

RESEARCH ARTICLE

Digesting pythons quickly oxidize the proteins in their meals and save the lipids for later

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ABSTRACT

Pythons digesting rodent meals exhibit up to 10-fold increases in their resting metabolic rate (RMR); this increase in RMR is termed specific dynamic action (SDA). Studies have shown that SDA is partially fueled by oxidizing dietary nutrients, yet it remains unclear whether the proteins and the lipids in their meals contribute equally to this energy demand. We raised two populations of mice on diets labeled with either [^{13}C]leucine or [^{13}C]palmitic acid to intrinsically enrich the proteins and lipids in their bodies, respectively. Ball pythons (*Python regius*) were fed whole mice (and pureed mice 3 weeks later), after which we measured their metabolic rates and the $\delta^{13}\text{C}$ in the breath. The $\delta^{13}\text{C}$ values in the whole bodies of the protein- and lipid-labeled mice were generally similar (i.e. $5.7 \pm 4.7\%$ and $2.8 \pm 5.4\%$, respectively) but the oxidative kinetics of these two macronutrient pools were quite different. We found that the snakes oxidized 5% of the protein and only 0.24% of the lipids in their meals within 14 days. Oxidation of the dietary proteins peaked 24 h after ingestion, at which point these proteins provided $\sim 90\%$ of the metabolic requirement of the snakes, and by 14 days the oxidation of these proteins decreased to nearly zero. The oxidation of the dietary lipids peaked 1 day later, at which point these lipids supplied $\sim 25\%$ of the energy demand. Fourteen days after ingestion, these lipids were still being oxidized and continued to account for $\sim 25\%$ of the metabolic rate. Pureeing the mice reduced the cost of gastric digestion and decreased SDA by 24%. Pureeing also reduced the oxidation of dietary proteins by 43%, but it had no effect on the rates of dietary lipid oxidation. Collectively, these results demonstrate that pythons are able to effectively partition the two primary metabolic fuels in their meals. This approach of uniquely labeling the different components of the diet will allow researchers to examine new questions about how and when animals use the nutrients in their meals.

KEY WORDS: Metabolism, Nutrition, Snakes, Specific dynamic action, Stable isotopes

INTRODUCTION

Pythons have become popular models to explore the morphological and physiological responses to digestion (reviewed in Secor, 2008). During digestion of a typical meal they double the size of their intestine (Secor and Diamond, 1998) and exhibit increases in resting metabolic rate (RMR) similar in magnitude (e.g. up to 10-fold) to those caused by vigorous locomotor activity (Secor et al., 2000). Their metabolic rates peak ~ 24 – 48 h after feeding and then gradually return to pre-feeding values over the following week (reviewed in Wang et al., 2001; McCue, 2006; Secor, 2009). The

cumulative energy expended on meal digestion and assimilation is called specific dynamic action (SDA). The magnitude and timing of the SDA response in pythons depend on the relative size of the meal (Secor and Diamond, 1997, 1998; Cox and Secor, 2007), the macronutrient composition of the meal (McCue et al., 2005; Enok et al., 2013; Henriksen et al., 2015), and body temperature (Bedford and Christian, 2000; Toledo et al., 2003; Wang et al., 2003). Nevertheless, under similar feeding conditions the SDA response is highly repeatable (Secor, 1995; Overgaard et al., 2002; Starck and Wimmer, 2005).

Pythons generally consume rodent meals, which are high in protein ($\sim 17\%$ wet mass) and fat ($\sim 10\%$ wet mass), and low in carbohydrates (Secor et al., 1994; McCue et al., 2005). Although pythons can assimilate $>90\%$ of the energy from their meals (Bedford and Christian, 2000; Cox and Secor, 2007), SDA typically accounts for 20–30% of this energy (Secor, 2009). For over a century researchers have worked to quantify the relative energetic components of mechanical, physiological and biochemical processes associated with the costs of SDA (reviewed in Jobling, 1983; McCue, 2006; Secor, 2009). We now know that the costs of gastric digestion (Secor, 2003; Andrade et al., 2004) and protein synthesis (McCue et al., 2005; Enok et al., 2013) account for most of the SDA in pythons. Unfortunately, the source of this energy (i.e. endogenous – from nutrient stores, or exogenous – from the meal itself) is less well understood (Secor and Diamond, 1995; Starck et al., 2004).

In order to investigate the time course of macrodigestion, Cox and Secor (2008) killed Burmese pythons (*Python bivittatus*) 12 h after they had ingested a rodent meal and found that 18% of the meal had moved into the small intestine. X-ray imaging also revealed that 78% of a meal remained in the stomach by 24 h and only 28% of the meal by 72 h (Secor and Diamond, 1995; Secor, 2003). Recognizing that the SDA response peaked around the time when most of the meal was still in the stomach, researchers originally assumed that pythons were primarily mobilizing and oxidizing endogenous nutrient stores to fuel SDA. To examine this possibility, Starck et al. (2004) raised laboratory mice on a corn-based diet for 2 weeks in order to allow their tissues to become naturally enriched in ^{13}C (compared with mice raised on control diets). They fed the ^{13}C -enriched mice to pythons and measured the exhaled ^{13}C each day during digestion and found that it increased at around 24 h, confirming that at least some of the nutrients in the mice were being oxidized. The highest $\delta^{13}\text{C}$ values in the breath were observed at 48 h ($\sim 7\%$ above control treatments) after which they gradually declined during the subsequent 10 days.

In a follow-up study Waas et al. (2010) fed mice raised on corn-based diets to pythons. They collected the breath at 30 min intervals during the first 12 h of digestion (and then hourly over the following 2 days) and found that the $\delta^{13}\text{C}$ increased within 4 h of ingestion. By 12 h it was significantly higher than in snakes

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consuming control mice, whose breath $\delta^{13}\text{C}$ remained relatively constant. They concluded that the SDA was fueled using a mixture of endogenous and exogenous nutrients, and that at peak SDA (~24 h) the nutrients in the meal contributed to 75% of their metabolic requirement; although they did not measure the breath during the second half of the SDA response, they ultimately estimated that 60% of the SDA was fueled from nutrients in the meal.

The corn-based diet in the two aforementioned studies caused the tissues of the mice, and subsequently the breath of the snakes, to exhibit increased $\delta^{13}\text{C}$. Unfortunately, the approach of using naturally occurring differences in ^{13}C (e.g. C_4 plant-derived diets) to experimentally label the mice suffers from two limitations. The first is that the magnitude of the final isotopic enrichment is much smaller than if artificial ^{13}C -labeled tracers are used (Welch et al., 2015). The second limitation is what has been called the ‘scrambled-egg’ premise (Van Der Merwe, 1982); it is impossible to distinguish the extent to which animals oxidize different classes of macronutrients (e.g. proteins or lipids) in their meals.

Recent studies employing ^{13}C -breath testing have revealed that different classes of purified nutrients exhibit unique oxidative kinetics when fed to birds (McCue et al., 2010, 2011a), rodents (McCue et al., 2014) and bats (Voigt et al., 2012). The general pattern observed is that exogenous amino acids are oxidized much sooner and to a greater extent than exogenous fatty acids during the postprandial period (Welch et al., 2015). Dietary carbohydrates exhibit some intermediate response, but are not a major component of the diet of carnivores like pythons.

It has been noted that purified ^{13}C -tracer molecules (e.g. amino acids and fatty acids) may not provide the best proxy of the oxidative fates of ^{13}C -tracers that are biochemically integrated into larger dietary macromolecules (e.g. proteins and triacylglycerols) (McCue, 2007b; McCue et al., 2013; McCue and Cardentey, 2014; Welch et al., 2015). In order to avoid this issue in the present study, we allowed the ^{13}C -tracers (e.g. amino acids and fatty acids) to become deeply integrated into the tissues and organs of the mouse prey over the course of their lives. This allowed us to test whether pythons, *Python regius* (Shaw 1802), differentially oxidize different classes of macronutrients during digestion, similar to what is typically seen using purified tracers. In particular, we predicted that the exogenous proteins would contribute more to SDA than lipids.

Pythons and other ambush foraging snakes rely extensively on endogenous lipids during prolonged fasting (McCue, 2007a; Leite et al., 2014), but like most other vertebrates they do not have specialized organs and tissues for storing proteins that are comparable to those for lipid storage. As such, we also predicted that pythons would diminish their reliance on recently assimilated proteins as they became post-absorptive while continuing to oxidize the recently assimilated lipids.

Researchers examining the costs of gastric digestion compared the metabolic rates of snakes digesting intact versus pureed rodent meals and found that SDA was reduced by ~25% when the meal was pureed (reviewed in Secor, 2009). In the present study, we examined whether reducing the costs of gastric digestion by pureeing the meal would also alter the extent to which different classes of dietary nutrients were used for SDA. In order to test this, we compared the oxidative kinetics of exogenous amino acids and lipids in intact and pureed mouse meals.

RESULTS

Mice

The $\delta^{13}\text{C}$ of the bulk tissues of the protein- and lipid-labeled mice was $5.7\pm 4.7\%$ and $2.8\pm 5.4\%$, respectively (Table 1). However, the

Table 1. $\delta^{13}\text{C}$ values of whole carcasses and the lean and lipid fractions of the feeder mice described in Table 2

Tracer	Tissue fraction	^{13}C (means \pm s.d.)
None	Lean	-18.5 ± 0.3
	Lipid	-24.5 ± 0.7
	Whole	-21.4 ± 0.6
Leucine	Lean	38.1 ± 5.4
	Lipid	-16.3 ± 4.8
	Whole	5.7 ± 4.7
Palmitic acid	Lean	-16.8 ± 0.9
	Lipid	28.3 ± 9.6
	Whole	2.8 ± 5.4

Three mice were used for each determination and samples were analyzed in triplicate.

lean fraction of the protein-labeled mice and the lipid fraction of the lipid-labeled mice were substantially more enriched in ^{13}C than the bulk tissues, with values of $38.1\pm 5.4\%$ and $28.3\pm 9.6\%$, respectively (Table 1). The biochemical losses of the ^{13}C from the palmitic acid tracer were minimal and caused the lean fraction of the palmitic acid mice to become 1.7‰ more enriched than the lean fraction of the control mice. The losses of the ^{13}C from the leucine tracer into the lipid pool were larger, causing the lipid fraction of the protein-labeled mice to become 8.2‰ more enriched than the lipids in the control mice. Nevertheless, most of the ^{13}C atoms from both the leucine and palmitic acid tracers remained in their respective parent macronutrient pools (i.e. proteins and lipids) within the mice. As such, we assumed that the ^{13}C in the breath of snakes that had consumed the protein- and lipid-labeled mice was an accurate proxy of the rates of total protein and lipid oxidation.

The rate of CO_2 production (\dot{V}_{CO_2}) of dead mice that were not being actively digested and were instead allowed to decompose inside metabolic chambers gradually increased over time. The mean values were 0.013, 0.038 and 0.052 ml min^{-1} at 0, 24 and 48 h, respectively. However, because these rates of CO_2 production were only 5%, 6.5% and 11.7% of the \dot{V}_{CO_2} of the postprandial snakes at the same time points, respectively, and we could not confirm whether the same level of decomposition occurs inside the lumen of the stomach of the snakes, where $\text{pH}<2.0$ (reviewed in Secor, 2008), we assumed that all of the $^{13}\text{CO}_2$ production of the pythons was the result of digestive processes and not decomposition.

Snakes

The \dot{V}_{CO_2} of post-absorptive pythons did not differ between the ^{13}C -tracer treatment groups (protein: 0.023 ± 0.007 ml h^{-1} g^{-1} and lipid: 0.024 ± 0.004 ml h^{-1} g^{-1} ; t -test, d.f.=10, $P=0.841$). In contrast, the metabolic rates did differ when pythons processed intact versus pureed meals. Specifically, the \dot{V}_{CO_2} of the pythons digesting whole mice peaked at 30 h at a magnitude that was 6.0-fold higher than in the post-absorptive state (Fig. 1), whereas the \dot{V}_{CO_2} of pythons digesting pureed mice peaked sooner (i.e. at 24 h) and at a lower

Table 2. Isotope supplements used to raise the three populations of CD-1 mice used for snake feeding trials

Mice (N)	Treatment	Tracer	Dose	Medium
100	Control	None	None	None
20	Protein	L-[1- ^{13}C] leucine	1 g l^{-1}	Drinking water
80	Lipid	[1- ^{13}C] palmitic acid	5 mg day^{-1} mouse $^{-1}$	Peanut butter

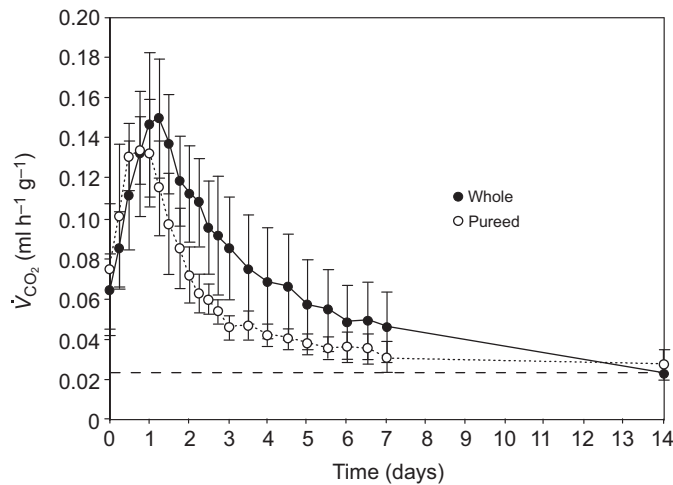


Fig. 1. Mass-specific rates of CO₂ production (\dot{V}_{CO_2}) in *Python regius*. Pythons consumed either whole (solid line) or pureed (dotted line) mouse meals equal to 13% of their body mass. Pureed mice caused the postprandial metabolic rates to peak sooner and reduced specific dynamic action (SDA) by an average of 24% (see Results for details). Each data point represents the mean of $N=14$ snakes. Error bars refer to \pm s.d. The dashed line represents the mean post-absorptive \dot{V}_{CO_2} for comparison.

magnitude (i.e. 5.3-fold). The SDA coefficients (a measure of the relative cost of SDA for a given meal size) of the whole mice did not differ between the ¹³C-tracer treatment groups (protein: 0.221 ± 0.045 and lipid: 0.203 ± 0.019 ; t -test, d.f.=10, $P=0.524$); however, the SDA coefficient did differ between the whole and the pureed mouse meals (whole: 0.211 ± 0.039 , pureed: 0.160 ± 0.025 ; t -test, d.f.=20, $P<0.001$). In total, the cost of digestion of the pureed mice was reduced by 24%.

Increases in the $\delta^{13}C$ in the breath of pythons in both the protein and lipid treatment groups were observed within 6 h of ingesting the mice. The $\delta^{13}C$ in the breath of the protein group pythons continued to rapidly increase until reaching a peak value of $35.6 \pm 11.7\%$ at 30 h (Fig. 2A). Because the ¹³C of the lean fraction of the protein-labeled mice was 38‰ (see Table 1), we calculated that approximately 96% of the metabolic energy at that time point was derived from oxidizing the exogenous proteins in the mice. Thereafter, the $\delta^{13}C$ value of the breath gradually decreased, reaching a minimum at 14 days, at which point we calculated that less than 1% of the energy requirement was derived from the exogenous mouse proteins.

The breath of the lipid group pythons reached a peak $\delta^{13}C$ value of -5.1% at 120 h (Fig. 2B). Given that the ¹³C of the lipids of the lipid-labeled mice was 28‰ (see Table 1), we calculated that the oxidation of the exogenous lipids accounted for approximately 36% of the energy demand at that time point. Thereafter, the ¹³C values gradually decreased, but never fell below a mean value of -10.7% during the 14 day measurement period, at which point we calculated that approximately 25% of the energy demand was still met by oxidizing the exogenous lipids from the mouse meal.

Pureeing the mice affected the $\delta^{13}C$ in the breath of the pythons, and we noted three different responses when snakes processed the pureed meals. First, the inter-individual variances in $\delta^{13}C$ at any given time point were smaller when the snakes consumed the pureed meals (Fig. 2). For example, 72 h into digestion the standard deviations of the protein and lipid group snakes was 5.9- and 5.3-fold larger when the snakes were processing intact mice. Second, the timing of the peak $\delta^{13}C$ in the protein group pythons occurred

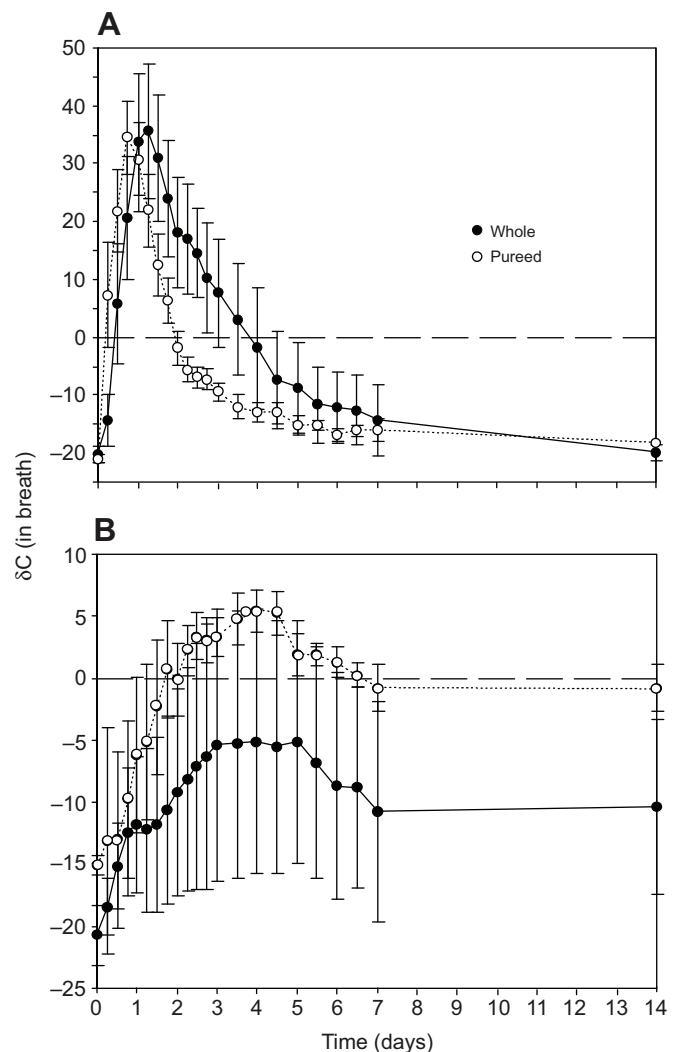


Fig. 2. The ¹³C-values in the exhaled breath of pythons digesting isotopically labeled whole and pureed mice. Mice were raised on diets supplemented with either (A) [¹³C]leucine to enrich the proteins in their bodies or (B) [¹³C]palmitic acid to enrich the lipids in their bodies. Note that pureeing the mice caused the snakes consuming the protein-labeled mice to have lower $\delta^{13}C$ in their breath after 48 h, and that the snakes digesting the pureed, lipid-labeled mice (3 weeks later) had $\delta^{13}C$ -values that were constantly higher than the previous feeding (see Discussion for more details). Each data point represents the mean of $N=7$ snakes. Error bars refer to \pm s.d.

earlier (i.e. at 18 h versus 30 h) when the meal was pureed (Fig. 2A). Third, the $\delta^{13}C$ values of the lipid pythons were consistently higher throughout the SDA response after consuming the pureed meals (Fig. 2B). Below, we discuss the mechanisms responsible for each of these differences.

The instantaneous rates of tracer oxidation (T) are a product of both the \dot{V}_{CO_2} of the python and the $\delta^{13}C$ of the breath at any given time point (see Eqn 1, Materials and methods). The T of the protein group snakes consuming whole and pureed mice exhibited a general pattern whereby values increased from zero and peaked at 18–24 h, then returned to near zero after 14 days (Fig. 3A). We calculated that the mean $\delta^{13}C$ of the leucine residues in the mice (i.e. both whole and pureed) was 418‰, and therefore each protein-labeled mouse contained 226 μ mol of [¹³C]leucine molecules. The snakes consuming the whole mice therefore oxidized 10.9 μ mol of the tracer molecules over 14 days (i.e. 4.7% of those contained in

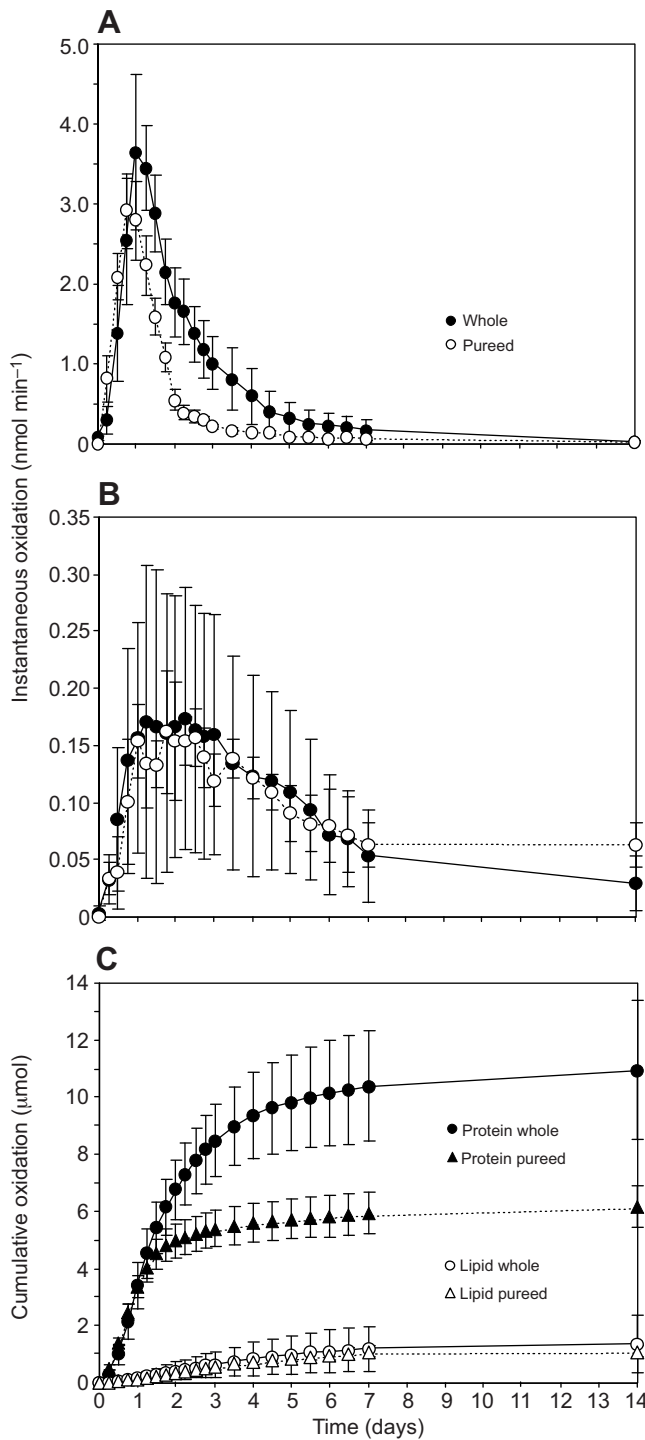


Fig. 3. Instantaneous rates of ^{13}C -tracer oxidation in pythons digesting isotopically labeled whole or pureed mice. Mice were raised on diets supplemented with either (A) [^{13}C]leucine to enrich the proteins in their bodies or (B) [^{13}C]palmitic acid to enrich the lipids in their bodies. The rates of tracer oxidation were calculated from \dot{V}_{CO_2} and $\delta^{13}\text{C}$ (see Eqns 1 and 2, Materials and methods). Note that the exogenous leucine was oxidized to a greater extent than the palmitic acid. (C) The cumulative oxidation of the exogenous ^{13}C -tracers in mice that were fed whole or pureed mice. Note that pureeing the mice affected the extent to which the exogenous leucine was oxidized but not the palmitic acid. Each data point represents the mean of $N=7$ snakes. Error bars refer to \pm s.d.

the meal), whereas the snakes consuming the pureed mice oxidized $6.2 \mu\text{mol}$ (i.e. 2.7% of those contained in the meal; Fig. 3C). In other words, pureeing the mice caused the exogenous protein oxidation to

peak sooner after ingestion (i.e. 18 h versus 24 h; Fig. 3A) and also reduced the cumulative protein oxidation by 43% (Fig. 3C).

The T of the lipid group snakes was lower than that of the protein group snakes during the first week, but as snakes became post-absorptive, T became higher in the lipid group snakes. Values peaked 54 h into digestion, notably later than peak values for the protein group snakes. Below, we propose a mechanistic explanation for this difference. Unlike for the protein group snakes, the T of the whole and pureed mice treatments was nearly identical in the lipid group snakes (Fig. 3B). We calculated that the mean $\delta^{13}\text{C}$ of the palmitic acid residues in the mice (i.e. both whole and pureed) was 173‰, and therefore each lipid-labeled mouse contained $531 \mu\text{mol}$ of [^{13}C]palmitic acid molecules. Ultimately, the snakes consuming the whole mice oxidized $1.3 \mu\text{mol}$ of the tracer molecules over 14 days (i.e. 0.24% of those contained in the meal), whereas the snakes consuming the pureed mice oxidized $1.2 \mu\text{mol}$ (i.e. 0.23% of those contained in the meal; Fig. 3C).

DISCUSSION

The fact that the bulk $\delta^{13}\text{C}$ values of the protein- and lipid-labeled mice in this study were similar (see Table 1) underscores one of the limitations in isotopically labeling all of the tissues in the mice by raising them on C_4 plant-derived diets. This targeted (sensu McCue et al., 2013) isotopic labeling of the protein and lipid pools in the mice allows us to quantify the different oxidative fates of these nutrients. Starck et al. (2004) and Waas et al. (2010) fed pythons with ^{13}C -enriched mice and observed peak $\delta^{13}\text{C}$ at 48 h. Although they did not directly measure the $\delta^{13}\text{C}$ in the protein and lipid pools of those mice, they likely had roughly the same $\delta^{13}\text{C}$ -enrichment (Guzman et al., 2015). It follows that this 48 h time point represents cumulative oxidation of both proteins and lipids in the meal. Here, we show that these two classes of nutrients actually exhibit unique oxidative kinetics during the postprandial period and that the relative contribution of these dietary fuels to SDA changes during digestion. Although some of the ^{13}C -atoms from the [^{13}C]leucine and the [^{13}C]palmitic acid tracers were recovered in other macronutrient pools in the body, the vast majority of the ^{13}C atoms remained in their parent macronutrient pools, thereby providing new insights into how and when these animals use their dietary proteins and lipids.

Dietary proteins

Overall, the dietary proteins contributed to SDA much more than the dietary lipids, especially during the first several days of digestion. In the protein group pythons fed intact mice, the $\delta^{13}\text{C}$ of the breath peaked at 24 h and the instantaneous rates of oxidation peaked slightly later at 30 h ($3.65 \text{ nmol min}^{-1}$). It is noteworthy that the $\delta^{13}\text{C}$ does not necessarily coincide with the peak rate of tracer oxidation (T), because T is also dependent on \dot{V}_{CO_2} at that time point. Nevertheless, the peak $\delta^{13}\text{C}$ is indicative of the time at which the tracer (and thus the macronutrient pool for which it is a proxy) makes the greatest relative contribution to the oxidative fuel mixture.

Mice are usually ingested head first (Feder and Arnold, 1982; de Queiroz and de Queiroz, 1987; Rodriguez-Robles et al., 1999; Secor, 2008) and thus the head is the first part of the mouse to be broken down and pass into the small intestine (Secor, 2008); therefore, it is possible that regional differences in the protein and lipid content of the mouse could drive the changes in the $\delta^{13}\text{C}$ of the breath over time. To test for this we compared the macronutrient composition of the head of the mouse with that of the rest of the body. We found that the head region of the mouse had no higher

protein content ($47.4 \pm 1.0\%$) than the remainder of the mouse carcass ($48.2 \pm 0.9\%$; *t*-test, d.f.=8, $P=0.370$). Similarly, the lipid content ($24.9 \pm 1.8\%$) of the head did not differ from that of the rest of the body ($29.8 \pm 4.6\%$; *t*-test, d.f.=8, $P=0.057$). As such, the finding that the dietary proteins were oxidized more extensively in the early phases of digestion is not an artifact of meal composition. This conclusion is also supported by the fact that the same general pattern of oxidative kinetics of dietary protein was seen in the pureed meals where the head and body were combined (see below).

Fourteen days after ingestion, as the snakes became post-absorptive, the rate of exogenous protein oxidation fell to nearly zero and thus the proteins were not providing a significant portion of the energy budget. If fasting continued, however, these proteins would again become an important source of energy (McCue, 2007a; McCue and Pollock, 2008), and future studies could take advantage of this phenomenon to quantify the timing and magnitude of increased protein oxidation during prolonged fasting (McCue, 2012; McCue and Pollock, 2013).

Dietary lipids

Although the breath of snakes consuming lipid-labeled mice became isotopically enriched within a few hours of feeding, the dietary lipids did not provide a substantial source of metabolic energy during the first day of digestion. The instantaneous rate of lipid oxidation peaked at 54 h ($0.17 \text{ nmol min}^{-1}$). Interestingly, the ^{13}C -values in the breath continued to increase thereafter, peaking at 120 h, at which point dietary lipids fueled 45% of the metabolic rate. The continued increase in ^{13}C in the breath of the lipid group snakes after 54 h occurred concomitantly with a decrease in the $\delta^{13}\text{C}$ of the protein group snakes and reflects an increase in the relative importance of lipid as an oxidative substrate during this later phase of digestion. Fourteen days after ingestion, as the snakes became post-absorptive, the ^{13}C of the lipid group snakes remained elevated, suggesting continued oxidation of the dietary lipids. Although the metabolic rate had returned to pre-feeding levels, the lipids from the meal were still supplying 25% of the energy requirement at 14 days. As fasting continues, lipids become a critical source of fuel and one study showed that pythons fasted for 6 months met >75% of their total energy demands by oxidizing their lipid stores (McCue, 2007a). Future studies could use this experimental approach to document the changes in the oxidation of endogenous lipid stores during prolonged food limitation.

It is noteworthy that while the $\delta^{13}\text{C}$ of the breath of the protein group snakes at the start of the initial experimental feeding (i.e. the intact mice) did not differ from their breath at the start of the second experimental feeding (i.e. the pureed mouse), this was not the case for the lipid group snakes. At the start of the second experimental feeding, 3 weeks after their first feeding, the breath of the lipid snakes was 7‰ more enriched than when they were initially fed the intact mice. After digesting the second experimental meal (i.e. the pureed meal), their breath was 20‰ more enriched. This progressive ^{13}C enrichment of the breath indicates that at this time the snakes were oxidizing a mixture of lipids from the two most recent meals. Future studies using a series of repeated feedings could be useful to study the rate of carbon turnover in the lipid stores of these animals. Presently, most of our knowledge of carbon turnover in vertebrates comes from endothermic models (e.g. Voigt et al., 2003; MacAvoy et al., 2006; Bauchinger and McWilliams, 2010).

What is happening to the dietary lipids if they are not immediately being oxidized? Within 24 h of ingestion of a meal, a python's small intestine doubles in mass (Secor, 1995; Secor and Diamond, 1995;

Lignot et al., 2005) and volume (Hansen et al., 2013), and increases its mass-specific lipid content by 75% (Henriksen et al., 2015). A portion of this increase in lipid content is caused by the accumulation of lipid droplets in the apical side of the enterocytes during the first day of digestion (Starck and Beese, 2001; Lignot et al., 2005; Starck and Wimmer, 2005; Helmstetter et al., 2009). It is generally believed that the meal is the primary source of these lipids (Holmberg et al., 2003; Starck and Wimmer, 2005; Secor, 2008; Henriksen et al., 2015), but this phenomenon has not been formally documented using any tracer methods. A previous study showed that when pit-vipers were fed with mice that had been intravenously injected with [^{13}C]glucose and [^{15}N]leucine, the peak isotopic enrichments in the small intestine and the liver occurred between 48 and 96 h (McCue, 2007b). Unfortunately, lipid tracers were not used, nor were any of the tracers biochemically integrated into the mouse tissues as in the present study. Future studies using mice whose lipids are intrinsically labeled could be used to quantify the temporal and spatial patterns of allocation of dietary lipids into the visceral organs as has been done in birds (McCue et al., 2011b).

Reducing the costs of gastric digestion

Pureeing the meals reduced the SDA of ball pythons to a similar extent to that previously reported for Burmese pythons (Secor, 2009). However, the present study revealed that pureeing the meals affected the timing and magnitude of exogenous protein oxidation but had no effect on the oxidation of the lipids in the meal. In particular, pureeing the meal caused the peak rate of exogenous protein oxidation to occur several hours sooner compared with intact mice meals (i.e. 18 h versus 24 h). This outcome was likely permitted by increased gastric passage rates, a phenomenon that could be verified using various imaging techniques, but was beyond the scope of this study. Pureeing the meal also reduced the magnitude of the peak SDA response by 40% and reduced the cumulative exogenous protein oxidation by 43% (Fig. 3C). The reduction in the dietary protein oxidation likely reflects a portion of the energetic costs of gastric digestion including mechanical activity, enzyme production and HCl secretion as described by Secor (2003).

The fact that pureeing did not affect the extent to which the dietary lipids were oxidized (Fig. 3C) was initially surprising given the magnitude of the changes in dietary protein oxidation. It is possible that because the costs of gastric phase digestion are incurred early on in the digestion process (i.e. at the time when exogenous proteins are the primary metabolic fuel), the energetic 'savings' during the later phases of digestion (i.e. when lipids are the predominant fuel) are minimal. Nevertheless, the differential effect that pureeing had on dietary protein and lipid oxidation underscores the idea that pythons use these two types of metabolic substrates differently. The present study used a relative prey mass (RPM) of 13%, a value about half that used in most studies of SDA in pythons (Secor, 2008) that are capable of consuming a RPM greater than 50% (Greene, 1983; Secor and Diamond, 1997, 1998). In the future it could be informative to use this experimental approach to explore the mechanistic basis for the relationships between SDA and body mass and meal size in snakes (e.g. Beaupre, 2005; Crocker-Buta and Secor, 2014).

It is noteworthy that the variation around the means of the $\delta^{13}\text{C}$ values (Fig. 2) was lower when snakes were fed either of the pureed meals. We did not expect this but attribute this outcome to the fact that the mice were raised in small groups rather than individually and some individuals were likely to have consumed a disproportionate amount of drinking water (and thus the [^{13}C]

leucine tracer) or peanut butter (and thus the [^{13}C]palmitic acid tracer). This scenario could explain the high inter-individual variation in the $\delta^{13}\text{C}$ values among the mice (Table 1). We suspect that physically pureeing several mice together before feeding them to the snakes reduced the differences in the $\delta^{13}\text{C}$ meals, thereby resulting in lower variance in the $\delta^{13}\text{C}$ of the breath of the snakes. In future studies where researchers are examining small differences in nutrient oxidation, it may be prudent to homogenize several ^{13}C -enriched prey items to minimize this unwanted variance.

^{13}C -Breath testing versus respiratory exchange ratios

Do respiratory exchange ratios (RERs) provide insight into the extent to which digesting pythons rely on different classes of macronutrients? Benedict (1932) conducted the first measurements of RER in snakes digesting rodent meals, but because of the similarities in the respiratory quotient (RQ) of proteins (~0.8) and lipids (~0.7) he did not report any systematic changes in RER during digestion. More recent studies of RER in postprandial pythons are mixed. Overgaard et al. (1999) reported a general increase in RER of digesting snakes from values of ~0.7 to ~0.8, but most studies including the present study (data not shown) report that RER is highly variable from day to day and showed no patterns that provide useful insight into oxidative fuel use (Secor et al., 2000; Overgaard et al., 2002; Wang et al., 2003; Henriksen et al., 2015).

Digestion-induced changes in acid–base values combined with changes in ventilation (Secor and Diamond, 1995; Overgaard et al., 1999, 2002; Secor et al., 2000; Wang et al., 2001) further preclude the use of RER as an accurate proxy of fuel use. Digesting pythons have been shown to increase nitrogenous waste during digestion (Secor and Diamond, 1997; Overgaard et al., 1999; Wang et al., 2001), but the source of these amino acids (i.e. whether they are endogenous or exogenous) remains unclear. While labeling feeder mice with ^{15}N -amino acids could be useful to quantify the source of that nitrogen, it is now clear that exogenous proteins provide a great deal of this nitrogen – even during the first hours of SDA. Future studies where snakes are raised on different ^{13}C -tracers could be used to quantify digestion-induced changes in endogenous metabolic fuels.

Conclusions

The intrinsic labeling of the proteins and lipids in feeder mice over the course of their lives allowed us to determine that exogenous nutrients in the meal are used to fuel the SDA response even within the first few hours of ingestion – long before the gastrointestinal tract has been fully upregulated for digestion. More importantly, we were able to determine that dietary proteins provided the majority of the fuel for SDA during the first half of the postprandial period. The rates of dietary lipid oxidation did not peak until after the peak SDA response had passed. These lipids remained an important source of energy between meals and even during digestion of the next meal. We also found that pureeing the meal to reduce the cost of gastric digestion did not affect the extent to which exogenous lipids were oxidized but sharply reduced the exogenous protein requirement of digestion. Overall, we have shown that intrinsically labeling the different macronutrient pools in the diet allows us to test new hypotheses about how and when animals use the nutrients they consume.

MATERIALS AND METHODS

This project was conducted under the auspices of St Mary's University IACUC (StMU-IACUC protocol no. 2014-6).

Mice

Juvenile mice (*Mus musculus*, $N=200$, male, age 3 weeks; CD-1, Harlan Laboratories) were raised in the laboratory (28°C, 14 h:10 h light:dark) on one of three isotopically distinct diets with the aim of generating adult mice whose bodies contained normal (i.e. natural abundance) levels of ^{13}C (i.e. control mice) or whose proteins or lipids were selectively enriched in ^{13}C using engineered diets (Table 2). At 8 weeks of age (~31 g), the mice were killed via exposure to 100% CO_2 followed by cervical dislocation (AVMA, 2007) and then stored at -20°C for later use.

Subsets of six mice from each treatment group were skinned and then dried to a constant mass (70°C). Their carcasses were homogenized and separated into lean and lipid fractions and analyzed for $\delta^{13}\text{C}$ as described by McCue and Pollock (2013) at the University of Arkansas Stable Isotope Laboratory. Mice from each of the two ^{13}C -enriched treatment groups ($N=8$ each) were pureed and later used to test the prediction about the costs of gastric digestion. The nitrogen and lipid content of $N=12$ control mice were determined separately for the head region and the remaining carcass as described by McWilliams and Whitman (2013) and values were expressed in terms of percentage dry mass. The nitrogen content was further converted to protein content assuming a conversion factor of 5.4 (Sriperum et al., 2011). For our calculations of the ^{13}C distribution within the bodies of the mice, the leucine and palmitic acid residues were assumed to account for 8% and 25% of the amino acid and fatty acid composition of the protein and lipid pools, respectively (McCue, 2008). We assumed that all of the [^{13}C]leucine and [^{13}C]palmitic acid residues in the bodies of the mice were incorporated into larger macromolecules (i.e. proteins and larger lipids, respectively) and not occurring in free-form in the body. The carbon content of proteins and lipids was assumed to be 46% and 70% by mass, respectively (McCue et al., 2013).

Snakes

Juvenile ball pythons (*P. regius*, $N=14$, ~100 g) were obtained from a commercial supplier (LLL Reptiles, Oceanside, CA, USA), and raised in the laboratory (28°C, 14 h:10 h light:dark) on pre-killed control mice for 6 months. The pythons were then divided into two size-matched treatment groups (protein: 235 ± 42 g and lipid: 259 ± 71 g; t -test, d.f.=12; $P=0.492$). Post-absorptive snakes (i.e. fasted for 3 weeks) were placed into 11 metabolic chambers and rates of CO_2 production (\dot{V}_{CO_2}) and O_2 consumption (\dot{V}_{O_2}) were measured at 28°C for 24 h using an 8-channel multiplexing, flow-through respirometry system that supplied metabolic chambers with dry, CO_2 -free air as previously described (McCue, 2010). Rates of gas exchange were calculated using standard equations (Lighton, 2008). The multiplexer cycled through each chamber every hour with a dwell time of 8 min. One metabolic chamber always remained empty to serve as a baseline for O_2 and CO_2 levels.

Snakes assigned to the protein and lipid treatment groups were offered whole (intact), protein- or lipid-labeled mice, respectively, and returned to their metabolic chambers for the following 7 days during which we measured metabolic rates as described above. Snakes were returned to the chambers to obtain metabolic rates 14 days after ingestion for a final SDA measurement. The sizes of the whole mouse meals did not differ between the treatment groups (protein: 31.2 ± 2.0 g, lipid: 31.9 ± 1.6 g; t -test, d.f.=10, $P=0.841$) and represented a RPM of $13\pm 1\%$. This RPM is smaller than that used for most SDA experiments in pythons (~25%; Secor and Diamond, 2000; Secor, 2008), but because we had a limited number of the isotopically engineered mice, we used the smaller RPM to minimize the chance that a snake would regurgitate the meal. In the Discussion we consider how different RPMs could impact the observed responses.

If a snake did not voluntarily consume the mouse within 1 h, it was force fed – a procedure that does not significantly affect the SDA response (Enok et al., 2013). Every 6 h for the first 72 h, and every 12 h during the remaining 96 h, the air flow to chambers was temporarily stopped for 60 min to allow the CO_2 within the chambers to accumulate to ~1.5–2.5%; this was the minimum concentration of CO_2 required for $\delta^{13}\text{C}$ analysis using the system described below. Subsamples of the chamber air were then collected into a 20 ml gas-tight syringe through resealable injection ports and transferred into evacuated 12 ml Exetainer (Labco Limited, Ceredigion, UK) vials. The contents of the vials were analyzed for $\delta^{13}\text{C}$ within 36 h of collection using a HeliFan Plus (Fischer, ANALYSEN Instrumente GmbH,

Germany) non-dispersive infrared spectrometer interfaced with a FanAS autosampler (McCue et al., 2015). Vials containing CO₂ samples with known δ¹³C were analyzed in parallel with the unknown samples to detect and correct for analytical drift. All ¹³C-values are presented in units of δ¹³C_{VPDB} (Slater et al., 2001). Three weeks after consuming the intact experimental mice, the protein and lipid group snakes were given an oral gavage (31 g) of pureed protein- or lipid-labeled mice, respectively. Again, we measured metabolic rates and the δ¹³C of the breath as described above.

Because CO₂ is a normal by-product of decomposition that may be occurring within the stomachs of postprandial pythons, we also measured the \dot{V}_{CO_2} generated by dead mice for 48 h to determine the potential effect that decomposition could have on the apparent ¹³CO₂ production by the snakes.

Calculations

The energy devoted to SDA was calculated assuming 20.13 kJ l⁻¹ O₂ (Jobling, 1981; Chappell and Ellis, 1987), and the SDA coefficient (i.e. a measure of the relative cost of SDA) was calculated assuming the energy content of 7.02 kJ g⁻¹ wet mass (McCue, 2003). In order to quantify the instantaneous rates of ¹³C-tracer oxidation (e.g. in terms of mol min⁻¹), it is critical to know the \dot{V}_{CO_2} and the tracer-induced δ¹³C enrichment in the exhaled CO₂ (McCue, 2011). The increase in δ¹³C at any given time point was calculated in terms of atom fraction excess (AFE) where δ¹³C_{fast} and δ¹³C_{fed} refer to the ¹³C in the breath during post-absorptive and postprandial states, respectively, according to Eqn 1:

$$AFE = \frac{\left[\frac{VPDB \cdot \left(\frac{\delta^{13}C_{fed}}{1000} + 1 \right)}{1 + \left[VPDB \cdot \left(\frac{\delta^{13}C_{fed}}{1000} + 1 \right) \right]} \right]}{\left[\frac{VPDB \cdot \left(\frac{\delta^{13}C_{fast}}{1000} + 1 \right)}{1 + \left[VPDB \cdot \left(\frac{\delta^{13}C_{fast}}{1000} + 1 \right) \right]} \right]}, \quad (1)$$

where VPDB is a constant (i.e. the absolute mole fraction ratio of the heavy to light isotopes: 0.0112372) (Slater et al., 2001).

The instantaneous rate of tracer oxidation (T) was then calculated in terms of nmol min⁻¹ according to Eqn 2:

$$T = \left(\frac{\dot{V}_{CO_2} \cdot AFE}{m \cdot K} \right), \quad (2)$$

where m is the molar mass of the tracer and K is the volume of CO₂ produced per unit mass of tracer oxidized ($K_{leucine}=0.820$ ml CO₂ mg⁻¹ and $K_{palmitic\ acid}=1.398$ ml CO₂ mg⁻¹) (McCue et al., 2010). Cumulative tracer oxidation was later calculated by integrating T across time.

We also calculated the proportional contributions of the metabolic expenditure that were derived from using the oxidation of the exogenous mouse proteins ($P_{protein}$) and lipids (P_{lipid}), respectively, using linear mixing models of the form:

$$P_{protein} = \frac{B_{protein} - i_{protein}}{M_{protein} - i_{protein}}, \quad (3)$$

$$P_{lipid} = \frac{B_{lipid} - i_{lipid}}{M_{lipid} - i_{lipid}}, \quad (4)$$

where B refers to the δ¹³C in the breath of the protein or lipid group pythons, M refers to the δ¹³C in the protein or lipid pools of the mouse meals and i refers to the naturally occurring δ¹³C in the protein or lipid fractions of the control mice. These mixing models function on the premise that if the δ¹³C in the breath of a snake is identical to that of the control mice on which it was raised, then we conclude that the snake is oxidizing 0% of the protein or lipid from the meal at that time. Similarly, if the δ¹³C in the breath of a snake is identical to the δ¹³C of the lean or lipid fraction of the protein- and lipid-labeled mice, respectively, then we conclude that 100% of the snake's metabolic energy at that time point is derived from the dietary protein or lipid. We did not calculate $P_{protein}$ and P_{lipid} for the pureed meals as each

snake had prior exposure to ¹³C-mice and their breath may therefore not reflect the background δ¹³C values – particularly in the case of the lipid mice (see Discussion). Statistical comparisons were conducted using SigmaPlot 12.2 and significance was determined when $\alpha < 0.05$. Means are reported \pm s.d.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.D.M. conceived of the experiment, conducted the experiment, executed statistical analyses, and prepared the manuscript. R.M.G. conducted the experiment and assisted with manuscript preparation. C.A.P. assisted in experimental design and conducted the experiment.

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