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

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Running Head: Effects of measurement duration and timing

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.

Key words Basal metabolic rate, evaporative water loss, measurement, respirometry, rodent

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 Wolf 2004; Withers et al. 2006). Such studies commonly involve intra- and/or inter-specific 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; McKecknie and Wolf 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 hours. 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 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 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 Wolf 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 07:00h). 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 770cm^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 seconds throughout the experimental period by a custom written data acquisition program (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.

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149 Each bush rat was weighed (to ± 0.1g) immediately before and after each experimental
150 period, with the mean mass used for calculations. MR and EWL of each individual bush rat
151 was measured 5 times (on 5 separate days) at experimental start times of 03:00 h, 05:00 h,
152 07:00 h, 09:00 h and 11:00 h, in random order, with each measurement period lasting 12
153 hours. Individual rats were allowed at least four days between measurements. All
154 measurements were at a thermoneutral T_a of 30°C (Collins 1973).

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156 Minimal 20 min mean values for $\dot{V}O_2$, $\dot{V}CO_2$ and EWL were calculated (after Withers 2001)
157 for each hour of each measurement period using a custom-written programme (Visual Basic
158 v5; P Withers). These minimal 20 min mean values were converted to a percentage of the
159 overall lowest hourly value for that experiment. Once a value that was 100% of the overall
160 experimental minimum was reached, all subsequent values were set to 100%. Percentages
161 were ranked highest to lowest and the ranks analysed by ANOVA (equivalent to a Kruskal-
162 Wallis non-parametric test) to examine the time taken to reach minimal values for $\dot{V}O_2$,
163 $\dot{V}CO_2$ and EWL for each start time separately. Simple *a priori* contrasts were used to
164 compare each hour with the last (i.e. with 100%) to determine which hours were significantly
165 higher than 100%.

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167 Random re-assortment (10 000 times) of hourly $\dot{V}O_2$, $\dot{V}CO_2$ and EWL minima (using a
168 custom written Excel macro; Cooper & Withers 2009) determined whether any decrease in
169 mean hourly percentages during an experiment was due to an animal settling effect or the
170 mathematical effect of a greater probability of getting a lower value from a great number of
171 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 07:38 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.

3.1 Measurement duration

Measurement duration had a significant effect for all start times. The general pattern was an exponential decline as the experiment progressed, to an overall minimal value (Fig. 1) for

$\dot{V}O_2$ ($F_{11,84} \geq 3.11$, $P \leq 0.001$), $\dot{V}CO_2$ ($F_{11,84} \geq 33.11$, $P < 0.001$) and EWL ($F_{11,84} \geq 51.2$, $P < 0.001$). Simple contrasts between each hour with the last hour (the overall experimental minimal value, or 100%) indicated that hourly minimal values for $\dot{V}O_2$ were significantly different to the overall minimal value for the first 2-7 hours dependant on start time (e.g. $P \leq 0.013$ for hours 1-2 and $P \geq 0.203$ for hours 3-11 for start time 09:00h compared to $P < 0.001$ for hours 1-7 and $P \geq 0.466$ for hour 8-11 at start time 03:00). The first 4-8 hours were significantly different from the experimental minimum for $\dot{V}CO_2$ (e.g. $P < 0.001$ for hours 1-4 and $P \geq 0.334$ for hours 5-11 at start time 11:00 and $P \leq 0.001$ for hours 1-8 and $P \geq 0.104$ for hours 9-11 at start time 03:00). EWL during the first 5-10 hours was significantly higher than the experimental minimal ($P < 0.001$ for hours 1-5 and $P \geq 0.077$ for hours 6-11 at start time 11:00h, and $P \leq 0.001$ for hours 1-10 and $P \geq 0.638$ for hour 11 at start time 03:00h; Fig. 1).

Random re-assortment of hourly $\dot{V}O_2$, $\dot{V}CO_2$ and EWL minima indicated significant animal effects on measurement of minimal values at the start of experiments for all start times (Fig. 1). Measured hourly minimal $\dot{V}O_2$ means were significantly higher than randomised re-assorted means for between 3 h (at start time 07:00 h; $P < 0.001$) and 11 h (at start time 11:00 h; $P < 0.001$). Hourly minimal $\dot{V}CO_2$ experimental means were significantly higher than randomised re-assorted means for between 5 h (at start time 11:00 h; $P = 0.0015$), and 10 h (at start times 03:00 h and 05:00 h; $P = 0.0002$ and $P = 0.0156$ respectively). For EWL, random re-assortment of hourly EWL minima indicated a significant animal effect for 6 h (at start time 11:00 h; $P < 0.001$) to 11 h (at start time 3:00 h; $P = 0.0135$).

3.2 Experimental start time

221 Time taken for bush rats to reach minimal $\dot{V}O_2$ generally decreased with later start times ($F_{4,4}$
222 = 48.2, P = 0.001; Fig. 2) ranging from 3:23 h ± 16 min for a start time of 07:00 h to 7:38 h ±
223 23 min for a start time of 03:00 h. Polynomial contrasts indicated a quadratic effect (t_7 = 3.23,
224 P = 0.014) where the time taken to reach minimal $\dot{V}O_2$ decreased with later start times until
225 09:00 h, after which time taken to reach minimal values began to increase and become more
226 variable. Time taken to obtain minimal $\dot{V}CO_2$ was also significantly influenced by
227 experimental start time ($F_{4,4}$ = 8.51, P = 0.03), ranging from 4:15 h ± 25 min for a start time
228 of 11:00 h, to 9:15 h ± 42 min for a start time of 03:00 h. Polynomial contrasts indicated a
229 negative linear effect of start time (t_7 = 6.80, P < 0.001). Time taken for EWL to become
230 minimal ranged from 5:45 h ± 22 min for a start time of 11:00 h, to 10:45 h ± 22 min for a
231 start time of 03:00 h, with start time having a significant overall effect ($F_{4,4}$ = 16.5, P =
232 0.009). Polynomial contrasts indicated a negative linear effect (t_7 = 9.4, P < 0.001).
233
234 The time of day that bush rats reached minimal $\dot{V}O_2$ ranged from 10:23 h ± 16 min for a start
235 time of 07:00 h, to 17:00 h ± 106 min for a start time of 11:00 h. Although there was no
236 overall significant influence by RMANOVA ($F_{4,4}$ = 2.26, P = 0.223; Fig. 3), polynomial
237 contrasts indicated both significant positive linear (t_7 = 3.84, P = 0.006) and quadratic effects
238 (t_7 = 3.23, P =0.014). The time of day that bush rats reached minimal $\dot{V}CO_2$ ranged from
239 12:15 h ± 42 min at start time 03:00 h, to 15:38 h ± 32 min for a start time of 09:00 h. There
240 was no overall significant influence of start time by RMANOVA ($F_{4,4}$ = 5.74, P = 0.059) but
241 polynomial contrasts indicated a significant positive linear effect (t_7 = 4.65, P = 0.002). The
242 time of day that bush rats reached minimal EWL differed significantly with start time
243 (RMANOVA $F_{4,4}$ = 7.18, P = 0.041) and ranged from 13:45 h ± 35 min at start time 05:00 h,
244 to 16:45 h ± 22 min at start time 11:00 h. Polynomial contrasts indicated a positive linear
245 effect (t_7 = 6.60, P < 0.001).

Experimental start time also had a significant effect on the overall minimal value for $\dot{V}O_2$ ($F_{4,4}$ = 37.5, P = 0.002; Fig. 4), which ranged from 0.885 ± 0.060 mL O_2 g^{-1} h^{-1} at start time 03:00 h to 1.31 ± 0.038 mL O_2 g^{-1} h^{-1} at start time 11:00 h. Polynomial contrasts indicated both positive linear (t_7 = 7.65, P < 0.001) and cubic (t_7 = 2.99, P = 0.020) effects. Minimal $\dot{V}CO_2$ ranged from 0.863 ± 0.029 mL CO_2 g^{-1} h^{-1} at start time 03:00 h to 0.920 ± 0.033 mL CO_2 g^{-1} h^{-1} at start time 09:00 h. Although there was no overall significant effect by RMANOVA ($F_{4,4}$ = 2.33, P = 0.216) there was a significant polynomial (quadratic) contrast (t_7 = 2.99, P = 0.020). Minimal EWL ranged from 1.38 ± 0.067 mg H_2O g^{-1} h^{-1} at start time 05:00 h to 1.52 ± 0.086 mg H_2O g^{-1} h^{-1} at start time 07:00 h. There was no overall significant effect of start time on minimal EWL by RMANOVA ($F_{4,4}$ = 1.14, P = 0.451), and no significant polynomial contrasts.

4. Discussion

This study has shown that both experimental duration and experimental start time are important factors that significantly affect the measurement of standard physiological variables for a small nocturnal mammal. Both an animal alertness effect in the early stages of an experiment and a time of day effect can result in elevated (non-basal) rates for physiological variables and as such appropriate measurement duration and experimental timing needs to be incorporated into the measurement protocol for BMR and standard EWL to obtain truly basal and thus comparable data. An analysis of recently published studies measuring standardised physiological variables indicates that the data of a large proportion of these studies are unlikely to be standardised.

We found that measurement duration had a significant effect on values for minimal $\dot{V}O_2$, $\dot{V}CO_2$ and EWL for bush rats, consistent with other studies of mammals and birds (Gallivan 1992; Hayes et al. 1992; Cooper and Withers 2009; Page et al. 2011). Bush rats required up to 8 h for $\dot{V}O_2$, 9 h for $\dot{V}CO_2$ and 11 h for EWL to attain values that did not differ significantly from the overall experimental minimum values for these variables. This requirement for long measurement durations may occur due to the mathematical inevitability of achieving a lower value if measurements occur for a longer time period (Cooper and Withers 2009, Page et al 2011). However, comparison of actual measured values with values from random re-assortment of hourly minimum data indicates that the impact on animals from handling and being in a new environment elevates MR and EWL above randomly reallocated mean values. This significant animal effect occurred for up to 11 h into measurements for $\dot{V}O_2$, up to 10 h for $\dot{V}CO_2$ and up to 11 h for EWL.

A circadian rhythm of MR and T_b is well documented for mammals (and other animals), with these and other physiological variables lower during the ρ phase and higher during the α phase (Aschoff and Pohl 1970; Kenagy and Vleck 1982; Aschoff 1983; Refinetti and Menaker 1992; Green et al. 2008). Minimal MR and EWL occurring after 10:00 h for the bush rats were consistent with circadian timing of minima for other small nocturnal rodents (Chew et al. 1965; Heusner et al. 1971; Rubal et al. 1992; Riccio and Goldman 2000). A significant time of day effect could also contribute to the animal effect of declining MR and EWL throughout the experimental period, observed here and in previous studies of small mammals (Hayes et al. 1992; Cooper and Withers 2009). Indeed, significant negative linear (and for $\dot{V}O_2$ also quadratic) effects of experimental start time on the time taken to attain minimal MR and EWL are clear evidence of a time of day effect on these physiological variables. If measurement duration was the only factor to influence measurement of minimal

MR and EWL, then the time taken to attain minimal values would be independent of experimental start time. We observed that start times earlier in the day required longer measurement durations to obtain minimal values that those later in the day. However, we also observed a significant influence of experiment start time on the time of day at which minimal values for all physiological variables were measured, suggesting an actual measurement duration effect in addition to this time of day effect. If there was only a time of day effect, then minimal values would have been measured at the same time of day regardless of experimental start time. Commencing experiments close to the bush rat's circadian minimum did not allow them sufficient time to attain a resting state after the activity and alertness resulting from being handled and placed in the metabolic chamber, before their circadian minimum.

The combination of measurement duration and timing effects that we show here has important consequences for experimental design to measure BMR. Just as it is necessary to consider both these factors when measuring standard physiological variables of diurnal birds (Page et al. 2011), both measurement duration and circadian phase must be considered when measuring similar variables for nocturnal mammals. It is necessary to measure animals for a sufficient experimental period to allow them to attain a resting state in the metabolic chamber; the experimental duration must exceed the period required for the animal to attain a resting state. Shorter measurement durations significantly overestimate BMR and EWL. For example measuring for only the first hour would result in overestimates of 210 ± 15.9% for $\dot{V}O_2$, 162 ± 11.4% for $\dot{V}CO_2$ and 333 ± 31.8% for EWL (compared to minimal values). EWL consistently required a longer period to reach basal values compared to $\dot{V}O_2$ and $\dot{V}CO_2$, indicating that if EWL is measured in conjunction with BMR, longer measurement durations are required than for BMR alone, and the consequences of short measurement durations are

320 greater for EWL than for $\dot{V}O_2$ or $\dot{V}CO_2$. This is likely to be due to the adhesion of water and
321 water vapour to the tubing and metabolic chamber (Cooper and Withers 2009; Page et al.
322 2011) resulting in longer washout periods for water vapour. Minimising the length of all
323 excurrent tubing and the use of glass rather than plastic chambers minimises this washout, but
324 longer washout characteristics are an inherit characteristic of measuring EWL compared to
325 MR. Despite reduced experimental times required to reach experimental minima with
326 experimental start times closer to the circadian minimum, delaying the start of the experiment
327 to close to this minima overestimated BMR, by up to 148% (compared to the minimal BMR
328 measured with an early start time), as animals never achieved a truly minimal state, still
329 showing the effects of prior handling during their circadian minimum. Based on our data, for
330 small nocturnal rodents like the bush rat, we recommend that experiments should commence
331 between 03:00 h and 05:00 h and last for at least 8 h for measurement of BMR, or 10 h for
332 measurement of standard EWL to ensure minimal standardised values are obtained. The
333 effects of even longer measurement durations and early start times, such as placing nocturnal
334 animals in the metabolic chamber overnight and continuing the measurements into the next
335 day, are worthy of further investigation. This approach will extend acclimation times and can
336 also facilitate pre-experimental fasting. However, confining animals to a small metabolic
337 chamber for a large proportion of their active period could raise ethical issues for some
338 species, may lead to compromises in air flow rate (e.g. a higher flow rate required for active
339 compared to resting animals) and may result in increased urinary/faecal contamination of the
340 chamber.
341
342 We can assess here the potential impact of short measurement duration on measurement of
343 BMR for the bush rat. Collins (1973) measured a minimal $\dot{V}CO_2$ of 1.00 ± 0.061 ml CO_2 g^{-1}
344 h^{-1} for bush rats also from the Albany region. This value was 116% of our minimal value of

345 0.863 ± 0.029 ml CO_2 g^{-1} $h^{-1}$and was significantly higher (one sample T-test; $t_7 = 4.72$, $P =$
346 0.002). Collins' (1973) experimental protocol measured MR between 1100 h and 1700 h, a
347 maximal measurement duration of 6 h, commencing close to the species' circadian minima.
348 Our data suggest minimal values for $\dot{V}CO_2$ after a 6 h measurement period for an experiment
349 beginning at 11:00 would result in an estimation of basal $\dot{V}CO_2$ of 0.915 ± 0.031, 106% of
350 our estimated actual minimal value. Methodological differences (a gravimetric method for
351 measuring CO_2 consumption as opposed to our use of an electronic gas analyser) probably
352 account for the difference between our predictions for Collin's measurement protocol and his
353 actual values.
354
355 To determine the wider significance of measurement duration variably on published data for
356 small mammals, we assessed the measurement duration from a sample of 40 peer-reviewed
357 articles published in leading zoological and physiological journals (e.g. Comparative
358 Biochemistry and Physiology, Journal of Comparative Physiology B, Journal of Experimental
359 Biology, Physiological and Biochemical Zoology) during the period 2002 to 2012 (most
360 articles do not explicitly state experimental start times, so it was not possible to assess this
361 measurement criteria). Thirty two (80 %) of the forty studies measured BMR/EWL for 7 h or
362 less, while 22 (55 %) of the studies actually measured BMR and/or EWL for 3 h or less. This
363 suggests that experimental duration is a real and current issue impacting on the interpretation
364 and validity of published standard data for small mammals, as it is for small birds (Page et al.
365 2011). As only published studies are available for analysis, presumably there are even more
366 studies of short duration that have not proceeded beyond the review process. Measurement
367 duration and timing are clearly issues that must be addressed by authors and reviewers of
368 respirometry data for small endotherms if truly standardised physiological variables such as
369 BMR are to be of value for comparative studies.

In summary, the findings of this study support those of Hayes et al. (1992) and Cooper and Withers (2009), who identified that sufficient measurement duration is required to accurately measure standard BMR and EWL of small mammals; short measurement periods may significantly overestimate these values. However, this study also demonstrated that the time of day effect identified by Page et al. (2011) for diurnal budgerigars is also a factor influencing measurement protocol for nocturnal mammals, and so both measurement duration and time of day need to be considered when designing and interpreting physiological studies that aim to produce comparable data.

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451 **Figure Legends**

452 Figure 1. Hourly minimal experimental means as a percentage of the overall experimental
453 minimum of oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), and evaporative
454 water loss (EWL) at different start times (circles). Squares indicate the mean of 10 000
455 random reallocations of minimal values for these variables. Black circles indicate where
456 experimental means are significantly different from the overall experimental mean, while
457 white circles indicate where the difference is no longer significant. An asterisk indicates
458 where experimental means are no longer significantly different to randomly re-allocated
459 means. Values are mean ± SE, n = 8.

460

461 Figure 2 Time taken (h) to reach minimal oxygen consumption ($\dot{V}O_2$), carbon dioxide
462 production ($\dot{V}CO_2$), and evaporative water loss (EWL), at different start times. A line is
463 included where polynomial contrasts have indicated a significant relationship. Values are
464 mean ± SE, n = 8.

465

466 Figure 3 Time of day that bush rats obtained minimal oxygen consumption ($\dot{V}O_2$), carbon
467 dioxide production ($\dot{V}CO_2$), and evaporative water loss (EWL) at different start times. A line
468 is included where polynomial contrasts have indicated a significant relationship. Values are
469 mean ± SE, n = 8.

470

471 Figure 4 Minimal oxygen consumption ($\dot{V}O_2$), carbon dioxide consumption ($\dot{V}CO_2$), and
472 evaporative water loss (EWL) at different experimental start times. A line is included where
473 polynomial contrasts have indicated a significant relationship. Values are mean ± SE, n = 8.

474

475

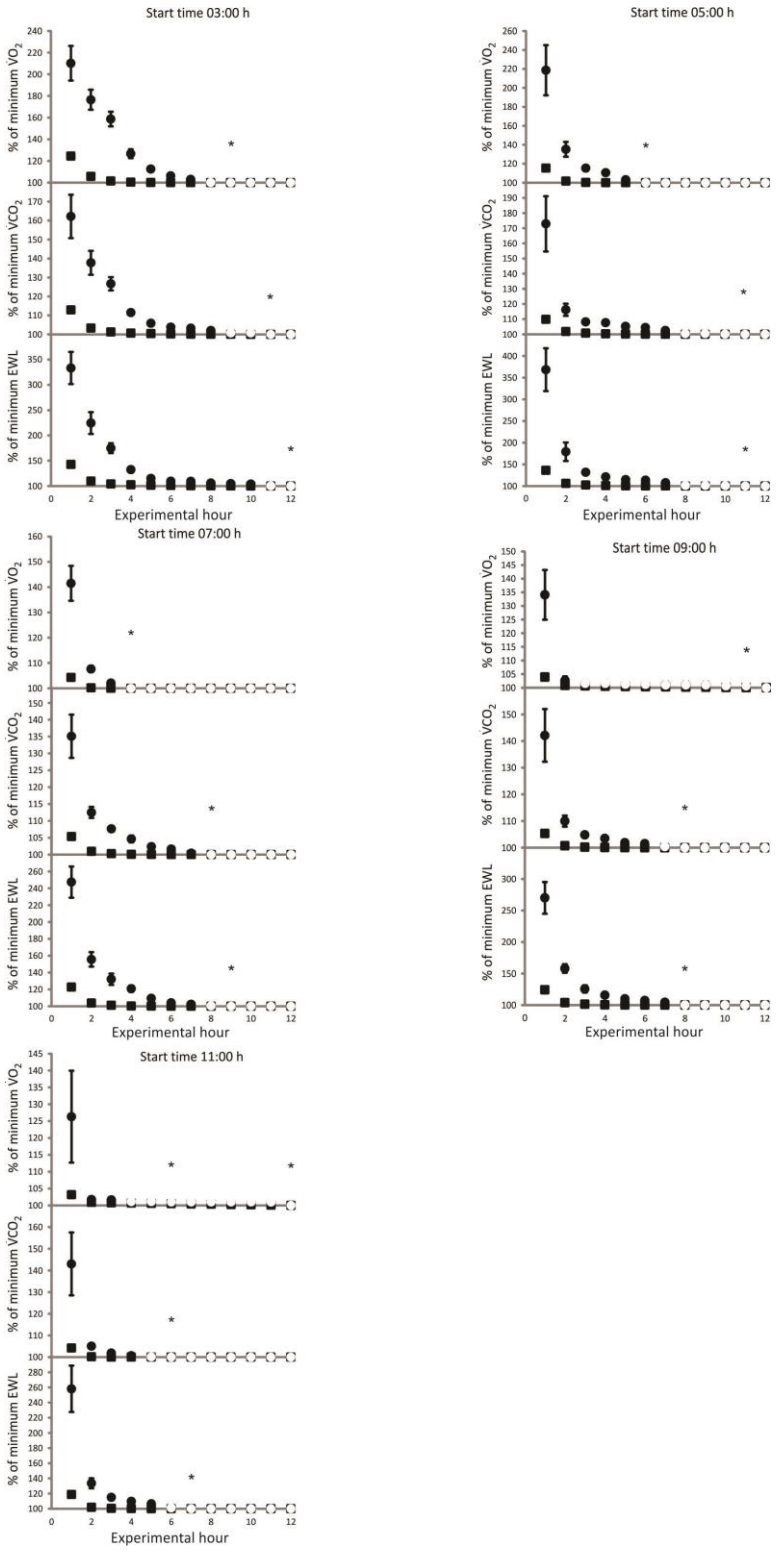
Start time 03:00 h
Start time 05:00 h
Start time 07:00 h
Start time 09:00 h
Start time 11:00 h
% of minimum VO2
% of minimum VCO2
% of minimum EWL
Experimental hour

Figure One

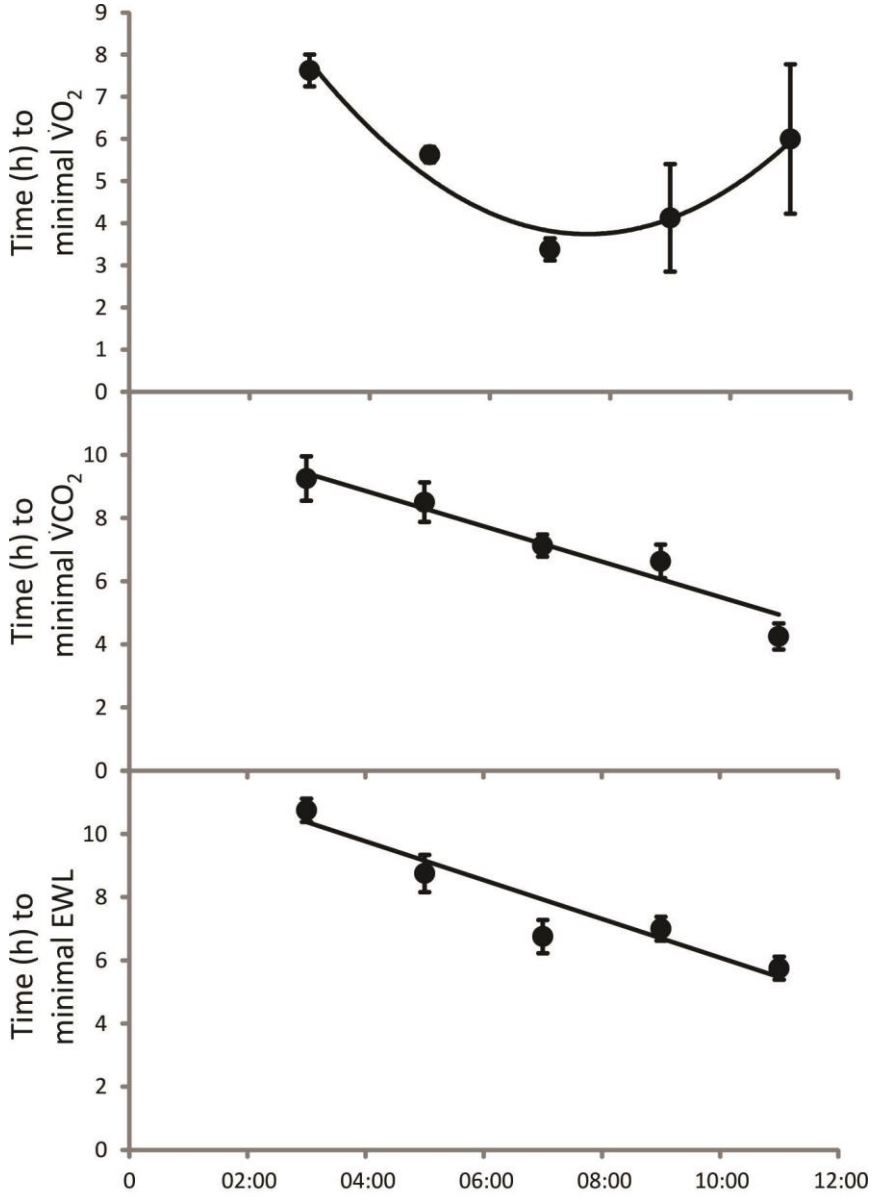
Time (h) to minimal $\dot{V}O_2$
Time (h) to minimal $\dot{V}CO_2$
Time (h) to minimal EWL
0
1
2
3
4
5
6
7
8
9
10
02:00
04:00
06:00
08:00
10:00
12:00

Figure Two

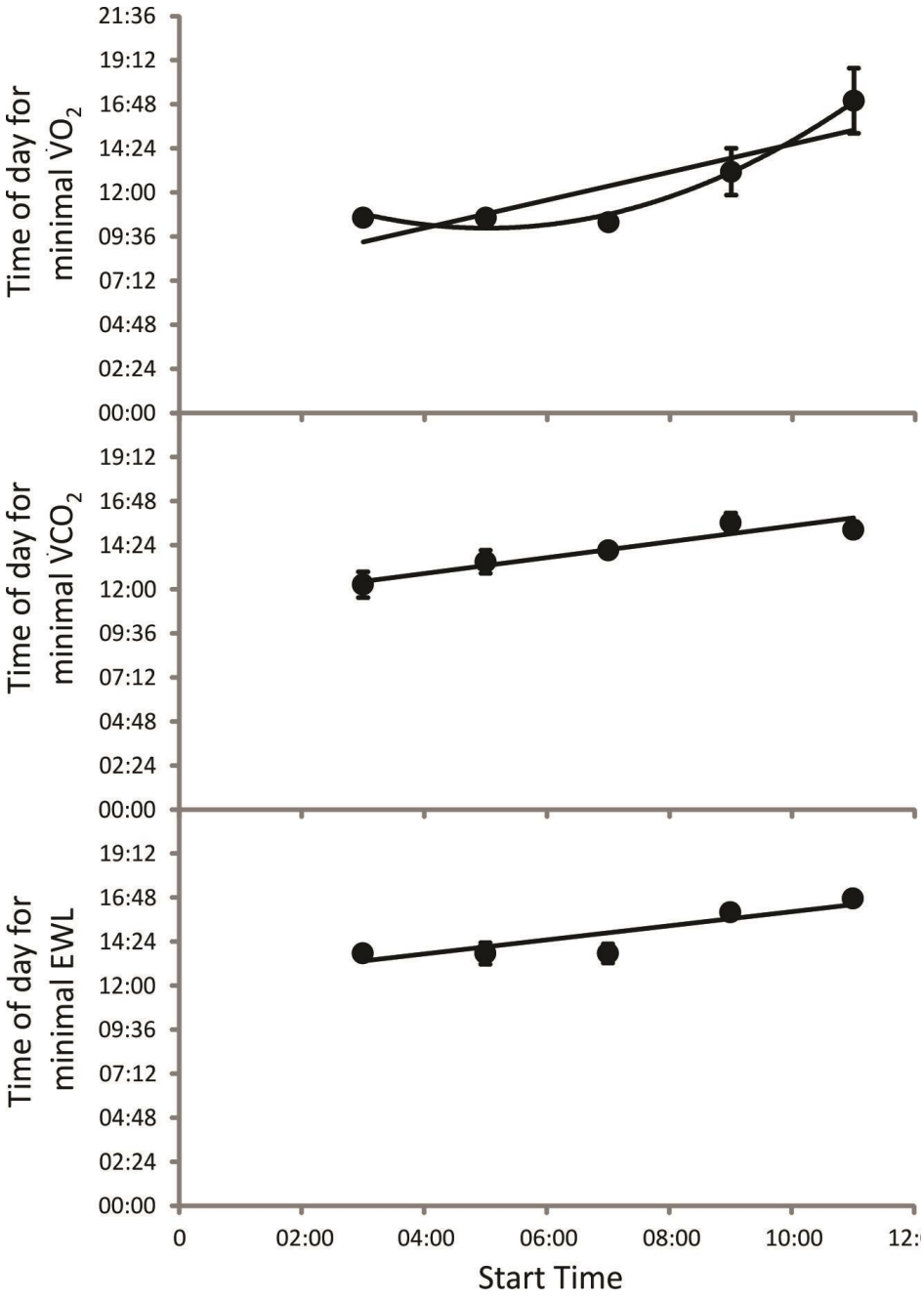

Figure Three

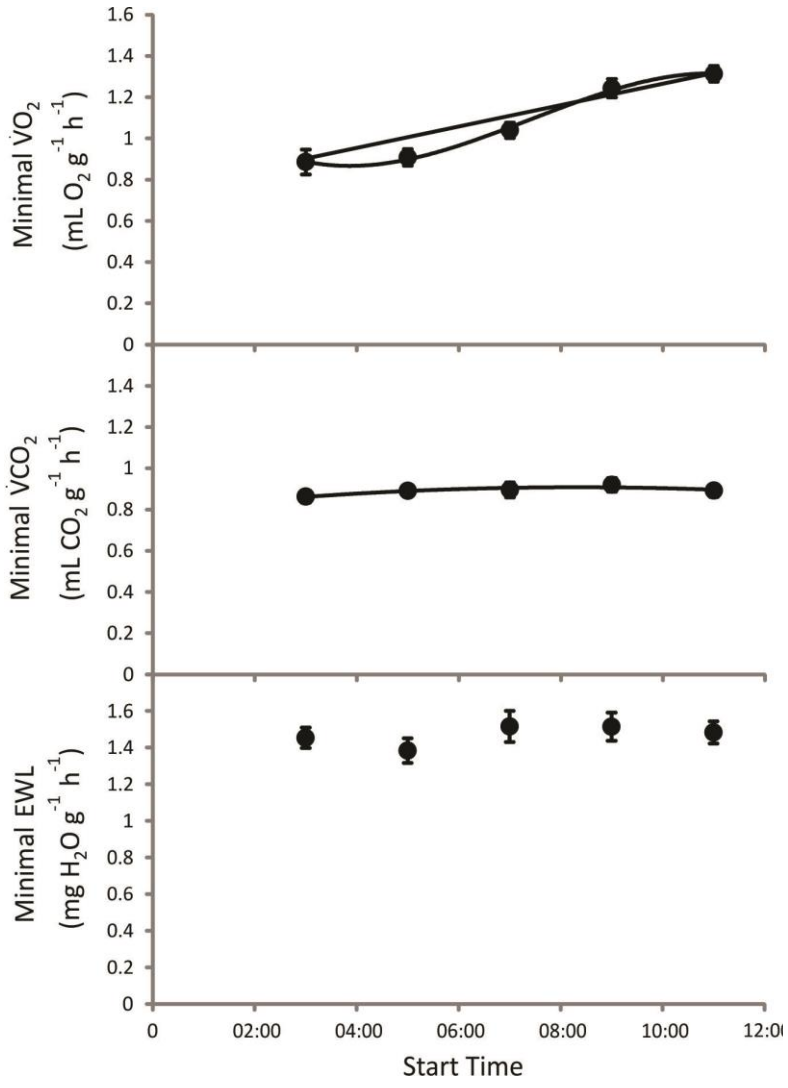
Minimal VO_2 (mL O_2 g^{-1} h^{-1})
Minimal VCO_2 (mL CO_2 g^{-1} h^{-1})
Minimal EWL (mg H_2O g^{-1} h^{-1})
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
02:00
04:00
06:00
08:00
10:00
Start Time

Figure Four