of feces were based on masses of similar fecal volumes obtained from other captive brown treesnakes (1). Because of the lack of precision of the fecal mass estimates (±2 g) and the large volume of urine associated with feces, we assumed that fecal masses represented fecal water loss.

Each snake was returned to the metabolism chamber and remained there until *day 8*, when we took a final blood sample. We converted ppm CO_2 for each 5-min period into measurements of $\dot{V}CO_2$ by using standard equations (11, 12) and then summed these to calculate the total mass of CO_2 produced per 4-day period and for the entire 8-day period. Data are presented as $ml \cdot g^{-1} \cdot h^{-1}$ to allow comparison with data from other studies.

Data Analysis

Statistics. We used Minitab Release 13 (Minitab, State College, PA) to perform linear regression and basic statistics. Statistical significance was set at $P < 0.05$. For snakes that defecated during trials, DLW estimates for TWE − (water lost in feces) were compared with indirect calorimetry measurements of net TEWL (or estimates of net TEWL for the high-humidity trial). Mean error was calculated as (Estimated mean using DLW − control measurement mean)/control measurement mean. In addition to *t*-tests (paired when appropriate), we used linear regression (zero intercept) to compare DLW estimates of $\dot{V}CO_2$ and TWE to indirect calorimetry measurements and to determine whether equations correcting for fractionation improved DLW estimates.

Preliminary Studies and Results Performed to Ensure Appropriate Use of the DLW Technique

Slope of D-to-slope of ^{18}O ratio. The rate of decline of D and ^{18}O in the TBW pool of an animal injected with DLW is exponential (32). A logarithmic plot of this decline provides a slope that is called the fractional turnover rate (*k*). The slope of the D washout curve (k_d) is used to calculate water influx and the difference between k_d and ^{18}O (k_o) washout curves is used to calculate $\dot{V}CO_2$. When water turnover is high relative to $\dot{V}CO_2$, the ratio of the slope of the washout curves (k_o/k_d) approaches one, and the precision of the technique rapidly decreases (26). A k_o/k_d of >1.1 is necessary to ensure that errors inherent in measurement of isotope concentrations will not significantly influence results of DLW estimates (32). Because previous independent indirect calorimetry studies (1) found that brown treesnakes had high net TEWL rates compared with $\dot{V}CO_2$, we calculated k_o/k_d for all time periods (0–4, 4–8, and 0–8 days) and humidity trials. All ratios (1.15–1.3) were >1.1.

Determination of Percent TBW of Brown Treesnakes

To determine whether the percent of TBW (%TBW) varied with body mass, five brown treesnakes (78.35–159.00 g) were killed with <0.2 ml of pentobarbital sodium solution, weighed (OHaus scale model no. ts400s), cut open, dried at 65°C for 6 days, and reweighed. We found no relationship between body mass and %TBW in brown treesnakes (mean %TBW: 66.2 ± 1.05%; $P > 0.24$).

TBW has been predicted by using dilution space of D and ^{18}O (32). We compared dilution space of both isotopes with measured values, and we evaluated whether TBW changed between humidity trials. We found no difference in %TBW determined by drying between the high- and low-humidity trials ($P > 0.25$). For the low-humidity trial, mean %TBW determined by either ^{18}O (66.3%) or D (68.2%) did not differ significantly from 66.2% ($P > 0.11$). For the high-humidity trial, mean %TBW determined by either ^{18}O (67.7%) or D (69.4%) was >66.2% ($P < 0.047$). The difference was due to one snake (no. 2,719; 70.48% TBW) that was tested only in the high-humidity trial. Deuterium overestimated the %TBW determined by O^{18} by 2.77% in the low-humidity trial and by 2.60% in the high-humidity trial. We used the value of 66.2% to calculate TBW in this study.

DLW Technique

Low-humidity trial $\dot{V}CO_2$ and TWE and high-humidity trial $\dot{V}CO_2$. We calculated TWE and $\dot{V}CO_2$ by using equations that did not correct for a change in body mass, that corrected for a linear change in body mass, and that corrected for an exponential change in body mass (16, 21, 32). In addition, we used several equations to investigate the effects of correction for fractionation (APPENDIX A).

High-humidity trial TWE. A validation of DLW estimates of TWE under humid conditions was problematic by using standard indirect calorimetry techniques because changes in isotope concentrations reflected total exchange of water molecules between the animal and the environment, whereas indirect calorimetry measured the net difference in water produced by the snake over the amount of water added to the system. We assumed that, at any given T_a, the rate of evaporation of water through skin was a first-order reaction [i.e., a constant property of the skin and not influenced by the concentration of water molecules (i.e., humidity) at the skin surface (24, 36)]. Therefore, we predicted that the total number of water molecules lost through evaporation through the skin in a normally hydrated snake should remain the same between high- and low-humidity trials. Thus net TEWL measured during the first 24 h of the low-humidity trial (multiplied by 4 to equal the 4-day DLW estimate period) should be an approximation of TWE in the high-humidity trials. We compared DLW estimates of TWE corrected for fecal water loss to average net TEWL (0.1301 g H_2O/g snake per 4 days) and maximum net TEWL ($TEWL_{max}$; 0.2007 g H_2O/g snake per 4 days).

Comparison of TWE, TEWL, and $\dot{V}CO_2$ Between Humidity Trials and Time Periods

We compared DLW estimates of TWE, net TEWL measured gravimetrically, and $\dot{V}CO_2$ measured by the CO_2 analyzer for differences between the humidity trials (CO_2 only) and time periods (0–4 and 5–8 days).

RESULTS

Effects of Fractionation and Correction for Body Mass

Use of equations to correct for fractionation increased the mean error, decreased r^2 values, and increased the range of 95% confidence interval of regression coefficients of DLW estimates of TWE and $\dot{V}CO_2$ (33). We found that use of a linear correction for body mass change minimized the mean error and maximized r^2 values compared with other equations for both TWE and $\dot{V}CO_2$ (Table 1). Therefore, we used *Eq. 4* of Nagy and Costa (21) and *Eq. 2* of Nagy (16) to calculate TWE and $\dot{V}CO_2$, respectively.

Accuracy of DLW Estimates of TWE

Low humidity. For all time periods, the linear DLW model yielded a mean error of −3.7 to −1.0% and regression coefficients that were not significantly different than unity (Table 1). With the use of paired *t*-tests, we found no difference between gravimetric and DLW estimates of water efflux (Table 1) for any time period ($P > 0.14$). In dry air, DLW estimates of TWE − fecal water loss were equivalent to gravimetric estimates of net TEWL.

High humidity. During high-humidity trials, average net TEWL accounted for 63–95% and $TEWL_{max}$ accounted for 88–132% of DLW estimates of TWE − fecal losses. For all time periods, the linear DLW model − fecal water loss vs. $TEWL_{max}$ produced mean errors from −5.2–6.8% and regression coefficients that were not significantly different than unity (Table 1). With the use of paired *t*-tests, we found that linear model DLW estimates of TWE − fecal water loss (Table 1) were not significantly different from $TEWL_{max}$ for any time period ($P > 0.32$).

Accuracy of DLW Estimates of $\dot{V}_{CO_2}$

Low humidity. When compared with open-system respirometry measures of $\dot{V}_{CO_2}$, DLW estimates produced mean errors of 78–168% with standard deviations of >63% for all time periods. Regression coefficients ranged from 1.7 to 2.7 (mean 2.2), and r^2 values ranged from 67 to 94% (Table 1). Because the regression was forced through the origin, the regression coefficients indicated that the DLW technique overestimated the $\dot{V}_{CO_2}$ of brown treesnakes by 1.7–2.7 times.

High humidity. When compared with the CO_2 analyzer measurements, the DLW technique produced mean errors of 308–415% for all time periods. Regression coefficients ranged from 4.1 to 5.2 (mean 4.6). Values for r^2 were 98.2–99.6%, suggesting a tight correlation (Table 1). Overall, the DLW technique overestimated the $\dot{V}_{CO_2}$ of brown treesnakes by a factor of 4.6.

Comparison of TWE and $\dot{V}_{CO_2}$ Between Low- and High-Humidity Trials

TWE estimated by the DLW technique for high-humidity trials was greater than low-humidity trials ($P < 0.0001$) (Table 1). Mean $\dot{V}_{CO_2}$ measured with the CO_2 analyzer for high-humidity trials was greater than low-humidity trials for 0–4 days ($P < 0.0001$) (Table 1). There was no difference between the humidity trials for 5–8 days ($P > 0.10$) (Table 1).

Comparison of TWE, TEWL, and $\dot{V}_{CO_2}$ Between Days 0–4 and 5–8

TWEs estimated by the DLW technique were not different between 0–4 and 5–8 days for either humidity trial ($P > 0.138$) (Table 1). For the low-humidity trial, net TEWL measured gravimetrically showed that snakes lost 7% more water during 5–8 days than during 0–4 days ($P = 0.02$) (Table 1). The rate of $\dot{V}_{CO_2}$ measured by the CO_2 analyzer was not different between 0–4 and 5–8 days for the high-humidity trial

Table 1. *Comparison of CO_2 production and TEWL*

Humidity Trial	Control	*n*	Mean BM, g	TEWL, mg/gh	DLW-fecal. water, mg/gh	r^2	*b*	95% CI	*P* Value	Mean Error	SD
					Water turnover						
Low, 0–4 days	Drierite	6	134.2	0.9445	0.9224	99	0.98	0.87–1.09	0.61	−2.2	11
Low, 5–8 days	Drierite	6	133.2	1.0231	0.9850	99.8	0.97	0.91–1.02	0.14	−3.7	5.4
Low, 0–8 days	Drierite	6	133.7	0.9837	0.9721	99	1.01	0.97–1.01	0.75	−1	4.7
High, 0–4 days	$TEWL_{max}$	8	129.8	2.0906	2.0538	98	0.98	.87–1.11	0.73	−1.8	16.3
High, 5–8 days	$TEWL_{max}$	8	129.4	2.0906	2.2320	97.5	1.07	0.92–1.22	0.33	6.8	20.8
High, 0–8 days	$TEWL_{max}$	8	129.6	2.0906	1.9827	98	0.95	0.83–1.07	0.32	−5.2	15.4
High, 0–4 days	$TEWL_{avg}$	8	129.8	1.3552	2.0538	98	1.51	1.32–1.71	<0.0001	51.5	25.2
High, 5–8 days	$TEWL_{avg}$	8	129.4	1.3552	2.2320	97.5	1.65	1.41–1.88	<0.0001	64.7	32
High, 0–8 days	$TEWL_{avg}$	8	129.6	1.3552	1.9827	98	1.46	1.28–1.64	<0.0001	46.3	23.7

Humidity Trial	*n*	Mean BM, g	Li-cor, ml/gh	DLW, ml/gh	r^2	*b*	95% CI	*P* Value	Mean Error	SD
				CO_2 production						
Low, 0–4 days	5	133.6	0.0379	0.1025	87.9	2.71	1.31–4.11	0.03	168.2	109.4
Low, 5–8 days	5	132.8	0.0440	0.0746	67	1.7	0.07–3.30	0.27	78.1	133.4
Low, 0–8 days	5	133.2	0.0409	0.0887	93.7	2.17	1.39–2.91	0.01	120.2	62.6
High, 0–4 days	7	130.1	0.0491	0.2000	98.2	4.09	3.53–4.64	<0.0001	308	61.6
High, 5–8 days	7	129.1	0.0491	0.2521	97.6	5.15	4.44–5.89	<0.0001	415.3	100.3
High, 0–8 days	7	129.7	0.0491	0.2261	99.6	4.59	4.07–4.89	<0.0001	361.7	36.5

Comparison of CO_2 production and total evaporative water loss (TEWL) by brown treesnakes (*Boiga irregularis*) measured by indirect calorimetry at 26°C under low- and high-humidity conditions to CO_2 production and TEWL estimated by the doubly labeled water (DLW) technique. TEWL for DLW estimates = total water efflux − fecal water loss. Indirect calorimetry values for CO_2 production were measured with an infrared CO_2 analyzer. During the low-humidity trial, indirect calorimetry measurements of TEWL were performed gravimetrically. During the high-humidity trial, DLW estimates of TEWL were compared with maximum ($TEWL_{max}$) and average TEWL ($TEWL_{avg}$) measured in low-humidity air for hydrated brown treesnakes. Comparison of indirect calorimetry and DLW estimates was performed by using linear regression [r^2 values, regression coefficients (*b*), 95% confidence intervals (CI)], mean error, and paired *t*-tests *(P* value). BM, body mass, *n,* no. of animals.

($P = 0.88$) (Table 1). For the low-humidity trial, $\dot{V}_{CO_2}$ was 13% higher during 5–8 days than during 0–4 days ($P = 0.03$) (Table 1).

DISCUSSION

Indirect Calorimetry Measurements Recorded for Other Colubrid Snakes

To evaluate the validity of the errors in the DLW method reported in this study for brown treesnakes, we first established that our indirect calorimetry measurements were reasonable for this species. Mean $\dot{V}_{CO_2}$ measured via indirect calorimetry measurements during the first 4 days of this study was 0.0379 ml $CO_2 \cdot g^{-1} \cdot h^{-1}$, or 0.0519 ml $O_2 \cdot g^{-1} \cdot h^{-1}$ if we assume a respiratory quotient of 0.73 (11). Our value of oxygen consumption ($\dot{V}_{O_2}$) is within the range reported for standard metabolic rate (SMR) in other colubrid snakes of similar body mass at 25°C [*Thamnophis elegans vagrans*, 107.5 g, 0.03 ml $O_2 \cdot g^{-1} \cdot h^{-1}$ (34); *Elaphe guttata*, 144 g, 0.054 ml $O_2 \cdot g^{-1} \cdot h^{-1}$ (31); *Natrix rhombifera*, 237.5 g, 0.084 ml $O_2 \cdot g^{-1} \cdot h^{-1}$ (13)]. This concordance suggests that our measurements of metabolic rate were reasonable.

However, because snakes were fed during the study, we also considered the effects of feeding on metabolism, called the specific dynamic action (SDA). Secor showed that increase in $\dot{V}_{O_2}$ as a result of SDA was positively correlated with meal size and ranged from 1.4 to 5 times SMR in pythons fed 0% (allowed to catch and constrict a prey item, but not swallow it) to 5% of their body mass (28). Increases in $\dot{V}_{O_2}$ due to SDA are 2.4 times higher in infrequently feeding species such as pythons when compared with frequently feeding species such as colubrids (29). Because brown treesnakes are frequent feeders, and because we fed meals 2–3% of body mass, we expected peak $\dot{V}_{O_2}$ associated with digestion to be increased by a maximum factor of 2 (5/2.4). $\dot{V}_{CO_2}$ values that we recorded every 5 min showed that peak $\dot{V}_{CO_2}$ associated with digestion occurred 24 h after feeding and returned to fasting levels after 2.6 days (1). From this information, we calculated mean SMR to be 0.037 ml $O_2 \cdot g^{-1} \cdot h^{-1}$ and peak $\dot{V}_{O_2}$ associated with digestion to be 0.067 ml $O_2 \cdot g^{-1} \cdot h^{-1}$ (1), the latter representing an increase by a factor of 1.8. These calculations indicate that the large discrepancies found in this study between DLW estimates and indirect calorimetry measurements of $\dot{V}_{CO_2}$ were not the result of failure of the indirect calorimetry technique to measure increases in metabolism associated with SDA.

Snakes living in dry environments have lower rates of TEWL than those living in moist environments (10). Hence, comparisons with other snakes need to be habitat specific. The range for indirect calorimetry measurements for mean TEWL in brown treesnakes for the low-humidity trial was 0.94–1.02 mg $H_2O \cdot g^{-1} \cdot h^{-1}$. These values are within the range reported for other colubrid snakes living in mesic habitats, having similar body mass, measured between 25 and 30°C, and tested in dry air [*Pituophis melanoleucus catenifer*, 46.6 g, 0.80 mg $H_2O \cdot g^{-1} \cdot h^{-1}$ (10); *Elaphe climacophora*, 70.0 g, 0.92 mg $H_2O \cdot g^{-1} \cdot h^{-1}$ (10); *Lampropeltis doliata triangulum*, 119.8 g, 1.07 mg $H_2O \cdot g^{-1} \cdot h^{-1}$ (10); *Lampropeltis getulus*, 81.7 g, 2.10 mg $H_2O \cdot g^{-1} \cdot h^{-1}$ (8)].

Accuracy of the DLW Technique in Measuring TWE

Under low-humidity conditions, the linear model for DLW accurately predicted net water efflux in brown treesnakes (regression coefficient = 1; mean error < 4%). This accuracy is similar to findings in birds, mammals, and other reptiles (19, 20, 32). Although TWE in brown treesnakes was mostly through evaporative losses, suggesting that fractionation effects should be significant, traditional methods to correct for fractionation decreased accuracy. Under high-humidity conditions, DLW estimates of TWE in brown tree snakes were equivalent to maximum net TEWL values measured in normally hydrated individuals tested under low-humidity conditions.

Accuracy of the DLW technique in measuring $\dot{V}_{CO_2}$

In the low-humidity trial, the DLW method overestimated $\dot{V}_{O_2}$ as measured by indirect calorimetry by ~200%. The error was not a result of fractionation effects because fractionation can account for a maximum error of ~40% and the resulting error is to underestimate isotope turnover. The error was the result of violation of the assumption that the TBW pool and $\dot{V}_{CO_2}$ remained constant (16) and illustrates the difficulties inherent in validating DLW estimates of $\dot{V}_{CO_2}$ in animals with low metabolic rates and high water losses. Because only a small proportion of the total decline of the oxygen isotope is due to $\dot{V}_{CO_2}$, it is problematic to obtain a measurable drop in the oxygen isotope attributable to metabolism before an animal becomes dehydrated. Our solution was to replace water losses with intraperitoneal saline administered when snakes were fed. Although this technique allowed us to minimize handling effects and accurately measure TWE, fluid losses were only replaced every 4 days, and the TBW pool changed by 12%. This change in TBW combined with variations in $\dot{V}_{CO_2}$ associated with handling and SDA contributed to the observed error of 200% (16).

Solutions to the dilemma of balancing high rates of water loss with low metabolic rate include increasing metabolic rate, decreasing water loss, or allowing more frequent access to water to maintain a stable TBW pool. Larger meals would have resulted in a higher metabolic rate and a larger water influx. Increasing T_a would have increased metabolic rate but would also have increased evaporative water losses. Applying a water-impermeable coating to the skin (such as mineral oil or petroleum jelly) would have decreased evaporative water losses. More frequent handling of the snake to inject or administer fluids would have resulted in more variation in $\dot{V}_{CO_2}$ but would have minimized changes in the TBW pool.

In the humid trial, body mass of snakes changed by <1.5%. The linear model for DLW overestimated $\dot{V}CO_2$ by a mean factor of 4.6 (range 4.1–5.2). None of the equations traditionally used to calculate fractionation effects accounted for this difference. One hypothesis for this overestimate was continuous diffusion of ^{18}O molecules from labeled CO_2 molecules to water molecules because the concentration of ^{18}O molecules was decreasing disproportionately with the rapid turnover of the TBW pool (Nagy, personal communication). A correction factor of 4–5 is likely inappropriate for DLW work conducted on other reptiles in high-humidity conditions because errors are likely to be dependent on the rate of evaporative water turnover compared with metabolic rate and, therefore, influenced by humidity, physiological status of the organism, wind speed, physical characteristics of the skin, and other factors. However, the error reported in this study does emphasize the need to validate the DLW technique when used in humid conditions.

In the past, researchers using DLW have found that field metabolic rates (FMR) of reptiles are higher when animals are tested in high-humidity environmental conditions than when they are tested in low-humidity conditions (3–7). In Christian et al.'s (5) study on marbled geckoes (*Oedura marmorata*), SMR from populations living in high-humidity vs. arid habitats were similar, whereas FMR measured in the field with DLW was uniformly higher for the geckoes living in the moist conditions. Differences in body temperature did not account for the difference in FMR. Most researchers have assumed that FMR is higher in wet conditions because animals increase their food consumption/activity levels because of greater food availability. Whereas this assumption may be warranted, our study indicates that FMR values may be inflated when large amounts of water vapor are absorbed through the skin or respiratory passages. With the use of DLW, Peterson et al. (23) found that garter snakes (*Thamnophis sirtalis*) living in a semiaquatic environment had a FMR that was ~2.5 times greater than other colubrid species, such as northern racer (*Coluber constrictor*) (25) and coachwhip snake (*Masticophis flagellum*) (30), which live in mesic and arid environments, respectively. Peterson et al.'s study (23) on garter snakes ruled out dilutional amplification of FMR by showing that feeding rates calculated from water influx rates were equivalent to feeding rates calculated from metabolic and growth rates. Peterson et al. (23) attributed the high FMR to high feeding rates (continuous addition of energy expended on digestion, SDA) resulting from a limited foraging season.

Comparison of TWE, TEWL, and $\dot{V}CO_2$ Between Humidity Trials and Time Periods

Snakes in high-humidity trials turned over approximately twice as many water molecules than snakes in low-humidity trials. This finding may seem counterintuitive at first, since snakes lost more body mass in low humidity trials. However, although the loss of body mass in the low-humidity trial was a good indication of net water turnover (i.e., true water loss) because there was no moisture available to replace evaporated water molecules, this was not true for the high-humidity trial. In the high-humidity trial, the DLW technique measured the total turnover of water molecules rather than the net turnover. In the high-humidity trial, snakes lost more water but were able to replace losses by absorption of water vapor through their mucous membranes and/or skin, so there was no concurrent body mass loss or net loss of water. Caution should be used when the DLW technique is used to estimate net water requirements for animals in humid environments because it is likely to result in overestimates.

The rate of CO_2 production in brown treesnakes was greater during high-humidity trials than low-humidity trials, likely because snakes were less active in the latter. A reduction of activity would decrease water loss through the respiratory passages and, probably more importantly, allow snakes to position their coils to minimize exposed surface area and thereby reduce transcutaneous water loss. The lack of fecal production during the low-humidity trial (1 scant defecation in twelve 4-day trials) compared with the high-humidity trial (11 defecations in sixteen 4-day trials) is evidence that snakes in the low-humidity trials were using strategies to conserve water.

Suitability of the DLW Technique for Humid Environments

Although the DLW method has been widely used, its reliability is in doubt when used on snakes in humid environments. This study shows that without a validation study, errors of up to 500% are possible when the DLW technique is used on snakes living in moist, tropical environments. It is reasonable to assume that errors of this magnitude are likely in other small ectotherms living in humid environments.

APPENDIX

Conversion of Voltages Recorded by the Campbell Data Logger to T_a and CO_2 Concentration

Voltages from the thermocouples were converted by the Campbell data logger to temperatures within 0.1°C (Campbell Scientific User manual).

The voltages from the Li-cor CO_2 analyzer were converted into CO_2 concentration ([CO_2]; in ppm) in the air stream by the following equation (Li-cor User's Manual)

$$[CO_2] = 30V$$

Parts per million CO_2 derived from voltages were compared with readings against a known standard and found to be precise to within 10 ppm.

Percent TBW

After a blood draw to determine isotope levels, snakes that lost >2 g of body mass after the first 4-day trial received injections of saline. Because it was rapidly absorbed, this saline diluted the concentration of isotope from the preinjection concentration. To account for this, we calculated the starting TBW pool for 4–8 days as %body water (original body mass at 4 days + mass NaCl + mass skink fed) entered into equations for DLW calculations. This equation modeled the expected concentration of isotope once the percentage of TBW had normalized back to 66.2% (i.e., after dehydration was corrected by absorption of water in the saline).

Calculations

D and ^{18}O concentrations were reported in delta SMOW units. In general, three values for background, initial, *day 4*, and *day 8* concentrations were reported for each snake. We determined the mean delta SMOW [difference in isotope concentration from standard mean ocean water (32)] for each time period and each snake. Mean delta SMOW units were converted to absolute ratio for deuterium by using *Eq. 14.4* of Speakman (32) {$\text{Ratio}_{\text{sample}} = [(\text{delta sample}\ _{\text{SMOW}}/1000) + 1]\cdot 0.00015595$} and *Eq. 14.9* of Speakman (32) {$\text{Ratio}_{\text{sample}} = [(\text{delta sample}\ ^{18}{}_{\text{SMOW}}/1000) + 1]\cdot 0.0020052$}. Absolute ratios were converted to ppm by using *Eq. 14.9* {$[\text{Ratio}_{\text{sample}}/(\text{Ratio}_{\text{sample}} + 0.000373 + 1)]\cdot 1000000$} for ^{18}O and *Eq. 14.8* from Speakman {$[\text{Ratio}_{\text{sample}}/(\text{Ratio}_{\text{sample}} + 1)]/0.000001$} for D (32). The background concentration of isotopes was subtracted from initial, *day 4*, and *day 8* values to give concentrations in ppm over background.

Nagy and Costas' (21) equations for constant body water (*Eq. 3*), linear (*Eq. 4*), and exponential (*Eq. 5*) changes in body water volume were used to calculate g H_2O efflux$\cdot g^{-1}\cdot h^{-1}$

$$\text{Constant ml } H_2O\cdot g^{-1}\cdot h^{-1} = [W\cdot LN(H_1^2/H_2^2)]/(M\cdot t\cdot 24)$$

$$\text{Linear ml } H_2O\cdot g^{-1}\cdot h^{-1} = \{2\cdot(W_2 - W_1)\cdot LN[(H_2^2\cdot W_1)/(H_2^2\cdot W_2)]\}/[(M_1 + M_2)\cdot LN(W_2/W_1)\cdot t\cdot 24]$$

$$\text{Exponential ml } H_2O\cdot g^{-1}\cdot h^{-1} = \{2\cdot W_1\cdot LN(W_2/W_1)\cdot LN[(H_1^2\cdot W_1)/(H_2^2\cdot W_2)]\}/\{(M_1 + M_2)\cdot[1 - (W_1/W_2)]\cdot t\cdot 24\}$$

where W is mean body water, H_2^1 is the initial concentration of D, H_2^2 is the final concentration of D, M is the body mass, LN is natural logarithm, and t is the length of the trial.

Nagy's (16) equations for constant body water (*Eq. 1*), linear (*Eq. 2*), and exponential (*Eq. 3*) changes in body water volume were used to calculate milliliters of CO_2 efflux per gram per hour

$$\text{Constant ml } CO_2\cdot g^{-1}\cdot h^{-1} = [25.93\cdot W\cdot LN(O_1^{18}\cdot H_2^2/O_2^{18}\cdot H_1^2)]/(M\cdot t)$$

$$\text{Linear ml } CO_2\cdot g^{-1}\cdot h^{-1} = \{51.86\cdot(W_2 - W_1)\cdot LN[(O_1^{18}\cdot H_2^2)/(O_2^{18}\cdot H_1^2]\}/[(M_1 + M_2)\cdot LN(W_2/W_1)\cdot t]\}$$

$$\text{Exponential ml } CO_2\cdot g^{-1}\cdot h^{-1} = \{51.86\cdot(W_2 - W_1)\cdot LN(W_2/W_1)\cdot LN[(O_1^{18}\cdot H_2^2)/(O_2^{18}\cdot H_1^2)]\}/\{(M_1 + M_2)\cdot[1 - (W_2/W_1)]\cdot t\}$$

where M_1 is initial wet body mass, M_2 is final wet body mass, W_1 is initial weight body water, and W_2 is final weight body water.

For TEWL, each of the values for the constant, linear, exponential models was used in Coward and Cole's equation [from Speakman (32)] for fractionation. This equation allows the investigator to enter fractionation effects ranging from 0 to 100%. The investigator can also vary the value of the fractionation factor (F_1) between the lowest and highest values reported. In the Excel spread sheet, we calculated fractionation effects every 10% from 0 to 100% at the F_1 of 0.941 (assuming 100% equilibrium fractionation), 0.917 (the lowest recorded value assuming 100% kinetic fractionation), and 0.93225 (assuming 50% equilibrium and 50% kinetic fractionation). The maximum TEWL and the minimum TWE from this matrix were used to examine the greatest correction possible due to fractionation

$$rH_2O = k_d\cdot N/(xF_1 + 1 - x)$$

where x is fractionated proportion of water, N is moles of body water and F_1 is of D/H_2O vapor over water.

For CO_2 production, of the three analyses, the linear equation had the smallest mean error when compared with indirect calorimetry data. To limit the number of permutations to a manageable level, the linear model was used as a basis to investigate the effects that correction for fractionation would have on relative error. The values for $\dot{V}CO_2$ and TEWL from the linear models were entered into Lifson and McClintocks' (14) *Eq. 35* (assumes 50% H_2O loss is fractionated, 100% equilibrium losses, average body temperature of 25°C)

$$rCO_2 = N/2.08\cdot(k_o - k_d) - 0.015k_dN$$

Speakman's (32) *Eq. 7.17* (assumes 25% fractionated water losses, 3:1 equilibrium-to-kinetic ratio, body temperature of 37°C) shows

$$rCO_2 = N/2.078\cdot(k_o - k_d) - 0.0062k_dN$$

and Coward and Cole from Speakman (32) show

$$rCO_2 = k_o\cdot N/2F_3 - k_d\cdot N/2F_3 - rH_2O\cdot x\cdot(F_2 - F_1)/2F_3$$

where F_2 is fractionation factor of $^{18}O\ H_2O$ vapor over water, F_3 is fractionation factor of $^{18}O\ CO_2$ over water, and x is fractionated proportion of water.

For Coward and Coles' equation (32), we simulated the fractionated proportion of water to vary in 10% increments from 0 to 100%. There is only one value for F_3 (1.039). The possible values for $(F_2 - F_1)/2F_3$ can only vary from 0.02478 to 0.02526. Because the magnitude of change of this coefficient is small and its variable is subtracted from a much larger number, use of the minimum vs. maximum value has minimal effects on the estimated $\dot{V}CO_2$. We chose to use a value of 0.0249 that is the most commonly used value that assumes a 3:1 contribution of equilibrium to kinetic fractionation. The maximum TWE and the minimum TWE from this matrix were used to examine the greatest correction possible due to fractionation.

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