Effects of experiment start time and duration on measurement of standard physiological variables

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Abstract

Duration and start time of respirometry experiments have significant effects on the measurement of basal values for several commonly measured physiological variables (metabolic rate, evaporative water loss and body temperature). A longer measurement duration reduced values for all variables for all start times, and this was an effect of reduced animal activity rather than random sampling. However, there was also an effect of circadian rhythm on the timing of minimal physiological values. Experiment start time had a significant effect on time taken to reach minimal values for all variables, ranging from 4:00 h ± 38 min (body temperature, start time 23:00 h) to 8:54 h ± 52 min (evaporative water loss, start time 17:00 h). It also influenced the time of day that minimal values were obtained, ranging from 22:24 h ± 40 min (carbon dioxide production, start time 15:00 h) to 06:00 h ± 57 min (oxygen consumption, start time 23:00 h), and the minimum values measured. Consequently both measurement duration and experiment start time should be considered in experimental design to account for both a handling and a circadian effect on the animal's physiology. We suggest that experiments to measure standard physiological variables for small diurnal birds should commence between 17:00 h and 21:00 h, and measurement duration should be at least 9 h.

Key words: basal metabolic rate, evaporative water loss, circadian effect, measurement duration, budgerigar, respirometry

Abbreviations

α	active phase of the circadian cycle
ρ	inactive phase of the circadian cycle
BMR	basal metabolic rate
D_{exp}	time of day at the minimal value
EWL	evaporative water loss
S_{exp}	experimental start time
T_b	body temperature
T_{exp}	time taken to reach the minimal value
VO_2	rate of oxygen consumption
VCO_2	rate of carbon dioxide production

Introduction

Comparative physiology examines processes or responses of different species under similar conditions, or of a single species under differing conditions (Withers 1992). Physiologists commonly compare energy expenditure and water requirements of animals, for example to compare requirements of particular species in different ecosystems (Hill et al. 2004), or adaptations of species to particular diets or other ecological or environmental variables (e.g. Elgar and Harvey 1987; McNab 1988; Williams et al. 1991; Lovegrove 2003; Cruz-Neto and Bozinovic 2004; Withers et al. 2006; McNab 2009). Although animals in the field probably rarely function at basal levels of energy expenditure and water loss (Williams and Tielman 2000), basal measures made under standardised laboratory conditions are important because they are consistent measures that allow robust inter- and intra-specific comparisons (Koteja 1991; McNab 1997; Hulbert and Else 2004; Speakman et al. 2004; Cooper and Withers 2009). However, for these standard measures of physiological variables to be of value, it is important that strict criteria for their measurement be rigorously adhered to. Accepted standard conditions for measurements of an endothermic animal to be considered basal are that it should be a non-reproductive adult, measured at rest and in a postabsorptive state, during its inactive period and within its thermoneutral zone (McNab 1997).

Activity is one of the most important factors influencing the metabolic rate (MR) of an individual animal (Withers 1992), therefore one of the key conditions when measuring basal metabolic rate (BMR) is that the animal must be at rest in its inactive (ρ), rather than active (α) phase (Aschoff and Pohl 1970). Activity, and alertness

associated with handling and being placed in an unfamiliar environment, will increase MR. A conscious state implies a certain degree of muscular tension, which will increase MR, so a degree of wakefulness will have a variable effect on BMR (Benedict 1938). Benedict (1938) suggested that animals should be allowed a period of 20-30 min within the experimental system immediately before BMR measurements, to ensure that they are at rest. Hayes et al. (1992) found that for short-tailed field voles (*Microtus agrestis*), a measurement duration of only 30 min overestimated minimum oxygen consumption (VO_2) by 13% compared to a duration of 6 h. For a variety of small marsupial species, VO_2 was basal after an average of 4.3 h, carbon dioxide production (VCO_2) after 4.5 h, and evaporative water loss (EWL) after 5.2 h (Cooper and Withers 2009), and shorter measurement durations significantly overestimated these variables. However, these studies only examined the effect of experimental duration, and did not determine if the observed effects were due to the experimental duration *per se*, or to confounding effects of a circadian rhythm.

The aim of our study was to measure standard physiological variables (VO_2, VCO_2, EWL, and body temperature; T_b) of a small diurnal bird, the budgerigar (*Melopsittacus undulatus*), to determine if there was an effect of measurement duration on estimates of these standard variables, and if this effect was due to the experimental duration, to the circadian rhythm, or to a combination of the two. We make recommendations from our results for the appropriate timing of experiments for small diurnal birds to obtain standardised minimum values of these commonly measured physiological variables.

Material and Methods

Eleven adult budgerigars were obtained from a commercial aviary, and were housed indoors at an ambient temperature of approximately 22°C, with a 12:12 light:dark cycle (lights on at 07:00 h). Budgerigars were allowed to adjust to indoor conditions for at least two weeks prior to commencement of experiments. Except for 24 h before and during measurements, budgerigars were provided with *ad lib* budgerigar seed mix and water, and their diet was supplemented with fresh fruit, greens and cuttlebone. All birds conformed to the criteria for measuring BMR and standard EWL – they were non-reproductive adults, and for experiments were postabsorptive (McNab 1997).

Prior to the commencement of experiments, five of the budgerigars were implanted with a passive implantable temperature transponder (TA E-mitter, Respironics) to continuously monitor T_b during experiments. Transponders were calibrated with a mercury thermometer (resolution 0.5°C) traceable to a national standard, at T_as from 20°C to 40°C, in increments of 5°C, and corrected for drift over time, assuming linear drift between calibration periods. The transponders were sterilised in hibitane for 24 h, then were rinsed with sterile saline prior to implantation. They were surgically implanted in the abdominal cavity under general anaesthesia (isoflurane in O_2; 3% induction, 2% maintenance). Budgerigars were then given a recovery period of at least one week before experiments commenced.

BMR (VO_2 and VCO_2) and standard EWL were measured using standard open flow-through respirometry as described by Withers (2001). An individual budgerigar was

placed in an air-tight, 500 mL glass chamber, inside a dark temperature-controlled room, at 30°C (within the budgerigars' thermoneutral zone; Weathers and Schoenbaechler 1976). Dry compressed air passed through the animal chamber at 400 mL^{-1}, controlled by an Aalborg 0-500 mL min^{-1} GFC17 or 0-1000 mL min^{-1} GFC17 mass flow controller, to maintain levels of O_2 above 20% and CO_2 below 1%. Excurrent air passed over a Vaisala HMP45A temperature and relative humidity (RH) probe, then a 100 ml min^{-1} subsample was dried with a column of Drierite before passing through a Sable Systems CA-10A or Qubit S153 CO_2 analyser, and a Taylor Servomex OA184 O_2 analyser.

Each budgerigar was measured at various experimental start times (see below), for a duration of 12 h. Individuals were measured in random order, with a minimum of four days to recover between each measurement; experimental start times were also randomised. Budgerigars were weighed immediately before being placed in the metabolic chamber, and again immediately after they were taken out, and the mean of the two masses was used for calculations. The O_2 analyser was calibrated using compressed nitrogen (0% O_2) and dry ambient air (20.95% O_2); the CO_2 analyser was calibrated with compressed nitrogen (0% CO_2) and a certified gas mix (0.53% CO_2; BOC, Perth, Western Australia); the calibration of the RH probe was confirmed with dried atmospheric air (<1% RH using Drierite) and by breathing on the sensor (100% RH).

Analog outputs from the O_2 analyser, CO_2 analyser, and RH probe were interfaced to a PC using a Sable Systems UI2 A/D converter and recorded every 20 sec, using custom-written data acquisition software (Visual Basic v6; P. Withers). A baseline of

background O_2, CO_2 and H_2O was measured for an hour before and after the budgerigar measurement period. Calculations of VO_2, VCO_2 and EWL were made using a custom-written program (Visual Basic v6; P. Withers) after Withers (2001). T_b was recorded every 10 sec throughout the experimental period, using Respironics VitalView ® data acquisition software (v4.2). The minimum stable 20-min mean VO_2, VCO_2, EWL and T_b were determined for each hour of the experiment (Withers 2001; Cooper and Withers 2010).

Measurement duration

Time taken to obtain minimum values within the 12 h period was used to determine the effect of experimental duration on standard physiological variables. Minimum 20-min mean VO_2, VCO_2, EWL and T_b values were converted to a percentage of the lowest hourly value. Longer experimental durations will inevitably result in lower values due to both a reduction in activity/alertness of the budgerigars over time (animal effect) and a higher probability of obtaining a lower value when more values are measured (sampling effect). To determine if the effect of measurement duration was due to animal or random sampling effects we compared the actual hourly minimum values with those obtained from random re-assortment of data (Cooper and Withers 2009). Data were randomly re-assorted 10,000 times using a custom-written Excel macro. The number of times that the actual mean hourly percentages were higher than the randomly re-assorted mean hourly percentage was interpreted as the probability that there was a significant animal effect. This would indicate whether the decline in hourly minimal values over time was the result of random fluctuations in measurements, or a systematic pattern of decline as a result of budgerigars being more

alert and awake at the commencement of experiments. We interpret $P < 0.05$ as indicating significance of a non-random animal effect, that is, evidence of a higher VO_2 (or VCO_2, EWL, T_b) for that measurement period than expected based on random fluctuations in metabolic rate.

Experiment start time

Each budgerigar was measured for 12 h at start times (S_{exp}) of 15:00 h, 17:00 h, 19:00 h, 21:00 h and 23:00 h in random order. For analysis of circadian effects on the experimental duration required to attain minimal values, the actual time of day that minimum values occurred was determined for each S_{exp}. To analyse these actual times of day when minimal values were measured, we converted hours of the day to a linear rather than circular scale, from 12 to 35, with 12:00 h = 12, 13:00 h = 13, 00:00 h = 24, 01:00 h = 25, etc.

Statistics

The effect of measurement duration was further examined by ANOVA using ranked data, with simple a priori contrasts (comparing each hour in turn with the final hour) to determine which hours were significantly different from 100%. For analysis of the effect of S_{exp}, multivariate repeated measures analysis of variance (RMANOVA) was used separately for VO_2, VCO_2, standard EWL and T_b, and polynomial contrasts were used to examine linear and quadratic effects over time (Rencher 1998). Polynomial contrast equations are presented for VO_2, VCO_2, and EWL, but could not be calculated for T_b.

SPSS (v17.0) and *statisti*XL (v1.8) were used for statistical analyses. A custom-written Excel macro (P. Withers) was used for contrast analysis of the repeated measures. Statistical significance was judged at a significance level of $P < 0.05$. Results are reported as mean ± standard error, with sample size (n) = 11 for VO_2, VCO_2 and EWL, and $n = 5$ for T_b (unless stated otherwise). This research was approved by Curtin University's Animal Ethics Committee (approval number N48/08).

Results

The body mass of the budgerigars ranged from 30.8 ± 0.47 to 47.5 ± 0.99 g; mean = 36.8 ± 1.58 g. There was no significant difference in body mass of budgerigars between experimental treatments ($F_{4,7} = 1.45$, P = 0.312), but there was a difference between individuals ($F_{10,44} = 24.04$, P = < 0.001).

Budgerigars were typically alert, with a high MR, EWL and T_b at the commencement of the experiment. Physiological variables declined sharply, and then more gradually as the experiment progressed (e.g. Fig. 1).

Measurement duration

Measurement duration had a significant effect on minimal VO_2, VCO_2, EWL and T_b for all start times (Fig. 2). Hourly minimum values were statistically indistinguishable from 100% by 7 h (S_{exp} 21:00 h) to 9 h (S_{exp} 15:00 h) for VO_2, 6 h (S_{exp} 23:00 h) to 9

h (S_{exp} 17:00 h) for VCO_2, 6 h (S_{exp} 23:00 h) to 10 h (S_{exp} 15:00 h and 17:00 h) for EWL, and 5 h (S_{exp} 23:00 h) to 8 h (S_{exp} 15:00 h and 17:00 h) for T_b.

Random re-assortment of hourly minima indicated that there was a significant animal effect for all S_{exp} on measurement duration for VO_2, VCO_2, EWL and T_b (Fig. 2). Hourly minimal VO_2 remained significantly higher than randomised means for between 4 h (S_{exp} 21:00 h; $P = 0.022$) and 10 h (S_{exp} 17:00 h; $P = 0.042$), and for VCO_2 between 2 h (S_{exp} 21:00 h and 23:00 h; $P < 0.039$) and 8 h (S_{exp} 15:00 h and 17:00 h; $P < 0.021$). For EWL, experimental means remained significantly higher than randomised means for between 1 h (S_{exp} 23:00 h; $P < 0.001$) and 10 h (S_{exp} 15:00 h and 17:00 h; $P = 0.002$) and for T_b between 5 h (S_{exp} 23:00 h; $P = 0.002$) and 10 h (S_{exp} 15:00 h; $P = 0.015$).

Experiment start time

Minimal VO_2 was significantly influenced by S_{exp} (RMANOVA $F_{4,7} = 4.22$, $P = 0.047$) ranging from 1.8 ± 0.11 mL O_2 g^{-1} h^{-1} (S_{exp} 17:00 h) to 2.2 ± 0.11 mL O_2 g^{-1} h^{-1} (S_{exp} 19:00 h; Fig. 3). There was a significant positive, linear relationship between S_{exp} and minimal VO_2 by polynomial contrasts ($t_{10} = 2.87$, $P = 0.016$; $VO_2 = 1.65 + 0.052\ S_{exp}$). There was also a significant difference in minimal VCO_2 between the different S_{exp} ($F_{4,7} = 5.19$, $P = 0.029$), which ranged from 1.5 ± 0.05 mL CO_2 g^{-1} h^{-1} (S_{exp} 1500 h) to 1.8 ± 0.09 mL CO_2 g^{-1} h^{-1} (S_{exp} 1900 h; Fig. 3). Polynomial contrasts indicated that the relationship between VCO_2 and S_{exp} was positive and linear ($t_{10} = 4.71$, $P < 0.001$; $VCO_2 = 1.46 + 0.029\ S_{exp}$). Minimal EWL ranged from 1.4 ± 0.12 mg H_2O g^{-1} h^{-1} (S_{exp} 21:00 h) to 1.6 ± 0.15 mg H_2O g^{-1} h^{-1} (S_{exp} 17:00 h), but there

was no significant effect of S_{exp} ($F_{4,7}$ = 0.84, P = 0.540; Fig. 3), and no significant polynomial contrasts. Body temperature ranged from 38.8 ± 0.36°C (S_{exp} 15:00 h) to 39.2 ± 0.25°C (S_{exp} 23:00 h; Fig. 3). The overall RMANOVA model did not detect a difference in minimal T_b at different S_{exp} ($F_{4,1}$ = 3.25, P = 0.392), but polynomial contrasts indicated a significant linear effect (t_4 = 3.1, P = 0.036). Individual budgerigars did not differ in minimal VO_2 (P = 0.056), but did differ significantly in minimal VCO_2, EWL and T_b ($P \leq 0.010$).

Time taken to reach minimal (T_{exp}) VO_2 differed significantly with S_{exp} for budgerigars (RMANOVA $F_{4,7}$ = 7.63, P = 0.011; Fig. 4), although polynomial contrasts did not reveal a significant linear, quadratic or cubic effect. T_{exp} for VCO_2 and T_b did not differ with S_{exp} by RMANOVA (T_b, $F_{4,7}$ = 3.07, P = 0.093; VCO_2, $F_{4,1}$ = 13.50, P = 0.201; Fig. 4), but polynomial contrasts indicated a significant negative linear effect (T_b, t_{10} = 2.42, P = 0.036; VCO_2, T_{exp} = 8.33 – 0.34 S_{exp}; t_4 = 7.59, P = 0.002). T_{exp} for EWL differed significantly with S_{exp} (RMANOVA $F_{4,7}$ = 15.01, P = 0.002; Fig. 4). Polynomial contrasts indicated that the relationship between S_{exp} and T_{exp} to minimal EWL was negative and linear (t_{10} = 6.21, P < 0.001; T_{exp} = 10.74 – 0.48S_{exp}). T_{exp} for T_b ranged from 4:00 h ± 38 min (S_{exp} 23:00 h) to 8:48 h ± 52 min (S_{exp} of 15:00 h; Fig. 4).

The actual time of day at the minimum (D_{exp}) for VO_2 ranged from 23:30 h ± 48 min (S_{exp} 17:00 h) to 06:00 h ± 60 min (S_{exp} 23:00 h), and this differed significantly with S_{exp} (RMANOVA $F_{4,7}$ = 31.76, P < 0.001; Fig. 5). Polynomial contrasts indicated that the relationship between S_{exp} and D_{exp} to obtain minimal VO_2, was positive and linear (t_{10} = 6.59, P < 0.001; D_{exp} = 21.04 + 0.68 S_{exp}). D_{exp} for VCO_2 ranged from 22:24 h ±

40 min (S_{exp} 15:00 h) to 04:18 h ± 51 min (S_{exp} 23:00 h), and this differed significantly with S_{exp} ($F_{4,7}$ = 11.22, P = 0.004; Fig. 5). Polynomial contrasts indicated a significant positive linear relationship between S_{exp} and D_{exp} for VCO_2 [t_{10} = 6.46, P < 0.001; D_{exp} = 20.3 + 0.66 S_{exp}). D_{exp} for EWL ranged from 23:48 h ± 41 min (S_{exp} 15:00 h and 17:00 h) to 06:00 h ± 58 min (S_{exp} 23:00 h), and this differed significantly with S_{exp} ($F_{4,7}$ = 17.69, P = 0.001; Fig. 5). Polynomial contrasts indicated that this effect was positive and linear (t_{10} = 7.88, P < 0.001; S_{exp} = 22.7 + 0.52 S_{exp}). D_{exp} for T_b ranged from 23:48 h ± 52 min (S_{exp} of 15:00 h) to 04.00 h ± 38 min (S_{exp} of 23:00 h; Fig. 5). Although these differences were not significant by RMANOVA ($F_{4,1}$ = 10.35, P = 0.229), polynomial contrasts indicated a significant quadratic effect (t_4 = 7.59, P = 0.002). D_{exp} did not differ between individual budgerigars ($P \geq 0.062$).

Discussion

Our study is the first to investigate the effect of experiment duration on measurement of BMR, standard EWL and standard T_b for birds. As for mammals (Hayes et al. 1992; Cooper and Withers 2009), we found highly significant effects of experimental duration on estimates of standard physiological variables. A significant animal effect, as indicated by variables being elevated significantly above randomised data, occurred for up to 10 h into measurement for VO_2, up 8 h for VCO_2, up to 10 h for EWL, and up to 5 h for T_b (depending on experiment start time). This demonstrates that alertness due to initial handling and becoming accustomed to the experimental environment has a significant effect on these physiological variables. A sufficient experimental duration is necessary to allow an animal to settle and rest in the experimental chamber for measurement of minimal values, and lower values over time are not simply a

mathematical inevitability from random sampling effects. The actual time required to obtain minimal values ranged from 4:00 h ± 38 min (T_b, S_{exp} 23:00 h) to 8:54 h ± 54 min (EWL, S_{exp} 17:00 h). Therefore experimental durations greater than these periods are necessary to obtain reliable estimates, and shorter measurement durations will substantially overestimate BMR, EWL and T_b.

The time required to measure the budgerigars' BMR and standard T_b was not necessarily sufficient to obtain standard EWL; for example at start time 17:00 h, 6.6 ± 0.84 h was required to obtain minimal VO_2, but 8.9 ± 0.87 h was required for minimal EWL. This discrepancy between required duration for BMR and standard EWL was also demonstrated by Cooper and Withers (2009) for six marsupial species. The longer time required to obtain standard EWL in respirometry systems can be affected by air flow rate (and thus washout of water vapour) and whether the animal urinates or defecates during the experiment, which may uncouple EWL and MR (Cooper and Withers 2009). Use of mineral oil may reduce the time required to obtain minimal EWL during an experiment, but the relative "stickiness" of water vapour in a system, particularly if there are plastic components, may still increase washout time. Therefore, a sufficient experimental duration is even more important for obtaining minimal EWL, and short measurement durations are likely to produce more severe overestimations of EWL than other physiological variables (Cooper and Withers 2009).

The effects of measurement duration described by Hayes et al. (1992), Cooper and Withers (2009) and this study may interact with effects of circadian rhythm. Hayes et al. (1992) and Cooper and Withers (2009) did not consider the circadian rhythm as a

confounding factor; they attributed differences in the time to minimal values to effects of measurement duration when it could also have been influenced by timing of measurements with respect to the animal's circadian rhythm. By commencing experiments at different times throughout the budgerigar's rest phase, we have demonstrated here that circadian rhythms also influence the timing and magnitude of these minimum physiological values.

A circadian rhythm of MR and core T_b is well documented, being lower during the ρ phase (Sturkie 1965; Aschoff and Pohl 1970; McNab 1966; Prinzinger and Hanssler 1980; McKechnie and Lovegrove 1999; Ellis and Gabrielsen 2001; Krauchi 2002). As T_b in endotherms is related to MR, we would expect minimal T_b to occur around the same time as minimal VO_2. Budgerigars attained minimal T_b between 23:00 h and 04:00 h, which is similar to when minimal VO_2 occurred, and is consistent with circadian minima in other species (e.g. Williams et al. 1991). Budgerigars required a shorter period of time to obtain minimal values for physiological variables at later S_{exp}, where they were closer to the minimal point in their circadian rhythm at the commencement of the experiment. This demonstrates a clear influence of circadian rhythm on the timing of measurement of standard variables.

The timing of minimal physiological variables for budgerigars is a combination of the significant effects of both measurement duration and circadian rhythm. S_{exp} influenced the time of day at which minimal values were obtained for all physiological variables measured, suggesting a handling effect. If there was no handling effect and only a circadian effect, then minimal values would have been obtained at the same time of day, regardless of experimental S_{exp}. This did not happen,

and there was clearly a disturbance effect with the budgerigars taking a number of hours to settle after introduction into the chamber despite variation in the time the experiment commenced. However, a shorter period of time was required to obtain minimal values for later S_{exp}, reflecting the circadian effect. Therefore, both circadian rhythm and measurement duration clearly play a role in the time required to obtain minimum values for physiological variables, and both need to be considered carefully when designing repiromentry experiments to measure standard variables. Overall, minimal values were lower for those experiments that commenced earlier. This presumably resulted from a late S_{exp} being close to the budgerigars' circadian minimum which combined with an experiment duration effect meant that variables were still elevated due to the disturbance effect when the circadian minimum was reached. This resulted in higher values for standard variables than if birds were allowed to settle well before the minimum point in their circadian cycle.

Previous measures for budgerigars of BMR (1.96 ml O_2 g^{-1} $h^{-}1$), T_b (39°C) and standard EWL (2.32 mg H_2O g^{-1} h^{-1}; Weathers and Schoenbaechler 1976) are higher than our estimates of BMR (1.83 ± 0.107 ml O_2 g^{-1} h^{-1}), T_b (38.8 ± 0.29 °C) and standard EWL (1.37 ± 0.109 mg H_2O g^{-1} h^{-1}), being 107 % (BMR) to 169 % (EWL) of our values. As Weathers and Schoenbaechler's (1976) data were also for postabsorptive adult birds measured during the ρ phase, it is likely that their short measurement duration (birds were allowed to rest for at least an hour before VO_2, VCO_2 and EWL were calculated) contributed to these differences, although gravimetric measurement of EWL presumably contributed as well. Other estimates of physiological variables for budgerigars are difficult to compare to these data as the conditions for standard measures were not met. Prinzinger and Hänssler (1980)

measured their budgerigars at T_a below thermoneutrality (20-25°C) and did not state if the birds were postabsorptive, and Greenwald et al. (1967) conducted their experiments during the α phase.

We conclude that both measurement duration and circadian rhythm have a significant effect on measurement of standard physiological variables, and both need to be considered in experimental design. Based on our study of budgerigars, experiments for small diurnal birds should commence between 17:00 h and 21:00 h, and measurement duration should be at least 9 h, particularly when making initial measurements for a previously unmeasured species, to ensure minimal BMR and standard EWL and T_b are obtained. Experimental durations less than this are likely to overestimate BMR, standard EWL and $T_{b,}$ and commencing experiments later may disrupt the bird too close to their minimum circadian point to achieve a true resting value.

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Figures

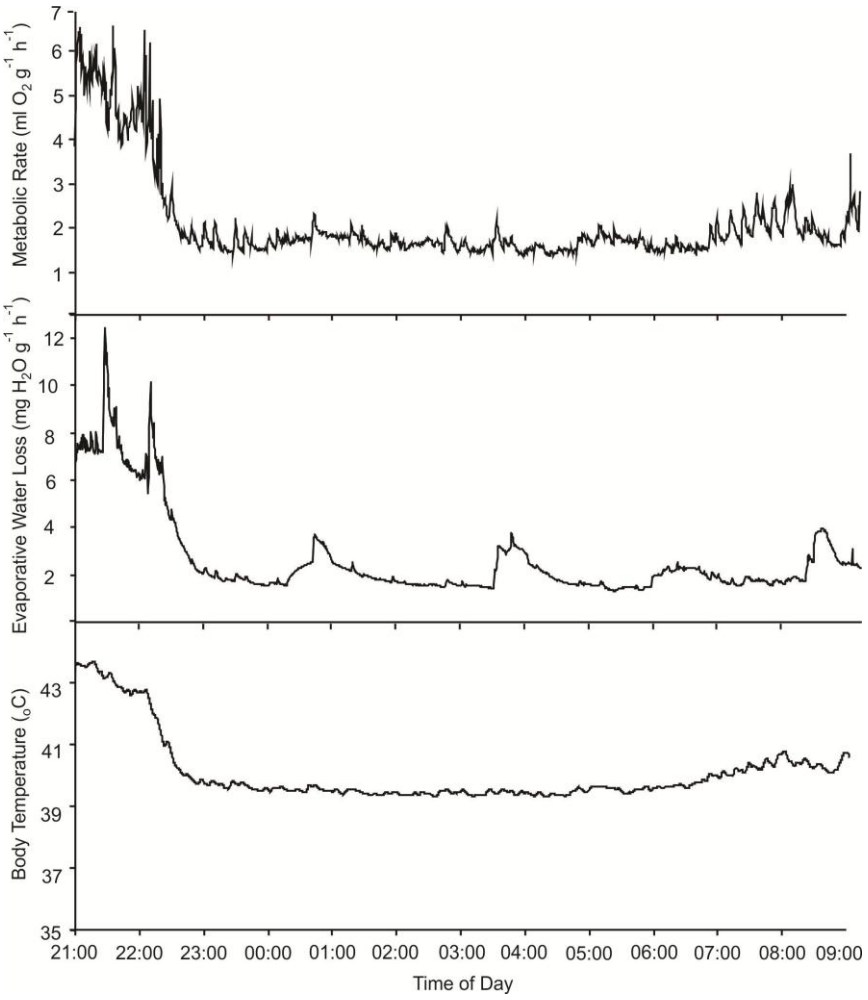

Fig. 1 Example of an experimental time course for metabolic rate (measured as oxygen consumption), evaporative water loss and body temperature for a budgerigar during a 12 hour experiment commencing at 21:00 h.

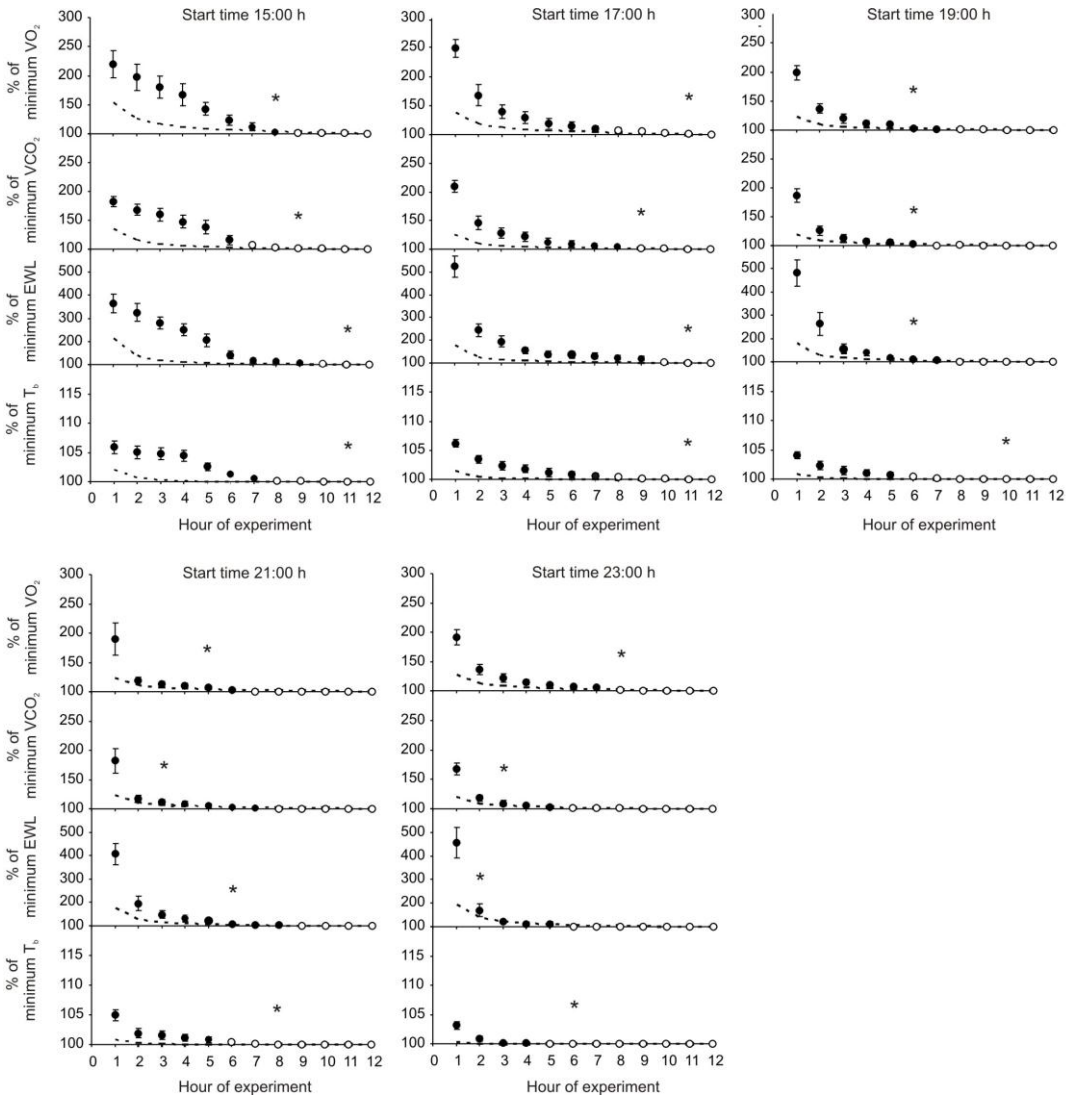

Fig. 2 Effect of experimental duration (h) on the percent of experimental minimum for oxygen consumption (VO_2), carbon dioxide production (VCO_2), evaporative water loss (EWL), and body temperature (T_b) at different start times. Black circles are significantly different from the experimental minimum; white circles are not significantly different from the experimental minimum. Dashed lines are the mean percent of the experimental minimum for 10,000 random reallocations of hourly minimum values. An asterisk (*) indicates where experimental means become statistically indistinguishable from randomised means. Values are mean ± SE; n = 11 for VO_2, VCO_2 and EWL, and n = 5 for T_b

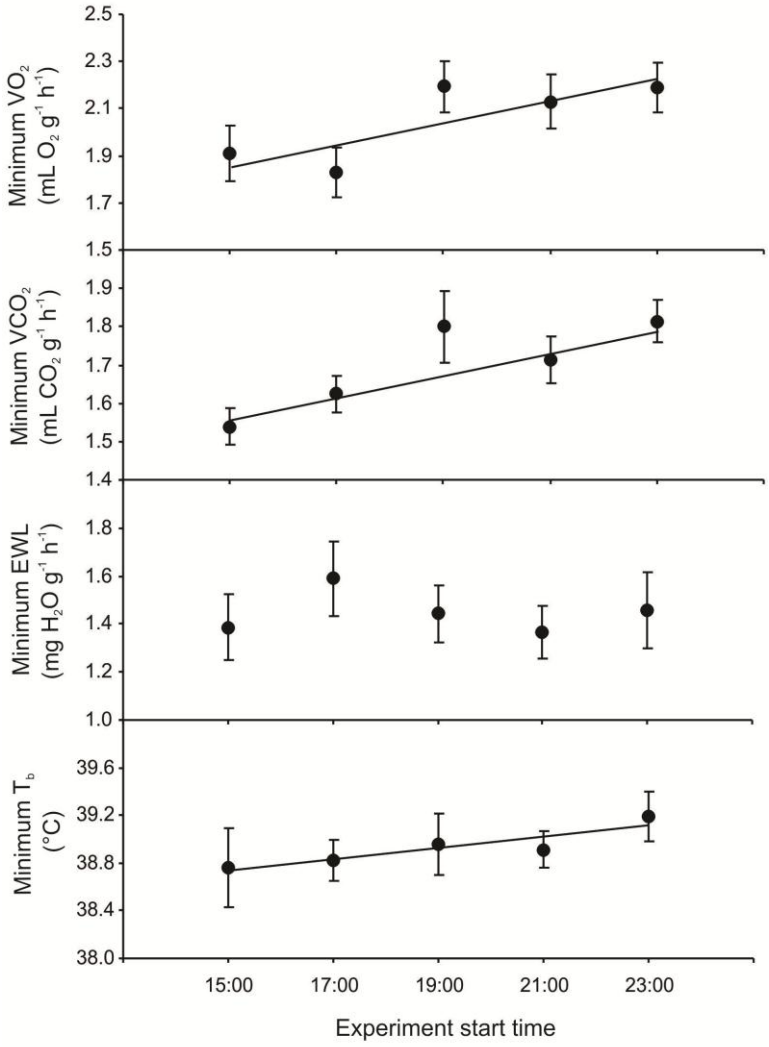

Fig. 3 Effect of experimental start time on minimal oxygen consumption (VO_2), carbon dioxide production (VCO_2), evaporative water loss (EWL), and body temperature (T_b) of budgerigars. A line representing the repeated measures polynomial contrast is included where it is significant. Values are mean ± SE, n = 11 for VO_2, VCO_2 and EWL, and n = 5 for T_b

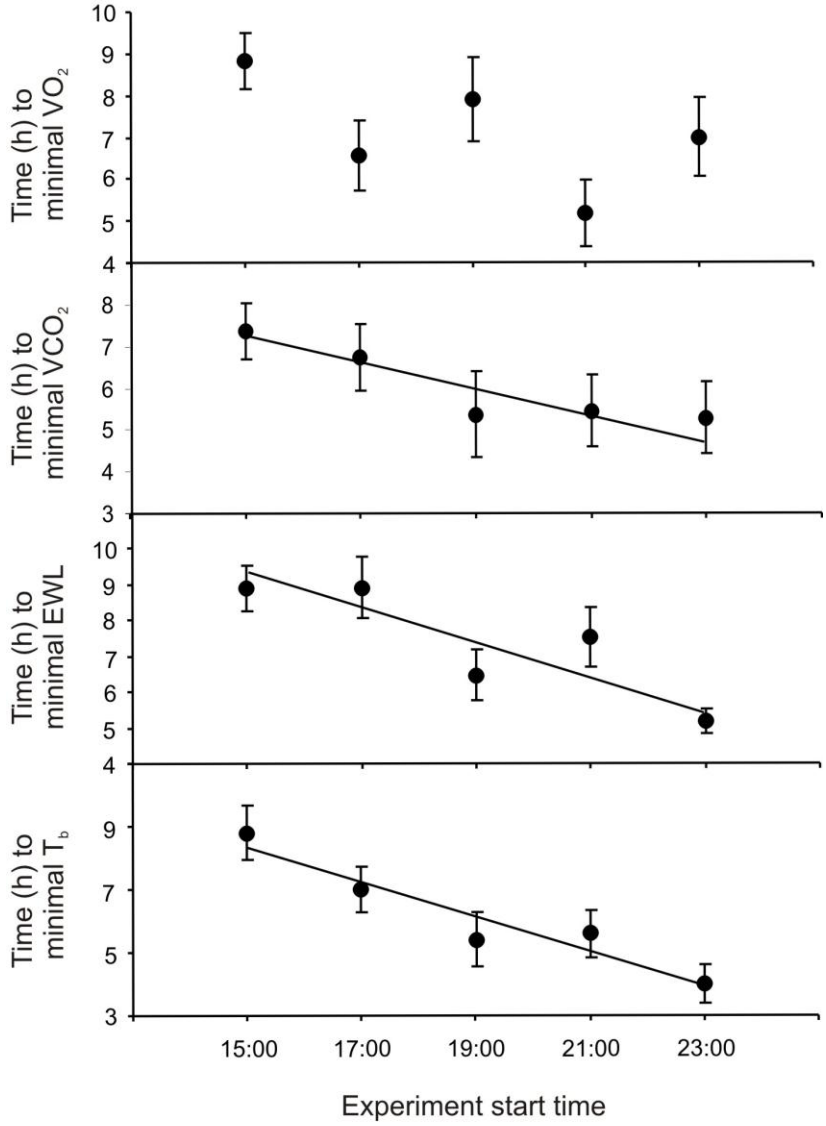

Fig. 4 Time taken (h) to reach minimal oxygen consumption (VO_2), carbon dioxide production (VCO_2), evaporative water loss (EWL), and body temperature (T_b), at different experimental start times. A line representing the repeated measures polynomial contrast is included where it is significant. Values are mean ± SE., n = 11 for VO_2, VCO_2 and EWL, and n = 5 for T_b

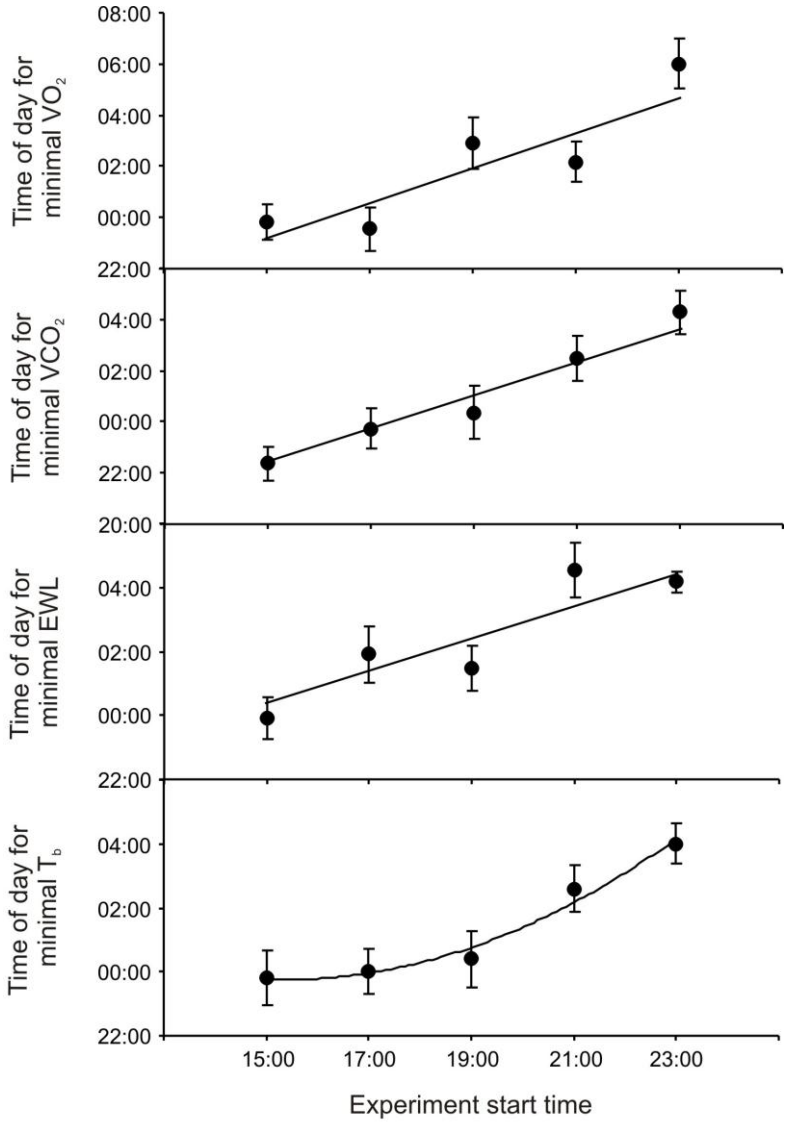

Fig. 5 Time of day that budgerigars attained minimal oxygen consumption (VO_2), carbon dioxide production (VCO_2), evaporative water loss (EWL), and body temperature (T_b), at different experimental start times. A line representing the repeated measures polynomial contrast is included for VO_2, VCO_2 and EWL, and a conventional quadratic line included for T_b. Values are mean ± SE, n = 11 for VO_2, VCO_2 and EWL, and n = 5 for T_b