



## Energy use and management of energy reserves in hatchling turtles (*Chrysemys picta*) exposed to variable winter conditions

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### ABSTRACT

The painted turtle (*Chrysemys picta*) is an especially useful organism in the study of metabolic regulation during dormancy because it is sustained by finite energy reserves from hatching until emerging from its nest, about nine months later. In this study we subjected overwintering *C. picta* hatchlings to 4, 10, or 15 °C, temperatures simulating cold, mild, and warm winters, respectively, to investigate how various energy reserves are impacted by differential metabolic demands. An energy budget based on seasonal changes in caloric content showed that these turtles consumed an average of 0.39, 0.75, or 1.21 kJ g<sup>-1</sup>, respectively, during the 6-month period of simulated hibernation. These estimates of energy use agreed reasonably well with estimates based solely on respirometric data. Unexpectedly, turtles in autumn contained little residual yolk, none of which was consumed by turtles in the cold- and mild-winter groups, this finding contradicting the widely held belief that residual yolk plays an important, direct role in the survival of turtles that overwinter inside their natal nest. By contrast, a marked reduction in dry mass of both liver and carcass attested to their importance in fueling metabolism and, indeed, catabolism of substrates from these components accounted for 31–52 and 35–63%, respectively, of the energetic cost of overwintering. The greater dependence on carcass reserves and relatively poor physiological condition of turtles in the mild- and warm-winter groups implies that metabolic demands imposed by high environmental temperatures would likely constrain post-emergence fitness.

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### 1. Introduction

Many animals endure extended bouts of dormancy during which they are aphagic and thus must rely on endogenous energy stores. Frugal use of stored energy is necessary not only to survive long-term dormancy, but also to fuel post-arousal activities in cases when food is not readily available upon emergence. For ectothermic animals, environmental temperature is a major factor in determining the rate and extent of energy use, and hibernating ectotherms presumably benefit from the effect of cold on catabolic rates (Irwin and Lee, 2000, 2003; Marshall and Sinclair, 2012; Zani et al., 2012). However, temperature is not uniformly low during hibernation and, indeed, can vary greatly over a species' geographic range, among microhabitats in the same general area, and from year to year. Exposure to relatively warm winter conditions hastens depletion of energy stores in ectotherms (Hahn and Denlinger, 2007) and can negatively impact their survival (Zani, 2008) and fitness upon arousal (Irwin and Lee, 2000, 2003;

Williams et al., 2003). Though the detrimental effects of high temperature on a dormant ectotherm's overall energy conservation are well known, its impact on energy management within different somatic storage compartments is less well understood. Hatchling turtles that hibernate terrestrially are especially useful subjects in such studies because they inhabit thermally variable microhabitats and are provisioned with finite energy reserves (Costanzo et al., 2008). Although a few studies of hatchling turtles (Costanzo et al., 2004; Willette et al., 2005) have linked mild winters to lower energy reserves upon spring arousal, more study is needed to determine how energy use is compartmentalized within the body.

We studied hatchling painted turtles (*Chrysemys picta*) in an effort to better understand the energetic costs of overwintering under different thermal regimes. Painted turtles have a broad latitudinal distribution, from southern Canada to southern Louisiana (Conant and Collins, 1998), and are subjected to frigid winter conditions in the northern part of their range, but mild winter conditions in the southern part of their range. Painted turtles hatch in late summer and usually remain in their subterranean natal nests until spring (Gibbons and Nelson, 1978). These hatchlings are aphagic during that period and thus rely on endogenous energy reserves, the bulk of which is presumably stored in residual

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yolk and liver, to meet metabolic demands (Costanzo et al., 2008). Hibernaculum temperature is dependent on weather, latitude, nest depth, snow cover, and edaphics and can vary substantially among nests (Costanzo et al., 2008), suggesting that energy use during hibernation may also vary greatly among individuals occupying different nests, especially if metabolic rate is particularly sensitive to temperature. Additionally, the extent to which metabolism can be fueled by energy stored in the yolk and liver may be low at relatively high temperatures, necessitating increased reliance on energy derived from other sources, including lean tissue. Upon emergence, this variation may contribute to differences in fitness, which, in hatchling turtles, has been positively linked to body mass (Janzen, 1993; Janzen et al., 2000). In this study, we compared the energetic costs of overwintering by shepherding hatchling turtles through a ~6-month simulated hibernation under cold-, mild-, and warm-winter conditions. For these turtles we determined changes in the mass of the body and its key components (residual yolk, liver, and carcass), and compared energy budgets based on changes in tissue caloric content and rates of oxygen consumption.

## 2. Material and methods

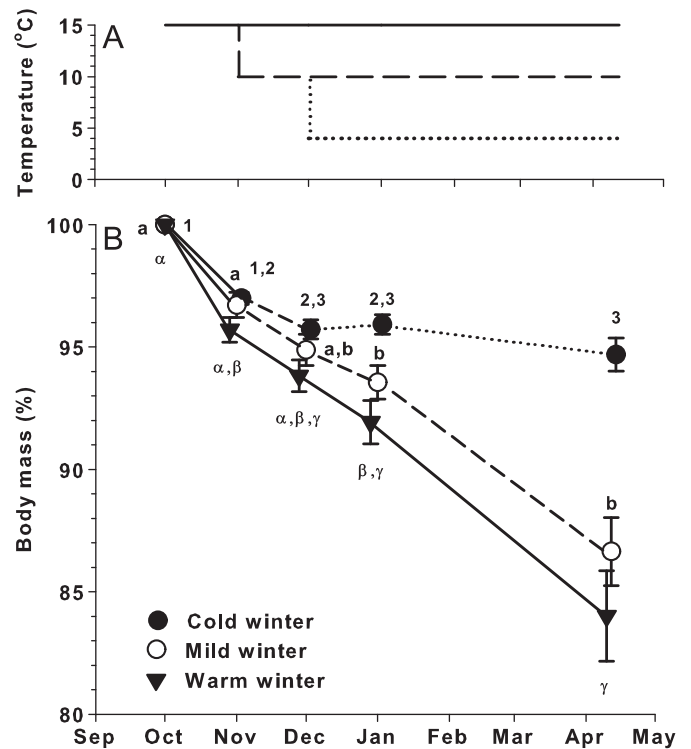
### 2.1. Acquisition of turtles

Eggs of the western painted turtle (*C. picta bellii*) were harvested from oxytocin-treated females (Ewert and Legler, 1978) collected from areas surrounding Crescent Lake National Wildlife Refuge (41°N, 102°W), Garden County, NE. Eggs were placed on moist vermiculite (1 g water: 1 g dry vermiculite) and delivered to Miami University where they were incubated at 28.5 °C until hatching in early August. Eggshells were discarded and neonates were transferred to fresh vermiculite (0.5 g water: 1 g dry vermiculite) and held in plastic boxes at 20 °C in a darkened incubator (Percival I-35X, Boone, IA, USA) until the 1st of October. Throughout the study, moisture content of the vermiculite was maintained by monitoring the mass of each box and replacing any water lost to evaporation.

### 2.2. Simulated hibernation

Using four offspring collected from each of eight clutches, we apportioned 32 hatchlings into a reference group ( $n=8$ ), to be sampled at the onset of the experiment, and three groups ( $n=8$  each) to undergo simulated hibernation under three different thermal regimes. Assignments were made such that each group had a single representative from each clutch. On the 1st of October, turtles in the reference group (hereafter, "fall group") were weighed, euthanized, and dissected (see below), whereas the remaining 24 turtles were kept in their plastic boxes at 15 °C until the 1st of November, when 16 of them were transferred to 10 °C. On the 1st of December, 8 of those 16 turtles were transferred to 4 °C (Fig. 1A). All turtles were held in the dark in simulated hibernation, within 0.5 °C of the prescribed minimum temperature (15, 10, or 4 °C) until the 12th of April, the approximate date at which they emerge from natural nests (Costanzo et al., 2004), at which time they were euthanized and dissected (see below). Accordingly, we exposed three groups of turtles to distinctly different thermal regimes intended to simulate nest conditions experienced by hatchling *C. picta* during cold, mild, and warm winters.

Turtles undergoing simulated hibernation were weighed to the nearest 0.01 g on the first day of October, November, December, and January, and finally on the 12th of April. Using a dial calipers,



**Fig. 1.** Thermal conditions (A) and mean ( $\pm$  SEM;  $n=8$ ) body mass (B) of hatchling painted turtles (*Chrysemys picta*) during simulated hibernation. Within a group, values not sharing a number (cold-winter group), letter (mild-winter group), or symbol (warm-winter group) are significantly different ( $P < 0.05$ ) based on a Friedman repeated-measures ANOVA on ranks and Tukey *post hoc* tests. Actual mass values were used in the statistical analyses, whereas the values presented are percentages of the fall values measured on the 1st of October.

carapace length was measured to the nearest 0.1 mm on the 1st of October and the 12th of April.

### 2.3. Respirometry

Resting rate of oxygen consumption ( $\dot{V}O_2$ ) was determined for individual turtles via closed-system respirometry. Measurements were made after each turtle had been held at its prescribed minimum hibernation temperature for at least 9 weeks, consistent with the recommendation of Ultsch (2013). Trials using animals in all three groups were conducted between late January and early March. Each turtle was gently brushed clean of adherent vermiculite and loosely secured in cotton mesh, except around the anterior, to simulate its envelopment in nest substrate and encourage quiescence. We took care to minimize light exposure and limit handling time to less than one minute. Each turtle was then habituated at its minimum hibernation temperature (cold-winter group, 4 °C; mild-winter group, 10 °C; warm-winter group, 15 °C), in darkness, for ~24 h inside a ~180-ml acrylic metabolic chamber (Qubit Systems G115, Kingston, ON, Canada) with valved incurrent and excurrent ports. During this time, the port valves were kept open and the partial pressure of oxygen ( $PO_2$ ) inside the chamber approximated the atmospheric level (~21%).  $\dot{V}O_2$  was determined following the methods of Vleck (1987). Briefly, each chamber was ventilated with thermally equilibrated room air, and an initial gas sample was drawn into a 60-ml Hamilton syringe fitted with a Teflon stopcock. The incurrent and excurrent valves were then closed thereby beginning the respirometry trial. Although we did not monitor movement during the respirometry trials, we found that each turtle was still wrapped in its cotton mesh at the conclusion of the trial, suggesting that the turtles remained quiescent. After a period of 2 d (warm-winter group),

4 d (mild-winter group), or 9 d (cold-winter group), a final gas sample was withdrawn from the chamber and the turtle was weighed to the nearest 0.01 g and returned to its holding box. Each gas sample was thermally equilibrated with room air and analyzed for PO<sub>2</sub> using a single-cell O<sub>2</sub> analyzer (Ametek S-3A, Newark, DE, USA). Oxygen concentration within the chamber decreased from ~21% to ~20.3% over the course of each trial.  $\dot{V}O_2$  was calculated for each turtle from the difference in readings from the two gas samples using the following equations from Vleck (1987)

$$\begin{aligned} V_{iH_2O} &= V(F_i - F_i)/F_i \\ V_{iCO_2} &= V(F_i)[(1/F_i) - (1/F'_i)] \\ VO_2 &= [(V - V_{iH_2O} - V_{iCO_2}) \cdot (F'_i - F''_e)] / (1 - F''_e) \end{aligned}$$

where  $V$  is the total volume of gas in the chamber adjusted to standard temperature and pressure and less the volume of the turtle,  $V_{iH_2O}$  is the initial volume of water vapor in the chamber,  $V_{iCO_2}$  is the initial volume of CO<sub>2</sub> in the chamber,  $F'_i$  is the percent fraction of O<sub>2</sub> in the initial sample after CO<sub>2</sub> and water vapor have been removed,  $F_i$  is the percent fraction of O<sub>2</sub> in the initial sample after only water vapor has been removed,  $F_i$  is the percent fraction of O<sub>2</sub> in the initial sample without removal of CO<sub>2</sub> or water vapor,  $F''_e$  is the percent fraction of O<sub>2</sub> in the final sample after CO<sub>2</sub> and water vapor have been removed, and  $VO_2$  is the volume of O<sub>2</sub> consumed over the course of the trial. Chambers not containing turtles were used as controls and exhibited no reduction in PO<sub>2</sub>. A leak in the respirometry chamber for one of the turtles in the mild-winter group caused us to discard its datum.  $\dot{V}O_2$  is expressed as microliters of O<sub>2</sub> consumed per gram fresh mass per hour at STPD. We calculated the temperature coefficient ( $Q_{10}$ ) for  $\dot{V}O_2$  using the equation

$$Q_{10} = \frac{R_2^{(10/T_2 - T_1)}}{R_1}$$

where  $R_1$  is  $\dot{V}O_2$  at temperature  $T_1$  and  $R_2$  is  $\dot{V}O_2$  at temperature  $T_2$ .

#### 2.4. Body composition and energy content

Each turtle was euthanized by decapitation and its residual yolk and liver were excised. The yolk, liver, and remaining carcass were immediately weighed to the nearest 0.1 mg, dried to constant mass at 65 °C, and reweighed. Water content of each sample was calculated by dividing the amount of mass lost during drying by the dry mass and is expressed as grams of water per gram of dry tissue. Body water content of each intact turtle was estimated by summing the mass lost from each body component upon drying and dividing that value by the pooled mass of each body component measured after drying.

We further analyzed the tissues to determine the overwintering reduction in total body organic content and energy concentration. The dried yolk, liver, and carcass of each turtle were combined, homogenized in a clean coffee grinder, and then pulverized with a mortar and pestle. Organic content of each pulverized sample was determined using a muffle furnace (Thermolyne 48000, Waltham, MA, USA). A ~125-mg subsample was placed in a pre-weighed crucible, weighed to the nearest 0.1 mg, and burned in the furnace at 550 °C for ~18 h. After being cooled to room temperature in a desiccator, the crucible and its contents were again weighed to the nearest 0.1 mg. Organic content was calculated by dividing the mass lost during burning by the mass of the initial aliquot and is expressed as a percentage thereof. Total caloric content in separate samples of the dried tissues was determined by measuring the heat of combustion of samples through standard bomb calorimetry, which was carried out under contractual agreement with the University of Arkansas Poultry Science Central Analytical Laboratory (Fayetteville, Arkansas, USA).

Energy concentration is expressed as kilojoules per gram of dry sample. Ash-free energy concentration was calculated by dividing the energy concentration by the proportion of organic material. Total caloric content was calculated by multiplying the energy concentration in the sample by the mass of the dry hatchling. This project (no. 662) was approved by the Miami University Institutional Animal Care and Use Committee.

#### 2.5. Statistical analyses

Statistical comparisons were performed with SAS 9.2. Means are reported  $\pm$  SEM and were compared among groups or between time points within a group using repeated-measures ANOVAs with Bonferroni *post hoc* tests. Data that did not meet the normality and homogeneity of variance assumptions were analyzed using Friedman repeated-measures ANOVAs on ranks with Tukey *post hoc* tests. Organic content values were arcsine square-root transformed prior to analysis. Mean carapace lengths of individuals measured at the start and end of simulated hibernation were compared using paired *t*-tests.

### 3. Results

#### 3.1. Morphometrics

All turtles survived the ~6-month period of simulated hibernation. Whereas those in the cold-winter group appeared healthy, several turtles in the mild- and warm-winter groups exhibited abnormal posture and were lethargic when sampled in mid April, at the end of the experiment. Carapace lengths of turtles in the cold-winter group (October: 24.3  $\pm$  0.5 mm; April: 24.3  $\pm$  0.4 mm) and mild-winter group (October: 24.5  $\pm$  0.4 mm; April: 24.5  $\pm$  0.5 mm) did not change (cold winter:  $t_7 = -0.14$ ,  $P = 0.890$ ; mild winter:  $t_7 = 0.94$ ,  $P = 0.381$ ) over the course of the experiment. However, carapace length of turtles in the warm-winter group (October: 24.6  $\pm$  0.3 mm; April: 24.4  $\pm$  0.3 mm) decreased significantly ( $t_7 = 2.60$ ,  $P = 0.035$ ), albeit slightly, during simulated hibernation. The mass of turtles sampled in fall and following simulated hibernation under cold-, mild-, and warm-winter regimes was 3.75  $\pm$  0.17, 3.57  $\pm$  0.17, 3.28  $\pm$  0.16, and 3.15  $\pm$  0.15 g, respectively. Turtles in the cold-winter group lost only 5.3% of their initial body mass during simulated hibernation, the majority of which occurred before they were transferred to 4 °C, on the 1st of December (Fig. 1B). In contrast, turtles in the mild- and warm-winter groups lost 13.4% and 16.0%, respectively, of their initial body mass during simulated hibernation, the decrease occurring at a fairly uniform rate over the entire experiment (Fig. 1B).

#### 3.2. Body composition

Body water content was uniformly 3.6–3.7 g g<sup>-1</sup> among all groups ( $F_{3,21} = 1.55$ ,  $P = 0.231$ ), suggesting that water was not differentially gained or lost in simulated hibernation. Water content of yolk (1.4–2.1 g g<sup>-1</sup>) and carcass (3.8–3.9 g g<sup>-1</sup>) also did not vary among groups (yolk:  $F_{3,21} = 1.11$ ,  $P = 0.368$ ; carcass:  $F_{3,21} = 0.38$ ,  $P = 0.766$ ). However, water content of liver did vary significantly ( $\chi^2_3 = 14.55$ ,  $P = 0.002$ ) among groups such that it was significantly ( $P < 0.05$ ) lower in the fall group (2.0  $\pm$  0.1 g g<sup>-1</sup>) than in the mild-winter group (2.8  $\pm$  0.4 g g<sup>-1</sup>) and warm-winter group (2.7  $\pm$  0.1 g g<sup>-1</sup>), whereas the value for the cold-winter group (2.5  $\pm$  0.2 g g<sup>-1</sup>) was intermediate and statistically indistinguishable from that of the other groups ( $P > 0.05$ ).

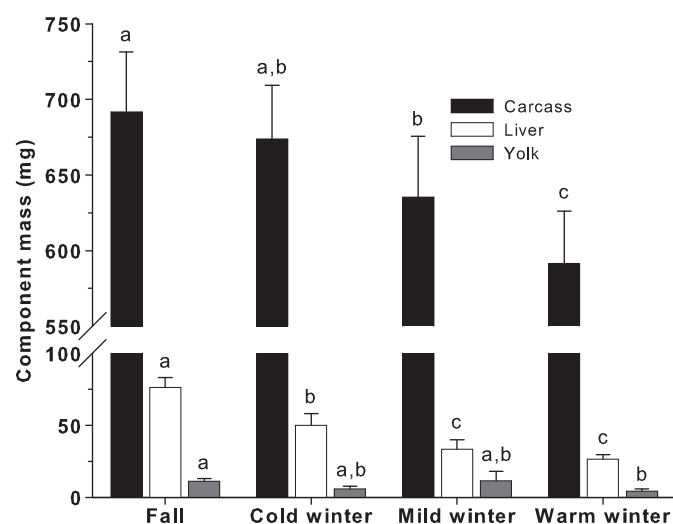
Mass of the residual yolk, which accounted for less than 2% of the dry body mass, varied significantly ( $\chi^2_3 = 10.80$ ,  $P = 0.013$ ) among groups such that it was significantly ( $P < 0.05$ ) higher in

fall turtles than in warm-winter turtles, but was statistically indistinguishable among other groups (Fig. 2). Liver and carcass mass varied significantly (liver:  $F_{3,21}=48.86$ ,  $P<0.001$ ; carcass:  $F_{3,21}=21.90$ ,  $P<0.001$ ) among groups, with warmer conditions generally resulting in lower component masses (Fig. 2). Notably, turtles in the cold-, mild-, and warm-winter groups had liver masses that were 34, 55, and 66% lower, respectively, than that of fall turtles. Liver represented  $9.7 \pm 0.4\%$  of dry body mass in fall, but this metric was reduced to  $6.6 \pm 0.7$ ,  $4.7 \pm 0.7$ , and  $4.1 \pm 0.3\%$  following simulated hibernation under respective cold-, mild-, and warm-winter thermal regimes.

Analysis of dried turtles showed marked variation among groups in organic content ( $F_{3,21}=24.13$ ,  $P<0.001$ ), energy concentration of whole tissue ( $F_{3,21}=109.61$ ,  $P<0.001$ ), energy concentration of ash-free tissue ( $F_{3,21}=24.09$ ,  $P<0.001$ ), and total caloric content ( $F_{3,21}=72.38$ ,  $P<0.001$ ), with lower values being characteristic of turtles hibernating under warmer conditions (Table 1). Notably, energy concentration of ash-free tissue of turtles in the mild- and warm-winter groups was significantly ( $P<0.001$ ) lower than that of fall turtles, but no such difference was found for turtles in the cold-winter group ( $P=0.190$ ). Caloric content of turtles in the cold-, mild-, and warm-winter groups were 9, 18, and 28% lower, respectively, than that (16.1 kJ) of fall turtles; on average, these turtles consumed 1.46, 2.81, and 4.52 kJ during the period of simulated hibernation.

### 3.3. Metabolic rates

Turtles remained within the cotton mesh throughout the respirometry trials, suggesting that the resultant data reflected



**Fig. 2.** Mean ( $\pm$  SEM;  $n=8$ ) mass of dried carcass, liver, and residual yolk from hatchling painted turtles (*Chrysemys picta*) measured on the 1st of October (fall) or on the 12th of April, following simulated hibernation under cold-, mild-, or warm-winter regimes characterized by final winter temperatures of 4, 10, or 15 °C, respectively. Between-group differences in values for carcass and liver were investigated using repeated-measures ANOVAs and Bonferroni *post hoc* tests. Between-group differences in values for residual yolk were investigated using a Friedman repeated-measures ANOVA on ranks and Tukey *post-hoc* tests. Values not sharing a letter are significantly different ( $P<0.05$ ).

**Table 1**

Analysis of hatchling painted turtles (*Chrysemys picta*) in fall and following simulated hibernation under three thermal regimes (final winter temperature in italics).

	Fall	Cold winter 4 °C	Mild winter 10 °C	Warm winter 15 °C
Organic content, %	87.8 $\pm$ 0.2 <sup>a</sup>	86.9 $\pm$ 0.4 <sup>a,b</sup>	86.2 $\pm$ 0.3 <sup>b</sup>	84.3 $\pm$ 0.5 <sup>c</sup>
Energy concentration, kJ g <sup>-1</sup> whole tissue	20.63 $\pm$ 0.11 <sup>a</sup>	20.06 $\pm$ 0.14 <sup>b</sup>	19.53 $\pm$ 0.08 <sup>c</sup>	18.58 $\pm$ 0.12 <sup>d</sup>
Energy concentration, kJ g <sup>-1</sup> ash-free tissue	23.51 $\pm$ 0.14 <sup>a</sup>	23.09 $\pm$ 0.20 <sup>a,b</sup>	22.65 $\pm$ 0.08 <sup>b</sup>	22.05 $\pm$ 0.12 <sup>c</sup>
Caloric content, kJ	16.10 $\pm$ 1.04 <sup>a</sup>	14.64 $\pm$ 0.89 <sup>b</sup>	13.29 $\pm$ 0.80 <sup>c</sup>	11.58 $\pm$ 0.75 <sup>d</sup>

Means ( $\pm$  SE) in the same row not sharing a letter were significantly different (repeated-measures ANOVA; Bonferroni *post hoc* tests).

resting levels of metabolism.  $\dot{V}O_2$  of the turtles was  $1.1 \pm 0.2$ ,  $4.2 \pm 0.6$ , and  $12.6 \pm 1.0 \mu\text{l g}^{-1} \text{h}^{-1}$  at 4, 10, and 15 °C, respectively and varied significantly ( $F_{2,13}=129.06$ ,  $P<0.001$ ) with test temperature. Turtles in the mild-winter group (tested at 10 °C) and warm-winter group (tested at 15 °C) had a mean  $\dot{V}O_2$  that was 4- and 11-fold higher, respectively, than that of turtles in the cold-winter group, which were tested at 4 °C. The resulting  $Q_{10}$  was 9.2 for the range 4–10 °C, 8.9 for the range 10–15 °C, and 9.1 for the range 4–15 °C. An estimate of the total volume of oxygen consumed in simulated hibernation was obtained using the mean  $\dot{V}O_2$  values determined at each temperature and the amount of time turtles in each group spent at each temperature over the entire 6-month period. We then separately used oxycaloric coefficients for lipid, protein, and carbohydrate catabolism (Willmer et al., 2005) to gauge hypothetical caloric consumption had the turtles been supported entirely by a single energy substrate (Table 2). Presumably, turtles were using a mixture of substrates and their caloric consumption based on  $\dot{V}O_2$  would be intermediate among the values presented in Table 2. We also acknowledge that the resulting energy budgets necessarily underestimate the actual costs of simulated hibernation because the  $\dot{V}O_2$  values used in the calculations were obtained from fully-acclimatized turtles; somewhat higher rates likely persisted for some time following the temperature transitions. This caveat notwithstanding, predicted energy use over the entire simulated hibernation was 1.6- and 3.6-fold higher for turtles in the mild- and warm-winter groups, respectively, compared to that (1.18 kJ) for turtles in the cold-winter group; these values compared favorably with estimates of energy consumption based on changes in caloric content of turtle tissues (Table 2).

## 4. Discussion

Because hatchling turtles must rely on finite energy reserves and because they inhabit thermally-variable microhabitats during winter, we used them as experimental subjects to better understand the energetic implications of cold, mild, and warm winters by quantifying the energetic costs incurred under different thermal regimes. Turtles in the cold-winter group were exposed to diminishing temperatures such as occur in natural nests throughout late summer, autumn, and winter, with the minimum temperature attained, 4 °C, being more or less typical of northern locales where snow cover is persistent (Costanzo et al., 2000, 2008). For simplicity, this regime did not simulate short-term variations in environmental temperature, which can impact energetic demands by altering thermal sensitivity of metabolic rate in dormant ectotherms (Williams et al., 2012). Neither did it simulate the occasional, brief excursions to subfreezing temperatures that may occur from mid-December until late January, nor the gradual rise in temperature that precedes spring emergence (Costanzo et al., 1995). Such events could influence the overall energy expenditures of turtles overwintering in nature, as, for example, bouts of chilling to temperatures below 4 °C could substantially reduce metabolic rate. On the other hand, our results indicate that

**Table 2**  
Energy budgets of hatchling painted turtles (*Chrysemys picta*) in simulated hibernation under three thermal regimes (final winter temperature in italics) based on changes in tissue caloric content and rates of oxygen consumption ( $\dot{V}O_2$ ).

Group	Energy usage, based on change in caloric content (kJ) <sup>a</sup>	Energy usage, based on $\dot{V}O_2$ (kJ) <sup>b</sup>		
		Protein catabolism	Lipid catabolism	Carbohydrate catabolism
Cold winter 4 °C	1.46	1.12	1.18	1.25
Mild winter 10 °C	2.81	1.81	1.91	2.04
Warm winter 15 °C	4.52	4.09	4.30	4.59

<sup>a</sup> Calculated as the difference in mean caloric content of tissues in overwintered turtles from that of turtles in the fall group, as given in Table 1.

<sup>b</sup> Calculated from mean  $\dot{V}O_2$  and an oxy-caloric coefficient of 18.6 J ml<sup>-1</sup> O<sub>2</sub> for protein, 19.6 J ml<sup>-1</sup> O<sub>2</sub> for lipid, and 20.9 J ml<sup>-1</sup> O<sub>2</sub> for carbohydrate.

the vast majority (~78%, see below) of the energy used by turtles in the cold-winter group was consumed even before they reached 4 °C. Turtles in the mild-winter group, which attained a minimum temperature of 10 °C, experienced a thermal regime simulating the nest environment in a moderate winter in temperate locales and, presumably, a typical winter in nests of turtles from more southerly populations. Turtles in the warm-winter group were continuously exposed to 15 °C, a temperature commonly prevailing in *C. picta* nests during early autumn. Such temperatures are unlikely to prevail in nests at high latitudes and thus exaggerate responses of turtles in northern populations, although they potentially could occur during warm winters in southern locales. Indeed, hatchlings of this species reportedly overwinter within the natal nest throughout their range, even as far south as Louisiana (Cagle, 1954).

#### 4.1. Somatic changes in overwintering turtles

Turtles overwintering within natal nests or other terrestrial situations are in negative energy balance, as they are unable to feed or digest food. Little is known about the dynamics of body mass of hatchling turtles during hibernation, although a few studies indicate that the mass lost from the time of hatching until spring emergence often is on the order of 15–25% (Costanzo et al., 2008). The comparatively small decrease occurring between October and mid April in our turtles, including ones exposed to relatively high environmental temperatures, suggests that, generally, hatchling turtles lose much of this mass during the period preceding winter. Unfortunately, few investigators have reported the extent to which any decrease in mass reflects water loss rather than nutrient consumption; however, this was not a factor in the present study, as we observed no change in hydration state of overwintering turtles.

It is generally believed that residual yolk, the portion of ovum yolk that is not reabsorbed during embryogenesis, plays an integral role in maintenance metabolism and, potentially, somatic growth in hibernating hatchling turtles (Costanzo et al., 2008). This supposition is promulgated by studies documenting marked differences in the quantities of residual yolk between recently-hatched turtles and those examined at winter's end. For example, Costanzo et al. (2003) found that neonatal *C. picta* contained a substantial quantity (up to 177 mg, fresh tissue) of residual yolk, but this amount was reduced by 75–90% before the end of dormancy. Other studies (Tucker et al., 1998a; Filoramo and

Janzen, 1999) determined that hatchling *T. scripta* consumed about 85% of their residual yolk from the time of hatching to winter's end, leading the investigators to conclude that this tissue is a primary fuel during hibernation. To the contrary, our present results showing that *C. picta* had little residual yolk remaining by October suggest that the predominant use of this material occurs within the first weeks after hatching, as seems true for other species (Lee et al., 2007). Energy from this tissue therefore would not be directly available to fuel metabolism in hibernation. It is possible that, in autumn, yolk materials are converted to other substrates, such as fat body and glycogen, that are stored for later use, although further research is necessary to determine whether or not this actually occurs. Regenerating energy substrates from mobilized yolk obviously incurs an energetic cost, although potentially this cost is offset by obviating the need to maintain the gut in a functional state during dormancy, which would otherwise be necessary for nutrient absorption from the yolk. Our present results contradict the widely held belief that residual yolk directly supports metabolism in hibernating hatchling turtles (Costanzo et al., 2008).

What, then, is the source of the energy that sustains hatchling turtles in hibernation? Significant amounts of glycogen are found in organs of hatchling *C. picta* (Storey et al., 1988) and this is especially true of liver, in which glycogen represents ~7% of wet tissue mass (Hemmings and Storey, 2000). The subsequent reduction in liver mass (up to 66%) over the winter attests to the importance of this organ in supporting metabolic activity during hibernation. To our knowledge, such a role for liver in hatchling turtles has not been recognized previously.

We observed a substantial decrease in mass of the carcass, particularly amongst turtles hibernating at higher temperatures; thus, tissues other than yolk and liver apparently contribute to energy supply during dormancy. Among hatchlings of various species of turtles, a substantial percentage (16–26%) of the dry tissue is non-polar lipid, primarily in the form of triacylglycerol, an important metabolic fuel in neonatal reptiles (Nagle et al., 2003). Hatchling *C. picta* treated identically to those in our cold-winter group consumed about half their total nonpolar lipid between mid-August and December, and an additional 15% during the ensuing two months, during which they were held at 4 °C (Costanzo et al., 2000). The extent to which catabolism of structural proteins contributes to the energy supply is unknown; however, that a substantial catabolism of proteins occurs during hibernation is evidenced by a marked accumulation of urea (Costanzo et al., 2000).

In our study, organic matter comprised 85–88% of dry tissue, a finding consistent with an earlier report (Costanzo et al., 2000). This fraction was diminished during simulated hibernation, the reduction being greater in turtles subjected to higher environmental temperatures (Table 1), undoubtedly as a consequence of greater metabolic demands. Similarly, hatchling *C. picta* hibernating in natural nests lost more organic matter during a mild winter than during a cold one (Costanzo et al., 2004). Given that 40–45% of the depleted organic matter is nonpolar lipid (Costanzo et al., 2000), it follows that the turtles subjected to higher temperatures had substantially lower caloric contents at the end of simulated hibernation. However, the inter-group variation in caloric concentration in ash-free tissue suggests that these turtles used proportionately more of their remaining high-energy substrates, as their tissues were of relatively low energy density.

#### 4.2. Energy budget for overwintering turtles

Results of our respirometry trials and whole-body caloric analyses were in generally good agreement in predicting the energy use of turtles overwintering under three distinct thermal

regimes. Although the effect of environmental temperature on energy use is evident, it would be instructive to know the extent to which individual body components contribute to the overall energy budget. Unfortunately, caloric analyses could not be made on such small tissue samples; however, we generated similar information by mathematically analyzing the available data. Using the following equation, we estimated for each group of turtles the energy lost from each major body component during simulated hibernation:

$$\Delta E_{\text{Total}} = (\Delta M_{\text{Yolk}} \times EC_{\text{Yolk}}) + (\Delta M_{\text{Liver}} \times EC_{\text{Liver}}) + (\Delta M_{\text{Carcass}} \times EC_{\text{Carcass}})$$

where  $\Delta E_{\text{Total}}$  (kJ) is the difference in mean total caloric content between fall turtles and overwintered turtles (values as given in Table 1);  $\Delta M_{\text{Yolk}}$  (g),  $\Delta M_{\text{Liver}}$  (g), and  $\Delta M_{\text{Carcass}}$  (g) are the differences in mean dry mass of the indicated components between fall turtles and overwintered turtles (values as represented in Fig. 2); and  $EC_{\text{Yolk}}$  (kJ g<sup>-1</sup>),  $EC_{\text{Liver}}$  (kJ g<sup>-1</sup>), and  $EC_{\text{Carcass}}$  (kJ g<sup>-1</sup>) are energy concentrations of the mass that was lost from the indicated component during simulated hibernation. Substituting terms with the available data generated the following series of equations: Cold winter:  $1.46 = (0.005 \times EC_{\text{Yolk}}) + (0.027 \times EC_{\text{Liver}}) + (0.018 \times EC_{\text{Carcass}})$  Mild winter:  $2.81 = (0.000 \times EC_{\text{Yolk}}) + (0.043 \times EC_{\text{Liver}}) + (0.056 \times EC_{\text{Carcass}})$  Warm winter:  $4.52 = (0.007 \times EC_{\text{Yolk}}) + (0.050 \times EC_{\text{Liver}}) + (0.100 \times EC_{\text{Carcass}})$ .

Using an iterative algebraic algorithm to solve for the EC terms, we obtained the following values:  $EC_{\text{Yolk}} = 36.93$  kJ g<sup>-1</sup>,  $EC_{\text{Liver}} = 28.24$  kJ g<sup>-1</sup>,  $EC_{\text{Carcass}} = 28.50$  kJ g<sup>-1</sup>. One constraint of the algorithm is that the aforementioned values are composites and necessarily represent EC values for all treatment groups. Despite this limitation, our estimate of  $EC_{\text{Yolk}}$  is comparable to the energy concentration (on an ash-free, dry mass basis) of residual yolk, and our estimates of  $EC_{\text{Liver}}$  and  $EC_{\text{Carcass}}$  are comparable to the energy concentration of yolk-free tissue, as determined by Kraemer and Bennett (1981) for hatchling loggerhead turtles (*Caretta caretta*). The similarity between our estimate of  $EC_{\text{Yolk}}$  and the energy density of pure lipid (~38 kJ g<sup>-1</sup>) suggests that any energy derived from yolk during simulated hibernation was almost exclusively from this substrate. Our estimates of  $EC_{\text{Liver}}$  and  $EC_{\text{Carcass}}$  are lower than the energy density of lipid, but considerably higher than caloric values for pure protein (~20 kJ g<sup>-1</sup>) and carbohydrate (~17 kJ g<sup>-1</sup>); thus, although much of the energy generated from liver and carcass during overwintering was derived from lipid oxidation, catabolism of protein and/or carbohydrate is also indicated.

Taking together the EC values calculated as above and our estimates of mass loss, we determined the absolute and relative contribution of each body component to the energy budget of overwintering turtles (Table 3). These results underscore the notion that, despite its exceptionally high energy density, residual yolk contributed relatively little to maintenance metabolism. Liver was a particularly important energy source in the cold-winter group, contributing over half of the total energy consumed, whereas carcass was the predominant source in turtles overwintering at the higher temperatures. Although we did not measure the substrate composition of each body component, liver presumably contained a high proportion of carbohydrate and lipid

(Derickson, 1976; Hemmings and Storey, 2000), whereas carcass would have also contained substantial protein (Crawford, 1994), especially in skeletal muscle. Because fasting and starving animals tend to catabolize stored carbohydrate and lipid before relying on energy from body protein (Wang et al., 2006; McCue, 2010), such a heavy reliance on carcass-derived fuel reserves by mild- and warm-winter turtles may have become necessary as they depleted carbohydrate and lipid stores and switched to protein-dominated catabolism. That increased use of carcass-derived fuel may, in turn, have reduced fitness is evidenced by the several moribund turtles in those groups.

Physiological responses to fasting and starvation have garnered considerable scientific interest (McCue, 2010), and there are obvious parallels between these states and aphagia during dormancy. However, the extent to which energy-use pathways during hibernation match those during fasting or starvation is unclear. In these processes, organismal energy demand is reduced by a suite of behavioral and physiological mechanisms that often includes a decrease in the function and even size of the gut (Wang et al., 2006). However, the role of the gut in pre-gustatory hatchling turtles is not well understood, and it is unclear whether such a change in this organ, if it indeed occurs, would substantially reduce energy demand. Nonetheless, the results of our study do suggest a fasting- or starvation-like shift in energetic strategy from that of acquiring and storing energy from the yolk soon after hatching to one of relying on endogenous energy stores throughout the winter.

Metabolic rates of our hatchling *C. picta* in simulated hibernation were markedly sensitive to temperature. Relatively high  $Q_{10}$  values (5–10) are not uncommon for reptiles at low temperature (Aleksiuk, 1976; Morris, 1981; Gregory, 1982), and the high  $Q_{10}$  we found for *C. picta* may be indicative of pronounced metabolic depression during hibernation. Alternatively, the high  $Q_{10}$  may be the result of greatly increased metabolic rate at relatively high temperatures. Although the  $\dot{V}O_2$  of turtles in the cold-winter group was many-fold lower than that of turtles overwintering at higher temperatures, the overall energetic savings they gained was tempered by the high metabolic costs they incurred during autumn; indeed, we estimate that 78% of the energy that turtles in the cold-winter group consumed in simulated hibernation was expended during October and November.

The high  $Q_{10}$  apparent in our respirometry data probably benefits turtles by greatly reducing their metabolism during winter dormancy whilst also allowing them to increase metabolism and activity necessary for nest emergence when the weather warms in spring (Costanzo et al., 2008). However, because hatchling *C. picta* overwinter in shallow nests, the temperatures they experience are influenced by the predominating weather, among other things. Our present results indicate that unusually warm conditions may have profound consequences for energy use in dormancy. This finding is bolstered by results of field studies (Costanzo et al., 2004; Willette et al., 2005). In hatchling *C. picta*, for example, Costanzo et al. (2004) found that the decrease in lipids and other energy-yielding substrates was greater in a warmer, as compared to a cooler, winter, and also in a population inhabiting a more temperate locale. Even if survival in winter is not energy limited, retaining sufficient energy reserves to fuel the spring migration from the nest to permanent habitat is critical to making a successful transition to the next life history stage (Tucker et al., 1998b). Our finding that higher winter temperatures are energetically unfavorable for terrestrially hibernating hatchling turtles seemingly has implications for predicting the energetic effects of global climate change. Specifically, the high thermal sensitivity of these turtles' metabolic rates suggests that even a modest increase in winter soil temperature may result in a substantial increase in energy use. For hatchling *C. picta* and other

**Table 3**

Absolute and relative contributions of three body components to total energy use (kJ) in hatchling painted turtles (*Chrysemys picta*) during simulated hibernation under three thermal regimes (final winter temperature in italics).

	Yolk	Liver	Carcass	Total
Cold winter 4 °C	0.19 (13.0%)	0.76 (52.1%)	0.51 (34.9%)	1.46 (100.0%)
Mild winter 10 °C	0.00 (0.0%)	1.21 (43.1%)	1.60 (56.9%)	2.81 (100.0%)
Warm winter 15 °C	0.26 (5.7%)	1.41 (31.2%)	2.85 (63.1%)	4.52 (100.0%)

temperate ectotherms, a marked increase in energy demand has the potential to limit the survival of individuals (Irwin and Lee, 2000, 2003; Marshall and Sinclair, 2012; Zani et al., 2012) as well as threaten the persistence of populations.

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