TECHNICAL COMMENT

Using Direct Calorimetry to Test the Accuracy of Indirect Calorimetry in an Ectotherm

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ABSTRACT

We previously demonstrated that the relationship between respiratory gas exchange and metabolic heat production is unexpectedly variable and that conventional approaches to estimating energy expenditure by indirect calorimetry can incorporate large errors. Prior studies, however, comparing direct and indirect calorimetry of animals focused only on endothermic organisms. Given that endothermy and ectothermy represent a fundamental dichotomy of animal energetics, in this analysis we explore how these contrasting physiologies correlate with the relationship between heat production and respiratory gas exchange. Simultaneous indirect and direct calorimetry in an ectotherm, the ball python (*Python regius* Shaw), revealed that the relationships between gas exchange and heat production were within 1% of those expected when analyses using indirect calorimetry were based on the assumption that the fasting animal catabolized only protein. This accuracy of indirect calorimetry contrasts sharply with our previous conclusions for three species of birds and mammals.

Introduction

Indirect calorimetry by respirometry has provided the foundation for our understanding of organismal energetics. Though respirometry is very broadly used, there are two important and potentially constraining requirements for obtaining meaningful data from this set of techniques. First, accurate measurement must be made of the volumetric rate of change of at least one of the respiratory gases, oxygen and carbon dioxide. Second, rates of metabolic heat production must then be calculated from those measured rates of gas exchange and from estimates of the thermal equivalents of such gas exchange. The first of these requirements is relatively nonproblematic; readily available modern equipment, if properly calibrated, allows for accurate measurement of oxygen consumption ($\dot{V}O_2$) or carbon dioxide production ($\dot{V}CO_2$). But estimating thermal equivalents requires assumptions regarding the composition of the catabolic substrate and end products. Workers estimate the substrate's proportional representation of lipids, carbohydrates, and proteins using a variety of approaches (Walsberg and Hoffman 2005). The overall thermal equivalents of oxygen consumption or carbon dioxide production are then calculated on the basis of values characteristic of each class of nutrient, using the assumption that the substrate is completely oxidized except for the production of nitrogenous wastes (e.g., lipid, 19.8 kJ L^{-1} O_2 or 27.8 kJ L^{-1} CO_2: Brouwer 1957; carbohydrate, 21.1 kJ L^{-1} for either gas: Brouwer 1957; protein in uricotelic species, 18.7 kJ L^{-1} O_2 or 25.4 kJ L^{-1} CO_2: King 1957; protein in ureotelic species, 19.2 kJ L^{-1} O_2 or 23.8 kJ L^{-1} CO_2: Brouwer 1957).

Unfortunately, the assumptions used to estimate these thermal equivalents can readily be violated (Walsberg and Hoffman 2005). Estimated values derived from indirect calorimetry have been compared with empirical values derived by direct calorimetry (i.e., by directly measuring metabolic heat production) only for a very limited set of taxa and only under highly restricted experimental conditions that affect energy metabolism in important ways. Recognizing this, we previously (Walsberg and Hoffman 2005) tested the accuracy of respirometric estimates of energy expenditure by simultaneous indirect and direct calorimetry in a small mammal (the kangaroo rat *Dipodomys merriami* Mearns), a small bird (the dove *Columbina inca* Lesson), and a medium-sized bird (the quail *Coturnix communis* Linnaeus). We demonstrated that the relationship between gas exchange and heat production is much more variable than previously appreciated and that major gaps exist in our knowledge of these relations. Indeed, conventional approaches to estimating energy expenditure from respiratory gas exchange can incorporate large errors (up to at least 38%) that are sufficient to call into question generalizations regarding animal energy use in many studies (Walsberg and Hoffman 2005).

That study and all previous experiments quantitatively comparing direct and indirect calorimetry of animals focused solely on endothermic organisms. Given that endothermy and ec-

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tothermy represent a fundamental dichotomy of animal energetics, with the high resting rates of heat production in endotherms being significantly attributed to a greater allocation of energy to futile cycles of proton pumping and leaking (e.g., Rolfe and Brown 1997), it clearly is important to explore how these contrasting physiologies might correlate with the relationship between metabolic heat production and respiratory gas exchange. In this study, we report the first simultaneous indirect and direct calorimetry in an ectothermic animal.

These analyses focus on ball pythons *Python regius* Shaw. Pythons represent an extreme contrast to typical mammals or birds, in that they are ectothermic when not brooding eggs and are also adapted to fasting for very long periods after consuming large meals at erratic intervals (Secor and Diamond 1998). Our focus in this initial analysis of ectotherms is on fasting pythons, because fasting is the standard physiological condition most commonly used in metabolic analyses and because nonfasting snakes exhibit rapid and dramatic changes in their physiological status that could significantly complicate both experimental design and data analysis. For example, the postprandial response of pythons is characterized by a rapid increase in organ size and by metabolic upregulation within 24–48 h after feeding (Overgaard et al. 1999, 2002; Thompson and Withers 1999; Bedford and Christian 2001; Starck and Beese 2001; Secor 2003). Following feeding, indicators of metabolic heat production decline to fasting levels within about 10 d (Starck and Wimmer 2005).

Material and Methods

Animal Use

We used eight ball pythons *Python regius* on loan to us by Dr. Dale DeNardo, and all research was conducted under the auspices of the Arizona State University Institutional Animal Care and Use Committee. These snakes were maintained in a thermal gradient produced by placing a subsurface heating element at one end of each individual's cage, providing a gradient of temperatures ranging from 25° to 40°C. All measurements are for animals that had been fasting at least 5 wk before experimental trials. Snake mass averaged 856 g (range ± 95% confidence interval: 634–976 ± 97.9 g).

Experimental trials lasted 21 h, during which the animal was kept in darkness in the calorimetry chamber at 25°C ambient temperature (T_a), with no access to food or water. All trials began between 1300 and 1500 hours.

Direct Calorimetry

Our combined direct calorimeter/respirometry chamber allowed simultaneous measurements of animal heat production and respiratory gas exchange; see Walsberg and Hoffman (2005) for details of this device's construction, operation, and calibration. We calculated animal heat production as the sum of conductive heat flux (through the surface of the calorimetry chamber), convective heat flux (from the product of air temperature change, the specific heat capacity of the air, and airflow rate), and evaporative heat flux (from the product of airflow rate, hygrometric measurements, and latent heat of vaporization). To ensure accurate calorimetry, the chamber was submerged in a 200-L water bath that was continuously stirred and maintained by proportional thermostatic control at 25.000° ± 0.005°C.

Indirect Calorimetry

We used flow-through respirometry to measure oxygen consumption and carbon dioxide production, concurrent with direct measurements of heat production (Walsberg and Hoffman 2005). Air supplied to the chamber was first dried and scrubbed of carbon dioxide by an industrial air purifier (PureGas model CDA 1112), and airflow through the chamber was maintained at 206 mL min^{-1} by a mass flow controller (Unit Instruments UFC-2550) that we calibrated using soap film flowmeters. Air exiting the chamber was passed through a dew point hygrometer (Sable Systems RH200, calibrated using bottled nitrogen for the zero gas and air bubbled though ca. 450 vertical centimeters of temperature-controlled water for the span gas) and then dried by anhydrous calcium sulfate before flowing through a carbon dioxide analyzer (Li-Cor LI-6252) and then an oxygen analyzer (Sable Systems FC-1B). Both the oxygen and carbon dioxide analyzers were calibrated using bottled gases at high and low extremes that exceeded those of measurements. The overall system was then verified against several replicates of a known combustion reaction. For each replicate, a measured mass of 100% ethanol was burned inside the calorimetry chamber using a microscale ethanol lamp whose mass was determined at the start and finish of a 6-h period. Measurements of water vapor, CO_2 production, and O_2 consumption were within 0.4% of values expected from the stoichiometry of ethanol combustion (Walsberg and Hoffman 2005).

Gas Exchange, Heat Flux, and Thermal Equivalents

For the three gases that were analyzed, we used equations from the following derivation:

$$\dot{V}_O = \dot{V}'_A F'_O - \dot{V}_A F_O, \tag{1}$$

$$\dot{V}_C = \dot{V}_A F_C - \dot{V}'_A F'_C, \tag{2}$$

$$\dot{V}_W = \dot{V}_A F_W - \dot{V}'_A F'_W, \tag{3}$$

$$\dot{V}_A = \dot{V}'_A - \dot{V}_O + \dot{V}_C + \dot{V}_W = \dot{V}'_A \frac{(1 - F'_O - F'_C - F'_W)}{(1 - F_O - F_C - F_W)}, \tag{4}$$

$$\dot{V}_O = \dot{V}_A \left[F'_O - F_O \frac{(1 - F'_O - F'_C - F'_W)}{(1 - F_O - F_C - F_W)} \right], \tag{5}$$

$$\dot{V}_C = \dot{V}'_A\left[F_C\frac{(1 - F'_O - F'_C - F'_W)}{(1 - F_O - F_C - F_W)} - F'_C\right], \quad (6)$$

$$\dot{V}_W = \dot{V}'_A\left[F_W\frac{(1 - F'_O - F'_C - F'_W)}{(1 - F_O - F_C - F_W)} - F'_W\right]. \quad (7)$$

Here, $\dot{V}_O$, $\dot{V}_C$, and $\dot{V}_W$ are the volumetric rates of change in oxygen, carbon dioxide, and water vapor, respectively; $\dot{V}'_A$ and $\dot{V}_A$ are the rates of flow of influent air and effluent air, respectively; F_O, F_C, and F_W are the effluent air's fractional content of O_2, CO_2, and H_2O, respectively; and corresponding symbols with primes denote respective fractional contents in the influent air. We calculated conductive heat flux directly from our empirical calibration equation, which is a linear fit of heat flux (mW) through the thermopile as a function of electrical potential difference (mV) measured across the thermopile. For the other two heat fluxes, convective and evaporative, we used the following:

$$Q_{CONV} = \dot{V}_W\rho_S c_{P,S}T_A + (\dot{V}'_A + \dot{V}_C - \dot{V}_O)\rho_{DRY}c_{P,DRY}T_A - \dot{V}'_A\rho_{DRY}c_{P,DRY}T'_A, \quad (8)$$

$$Q_{EVAP} = \lambda[(\dot{V}'_A + \dot{V}_W + \dot{V}_C - \dot{V}_O)\rho_V - \dot{V}'_A\rho'_V], \quad (9)$$

where $\dot{V}$ denotes flow (L s^{-1}) of gases indicated by subscripts; ρ_S and ρ_{DRY} are the densities (kg L^{-1}) of water vapor and dry air, respectively; $c_{P,S}$ and $c_{P,DRY}$ are the isobaric specific heat capacities (J kg^{-1} °C^{-1}) of water vapor and dry air, respectively; T_A and T'_A are the centigrade temperatures of effluent air and influent air, respectively; λ is the latent heat (J kg^{-1}) of vaporization of water; and ρ_V and ρ'_V are the vapor densities (kg L^{-1}) of effluent and influent air, respectively.

Although signal output from the direct calorimeter reached 99% of its equilibrium value within 28 min, the time required for fractional gas concentrations to reach 99% of their equilibrium values was 3 h with flow rate maintained at 206 mL min^{-1} (on the basis of data and computations by Walsberg and Hoffman [2005]). Such a low airflow rate was required to obtain adequate precision in our measurements of gas exchange, owing to the much reduced rates of metabolism in ectotherms as compared with endotherms. We therefore calculated values for gas concentrations and thermal equivalents of CO_2 production and O_2 consumption as values averaged over 3-h periods, and we discarded data from the initial 3-h period. Thermal equivalents of gas exchange were calculated as 3-h average heat production divided by 3-h average gas production or consumption.

Effects of Heat Storage

In an ectothermic animal, slight changes in the quantity of heat stored in the body can produce a large effect compared with typically low levels of metabolic heat production. Determining such effects would require quantifying changes in average whole-body temperature. Given the potential for important temperature gradients within a large ectotherm's body, especially considering a snake's geometry, measurements of body temperature (T_b) by one or a few implanted sensors may well be misleading. Therefore, we took an alternative approach of allowing the animal to equilibrate with the thermally uniform environment within the calorimeter for a 15-h period that allowed animal heat loss to stabilize.

The relative contributions to total heat loss of metabolic heat production and changes in heat storage were subsequently estimated by assuming that the catabolic substrate did not change during the experimental trial. That is, the mean thermal equivalents of respiratory gas exchange measured during the final 6 h of experimental trials were separately multiplied by mean measurements of oxygen consumption and carbon dioxide production to estimate metabolic heat production during the initial 15 h of experimental trials. We thus derived two estimates of metabolic heat production, one based on CO_2 production and one based on O_2 consumption. Subtracting these values for heat production from directly measured values for heat loss yields estimates of heat flux produced by changes in body storage of heat.

The change in average body temperature associated with these heat storage changes was estimated by assuming a specific heat capacity of 3.47 J g^{-1} °C^{-1}, the value that is used in essentially all modern analyses (Blaxter 1989). Apparently, no measurements of heat capacity are available for snakes, nor are appropriate measurements available for body composition of pythons that would allow us to produce an alternative estimate. Our assumed value is very unlikely to produce significant errors, however. For example, the nonlipid composition of garter snakes is typically 80% water, 15% protein, and 4% mineral (Aleksiuk and Stewart 1971). Assuming that the ratio of protein to mineral is conserved with the addition of lipids, a very lean snake might be composed of 4% lipid, 76% water, 15% protein, and 4% mineral. Using values from Blaxter (1989) for the specific heats of these materials predicts an average specific heat of 3.52 J g^{-1} °C^{-1}. Increasing lipid stores to 20% of total mass and conserving the ratios of other materials yield a predicted specific heat of 3.29 J g^{-1} °C^{-1}. That is, a fivefold increase in fat stores is likely to produce only a 7% decrease in specific heat and in the body temperature changes calculated below (i.e., <0.02°C). We thus ignore such effects.

Statistical Analyses

We determined experimental values to be statistically different from conventionally expected values if the empirical 95% con-

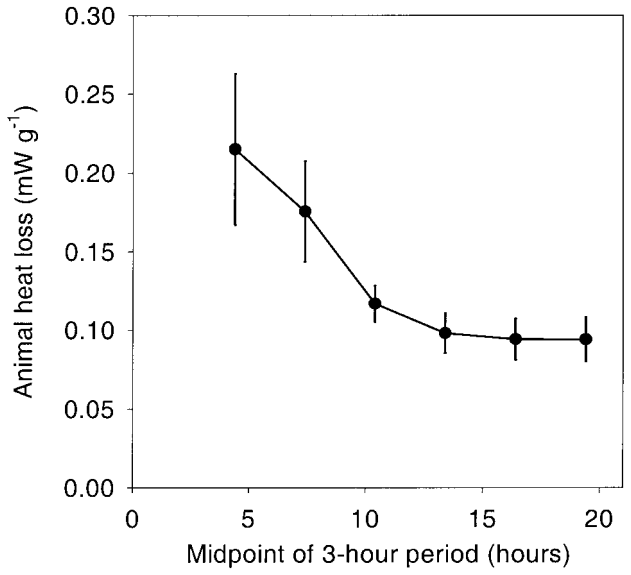

Figure 1. Heat loss from ball pythons over 21 h. Values are means ± 95% confidence intervals ($N = 8$) and are plotted against the midpoints of 3-h periods.

fidence intervals did not overlap the expected range. We applied linear regression to test whether rates of respiratory gas exchange or heat loss from the animal underwent progressive changes with time. The significance of the slope of the regression equation was tested by ANOVA and accepted as significant if $P < 0.05$.

Results

Animal heat loss was comparatively high at the beginning of experimental trials and declined to values that were essentially stable only after 12 h (Fig. 1). Such a decline in heat loss can have either or both of two causes: a decline in the quantity of heat stored in the body and a reduction in the rate of metabolic heat production. It is not possible to confidently discriminate the two in this study. Concurrent changes in respiratory exchange rates do indicate, however, that there was some reduction in heat storage. From the first 3-h period to the last, carbon dioxide production and oxygen consumption declined 31% and 34%, respectively (Fig. 2). Animal heat loss in the same period declined 56%. This greater decline in heat loss suggests reductions in stored heat that extended over the initial 12 h of experimental trials. Values for O_2 consumption, CO_2 production, and animal heat loss stabilized and did not change within the final two 3-h periods. Values for this latter 6-h period were, therefore, combined for subsequent analyses. During this period, the respiratory exchange ratio was 0.739 ± 0.035. The thermal equivalent of carbon dioxide production, calculated by dividing average CO_2 production (L s^{-1}) by average heat production (W), was 25.1 ± 1.71 kJ L^{-1}, and that for oxygen consumption was 18.9 ± 1.88 kJ L^{-1}.

Discussion

Our results indicate that, given appropriate assumptions, indirect calorimetry provides accurate estimates of metabolic heat production in these fasting ectotherms. This contrasts sharply with the conclusions of our previous analyses for three species of birds and mammals (Walsberg and Hoffman 2005). Thus, the relationships between respiratory gas exchange and metabolic heat production in ball pythons conform closely to those that might be expected in analyses relying solely on indirect calorimetry, but only if the worker makes a correct assumption regarding the catabolic substrate.

The latter is a critical point, because an error of only about 1% in indirect estimates derived from measurements of either oxygen consumption or carbon dioxide production results only if a worker assumes that the animal is relying entirely on protein as a catabolic substrate. After heat loss had stabilized, the values for three critical parameters describing an animal's respiratory and energy metabolism strongly suggested extensive reliance on catabolism of a protein substrate (Fig. 3). The respiratory exchange ratio was 0.739 ± 0.02, essentially identical to the value expected (0.74) for a mixed protein substrate in a uricotelic animal (King 1957). The thermal equivalent of oxygen consumption was 18.9 ± 1.9 kJ L^{-1}, only 1.1% greater than the value expected (18.7 kJ L^{-1}) for a protein substrate (King 1957). The thermal equivalent of carbon dioxide production was

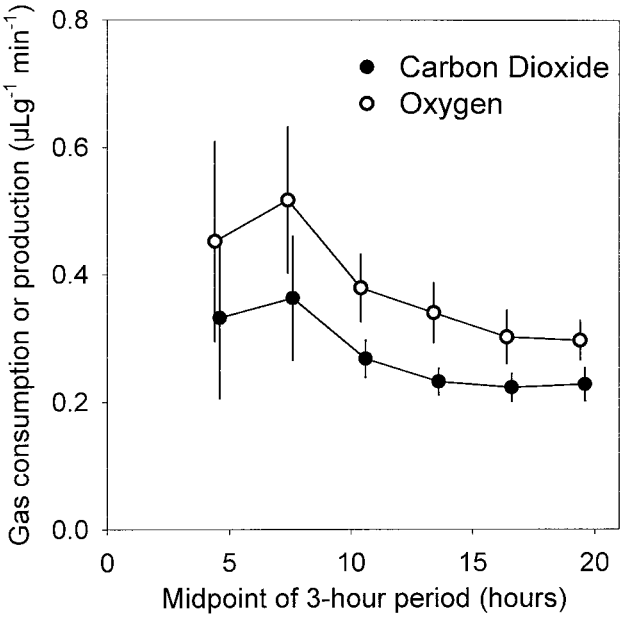

Figure 2. Oxygen consumption and carbon dioxide production of ball pythons over 21 h. Values are means ± 95% confidence intervals ($N = 8$) and are plotted against the midpoints of 3-h periods.

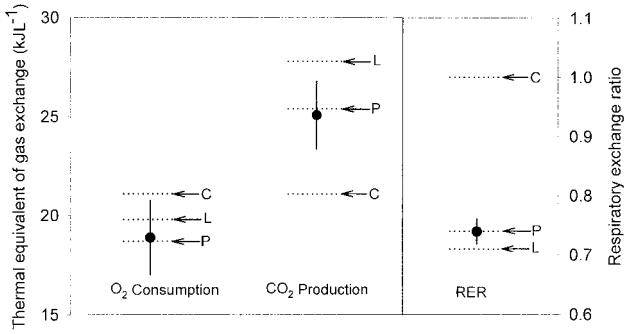

Figure 3. Thermal equivalents of gas exchange and the respiratory exchange ratio in ball pythons during the final 6 h of measurements. Expected values for substrates composed entirely of carbohydrate (*C*), lipid (*L*), and protein (*P*) are also shown (Brouwer 1957; King 1957). Values are means ± 95% confidence intervals ($N = 8$).

25.1 ± 1.7 kJ L^{-1}, or 1.2% less than the value expected (25.4 kJ L^{-1}) for such a substrate (King 1957). Extensive reliance on catabolism of body protein is consistent, of course, with expectations for an animal that has fasted more than 5 wk and presumably has exhausted its lipid and carbohydrate stores.

It is important to note, however, that workers rarely presume an exclusive metabolic reliance on protein catabolism and that more typical assumptions would yield much larger errors. For fasting animals, the dominant substrate usually is assumed to be lipid, and thus thermal equivalents of respiratory gas exchange are estimated as either 19.8 kJ L^{-1} O_2 or 27.8 kJ L^{-1} CO_2 (Brouwer 1957). In this study, respirometric calculations of metabolic heat production obtained using these assumptions yield values that differ from directly measured values by 15% if carbon dioxide were measured or 19% if oxygen were measured. This illustrates the errors that can be produced using indirect calorimetry if workers measure only a single respiratory gas.

Even if both gases are measured, however, the worker is left with the challenge of estimating the thermal equivalents of gas exchange. Our observed respiratory exchange ratio value of 0.74 could be produced by various mixtures of lipid, carbohydrate, and protein. Workers typically ignore the possibility of substantial protein catabolism and assume that the animal is catabolizing materials with a carbohydrate : lipid ratio that would produce an observed respiratory exchange ratio (e.g., Duncan and Lighton 1994; Hansen et al. 1995; Lighton and Fielden 1995). Doing so and using our respiratory data would produce a 7% error in the estimate of metabolic heat production.

It is also notable that heat loss required more than 10 h to reach stable values, due at least partially to reductions in stored heat. After the initial 3-h period, average body temperature is estimated to have declined a total of only about 0.3°C (Fig. 4). However, the rate of metabolic heat production is so low that even such small changes in body temperature produce substantial effects on the animal's heat budget. For example, heat loss associated with decreased body temperature accounted for about 34% of total flux from the animal during the period from 3 to 6 h after the start of experimental trials (Fig. 4). Such a long period required to reach thermal equilibrium and the important effect produced by even slight changes in body temperature during the initial period of experimental trials thus provide a cautionary note for future analyses of heat budgets in similar ectotherms.

Does the contrast between the apparent accuracy of indirect calorimetry in these snakes and the large-scale errors observed in birds and mammals result from fundamental differences in the energy physiology of endotherms and ectotherms? Before considering such a conclusion, several major caveats must be recognized. Data exist only for a single species of ectotherm, and for both endotherms and ectotherms, data are largely restricted to fasting animals (Walsberg and Hoffman 2005). In addition, workers relying on indirect calorimetry commonly ignore the possibility of extensive catabolism of protein in a fasting animal. Rather, they typically assume that lipids supply the primary metabolic fuel (Blaxter 1989). Although the possibility is intriguing, we therefore consider it premature to conclude that the differences we observe in pythons compared with birds and mammals reflect a fundamental contrast between endotherms and ectotherms. Similarly, it is premature to generalize that, for ectotherms, indirect calorimetry as conventionally applied yields reliably accurate estimates of metabolic

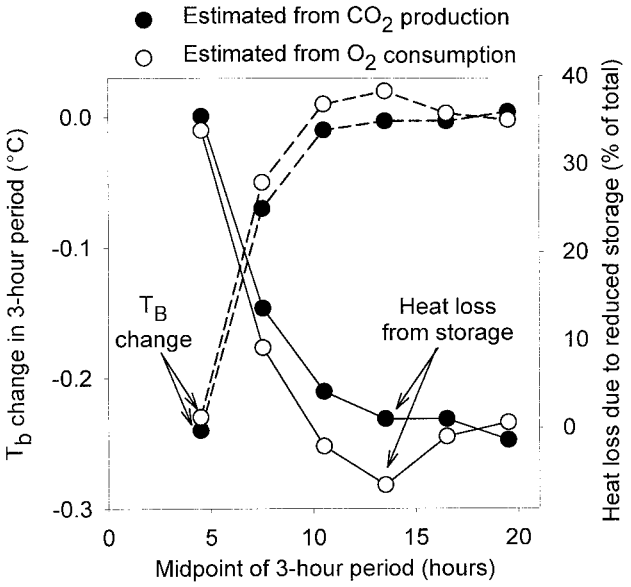

Figure 4. Estimated change in average body temperature and the contribution of changes in heat stores to total heat loss. Heat lost because of change in body storage is expressed as a percentage of total heat flow. Values are calculated from mean measured heat loss combined with estimates of metabolic heat production derived from either mean carbon dioxide production or mean oxygen consumption.

energy expenditure. Much further testing, including concurrent indirect and direct calorimetry of other ectotherm taxa and of postprandial animals, clearly is needed.

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