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METABOLIC AND BEHAVIOURAL CONSEQUENCES OF THE PROCEDURES OF THE DOUBLY LABELLED WATER TECHNIQUE ON WHITE (MF1) MICE

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Summary

The effects of the doubly labelled water technique (intraperitoneal injection, temporary food deprivation and blood sampling) on the energy expenditure, food intake and behaviour of 18 white (MF1) mice was investigated.

There were no significant differences in mean energy expenditure or food intake between experimental and control animals, on which the techniques were not performed, over the first 24 h after manipulation. These data indicate that there are no direct metabolic consequences associated with the procedures.

During the 100 min immediately after blood sampling, the behaviour of experimental animals involved significantly more grooming, mostly at the site of the blood sample wound, more feeding and more general activity, at the expense of resting, when compared with controls. Twenty hours later the behavioural differences were less marked, but still statistically significantly different, and reversed: experimental animals spent more time resting and less in general activity or feeding.

The effects of the technique on the behaviour of white mice had trivial consequences for their daily energy expenditure. This may reflect the restricted behavioural repertoire of these captive animals within respirometry chambers. The effect on wild animals may be more profound and requires investigation.

Introduction

The doubly labelled water (DLW) technique is a non-invasive method for measuring the CO_2 production and hence energy expenditure of free-living animals (Lifson and McClintock, 1966; Nagy, 1980; Speakman and Racey, 1988*a*). The technique depends on the fact that the oxygen in respiratory carbon dioxide comes rapidly to isotopic exchange equilibrium with the oxygen in body water. A measure of CO_2 production is therefore possible from the difference in turnover of two isotopic labels, one of oxygen and one of hydrogen, which are introduced into the body water. Validation studies, in which the estimate of CO_2 production is

Key words: energy expenditure, oxygen consumption, respiratory quotient, blood sampling, mouse.

compared with simultaneous indirect calorimetry (reviewed in Speakman and Racey, 1988*b*), indicate that the technique has an average accuracy of about 3 % in vertebrates.

The biological usefulness of the information provided by the technique, however, depends not only on its analytical accuracy, but also on the extent to which the procedures of the technique modify the animals' energy expenditure during the measurement period. This might occur in two ways. First, there may be a direct metabolic effect of labelling the body water with the heavy isotopes and, in small animals, of taking a relatively large blood sample (usually 20–100 μl) at the start of the measurement period. Second, the procedures may influence the energy expended indirectly *via* their effects on behaviour. It is already well established that providing animals with deuterated drinking water affects the duration of their circadian activity cycles (Suter and Rawson, 1968; Enright, 1971; Dowse and Palmer, 1972; Daan and Pittendrigh, 1976) and that anaemia may lead to reduction in appetite and hence feeding activity (McDonald *et al.* 1981).

These latter problems have received only minimal attention. Bryant and Westerterp (1983) found that the energy expenditure of a single house martin (*Delichon urbica*) measured overnight differed by only 2 % from that of another individual on which the procedures of the technique had been performed, and concluded that the effects of the procedures were negligible. In this study we explore the effects of the DLW procedures on the behaviour and metabolism of white mice.

The choice of experimental animal for this study was difficult. It could be argued that laboratory mice are acclimatized to handling stress and thus the behavioural response would be lower than in a wild animal. Conversely, it might be argued that the procedures used to assess the effects (respirometry) might themselves be stressful. If the stress of confinement in a chamber exceeded the effects of the DLW procedures, as might be the case for a wild animal, then any effect of the DLW procedures might be swamped. We chose laboratory mice because they are routinely kept in small plastic cages, similar to our respirometry chambers, and the effects of the respirometry would consequently be small relative to the effects of the DLW procedures to which they were completely naive.

Materials and methods

Eighteen white mice (strain MF1) were involved in the experiments. Nine of these were allocated at random to a control group and nine to an experimental group. The control animals were weighed to an accuracy of 0.01 g using a top-pan balance (Sartorius Ltd) and placed into a respirometry chamber with nesting material (a paper towel) and a weighed quantity of between 5 and 7 g of food (CRM rat and mouse pellets). The chamber was immersed in a water bath and the temperature was regulated at 18°C. After 90 min in the chamber, the animals' behaviour was recorded at 1 min intervals for 100 min. Behaviour was recorded as one of the following classes: resting, grooming, feeding and non-specific activity –

for example, moving around or rearranging the nesting material. Subjects remained in the chambers for a further 24 h, 12 h of which were spent in darkness. Approximately 20 h after the first set of behavioural observations, a second set of behavioural observations were made. After 25.5 h in the chamber the subjects were removed and reweighed. The remaining food and nesting material and faeces produced were collected and separated. The food and faeces were then dried at 60 °C for 14 days to obtain the dry masses. The experimental animals were weighed using a top-pan balance and then placed in a cotton bag, routinely used to transport small mammals in the field, and weighed using a field spring balance (Pesola). After removal from the bag the mice were injected intraperitoneally with approximately 0.4 ml of a mixture of ^{2}H (10 atom percent excess) and ^{18}O (19.5 atom percent excess) in water. The subjects were then replaced in the cotton bags and left in a quiet room for 90 min to allow the isotopes to reach equilibrium in the body water. After this period, the subjects were removed and a blood sample (approx. 100 μl) was collected by puncturing the tail vein. The animal was then placed into a respirometry chamber containing both nesting material and food. Behaviour of the animal was recorded at 1 min intervals over the initial 100 min in the chamber, using the same catagories as for controls. By delaying the start of the behaviour monitoring of the controls for 90 min, we ensured that the time from initial handling to remove the animals from their cages, to the start of behaviour monitoring, was the same for both groups. After these initial differences, experimental animals were treated exactly as controls.

The respirometry system was identical to that described in Racey and Speakman (1987) and Speakman and Racey (1988*b*) and consisted of a respirometry chamber in series with a CO_2 analyser (Lira 3000: Mine Safety Instruments Ltd) and a dual-channel oxygen analyser (Servomex OM 184: Taylor Servomex Ltd). One channel of the analyser continuously measured the oxygen content of air entering the chamber and the second channel monitored the exhaust stream. Voltage outputs from the analysers and a high-precision flowmeter (A. Wrights Ltd) were sampled at roughly 40 ms intervals and digitized using the A/D convertor (μPD 7002) of a BBC microcomputer (Acorn Computers Ltd). The accumulated digitized data were averaged over a preset interval (3–12 min) and stored directly on disc. At the time these measurements were made some problems were experienced with the data storage and only seven of the control runs and six of the experimental runs were saved in their entirety. We have restricted our analysis of the metabolic responses to these 13 complete data sets. Energy expenditures were evaluated using oxycalorific coefficients derived from the measured respiratory quotients (RQs) for each individual (Lusk, 1926).

Results

Energy expenditure and food intake

The food intake and faecal production of all the animals are summarized in Table 1. Net consumption of food averaged 2.065±0.654 g (S.D., N=9) in the

Table 1. *Food intake, faecal production, assimilation efficiency and net food intake (intake minus faeces) of control mice and experimental mice on which procedures of the double labelled water technique had been performed prior to placing them in the respirometers, over the time spent in the respirometers*

	Animal number								
	1	2	3	4	5	6	7	8	9
Control animals									
Food intake (dry g)	3.8	3.5	4.2	1.6	1.9	2.8	2.6	3.2	3.2
Faecal production (dry g)	1.1	1.2	1.6	0.8	0.7	0.7	0.8	0.9	0.6
Assimilation efficiency (%)	70.2	66.8	62.4	50.0	64.7	75.3	69.1	72.9	82.6
Net food intake (food minus faeces) (dry g)	2.7	2.3	2.6	0.8	1.2	2.1	1.8	2.3	2.6
Experimental animals									
Food intake (dry g)	3.8	3.2	3.1	3.8	2.6	3.0	3.7	3.2	3.4
Faecal production (dry g)	0.9	1.2	0.7	1.3	1.5	0.6	0.8	0.5	0.8
Assimilation efficiency (%)	76.1	61.9	76.2	64.2	40.7	79.5	83.5	85.3	76.9
Net food intake (food minus faeces) (dry g)	2.9	2.0	2.4	2.4	1.1	2.4	3.1	2.7	2.6

controls and 2.387±0.595 g (S.D., $N=9$) in the experimental group, over the time spent in the chambers. Although this represents an increase in consumption by the experimental group of 15.6% over the controls, the wide degree of individual variation in consumption, reflected in the high coefficients of variation (31.6% in controls and 24.9% in experimental animals), meant that the difference was not significant ($t=1.09$, $P=0.29$, d.f.=16). Mean oxygen consumption, RQ and the calculated energy expenditure from indirect calorimetry for all the animals for which complete energy expenditure records were available are shown in Table 2.

Across all individuals there was a significant positive relationship between the average energy expenditure over the 24 h and the average body mass of the individual (Fig. 1). The least-squares regression, energy expenditure (W)= $0.3605+0.0113M$, where M is the body mass in grams, explained 72.5% of the variability in energy expenditure. Logarithmic transformation of either or both variables did not improve the fit. Energy expenditures were corrected to a standard mass (43.3 g) to remove the mass effect prior to analysis for the effects of the treatment variable (after Packard and Boardman, 1987). All subsequent analyses refer to mass-corrected data. Once the effects of mass had been removed, there was no significant difference in the average energy expenditure over the 24 h between the control and experimental groups [control, mean=0.861±0.019 W

Table 2. *Oxygen consumption, respiratory quotient, energy expenditure and body masses of control and experimental mice averaged over the 24 h spent in the respirometers*

	Animal number						
	1	2	3	4	5	6	7
Control animals							
Mean oxygen consumption ($ml\,min^{-1}$)	2.66	2.61	2.39	2.48	2.26	2.27	2.14
S.E.	0.02	0.02	0.03	0.02	0.07	0.07	0.10
Mean RQ	0.65	1.01	0.90	0.83	0.72	0.76	0.73
S.E.	0.01	0.01	0.01	0.01	0.04	0.02	0.03
Mean energy expenditure (W)	0.87	0.92	0.82	0.84	0.74	0.76	0.73
N	466	431	429	455	101	112	103
Body mass (g)	42.4	46.2	39.5	41.8	33.5	33.9	34.2
Experimental animals							
Mean oxygen consumption ($ml\,min^{-1}$)	2.32	2.75	2.44	2.17	3.01	2.62	
S.E.	0.01	0.02	0.02	0.02	0.03	0.02	
Mean RQ	0.67	0.75	0.74	0.83	0.71	0.72	
S.E.	0.01	0.01	0.01	0.01	0.01	0.01	
Mean energy expenditure (W)	0.76	0.92	0.81	0.73	0.99	0.86	
N	442	468	437	464	353	363	
Body mass (g)	43.6	45.3	40.9	38.4	56.2	38.7	

The duration of measurement for controls starts 1.5 h after entry into the chambers.
N values refer to numbers of readings taken over 24 h at intervals of between 3 and 12 min.
RQ, respiratory quotient.

(S.D., $N=7$) experimental, mean=0.839±0.06 W (S.D., $N=6$), t-test, $t=0.93$, $P=0.37$, d.f.=11)].

The energy expenditures of control and experimental groups averaged across all individuals throughout the 24 h experiments are shown in Fig. 2. A two-way analysis of variance revealed that the effects of time and the treatment on energy expenditure were both highly significant and that there was also a significant interaction between the two (F-values for sources of variation: treatment 1.52, $P<0.0001$; time 23.26, $P<0.001$; interaction 9.26, $P<0.0001$). There was no consistent pattern in the different responses of the control and experimental groups and overall the positive differences balanced the negative differences such that throughout the 24 h there was no significant effect. The magnitude of the difference in any hour was, however, small, and only exceeded 10 % of the control value during three of the 24 h.

Behaviour

The behaviour of control and experimental groups of animals, summed across

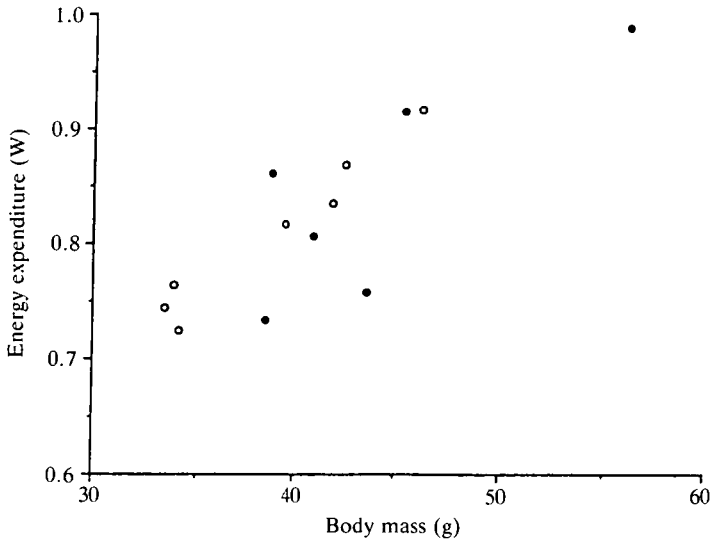

Fig. 1. Mean energy expenditure of white mice over the 24 h spent in the respirometry chambers plotted against body mass. Open symbols represent control animals and closed symbols represent experimental animals on which the procedures of the doubly labelled water technique had been performed prior to entry into the chamber.

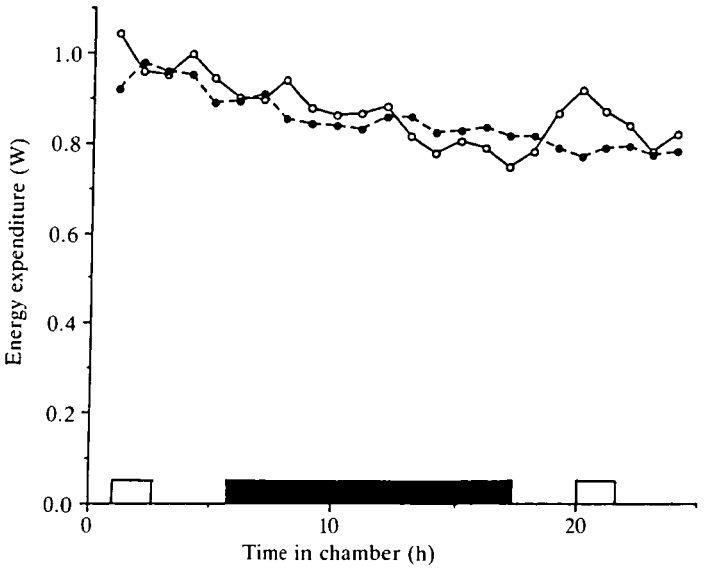

Fig. 2. Mean energy expenditure over 24 h for control (open symbols) and experimental (closed symbols) mice on which the procedures of the doubly labelled water technique had been performed immediately prior to the first measurements. For control animals, entry to the chamber was 1.5 h prior to the first measurements. The open bars on the abscissa indicate the intervals during which behaviour was recorded and the solid bar indicates the time when the lights were out.

Table 3. *Behaviour of control and experimental animals at the start of the measurement period (immediately after bleeding for experimental animals and 1.5 h after entry to the chamber for controls) and 20 h later*

	Control				Experimental			
	All		Restricted		All		Restricted	
	Frequency	%	Frequency	%	Frequency	%	Frequency	%
Initial behaviour								
Resting	537	59.6	404	57.7	313	34.8	245	40.8
Grooming	142	15.8	120	17.1	192	21.3	124	20.6
Feeding	100	11.1	72	10.3	145	16.1	96	16.0
General activity	121	13.4	104	14.8	250	27.8	135	22.5
Behaviour after 20 h								
Resting	670	74.4	540	77.1	777	86.3	524	87.3
Grooming	81	9.0	61	8.7	83	9.2	58	9.7
Feeding	50	5.5	32	4.6	16	1.8	10	1.7
General activity	99	11.1	67	11.2	24	2.7	8	1.3

Values represent summed assignments to given behaviour classes for nine individuals in each group monitored for 100 min each: individuals values are not presented. The average percentage representation of different behaviours is also shown. In addition, the summed assignments and average representation of the classes for a restricted subset, including only the seven control and six experimental animals for which we also had data on energy expenditure, are shown.

all individuals at the start and after 20 h within the respirometry chambers, is summarized in Table 3. The behaviour of both control and experimental groups differed significantly between the start and later in the measurement period (control: start–20 h, $\chi^2=52.6$, $P<0.001$; experimental: start–20 h, $\chi^2=584.4$, $P<0.001$). In general, both control and experimental animals increased the amount of resting behaviour at the expense of all other activities between early and late in the session. In addition, significant differences were apparent between the control and experimental groups both at the start and after 20 h (start: control–experimental, $\chi^2=144.2$, $P<0.001$, 20 h: control–experimental, $\chi^2=71.2$, $P<0.001$). At the start of the session the experimental animals were much more active than controls. This increased activity involved increases in grooming, from 15.8±0.4 % (S.E. based on sampling unit of 900 observations) of the time to 21.3±0.6 %, increased general activity from 13.4±0.4 % to 27.8±0.7 %, and increased feeding from 11.1±0.3 % to 16.1±0.4 %. The large increase in grooming behaviour involved mostly grooming attention focused at the site of the wound where blood had been collected. Control animals were only active for 40.4±0.75 % of the time, compared with 65.2±0.8 % of the time in experimental animals, at the start of the measurement period. After 20 h, the differences between control and experimental animals were reversed: control animals were more active than experimental ones. This activity involved only general activity

which occupied 11.1±0.4% of the time in controls compared with 2.7±0.1% in the experimental group, and feeding which occupied 5.5±0.2% of the time in controls but only 1.8±0.1% of the time in experimental animals. There was no significant difference in grooming behaviour at this later stage of the experiment (9.0±0.3% in controls and 9.2±0.3% in experimental animals). The same significant differences were found if the data were restricted to include only the seven control animals and the six experimental animals for which we had indirect calorimetry evaluations of energy expenditure (Table 3).

Discussion

The absence of a significant effect of the procedures of the doubly labelled water technique on food intake and energy expenditure, in the current study, supports the earlier observation of Bryant and Westerterp (1983) that energy expenditure of a single house martin, on which DLW was used, differed by only 2% from that of another bird on which the procedures were not employed. Taken together the results of the present study and that of Bryant and Westerterp (1983) suggest that there is no direct metabolic effect of the procedures on energy expenditure. However, the absence of an overall difference in the average energy expenditure over the first 24 h of the measurements cannot rule out the possibility of an indirect effect. This is because the absence of an overall effect might reflect the fortuitous consequence of two behavioural effects of the procedures occurring at different times, and their associated energy demands cancelling each other out. In other words, a high initial energy expenditure resulting from disturbance, grooming and feeding might be offset by a later increase in resting behaviour. The directly observed behavioural differences between control and experimental groups support this possibility. The behavioural differences, during the period immediately following collection of blood samples from the experimental subjects, were evidently a direct consequence of the handling procedures. Hence, the increase in grooming involved behaviour directed predominantly at the wound from which blood had been collected. The increased feeding behaviour probably reflected the temporary food deprivation over the 90 min prior to being placed in the chamber. In contrast, the behavioural differences 20 h later suggest that the experimental animals had generally reduced their activity and increased their resting behaviour when compared with controls.

The time course of the energy demands of both experimental and control groups revealed, however, that there was no high initial energy expenditure of experimental animals compared with controls and that, similarly, there was no prolonged suppression of energy expenditure later in the 24 h. Indeed, throughout the measurement period the energy demands of the control and experimental groups hardly differed. This might reflect the fact that the data on energy expenditure refer to a more restricted group than do the behavioural observations. Restricting the behavioural observations only to those individuals for which we had metabolic data nevertheless reveals the same significant pattern of behav-

ioural differences (Table 3). These data suggest that, although the technique produced some indirect effects on behaviour, these differences were insufficient to produce any gross effects on energy expenditure. This may, in part, reflect the choice of the experimental animal and the possibility that the behaviour exhibited by white mice in respirometry chambers, and which was altered by the procedures (grooming, feeding and general movement around the chamber), only involved expenditure of energy at low levels. An alternative explanation for the small differences in energy expenditure is that, since these animals were kept at temperatures below thermoneutral (18°C), any heat generated by the increased activity may have been substituted for thermoregulatory demands (Webster and Weathers, 1990) which had to be paid by the resting comparison group. Clearly, the indirect behavioural effects of the technique on wild animals may have more profound consequences for the measured energy expenditure than the effects reported here on white laboratory mice. Studies quantifying these effects are urgently required.

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