GUT LOADING AS A METHOD TO EFFECTIVELY SUPPLEMENT CRICKETS WITH CALCIUM AND VITAMIN A.

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Abstract

Limited quantities of vital nutrients such as calcium[2,5,12] and vitamin A[5,9,13] in crickets have led to the occurrence of diseases such as rickets, osteomalacia, metabolic bone disease (calcium deficiency) and more recently, squamous metaplasia or "short tongue syndrome" in amphibians.[1,2,10,20,21] Gut loading has been an effective method to supplement feeder crickets with both calcium and vitamin A.[2,9] A dose of 0.4 ug vitamin A/g bodyweight/week given orally maintained captive Puerto Rican crested toads (*Bufo lemur*) without signs of vitamin A deficiency and squamous metaplasia at the Fort Worth Zoo. To achieve a similar supplementation level and a 1:1 calcium to phosphorus ratio, a gut loading diet 9.07% calcium and 429 ug/g retinol on a dry matter basis was fed for seven days. Crickets were removed at days two, four, and seven for subsequent dry matter, calcium, phosphorus, magnesium, potassium, sodium, iron, zinc, copper, manganese, molybdenum, vitamin A, vitamin E, beta carotene, lutein, canthaxanthin, echinenone, beta-cryptoxanthin, and zeaxanthin analysis. At day zero, neither calcium (0.15%) nor vitamin A (0.20 ug/g cricket) met minimum carnivore requirements. The desired 1:1 calcium:phosphorus ratio, calcium (0.6%) and vitamin A (12 ug/g cricket) levels were achieved by day two (1.60% and 17.0 ug/g cricket, respectively) on the gut-loading diet. This study supports the hypothesis that gut loading is an effective method of vitamin A supplementation.

Introduction

The house cricket, *Acheta domestica*, is commercially available in large quantities and often used as the sole diet of insectivorous reptiles and amphibians held in captivity. Though nutrient requirements of amphibians have not been determined, limited quantities of vital nutrients such as calcium[2,5,12] and vitamin A[5,9,13] have led to the occurrence of diseases such as rickets, osteomalacia, metabolic bone disease (calcium deficiency) and more recently, squamous metaplasia or "short tongue syndrome".[1,2,10,20,21]

Hypovitaminosis A is diagnosed based on the occurrence of squamous metaplasia of mucus secreting glands of the tongue and has been recently identified in species including Wyoming toads (*Bufo baxteri*), Puerto Rican crested toads (*Bufo lemur*), Kihansi spray toads (*Nectophrynoides asperginis)* and Panamanian golden frogs (*Atelopus zeteki*).[20,21] In addition to squamous metaplasia, vitamin A deficiency may also decrease reproductive success and increase susceptibility to infectious disease.[7,23]

The low level of calcium in crickets and possible methods of supplementation has been widely investigated.[1,2,8,9,11,12,13] Methods of calcium supplementation include dusting and gut loading. Though dusting may be a viable option for calcium supplementation, if the insect is not consumed immediately, grooming may decrease the amount of calcium on the insect.[25] In addition, this method does not facilitate the ability to quantify calcium intake.[22,25] Due to these limitations, as well as potential toxicity of vitamin A, [15,16,19] dusting is not an ideal method. Gut loading has been shown to be an effective method to supplement feeder crickets with both calcium and vitamin A.[2,9]

Puerto Rican crested toads (*Bufo lemur*) at the Fort Worth Zoo exhibiting signs of squamous metaplasia including blackening of the tongue and an inability to use the tongue to capture prey were given an initial treatment dose of 0.8 ug/g bodyweight vitamin A palmitate (Aquasol A®, manufactured by AstraZeneca LP for Hospira™, Inc., Lake Forest, IL). This dose was approximately half the recommended dose for severe cases of squamous metaplasia (Fleming and Valdes, 2008, unpublished data). This level resulted in regained use of the tongue and subsequent weight gain. Following the treatment dose, a maintenance dose of 0.4 ug vitamin A/g bodyweight/week was given orally to maintain captive Puerto Rican crested toads (*Bufo lemur*) without signs of vitamin A deficiency. In both cases, providing oral supplementation required holding the toads and putting the supplement in the mouth. Providing the supplement via a food item is a preferable method of distribution.

The objective of this study was to gut load vitamin A in crickets to a level similar to an oral dose which maintained Puerto Rican crested toads without visible signs of vitamin A deficiency in addition to obtaining a 1:1 calcium:phosphorus (Ca:P) ratio. Vitamin E, carotenoid, and mineral levels were also analyzed throughout the experimental period considering they have not been previously quantified for changes over time.

Methods

Half grown crickets, *Acheta domestica*, (0.2 – 0.3 g) were obtained from the Armstrong Cricket farm, West Monroe, LA. Upon arrival, approximately 200 g of crickets were removed and frozen at -80ºC for subsequent dry matter, mineral, vitamin, and carotenoid analysis. Remaining crickets were divided into six, 26.5" x 18.5" x 16.5" plastic terrariums with ventilated tops (Container Store®, Fort Worth, TX), 75 grams per terrarium. Several squares of egg cartons provided shelter and distilled water was offered fresh daily in two plastic containers per terrarium with wicks in order to prevent drowning.

The diet was formulated using the Animal Nutritionist™ (N-squared Inc. Durango Software, Silverton, OR, USA) to contain approximately 9% calcium and 450 ug/g retinol on a dry matter basis. At this concentration, appropriate calcium and Ca:P ratios were achieved in a previous study.[1] The level of vitamin A was a starting point to achieve a cricket concentration of 12 ug/g. An average toad (90 g) consumes approximately twelve crickets per week, 1% bodyweight per feed, thus the desired concentration of vitamin A was 12 ug vitamin A/ g cricket. Zeigler™ Hi-Cal Cricket

Monster® insect diet™ (Gardners, PA) was supplemented with vitamin A palmitate (DSM Nutritional Products Ltd®, Parsippany, NJ) and calcium carbonate (Tri Ag Supply, Inc.®, Harvard, IL) in order to obtain the experimental nutrient concentrations. The diet was pre-made in bulk and a 200 g sample removed immediately and frozen at -80ºC for subsequent baseline dry matter, mineral, vitamin, and carotenoid analysis. Analyzed nutrient concentrations of the diet are provided in table 1. Remaining diet was stored in a freezer at -20ºC for use during the experimental period.

During the seven-day experiment, 25 grams of food was offered each day and the remaining diet was removed. At days two, four and seven, ten grams of crickets were samples and frozen at -80ºC for subsequent analysis. Insects were held on a 12/12 light/dark cycle and temperature was maintained at 24ºC.

Laboratory analysis

Insect and diet samples were pulverized (size = one mm) using a coffee grinder (Mr. Coffee® coffee mill, Model 3164, Cleveland, OH). All samples were split into two portions, one to be dried for dry matter and mineral analysis and the other frozen at -80°C for vitamin and carotenoid analysis.

Mineral analysis

Samples were dried in a Fisher Scientific Isotemp Oven at 60°C to determine dry matter content. Once dry, they were reground using a coffee grinder (Mr. Coffee® coffee mill, Model 3164, Cleveland, OH) into a homogenous mixture (size = one mm). Samples of approximately a half gram were dried at 80°C to a constant weight for determination of a dry matter correction factor. Samples were sent to Dairy One Forage Lab (Dairy One Cooperative, Inc. Ithaca, NY) to determine calcium, phosphorus, magnesium, sodium, potassium, iron, zinc, copper, manganese, and molybdenum concentrations according to AOAC methods.[4]

Vitamin and carotenoid analysis

Approximately two grams of each sample was homogenized with a 2% pyrogallol methanol solution and then alloquoted at one and a half grams each into three test tubes. Samples were saponified in a 60% v/v potassium hydroxide solution and a 70ºC water bath for 60 minutes. Following saponification, samples were extracted according to methods previously described[24] and analyzed for retinol, alpha- and gamma-tocopherol, beta carotene, lutein, canthaxanthin, echinenone, beta-cryptoxanthin, and zeaxanthin using a Waters™ 2695® separations module HPLC, with a Grace/Vydac 201TP54® column and a Waters™ 2487® dual wavelength absorbance detector.

Statistical analysis

Values for nutrient concentrations were compared by ANOVA with repeated measures using day as the repeated variable and including tank as the block variable in a

randomized complete block design. P values were set at 0.05 using Box's conservative epsilon values. Tukey Kramer multiple comparison tests were used to compare across day nutrient concentrations. Statistics were performed using STATA® software (Intercooled Version 9.2, College Station, TX).

Results:

There was a significant increase in the level of calcium from day zero to day two and a 1:1 Ca:P ratio was obtained by day two of the experimental period and maintained until day four. There was a significant decrease in the level of calcium from day two to day four and from day four to day seven. By day seven, the Ca:P ratio dropped below 1:1 (Figure 1). There was a significant increase in the level of vitamin A from day zero to day two, however, there were significant decreases in the level of vitamin A from both day two to four and day four to seven of the experimental period (Table 2).

With the exception of copper, there were significant changes in the levels of all nutrients from day zero to day two. There was a significant increase in the levels of dry matter, vitamin E, lutein, calcium, magnesium, iron, manganese and molybdenum and a significant decrease in the levels of beta carotene, phosphorus, potassium, sodium and zinc from day zero to day two. There were no significant changes in the level of copper throughout the seven day period. There was a significant decrease in the levels of vitamin E, beta carotene, lutein, magnesium, iron and manganese from day two to day seven. There was a significant decrease over time in the levels of beta carotene, lutein, magnesium, iron and manganese. The levels of potassium, sodium and zinc increased significantly over time and phosphorous varied slightly over the seven day period. There were no significant changes in the levels of dry matter, copper and molybdenum from day two to seven. (Table 2). Levels of gamma tocopherol, canthaxanthin, echinenone, beta-cryptoxanthin, and zeaxanthin were not detectable.

Discussion

This study, in agreement with previous work, confirmed that crickets do not meet the estimated amphibian requirements for either calcium or vitamin A unless supplementated.[1,5,9,12,13] These estimated requirements are based primarily on carnivore requirements considering the general lack of published insectivore nutrient requirements and the assumption that amphibians exhibiting signs of vitamin A deficiency consume only animal matter.

Cricket vitamin A concentrations in this study agree with previous work confirming gut loading to be most effective between days two and four after feeding.[1] The desired Ca:P ratio, calcium, and vitamin A levels were achieved by day two on the gut-loading diet. At day zero, neither calcium nor vitamin A met minimum carnivore requirements (Table 2). By day two, the levels of vitamin A and calcium exceeded minimum carnivore requirements. From day two through day seven levels dropped significantly for both, however, still exceeded carnivore requirements.

The diet used in this study contained 429 ug/g vitamin A. At this concentration, the diet was successful in gut-loading half grown crickets to a level that should maintain captive amphibians without signs of vitamin A deficiency and without the need to handle animals for oral supplementation. Finke[9] determined regression equations for dietary vitamin A that may be used to estimate effective dietary gut loading concentrations. Utilizing Finke's regression equation, the crickets in this study should have contained approximately 24 ug/g retinol.[9] However, the highest analyzed concentration was 17.0 ug/g cricket. The levels of vitamin A fed in this study significantly exceed those levels used by Finke[9] to determine his regression. It is possible, at such high levels, a linear relationship no longer exists. A possible difference in palatability and thus consumption of this diet should be considered.

There are no published requirements for carotenoids for carnivores. In addition, it is unknown if most reptiles and amphibians have the enzyme required to convert beta carotene to vitamin A. The presence of carotenoids in the liver of frogs, *Rana pipiens,* warrants investigation,[14] however, until further research is conducted, it is important to supplement vitamin A.

Levels of the carotenoids beta carotene and lutein decreased significantly during the experimental period. This is most likely due to the lack of carotenoids in the experimental diet. It is possible that prior to shipping, crickets were fed a produce diet containing more carotenoids than the experimental diet. Fruit or vegetable based diets lead to increased levels of carotenoids in crickets.[13]

Required levels of vitamin E for carnivores were never reached in this study. Due to the lack of information on vitamin E requirements for reptiles and amphibians, recommendations for levels of dietary vitamin E are difficult to make.

Phosphorus, magnesium, potassium, sodium, iron, zinc, copper and manganese all met or exceeded carnivore requirements through out the experimental period. A few minerals, such as iron and manganese, increased significantly from the initial (day zero) concentration of the cricket. High levels of these minerals in the gut loading diet may explain the subsequent higher levels in the cricket whereas lower levels of minerals in the diet may explain decreases in other mineral concentrations.

In conclusion, a gut loading diet with 9.07% calcium and 429 ug/g retinol on a dry matter basis successfully gut loaded half grown crickets to a 1:1 Ca-to-P ratio until at least day four of feeding. In addition, the experimental diet was successful in gut-loading half grown crickets to a level of vitamin A exceeding the amount proposed to be effect to maintain captive Puerto Rican toads without signs of vitamin A deficiency. Though the level of vitamin A sought in this study was based on the maintenance of toads held at the Fort Worth Zoo, it is important to note this does not imply a true requirement level. No other levels of vitamin A were tested. In addition, long term reassessment is required. This study supports the hypothesis that gut loading is an effective method of vitamin A supplementation.

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Table 1: Dry matter, mineral, and vitamin content of a gut loading diet formulated to contain 9.0 % calcium and 450 ug/g retinol on a dry matter basis ((1190 grams Zeigler™ Hi-Cal Cricket Monster diet™ (Gardners, PA), three grams calcium carbonate (Tri Ag Supply, Inc.®, Harvard, IL), and seven grams vitamin A palmitate (DSM Nutritional Products Ltd ®, Parsippany, NJ)). Mineral and vitamin concentrations are on a dry matter basis.

Dry matter, %	92.10	Zinc, ug/g	199
Calcium, %	9.07	Copper, ug/g	23
Phosphorous, %	0.99	Molybdenum, ug/g	2.9
Potassium, %	1.02	Retinol, ug/g	429
Magnesium, %	0.32	Alpha Tocopherol, ug/g	354
Sodium, %	0.21	Lutein, ug/g	3.97
Iron, ug/g	644	Beta carotene, ug/g	1.15
Manganese, ug/g	293		

Table 2: Recommended minimum nutrient concentrations for diets fed to carnivores compared to mean nutrient concentrations (n=six) of half grown crickets (*Acheta domestica*) fed a gut loading diet containing 9.07% calcium and 429 ug/g retinol on a dry matter basis ((1190 grams Zeigler™ Hi-Cal Cricket Monster diet™ (Gardners, PA), three grams calcium carbonate (Tri Ag Supply, Inc.®, Harvard, IL), and seven grams vitamin A palmitate (DSM Nutritional Products Ltd ®, Parsippany, NJ)) at days zero, two, four, and seven.

Nutrient	Reqt.*	Day 0	Day 2	Day 4	Day 7
Dry matter, %		26.7^{a**}	28.3 ± 0.86^{b}	28.3 ± 0.87^{b}	28.3 ± 0.85^{b}
Vitamin A, ug/g	1.50	0.20^{a}	17.0 ± 4.08^{b}	11.0 ± 1.37^{c}	6.48 ± 1.92^{d}
Vitamin E, ug/g	67	0.67^{a}	11.2 ± 2.94^{bc}	9.10 ± 0.89^{b}	6.89 ± 0.83^{d}
Beta Carotene ug/g		0.57^{a}	0.12 ± 0.06^{b}	0.05 ± 0.01^{c}	0.03 ± 0.00^{c}
Lutein, ug/g		0.01^{a}	0.05 ± 0.01^{b}	0.03 ± 0.00^{c}	0.02 ± 0.00^{c}
Calcium, %	0.6	0.15^{a}	1.60 ± 0.15^{b}	1.20 ± 0.11^{c}	0.87 ± 0.12^{d}
Phosphorus, %	0.5	1.07^{a}	0.95 ± 0.02^{bc}	0.91 ± 0.01^{b}	0.96 ± 0.03^{c}
Magnesium, %	0.04	0.10^{a}	0.12 ± 0.01^{b}	0.11 ± 0.01^{a}	0.10 ± 0.01^{a}
Potassium, %	0.6	1.38^{a}	1.16 ± 0.02^{b}	1.09 ± 0.03^{c}	1.16 ± 0.04^{b}
Sodium, %	0.2	0.54^{a}	0.41 ± 0.01^{b}	0.41 ± 0.01^{b}	0.45 ± 0.02^{c}
Iron, ug/g	80	112^{a}	218 ± 11.9^{b}	178 ± 14.5^{c}	162 ± 14.8^{c}
Zinc, ug/g	75	241^{a}	205 ± 7.45^{b}	204 ± 7.77^{b}	228 ± 10.1^{c}
Copper, ug/g	15	22.0^{a}	23.3 ± 0.82^{a}	22.5 ± 1.38^{a}	22.7 ± 1.51^{a}
Manganese, ug/g	7.5	43.0^{a}	103 ± 8.78^{b}	81.2 ± 4.54^{c}	75.0 ± 5.14^{c}
Molybdenum, ug/g		1.40^{a}	1.98 ± 0.25^{b}	1.88 ± 0.21^{b}	1.62 ± 0.35^{ab}

*National Research Council. Nutrient Requirements of Cats. 2006. Washington, DC: National Academy Press. 3-28.[18]

**Means within rows, with any identical letters are not significantly different using single factor ANOVA with Box's conservative epsilon set at 0.05.

Figure 1: Mean (n=six) calcium (%) and calcium:phosphorous ratio of half grown crickets (*Acheta domestica*) fed a gut loading diet containing 9.07% calcium and 429 ug/g retinol on a dry matter basis ((1190 grams Zeigler™ Hi-Cal Cricket Monster diet™ (Gardners, PA), three grams calcium carbonate (Tri Ag Supply, Inc.®, Harvard, IL), and seven grams vitamin A palmitate (DSM Nutritional Products Ltd ®, Parsippany, NJ)) at days zero, two, four, and seven.

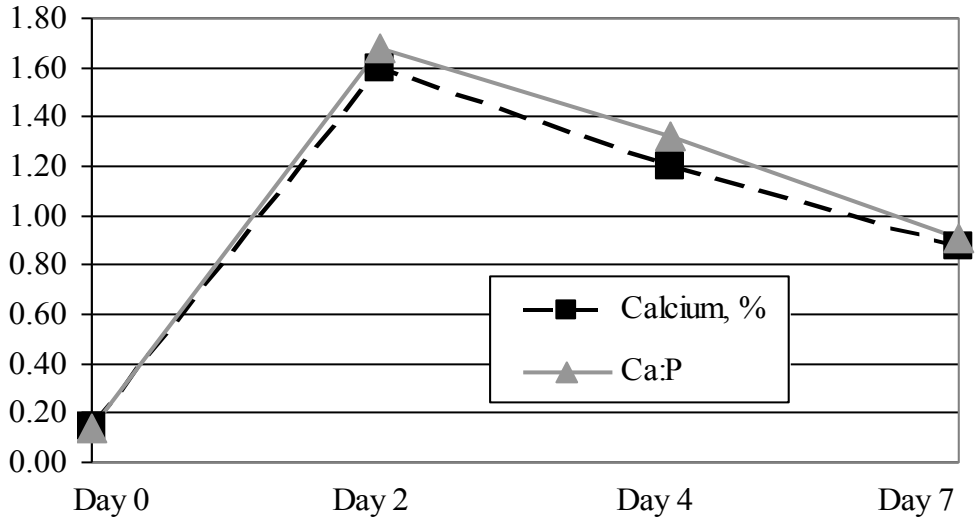

Figure 2: Mean (n=six) retinol (ug/g cricket) of half grown crickets (*Acheta domestica*) fed a gut loading diet containing 9.07% calcium and 429 ug/g retinol on a dry matter basis ((1190 grams Zeigler™ Hi-Cal Cricket Monster diet™ (Gardners, PA), three grams calcium carbonate (Tri Ag Supply, Inc.®, Harvard, IL), and seven grams vitamin A palmitate (DSM Nutritional Products Ltd ®, Parsippany, NJ)) at days zero, two, four, and seven.