The Journal of Experimental Biology 212, 2635-2644
Published by The Company of Biologists 2009
doi:10.1242/jeb.029330

Validation of the use of doubly labeled water for estimating metabolic rate in the green turtle (*Chelonia mydas* L.): a word of caution

T. Todd Jones[1,*], Mervin D. Hastings[1,2], Brian L. Bostrom[1], Russel D. Andrews[3] and David R. Jones[1]

[1]Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, BC, V6T 1Z4 Canada, [2]Conservation and Fisheries Department, Ministry of Natural Resources and Labour, Government of the British Virgin Islands, Road Town, Tortola, British Virgin Islands and [3]School of Fisheries and Ocean Sciences, University of Alaska Fairbanks and the Alaska SeaLife Center, Seward, AK 99664, USA

*Author for correspondence (e-mail: tjones@zoology.ubc.ca)

Accepted 21 May 2009

SUMMARY

Marine turtles often have extremely high water turnover accompanied by a low field metabolic rate (FMR), a combination that can contraindicate the use of doubly labelled water (DLW). Therefore, we conducted a validation study to assess the suitability of the DLW technique for determining FMR of marine turtles. Six green turtles (22.42±3.13 kg) were injected with DLW and placed in a tank of seawater with a respirometer for continuous monitoring of oxygen consumption (MR) over a 5-day period. Trials were conducted for turtles in both fed and fasted states. Respiratory exchange ratio (RER) was determined in a dry respirometer and used to calculate energy expenditure. For fed and fasted turtles, total body water (TBW) was 66.67±3.37% and 58.70±7.63% of body mass, and water flux rates were 9.57±1.33% and 6.14±0.65% TBW day^{-1}, respectively. Water turnover in fasted turtles was 36% lower than that of fed turtles but MR (from oxygen consumption) of fasted turtles (13.77±1.49 kJ kg^{-1} day^{-1}) was 52% lower than in fed turtles (28.66±5.31 kJ kg^{-1} day^{-1}). Deuterium to oxygen-18 turnover rate (k_d:k_o) ratios averaged 0.91±0.02 for fed turtles and 1.07±0.16 for fasted turtles. Fed turtles had a mean group difference of 8% and a mean individual difference of 53% between DLW and respirometry. The DLW method gave negative MR values in fasted turtles and could not be compared with respirometry data. Researchers should use caution when applying the DLW method in marine reptiles, especially when high water flux causes >90% of the labeled oxygen turnover to be due to water exchange.

Key words: doubly labeled water, validation, deuterium, fasting, marine turtle, metabolic rate, oxygen consumption, oxygen-18, respirometry, RER, total body water, water turnover.

INTRODUCTION

Daily energetic expenditure and time–energy budgets are useful for gaining insight into an animal's daily food requirements and allocation of energy to various activities (i.e. growth, reproduction, foraging, movement). Construction of time–energy budgets requires detailed observations of behavior in the wild and replication of observed activities in the laboratory while simultaneously measuring energy expenditure through indirect calorimetry for metabolic rate (MR) determinations (Speakman, 1997). Time–energy budgets have been determined for loggerhead and leatherback turtles (Kraemer and Bennett, 1981; Lutcavage and Lutz, 1986; Davenport, 1998; Jones et al., 2007), but the logistic difficulties of using this approach with marine turtles (i.e. simulating diving, swimming and feeding on natural foods) have led researchers to investigate other options. The use of doubly labeled water (DLW) to study the energetics of free-ranging animals (Lifson and McClintock, 1966) has become increasingly popular in the field of physiological ecology (Speakman, 1997; Nagy et al., 1999), and field metabolic rates (FMR) of a wide variety of taxa, including marine turtles, have been determined using this technique (Nagy et al., 1999; Wallace et al., 2005; Southwood et al., 2006; Trullas et al., 2006).

The DLW method requires capturing and dosing a study animal with water that has been enriched with isotopes of hydrogen (^{2}H, deuterium, or ^{3}H, tritium) and oxygen (^{18}O). A blood sample is taken before injection of DLW to determine background enrichment of the isotopes naturally occurring in the animal. Alternatively, for small animals, where taking multiple blood samples can be problematic, the background enrichment can be (i) assumed to be equal to the international natural abundance standard, (ii) assumed to be equal to the enrichment of drinking water in the habitat or (iii) measured (blood sample) from conspecific animals in the habitat (Speakman and Racey, 1987; Speakman, 1997). A second blood sample is taken when the injected isotopes reach equilibration with the animal's body water, thus giving the isotope dilution space for oxygen (N_o) and hydrogen (N_d). The isotope dilution space for oxygen or hydrogen can then be used to infer total body water (TBW). The animal is then released and recaptured for a final blood sample. During the period when the animal is at large, water added to the animal's TBW (i.e. water influx) due to drinking and metabolic water production is unlabeled, while the labeled isotopes that have equilibrated with the animal's TBW, ^{2}H and ^{18}O, are lost through water efflux due to urination, defecation, evaporation and tear production (salt gland secretion). ^{18}O is also lost as CO_2 due to cellular respiration, and the difference in slopes of ^{2}H and ^{18}O washout, over time, yields a value for CO_2 production. If the animal's respiratory quotient (RQ) or measured respiratory exchange ratio (RER) is known, the estimate of CO_2 production provided by the DLW method can be used to calculate MR. Many reptiles, however, excrete respiratory derived CO_2 as bicarbonate from the cloaca (Coulson and Hernandez, 1964). This is reflected in the DLW washout but not detected in RER measurements;

therefore causing errors in the estimated RER used to convert CO_2 production to energy expenditure. Additionally, the washout slope of ^2H multiplied by TBW gives an estimate of daily water flux. The procedures and assumptions of the DLW method are described in detail by Nagy (Nagy, 1989) and Speakman (Speakman, 1997).

The use of DLW does not give meaningful results in some animals, and validation of the technique is recommended for studies involving species that have potentially unique physiologies or habitats (Nagy, 1980; Speakman, 1997; Nagy et al., 1999). The effectiveness of the DLW method for use with a given species can be determined by simultaneously measuring metabolic rate using DLW and respirometry or calorimetry. The DLW method has been shown to work in some reptiles (Nagy, 1983a; Nagy, 1983b; Nagy et al., 1999; Anderson et al., 2003) but these studies were performed on terrestrial reptiles that could be considered water conservers. Field metabolic rates for marine iguanas have been determined with DLW (Nagy and Shoemaker, 1984; Drent et al., 1999); however, the iguanas spent substantial time on land and had moderate water turnover rates. The problem associated with using DLW in aquatic animals is that high water turnover rates and subsequent rapid washout of isotopes reduces the difference between the ^{18}O and ^2H washout curves to less than the variability in mass spectrometry measurements. In animals with large water turnover rates it is possible that >90 % of ^{18}O is washed out as water flux with ^2H. When >90% of labeled oxygen turnover is due to water exchange, the difference in isotope washouts does not give an accurate measurement of CO_2 production (Speakman, 1997). Despite the risk that the DLW may give inaccurate results for aquatic animals, DLW has been used in metabolic determinations of marine turtles without validation (Wallace et al., 2005; Southwood et al., 2006; Trullas et al., 2006).

Use of DLW in marine mammal studies has been similarly controversial as DLW-derived FMRs have been estimated at 5–7 times higher than when measured by time–energy budgets (Costa et al., 1989; Reilly and Fedak, 1991; Arnould et al., 1996). The FMRs from DLW studies are close to physiologically attainable maximums, leading to questions about the validity of the method. As the use of the DLW method is expanding in marine organisms, there is a likewise increase in the necessity of validation studies. A recent study by Sparling et al. (Sparling et al., 2008) validated the DLW method against open-flow respirometry in grey seals, and the data indicated that the % difference between average MRs for the two methods (i.e. DLW and respirometry) were <1% but the error of the methods within individual seals can range by ±40%.

We conducted a study to determine the validity of using the DLW method for estimating metabolic rate of green turtles (*Chelonia mydas* L.). Turtles were injected with DLW, and washout of isotopes was monitored by taking daily blood samples. We simultaneously recorded oxygen consumption using open-flow respirometry, and MRs calculated using DLW and respirometry were compared. Although DLW has been used previously in marine turtles, this study represents the first validation of its use in these turtles.

MATERIALS AND METHODS

Animals

Eight green turtles were imported from the Cayman Turtle Farm (1983; Grand Cayman, British West Indies; CITES Export Permit 2002/ky/000112) to the Zoology Animal Care Center, Department of Zoology, University of British Columbia (CITES Import Permit CA02CWIM0129). Turtles were maintained and research was conducted under Animal Care Protocol A03-0255 from the UBC Animal Care Committee.

Turtles were kept in a large oval fiberglass tank (10 m×3 m×1.5 m) filled with seawater (holding tank) except when they were kept in isolation (isolation tanks) for an experiment. Water quality for the pool was maintained by two filter systems: (1) a biological/mechanical filter (built by UBC – Zoology Workshop staff) containing a protein skimmer, bio-balls™ and fiberglass mat and (2) two sand filtration systems (TRITON® II TR 100; Pentair Pool Products™, Sanford, NC, USA) designed for large pools. The water temperature was maintained at 24±1°C. Fluorescent light fixtures (40 W UVA/B; Repti-Glow® 8) suspended above each tank provided full-spectrum radiation for 12 h each day; the tanks were also exposed to ambient light. Water quality was maintained between the following levels: pH=8.0–8.3, salinity=33–35 and ammonia <0.1 mg^{-1}. Monthly water changes prevented accumulation of high levels of ammonia, bacteria and fungi.

Turtles were fed a diet of Purina Trout Chow® 5D-VO5 (Purina Mills, LLC, St Louis, MO, USA) mixed with an aqueous solution of flavorless gelatin, Reptavite® and Reptamin® (vitamin and mineral supplements). Dried homogenized samples of the food were analyzed by bomb calorimetry (Parr Instrument, Moline, IL, USA) at the Southwest Fisheries Science Center of the National Oceanic and Atmospheric Administration (NOAA, La Jolla, CA, USA). The diet contained 41% protein, 12% lipids, 4% fiber and had ~17,000 kJ kg^{-1} dry mass (DM). The turtles were fed 1–2% of body mass every other day. Food quantities were based on presumed daily calorific intake of wild green turtles (Bjorndal, 1996).

Experimental design

A turtle was removed from the holding tank and a background blood sample was drawn from the cervical venous sinus. A measured dose of DLW (oxygen-18, ^{18}O; deuterium, ^2H) was injected into the turtle's coelomic cavity (intra-coelomic; IC) (see below). The equilibration time curve was followed over 10 h after DLW injection (Fig. 1). Blood was drawn every hour up to 5 h to determine time to isotopic equilibration with body water. A final blood sample was drawn at 10 h (Fig. 1) and the turtle was placed in an isolation tank. At 24 h, another blood sample was drawn and this sample served as the initial isotope level for the start of the validation experiment. The turtle was then placed inside another isolation tank equipped with a respirometer. Blood samples were drawn once a day for 5 days while the turtle's oxygen consumption rate was measured continuously. Turtles were fed during this period using the normal feeding regime. To prevent possible isotope re-entry from drinking, the tank was flushed with fresh seawater every other day. The turtle was then fasted for 10 days. On day 15 (5 days of fed trial and 10 days of fasting), a blood sample was drawn, the turtle was injected with a ^2H boost (IC), and a blood sample was drawn after 5 h (to determine N_d and initial isotope levels for the fasting trial). A blood sample was drawn on each day of the fasting trial (days 16–20) while the turtle's oxygen consumption rate was continuously recorded. As in the feeding trial, complete tank flushes were performed every other day to prevent isotope re-entry. After the last blood sample, the turtle was given a second ^2H boost (IC), and 5 h later a blood sample was taken (to determine N_d) (Fig. 2). The normal feeding regime was then resumed. Blood samples were drawn for 5 days after the end of the fasting trial to determine water turnover rates post-fasting. The time course of a complete experiment is shown in Figs 1 and 2.

There is a possibility that labeled isotopes could be stored in the body through anabolic pathways during the feeding trial (when the animal is in energetic equilibrium) and then released during the fasting trial (J. Speakman, personal communication), causing error

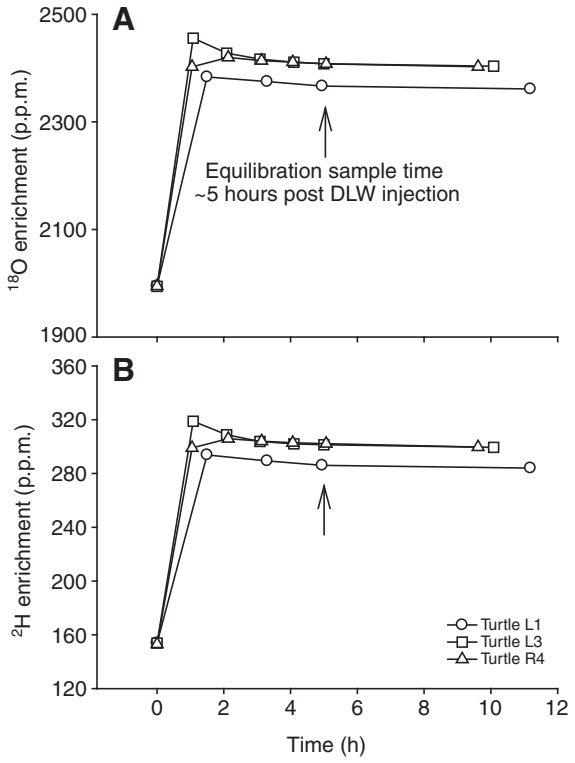

Fig. 1. Isotopic enrichment values of the equilibration time course for (A) oxygen-18 (^{18}O) and (B) deuterium (^{2}H) of three green turtles (*Chelonia mydas*). These enrichments represent the background enrichment levels (day 0) and the enrichment levels post intra-coelomic injection and during the plateau period.

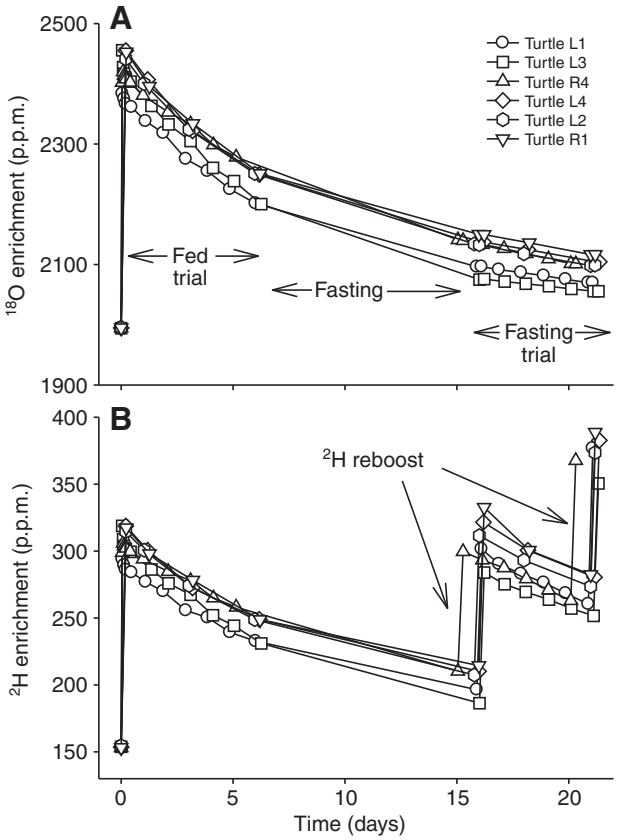

Fig. 2. Isotopic enrichment values for (A) oxygen-18 (^{18}O) and (B) deuterium (^{2}H) during the course of the doubly labeled water (DLW) validation experiment for six green turtles (*Chelonia mydas*). Background enrichment levels given at day 0, equilibration, fed DLW trials (days 1–6), fasting period (days 6–16), deuterium reboost (day 16), fasting DLW trials (days 16–21) and final deuterium reboost (day 21).

in the fasting DLW measurements. If ^{18}O were released, this would cause enrichment of isotope in the turtle and thus mask loss of ^{18}O through respiration. Therefore, we performed a separate fasting trial with two turtles. The turtles were fasted for 10 days before a blood sample was taken to determine background isotopic levels. A measured dose of DLW was injected and an equilibration sample was taken 5 h later. At 24 h, a blood sample was drawn to determine the initial isotope level for the start of the fasting trial. Blood samples were drawn for the next 3 days while the turtle's oxygen consumption was measured. On the third day of the trial, a final blood sample was taken and then the turtle was given a ^{2}H boost; 5 h later another blood sample was drawn to obtain the equilibration sample for the ^{2}H boost.

Respirometry

The isolation tank (1.5 m×1 m×1.5 m), filled with seawater, was covered by an acrylic respirometry dome to trap expired gases. The salinity and temperature of the seawater were 34.7±0.4 and 25.8±0.7°C for fed turtles and 34.9±0.4 and 25.1±1.0°C for fasted turtles, respectively. Turtles were able to move freely inside the tank. Turtles were trained to breath into the respirometry dome, which had an air space of ~10 liters. Air of known partial pressures of O_2 and N_2 (CO_2 and water vapor free) flowed through a Sierra Side-Trak 840 Mass Flow Controller (MFC) (Sierra Instruments, Monterey, CA, USA) into the dome. The MFC regulated flow to 8 l min^{-1}. Ex-current air was sub-sampled at 250 ml min^{-1} and scrubbed of water vapor (Drierite® water absorbent, W. A. Hammond DRIERITE, Xenia, OH, USA) before being drawn through an Applied Electrochemistry O_2 Analyzer S-3A (AEI Technologies, Pittsburg, PN, USA). Data from the MFC and O_2 analyzer were recorded at a frequency of 1 Hz and later analyzed using Sable Systems® DataCan V Data Acquisition and Analysis Software and Hardware (Sable Systems International, Las Vegas, NV, USA). The open-flow respirometer was calibrated using the nitrogen dilution technique (Fedak et al., 1981). Oxygen consumption data was corrected to STPD.

RER was measured using the recording system described above but the turtles were kept in an acrylic dry box. The box was 1 m×0.5 m×0.5 m, with a clamp-down lid and a thin rubber section on one side that acted as a pressure damper. Turtles were placed in the respirometer and both O_2 consumption and CO_2 production were measured, the latter using an Applied Electrochemistry CO_2 Analyzer CD-3A. Measurements were made over 1.5 h or more, which allowed sufficient time for gases to equilibrate and to establish stable gas exchange values from the animal. RER trials were done in the fed state and after 10 and 15 days of fasting. The dry box respirometer was calibrated using the nitrogen dilution technique.

Doubly labeled water determinations

Turtles were weighed on an ADAM CPW-60 0–60 kg±0.02 kg digital scale (Dynamic Scales, Terre Haute, IN, USA). For blood-sampling purposes, turtles were placed head down on a bench with a 45 deg. declination (to aid venous pooling of the blood) and held with straps.

Table 1. Mass background isotope levels, injectate details, isotope dilution space, washout ratios, metabolic rate and water turnover rate for 6 green turtles used in DLW validation

Turtle	L1	L2	L3	L4	R1	R4	Mean ± s.d.	Sig. diff.
Mass (kg)								
Fed	19.60	25.04	19.76	25.88	24.84	19.38	22.42±3.13	a
Fasted	19.04	25.18	19.10	25.30	24.10	19.20	21.99±3.18	a
Post-fast 21 days	19.48	25.06	19.44	25.50	24.38	19.46	22.22±3.04	a
Post-fast 23 days	19.42	25.28	19.92	26.20	24.92	19.82	22.59±3.18	a
Post-fast 25 days	19.92	25.38	20.10	26.20	25.22	19.90	22.79±3.10	a
Background ^{2}H	153.85	154.59	153.82	153.57	153.21	153.12	153.69±0.53	
Background ^{18}O	1994.64	1995.18	1994.21	1994.67	1994.82	1994.51	1994.67±0.32	
Initial injectate (moles)	0.37	0.55	0.42	0.52	0.53	0.42		
Injectate enrichment								
^{2}H APE	260611.09	260611.09	260611.09	260611.09	260611.09	260611.09		
^{18}O APE	695197.53	695197.53	695197.53	695197.53	695197.53	695197.53		
N_d (ml)	14047.42	17593.36	14821.41	16709.71	17019.68	14014.79	15701.06±1593.03	
N_o (ml)	13398.16	16887.24	14152.22	15862.79	16365.00	13608.91	15045.72±1508.41	
Dilution ratio N_d:N_o	1.048	1.042	1.047	1.053	1.040	1.030	1.048±0.005	
10 day fast ^{2}H boost								
Injectate (moles)	0.07	0.10	0.08	0.08	0.09	0.07	0.08±0.01	
15 day fast ^{2}H boost								
Injectate (moles)	0.08	0.09	0.07	0.08	0.08	0.07	0.08±0.01	
^{2}H boost enrichment								
^{2}H APE	952572.52	952572.52	952572.52	952572.52	952572.52	952572.52		
Fed – k_d	6.399E–05	6.154E–05	8.009E–05	6.024E–05	5.836E–05	4.913E–05	6.223E–05±1.013E–05	
Fed – k_o	7.297E–05	6.452E–05	8.653E–05	6.704E–05	6.298E–05	5.143E–05	6.758E–05±1.166E–05	
Fasted – k_d	4.832E–05	3.799E–05	4.057E–05	3.925E–05	4.786E–05	4.218E–05	4.270E–05±4.406E–06	
Fasted – k_o	4.462E–05	3.969E–05	3.997E–05	3.616E–05	3.500E–05	4.490E–05	4.006E–05±4.126E–06	
Fed – *kd*:*ko*	0.88	0.95*	0.93	0.90	0.93	0.96*	0.91±0.02	a
Fasted – *kd*:*ko*	1.08	0.96	1.02	1.09	1.37	0.94	1.07±0.16	b
Fed – respirometry (kJ kg^{-1} day^{-1})	32.70	24.14*	33.80	23.98	24.17	22.86*	28.66±5.31	a
Fed – DLW (kJ kg^{-1} day^{-1})	59.56	–8.76*	19.22	32.78	11.85	–8.06*	30.85±21.01	a
Fasted – respirometry (kJ kg^{-1} day^{-1})	15.89	14.40	12.43	14.17	11.73	14.01	13.77±1.49	b
Fasted – DLW (kJ kg^{-1} day^{-1})	–65.80	–6.60	–34.19	–44.01	–130.35	1.63	–46.55±47.90	n/a
Fed – RER	0.88	0.82	0.78	0.93	0.74	0.85	0.83±0.07	a
Fast (10 days) – RER	0.58	0.60	0.24	0.46	0.50	0.78	0.53±0.18	b
Fast (15 days) – RER	0.69	0.69	0.58	0.58	0.48	0.52	0.59±0.09	b
Fed – breathing frequency	0.14	0.09	0.1	0.09	0.1	0.07	0.10±0.02	a
Fasted – breathing frequency	0.11	0.06	0.09	0.08	0.06	0.12	0.09±0.03	a
% TBW (^{2}H)								
Fed	67.06	67.07	71.48	61.10	65.51	67.77	66.67±3.37	a
Fasted (10 days)	59.48	62.49	66.37	48.18	50.59	65.07	58.70±7.63	b
Fasted (15 days)	60.31	60.61	61.72	53.51	50.31	61.54	58.00±4.85	b
Water turnover (l day^{-1})								
Fed	1.24	1.49	1.63	1.38	1.36	0.95	1.43±0.17	a
Fasted (10 days)	0.79	0.86	0.75	0.69	0.83	0.76	0.78±0.06	b
Fasted (15 days)	0.79	0.83	0.68	0.76	0.83	0.71	0.77±0.06	b
Fed (21 days)	1.57	1.43	1.67	1.55	1.35	1.24	1.47±0.16	a
Fed (25 days)	1.32	1.25	1.67	1.34	1.36	1.10	1.34±0.19	a
% TBW day^{-1}								
Fed	9.21	8.86	11.53	8.68	8.40	7.08	9.57±1.33	a
Fasted (10 days)	7.00	5.45	5.82	5.64	6.87	6.07	6.14±0.65	b
Fasted (15 days)	6.94	5.48	5.83	5.68	6.86	6.07	6.14±0.62	b
Fed (21 days)	13.74	9.46	14.34	11.52	11.14	10.65	11.81±1.87	c
Fed (25 days)	9.82	7.47	11.79	8.41	8.35	8.25	9.02±1.56	a

Mass for fasted turtles is the average between days 10 and 15; post-fast 21 days is 21 days from start of trial and so on. The DLW MRs are from Eqn 7, see Materials and methods.

Mean ± s.d. and significant differences are given among related data groups (i.e. values within the lines of sig. diff. column). In the final column, letters (a,b,c) are used to denote significant differences. If the same letter is given then there is no difference, i.e. 'a' is significantly different from 'b' and 'c' but not from another 'a'. Values with an asterisk were not used in the determination of the mean.

All blood samples (2–5 ml) were drawn from the cervical venous sinus using 21 gauge×1.5 inch BD needles and BD SST Gel and Clot Activator Vacutainers® (BD; Becton Dickinson and Co., Franklin Lakes, NJ, USA). All blood samples were left to clot for 30 min before centrifuging for 30 min at 1509 ***g***. Serum was removed and transferred to Nalgene™ cryo-safe plastic tubes and frozen. For DLW injections, a 21 gauge×3.5 inch needle (BD) was used to penetrate the body cavity (coelomic cavity) just anterior to the rear flipper and angled 45 deg. towards the midline (Southwood et al., 2006). All blood and injectate (^{18}O and ^{2}H) samples were later analyzed for ^{2}H and ^{18}O isotope concentrations by Metabolic Solutions (Nashua, NH, USA), which reports the accuracy of their analyses to 2% of 1 s.d. for ^{2}H and 0.4% of 1 s.d. for ^{18}O. For the injectate samples, a known quantity of injectate was diluted 2000:1 prior to analysis. The enrichment of the dilution water and the diluted injectate was measured by isotope ratio mass spectrometry, as were the blood samples.

DLW dose administered to each turtle was determined using equation 12.1 from Speakman (Speakman, 1997). TBW was assumed to be 66% (Thorson, 1968) in the initial calculation. TBW of our animals was confirmed when we analyzed data from the first turtles. Desired initial enrichment (DIE) was determined from published curves for mammals (Speakman, 1997), preliminary green turtle washout estimates and Southwood et al. (Southwood et al., 2006) with the goal of having enrichment levels at least 150 p.p.m. above background levels at the end of 20 days. Injectate enrichment from the mixture high-enrichment ^{2}H (99.9 atom%; Isotec, Miamisburg, OH, USA) and ^{18}O (95.1 atom%; Isochem UK, Banstead, UK) and the dose given are shown in Table 1. The actual dose administered to a turtle was determined by weighing the injectate syringe before and after drawing the mixed DLW into the syringe (Sartorius bp2105 digital scale ±0.0001 g; Goettingen, Germany). A three-way stopcock and a separate syringe filled with 0.9% NaCl solution (two times the volume of the DLW dose) were used to flush out the injectate syringe into the turtle's body cavity. The total injection (dose + flush) was less than 0.1% of TBW for all turtles.

Analysis of isotopic data

The turnover (washout) rates for ^{2}H and ^{18}O (k_d and k_o, respectively) were determined using the two-sample technique (Speakman, 1997), measuring isotope decay over the time period from the first and last isotope determination (day 1 and day 6, fed DLW trials; day 16 and day 21, fasted DLW trials). The two-sample approach was used and reported in Table 1 as this is the common, and typically only, method available to researchers working in the field. For comparative purposes, and to use all available data, we also used the multiple-sample approach (MSA) (Speakman, 1997) where k_d and k_o are determined from a curve fitted to the $\log_e$-transformed daily isotope determinations (after subtracting background levels to obtain excess isotope levels) (Fig. 3).

The plateau method was used to determine N_d and was used to infer TBW by dividing N_d by the dilution ratio (N_d:N_o). Typically, N_o is used as it has been shown to be closer to real TBW values in desiccation studies (Speakman, 1997); however, as we re-boosted the turtles with ^{2}H (cheaper than ^{18}O) to determine isotope dilution space, to obtain TBW before and after the fasting trial, we used the converted N_d throughout for consistency. Water turnover rates were determined by multiplying the converted N_d by k_d (Speakman, 1997). Body water pools for fed turtles were determined by measuring the converted N_d pre-trial and averaging this with the converted N_d post-trial. The post-trial N_d was calculated as a percentage of the mass of the turtle at the end of the experiment. N_d measured pre- and post-trials showed that body water pools were stable. CO_2 production

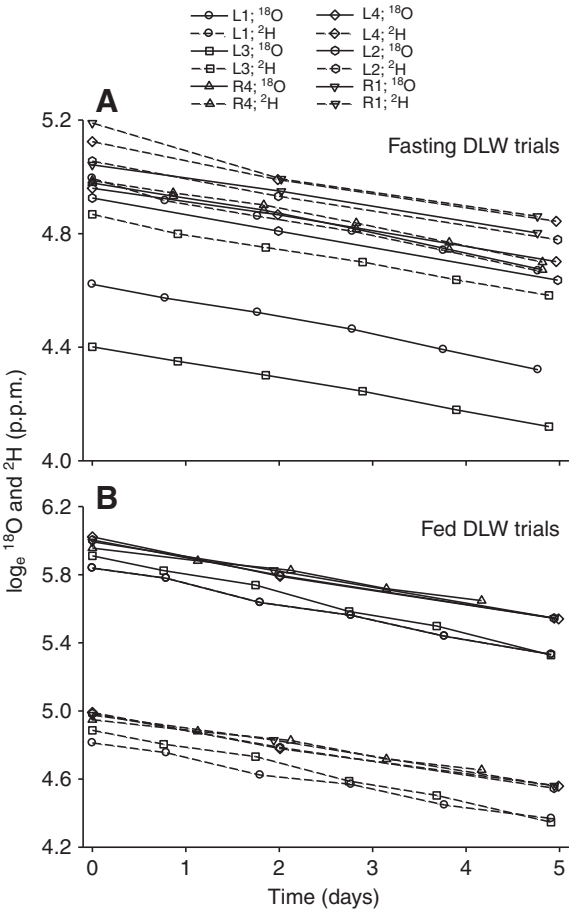

Fig. 3. Natural log of isotopic enrichment above background levels used for doubly labeled water (DLW) metabolic measurements using the multiple-sample approach. (A) Washout during the fasting trials; (B) washout during fed trials. Solid lines are for ^{18}O and broken lines for ^{2}H.

was determined using several equations that allowed us to determine a best practice for DLW studies of marine turtles. The equations, only three of which have been used in marine turtle studies, were as follows (where rCO_2=CO_2 production).

One-pool method by Lifson and McClintock (Lifson and McClintock, 1966) (equation 36), used by Trullas et al. (Trullas et al., 2006) in emergent, hatchling olive ridleys:

$$rCO_2 = (N / 2.08)\,(k_o - k_d) - (0.015\,k_d\,N), \quad (1)$$

where N=N_o.

One-pool method by Speakman (Speakman, 1997) (equation 7.17):

$$rCO_2 = (N / 2.078)\,(k_o - k_d) - (0.0062\,k_d\,N), \quad (2)$$

where N=N_o.

Two-pool method by Coward et al. (Coward et al., 1985):

$$rCO_2 = (1 / 2.08)\,(N_o k_o - N_d k_d) - (0.015\,N_d\,k_d). \quad (3)$$

Two-pool method by Schoeller (Schoeller, 1988) (equation 4), used by Southwood et al. (Southwood et al., 2006) for green turtles:

$$rCO_2 = (N / 2.078)\,(1.01k_o - 1.04k_d) - (0.0246\,N\,1.05)\,(1.01\,k_o - 1.04\,k_d), \quad (4)$$

where N=[(N_o/1.01)+(N_d/1.04)]/2.

Two-pool method by Speakman et al. (Speakman et al., 1993):

$$rCO_2 = (N / 2.078)\,(1.01k_o - 1.0532k_d) - (0.0246\,N\,1.05)\,(1.01k_o - 1.0532k_d), \quad (5)$$

where $N=[(N_o/1.01)+(N_d/1.0532)]/2$.

Two-pool method by Speakman (Speakman, 1993) (equation 4), used by Wallace et al. (Wallace et al., 2005) for inter-nesting leatherbacks:

$$rCO_2 = (N / 2.078)\,(k_o - R_{dilspace}\,k_d) - (0.0246\,N\,1.05)\,(k_o - R_{dilspace}\,k_d), \quad (6)$$

where $N=[(N_o+N_d/R_{dilspace})]/2$ and $R_{dilspace}=N_d/N_o$.

Two-pool method by Speakman (Speakman, 1997):

$$rCO_2 = (N / 2.078)\,(k_o - R_{dilspace}\,k_d) - (0.006\,N\,R_{dilspace}\,k_d), \quad (7)$$

for N see Eqn 6 above.

The complete reference is given for each equation above; however, from this point forward we will refer to the equations by the equation number assigned above and not that in the original publication, e.g. equation 36 from Lifson and McClintock (Lifson and McClintock, 1966) will be referred to as Eqn 1. CO_2 production was converted to energy expenditure using the measured RER for fed and fasted turtles (fasted measurements made at both 10 and 15 days of fasting). All values are listed in Table 1; thus, our data may be re-analyzed by other researchers for comparative purposes or revised as the techniques and equations advance and change (Speakman, 1997). Statistical comparisons between two treatment groups were done with Student's *t*-test. For more than two treatment groups, a one-way ANOVA was used to determine if significant differences existed between treatment groups and a Tukey-Kramer *post-hoc* test was used to determine where significant differences lay. A Welch ANOVA, testing equality of means when standard deviations are not equal (correcting for unequal variances), was used for comparisons between MRs obtained from respirometry and DLW. In all statistical analyses, α was set to 0.05. All statistical analyses were done on JMP® 4 statistical software program (SAS Institute, Cary, NC, USA). All values are given as means ± 1 s.d.

RESULTS

The results from the isotopic as well as the respirometric analyses are summarized in Tables 1 and 2. All data for individual turtles, including N_o and N_d, k_d and k_o, injectate quantity and enrichment, and RER are given in order to keep the validation transparent. There was a trend for body mass to drop with fasting and increase post-fasting; however, any changes from initial values were <2% and not significant (F=0.059, P=0.9929). Turtles L2 and R4 were not used in the calculation of MR (DLW or respirometry) for the feeding trials as they had k_d:k_o ratios of 0.95 and 0.96, respectively (Table 1), and were thus outside the range of acceptable k_d:k_o values (Speakman, 1997).

Respirometry

Metabolic rate dropped 51.95% from 28.66±5.31 to 13.77±1.49 kJ kg^{-1} day^{-1} during fasting (t-test=–6.273, P<0.0001). Interestingly, while there was a significant drop in MR, the turtles showed no change in breathing frequency (0.10±0.02 and 0.09±0.03 breaths min^{-1}, for fed and fasted, respectively; t-test=0.838, P=0.4217). RER showed a significant drop from fed (0.83±0.07) to fasted states at 10 days (0.53±0.18) and 15 days (0.59±0.09) (F=8.2255, P=0.0039). There was no significant change in RER during fasting from 10 to 15 days.

Doubly labeled water determinations

Using the plateau method, the ^{2}H and ^{18}O dilution spaces (N_d and N_o) were 15.7±1.6 and 15.0±1.5 liters, respectively. The isotopes equilibrated with the body water by 5 h post-injection (Fig. 1). The dilution space ratio (N_d:N_o) was 1.048±0.005, which is within the range typically found across taxa (Speakman, 1997). The k_d:k_o ratio was 0.91±0.02 for fed turtles (excluding L2 and R4) and increased significantly to 1.07±0.16 for fasted turtles (t=2.338, P=0.0415). Estimating TBW from the ^{2}H dilution space (divided by the dilution ratio 1.048) gave values of 66.64±3.37% of body mass, which decreased significantly to 58.70±7.63 and 58.00±4.85% of body mass after 10 and 15 days fasting, respectively (F=4.3517, P=0.0323). Water turnover dropped 45.33% from the fed to fasted state and returned to pre-fasting levels within 1 day of post-fasting trials. All these changes were significant (F=27.9044, P<0.0001). Similar differences were seen in water flux as % TBW day^{-1}. However, the decrease from fed to fasted was 35.84% and at the end of the fasting trial the turtles showed a compensatory rebound; water flux increased 18.97% above pre-fasting levels, returning to normal feeding levels by 5 days post-fasting (F=19.0584, P<0.0001).

The seven equations compared in the analysis are listed in Table 2. The two-sample technique gave MRs ranging from 16.40±19.83 to 66.91±21.56 kJ kg^{-1} day^{-1}, a fourfold difference, depending on the equation used. Eqn 7, the two-pool method of Speakman (Speakman, 1997), gave the closest MR to that obtained through respirometry (mean values 30.85±21.01 and 28.66±5.31 kJ kg^{-1} day^{-1}, respectively), a mean absolute difference of only 7.67%; the other equations differed from respirometry by 34–133%. The difference between MR derived from Eqn 7 and respirometry was not significantly different (using a Welch ANOVA for unequal variances, t-test=0.0601, P=0.952). Furthermore, when the multiple-sample approach was used for determining k_d and k_o, Eqn 7 gave an MR of

Table 2. Body pool estimate (N) and doubly labelled water (DLW) derived metabolic rate (MR) for the seven equations listed in the methods section for the fed trials

Equation	Pool(s)	N (ml)	MR (kJ kg^{-1} day^{-1})	% diff. from resp.
1	1	15045.72±1508.41	53.07±20.98	85.17
2	1	15045.72±1508.41	66.91±21.56	133.46
3	2	Table 1	16.4±19.83	42.78
4	2	14996.96±1511.58	51.79±20.13	80.70
5	2	14902.35±1501.99	42.13±19.83	47.00
6	2	15045.72±1508.41	38.5±19.77	34.33
7	2	15045.72±1508.41	30.85±21.01	7.67

Values are means ± s.d. MRs are from the DLW two-sample technique (Speakman, 1997). Final column gives the absolute percent difference between the DLW-derived MR and the respirometry-derived MR. For Eqn 3, the body pool estimate (N_o and N_d) are used for each individual turtle and these can be found in Table 1.

29.86±20.72 kJ kg^{-1} day^{-1}, which was only 4.19% above the respirometry value. These percentage differences were from comparing group means whereas the averages of the % differences between individuals for Eqn 7 and respirometry are 53.24±20.14 and 54.70±15.80% for the two-sample technique and MSA, respectively.

All of the above DLW energetic determinations and comparisons are for fed trials only. DLW water comparisons cannot be made for the fasting trials as negative MRs were obtained (i.e. –46.55±47.90 kJ kg^{-1} day^{-1}, for Eqn 7). We conducted a second trial on a subsample (n=2) of turtles, fasting them first and injecting DLW just before the respirometry trials to eliminate the possibility that the isotope could be stored in fed animals and released later during fasting. Again, we obtained negative MR determinations, –9.75±5.18 kJ kg^{-1} day^{-1} (Eqn 7). In all fasting trials, k_d was greater than k_o.

DISCUSSION

Application of the DLW method in the present study gave MR estimates that were greater than respirometry by 8% and 4% for the two-sample and multiple-sample approaches, respectively, when using Eqn 7 with fed turtles. The difference between DLW and respirometry MR estimates in both cases was not significant (Table 1). The two-sample technique is typically the only approach available to field researchers as recapturing the study animals multiple times can be difficult and disrupts the animal's natural behavior, but our data suggest that the two-sample technique results in twice the error of the multiple-sample approach in marine turtles. Overestimation by ≤8% for energy expenditure by the DLW method, when compared with respirometry, is common when group means are used (Speakman, 1997; Butler et al., 2004); however, the percentage difference between DLW and respirometry MR estimates for individuals ranged from –51 to 82%. As stated by Speakman (Speakman, 1997), the DLW technique is extremely limited in its ability to determine the MR of single individuals, or to make comparisons of individuals in different activities or metabolic state; however, the technique is quite adequate to determine group energetic demands.

The different equations listed in Table 2 gave MRs that deviated from respirometry by 8–133%. The worst estimates were derived from Eqn 2, the single-pool equation of Speakman (Speakman, 1997). While it is generally accepted that the two-pool method is more accurate in estimating water turnover in large animals, there is uncertainty about what constitutes 'large' body mass, especially in reptiles. Thus, we calculated MR using all seven equations (two single-pool and five two-pool) to illustrate equation-dependent effects on the calculations of MR (Table 2).

A major source of error in DLW calculations can lie in the estimate of fractionation. For instance, Eqns 6 and 7 differ in their fractionation correction, and MR output differs by nearly 25%. Fractionation is a measure of the degree of discrimination that exists between isotopes when released by various routes from the body. A major problem in reptile studies is that corrections for fractionation are derived mostly from studies of humans and other mammals. For instance, Schoeller et al. (Schoeller et al., 1986) corrected for fractionation assuming a body temperature of 37°C. As marine turtles have lower body temperature (ectothermic), except for perhaps the leatherback, and probably near-zero transcutaneous water loss, it is imperative that the equation used in calculation of MR does not over-correct for fractionation based on mammalian studies. Therefore, it is no surprise that Eqn 7 gave the best DLW-derived MR (Table 2), as this equation reduced the overcorrection of fractionation of Lifson and McClintock (Lifson and McClintock, 1966) (equation 36) by 50%. The other two-pool equations used in our calculations incorporate the higher fractionation correction originally established by Lifson and McClintock (Lifson and McClintock, 1966).

None of the three previous marine turtle DLW studies [leatherbacks (Wallace et al., 2005); green turtles (Southwood et al., 2006); olive ridley hatchlings (Trullas et al., 2006)] used Eqn 7. While the DLW-derived MR for Wallace et al. (Wallace et al., 2005) and Southwood et al. (Southwood et al., 2006) are in plausible ranges, the use of Eqn 7 could lower Wallace et al.'s MR by 25% and Southwood et al.'s MR by nearly 37%. This would reduce Southwood et al.'s (Southwood et al., 2006) MRs to 1.8–3.2 times resting, which are probably more typical of FMRs, and place Wallace et al.'s FMRs in the middle of the range for RMR and diving metabolic rates in leatherbacks (Wallace and Jones, 2008). Trullas et al. (Trullas et al., 2006) used Eqn 1, a single-pool equation that is probably correct for a study of ~18 g hatchlings. However, in attempting to determine the metabolic costs of three distinct phases of hatchling dispersal, the experimental design was perhaps outside the attainable scope (i.e. capabilities) of the DLW method. For instance, Jones et al. (Jones et al., 2007) found 176 kJ kg^{-1} day^{-1} for the maximum metabolic rate (MMR) in frenzied swimming by olive ridleys (measured by respirometry), a value 4.6 times lower than Trullas et al. found for swimming MR estimated by DLW (812 kJ kg^{-1} day^{-1}) (Trullas et al., 2006).

Isotopes injected intravenously (IV) have the shortest time to equilibration with the subject's body water. Intramuscular/intraperitoneal (IM/IP) injections are intermediate while oral dosing produces the longest times to equilibration. Other factors such as metabolic rate and body mass also affect time to equilibration; for instance, smaller animals with higher metabolic rates have shortened equilibration times. As marine turtles do not have a peritoneal cavity, we use the term IC to indicate intra-coelomic, and IC injections in turtles are equivalent to IP in other species. Speakman (Speakman, 1997) derived an equation to determine time to equilibration for IM/IP injections (equilibration time in hours=2.555+0.360log$_e$) based on data from 41 studies on mammals, marsupials, birds and reptiles ranging in mass from 2.6 g to 108 kg. IV injections accelerate and oral dosing retards the time to equilibration as derived from the above equation. Our green turtles (mass 22.42±3.13 kg), injected IC, had equilibration times of ~5 h (Fig. 1). This compares with a time of 3.7 h from the Speakman (Speakman, 1997) equilibration time equation, and the increase in time (1.3 h) for equilibration in our study is probably due to the lower metabolic rate of reptiles as the equation is derived from 41 species but only two are reptilian so it is biased towards animals with higher MRs. Southwood et al. (Southwood et al., 2006) used 12 h post-injection for their equilibration sample of IC-injected green turtles (15.9±4.7 kg) and did not perform an equilibration time curve. According to the equation above and our equilibration time curve for green turtles (Fig. 1), equilibration for Southwood et al. should have occurred within 3.6–5 h post-injection. Therefore, at 12 h post-injection their sample is probably on the washout curve, leading to an overestimate of the body water pool and consequently overestimation of CO_2 production. Wallace et al. (Wallace et al., 2005) injected leatherback turtles IV, and their equilibration time curve suggests that isotopes equilibrated with the turtles' body water in 2–4 h post-injection. IV injections in large marine mammals equilibrate in 1–3 h (Lydersen et al., 1992; Aquarone et al., 2006), and a somewhat longer equilibrium time is expected in reptiles.

Trullas et al. (Trullas et al., 2006) injected ~18 g hatchlings IV with DLW and took the equilibration sample 2 h later. They based

the equilibration time of 2 h on Speakman's equation and added an hour as they were using reptiles. Nagy and Knight (Nagy and Knight, 1989) found 16.6–19.5 g geckos and skinks equilibrated in 1 h with IP injections. As previously mentioned, Wallace et al. (Wallace et al., 2005) found ~268 kg leatherbacks equilibrated with body water in 2–4 h for IV injections; thus, Trullas et al. (Trullas et al., 2006) probably took their equilibration sample on the washout curve and therefore overestimated the isotope dilution space and subsequently CO_2 production. Their TBW measurements confirm this because the hatchlings were injected as they emerged from the nest or incubator, before ingesting any water, yet TBWs were 85% – values usually associated with well-hydrated turtles (see Ortiz et al., 2001; Wallace et al., 2005; Southwood et al., 2006) (present study; Table 1). Turtles emerge from the nest dehydrated and drink seawater upon entering the ocean (Reina et al., 2002). For hatchlings of this size, we suggest that the intercept method to determine isotope dilution space from body water equilibration is more appropriate (see Speakman, 1997) than the plateau method as it is hard to take multiple blood samples from animals weighing <100 g. Alternatively, equilibration could be determined using breath sample analysis of the expired CO_2 (Kroll and Speakman, 1999). All sea turtle species are considered threatened or endangered, which calls into question the use of the whole-body desiccation method for determination of TBW.

The DLW method gave negative MR determinations for fasted turtles (Table 1). Five of six turtles had negative MRs, and the one positive MR value was 88% less than the respirometry MR value. Speakman suggested that isotopes sequestered during the fed trials could be released during the fasting trial (J. Speakman, personal communication). Therefore, we conducted a revised fasting trial, fasting two turtles for 10 days then injecting with isotope just before the start of the fasting trial. We again obtained negative MRs (-9.75 ± 5.18 kJ kg^{-1} day^{-1}). A possible explanation for some of the error in DLW-derived MRs for fasted turtles is that there is a 52% drop in MR (based on oxygen consumption) with fasting (28.66 ± 5.31 to 13.77 ± 1.49 kJ kg^{-1} day^{-1}) while only a 36% drop in water flux (9.57 ± 1.33 to 6.14 ± 0.65% TBW day^{-1}), which decreases the difference between the ^{18}O and ^{2}H washout. Deuterium washout (k_d) was greater than k_o for four of the six turtles (Table 1). Southwood et al.'s field study (Southwood et al., 2006) had the lowest k_d:k_o ratios (i.e. 0.81 ± 0.03 and 0.84 ± 0.02 for summer and winter, respectively) of any marine turtle study to date. Obviously, the study turtles did not have the typical low MR accompanied with high water turnover as seen in the other marine turtle studies (Wallace et al., 2005; Trullas et al., 2006) (this study). Water turnover of green turtles is lower than other marine turtles (Ortiz et al., 2001; Wallace et al., 2005) (Table 1), and actively foraging green turtles probably have an elevated mass-specific MR compared with inter-nesting leatherbacks (Wallace et al., 2005) and our captive green turtles with limited mobility.

Wallace et al. (Wallace et al., 2005) did not report any negative MRs but could not calculate MR for two turtles due to high k_d:k_o ratios. Leatherbacks have the highest water flux values of marine turtles, and Wallace and Jones (Wallace and Jones, 2008) have recently shown that, contrary to popular belief, leatherback MR is not elevated relative to other marine turtles. Wallace et al. (Wallace et al., 2005), however, did find that leatherbacks remained active (swimming) during the inter-nesting period and this may have been enough to keep the MR to water flux ratio in check (k_d:k_o=0.7, 0.86, 0.92 and 0.93 in the other four animals).

Another source of error in Wallace et al.'s determination of leatherback MR may lie in selection of 0.7 for RQ in inter-nesting leatherbacks. Evidence from other studies suggests that leatherbacks forage during the inter-nesting interval (Southwood et al., 2005; Fossette et al., 2008). If leatherbacks were ingesting prey then, as RQ is higher for feeding animals, this could lead to an overestimation of MR. On the other hand, if turtles are fasting then perhaps RQ=0.7 is too high. We measured RER as <0.7 for fasted turtles in our study (Table 1). These low RERs, however, could be the result of respiratory-derived CO_2 being incorporated in the urine as ammonium bicarbonate, buffering ammonia excretion, but whether this even occurs in leatherbacks is unknown.

The DLW method accurately measures MR in the three non-Chelonian members of the marine reptile group. For instance, marine iguanas and crocodiles have water flux rates of <10% TBW day^{-1} and MRs of 30–70 kJ kg^{-1} day^{-1} (Nagy and Shoemaker, 1984; Christian et al., 1996; Drent et al., 1999). While there is no record of DLW use in sea snakes, their water flux is as low as 1.2% TBW day^{-1} (Schmidt-Nielsen and Skadhauge, 1967). Freshwater reptiles, however, are probably bad candidates for DLW studies. Booth (Booth, 2002) found freshwater turtles turnover their body water 1.6 to 4.3 times per day (160–430% TBW day^{-1}) and concluded that the use of DLW is impractical, unless the freshwater turtles are hauling over land or in terrestrial estivation (Roe et al., 2008), when water turnover rates will be reduced.

Green turtles in this study showed a significant decrease in TBW content when fasted. Yet there was not a significant drop in body mass (Table 1). There was a trend for a ~600 g drop in body mass but this does not account for all the water lost if TBW decreased 8%. However, a turtle body mass measurement may include water in the intestinal tract and bladder that is temporarily stored or moving through the turtle and is not incorporated in the TBW measurement. This is perplexing and we do not have a concrete explanation. Water turnover rates decreased with fasting and then returned to normal levels post-fasting or showed a compensatory increase for the first 24 h post-fasting. Green turtle water flux rates are low compared with other marine turtles. Southwood et al. (Southwood et al., 2006) found that green turtles had water flux rates of 6–8% TBW day^{-1}, which corresponds with our finding of 6–10% TBW day^{-1}. Ortiz et al. (Ortiz et al., 2000) found that Kemp's ridley turtles have flux rates of 16% TBW day^{-1}, while for leatherbacks water flux is as high as 24% TBW day^{-1} (Wallace et al., 2005). Hatchling water flux rates can be anywhere from 20 to 90% TBW day^{-1} for green, leatherback and olive ridley turtles (Reina, 2000; Reina et al., 2002; Wallace et al., 2005). The differences in adult water flux rates are most likely due to water content of diet and MRs (e.g. how active the turtle is, how much food intake per day, how rapidly wastes are voided).

Our green turtle RER during feeding was 0.83 ± 0.07, which implies a combination of fat, carbohydrate and protein burning. The fasted turtles, however, had RERs lower than expected (0.53 ± 0.18, 0.59 ± 0.09) (Table 1). RERs less than 0.7 may be due to the production of uric acid in the excreta or gluconeogenesis from fat (Kleiber, 1961). On the other hand, Coulson and Hernandez (Coulson and Hernandez, 1964) found that low RER measurements in alligators could be due to ammonium bicarbonate, in the urine, being derived from respiratory CO_2, thus reducing CO_2 excretion from the lungs. A similar observation was made by Grigg (Grigg, 1978) in crocodiles. Interestingly, we only recorded low RERs during fasting. If the decreased RER was due to CO_2 excretion in urine then we would expect a low RER for fed turtles as well as fasting, suggesting that gluconeogenesis from fat (during fasting) is the probable explanation. This leaves the researcher using the DLW method on reptiles with a conundrum: what RQ to use for fasted animals? In our study, the point was moot as fasting trials

gave negative DLW-derived MRs no matter whether an RER of 0.53 or 0.70–1.0 was used. Researchers working with reptiles and DLW should determine RER in either pre- or post-study validation experiments. Furthermore, urine bicarbonate levels should be tested as well to determine if these are from respiratory CO_2. Marine turtles are capable of urea, uric acid and ammonia excretion (Khahil, 1947), thus a further issue arises as these animals may shift their nitrogenous waste biochemistry and change RER depending on their situation (i.e. in salt water or respirometer dry box). However, as we found that DLW does not work in fasting turtles, the technique should only be used in foraging turtles that are in a steady-state (i.e. energy intake equals energy output) and RER should be measured or derived for the diet.

Interestingly, there was no significant difference in breathing frequency for fed and fasted turtles (0.10±0.02 and 0.09±0.03 breaths min^{-1}, respectively). Yet, there was a 52% drop in MR with fasting. This suggests that the turtles decreased tidal volume or oxygen extraction efficiency. Turtles lowering oxygen extraction efficiency by shunting blood away from the pulmonary system would reduce oxygen partial pressure (P_{O_2}) and increase CO_2 partial pressure (P_{CO_2}) in systemic blood, signaling the turtle to breath even though it would still have ample lung stores (causing the turtle to maintain an increased breathing frequency despite the MR drop). Alternatively, turtles could simply decrease tidal volume in response to lowered metabolic demand. With decreased O_2 stores, their internal chemoreceptors would signal them to breath, causing them to surface with the same frequency as fed turtles even with lowered oxygen demand.

Conclusions

This study shows that the DLW method gives valid MR determinations in marine turtles if certain criteria are met. For instance, the DLW method should not be used to estimate the MR of an individual and turtles should preferably be in steady-state or positive energy balance, where energy input is equal to or greater than energy output. It is imperative that the turtles' water flux rates are moderate and the turtles are active, thus reducing the ratio of ^2H isotope turnover to ^{18}O isotope turnover (reducing k_d:k_o). DLW method validation should be performed on a species level basis and RER should be measured or based on a known diet. Changing TBW and RQ issues arise with fasting turtles, causing complications in isotope dilution space and energy calculations, respectively. If low RERs are measured, further research should be done to determine the cause and whether urine bicarbonate is being derived from respiratory CO_2. Furthermore, if urine bicarbonate is found, this could affect fractionation factors, as CO_2 dissolved in liquid is not fractionated from body water with the same isotopic proportions as expired CO_2. And finally, researchers publishing papers using DLW should give complete details on individual animals for injectate enrichment and dose, N_o and N_d, as well as k_o and k_d, so that values can be recalculated as information on equations, isotope dilution space ratio and fractionation advance. In this regard, we have attempted to make our experimental design and results transparent in the hope that future researchers may re-work the numbers as information on DLW techniques evolve.

LIST OF ABBREVIATIONS

DLW	doubly labeled water
FMR	field metabolic rate
IC	intra-coelomic
k	isotopic washout
k_d	turnover (washout) rate for ^2H
k_o	turnover (washout) rate for ^{18}O
MR	metabolic rate
MSA	multiple-sample approach
N	body water pool
N_d	isotope dilution space for hydrogen
N_o	isotope dilution space for oxygen
RER	respiratory exchange ratio
RQ	respiratory quotient
TBW	total body water

We thank the Cayman Island Turtle Farm for graciously donating the green turtles to science and Ashley Houlihan for assisting in the experiments. We also thank T. Sellman, D. Lund, K. Kwon, A. Houlihan, A. Shamlou, C. Lefroy, A. Pei, J. Latinder, D. Miller, A. Stevenson, A. Yamada, V. Prilesky, R. Vaisman, E. Kume, O. Claque, E. Cheng, A. Todd, A. Kwong, B. Woo, S. Ibrahim and K. Sorenson for caring for the green turtles housed at the Zoology Animal Care Center, UBC, as well as A. Vanderhorst and S. Gopaul (turtle emergency care), B. Gillespie and V. Grant (for everything mechanical) and C. Harvey-Clark, B. George and T. Godbey for veterinary assistance. We thank John Speakman for invaluable insight and many answered late night emails as well as Amanda Southwood for logistical support and reviewing an early draft of this manuscript. This study was funded by an NSERC Discovery Grant to D.R.J. and from NMFS/NOAA-PIFSC.

REFERENCES

Anderson, N. L., Hetherington, T. E. and Williams, J. B. (2003). 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.

Aquarone, M., Born, E. W. and Speakman, J. R. (2006). Field metabolic rates of walrus (*Odobenus rosmarus*) measured by the doubly-labelled water method. *Aquat. Mamm.* **32**, 363-369.

Arnould, J. P. Y., Boyd, I. L. and Speakman, J. R. (1996). The relationship between foraging behaviour and energy expenditure in Antarctic Fur seals. *J. Zool. (Lond.)* **239**, 769-782.

Bjorndal, K. A. (1996). Foraging ecology and nutrition of sea turtles. In *The Biology of Sea Turtles* (ed. P. L. Lutz and J. Musick), pp. 199-231. Boca Raton, FL: CRC Press.

Booth, D. T. (2002). The doubly-labeled water technique is impractical for measurement of field metabolic rate in freshwater turtles. *Herpetol. Rev.* **33**, 105-107.

Butler, P. J., Green, J. A., Boyd, I. L. and Speakman, J. R. (2004). Measuring metabolic rate in the field: the pros and cons of the doubly labelled water and heart rate methods. *Funct. Ecol.* **18**, 168-183.

Christian, K., Green, B. and Kennett, R. (1996). Some physiological consequences of estivation by freshwater crocodiles, *Crocodylus johnstoni*. *J. Herpetol.* **30**, 1-9.

Costa, D., Croxal, J. and Duck, C. (1989). Foraging energetics of Antarctic fur seals in relation to changes in prey availability. *Ecology* **70**, 596-606.

Coulson, R. A. and Hernandez, T. (1964). *Biochemistry of the Alligator: a Study of Metabolism in Slow Motion*. Baton Rouge, LA: Louisiana State University Press.

Coward, W. A., Prentice, A. M., Murgatroyd, P. R., Davies, H. L., Cole, T. J., Sawyer, M., Goldberg, G. R., Halliday, D. and MacNamara, J. P. (1985). Measurement of CO_2 and water production rates in man using ^2H, ^{18}O labeled H2O: comparisons between calorimeter and isotope values. In *Human Energy Metabolism: Physical Activity and Energy Expenditure Measurements in Epidemiological Research Based upon Direct and Indirect Calorimetry*, vol. 5 (ed. A. J. H. van Es), pp. 126-268. The Hague: CIP-gegevens Koninklijke Bibliotheek.

Davenport, J. (1998). Sustaining endothermy on a diet of cold jelly: energetics of the leatherback turtle *Dermochelys coriacea*. *Br. Herpetol. Soc. Bull.* **62**, 4-8.

Drent, J., van Marken Lichtenbelt, W. D. and Wikelski, M. (1999). Effects of foraging mode on the energetics of the marine iguana, *Amblyrhyncus cristalus*. *Funct. Ecol.* **13**, 493-499.

Fedak, M. A., Rome, L. and Seeherman, H. J. (1981). One-step N_2-dilution technique for calibrating open-circuit VO_2 measuring systems. *J. Appl. Physiol.* **51**, 772-776.

Fossette, S., Corbel, H., Gaspar, P., LeMaho, Y. and Georges, J. (2008). An alternative technique for the long-term satellite tracking of leatherback turtles. *Endanger. Species Res.* **4**, 33-41.

Grigg, G. C. (1978). Metabolic rate, Q10 and respiratory quotient (RQ) in *Crocodylus porosus*, and some generalizations about low RQ in reptiles. *Physiol. Zool.* **51**, 354-360.

Jones, T. T., Reina, R. D., Darveau, C.-A. and Lutz, P. L. (2007). Ontogeny of energetics in leatherback (*Dermochelys coriacea*) and olive ridley (*Lepidochelys olivacea*) sea turtle hatchlings. *Comp. Biochem. Physiol.* **147A**, 313-322.

Khalil, F. (1947). Excretion in reptiles 1. Non-protein nitrogen constituents of the urine of the sea turtle, *Chelonia mydas* (L.). *J. Biol. Chem.* **171**, 611-616.

Kleiber, M. (1961). *The Fire of Life: An Introduction to Animal Energetics*. New York: John Wiley.

Kraemer, J. E. and Bennett, S. H. (1981). Utilization of posthatching yolk in loggerhead sea turtles, *Caretta caretta*. *Copeia* **1981**, 406-411.

Krol, E. and Speakman, J. R. (1999). Isotope dilution spaces of mice injected simultaneously with deuterium, tritium and oxygen-18. *J. Exp. Biol.* **202**, 2839-2849.

Lifson, N. and McClintock, R. (1966). Theory of use of the turnover rates of body water for measuring energy and material balance. *J. Theor. Biol.* **12**, 46-74.

Lutcavage, M. and Lutz, P. L. (1986). Metabolic rate and food energy requirements of the leatherback sea turtle, *Dermochelys coriaceia*. *Copeia* **1986**, 796-798.

Lydersen, C., Griffiths, D., Gjertz, I. and Wiig, O. (1992). A tritiated water experiment on a male Atlantic walrus (*Odobemus rosmarus rosmarus*). *Mar. Mamm. Sci.* **8**, 418-420.

Nagy, K. A. (1980). CO2 production in animals: analysis of potential errors in the doubly labeled water method. *Am. J. Physiol.* **238**, R466-R473.
Nagy, K. A. (1983a). *The Doubly Labeled Water (3HH18O) Method: A Guide to Its Use.* Los Angeles, CA: University of California Publ. No. 12-1417.
Nagy, K. A. (1983b). Ecological energetics. In *Lizard Ecology: Studies of a Model Organism* (ed. R. B. Huey, E. R. Pianka and T. W. Schoener), pp. 24-54. Cambridge, MA: Harvard University Press.
Nagy, K. A. (1989). Doubly-labeled water studies of vertebrate physiological ecology. In *Stable Isotopes in Ecolgical Research* (ed. P. W. Rundel, J. R. Ehleringer and K. A. Nagy), pp. 270-287. New York: Springer-Verlag.
Nagy, K. A. and Knight, M. H. (1989). Comparative field energetics of a kalahari skink (*Mahuya striata*) and gecko (*Pachydactylus bibroni*). *Copeia* **1989**, 13-17.
Nagy, K. A. and Shoemaker, V. H. (1984). Field energetics and food consumption of the Galapagos marine iguana, *Amblyrhyncus cristatus*. *Physiol. Zool.* **57**, 281-290.
Nagy, K. A., Girard, I. A. and Brown, T. K. (1999). Energetics of free-ranging mammals, reptiles, and birds. *Annu. Rev. Nutr.* **19**, 247-277.
Ortiz, R. M., Patterson, R. M., Wade, C. E. and Byers, F. M. (2000). Effects of acute fresh water exposure on water flux rates and osmotic responses in Kemp's ridley sea turtles (*Lepidochelys kempi*). *Comp. Biochem. Physiol.* **127A**, 81-87.
Reilly, J. J. and Fedak, M. A. (1991). Rates of water turnover and energy expenditure of free-living male common seals (*Phoca vitulina*). *J. Zool. (Lond.)* **223**, 431-468.
Reina, R. D. (2000). Salt gland blood flow in the hatchling green turtle, *Chelonia mydas*. *J. Comp. Physiol. B* **170**, 573-580.
Reina, R. D., Jones, T. T. and Spotila, J. R. (2002). Salt and water regulation by the leatherback sea turtle *Dermochelys coriacea*. *J. Exp. Biol.* **205**, 1853-1860.
Roe, J. H., Georges, A. and Green, B. (2008). Energy and water flux during terrestrial estivation and overland movement in a freshwater turtle. *Physiol. Biochem. Zool.* **81**, 570-583.
Schmidt-Nielsen, K. and Skadhauge, E. (1967). Function of the excretory system of the crocodile (*Crocodylus acutus*). *Am. J. Physiol.* **212**, 973-980.
Schoeller, D. A. (1988). Measurement of energy expenditure in free-living humans by using doubly labeled water. *J. Nutr.* **118**, 1278-1289.
Schoeller, D. A., Ravussin, E., Schutz, Y., Acheson, K. J., Baertschi, P. and Jéquier, E. (1986). Energy expenditure by doubly labeled water: validation in humans and proposed calculation. *Am. J. Physiol.* **250**, R823-R830.
Southwood, A. L., Andrews, R. D., Paladino, F. V. and Jones, D. R. (2005). Effects of swimming and diving behavior on body temperatures of Pacific leatherbacks in tropical seas. *Physiol. Biochem. Zool.* **78**, 285-297.
Southwood, A. L., Reina, R. D., Jones, V. S., Speakman, J. R. and Jones, D. R. (2006). Seasonal metabolism of juvenile green turtles (*Chelonia mydas*) at Heron Island, Australia. *Can. J. Zool.* **84**, 125-135.
Sparling, C. E., Thompson, D., Fedak, M. A., Gallon, S. M. and Speakman, J. R. (2008). Estimating the field metabolic rate of pinnipeds: doubly-labelled water gets the seal of approval. *Funct. Ecol.* **22**, 245-254.
Speakman, J. R. (1993). How should we calculate CO2 production in DLW studies of animals? *Funct. Ecol.* **7**, 746-750.
Speakman, J. R. (1997). *Doubly Labelled Water: Theory and Practice*. London: Chapman and Hall.
Speakman, J. R. and Racey, P. A. (1987). The energetics of pregnancy and lactation in the brown long-eared bat, *Plecotus auritus*. In *Recent Advances in the Study of Bats* (ed M. B. Fenton, P. A. Racey and J. M. V. Rayner), pp. 368-393. Cambridge: Cambridge University Press.
Speakman, J. R., Nair, K. S. and Goran, M. I. (1993). Revised equations for calculating CO_2 production from doubly labelled water in humans. *Am. J. Physiol.* 264, E912-E917.
Thorson, T. B. (1968). Body fluid partitioning in reptilia. *Copeia* **3**, 592-601.
Trullas, S., Spotila, J. R. and Paladino, F. V. (2006). Energetics during hatchling dispersal of the olive ridley turtle *Lepidochelys olivacea* using doubly labeled water. *Physiol. Biochem. Zool.* **79**, 389-399.
Wallace, B. P. and Jones, T. T. (2008). What makes marine turtles go: a review of metabolic rates and their consequences. *J. Exp. Mar. Biol. Ecol.* **356**, 8-24.
Wallace, B. P., Williams, C. L., Paladino, F. V., Morreale, S. J., Lindstrom, R. T. and Spotila, J. R. (2005). Bioenergetics and diving activity of interesting leatherback turtles *Dermochelys coriacea* at Parque Nacional Marino Las Baulas, Costa Rica. *J. Exp. Biol.* **208**, 3873-3884.