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Effect of feeding on circulating micronutrient concentrations in the Burmese python (*Python molurus*)

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Abstract

Burmese pythons (*Python molurus*) regulate digestive performance and metabolism with the ingestion of each meal. To explore the python's postprandial responses, we monitored the concentrations of blood micronutrients and homocysteine during fasting and for 15 days after feeding. Plasma folate concentrations peaked with a 270% increase over fasting levels 3 days after feeding, whereas plasma B-12 peaked with a 66% increase within 1 day. Erythrocyte folate concentrations were highest 15 days after feeding with a 44% increase. The major plasma folate was 5-methyltetrahydrofolate during fasting and was non-5-methyltetrahydrofolate during digestion, whereas erythrocytes contained polyglutamyl forms of non-5-methyltetrahydrofolate. Plasma homocysteine concentrations peaked with a 56% increase 3 days after feeding, and were markedly greater than those of mammals. Plasma zinc and copper did not change significantly. Plasma zinc concentrations were 20 times greater than plasma copper and approximately 30 times higher than those of mammals. Pythons showed a significant postprandial decline of 25% in hematocrit. Plasma pyridoxal 5′-phosphate (coenzyme form of vitamin B-6) was not detected probably due to its tight protein binding. Most micronutrient concentrations appear to plateau 3 days after feeding, suggesting that pythons have relatively rapid homeostasis of micronutrients despite the ingestion of large meals.

Keywords: Snake; *Python molurus*; Feeding; Folate; Homocysteine; Vitamin B-12; Pyridoxal 5′-phosphate; Zinc; Copper

1. Introduction

The Burmese python (*Python molurus* Linnaeus), native to the Indo-China region, is one of the largest snake species, weighing more than 100 kg and reaching a length in excess of 7.0 m at maturity (Pope, 1961). In the wild, these sit-and-wait foraging snakes feed relatively infrequently on prey as large as pigs, deer and leopards (Wall, 1912; Pope, 1961). As an apparent adaptation to the consumption of large and infrequent meals, Burmese pythons rapidly up-regulate the gastrointestinal system after feeding and down-regulate it upon the completion of digestion (Secor and Diamond, 1995). Their postprandial up-regulation includes a doubling of intestinal mass, a sixfold increase in microvillus length, and as much as a 40-fold increase in intestinal nutrient

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transport and metabolic rate (Secor and Diamond, 1998). As the magnitude of regulatory responses of digestion in the python greatly exceeds those of traditional mammalian laboratory models, such as mice, rats, and rabbits, the Burmese python has been proposed as a new vertebrate model to examine the regulatory mechanisms of digestion and metabolism (Secor and Diamond, 1998).

Various researchers have evaluated postprandial changes in blood chemistry, gases, and pH in pythons. The findings indicated significant postprandial changes in plasma levels of Na, Cl^-, phosphorous, CO_2, glucose, creatinine and HCO_3^- and arterial O_2 and CO_2 (Secor and Diamond, 1995, 1997; Overgaard et al., 1999). However, little information exists for the changes in blood miconutrient levels in snakes with the exception of plasma riboflavin concentrations (Villela and Prado, 1945; Villela and Thein, 1967). In this report, we present the postprandial responses of plasma micronutrients in the Burmese python. We assayed plasma concentrations of folate, vitamin B-12, pyridoxal 5′-phosphate (PLP, coenzyme form of vitamin B-6), zinc, copper and total homocysteine and erythrocyte concentrations of folate and zinc, and hematocrit in pythons during fasting and up to 15 days after feeding.

2. Materials and methods

2.1. Animals, care and blood sampling

The pythons used in this study were originally purchased as 100-g hatchlings from a commercial breeder (Captive Bred Reptiles, Oklahoma City, OK, USA). The pythons were housed individually, provided with water ad libitum and fed every 2 weeks a diet of rodents as previously described (Secor and Diamond, 1995). The seven pythons used in this study ranged in age from 4 to 15 years old [7.1 ± 1.5 (S.E.M.) years] and in body mass from 6.3 to 15.4 kg (10.1 ± 1.3 kg). Before feeding, snakes were fasted for 30 days to ensure that they had completed digestion. To induce a postprandial response, we fed snakes meals (intact pigs, rabbits, or laboratory rats) equivalent to 20.6 ($\pm 0.6\%$) of the snake's body mass. Although the nutrient contents of these meals were not measured, the variations of food intakes among pythons were minimal. The protocol was reviewed and approved by the University of Mississippi Animal Care and Use Committee.

Following the 30-day fast, we collected from each snake approximately 5.0 ml of blood drawn directly from the heart. Aliquots of whole blood (2.5 ml) were immediately transferred into trace-mineral-free evacuated tubes (Beckton Dickinson, Rutherford, NJ, USA) containing heparin and tubes containing EDTA, and placed on ice. Small portions of whole-blood samples were taken for hematocrit determination and frozen for erythrocyte folate and zinc measurements. The remaining whole-blood samples were centrifuged at $900 \times g$ for 10 min at 4°C. Plasma samples were immediately frozen in liquid nitrogen and stored at −80°C. We repeated this same procedure at 1, 3, 6, 10 and 15 days postfeeding, with the exception that one snake was not sampled at 10 and 15 days after feeding. All blood samples were frozen within 1 h of collection and all analyses were performed within 2 months after sampling.

2.2. Laboratory analyses

We measured plasma folate concentrations microbiologically using *Lactobacillus casei* and *Enterococcus hirae* as the assay organisms (Tamura, 1990). *L. casei* is known to respond to a wide spectrum of folates including 5-methyltetrahydrofolate, whereas *E. hirae* does not demonstrate a growth response to 5-methyltetrahydrofolate. Therefore, the combined use of these two microorganisms allowed us to approximate the fraction of 5-methyltetrahydrofolate in total folates. We used both microorganisms to assay erythrocyte folate in the following two preparations. In one, 0.1 ml of whole blood was mixed with 0.9 ml of 0.1 M potassium phosphate buffer containing 10 mg ascorbic acid/ml with a final pH of 4.3, and the mixture was incubated for 30 min at 37°C. For the second, an aliquot of whole blood was mixed with an equal volume of 0.1 M potassium phosphate buffer containing 10 mg ascorbic acid/ml with a final pH of 7.0. The mixture was incubated at 37°C for 60 min with rat plasma as a source of folate conjugase (Tamura, 1998). Folate conjugase hydrolyzes polyglutamyl forms of folates to monoglutamyl folates which can then be utilized by the assay organisms (Tamura, 1990). Plasma vitamin B-12 and PLP concentrations were determined by a radiobinding assay kit (Bayer, Tarrytown, NY, USA) and tyrosine-decarboxylase

assay method with ^{14}C-tyrosine as a substrate, respectively (Tamura et al., 1998; Camp et al., 1983). Plasma total homocysteine concentrations were measured by an HPLC-fluorescent detection method as previously described (Tamura et al., 1996). Plasma and whole-blood zinc and plasma copper concentrations were determined by a flame atomic absorption spectrophotometric method (Tamura et al., 1994). We calculated erythrocyte folate and zinc concentrations based on the relative volume using hematocrit values. The selection of these indices measured in the present study was made because the assay methods were available in our laboratory.

2.3. Statistical analyses

We used a repeated-design analysis of variance (ANOVA; proc mixed procedure of SAS) to test for significant effects of sampling time on micronutrient concentrations and hematocrit values. We subsequently tested, and found no significant covariation of sex, age and body mass on any of the measured variables. When sampling time was found to be significant by ANOVA, we compared mean values at days 1, 3, 6, 10 and 15 postfeeding to fasting values using pair-wise means comparison. We designate the level of statistical significance as $P < 0.05$. All statistical analyses were conducted using the microcomputer version of SAS (ver. 7.0, SAS Institute, Cary, NC, USA).

3. Results and discussion

We found significant variations in the concentrations of plasma folate ($F = 10.7$, $P < 0.0001$), vitamin B-12 ($F = 4.07$, $P = 0.007$) and homocysteine ($F = 8.71$, $P < 0.0001$), and erythrocyte folate ($F = 4.12$, $P = 0.005$), and the values of hematocrit ($F = 6.37$, $P = 0.0005$) among time points of blood sampling (Figs. 1–3). The concentration of plasma zinc and copper (Fig. 2), and erythrocyte zinc concentrations varied not significantly among sampling time points ($P > 0.17$). We were unable to detect PLP in the plasma of the python.

As shown in Fig. 1a,b, plasma folate concentrations increased significantly ($P = 0.0003$) within 1 day after feeding, and peaked at four times the

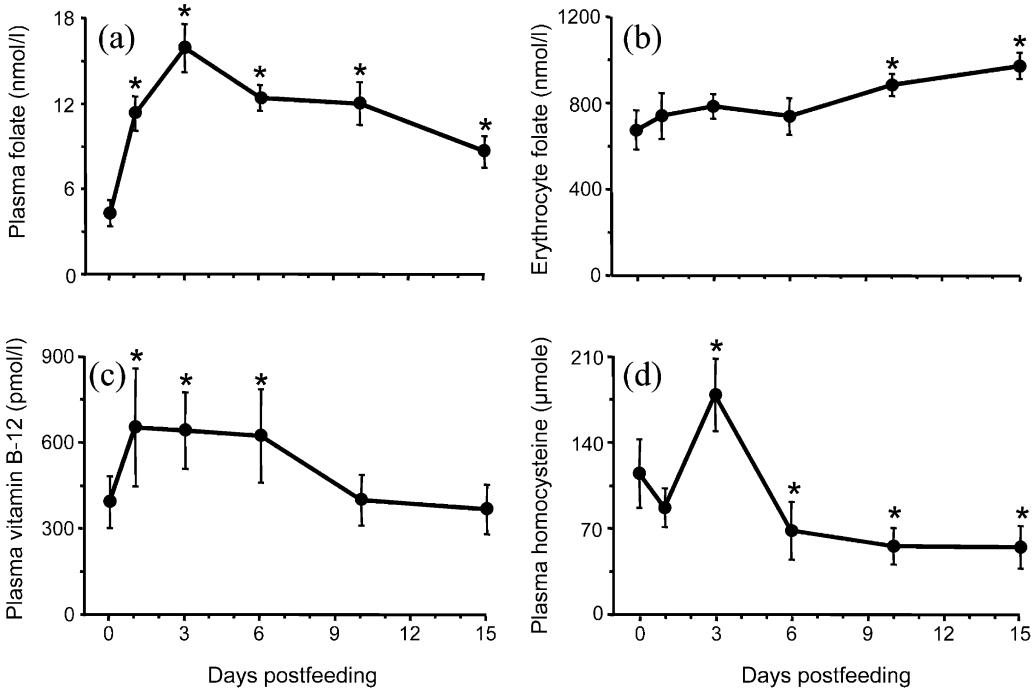

Fig. 1. Changes in plasma (a) and erythrocyte (b) folate, plasma vitamin B-12 (c) and homocysteine (d) concentrations of seven *P. molurus* between fasting (day 0) and day 15 after ingesting meals equivalent to approximately 21% of their body mass. Folate concentrations were assayed microbiologically using *L. casei*. Vertical bars represent S.E.M. An asterisk indicates that the mean value is significantly different from the mean on day 0.

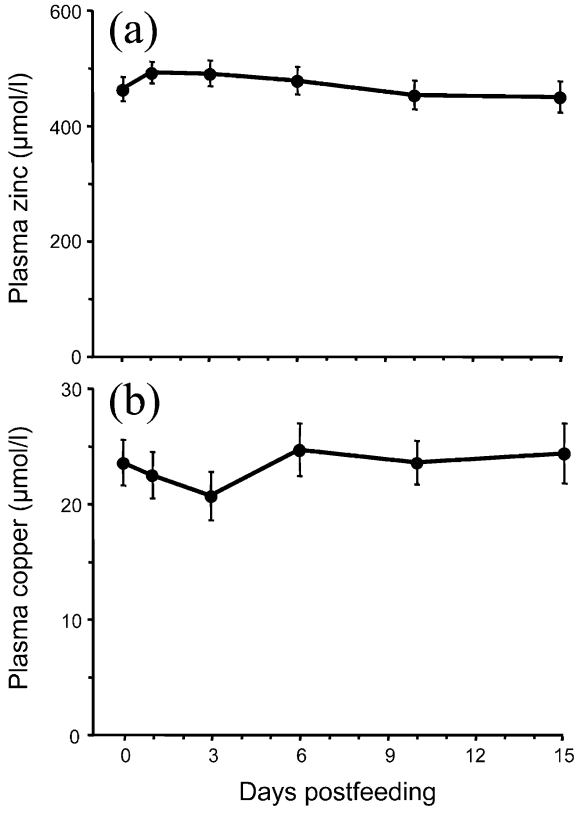

Fig. 2. Plasma concentration of zinc (a) and copper (b) during 15 days postfeeding in *P. molurus*. Vertical bars represent S.E.M.

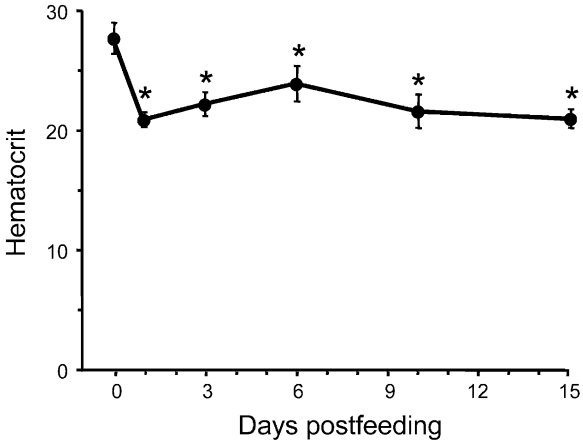

Fig. 3. Changes in hematocrit values as a function of time postfeeding in *P. molurus*. Vertical bars represent S.E.M. An asterisk indicates that the mean value is significantly different from the mean on day 0.

fasting value on day 3. Thereafter, plasma folate gradually declined, but still remained significantly elevated above fasting values by day 15 ($P = 0.02$). Erythrocyte folate concentrations did not significantly increase above fasting levels until day 10 and remained significantly elevated throughout the experimental period. This response suggests that the release of newly matured erythrocytes from the hematopoietic tissues, where the intracellular synthesis of polyglutamyl folates likely takes place, had started approximately 10–15 days after feeding. However, the sites of hematopoiesis and the life span of matured erythrocytes are unknown in the python.

Plasma and erythrocyte folate concentrations and the ratio between the two in pythons were generally comparable to those in humans and laboratory animals (Tamura et al., 1998; Branda, 1981). As shown in Table 1, mean folate concentration at fasting was 4.3 nmol/l as measured by *L. casei*, whereas *E. hirae* did not show a significant response to plasma folate. These findings indicate that 5-methyltetrahydrofolate constitutes the majority of plasma folate during the fasting state. This is similar to the findings of plasma folate in mammals. For example, only 5-methyltetrahydrofolate is generally identified in human plasma at fasting (Baggott and Tamura, 1999). However, between days 1 and 6 after feeding, nearly 100% of plasma folate was found as non-methyltetrahydrofolates (data not shown), sug-

Table 1
Mean (±S.E.M.) plasma and erythrocyte folate concentrations (μmol/l) measured by *L. casei* and *E. hirae* in six *P. molurus*[a]

Assay organism	Plasma	Erthrocytes	
		Without rat plasma folate conjugase	With rat plasma folate conjugase
L. casei	4.3 ± 0.9	98.8 ± 9.3	849 ± 78
E. hirae	0.2 ± 0.2	Not measured	859 ± 75

[a] Plasma values include only those from fasted snakes ($N = 6$). Erythrocyte folate concentrations were measured in the absence (pH 4.3) and in the presence (pH 7.0) of rat plasma folate conjugase. Because we found no difference in the ratios of 5-methyltetrahydrofolate to non-methyltetrahydrofolates in erythrocytes throughout the study, we present the mean values of all samples ($N = 36$).

gesting that the increase in plasma folate was due to the absorption of non-methyltetrahydrofolate from the meal. In contrast to plasma folate, the majority of erythrocyte folates in pythons were polyglutamyl non-methyltetrahydrofolates throughout the study period (Table 1). This finding is uniquely different from that in humans or laboratory mammals, where the majority of erythrocyte folates exist as polyglutamyl 5-methyltetrahydrofolates (Chanarin, 1969).

Table 1 shows that plasma folate conjugase did not sufficiently hydrolyze polyglutamyl forms of erythrocyte folate at pH 4.3. This pH was selected because human plasma folate conjugase is most active at this acidic pH (Tamura, 1990). Therefore, we do not know if python plasma folate conjugase is active enough to hydrolyze erythrocyte polyglutamyl folates, had we used other pH values. The addition of exogenous folate conjugase from rat plasma at pH 7.0 significantly increased the total amount of folate as measured by *L. casei*. These results suggest that the majority of python erythrocyte folates contain more than four- or five-glutamyl residues in its moiety, since *L. casei* was only 12% active in the absence of rat plasma folate conjugase compared to the treatment with this exogenous folate conjugase. Tamura et al. (1972) reported that *L. casei* responds to fully-oxidized folic acid containing four- and five-glutamyl residues at 66 and 20%, respectively compared to monoglutamyl folic acid.

We found that plasma vitamin B-12 concentrations peaked 1 day after feeding with a significant 65% increase over fasting levels ($P = 0.025$). Plasma vitamin B-12 remained significantly elevated above fasting concentrations until day 6. Thereafter, concentrations were not significantly different from fasting values (Fig. 1c). Plasma vitamin B-12 concentrations are similar to those found in human plasma (Tamura et al., 1998).

It was unexpected that PLP was not detected in python plasma. In mammals, PLP is synthesized in the liver and is released into plasma, where it binds to albumin. PLP is a coenzyme for approximately 100 enzymes in mammals (Leklem, 1996). Our inability to detect plasma PLP may be that intracellular PLP is tightly bound to enzymes and is released only in low concentrations from the cells. An alternative explanation is that python PLP is so tightly bound through a Schiff-base linkage to a plasma protein that free PLP is not available to bind to the tyrosine-decarboxylase used in our in vitro assay system. We suspect that the latter explanation is more likely the case. Given that the metabolism and functions of PLP are similar over a wide range of organisms from bacteria to mammals, it would seem unlikely for pythons to have different vitamin B-6 metabolism.

Homocysteine is metabolized by two metabolic routes, including the transmethylation and transsulfuration pathways (Finkelstein, 1990). In the former pathway, homocysteine is remethylated to methionine by methionine synthase which requires 5-methyltetrahydrofolate as a substrate and methylcobalamin as a cofactor. In the latter pathway the two enzymes, which catalyze the reactions from homocysteine to cystathionine, and cystathionine to cysteine, require PLP as a cofactor. Therefore, the nutritional status of these three B vitamins (folate, vitamin B-12 and vitamin B-6) are important regulators of homocysteine metabolism. In humans, mildly elevated plasma homocysteine concentrations (over 15 μmol/l) have been implicated as a risk factor for cardiovascular disease (Refsum et al., 1998). In pythons, plasma homocysteine concentrations were markedly greater than those levels (5–12 μmol/l) found in fasting mammals, including rats, pigs and humans (Miller et al., 1994; Rolland et al., 1995; Tamura et al., 1996, 1998). No information is available whether the incidence of vascular damage is high in pythons due to such high concentrations of plasma homocysteine. Plasma homocysteine significantly increased by day 3 ($P = 0.005$), peaking at 180 μmol/l, which is 56% above fasting values (Fig. 1d). After this time point, plasma homocysteine declined and actually attained levels on days 10 and 15 (55 μmol/l) that were significantly less than fasting concentrations of 115 μmol/l ($P < 0.05$). This increase probably originates from the absorption of dietary methionine, which is considered to be the sole source of homocysteine in mammals. A possible explanation as to why homocysteine concentrations were significantly less on days 10 and 15 compared to fasting values is that there existed a suboptimal folate nutriture during fasting as evidenced by the relatively low plasma folate concentrations. The steady increase in erythrocyte folate concentrations from day 0 to 15 might support this hypothesis. In addition, it is known that folic acid supplementation effectively lowers plasma homocysteine concentrations in humans (Refsum et al., 1998). In pythons following feed-

ing, the rate at which homocysteine is remethylated to methionine might have increased due to the liberation of folate and vitamin B-12 from the meal.

Plasma zinc concentrations did not significantly change after feeding (Fig. 2a). Mean plasma zinc concentration (470 μmol/l) is 30 times greater in pythons than those (10–20 μmol/l) in laboratory and domesticated mammals, such as mice, rats, dogs, cows, and horses and humans (Keen et al., 1981; Masters et al., 1983; Bell et al., 1987; Graham et al., 1994; Tamura et al., 1994). We found erythrocyte zinc concentrations to be similar to plasma values; thus, the ratio of erythrocyte to plasma zinc concentrations was approximately 1.0. This ratio is markedly different from that (15–20) found in humans (Tamura et al., 1994).

As shown in Fig. 2b, plasma copper concentrations did not significantly change after feeding. However, we are not certain whether this was due to the lack of synthesis of ceruloplasmin in response to copper absorption from the diets. In contrast to zinc, mean plasma copper concentration (23.3 μmol/l) in pythons was similar to that reported in mammals, whose values generally range between 14 and 40 μmol/l (Keen et al., 1981; Masters et al., 1983; Bell et al., 1987; Graham et al., 1994; Milne, 1998). Due to high zinc concentrations, the overall mean ratio of plasma zinc/copper concentrations was 20:1 in pythons, which is markedly higher than the ratio of approximately 1:1 found in mammals. In pythons, the physiological significance of possessing such a high plasma zinc concentration with an exceptionally high zinc/copper ratio remains obscure. Furthermore, no information is available in the literature whether such high zinc concentrations exist in other snakes or reptiles and whether zinc- or copper-binding ligands are present in python plasma.

Hematocrit values declined significantly by 25% within 1 day after feeding ($P < 0.0001$). Thereafter, the values did not change and remained significantly lower than fasting values (Fig. 3). Overgaard et al. (1999) also observed a significant postprandial decline in hematocrit values in *P. molurus*. One possible explanation for this phenomenon is that plasma volume increases relative to erythrocyte volume, as snakes absorb water from their meals.

In summary, plasma micronutrients, including folate and vitamin B-12, rapidly increased postprandially in pythons. These circulating micronutrient concentrations appear to reach a plateau at approximately 6 days after feeding, suggesting the relatively rapid homeostasis of micronutrients despite the digestion of a large meal. Distinct features of blood constituents include that the majority of plasma folate during fasting is 5-methyltetrahydrofolate, whereas erythrocyte folate consists of polyglutamyl non-methyltetrahydrofolates and plasma homocysteine and zinc concentrations that are 10 and 30 times greater, respectively, than those found in mammals. Further studies are warranted to investigate the mechanisms of the above findings in this unique python model and compare these in other animals.

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