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How do measurement duration and timing interact to influence estimation of basal physiological variables of a nocturnal rodent?

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ABSTRACT

Metabolic rate and evaporative water loss are two commonly measured physiological variables. It is therefore important, especially for comparative studies, that these variables (and others) are measured under standardised conditions, of which a resting state during the inactive phase is part of the accepted criteria. Here we show how measurement duration and timing affect these criteria and impact on the estimation of basal metabolic rate (oxygen consumption and carbon dioxide production) and standard evaporative water loss of a small nocturnal rodent. Oxygen consumption, carbon dioxide production and evaporative water loss all decreased over the duration of an experiment. Random assortment of hourly values indicated that this was an animal rather than a random effect for up to 11 h. Experimental start time also had a significant effect on measurement of physiological variables. A longer time period was required to achieve minimal carbon dioxide consumption and evaporative water loss when experiments commenced earlier in the day; however, experiments with earlier start times had a lower overall estimates of minimal oxygen consumption and carbon dioxide production. For this species, measurement duration of at least 8 h, ideally commencing between before the inactive phase at 03:00 h and 05:00 h, is required to obtain minimal standard values for physiological variables. Up to 80% of recently published studies measuring basal metabolic rate and/or evaporative water loss of small nocturnal mammals may overestimate basal values due to insufficiently long measurement duration.

1. Introduction

One of the central aims of the discipline of comparative physiology is to identify how physiological variables are influenced by factors such as body mass, climate, diet, habitat and life history, to better understand the selection pressures resulting in adaptive evolution of physiological processes (Lovegrove, 2003; McKechnie and Wolfe, 2004; Withers et al., 2006). Such studies commonly involve intra- and/or interspecific comparison of metabolic and hygric physiological parameters, such as basal metabolic rate (BMR) and standard evaporative water loss (EWL). To make comparable assessments of metabolic and hygric physiology for different species, and therefore assess the influence of environmental and ecological factors on a species' physiology, experiments must follow standardised measurement protocols that result in repeatable minimal measurement of the physiological variables in question (Careau et al., 2008). Standardisation is best achieved when any variance due to extraneous environmental factors is removed (Speakman et al., 2004). For comparative studies of endotherms, the conditions which must be met to ensure physiological data are truly standardised and comparable are those generally accepted for measuring BMR; the animal must be a post-absorptive, non-reproducing, non-growing adult measured at rest within their thermoneutral zone during the inactive phase of their circadian cycle (McNab, 1997; McKechnie and Wolfe, 2004; Speakman et al., 2004; Cooper and Withers, 2009).

Rest is one of the defining criteria for measurement of BMR (and other standard variables) as activity is one of the most important influences on metabolic rate (Withers, 1992). Activity and alertness caused by handling and unfamiliarity with surroundings will result in an increase in consciousness and muscle tension, significantly increasing metabolic rate above basal (Gallivan, 1992; Hayes et al., 1992; Cooper and Withers, 2009; Page et al., 2011). Therefore the experimental duration for measurement of BMR and other standardised physiological variables should be sufficiently long to allow for this increase in metabolic rate to subside, and to reduce the likelihood of overestimation of BMR and EWL. For example, Hayes et al. (1992) found that a measurement duration of 30 min overestimated minimum oxygen consumption ($\dot{V}O_2$) of short-tailed field voles (*Microtus agrestis*) by 13% compared to a measurement duration of 6 h. Cooper and Withers (2009) supported the idea that short measurement duration overestimated basal values for physiological variables.

Despite the evidence for increased measurement duration resulting in more reliable estimates of standard physiological variables, measurement duration per se is not the only important factor to consider when

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measuring and interpreting standardised physiological data. Most animals have a daily cycle of active (α) and inactive (ρ) phases aligned with their circadian rhythm. Circadian rhythm is the natural fluctuation of body functions driven by the body's internal biological clock (Turek, 1985). These fluctuations of physiological, biochemical, and behavioural phenomena are synchronised with a 24 h environmental cycle such as the light and dark cycle (Turek, 1985; Meijer and Rietveld, 1989; Edery, 2000), with photoperiod entraining the circadian rhythm (Bakken and Lee, 1992). While it is generally appreciated that standardised measurements must occur in the ρ phase (Aschoff and Pohl, 1970), the interaction between measurement duration and the timing of experiments has not been investigated for small nocturnal mammals.

Page et al. (2011) showed that both measurement duration and timing interacted to determine the time required to measure minimal values for standard physiological variables of a small diurnal bird, the budgerigar (*Melopsittacus undulatus*). However, previous studies of measurement duration effects for small mammals (e.g. Hayes et al., 1992; Cooper and Withers, 2009) neglected to examine the potential interaction of time of day and measurement duration on estimations of BMR, so it is unclear if it was experimental duration per se, time of day, or some interaction of the two factors that resulted in significant effects of time for measurement of standardised physiological variables. The importance of standardised measurements to the discipline of comparative physiology (McKechnie and Wolfe, 2004) means that understanding these potential methodological effects on estimates of these parameters is essential, both for the design of future studies and for interpretation of existing data. Cooper and Withers (2009) suggested that one half of the studies measuring BMR and three quarters of those measuring EWL for small marsupials overestimated these physiological parameters due to experimental protocol.

We investigate here the influence of experimental duration and start time on the measurement of basal metabolic rate (BMR, measured as oxygen consumption, $\dot{V}O_2$ and carbon dioxide production, $\dot{V}CO_2$) and standard EWL (EWL measured under the same conditions as BMR; Cooper and Withers, 2009) of a small nocturnal rodent, the bush rat (*Rattus fuscipes*), to determine the minimum experimental period, and appropriate time for measurement, necessary to achieve minimal and standardised measures of these physiological variables for a small nocturnal mammal.

2. Materials and methods

Eight bush rats were wild-caught near Albany (34° 58′S, 117° 55′E), approximately 390 km south-west of Perth, Western Australia. They were housed individually in plastic crates indoors in the animal facility at Curtin University, with a 12:12 light:dark cycle (lights on at 07:00 h). The bush rats were provided with seed, mouse cubes and fresh fruit and vegetables. Water was available ad libitum. Bush rats were fasted the night before measurement to ensure they were post-absorptive.

Metabolic rate (measured as $\dot{V}O_2$ and $\dot{V}CO_2$) and EWL were measured using standard open flow respirometry as described by Withers (2001). An individual bush rat was removed from its enclosure in the morning, and placed inside an air-tight metabolic chamber (a 770 cm^3 glass tube) kept within a temperature controlled cabinet. Compressed dry air (dried using drierite–anhydrous calcium sulphate) flowed through the metabolic chamber at a flow rate of 650 mL min^{-1}, controlled by either a Cole-Parmer 0–1000 mL min^{-1} 32708–26 or an Aalborg 0–1000 mL min^{-1} GFC17 mass flow controller. Excurrent air from the metabolic chamber passed through a Vaisala HMP 45A temperature and humidity probe, before passing through a further column of drierite to remove water vapour. The air then passed through a Sable Systems CA-10A CO_2 analyser and a PA-10 paramagnetic O_2 analyser, which were maintained in an insulated cabinet in the air-conditioned lab to control temperature-induced baseline drift in O_2 values. Airflow through the metabolic chambers and gas analysers was via Tygon laboratory tubing. The voltage outputs from the O_2 analyser, CO_2 analyser and RH probe were linked to a computer using a Sable Systems International UI2 Universal Interface II and recorded every 20 s throughout the experimental period by a custom written data acquisition programme (Visual Basic v6; P Withers). A baseline measurement for O_2, CO_2 and H_2O was recorded for approximately an hour before and after each experimental period.

Calibration of the O_2 analyser was achieved using compressed nitrogen gas (0% O_2) and dry ambient air (20.95% O_2); the CO_2 analyser was calibrated using compressed nitrogen (0% CO_2) and a gas mixture of 0.53% CO_2 in air (BOC gases). Calibration of the relative humidity (RH) probe was confirmed with dried air (<1% RH obtained using drierite) and by breathing on the sensor (for 100% RH). The mass flow controllers were calibrated using a Gilian Gilibrator, traceable to a national standard.

Each bush rat was weighed (to ±0.1 g) immediately before and after each experimental period, with the mean mass used for calculations. MR and EWL of each individual bush rat was measured 5 times (on 5 separate days) at experimental start times of 03:00 h, 05:00 h, 07:00 h, 09:00 h and 11:00 h, in random order, with each measurement period lasting 12 h. Individual rats were allowed at least four days between measurements. All measurements were at a thermoneutral T_a of 30 °C (Collins, 1973).

Minimal 20 min mean values for $\dot{V}O_2$, $\dot{V}CO_2$ and EWL were calculated (after Withers, 2001) for each hour of each measurement period using a custom-written programme (Visual Basic v5; P Withers). These minimal 20 min mean values were converted to a percentage of the overall lowest hourly value for that experiment. Once a value that was 100% of the overall experimental minimum was reached, all subsequent values were set to 100%. Percentages were ranked highest to lowest and the ranks analysed by ANOVA (equivalent to a Kruskal–Wallis non-parametric test) to examine the time taken to reach minimal values for $\dot{V}O_2$, $\dot{V}CO_2$ and EWL for each start time separately. Simple a priori contrasts were used to compare each hour with the last (i.e. with 100%) to determine which hours were significantly higher than 100%.

Random re-assortment (10 000 times) of hourly $\dot{V}O_2$, $\dot{V}CO_2$ and EWL minima (using a custom written Excel macro; Cooper and Withers, 2009) determined whether any decrease in mean hourly percentages during an experiment was due to an animal settling effect or the mathematical effect of a greater probability of getting a lower value from a great number of possible values over time. This indicated if the expected decline in hourly minimal values over time was the result of random fluctuations in measurement or a systematic pattern of decline as a result of bush rats being more alert at the beginning of the experiments.

Overall minimal values, time taken to reach the overall minimal values and the actual time of day these minimal values occurred were determined for each start time. To analyse the effect of experimental start time on these variables, a multivariate repeated measures ANOVA (RMANOVA) was used for $\dot{V}O_2$, $\dot{V}CO_2$ and EWL separately, with the experimental start time as the repeat variable and the bush rat as the subject. Polynomial contrasts were used to determine any pattern of response to start time after Withers and Cooper (2011).

Values are mean ± SE, with sample size N = number of individuals and n = number of measurements. StatistiXL (v1.8) and custom-written Excel macros (Cooper and Withers, 2009; Withers and Cooper, 2011) were used for statistical analyses.

3. Results

Measurement duration and experimental start time both had significant effects on minimal physiological variables of the Australian bush rat (mean body mass over all experiments 77.4 ± 1.85 g; $N = 8$, $n = 40$). Overall experimental minima were recorded at 10:37 h, after an experimental duration of 07:37 h from a start time of 03:00 h for $\dot{V}O_2$, at 12:15 h, after an experimental duration of 09:15 h from a start time of 03:00 h for $\dot{V}CO_2$, and at 13:45 h, after an experimental duration of 08:45 h from a start time of 05:00 h for EWL.