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

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19 Running Head: Effects of measurement duration and timing

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25

26 **Abstract**

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

45

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

47

48 **1. Introduction**

49 One of the central aims of the discipline of comparative physiology is to identify how
50 physiological variables are influenced by factors such as body mass, climate, diet, habitat and
51 life history, to better understand the selection pressures resulting in adaptive evolution of
52 physiological processes (Lovegrove 2003; McKechnie and Wolf 2004; Withers et al. 2006).
53 Such studies commonly involve intra- and/or inter-specific comparison of metabolic and
54 hygric physiological parameters, such as basal metabolic rate (BMR) and standard
55 evaporative water loss (EWL). To make comparable assessments of metabolic and hygric
56 physiology for different species, and therefore assess the influence of environmental and
57 ecological factors on a species' physiology, experiments must follow standardised
58 measurement protocols that result in repeatable minimal measurement of the physiological
59 variables in question (Careau *et al.* 2008). Standardisation is best achieved when any variance
60 due to extraneous environmental factors is removed (Speakman et al. 2004). For comparative
61 studies of endotherms, the conditions which must be met to ensure physiological data are
62 truly standardised and comparable are those generally accepted for measuring BMR; the
63 animal must be a post-absorptive, non-reproducing, non-growing adult measured at rest
64 within their thermoneutral zone during the inactive phase of their circadian cycle (McNab
65 1997; McKechnie and Wolf 2004; Speakman et al. 2004; Cooper and Withers 2009).

66

67 Rest is one of the defining criteria for measurement of BMR (and other standard variables) as
68 activity is one of the most important influences on metabolic rate (Withers 1992). Activity
69 and alertness caused by handling and unfamiliarity with surroundings will result in an
70 increase in consciousness and muscle tension, significantly increasing metabolic rate above
71 basal (Gallivan 1992; Hayes et al. 1992; Cooper and Withers 2009; Page et al. 2011).
72 Therefore the experimental duration for measurement of BMR and other standardised

73 physiological variables should be sufficiently long to allow for this increase in metabolic rate
74 to subside, and to reduce the likelihood of overestimation of BMR and EWL. For example,
75 Hayes et al. (1992) found that a measurement duration of 30 min overestimated minimum
76 oxygen consumption ($\dot{V}O_2$) of short-tailed field voles (*Microtus agrestis*) by 13% compared
77 to a measurement duration of 6 hours. Cooper and Withers (2009) supported the idea that
78 short measurement duration overestimated basal values for physiological variables.

79

80 Despite the evidence for increased measurement duration resulting in more reliable estimates
81 of standard physiological variables, measurement duration per se is not the only important
82 factor to consider when measuring and interpreting standardised physiological data. Most
83 animals have a daily cycle of active (α) and inactive (ρ) phases aligned with their circadian
84 rhythm. Circadian rhythm is the natural fluctuation of body functions driven by the body's
85 internal biological clock (Turek 1985). These fluctuations of physiological, biochemical, and
86 behavioural phenomena are synchronised with a 24 h environmental cycle such as the light
87 and dark cycle (Turek 1985; Meijer and Rietveld 1989; Edery 2000), with photoperiod
88 entraining the circadian rhythm (Bakken and Lee 1992). While it is generally appreciated that
89 standardised measurements must occur in the ρ phase (Aschoff and Pohl 1970), the
90 interaction between measurement duration and the timing of experiments has not been
91 investigated for small nocturnal mammals.

92

93 Page et al. (2011) showed that both measurement duration and timing interacted to determine
94 the time required to measure minimal values for standard physiological variables of a small
95 diurnal bird, the budgerigar (*Melopsittacus undulatus*). However, previous studies of
96 measurement duration effects for small mammals (e.g. Hayes et al 1992; Cooper and Withers
97 2009) neglected to examine the potential interaction of time of day and measurement duration

98 on estimations of BMR, so it is unclear if it was experimental duration per se, time of day, or
99 some interaction of the two factors that resulted in significant effects of time for measurement
100 of standardised physiological variables. The importance of standardised measurements to the
101 discipline of comparative physiology (McKechnie and Wolf 2004) means that understanding
102 these potential methodological effects on estimates of these parameters is essential, both for
103 the design of future studies and for interpretation of existing data. Cooper and Withers (2009)
104 suggested that one half of the studies measuring BMR and three quarters of those measuring
105 EWL for small marsupials overestimated these physiological parameters due to experimental
106 protocol.

107

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

115

116 **2. Materials and Methods**

117 Eight bush rats were wild-caught near Albany (34° 58'S, 117° 55'E), approximately 390 km
118 south-west of Perth, Western Australia. They were housed individually in plastic crates
119 indoors in the animal facility at Curtin University, with a 12:12 light:dark cycle (lights on at
120 07:00h). The bush rats were provided with seed, mouse cubes and fresh fruit and vegetables.
121 Water was available *ad libitum*. Bush rats were fasted the night before measurement to ensure
122 they were post-absorptive.

123

124 Metabolic rate (measured as $\dot{V}O_2$ and $\dot{V}CO_2$) and EWL were measured using standard open
125 flow respirometry as described by Withers (2001). An individual bush rat was removed from
126 its enclosure in the morning, and placed inside an air-tight metabolic chamber (a 770cm³
127 glass tube) kept within a temperature controlled cabinet. Compressed dry air (dried using
128 drierite – anhydrous calcium sulphate) flowed through the metabolic chamber at a flow rate
129 of 650 ml min⁻¹, controlled by either a Cole-Parmer 0-1000 ml min⁻¹ 32708-26 or an Aalborg
130 0-1000 ml min⁻¹ GFC17 mass flow controller. Excurrent air from the metabolic chamber
131 passed through a Vaisala HMP 45A temperature and humidity probe, before passing through
132 a further column of drierite to remove water vapour. The air then passed through a Sable
133 Systems CA-10A CO₂ analyser and a PA-10 paramagnetic O₂ analyser, which were
134 maintained in an insulated cabinet in the air-conditioned lab to control temperature-induced
135 baseline drift in O₂ values. Airflow through the metabolic chambers and gas analysers was
136 via Tygon laboratory tubing. The voltage outputs from the O₂ analyser, CO₂ analyser and RH
137 probe were linked to a computer using a Sable Systems International UI2 Universal Interface
138 II and recorded every 20 seconds throughout the experimental period by a custom written
139 data acquisition program (Visual Basic v6; P Withers). A baseline measurement for O₂, CO₂
140 and H₂O was recorded for approximately an hour before and after each experimental period.

141

142 Calibration of the O₂ analyser was achieved using compressed nitrogen gas (0% O₂) and dry
143 ambient air (20.95% O₂); the CO₂ analyser was calibrated using compressed nitrogen (0%
144 CO₂) and a gas mixture of 0.53% CO₂ in air (BOC gases). Calibration of the relative humidity
145 (RH) probe was confirmed with dried air (<1% RH obtained using drierite) and by breathing
146 on the sensor (for 100% RH). The mass flow controllers were calibrated using a Gilian
147 Gilibrator, traceable to a national standard.

148

149 Each bush rat was weighed (to $\pm 0.1\text{g}$) 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).

155

156 Minimal 20 min mean values for $\dot{V}\text{O}_2$, $\dot{V}\text{CO}_2$ 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 $\dot{V}\text{O}_2$,
163 $\dot{V}\text{CO}_2$ 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%.

166

167 Random re-assortment (10 000 times) of hourly $\dot{V}\text{O}_2$, $\dot{V}\text{CO}_2$ 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

172 over time was the result of random fluctuations in measurement or a systematic pattern of
173 decline as a result of bush rats being more alert at the beginning of the experiments.

174

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

181

182 Values are mean \pm SE, with sample size N = number of individuals and n = number of
183 measurements. StatistiXL (v1.8) and custom-written Excel macros (Cooper and Withers
184 2009; Withers and Cooper 2011) were used for statistical analyses.

185

186 **3. Results**

187 Measurement duration and experimental start time both had significant effects on minimal
188 physiological variables of the Australian bush rat (mean body mass over all experiments 77.4
189 ± 1.85 g; N = 8, n = 40). Overall experimental minima were recorded at 10:37 h, after an
190 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
191 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
192 an experimental duration of 08:45 h from a start time of 05:00 h for EWL.

193

194 *3.1 Measurement duration*

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

197 $\dot{V}O_2$ ($F_{11,84} \geq 3.11$, $P \leq 0.001$), $\dot{V}CO_2$ ($F_{11,84} \geq 33.11$, $P < 0.001$) and EWL ($F_{11,84} \geq 51.2$, $P <$
198 0.001). Simple contrasts between each hour with the last hour (the overall experimental
199 minimal value, or 100%) indicated that hourly minimal values for $\dot{V}O_2$ were significantly
200 different to the overall minimal value for the first 2-7 hours dependant on start time (e.g. $P \leq$
201 0.013 for hours 1-2 and $P \geq 0.203$ for hours 3-11 for start time 09:00h compared to $P < 0.001$
202 for hours 1-7 and $P \geq 0.466$ for hour 8-11 at start time 03:00). The first 4-8 hours were
203 significantly different from the experimental minimum for $\dot{V}CO_2$ (e.g. $P < 0.001$ for hours 1-
204 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 $P \geq 0.104$
205 for hours 9-11 at start time 03:00). EWL during the first 5-10 hours was significantly higher
206 than the experimental minimal ($P < 0.001$ for hours 1-5 and $P \geq 0.077$ for hours 6-11 at start
207 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.
208 1).

209
210 Random re-assortment of hourly $\dot{V}O_2$, $\dot{V}CO_2$ and EWL minima indicated significant animal
211 effects on measurement of minimal values at the start of experiments for all start times (Fig.
212 1). Measured hourly minimal $\dot{V}O_2$ means were significantly higher than randomised re-
213 assorted means for between 3 h (at start time 07:00 h; $P < 0.001$) and 11 h (at start time 11:00
214 h; $P < 0.001$). Hourly minimal $\dot{V}CO_2$ experimental means were significantly higher than
215 randomised re-assorted means for between 5 h (at start time 11:00 h; $P = 0.0015$), and 10 h
216 (at start times 03:00 h and 05:00 h; $P = 0.0002$ and $P = 0.0156$ respectively). For EWL,
217 random re-assortment of hourly EWL minima indicated a significant animal effect for 6 h (at
218 start time 11:00 h; $P < 0.001$) to 11 h (at start time 3:00 h; $P = 0.0135$).

219

220 *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 \pm 16 min for a start time of 07:00 h to 7:38 h \pm
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 \pm 25 min for a start time
228 of 11:00 h, to 9:15 h \pm 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 \pm 22 min for a start time of 11:00 h, to 10:45 h \pm 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 \pm 16 min for a start
235 time of 07:00 h, to 17:00 h \pm 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 \pm 42 min at start time 03:00 h, to 15:38 h \pm 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 \pm 35 min at start time 05:00 h,
244 to 16:45 h \pm 22 min at start time 11:00 h. Polynomial contrasts indicated a positive linear
245 effect ($t_7 = 6.60$, $P < 0.001$).

246

247 Experimental start time also had a significant effect on the overall minimal value for $\dot{V}O_2$
248 ($F_{4,4} = 37.5$, $P = 0.002$; Fig. 4), which ranged from 0.885 ± 0.060 mL O₂ g⁻¹ h⁻¹ at start time
249 03:00 h to 1.31 ± 0.038 mL O₂ g⁻¹ h⁻¹ at start time 11:00 h. Polynomial contrasts indicated
250 both positive linear ($t_7 = 7.65$, $P < 0.001$) and cubic ($t_7 = 2.99$, $P = 0.020$) effects. Minimal
251 $\dot{V}CO_2$ ranged from 0.863 ± 0.029 mL CO₂ g⁻¹ h⁻¹ at start time 03:00 h to 0.920 ± 0.033 mL
252 CO₂ g⁻¹ h⁻¹ at start time 09:00 h. Although there was no overall significant effect by
253 RMANOVA ($F_{4,4} = 2.33$, $P = 0.216$) there was a significant polynomial (quadratic) contrast
254 ($t_7 = 2.99$, $P = 0.020$). Minimal EWL ranged from 1.38 ± 0.067 mg H₂O g⁻¹ h⁻¹ at start time
255 05:00 h to 1.52 ± 0.086 mg H₂O g⁻¹ h⁻¹ at start time 07:00 h. There was no overall significant
256 effect of start time on minimal EWL by RMANOVA ($F_{4,4} = 1.14$, $P = 0.451$), and no
257 significant polynomial contrasts.

258

259 **4. Discussion**

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

269

270 We found that measurement duration had a significant effect on values for minimal $\dot{V}O_2$,
271 $\dot{V}CO_2$ and EWL for bush rats, consistent with other studies of mammals and birds (Gallivan
272 1992; Hayes et al. 1992; Cooper and Withers 2009; Page et al. 2011). Bush rats required up
273 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
274 significantly from the overall experimental minimum values for these variables. This
275 requirement for long measurement durations may occur due to the mathematical inevitability
276 of achieving a lower value if measurements occur for a longer time period (Cooper and
277 Withers 2009, Page et al 2011). However, comparison of actual measured values with values
278 from random re-assortment of hourly minimum data indicates that the impact on animals
279 from handling and being in a new environment elevates MR and EWL above randomly
280 reallocated mean values. This significant animal effect occurred for up to 11 h into
281 measurements for $\dot{V}O_2$, up to 10 h for $\dot{V}CO_2$ and up to 11 h for EWL.

282

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

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

306

307 The combination of measurement duration and timing effects that we show here has
308 important consequences for experimental design to measure BMR. Just as it is necessary to
309 consider both these factors when measuring standard physiological variables of diurnal birds
310 (Page et al. 2011), both measurement duration and circadian phase must be considered when
311 measuring similar variables for nocturnal mammals. It is necessary to measure animals for a
312 sufficient experimental period to allow them to attain a resting state in the metabolic
313 chamber; the experimental duration must exceed the period required for the animal to attain a
314 resting state. Shorter measurement durations significantly overestimate BMR and EWL. For
315 example measuring for only the first hour would result in overestimates of $210 \pm 15.9\%$ for
316 $\dot{V}O_2$, $162 \pm 11.4\%$ for $\dot{V}CO_2$ and $333 \pm 31.8\%$ for EWL (compared to minimal values). EWL
317 consistently required a longer period to reach basal values compared to $\dot{V}O_2$ and $\dot{V}CO_2$,
318 indicating that if EWL is measured in conjunction with BMR, longer measurement durations
319 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 inherent 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 $0.863 \pm 0.029 \text{ ml CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ 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 $\dot{V}\text{CO}_2$ after a 6 h measurement period for an experiment
349 beginning at 11:00 would result in an estimation of basal $\dot{V}\text{CO}_2$ 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.

370

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

379

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385 the Care and Use of Animals for Scientific Purposes and were approved by Curtin
386 University's Animal Ethics Committee. Bush rats were caught and held under licence from
387 the Western Australian Department of Environment and Conservation.

388

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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 \pm 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 \pm 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 \pm 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 \pm SE, $n = 8$.

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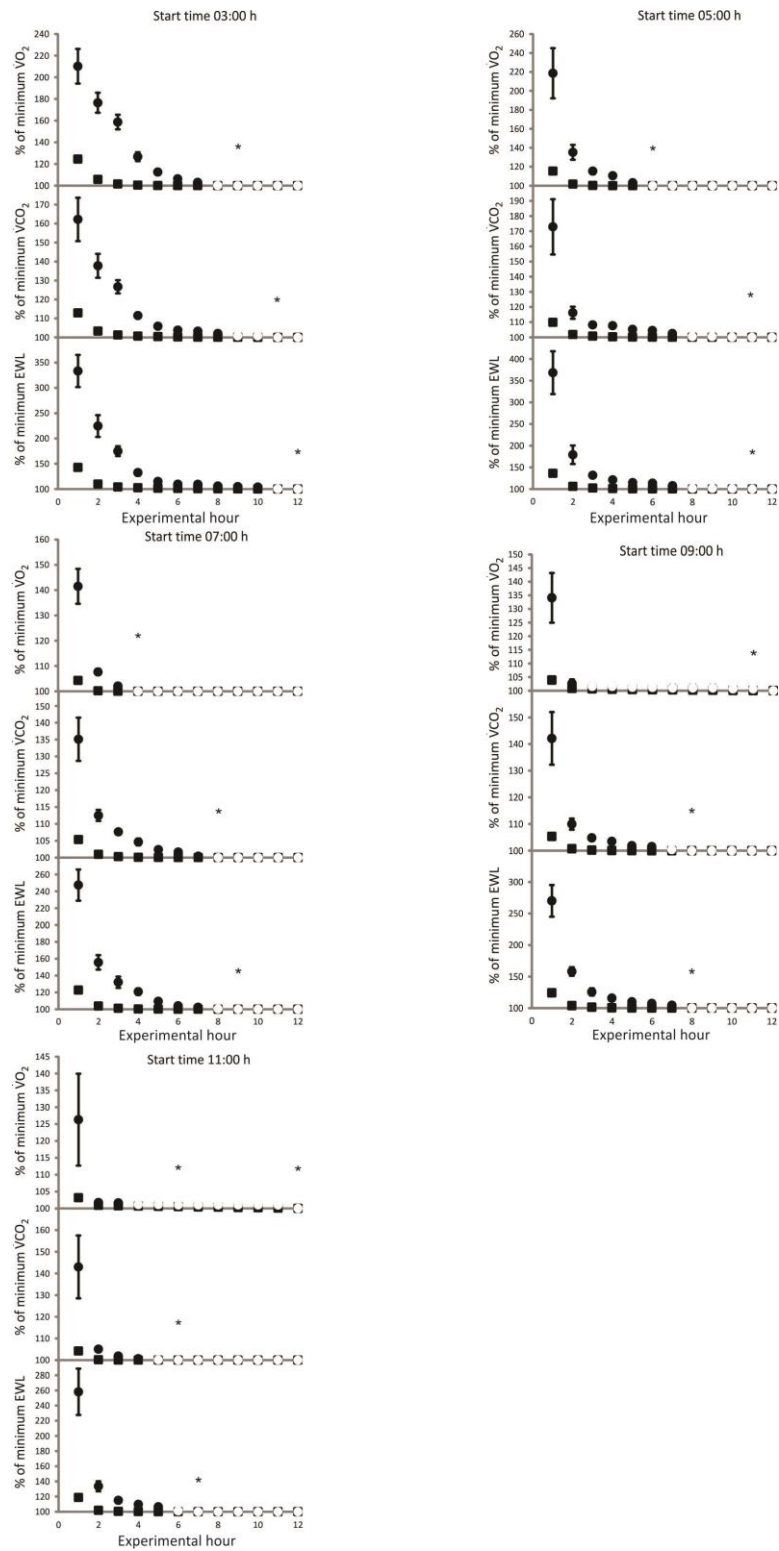


Figure One

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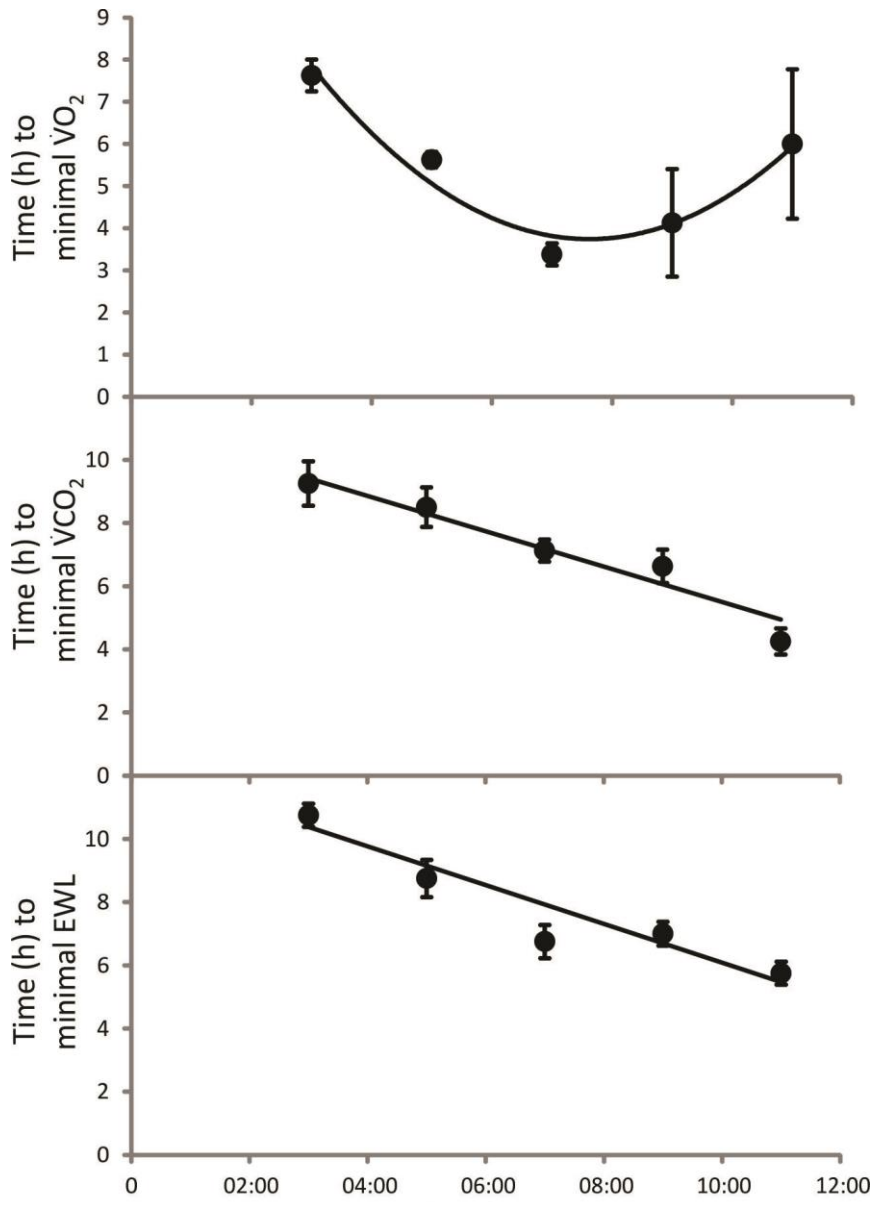
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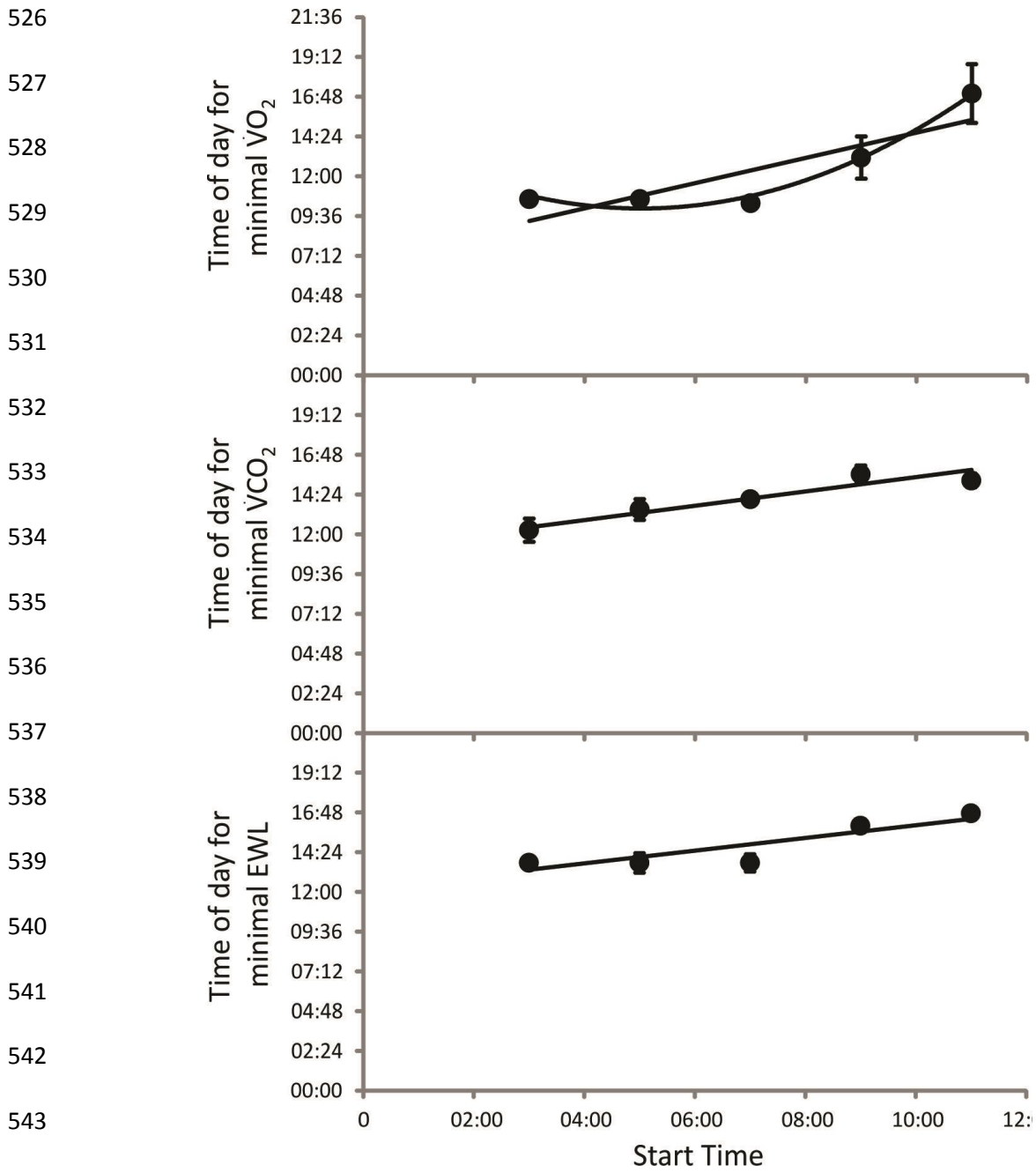
522 Figure Two

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546 Figure Three

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568 Figure Four

