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VALIDATION OF THE DOUBLY LABELLED WATER TECHNIQUE FOR BUMBLEBEES *BOMBUS TERRESTRIS* (L.)

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Summary

The doubly labelled water (DLW) technique was validated for the bumblebee *Bombus terrestris* (L.) using respirometry (RESP) from tethered roundabout flights. We injected small volumes (1 μl) of a mixture containing low concentrations of deuterium and ^{18}O and withdrew 1–2 μl of haemolymph to determine initial ^{18}O concentration. The injected isotopes were equilibrated with the body water pool after 10 min, and high material turnover allowed the analysis of final blood isotope concentrations after 5–7 h. On average (n=16), values measured using the DLW technique exceeded values measured using RESP by 3.1±9.9 %, a difference that was not statistically significant at the 99 % confidence level. The absolute error was 7.4±7.1 % (mean ± S.D.). Isotope dilution spaces of both deuterium and ^{18}O were almost identical with the body water pool. We corrected for isotope fractionation, using a slightly higher value than usual for one of the fractionation factors. The single most important variable to influence DLW results, which could not be measured with the desired accuracy, was the volume of the final body water pool N. An overestimate of final N possibly resulted in the DLW overestimate.

Key words: doubly labelled water, deuterium, oxygen-18, flight metabolism, bumblebee, *Bombus terrestris*, insect.

Introduction

Since its original formulation by Lifson and his colleagues in the 1950s and 1960s (Lifson *et al.* 1955; Lifson and McClintock, 1966), the doubly labelled water (DLW) technique has found wide application in studies of vertebrate physiological ecology. Its most outstanding feature is the ability to estimate the rates of body water turnover and the energy expenditure of free-living animals with minimal interference with their natural behaviour. This technique has liberated the study of animal energetics from the artificial environment of the laboratory and enabled researchers to examine species whose metabolic characteristics could not easily be analysed otherwise. Furthermore, because the DLW method is relatively simple to use in field studies, it has yielded metabolic data for many species within a short period. Its widespread application to avian energetics has even enabled comparative studies of intraspecific variation of daily energy expenditure (Bryant and Tatner, 1991) which would have been almost impossible without this technique (for overviews, see Nagy, 1988; Speakman and Racey, 1988; Tatner and Bryant, 1989).

In the DLW technique, hydrogen and oxygen isotopes (deuterium or tritium and ^{18}O, respectively) are added to an animal's body water. Isotope concentrations decrease exponentially with time through the natural 'wash-out' of CO_2 and water. The hydrogen isotope is lost as water only, and the oxygen isotope as both water and CO_2. Therefore, the apparent turnover rate of ^{18}O is higher than that of deuterium (or tritium) and the difference between the two apparent turnover rates reflects the CO_2 production rate. The method takes advantage of the isotopic equilibrium between the ^{18}O of respiratory CO_2 and the ^{18}O of body water, a reversible exchange which is realised through the hydration of CO_2 and the dehydration of bicarbonate ions by the enzyme carbonic anhydrase (Maren, 1967; Carter, 1972).

Despite its wide application to vertebrates, there have been only three DLW validation studies for arthropods: Buscarlet *et al.* (1978) using locusts (*Locusta migratoria migratorioides*), King and Hadley (1979) using scorpions (*Hadrurus arizonensis*) and Cooper (1983) using tenebrionid beetles (*Elodes armata* and *Cryptoglossa verrucosa*). The accuracy of the technique reported in these studies was, however, far below that of vertebrate studies (overestimates of 20 % or greater have been reported), and this has placed serious limitations on its use in field experiments. Thus, whereas successful DLW validation in vertebrates has spawned a rich variety of studies on field energetics, this has not been the case for arthropods.

We chose, therefore, to validate the DLW technique on the bumblebee *Bombus terrestris*. Colonies can be easily kept in

the laboratory and, as a result of commercial breeding, they are now available throughout the year. Bumblebees have a pure carbohydrate metabolism (respiratory quotient, RQ=1; Bertsch, 1984), and CO_2 production rates obtained from DLW in field experiments can be directly translated into units of energy consumption. Foragers can be trained to visit artificial flowers to collect nectar and thus can be kept under continuous observation during the course of a DLW experiment. High metabolic rates enable isotope turnover times (period between initial and final isotope samples) to be measured not in days or weeks, as in many vertebrate studies, but in hours. The application of the DLW technique to bumblebees in controlled 'artificial patch' experiments might make it possible not only to measure 'total energy consumption' over a given period but also to analyse the energetic costs of specific activities. Permutation experiments could show, for example, how 'expensive' it is for bumblebee foragers to fly between flowers and how much energy they use while feeding on the flower. Such information could be crucial in testing the basic underlying assumptions of optimal foraging models (e.g. Pyke, 1980; Bertsch, 1984; Schmid-Hempel *et al.* 1985; Surholt *et al.* 1988; Wolf and Schmid-Hempel, 1990) about the relative magnitudes of metabolic rates during different activities.

We preferred the use of deuterium to tritium as the hydrogen isotope because it is increasingly difficult to gain permission to use radioactive substances in field experiments. In addition, physical fractionation effects (differential phase transition rates of isotopes due to different atomic masses) could be more pronounced for tritium than for deuterium (Tiebout and Nagy, 1991), especially in animals with high turnover rates such as active insects. *Bombus terrestris* workers weigh between 70 and 400 mg (mean mass 250 mg) and mass-specific metabolic rates are around $70\,ml\,O_2\,g^{-1}\,h^{-1}$ (Ellington *et al.* 1990; Cooper, 1993). High turnover rates coupled with high thoracic temperatures (Heinrich, 1972) led us to examine the temperature-dependence of fractionation as part of the study.

The small size of insects meant that existing DLW protocols were unsuitable and so the DLW technique was modified accordingly. For most vertebrate studies, an initial blood sample of at least 20 μl is withdrawn for isotope analysis: 2×5 μl (including one replicate) for the initial deuterium and 2×5 μl for the initial ^{18}O concentrations. To our knowledge, there are no published data regarding haemolymph volumes in bumblebee workers, but Surholt *et al.* (1988) measured an average haemolymph volume of $149\,\mu l\,g^{-1}$ body mass for bumblebee (*Bombus terrestris*) males, which corresponds to 37 μl of haemolymph for a 250 mg worker. Crailsheim (1985) gives values for honeybee (*Apis mellifera*) workers which, when extrapolated to bumblebees, yield an estimate of 45–50 μl of haemolymph. Both estimates indicate that the available haemolymph is not sufficient for the withdrawal of an initial 20 μl sample without detrimental consequences. We therefore examined two alternative methods; the single-sample technique (Webster and Weathers, 1989; Tiebout and Nagy, 1991) and the withdrawal and analysis of very small (1–2 μl) haemolymph volumes.

Our final goal was to validate the DLW technique for bumblebees by comparing rates of CO_2 production derived from respirometry with results from isotope ratio mass spectrometry using the stable isotopes deuterium and ^{18}O. The technique has never been validated to date for active animals with mass-specific metabolic rates as high as those of flying insects, so we used tethered, flying bumblebees for the validation experiments.

Materials and methods

Study species

Bumblebee (*Bombus terrestris*) colonies from two sources were used. During spring and summer, wild colonies were excavated and installed either in small wooden boxes in the field or in plaster nest boxes (Plowright and Jay, 1966) in the laboratory. At other times, artificially bred bumblebee colonies (Biological Crop Protection Ltd, Kent, UK) were purchased and installed in the laboratory. The laboratory nest boxes were connected to small Perspex flight cages ($35\,cm\times25\,cm\times20\,cm$) through a 15 cm long plastic tube. The tube opened at the top of the flight cage, which forced the bees to fly to a gravity feeder in the opposite lower corner. The feeder contained 66 % (w/w) sucrose solution offered *ad libitum*, and pollen was added directly into the nest box when required. The field colonies were monitored regularly for pollen and nectar reserves, and pollen was added when reserves were low. For the DLW validation studies, only identified, individually marked (using numbered and coloured tags, Opalithplättchen; E. H. Thorne Ltd, Lincoln, UK) foragers were used.

Body water content

Bees (n=37) were taken from a colony, weighed on a Sartorius R 200 D semi-microbalance to the nearest 0.1 mg and kept in small glass tubes sealed with foam blocks. The foam blocks absorbed any excreta. Mass was monitored hourly for 6 h to estimate the rate of mass loss and the influence of crop and gut contents on body mass. Mass change due to excretion was estimated by weighing the foam blocks. A second group of bees (n=40) was used to establish the relationship between body water volume and body mass. They were treated as before but killed after 3 h and desiccated to constant mass for 48 h at 70 °C. The 3 h delay allowed for excretion of the gut contents.

The doubly labelled water technique

Isotope administration and withdrawal

Bees were injected directly into the haemolymph with 1 μl of 20 atom% excess $D_2{}^{18}O$ using a 2 μl Hamilton syringe equipped with a fine glass tip and a 'Chaney adapter' for repeatable volumes. The injection site was the intersegmental membrane between the third and fourth abdominal segments. The injection wound was not sealed but we used only bees which did not 'bleed' after injection (bleeding occurred in about 10 % of the animals). The success of the isotope

application was investigated by predicting initial isotope concentration from the previously derived body water volume and the known volume of injected isotopes. Repeatability and accuracy of the 'Chaney adapter' were checked by weighing (n=31, mean ± S.D.=1.0±0.03 mg). We used 5 μl microcapillaries with extended tips to withdraw an initial blood sample after equilibration. Capillary forces were usually strong enough to obtain a volume of 1–2 μl. The microcapillaries were flame-sealed (Tatner and Bryant, 1989) and refrigerated for later preparation and isotope analysis. For initial isotope concentrations, only ^{18}O was analysed by mass spectrometry; deuterium concentration was estimated from previously prepared standard dilution curves. To obtain the final blood sample at the end of the experiment, the bees were killed and the thorax and abdomen were separated and flame-sealed in soda glass tubes as before. For all experiments involving measurements of isotope concentration, additional bees were collected and the thorax was flame-sealed to analyse natural ^{18}O and deuterium levels.

Equilibration time

To examine the time required for the injected isotopes to equilibrate with the body water, bees (n=44) were taken out of the colony, starved for 3 h and injected with DLW as before. Six bees were killed by dissection through the petiole after 2 min, seven were killed after 5 min, six after 10 min, seven after 15 min, and six each after 30, 45 and 60 min. The thorax and abdomen were immediately flame-sealed in separate soda glass tubes. The difference in isotope concentrations between thorax and abdomen was then used to estimate the duration of the equilibration period. Approximately half the bees (n=23) were analysed for ^{18}O concentration and the remaining bees (n=21) for deuterium concentration. All samples were analysed in duplicate.

Sample preparation and isotope analysis

The procedure will be described in detail in a future paper (S. Davis, M. J. Feltham, T. J. Wolf and C. P. Ellington, in preparation), so we will give only a brief description of the techniques and equipment used. Additional information on the basic procedures and chemical reactions involved in the preparation of CO_2 and hydrogen gases is given by Tatner and Bryant (1989). We used specially constructed 'breakers' to break the soda glass tubes containing the thorax (abdomens were retained as back-up samples, with the exception of the equilibration study) under high vacuum ($\leqslant 10^{-3}$ Pa). Thoracic water was extracted under high vacuum and condensed into liquid nitrogen cold traps.

To determine the ^{18}O isotopic composition, samples were converted from H_2O to CO_2 for isotope ratio mass spectrometer (IRMS) analysis using the guanidine hydrochloride method (Wong *et al.* 1987). Following preparation (carried out as for the entire thorax) and cryogenic purification, the CO_2 samples were admitted directly to the inlet manifold of a VG SIRA 12 mass spectrometer for standard IRMS analysis.

Hydrogen gas was evolved from samples using the zinc reduction technique (Tanweer *et al.* 1988). In the present study, reduction was achieved using an experimental zinc reagent developed by Indiana University Geological Department. The samples were transferred under vacuum to a 6 mm 'Vycor' tube containing 20 mg of zinc per 1 μl of sample water. The reduction process was catalysed by placing the 'Vycor' tube into a muffle furnace at 500 °C for 30 min, and the evolved hydrogen gas was released under vacuum into a borosilicate gas collection vessel. The gas sample was then admitted directly to a VG SIRA 602 mass spectrometer. All isotope concentrations (expressed as delta values) were corrected against two international standards, V-SMOW (Vienna-Standard Mean Ocean Water) and SLAP (Standard Light Arctic Precipitation) according to Gonfiantini (1984), and corrected to p.p.m.

Fractionation during thoracic water extraction

Two different approaches were used to obtain initial and final haemolymph samples. Initial samples were obtained using abdominal withdrawal of small haemolyph volumes with microcapillaries; final samples were obtained by total thorax water extraction under high vacuum. If high-vacuum water extraction is incomplete, fractionation during extraction could lead to underestimates of isotope concentrations because heavier isotopes evaporate at a slower rate. To test the fractionation and the equivalence of the two techniques, bees (n=20) were injected with 1 μl of $D_2{}^{18}O$ and, after equilibration, an initial haemolymph sample was withdrawn from the abdomen using microcapillaries. The bees were killed immediately after sample withdrawal and the thoraces sealed in glass tubes. Thorax water was extracted under high vacuum and half of the bees were analysed for deuterium concentration, the other half for ^{18}O concentration. All samples were analysed in duplicate.

Calculation of CO_2 production rates

CO_2 production rates (r_{CO_2} in mmol h^{-1}) were calculated using equation 35 of Lifson and McClintock (1966), which corrects for physical fractionation assuming an ambient temperature (T_a) of 25 °C:

$$r_{CO_2} = \frac{N}{2.08}\,(k_O - k_D) - 0.015 k_D N\,, \qquad (1)$$

where N is the body water pool (mmol) and k_O and k_D are the apparent fractional turnover rates (h^{-1}) of the ^{18}O and D isotopes, respectively. The volume of the body water pool N was derived from the arithmetic mean of initial and final body masses (M_{av}, in mg) and the empirically derived relationship between body mass and body water. The value of 2.08 in the denominator of equation 1 is the product of 2 (a stoichiometric factor required because each mole of CO_2 contains oxygen equal to two moles of water) and the fractionation factor f_3=1.04 ($C^{18}O_2$ gas → $H_2{}^{18}O$ fluid; where → denotes phase transitions from gaseous to liquid phases, as here, or *vice*

versa). The value 0.015 represents a fractionation correction for water evaporating from the animal's body, given by

$$\frac{f_2 - f_1}{2f_3}\ \frac{1}{2}\ , \tag{2}$$

where f_1=0.93 (D_2O fluid → D_2O gas) and f_2=0.99 ($H_2{}^{18}O$ fluid → $H_2{}^{18}O$ gas) (Lifson and McClintock, 1966). The fraction 1/2 in equation 2 arises from the assumption that 50 % of the water is lost through evaporation and is thus subject to fractionation.

The apparent ^{18}O fractional turnover rate, k_O, was calculated using:

$$k_O = \frac{\ln(O_i - O_b) - \ln(O_f - O_b)}{t}\ , \tag{3}$$

with O_i and O_f are the initial and final enrichment of ^{18}O (p.p.m.), O_b is the background isotope concentration (p.p.m.) and t is the time (h) between taking the initial and final haemolymph samples. The apparent fractional turnover rate for the hydrogen pool, k_D, was calculated in the same manner.

Individual metabolic rates (ml CO_2 h^{-1}) were calculated using:

$$\dot{V}_{CO_2} = 22.4 r_{CO_2}\ . \tag{4}$$

Respirometry

Foragers leaving the colonies were caught, weighed and injected with DLW as described above. To facilitate handling, the bees were trapped under textile mesh and pinned onto a foam block using dissecting pins for the duration of the equilibration period (10 min). Meanwhile, a small pin was glued onto the thorax. After equilibration, an initial blood sample was withdrawn and the bee was attached *via* the pin to the arm of a rotating flight-mill installed inside a respirometry chamber (volume 2.5 l; see Fig. 1). The telescopic arm of the flight mill could be extended through the chamber porthole (5 cm in diameter), which facilitated initial attachment and feeding. The open porthole was blocked with a foam cube. The bees were fed *ad libitum* with 66 % (w/w) sucrose solution from nectar-filled syringes at the beginning of each experiment and occasionally during the trials to encourage flight. Nectar intake was monitored by weighing the syringes before and after feeding. Flight could also be initiated by movement of the flight arm using a magnet. At the end of the experimental period (4.5–7.5 h) the bee was again weighed, and the thorax and abdomen were separated and sealed in soda glass tubes as previously described.

An open-flow respirometry system (Fig. 1) was used for simultaneous measurements of CO_2 production rates. Room air was scrubbed of CO_2 and water vapour using soda lime and silica gel, respectively, and pumped through the chamber at 1 l min^{-1}. Flow rate was measured using a Honeywell (AWM3300 V) mass-flow sensor, and the pump (ADC type 124) was controlled by a custom-built flow-controller. The high flow rate relative to the volume of the chamber secured short response times. The exit port of the chamber was vented to the room at low resistance, keeping the chamber at atmospheric pressure. Gas samples were drawn from the exit port through an infrared CO_2 gas analyser (ADC 225 Mk3). The reference gas (dry, CO_2-free room air) was drawn directly into the second port of the CO_2 analyser. For calibration at the beginning and end of each experiment, 50 ml of 8±0.08 mol% CO_2 (BOC special gases) was injected into the system using a motorised pump (Harvard '33') at a flow rate of 4 ml min^{-1}. CO_2 levels were monitored on a ÖVE SE 460 chart recorder and integrated graphically to obtain total CO_2 production. Room temperature was controlled at 22–23 °C and ambient pressure monitored regularly. All results are corrected to STPD.

Results

Body water pool and equilibration period

Fig. 2 shows the relationship between body mass and body water content for bumblebees which had been starved for 3 h

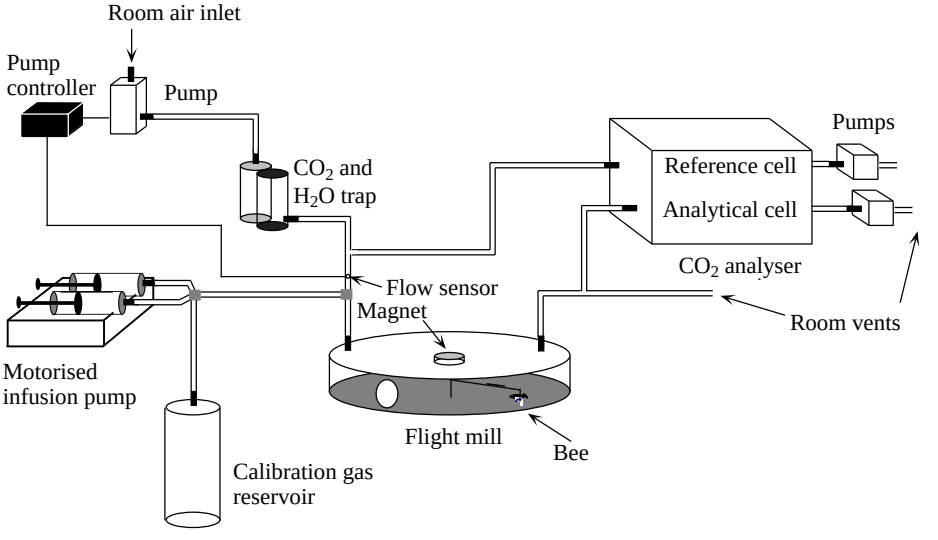

Fig. 1. Experimental arrangement for respirometry used to measure CO_2 production of bumblebees injected with doubly labelled water. The bee is attached to the rotating arm of a flight-mill installed inside a respirometry chamber (for further explanation see text).

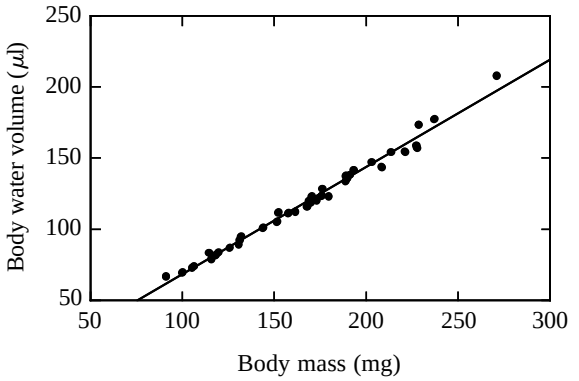

Fig. 2. Relationship between body mass (mg) and body water (μl) for *Bombus terrestris* workers (n=40) starved for 3 h, then killed and desiccated to constant mass for 48 h at 70 °C.

in small glass tubes. Least-squares linear regression analysis gave y=0.755x−7.1 (n=40, r^2=0.99, P<0.001), where y is body water volume (μl) and x is body mass (mg). This regression equation was used in all body water pool estimates for subsequent calculation of DLW CO_2 production rates. The 3 h 'starvation' period had been established beforehand by keeping bees (n=37), taken directly from the colony, separately in small glass tubes and monitoring their mass loss hourly. Any excretion during this period was noted and its mass could be determined by measuring the mass of the foam blocks on which the bees were sitting. Excretion only occurred during the first 3 h of the observation period, and the resulting body mass losses were between 18.9±7.5 % (mean ± S.D.) in the first hour and 14.4±7.5 % in the third hour. We therefore concluded that after a 'starvation' period of 3 h any gut contents left in the bee would not have a significant influence on the relationship between body mass and body water content.

The equilibration of both ^{18}O and D isotopes with the body water was complete 10 min after injection (Table 1). Although 5 min after injection the deuterium concentration was still 47.2 % higher in the abdomen than in the thorax, this difference

Table 1. *Mean percentage differences between abdominal and thoracic ^{18}O and deuterium concentrations at different times (min) after isotope injection (total* n=*44 bees)*

Time (min)*	Mean difference between abdominal and thoracic isotope concentrations (%)			
	Deuterium	*n*	Oxygen-18	*n*
2	45.1±16.3	3	66.9±7.1	3
5	47.2±13.4	3	57.2±11.9	4
10	−1.0±1.1	3	0.4±1.0	3
15	−1.1±0.3	3	0.3±1.2	4
30	−0.6±1.0	3	−2.6±4.1	3
45	−0.2±2.6	3	−0.1±0.3	3
60	−0.8±0.8	3	0.3±0.5	3

Values are means ± S.D.
*Time between isotope injection and initial isotope sample.

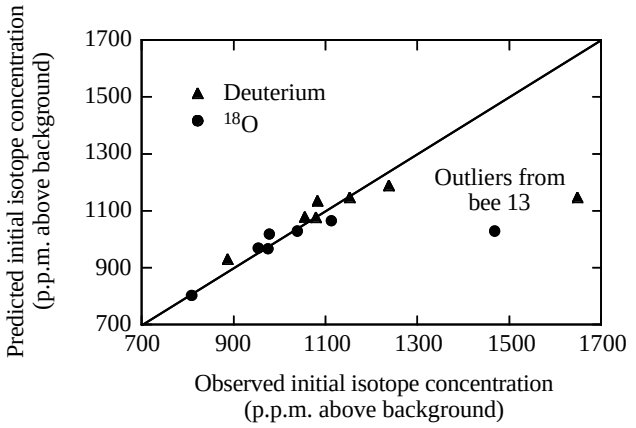

Fig. 3. Relationship between initial ^{18}O (circles) and deuterium (triangles) concentrations predicted from the body mass/body water relationship shown in Fig. 2 and the volume of the injected isotopes (1 μl 20 atom% excess) and those measured using isotope ratio mass spectrometry. The line drawn is the isoline.

had disappeared by 10 min after injection. We found a similar mixing pattern for ^{18}O and concluded that a period of 10 min was sufficient to allow injected isotopes to equilibrate with the body water pool.

Isotope dilution space

Predicted and observed values agreed well for six out of seven bees (Fig. 3). For bee 13, however, predicted ^{18}O and D concentrations were much lower than the measured values. As the deviation was in the same direction and of similar magnitude for both isotopes, we concluded that bee 13 was an outlier (most probably due to injection error) and therefore excluded it from further analysis. For the other bees, the mean (± S.D.) percentage difference between predicted and observed ^{18}O concentrations was −0.1±0.9 % (absolute mean, ignoring signs: 0.7±0.6 %), and the corresponding difference for deuterium was 0.9±2.8 % (absolute mean: 2.3±1.7 %). The data showed no statistically significant directional influence (sign test for ^{18}O, P>0.6; and for D, P=1.0). The inclusion of the magnitude of the deviations did not lead to a different result (Wilcoxon signed-rank test for ^{18}O, z=−0.31, P>0.7; for D, z=−0.52, P>0.6). The higher variation in the deuterium results was possibly due to the lower precision of the mass spectrometer used for the hydrogen ratio analysis (see below).

Fractionation during water extraction

There was no difference in the isotope concentrations of water extracted from abdominal haemolymph (procedure used to obtain initial samples) and of water extracted from the thorax under high vacuum (procedure used to obtain the final samples) (Fig. 4). Isotope concentrations of samples extracted under high vacuum were 0.11±0.2 % (mean ± S.D., n=10) higher in the case of ^{18}O and 0.26±1.11 % (n=10) higher in the case of deuterium than for samples drawn with microcapillaries. There was no directional error, which would have been expected if fractionation had occurred under vacuum

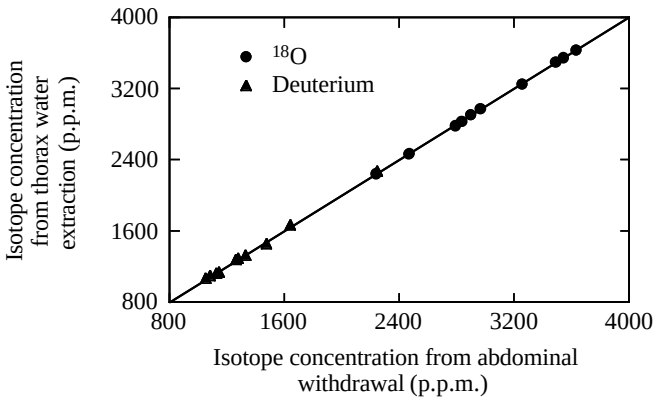

Fig. 4. The relationship between ^{18}O (circles) and deuterium (triangles) concentrations estimated from thoracic water extracted under high vacuum and from abdominal water withdrawn using microcapillaries. The line drawn is the isoline.

extraction (Wilcoxon signed-rank test for ^{18}O, $z=-1.53$, $P>0.1$; for D, $z=-0.97$, $P>0.3$).

Temperature-dependence of fractionation

Body temperature variation in insects can be considerable. Since fractionation is temperature-dependent, we examined the extent to which the numerical values used by Lifson and McClintock (1966) and by the majority of vertebrate validation studies (but see Schoeller *et al.* 1986*a*) can be used over a temperature range between 10 and 50 °C. The ^{18}O isotope fractionation between CO_2 and water is 1.0409 at T_a=25 °C with a variation of only 0.2 ‰ °C^{-1} (Bottinga and Craig, 1969), yielding f_3=1.0379 at 10 °C and f_3=1.046 at 50 °C. Schoeller *et al.* (1986*a*) used data by Pflug *et al.* (1979) to calculate f_3=1.038 at 37 °C, slightly lower than the value calculated from Bottinga and Craig's (1969) data at this temperature (f_3=1.043), but still within the rounding error. Thus, Lifson and McClintock's (1966) value of f_3=1.04 taken from Dole (1949) is appropriate for normal physiological temperatures.

Similarly, f_2 ($H_2{}^{18}O$ fluid $\rightarrow$ $H_2{}^{18}O$ gas) is little influenced by temperature variation. Kirshenbaum (1951) used experimental data to derive a relationship between temperature T (K) and the ratio of vapour pressures for $H_2{}^{16}O$ and $H_2{}^{18}O$. f_2 is equal to that ratio, so his result can be expressed as:

$$\log f_2 = 0.0068 - (3.2/T). \quad (5)$$

At 10 °C and 50 °C, f_2 will be 0.9897 and 0.9929, respectively. This is in close agreement with results from Schoeller *et al.* (1986*a*), who measured a fractionation factor of 0.991 at 37 °C for human respiration. This was close to the value of 0.9925 predicted by these authors from data by Dansgaard (1964). Lifson and McClintock's (1966) value of 0.99 for f_2 can therefore be used over the physiological temperature range.

For f_1 (D_2O fluid $\rightarrow$ D_2O gas), Lifson and McClintock (1966) used a value of 0.93 at T_a=25 °C, which was a compromise between theoretically expected values

Table 2. *Calculation of* f_1 *for low* D_2O *concentrations*

T (°C)	P_{H_2O} (kPa)	P_{D_2O} (kPa)	f_1 (rounded)
10	1.228	1.026	0.91
11–19			0.92*
20	2.339	1.999	0.92
21–29			0.93
30	4.246	3.701	0.93
31			0.93
32–39			0.94
40	7.381	6.549	0.94
41–43			0.94
44–49			0.95
50	12.34	11.12	0.95

Water vapour pressures (P) of H_2O and D_2O at different temperatures (T) are taken from Sengers and Watson (1986) and Matsunaga and Nagashima (1983), respectively.

*Intermediate values interpolated.

(Kirshenbaum, 1951) and experimental results from Wahl and Urey (1935; Table 1.15 in Kirshenbaum, 1951). We recalculated f_1 (Table 2) using more recent data from Matsunaga and Nagashima (1983) for D_2O and Sengers and Watson (1986) for H_2O. For low concentrations of deuterium, it was assumed that $P_{HDO}/P_{H_2O}=(P_{D_2O}/P_{H_2O})^{1/2}$, following Kirshenbaum (1951). The value for f_1 is 0.93 at T_a=25 °C, as used by Lifson and McClintock (1966), but f_1 is more temperature-dependent than are f_2 and f_3. At T_a=23 °C (this study), flying bumblebees have a thoracic temperature of 34 °C (Heinrich, 1972). At this temperature, f_1 increases to 0.94, which reduces the numerical value of the correction term in equation 1 from 0.015 to 0.012. The net effect on the calculations is that an initial error of ±1 % in f_1 will lead to errors of ±1.2 % in the final CO_2 production rates (see Table 4). Of course, this error will increase if the percentage of water lost through evaporation is higher than the 50 % assumed in our simulations.

The radioactive isotope tritium can be used as a hydrogen marker instead of deuterium, as used in the present study. Sepall and Mason (1960) measured some fractionation factors over a temperature range from 0 to 90 °C, and there are four values available for the temperature range considered here. A linear regression fitted to the values gave rounded f_1 values of 0.90 at temperatures between 11 °C and 18 °C, 0.91 between 19 °C and 24 °C, 0.92 between 25 °C and 32 °C, 0.93 between 33 °C and 39 °C, and 0.94 between 40 °C and 46 °C.

DLW validation

Flights were not continuous and were interrupted by periods of different durations. This resulted in mass-specific metabolic rates of only 20–50 % of those measured for free bumblebee flight (Ellington *et al.* 1990). Nevertheless, the metabolic rate was high enough to generate sufficient isotope turnover rates for the purpose of comparison with respirometric

measurements (RESP) of CO_2 production. We adjusted Lifson and McClintock's (1966) original equation (see also Materials and methods):

$$\dot{V}_{CO_2} = 22.4\ \frac{N}{2.08}\ (k_O - k_D) - 0.015 k_D N\,, \quad (DLW_1)$$

taking into account the higher fractionation factor (f_1=0.94) to calculate metabolic rates (ml CO_2 h^{-1}) as follows:

$$\dot{V}_{CO_2} = 22.4\ \frac{N}{2.08}\ (k_O - k_D) - 0.0125 k_D N\,. \quad (DLW_2)$$

All isotope concentrations (except initial deuterium levels) were obtained from analysis of duplicate initial and final haemolymph samples.

Differences between equation DLW_2 (using f_1=0.94) and RESP (Table 3) ranged from −8.4 % to +25.5 %, but in only three out of the 16 experiments did they differ by more than 10 %. The use of equation DLW_1 [with f_1 equal to 0.93 as in Lifson and McClintock's (1966) original equation] resulted in a slight decrease of the mean arithmetic difference from 3.1 % to 2.3 %. There was a highly significant correlation between the DLW and respirometric results (Fig. 5), and the slope of the regression was statistically not significantly different from 1 (95 % confidence limits of the slope are 0.75–1.01). There was no statistical difference between DLW_2 results (x) and RESP (y) [paired t-test; n=16, mean ($x-y$)=−0.09, t=−0.19, P>0.5].

Precision of IRMS results and sensitivity analysis

Precision of the IRMS analysis was generally very high, but better for ^{18}O than for deuterium. The lower precision of the deuterium analysis is indicated by the higher average

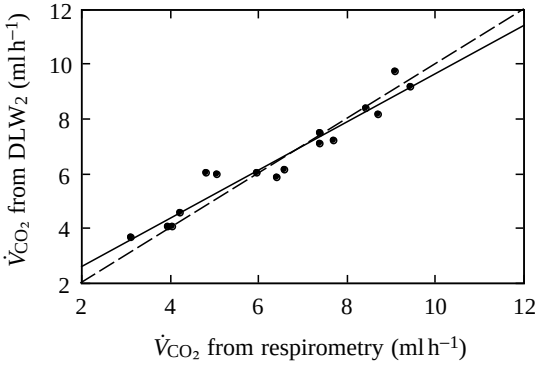

Fig. 5. The relationship between CO_2 production as measured by respirometry (x) and DLW (y). The DLW values were calculated using equation DLW_2 which incorporates a higher value than that used by Lifson and McClintock (1966) for one of the fractionation factors (f_1=0.94). The broken line is the isoline. The solid line is the least-squares regression line (y=0.86+0.88x; r^2=0.93, P<0.001).

Table 3. *Metabolic rates measured by respirometry (RESP) and the doubly labelled water (DLW) technique, using mean body mass to calculate the body water pool* N *(mmol)*

Bee	Initial body mass (mg)	Average body mass (mg)*	Turnover time for DLW (h)†	$\dot{V}_{CO_2}$ (ml h^{-1}) RESP	DLW_1	DLW_2	Percentage difference, DLW_1/RESP	Percentage difference, DLW_2/RESP
1	231.4	248.4	5.30	8.42	8.34	8.37	–1.0	–0.6
2	193.6	213.6	4.50	4.80	6.00	6.02	25.0	25.5
3	237.1	247.9	4.84	4.04	4.04	4.08	0	0.9
4	259.9	273.3	5.50	3.94	4.05	4.06	2.8	2.9
5	243.7	250.0	5.54	7.38	7.07	7.09	–4.2	–3.9
6	244.9	257.1	5.23	9.43	8.92	9.18	–5.4	–2.6
7	205.4	228.4	7.23	5.95	6.02	6.04	1.2	1.5
8	226.5	252.9	6.97	7.70	7.12	7.21	–7.5	–6.4
9	211.3	223.9	6.49	6.58	6.11	6.14	–7.2	–6.6
10	185.9	190.5	6.69	3.11	3.62	3.65	16.4	17.3
11	272.4	270.1	7.54	6.40	5.78	5.86	–9.7	–8.4
12	238.8	294.3	6.54	9.10	9.70	9.76	6.6	7.3
13	266.9	281.8	7.37	8.69	8.10	8.15	–6.8	–6.2
14	234.7	282.5	5.44	7.38	7.42	7.48	0.6	1.5
15	207.8	228.6	5.22	4.20	4.56	4.58	8.6	9.1
16	259.9	292.0	7.49	5.06	5.92	5.96	17.0	17.8
Mean ± S.D.	232.5±26.0	252.2±29.7	6.1±1.0	6.4±2.0	6.4±1.8	6.5±1.4	2.3±10.1	3.1±9.9
Mean absolute difference ± S.D.							7.5±6.9	7.4±7.1

Two equations were used for the DLW calculations (see text): DLW_1 is from Lifson and McClintock (1966, equation 35); DLW_2 is calculated using f_1=0.94 for a thoracic temperature of 34 °C.

*Mean of initial and final body mass.

†Time between initial and final isotope samples.

Table 4. *Magnitude of the output error (as a percentage of $\dot{V}_{CO_2}$) as a consequence of input errors of –5 % to +5 %*

Variable	Value	Percentage input error			
		–5	–1	+1	+5
Body water pool (mmol)	10.93	–5.0	–1.0	+1.0	+5.0
Initial ^{18}O concentration (p.p.m.)	3207.5	–33.9	–7.1	+7.3	+38.8
Initial D concentration (p.p.m.)	1494.8	+15.7	+3.2	–3.2	–16.6
Final ^{18}O concentration (p.p.m.)	2313.5	+86.9	+19.8	–21.3	–128.9
Final D concentration (p.p.m.)	692.9	–19.0	–3.9	+4.0	+20.4
Natural ^{18}O abundance (p.p.m.)	2008.8	–84.5	–13.9	+12.8	+55.2
Natural D abundance (p.p.m.)	203.6	+3.6	+0.7	–0.7	–3.5
Turnover time (h)	7.54	+5.1	+1.1	–1.1	–5.7
Evaporative water loss (%)*	50	+0.4	+0.08	–0.08	–0.4
f_1: D_2O (fluid)→D_2O (gas)	0.94	–6.1	–1.2	+1.2	+6.1
f_2: $H_2{}^{18}O$ (fluid)→$H_2{}^{18}O$ (gas)	0.99	+6.5	+1.3	–1.3	–6.5
f_3: $C^{18}O_2$ (gas)→$H_2{}^{18}O$ (body)	1.04	+4.8	+1.0	–1.0	–5.3

*Percentage of total water loss.

percentage difference between isotope concentrations of replicates from the same sample (data from the DLW validation study only: n=16, ^{18}O=0.06±0.71 %; D=0.12±1.7 %).

Data from bee 11, which are representative, were used to simulate the influence of errors in the measured and assumed variables on errors in the calculation of final CO_2 production rates (Table 4). With the exception of deuterium background levels, 5 % errors in all other isotope concentrations lead to double-figure errors in the metabolic rate. Clearly, careful treatment of the samples (e.g. sealing, water extraction) and accurate isotope analysis are of paramount importance for obtaining reliable data. Accurate values of turnover time and the three fractionation factors were also crucial, but their values could usually be obtained with the required accuracy. Evaporative water loss was the least influential variable, and the assumption that 50 % of the total water is lost through evaporation will lead to considerable errors only in extreme cases. The final input variable to be considered was the volume of the body water pool. Because of uncertainties in the assessment of this variable combined with a moderate influence on the DLW results, we shall discuss these problems in detail below.

Discussion

The bumblebee has the lowest body mass and the highest mass-specific metabolic rate of all species used in DLW validation studies so far (Table 5). Although its body mass is one-third of that of the tenebrionid beetles, the metabolic rates measured during the flight-mill experiments were almost twice as high as those measured for hummingbirds. The accuracy of the DLW technique found in this study is in the range reported from vertebrate validations and much better than those of previous arthropod studies. In two of the three arthropod studies, the DLW method overestimated metabolic rates by more than 20 % (accuracy), and only Buscarlet *et al.* (1978) produced accurate DLW predictions. However, the variation in their data (see Table 5) was quite pronounced.

The accuracy of DLW measurements in the present study allows further use of the technique in field experiments. This could help to liberate the study of insect flight energetics from the restricting and unnatural environment of the laboratory. The energetic assumptions underlying many theories in insect ecology could be tested directly. The small quantities of isotopes needed will help to restrict the costs of such studies, simultaneously allowing larger sample sizes. Recapturing labelled animals will impose little difficulty in species which return to a ‘central place’, such as provisioning bees or wasps, or in species which defend territories, such as male dragonflies. In cases where individuals can be trained to forage in artificial patches (such as honeybee and bumblebee workers), their activities can be monitored throughout the experiment and energetic expenditure directly correlated with activity patterns. The high material turnover did not seem to have influenced the accuracy of the DLW technique. A comparison with studies using vertebrate species with high metabolic rates shows similar accuracy values (Table 5).

Fractionation

Physical (isotopic) fractionation occurs when the heavier isotopes of body water evaporate at a slower rate than the lighter isotopes. As a consequence, final body water concentrations of labelled isotopes might be too high and CO_2 production rates thus underestimated. We used Lifson and McClintock’s (1966) equation 35, which corrects for physical fractionation at T_a=25 °C assuming that 50 % of the water lost from the body is evaporative water loss (EWL=0.5). They demonstrated that even a gross inaccuracy in EWL led to smaller errors in CO_2 production rates than neglect of physical fractionation. We confirmed this by simulations which showed that even if EWL was actually 0.9, the use of 0.5 would overestimate CO_2 production by only 3.0 %. Although the fractionation of $^{18}O/^{16}O$ isotopes is almost temperature-

Table 5. *Overview of DLW validation results from major taxonomic groups (and their smallest members) and from arthropods*

	Mass (g)	Metabolic rate (ml CO_2 g^{-1} h^{-1})	Accuracy (%)*	Absolute error (%)	Source
Mammals[1]	26[a]–70×10[3,b]	0.29[b]–2.94[a]	3.1	6.8	Speakman and Racey (1988)
Birds and reptiles[2]	7[c]–193[d]	0.17[c]–4.3[e]	–2.1	6.1	Speakman and Racey (1988)
Bats	5.6–8.3	10.1[f]	9.5±15.4	14.5±10.2[g]	Speakman and Racey (1988)
Hummingbirds	4.13	14.4[g]	0.1		Tiebout and Nagy (1991)
Locusts	≈1.2	2.09	–1.6±25.2	20.9±12.1	Buscarlet *et al.* (1978)
Scorpions	<3.0	0.32	36.5±10.8	36.5±10.8	King and Hadley (1979)
Tenebrionid beetles	0.8	0.23–0.46[h]	20.5–67.2[h]	20.5–67.2	Cooper (1983)
Bumblebees	0.25	25.2	3.1±9.9	7.4±7.1	This study

*Percentage difference between results from DLW and the comparative technique (e.g. respirometry), given as the arithmetic mean.
[1]25 studies on eight different species (including 12 studies with humans).
[2]14 studies with 12 different species.
[a]Mass and metabolic rate for mouse (Table 1 in Lifson *et al.* 1955).
[b]Data for adult humans (Table 4 in Schoeller *et al.* 1986*b*).
[c]Data for lizard (Congdon *et al.* 1978).
[d]Mass of kestrel (Masman and Klaassen, 1987).
[e]Metabolic rate for sparrow (Williams, 1985).
[f]Calculated from average value of active metabolism (Table 1 in Speakman and Racey, 1988).
[g]Calculated from Table 1 in Tiebout and Nagy (1991), average daily expenditure.
[h]Dependent on experimental conditions (temperature and relative humidity).

independent, there is more influence on the D/H system (Table 2). At temperatures between 21 and 31 °C the value of 0.93 for f_1, as originally used by Lifson and McClintock (1966), is appropriate, but at higher temperatures (32–43 °C) 0.94 should be used. The use of the higher value resulted in a slightly larger overestimation of metabolic rate by DLW compared with RESP (Table 3).

The three previous arthropod studies (King and Hadley, 1979; Cooper, 1983; Buscarlet *et al.* 1978) used tritium instead of deuterium. Tritium has a higher fractionation than deuterium (Sepall and Mason, 1960), and none of the studies corrected for fractionation, both of which are likely to have contributed substantially to the large overestimates of DLW in two of these three studies (King and Hadley, 1979; Cooper, 1983). Only Buscarlet *et al.* (1978) did not report a consistent DLW overestimate.

We found no indication of physical fractionation during thoracic water extraction when we compared isotope concentrations of samples from thoracic water extraction with those from microcapillary withdrawals (Fig. 4). This result can also be used to demonstrate that ambient water vapour adsorbed onto thoracic hair did not dilute isotope concentration and therefore did not bias our validation results. This might be expected, given the dry air and high flow rates in our respirometry chamber. However, external water adsorption should be considered in field experiments, especially in the tropics or in conditions of rapidly changing humidity.

Biological fractionation can also occur if rates of water influx and efflux are high relative to the mixing in the blood circulation. In bumblebees, for example, if water absorption rates through the midgut wall and excretion rates *via* the Malpighian tubules are both high, a low haemolymph circulation rate could result in incomplete isotope mixing after injection. Lifson and McClintock (1966) address this problem and suggest correction terms provided that the specific isotope activities in different body parts are determined. This has not been carried out to date and would be difficult to achieve in bees. The nectivorous hummingbirds used by Tiebout and Nagy (1991) have very short food transit times (Tiebout, 1989) involving possible active transport of glucose *via* the intestinal wall (Diamond *et al.* 1986). Tiebout and Nagy (1991) did not have direct evidence for complete isotope mixing, but concluded from their results that isotope turnover appeared not to have been affected by dynamic biological effects.

Bees also use carbohydrates to fuel their flight metabolism. Experiments with honeybees (*Apis mellifera*) showed that absorption takes place in the proximal two-thirds of the midgut (Crailsheim, 1988*a*) and involves only passive diffusion, which nevertheless can be very rapid. In hungry honeybees, 50 % of the glucose available in the midgut was absorbed after 5 min (Crailsheim, 1988*b*). Using ^{14}C markers, Crailsheim (1985) also showed that complete haemolymph mixing was accomplished after 2–5 min at a T_a of 22–25 °C. This is driven by very high pulsation rates of the heart, a dorsal vessel which pumps haemolymph from the abdomen into the head (Snodgrass, 1956). At a thoracic temperature of 35 °C, Heinrich (1980) measured rates of 200–450 beats min^{-1} in honeybees and 350–600 beats min^{-1} in bumblebees (Heinrich, 1976). Therefore, rapid diffusion of carbohydrates (and water) into the haemolymph is accompanied by rapid haemolymph mixing, which makes the influence of biological fractionation on DLW results relatively unlikely. Furthermore, the close agreement of the validation results suggests that biological fractionation is not a significant problem.

Isotope equilibration

Injected isotopes must be equilibrated with the body water before initial haemolymph samples can be withdrawn. 10 min after injection, we found no difference between thoracic and abdominal isotope concentations using total water extraction. Although this does not test for equilibration between extra- and intracellular compartments, we found no difference in isotope concentrations between abdominal haemolymph samples and samples from total thoracic water extraction (see Fig. 4). This indicates that both haemolymph and cellular mixing must be complete 10 min after injection. Furthermore, Crailsheim (1988*c*) showed that honeybees, fed glucose after flight to exhaustion, were able to resume flight 5 min after feeding. Thus, glucose was not only transported from the gut to the haemolymph but also from the haemolymph into the cells within a short period. It is therefore very probable that isotope mixing between extra- and intracellular spaces is a very fast process.

Body mass and its influence on the validation results

Bumblebee body mass varies widely both between and within individuals. The former variation is a life-history characteristic widespread among bumblebee species, but the estimation of body water from body mass is accurate provided that the crop is empty. Crop contents are one major source of within-individual body mass variation. The foragers used for the flight-mill experiments were captured upon leaving the colony for a foraging flight and weighed without a prior starvation period. This approach was used because it proved impractical, even in the laboratory, to postpone isotope injection for 3 h, and in field experiments it would be impossible. To test body mass variation in our 'validation bees', marked foragers leaving the colony were weighed on consecutive days before the validation experiment. We collected data from three out of the 16 bees used. Bee 7, for example, weighed 203, 210 and 209 mg (mean ± S.D.=207.3±3.8 mg) on three consecutive days, which is close to the exit mass on the validation day (205.4 mg; Table 3). We starved this bee for 3 h on the day before the experiment, and its mass decreased from 209 mg to 205 mg. Data for the other two bees are similar, showing that the inaccuracy in initial body mass (and hence in initial body water pool) is small if measured at the start of a foraging trip.

The body mass at the end of the experiment, and at the end of a foraging trip, will differ from the initial value as a result of variations in crop content and the occurrence of excretion, which is the other main source of within-individual variation. These factors are difficult enough to assess in the laboratory and impossible in the field. At the end of the experiments, the final mass of our bees was typically 17.0±12.7 % greater than the initial value. Thus, the average body water pool *N*, which was calculated from the mean of the initial and final body masses, was almost certainly overestimated because of partially filled crops and stored excreta. We therefore also calculated DLW metabolic rates using *N* calculated from the initial body mass only. This led to a −6.0±9.3 % underestimate of metabolic rate by the DLW technique using Lifson and McClintock's equation (1966) with f_1=0.93, and to a −5.2±9.5 % difference using f_1=0.94 instead. The corresponding mean absolute differences are 9.7±4.9 % (f_1=0.93) and 9.2±4.9 % (f_1=0.94). Thus, deriving *N* from initial instead of average body mass alters the differences between RESP and DLW_2 (using f_1=0.94) from +3.1 % to −5.2 %, a decline of 8.3 %. This is in good agreement with the results of the error simulations for the body water pool (Table 4). We stated above that initial and final body mass typically differed by 17.0 %, and thus the initial body mass was 8.5 % lower than the average mass. From Table 4 it can be seen that a change in the initial body water pool results in a change in the final DLW metabolic rates of the same direction and magnitude. Thus, an 8.5 % smaller body water pool will decrease the average DLW results by 8.5 % and, consequently, differences between RESP and DLW will change by −8.5 %: the difference between calculated (−8.3 %) and predicted (−8.5 %) changes is due to rounding errors.

To test the influence of an underestimate or overestimate of *N* on the validation results, we calculated a nominal body mass (M_0) that gave complete agreement between metabolic rates measured by DLW and RESP. In 12 out of 16 cases, M_0 lies between the initial and final body mass. There are only four bees for which we cannot conclude that a difference in the results from the two techniques might be due to body mass variation.

Water flux rates and constant body water volume

Body water volume (*N*) and body water flux rate (r_{H_2O}) are likely to influence the calculation of CO_2 production rates (r_{CO_2}) to different degrees. Lifson and McClintock (1966) showed that r_{CO_2} is not directly affected by variability in r_{H_2O}, because the turnover in both the oxygen and the hydrogen labels is influenced in the same way, and thus the difference in turnover rate remains unaffected. There was no influence of r_{H_2O} on DLW accuracy in our study; i.e. we did not find a correlation between deuterium fractional turnover rates and DLW accuracy (r^2=0.31, d.f.=14, P>0.2). Moreover, the relative importance of water turnover and CO_2 production rates in bumblebees is similar to that in birds. Tatner and Bryant (1989) compared average fractional isotope turnover rates in breeding birds (mass range 11.7–410.4 g) and concluded that the average deuterium fractional turnover rate was 79.4 % of the average ^{18}O turnover rate. In our study (mass range 190–295 mg), this figure was 76.6 %. Thus, average water turnover remains a constant fraction of ^{18}O turnover not only in birds (irrespective of body mass) but also in bumblebees.

Nagy (1980) simulated errors in CO_2 production rates as a result of changes (constant, linear and exponential) in the body water pool *N* with time. He concluded that the error in *mass-specific* r_{CO_2} exceeds 5 % only when the pool declines by more than 50 % or increases by more than 100 % over the measurement period. Such a weak influence of the pool on mass-specific rates is expected: total r_{CO_2} is proportional to *N* (equation 1), as is body mass, so their ratio must be relatively

independent of changes in *N*. We obtained a similar result for mass-specific rates with our error analysis: a 5 % error in *N* results in only a 0.2 % error in final CO_2 production rates. However, this apparent insensitivity is misleading. Total metabolic rates, not mass-specific values, are required for energy budgets. Any errors in the body water pool will affect the total rate by the same proportion.

The influence of body mass on the validation results was considered in detail above. Following convention, we used the average mass to calculate *N*. It was concluded that the final body mass overestimated the body water pool because of partially filled crops and stored excreta, and that use of the average mass resulted in high DLW results compared with RESP. The initial mass upon leaving the colony was relatively constant, because crops were empty, but estimating *N* from the initial mass led to underestimates for the DLW results. It was shown that complete agreement between DLW and RESP values could be obtained in most cases for a nominal body mass lying between the initial and final values.

From the metabolic rate and the volume of nectar fed, we can estimate the crop contents at the end of the experiments, subtract that from the final mass, and thus obtain a better estimate of the final body water pool. Average initial body mass was 232.5 mg (Table 3). We fed the bees an average of 94.0 mg of 66 % w/w nectar. Using the stoichiometry of carbohydrate metabolism, it can be calculated that the bees used 76.7 mg of the nectar to fuel their flight metabolism. This calculation rests on the assumption that haemolymph glucose levels are maintained by the passage of sugar and its solvent (water) from the crop into the midgut, where they are absorbed into the haemolymph. In honeybees, the food transfer from the crop into the midgut is regulated by the proventriculus (Crailsheim, 1985). The consumption of 76.7 mg of the 94.0 mg fed at the beginning of the flight leaves 17.3 mg of nectar in the crop at the end of the experiments. Because there is no material exchange between the crop and the haemolymph (Crailsheim, 1988*a*), the crop volume does not contribute to the body water pool. Thus, final body mass is 254.6 mg, and the 'new' average body mass 243.5 mg, 3.4 % lower than the one used in our calculations. The DLW accuracy would increase from 3.1 % to −0.3 %. We are aware of the shortcomings of this consideration (e.g. gut contents are not taken into account), but its shows how important accurate body water pool measurements are for successful validation studies in bees. Clearly, more information on body water pool fluctuations as a consequence of metabolic activities are needed.

Dilution space and isotope incorporation

If isotopes are incorporated into non-aqueous tissues, they are effectively removed from the body water pool. This will cause errors in two ways. If the body water pool (*N*) is estimated using the dilution method, rapid isotope incorporation during equilibration will lead to lower initial isotope concentrations and yield an overestimate of *N*. The second possible error concerns the calculation of the isotope turnover rates. If deuterium (or tritium) and ^{18}O are in exchange with non-aqueous tissues, their concentration in the body water pool is dependent not only on water and CO_2 turnover but also on the exchange rates with those tissues.

Nagy and Costa (1980) summarised studies in which the body water pool was estimated using tritiated water dilution and compared with estimates using drying to constant mass. They found that the dilution method overestimated *N* in 22 out of 23 studies and that the mean error could be as high as 13.1 %. They concluded that much of the overestimate was due to tritium incorporation into body compounds: considerable amounts were found in dried tissues of injected individuals. Nagy (1980) showed for three species of mammals that the ^{18}O dilution space provided a better estimate of *N*, with mean errors (as a percentage of *N*) between +0.4 % and 2.1 %. In a hummingbird study (Tiebout and Nagy, 1991), the ^{18}O dilution method overestimated *N* by 4 %. Speakman and Racey (1988) found that ^{18}O dilution space was accurate to less than 1 % for two species of small insectivorous bats. All arthropod studies used tritium instead of deuterium as the hydrogen marker. This might also have contributed to their large overestimates of *N* since tritium seems to be more readily incorporated than deuterium into non-aqueous tissues and therefore lost to the body water pool (Nagy and Costa, 1980). Indeed, Cooper (1983) and King and Hadley (1979) have found considerable tritium incorporation, whereas Buscarlet and Proux (1975) showed that tritium incorporation in locusts was negligible.

When isotope incorporation occurs, that of the hydrogen label is more pronounced than that of the oxygen label. For this reason, Schoeller *et al.* (1986*b*) proposed equations for the calculation of CO_2 rates where correction factors take account of the different isotope dilution spaces. We found that neither ^{18}O dilution space (0.1 % lower) nor deuterium dilution space (0.9 % higher) was significantly different from the body water pool (Fig. 3) and therefore used Lifson and McClintock's (1966) equation which does not correct for isotope incorporation.

A slow exchange of isotopes between the body water pool and non-aqueous tissues could overestimate CO_2 production if the exchange rate of the hydrogen label is higher than that of oxygen. Most studies have used tritium exchange and present varying results. Although Buscarlet and Proux (1975) did not detect slow tritium tissue binding in locusts (*Locusta migratoria*), it was found in scorpions (King, 1976; cited in Nagy and Costa, 1980). We do not know to what extent this process might have influenced our results, but the lack of any directional error in the DLW estimate makes this an unlikely source of inaccuracy.

The single-sample approach

A single-sample validation study dispenses with the withdrawal of an initial blood sample and relies instead on predicting initial isotope concentrations. Tiebout and Nagy (1991), for example, predicted initial concentrations for their hummingbird study from separate experiments on the body mass/body water relationship and on the relationship between

the amount of injected isotopes and body mass. This approach is used to prevent stress in sensitive species during isotope equilibration, during which individuals are restrained, or where animals are difficult to recapture after equilibration. Additionally, the sample volume may represent an unacceptably large fraction of the total blood volume (Webster and Weathers, 1989). Our bees did not seem to be affected by haemolymph withdrawal, but their small haemolymph volume led us to examine the feasibility of the single-sample approach. Fig. 2 shows that there is a good relationship between body mass and body water content. Furthermore, the accuracy and repeatability of isotope injection were high. However, the results of the experiment to examine the isotope dilution space (Fig. 3) show the unreliability of the single-sample approach. Predicted and observed initial isotope levels agreed well in only six out of the seven bees. The results for bee 13 are completely discordant with the others, and we can only speculate about the reasons for this. Predicted concentrations could have been too low because of an error in body mass measurement or, as is more likely, the observed values could be too high because the injected DLW volume was larger in this case. Whatever the reasons, without a control measurement for the initial isotope level(s), the results, especially from field experiments, might always be doubtful. Furthermore, even a small difference between estimated and actual initial isotope concentrations might lead to considerable errors in the calculation of r_{CO_2} because of the magnifying effect of errors in initial isotope concentration (Table 4).

We therefore used a compromise between the single- and double-sample approaches by withdrawing a small initial sample and analysing only one of the isotopes. We chose ^{18}O analysis for two reasons: first, the accuracy of that mass spectrometer was higher, and second, the effect of an initial error on final CO_2 production is much smaller for deuterium than for ^{18}O. There is an error inherent in this approach, because deuterium concentration was estimated using standard dilutions, whereas during equilibration ^{18}O isotopes are lost at a higher rate than deuterium isotopes. This might have led to an underestimate of initial deuterium concentration, but this inaccuracy was probably small because of very short equilibration times.

Carbonic anhydrase

Carbonic anhydrase catalyses the reversible reaction $H_2O + CO_2 \leftrightarrow H^+ + HCO_3^-$ and is involved in maintaining the isotopic equilibrium of oxygen in CO_2 and body water. Indeed, the discovery of the equilibrium in mice and rats (Lifson *et al.* 1949) led to the development of the DLW technique. The discovery of carbonic anhydrase by Meldrum and Roughton (1933) was followed by its detection in many other systems, ranging from bacteria to plants and mammals (Maren, 1967; Carter, 1972). There seems to be no carbonic anhydrase in arthropod blood (Maren, 1967), and in insects the enzyme seems to be of greater importance to larvae and pupae than to adults (Darlington *et al.* 1985). However, Edwards and Patton (1967) localised carbonic anhydrase in honeybee flight muscles. We do not know whether an uncatalysed reaction might be sufficient to account for our results, but the enzyme also seems to be active in bumblebee flight muscles. The overestimate of DLW CO_2 production rates reported by Cooper (1983) and King and Hadley (1978) cannot be explained by incomplete exchange of ^{18}O atoms between CO_2 and H_2O molecules. In such a case, ^{18}O would mainly be lost through water, and the difference between final ^{18}O and deuterium concentrations would be small, which would result in an underestimate rather than an overestimate of CO_2 production rates.

In conclusion, despite the small body size and high material turnover rates of flying bumblebees, the DLW technique produced estimates of metabolic rates which were only 3.1±9.9 % higher than respirometric measurements. Furthermore, we were able to demonstrate that at least part of this overestimate was due to an overestimate of the body water pool. Very small amounts of isotopes are needed, and the costs of field studies can be kept to a minimum. Turnover times of a few hours will allow energetic studies to be carried out over a short period, which is impossible for all but the smallest vertebrates.

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