The influence of circadian rhythms on pre- and post-prandial metabolism in the snake *Lamprophis fuliginosus*

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Abstract

Measuring standard metabolic rate (SMR) and specific dynamic action (SDA) has yielded insight into patterns of energy expenditure in snakes, but less emphasis has been placed on identifying metabolic variation and associated energy cost of circadian rhythms. To estimate SMR, SDA, and identify metabolic variation associated with circadian cycles in nocturnally active African house snakes (*Lamprophis fuliginosus*), we measured oxygen consumption rates (\(V\dot{O}_2\)) at frequent intervals before and during digestion of meals equaling 10%, 20% and 30% of their body mass. Circadian rhythms in metabolism were perceptible in the \(V\dot{O}_2\) data during fasting and after the initial stages of digestion. We estimated SMR of *L. fuliginosus* (mean mass=16.7 ± 0.3 g) to be 0.68 ± 0.02 (±SEM) mL O\(_2\)/h at 25 °C. Twenty-four hours after eating, \(V\dot{O}_2\) peaked at 3.2–5.3 times SMR. During digestion of meals equaling 10–30% of their body mass, the volume of oxygen consumed ranged from 109 to 119 mL O\(_2\) for SMR, whereas extra oxygen consumed for digestion and assimilation ranged from 68 to 256 mL O\(_2\) (equivalent to 14.5–17.0% of ingested energy). The oxygen consumed due to the rise in metabolism during the active phase of the daily cycle ranged from 55 to 66 mL O\(_2\) during digestion. Peak \(V\dot{O}_2\), digestive scope, and SDA increased with increasing meal size. Comparisons of our estimates to estimates derived from methods used in previous investigations resulted in wide variance of metabolic variables (up to 39%), likely due to the influence of circadian rhythms and activity on the selection of baseline metabolism. We suggest frequent \(V\dot{O}_2\) measurements over multiple days, coupled with mathematical methods that reduce the influence of undesired sources of \(V\dot{O}_2\) variation (e.g., activity, circadian cycles) are needed to reliably assess SMR and SDA in animals exhibiting strong circadian cycles.

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1. Introduction

Assessing patterns of energy utilization and developing energy budgets for individuals have provided a framework for investigating physiological, behavioral, and ecological adaptations of reptiles (Congdon et al., 1982). Metabolic energy expenditures can be estimated by measuring rates of oxygen consumption (\(V\dot{O}_2\)). Two commonly examined metabolic measures are standard metabolic rate (SMR), defined as the metabolic rate of a post-absorptive animal at rest at a specified temperature during the inactive phase of its circadian cycle (Bennett and Dawson, 1976), and specific dynamic action (SDA), defined as the increased energy expenditure associated with digestion, assimilation and biosynthesis (Kleiber, 1975). As SMR and SDA account for a large portion of annual energy budgets in reptiles, especially in snakes (Peterson et al., 1997; Beaupre and Duvall, 1998; McCue and Lillywhite, 2002), identifying variation in these factors is important for assessing patterns of overall energy expenditure. Consequently, measuring the costs of SMR and SDA has been a focus of recent investigations assessing...
variation in energy allocation patterns associated with many aspects of snake biology, including biogeography (Beaupre, 1993), foraging ecology (Secor and Diamond, 2000), and responses to xenobiotics (Hopkins et al., 1999).

Compared to SMR and SDA, less emphasis has been placed on quantifying the metabolic costs associated with circadian rhythms in snakes. However, energy used during active phases of the circadian cycle may also represent a large portion of energy expenditure in reptiles. For example, Niewiarowski and Waldshmidt (1992) estimated the annual metabolic cost of circadian variation to be roughly equal to SMR costs for the lizard Sceloporus merriami. While the circadian rhythm has been recognized as an important aspect of the biology of many lizards, comparatively few studies address circadian metabolic variation in snakes (Waldshmidt et al., 1987; Blem and Killeen, 1993).

Identifying metabolic variation associated with circadian rhythms may also be critical for obtaining accurate estimates of SMR and SDA. Animals may differ in activity levels, states of alertness, and presence or timing of circadian rhythms, and if such variation is not identified, comparisons of metabolism among studies may be difficult (Bennett and Dawson, 1976; Andrews and Pough, 1985). For instance, if respiratory measurements coincide with active phases of the circadian cycle or with spontaneous activity during typically inactive periods, metabolic estimates that require the animal to be in a resting state may be overestimated. By definition, SMR and SDA should not include the additional energy costs incurred during activity or daily metabolic oscillations (Kleiber, 1975; Bennett and Dawson, 1976). Despite the possibility that circadian cycles may confound SMR and SDA estimates, few researchers examining these measures have considered circadian cycles as potentially important sources of metabolic variation in snakes (but see Beaupre and Duvall, 1998; Beaupre and Zaidan, 2001; Zaidan and Beaupre, 2003).

The recent focus of metabolic studies on snakes has been on boas, pythons, and pitvipers, which are similar in being relatively inactive and typically ingesting large meals at infrequent intervals. Fewer studies have investigated metabolism in colubrids, which tend to be more active and to eat more frequent and smaller meals. In this study, we examine aspects of pre-and post-prandial metabolism in a nocturnally active colubrid snake, the African house snake (Lamprophis fuliginosus Bioe), to (1) determine the influence of circadian rhythms on daily metabolic variation and associated energy use, (2) assess the importance of detailed measures of circadian metabolic variation for estimating SMR and SDA, and (3) investigate the effect of meal size on SDA. As a requisite objective, we also describe a data acquisition and estimation technique that yields SMR and SDA estimates minimally influenced by activity and circadian cycles.

2. Materials and methods

2.1. Study species

*L. fuliginosus* (Colubridae) is a medium-sized terrestrial snake common in grassland and suburban habitats throughout Sub-Saharan Africa. It forages nocturnally for rodents and other small vertebrates, including bats (Branch, 1998; Marais, 1992). Captive snakes exhibit circadian patterns of activity, with greatest activity occurring in the scotophase (Luttershmidt et al., 2002).

Adult *L. fuliginosus* were obtained from a captive colony at the University of Texas in Tyler, TX, and a breeding colony was established at the Savannah River Ecology Laboratory in Aiken, SC. Snakes from 6 clutches were collected and hatched in August and September 1999. Snakes were housed individually in polyethylene containers (33.0 × 18.4 × 10.5 cm) with aspen shavings and maintained on a 12L:12D cycle (photophase starting at 0700 and scotophase at 1900) at 27 °C for 3–4 months. Snakes were given constant access to water and fed mice weighing 20–40% of snake body mass biweekly. To minimize variation due to age, size, and sex, we only used juvenile females within a narrow mass range \( n=24, \text{mean mass}=16.7\pm0.3 \text{ g, range}=14.3–18.6 \text{ g} \). Animal care and use protocols were approved by the University of Georgia’s Institutional Animal Care and Use Committee (#A1999-10024-c2).

2.2. Pre-prandial metabolic measurements

Twenty-four snakes were fasted for 11–14 days before metabolic measurements to ensure they were postabsorptive. Twenty-four hours prior to measurements, snakes were weighed and placed in individual 2780 ml respiratory chambers within an environmental cabinet (25 °C under constant dark conditions), where all metabolic measurements were taken. The exterior of each chamber was wrapped with paper to reduce external stimuli and chambers contained a heavily perforated hide-plated that provided refuge for the snake. Water was not provided during measurements of fasting metabolism.

We determined metabolic rate indirectly as \( VO_2 \) (adjusted for standard temperature and pressure) by connecting each respiratory chamber to a computer controlled, closed system respirometer (Micro Oxymax, Columbus Instruments, Columbus, OH, USA) described by Hopkins et al. (1999, 2004). Air was pumped from each respiratory chamber through a drying column containing Drierite before passing through an oxygen sensor. Metabolic measurements commenced at 1200 h, and each chamber was sampled 20 times over a 48-h period at alternating 1.4 and 3.2 h intervals. Sampling occurred at alternating intervals because the chambers were refreshed with ambient air equaling 3 × the volume of air in the chamber after every other sample. Snakes remained undisturbed during the 48-h fasting measurement period. Each snake was weighed prior to
and following metabolic measurements. Snakes lost only 1.9±0.2% of their initial body mass, indicating *L. fuliginosus* had low rates of water loss and that water deprivation likely was not stressful. After fasting measurements, snakes were held in the environmental cabinet and allowed constant access to water until digestive metabolic measurements began.

We developed a mathematical technique to estimate SMR using polynomial regression to model the variance in VO$_2$ due to circadian rhythms. Polynomial regression was chosen because this analysis can model variables that fluctuate cyclically over time, a pattern typical of circadian rhythms. Time elapsed since measurements began was the independent variable, and VO$_2$ was the dependent variable. We started with 6th-order polynomial models and dropped non-significant terms (*P* > 0.05) from the models. Higher-ordered (3rd–6th) regression models provided the best fit of observed and predicted values, whereas VO$_2$ variation for snakes that exhibited weak circadian rhythms was explained best with lower-ordered regression models (1st–2nd order; Fig. 1). In cases where snakes exhibited weak circadian rhythms, intermittent bouts of increased VO$_2$ of high magnitude or out of sequence relative to neighboring values were common, likely representative of spontaneous activity (Fig. 1). We used the mean $R^2$ value from models for snakes that fit 3rd–6th-ordered models as an estimate of the variance in VO$_2$ due to time of day (circadian rhythms). Based on this estimate of variance, we removed an upper percentage of measures corresponding to this mean $R^2$ value and calculated SMR as the mean of the remaining measures. The upper values were removed because we assumed estimator bias was in one direction and produced over-estimations of SMR. Of the 24 snakes used, 16 fit the 3rd–6th order polynomial regression models, five fit 1st and 2nd order models, and only three snakes showed metabolic variation not described by any model. The mean $R^2$ value of the 16 snakes fitting 3rd–6th order models was 0.76±0.03. We thus eliminated the upper 75% of VO$_2$ measurements and estimated baseline metabolism (SMR) as the mean of the lower 25% of measurements ($n$ = 5 of 20; AVG lower 25% VO$_2$) for all 24 snakes (Fig. 1). We used 75% rather than 76% because this number results in selection of a whole number from 20 measurements and is within the standard error variance of our mean $R^2$ value.

In addition to the procedure described above, we calculated SMR based on estimation techniques (hereafter referred to as SMR estimators) used to establish baseline metabolism in two other studies: (1) the average of the lower 50% of VO$_2$ measurements (AVG lower 50% VO$_2$; Hopkins et al., 1999), and (2) the single lowest VO$_2$ measurement (Lowest VO$_2$; Beaupre, 1993; Secor, 1995; Secor and Diamond, 1995, 1997a,b, 2000).

We also estimated the additional oxygen consumed (above SMR) due to circadian rhythms for the 48-h fasting period. Oxygen consumed during active phases of the circadian cycle was estimated by subtracting the volume of oxygen consumed for SMR from the total volume of oxygen consumed after a running median of five values (using a centered integration including each value and the two values on either side) was applied to the data. This technique normalizes each data point to proximate points, effectively eliminating isolated spikes in VO$_2$ of short duration resulting from sporadic activity (i.e., VO$_2$ of high magnitude or out of sequence relative to neighboring values), but adequately describing more sustained patterns of change in VO$_2$ (e.g., changes in VO$_2$ associated with circadian rhythms or digestion; see Fig. 2). Thus, our metabolic estimates during active phases of the circadian cycle likely reflect the combined influence of any rise in resting metabolism and periods of consistent activity, but not metabolism for isolated instances of sporadic activity.

### 2.3. Post-prandial metabolic variables

Twenty-four hours after fasting measurements were completed, snakes were fed a single dead mouse weighing 10%, 20% or 30% of snake body mass ($n$ = 8/feeding
treatment). Six snakes were run during each of four respiratory trials, and feeding levels were spread across all trials. Immediately after eating, snakes were placed in respiratory chambers and were sampled 119 times each at alternating 0.8 and 1.8 h intervals (with the additional hour between samples for refreshing chambers) for 7 days. Configuration of the respirometer and the conditions in the environmental chamber were the same as for fasting measurements, but because of the longer duration of chamber occupancy, snakes were given access to water (20 mL) for the first 48 h. Chambers were cleaned and snakes were weighed daily between 1100 and 1130 h and measurements recommenced at 1200 h each day. We assumed digestion was complete when VO₂ returned to pre-feeding SMR. If an individual failed to return to pre-feeding SMR, completion of digestion was determined to be at the end of day 7, which was at least 1 day following final defecation.

To reduce the influence of VO₂ variation due to spontaneous activity and circadian cycles on SDA estimates, we applied a two-part curve smoothing technique to the VO₂ measurements acquired for each snake. A two-part smoothing technique was used because temporal patterns in VO₂ variation differed between the first 48 h and the final 120 h of digestion. Variation in VO₂ attributable to spontaneous activity or circadian cycles was small, of short duration, and often undetectable during the first 48 h of digestion. Thus, for the first 48 h metabolic rate was described using the running median of five values (described above). For the final 120 h of measurements, circadian VO₂ variation similar to that of fasting periods returned. To exclude rises in metabolism associated with circadian rhythms from digestive metabolic estimates during the final 120 h of digestion, we used the AVG lower 25% VO₂ estimator to approximate oxygen consumed for SDA during each of the remaining 5 days of measurements.

We calculated the following variables during the 7 days of digestive metabolic measurements: peak VO₂ (maximal VO₂ after feeding), digestive scope (peak VO₂/SMR), and time to peak VO₂. We also calculated mL O₂ consumed for SMR (based on pre-feeding VO₂ measurements extrapolated over the digestive period), mL O₂ consumed for SDA (area under the SDA curve minus SMR), and mL O₂ consumed above SMR and SDA during active phases of the circadian cycle (area under the circadian curve minus SMR and SDA; Fig. 2). SDA was converted to energetic equivalents using a conversion factor of 19.8 J/mL O₂ (Secor and Diamond, 2000), and the cost of SDA was then calculated as a percentage of total ingested energy (hereafter referred to as SDA coefficient). Energy content of mice was determined by bomb calorimetry at the University of Georgia’s Poultry Science lab. For each meal size fed to snakes, we determined mean energy content (kJ/g wet mass) of 8–30 individual mice of similar size. Mean energy content (kJ/g wet mass) of mice in each feeding level was 4.7±0.1 (10%), 7.0±0.2 (20%), and 7.1±0.3 (30%).

2.4. Statistical analysis

Snake body mass and all metabolic variables were logarithmically (base 10) transformed prior to analyses to better approximate normal distributions and equal variances. The effects of the three SMR estimators on SMR values

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**Fig. 2.** Mean values for components of metabolism in *Lamprophis fuliginosus* (16.7±0.3 g) during digestion of meals equaling 10%, 20%, and 30% of snake body mass at 25 °C. Shaded regions represent mean volume of oxygen consumed for SMR, SDA, and active phases of the circadian cycle. See text for description of metabolic estimation procedures. Measurements began at 12:00 h (time “zero”).
were compared using repeated measures ANCOVA, with snake body mass as the covariate, and SMR estimator as the repeated variable in the model (PROC MIXED Model, SAS V 8.1, SAS Institute, 1999).

For all further comparisons of digestive metabolism dependent on SMR as a baseline value, we used SMR values derived from the AVG lower 25% VO2 SMR estimator. The timing and magnitude of the peak digestive metabolic response were compared among feeding levels using MANCOVA, with time to peak VO2, peak VO2, and digestive scope as the dependent variables, feeding level as the independent variable, and body mass as the covariate. Differences in SDA variables among feeding levels were investigated using MANCOVA, with SDA and SDA coefficient as the dependent variables, feeding level as the independent variable, and body mass as the covariate. To compare volume of oxygen consumed for SMR and for active phases of the circadian cycle among feeding levels we used ANCOVAs, with volume of oxygen consumed as the dependent variable, body mass as the covariate, and SMR estimator as the independent variable. We tested slopes of the relationships between body mass and all estimates of standard and digestive metabolic variables for homogeneity among feeding levels prior to covariance analyses by including an interaction term in the models. Slopes of the relationship between body mass and metabolic variables did not differ among feeding levels (log10 body mass×feeding level: P > 0.058 in all cases); thus, the interaction terms were dropped from the models. Each multivariate analysis was accompanied by univariate analyses (as a post-hoc test) to further examine the effects of feeding level and body mass for each dependent variable separately. The effects of feeding level (% body mass) on SDA and peak VO2 were also tested using regression analyses. We present results as the mean±1 SEM, and designate the level of statistical significance as P ≤ 0.05.

3. Results

3.1. Pre-Prandial metabolism

Fasted L. fuliginosus exhibited circadian cycles in metabolism (Figs. 1 and 3). Mean VO2 reached peaks of 1.4–1.8 mL O2/h around 1900 h on both days, and decreased to 0.66–0.82 mL O2/h between 0900 and 1400 h each day (Fig. 3). Fasted L. fuliginosus exhibited factorial increases in VO2 2.1–2.7 above SMR during the active phase of the circadian cycle. Total oxygen consumed over the 48-h period was 51.2 ± 2.2 mL O2, 62% of which was SMR (31.9 ± 1.7 mL O2), with the remaining 38% (19.3 ± 3.4 mL O2) accounted for by active phases of the circadian cycle.

Using the AVG lower 25% VO2 estimator, we estimated SMR of L. fuliginosus (mean mass=16.7±0.3 g) to be 0.68±0.02 mL O2/h at 25 °C. Use of the two other estimators resulted in a wide range of values, spanning 12.1% above and 10.4% below our AVG lower 25% VO2 estimate (Table 1, Fig. 3). The three estimators differed significantly, but due to the narrow mass range of snakes studied, the effect of mass on SMR was not significant (SAS PROC MIXED; log10 body mass: F1,20=1.46, P=0.239; estimator: F2,46=12.14, P<0.001; Table 1; Fig. 3).

3.2. Post-prandial metabolic variables

There was no clear evidence of the circadian cycle in metabolism during the initial two days of digestion, but for the last five days of digestion the cycle became evident again (Fig. 2). The timing of the circadian cycle during the last 5 days of digestion was similar to fasting periods, with the highest values occurring between 17:00 and 24:00 h, and the lowest values between 09:00 and 14:00 h (Fig. 2).

During the seven days of digestive measurements, L. fuliginosus consumed 108.8–118.7 mL O2 for SMR, plus an additional 54.6–66.0 mL O2 during active phases of its circadian cycle (Table 2). Snakes in the three feeding treatments had similar SMR, and neither meal nor body size influenced the volume of oxygen consumed for active phases of the circadian cycle (ANCOVA; SMR, log10 body mass: F1,20=0.04, P=0.853, feeding level: F2,20=0.66, P=0.528; circadian cycle, log10 body mass: F1,20=0.80, P=0.381, feeding level: F2,20=1.03, P=0.375).

Specific dynamic action ranged from 67.9 to 255.7 mL O2 (1.35–5.08 kJ) at 25 °C, increasing linearly with increasing meal size according to the following equation: mL O2=−24.92+9.37×feeding level (R2=0.92; P<0.001; Table 2; Fig. 4). Specific dynamic action coefficients ranged...
from 14.5% to 17.0% among feeding levels (Table 2). Taken together, aspects of SDA (SDA and SDA coefficient) differed significantly among feeding levels and body masses (MANCOVA; feeding level: Wilk’s $\lambda=0.020$, $F_{4,38}=56.94$, $P<0.001$; log10 body mass: Wilk’s $\lambda=0.464$, $F_{2,19}=10.95$, $P=0.001$). However, univariate analyses indicated that only SDA differed among feeding levels, whereas SDA coefficients did not (ANCOVA; SDA: $F_{2,20}=76.82$, $P<0.001$, SDA coefficient: $F_{2,20}=1.24$, $P=0.310$).

Aspects of peak digestive metabolism (timing, peak $V\dot{O}_2$, and digestive scope) differed among feeding levels, but not as a function of body mass (MANCOVA; feeding level: Wilk’s $\lambda=0.146$, $F_{6,36}=9.628$, $P<0.001$; log10 body mass: Wilk’s $\lambda=0.993$, $F_{3,18}=0.04$, $P=0.988$; Table 2, Fig. 4). However, univariate analyses indicated that only peak $V\dot{O}_2$ and digestive scope differed among feeding levels, whereas timing of peak $V\dot{O}_2$ did not (ANCOVA; peak $V\dot{O}_2$: $F_{2,20}=51.90$, $P<0.001$; digestive scope: $F_{2,20}=18.88$, $P<0.001$).

Fig. 4. Linear relationship between meal size and SDA (top) and curvilinear relationship between meal size and peak $V\dot{O}_2$ (bottom) for Lamprophis fuliginosus (16.7±0.3 g) at 25 °C. Values (points) are means±2 SEM. See text for equations and regression statistics.

### Table 1
Comparison of methods for estimating standard metabolic rate (SMR) for 24 Lamprophis fuliginosus (16.7±0.3 g) at 25 °C

<table>
<thead>
<tr>
<th>SMR estimator</th>
<th>$V\dot{O}_2$ (mL O2/h)</th>
<th>Mean</th>
<th>SEM</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVG lower 50% $V\dot{O}_2$</td>
<td>0.78</td>
<td>0.02</td>
<td></td>
<td>12.1</td>
</tr>
<tr>
<td>AVG lower 25% $V\dot{O}_2$</td>
<td>0.68</td>
<td>0.02</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Lowest $V\dot{O}_2$</td>
<td>0.61</td>
<td>0.02</td>
<td></td>
<td>–10.4</td>
</tr>
</tbody>
</table>

Right column indicates % difference from AVG lower 25% $V\dot{O}_2$ estimate (our estimate).

Sources for SMR estimators: AVG lower 50% $V\dot{O}_2$ (Hopkins et al., 1999); AVG lower 25% $V\dot{O}_2$ (this study); Lowest $V\dot{O}_2$ (Beaupre, 1993; Secor, 1995; Secor and Diamond, 1995, 1997a,b, 2000).

### Table 2
Lamprophis fuliginosus metabolism after ingesting different meal sizes at 25 °C

<table>
<thead>
<tr>
<th>Variable</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\eta$</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>–</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>17.1 (0.5)</td>
<td>16.3 (0.3)</td>
<td>16.6 (0.5)</td>
<td>–</td>
</tr>
<tr>
<td>Meal mass (% body mass)</td>
<td>10.1 (0.1)</td>
<td>20.0 (0.1)</td>
<td>30.1 (0.1)</td>
<td>–</td>
</tr>
<tr>
<td>Peak $V\dot{O}_2$ (mL O2/h)</td>
<td>2.2 (0.1)</td>
<td>3.2 (0.1)</td>
<td>3.6 (0.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Digestive scope</td>
<td>3.2 (0.2)</td>
<td>5.1 (0.4)</td>
<td>5.3 (0.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time to peak $V\dot{O}_2$ (h)</td>
<td>24.4 (2.1)</td>
<td>23.8 (1.6)</td>
<td>24.4 (1.7)</td>
<td>0.975</td>
</tr>
<tr>
<td>SDA</td>
<td>67.9 (8.3)</td>
<td>166.2 (8.3)</td>
<td>255.7 (8.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total KJ</td>
<td>1.4 (0.2)</td>
<td>3.3 (0.2)</td>
<td>5.1 (0.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>% Ingested energy</td>
<td>17.0 (2.5)</td>
<td>14.5 (0.9)</td>
<td>14.7 (1.0)</td>
<td>0.310</td>
</tr>
<tr>
<td>SMR (total mL O2)</td>
<td>118.7 (4.7)</td>
<td>108.8 (7.5)</td>
<td>114.5 (5.8)</td>
<td>0.528</td>
</tr>
<tr>
<td>Circadian rhythms (total mL O2)</td>
<td>56.4 (6.9)</td>
<td>54.6 (6.2)</td>
<td>66.0 (8.9)</td>
<td>0.375</td>
</tr>
</tbody>
</table>

Metabolic variables are described in the text. Values are mean (±1 SEM). $P$ values were determined by univariate ANOVA, and refer to the effect of meal size. Calculation of metabolic variables is based on the seven days following prey ingestion. SMR was calculated using the AVG lower 25% $V\dot{O}_2$ SMR estimator, and by extrapolating fasting measurements throughout the digestive period.

### Discussion
The presence of diel or circadian metabolic cycles in L. fuliginosus and other snakes suggests temporal $V\dot{O}_2$ variation may be common in snakes as a group. The two- to three-fold increase in $V\dot{O}_2$ above SMR during active periods is consistent with differences between daily maximum and minimum metabolic rates in other snakes (Gratz and Hutchison, 1977; Hicks and Riedesel, 1983; Blem and Killeen, 1993; Beaupre and Duvall, 1998;
Beaupre and Zaidan, 2001; McCue and Lillywhite, 2002; Zaidan, 2003). The oscillations in *L. fuliginosus* VO\(_2\) appear to be endogenous (i.e., circadian) because cyclic changes in VO\(_2\) occurred in constant dark. It is unlikely that VO\(_2\) oscillations were due to handling stress because (1) similar VO\(_2\) cycles persist even when snakes are left undisturbed (e.g., during fasting measurements), (2) maximum VO\(_2\) occurred several hours following handling during digestion, with elevated VO\(_2\) more closely coinciding with the photoperiod to which snakes were entrained, and (3) patterns of VO\(_2\) variation in this study closely mirror circadian patterns of activity in *L. fuliginosus* in a previous study (Luttershmidt et al., 2002). Thus our results suggest that cyclic VO\(_2\) variation is due to increased nocturnal activity and/or cyclic changes in other physiological or behavioral processes such as ventilatory patterns (Hicks and Riedesel, 1983) or state of alertness (Feder and Feder, 1981).

Compared to SMR and SDA, the additional oxygen consumed by *L. fuliginosus* for active phases of its circadian cycle accounts for a substantial amount of the total energy expenditures measured in this study. For instance, during the 7 days of digestive VO\(_2\) measurements, the cumulative oxygen consumed above SMR and SDA during active phases of the circadian cycle was nearly equal to the cost of digesting and assimilating a meal equal to 10% of snake body size and half that needed for SMR, which is the largest component of a reptile’s annual energy budget (Congdon et al., 1982; Table 2). While oxygen consumption during active phases of the circadian cycle was well below SDA for larger meals (Table 2), the energy cost of SDA is likely to be only periodic compared to additional energy used during circadian rhythms in snakes, which eat relatively infrequently compared to most other vertebrates. Consequently, the cumulative energy spent above SMR and SDA for active phases of the circadian cycle may approach or even surpass cumulative energy costs for SDA over long time periods. Failure to consider circadian and diel cycles in *L. fuliginosus* and perhaps many other snakes would ignore a substantial portion of their energy budget.

The presence of circadian metabolic variation in *L. fuliginosus* raises concerns for accurately estimating SDA. For instance, distinguishing increases in VO\(_2\) associated with activity and circadian rhythms from those attributable to digestion presents a major difficulty in estimating SDA. Following feeding, circadian rhythms in metabolism did not resemble rhythms observed during pre-prandial measurements until 48 h after feeding. However, if the circadian rhythm persists during this initial period, but remains concealed beneath the digestive response, we (and others) may be overestimating SDA. It is likely that the entrained circadian rhythms continue to some degree early in digestion, but the large addition of mass after feeding in snakes may also reduce activity immediately following ingestion. Large meals reduce locomotory speeds and endurance capacity in snakes, (Garland, 1983; Shine and Shetty, 2001), which may be related to changes in physiology that affect circadian activity and metabolic patterns as well. Determining the extent to which SDA estimates may be influenced by the circadian rhythm in the initial phases of digestion would yield potentially valuable insight into patterns of digestive energy expenditure in snakes.

The circadian cycles observed in this study also raise concerns about the timing and frequency of metabolic measurements. The majority of studies investigating SMR and SDA for snakes measure metabolic rates at infrequent intervals (Ruben, 1976; Abe and Mendes, 1980; Secor, 1995; Secor and Diamond, 1995, 1997a,b, 2000; Overgaard et al., 2002). For *L. fuliginosus*, very few measurements represent those of resting snakes during the inactive phase of their circadian cycle (Figs. 2 and 3), and the probability of measurements coinciding with inactive periods would decrease as fewer measurements are taken. Measuring VO\(_2\) at frequent intervals allowed us to reduce the risk of overestimating metabolic variables that assume animals are resting (e.g., SMR and SDA), and to avoid the complications of having to predict time frames of inactivity. Measuring VO\(_2\) less frequently could be effective if time frames for measurements are identified beforehand (e.g., Blem and Blem, 1990), but predicting such measurement
periods is difficult because patterns of VO$_2$ variation are not known for many species (e.g., *L. fuliginosus* prior to this study), and patterns often shift seasonally or as a result of other factors (e.g., temperature and light; Gibbons and Semlitsch, 1987; Underwood, 1992).

The estimation techniques used in this study were adopted because they were well suited for *L. fuliginosus* based on our assessment of its VO$_2$ variation, but the efficacy of estimation techniques will depend on species-specific patterns of VO$_2$ variation, such as the strength of the circadian cycle. Using a standardized lower proportion of the VO$_2$ data to estimate SMR is not unprecedented (Muusze et al., 1998; Hopkins et al., 1999, 2004; Dorcas et al., 2004), but the proportion of VO$_2$ values used is usually determined by visual inspection of data instead of mathematical procedures such as polynomial regression. Polynomial regression can be adopted by other researchers because it allows for an objective identification of values to remove, although the proportion of values to remove will not be consistent. The higher the proportion of VO$_2$ variance explained by the regression model, the fewer the values that can be used to estimate SMR for the species in question. Other techniques that account for daily metabolic variation and outlier values have been used to estimate digestive metabolic variables (Andrade et al., 1997; Powell et al., 1999; Zaidan and Beaupre, 2003), but the patterns of VO$_2$ variation specific to *L. fuliginosus* necessitated the development of other techniques. Some techniques rely on visual inspection of the animal’s activity level to determine which VO$_2$ measurements to discard, but VO$_2$ variation may arise from changes in alertness, endogenous rhythms, or other processes that may be imperceptible to the observer (Feder and Feder, 1981). Visual inspection may also disturb animals, and may be impractical when taking numerous VO$_2$ measurements (Zaidan, 2003). Another often-used technique is to report the single lowest measurement each day to estimate SMR and SDA, but this method is problematic because experimental error (e.g., respirometry equipment; Beaupre, 1993; McNab, 2003) and long apneic periods in snakes (Donnelly and Woolcock, 1977; Hicks and Riedesel, 1983; Andrade et al., 1997) may contribute to variation among measurements. Other estimation methods excluding too few values (e.g., AVG lower 50% VO$_2$) produced higher estimates of SMR that were likely influenced by activity or the circadian cycle of *L. fuliginosus*.

Although it is intuitive that different calculation methods for metabolic variables could result in variance among estimates, we demonstrate that these differences can be substantial. For instance, calculations of daily energy expenditure of SMR for a 16.7 g *L. fuliginosus* at constant temperature of 25 °C range from 0.371 kJ (AVG lower 50% VO$_2$ estimator) to 0.289 kJ (Lowest VO$_2$ estimator), a reduction of nearly 30%. Differences in SMR carry over to affect estimates of digestive metabolism as well. For instance, estimates of digestive scope for a meal equal to 20% of the snake’s body mass range from 4.3 (AVG lower 50% VO$_2$ estimator) to 6.0 (Lowest VO$_2$ estimator), an increase of nearly 40% (Table 3). In comparisons among studies using different methods, such differences could be misinterpreted as attributable to biological differences (e.g., ecology, phylogeny), when in fact differences resulted from methodology.

Direct comparison of metabolic values between *L. fuliginosus* and other snakes are not warranted due to methodological differences between this study and others. However, the complications of cross-study comparisons can be reduced by limiting comparisons to general trends described within studies, such as the relationship among meal size, peak VO$_2$ and SDA, because methods used within investigations typically do not differ. Similar to other snakes, peak VO$_2$ in *L. fuliginosus* increased curvilinearly with increasing meal size while SDA increased linearly (Andrade et al., 1997; Secor and Diamond, 1997a; Toledo et al., 2003; Fig. 4). SDA coefficient was not influenced by meal size, but our comparison of SDA coefficient across meal sizes is confounded by differences in energy content of prey items. It is not possible for us to ascertain whether *L. fuliginosus* reached an upper limit of VO$_2$ in digesting large meals because the range of meal sizes (10–30%) tested in this investigation was less extensive than that of previous studies (10–50%: Andrade et al., 1997; 5–111%: Secor and Diamond, 1997a; Toledo et al., 2003; Fig. 4). However, increases in peak VO$_2$ between 10% and 20% feeding levels were considerably larger than between 20% and 30% feeding levels (Fig. 4, Table 2). Further investigation of digestive responses to a wider range of meal sizes is needed to determine whether a plateau in peak VO$_2$ is attained with larger meal size in *L. fuliginosus*.

Because relationships between peak VO$_2$ and meal size appear to be curvilinear in many snakes, yet SDA linearly increases with meal size, the additional energy needed to digest larger meals may be partly manifest as longer duration of elevated VO$_2$ (Secor and Diamond, 1997a,b). However, examining duration of elevated VO$_2$ in this investigation was problematic because SMR of snakes failed to return to pre-feeding levels (Fig. 2). A confounding factor may be the high conversion efficiency for *L. fuliginosus*, as snakes were 3.8%, 9.6%, and 16.1% heavier than their pre-feeding mass after digestion of meals weighing 10%, 20%, and 30% of their body mass, respectively. As metabolic rate typically increases with increasing body size in reptiles (Bennett and Dawson, 1976; Andrews and Pough, 1985), larger body size after digestion is expected to increase VO$_2$. The narrow mass range of snakes used in our study precluded development of an equation describing the allometric relationship between mass and VO$_2$, and thus prevented us from assessing how much of the difference between pre- and post-feeding VO$_2$ may be attributable to mass gain. An additional confounding
factor may have been the difference in fasting times. For pre-feeding measurements, snakes had been fasted for 11–14 days, while measurements of digestive metabolism continued for only 7 days post-feeding. A decrease in VO₂ with increasing time of food deprivation has been detected in some reptiles (Belkin, 1965; Robert and Thompson, 2000), but not others (Beaupre et al., 1993).

The results of this investigation underscore the importance of identifying metabolic variation associated with circadian rhythms, yet such variation is rarely considered in studies of SDA in snakes. Failure to identify circadian VO₂ variation can have consequences for understanding important components of snake energy budgets. Most metabolic measurement and estimation techniques for snakes (e.g., few measurements and limited opportunity to identify daily variation) have been developed for boas, pythons, and pitvipers, which are typically considered relatively inactive. Our study demonstrates that these techniques may not be appropriate for species that exhibit high metabolic variation, such as L. fuliginosus. However, even some typically sedentary species have circadian or diel VO₂ cycles (Beaupre and Duvall, 1998; Beaupre and Zaidan, 2001; Zaidan, 2003), suggesting that researchers should carefully consider measurement regimes and estimation techniques, regardless of preconceived notions of the animal’s typical level of activity. Applying objective methods that allow researchers to confidently identify and minimize the influence of significant sources of VO₂ variation from SMR and SDA estimates would facilitate comparisons of metabolism across investigations and among the rich diversity of snake species.

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