ABSTRACT

FREEL, TARRA ANN. Cane Toad (*Rhinella marina*) Vitamin A, Vitamin E, and Carotenoid Kinetics with Potential Stress Measures (Under the direction of Dr. Kimberly Ange-van Heugten).

Approximately 33% of the total free-ranging amphibian species are threatened. Due to this threat of extinction, the nutrient requirements of all amphibians held within human care are of vital importance. Of these, vitamin A, vitamin E, and carotenoids are of particular interest because of their connection to breeding success and because of the deficiency symptoms that have been described when amphibians are kept under human care. To properly manage amphibian species under human care, management professionals need to know how to adequately care for them nutritionally. Therefore, determining normal circulating concentrations of vitamin A, vitamin E, and carotenoids in amphibians is vital so that their health status can be better assessed.

This current research contained two separate studies. The first study was a preliminary study conducted simultaneously with a veterinary amphibian healing trial to allow researchers to determine if cane toads (*Rhinella marina*) could be used as a successful nutrition model at the North Carolina Zoo. Liver samples were taken from free-ranging cane toads (n=5) and from the toads enrolled in the healing study at days 40 (n=7), 45 (n=6), 48 (n=6), 54 (n=7), 60 (n=6), and 69 (n=8) under human care. These liver samples were analyzed for vitamin A, vitamin E, and carotenoids. The preliminary trial allowed researchers to determine how to best assess cane toad nutrient changes over time while altering husbandry techniques for improved toad welfare.

The second study aimed to determine the kinetics of vitamin A, vitamin E, and carotenoid concentration changes in the liver and blood of free-ranging cane toads (*Rhinella marina*) as they adjusted to human care while being fed two different diets. Diet treatment 1 consisted of Mazuri® Hi Calcium Gut Loading Diet fed to feeder brown house crickets (*Acheta domestica*) supplemented

with small quantities of fresh sweet potato and carrots. Diet treatment 2 was the same feeder cricket diet except with vitamin A and vitamin E supplementation removed and with no produce supplementation. Additionally, the potential stress measures, corticosterone and lactate, were analyzed to evaluate how the animal's stress levels may have changed over time in human care. Liver, blood, and urine samples were taken from free-ranging cane toads (n=10) and toads kept under human care at days 22 (n=12), 50 (n=12), 81 (n=12), and 119 (n=14) to measure vitamin A, vitamin E, carotenoids, lactate, and corticosterone concentrations.

Results of the primary study showed that there were significant differences ($P<0.05$) between the free-ranging populations for vitamin A (retinol at 87.7±16.12µg/g), vitamin E (α-tocopherol at 9.2±0.54µg/g), and carotenoids (e.g. β-carotene at 6.1±1.37µg/g) and the toads kept in human care. Concentrations decreased over time in human care for all nutrients, despite dietary supplementation. Additionally, corticosterone values from the free-ranging population were higher (1.7±0.19ng/mL) compared to the groups in human care over time. Lactate also showed significant differences over time in human care but was inconsistent over time and treatment. Decay curves were fit to data to show the exponential nutrient changes over time. No correlations were found between liver and plasma values.

The results from this research imply that additional vitamin A, vitamin E, and carotenoid supplementation may be needed for cane toads in order to maintain the nutrient concentrations from their free-ranging diets. The findings from corticosterone suggest that cane toads may be less stressed under human care and thus is reconfirming that additional stress is not being added by keeping them under human care. Using the cane toad as a model for these nutrient values (vitamins A and E and carotenoids) and potential stress markers (corticosterone and lactate) can help other amphibian species in need by providing comparative data.

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Cane Toad (*Rhinella marina*) Vitamin A, Vitamin E, and Carotenoid Kinetics with Potential Stress Measures

by
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DEDICATION

To Nakita, for teaching me the love that will forever keep me driven with passion along this path I have chosen.

BIOGRAPHY

Tarra Freel was born in Goshen, Indiana and spent the majority of her life living in the small town of Milford, Indiana. Her fondest childhood memories were those spent outdoors in the summers catching tadpoles and turtles from the lake. Tarra grew up having many pets, but it was the special bonds formed with her two childhood dogs – Chance and Nakita – that really inspired her to pursue a career working with animals.

Tarra attended Purdue University in West Lafayette, Indiana for her undergraduate studies and earned her Bachelor of Animal Science with a minor in biology in May of 2015. She began her graduate work in Animal Science and Nutrition at NC State University in August of 2017. Tarra has had a variety of research and teaching experience during her time at NC State including helping to design the ANS590 graduate course, Advanced Canine and Feline Nutrition, and assisting Dr. Ange-van Heugten with the ANS400 Companion Animal Management course.

Tarra most enjoys working with companion animals and exotic species. She has had the opportunity to assist with nutrition consulting for multiple zoos and an aquarium. She wishes to pursue a career in nutrition consulting upon completion of her Masters in Animal Science & Nutrition.

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TABLE OF CONTENTS

LIST OF TABLES viii
LIST OF FIGURES x

Chapter 1: Literature Review 1
Introduction 1
Amphibian Classifications 4
Cane Toad 4
Current Knowledge of Diets of Free-Ranging Anura 5
Free-Ranging Cane Toad Diet 6
Nutritional Challenges in Human Care 6
Amphibian Nutritional Concerns 7
Calcium 8
Fat-soluble Vitamins 8
Vitamin A 9
Vitamin A in Wild Anura 10
Vitamin A Status in Wild Anura Versus Those in Human Care 11
Deficiency Symptoms 12
Vitamin A Toxicity and Symptoms 13
Ability of Amphibian Species to Use Carotenoids 14
Vitamin E 14
Deficiency Symptoms 15
Vitamin E Toxicity 15
Stress Measures 16
Corticosterone 17
Lactate 17
Rationale for Research 18
Chapter 2: Preliminary Study of Free-Ranging Cane Toad (*Rhinella Marina*) Vitamin A, Vitamin E, and Carotenoid Concentrations when Transitioned to the NC Zoo Amphibian Diet 19
Introduction 19
Materials and Methods 20
Results 23
Discussion 23
Conclusion 25
Chapter 3: Cane Toad (*Rhinella marina*) Vitamin A, Vitamin E, and Carotenoid Kinetics with Potential Stress Measures 27
Introduction 27
Materials and Methods 29
Animals 29
Euthanasia 30
Toad Husbandry 30
Toad Diets 31
Cricket Husbandry 32
Urine Collection and Processing 33
Blood Collection and Processing 33

Lactate 34
Liver Collection 34
Cricket Feed Analyses 35
Cricket Analyses 35
Stomach Content Analysis 35
Photographs 36
Experimental Design 36
Statistical Analysis 36
Results 37
Housing Data: Temperature and Humidity 37
Feed Intake of Toads 37
Toad Body Weights and Sex 37
Hepatic Vitamin A, Vitamin E, and Carotenoid Concentrations 38
Circulating Blood Plasma Vitamin A, Vitamin E, and Carotenoid Concentrations 38
Decay Curves for Vitamin A, Vitamin E, and Carotenoids 39
Correlation between Plasma and Hepatic Concentrations of Nutrients 39
Corticosterone 40
Lactate 40
Cricket Feed Analyses 40
Cricket Analyses 41
Stomach Contents 41
Photographs 41
Discussion 41
Hepatic Vitamin A, Vitamin E, and Carotenoid Concentrations 42
Circulating Blood Plasma Vitamin A, Vitamin E, and Carotenoid Concentrations 45
Decay Curves for Vitamin A, Vitamin E, and Carotenoids 47
Correlation between Plasma and Hepatic Concentrations of Nutrients 47
Corticosterone 48
Lactate 49
Cricket Feed Analyses 50
Cricket Analyses 52
Stomach Contents 53
Photographs 53
Conclusion 53
REFERENCES 55
TABLES 66
FIGURES 81
APPENDICES 93
Appendix A 94
Appendix B 98

LIST OF TABLES

Table 1a Cane Toad (*Rhinella marina*) Liver Vitamin A, Vitamin E, and Carotenoid Concentrations (LS Means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 69 in Human Care Consuming the NC Zoo Amphibian Diet 67

Table 1b NC Zoo Cane Toad (*Rhinella marina*) Mean Body Weights at Sample Collection Timepoints 68

Table 1c Cane Toad (*Rhinella marina*) Liver Vitamin A, Vitamin E, and Carotenoid Concentrations (LS Means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 69 in Human Care Consuming the NC Zoo Amphibian Diet, with Timepoints Day 40 and Day 60 Removed 69

Table 2 Sample Collection Description for Cane Toads (*Rhinella marina*) at Each Data Collection Point During the Primary Study 70

Table 3a Initial Housing Setup for Cane Toads (*Rhinella marina*) at NC Zoo from July 12, 2018 to August 31, 2018 71

Table 3b Housing Setup for Cane Toads (*Rhinella marina*) at NC Zoo from September 1, 2018 to October 1, 2018 72

Table 3c Housing Setup for Cane Toads (*Rhinella marina*) at NC Zoo from October 2, 2018 to November 7, 2018 73

Table 4 Cane Toad (*Rhinella marina*) Weight Ranges (Means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 119 in Human Care Consuming Two Different Diets 74

Table 5 Cane Toad (*Rhinella marina*) Liver Vitamin A, Vitamin E, and Carotenoid Concentrations (LS means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 119 in Human Care Consuming Two Different Diets. 75

Table 6 Cane Toad (*Rhinella marina*) Plasma Vitamin A, Vitamin E, and Carotenoid Concentrations (LS means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 119 in Human Care Consuming Two Different Diets. 76

Table 7 Cane Toad (*Rhinella marina*) Urinary Corticosterone (ng/mL) Concentrations (LS means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 119 in Human Care Consuming Two Different Diets 77

Table 8 Cane Toad (*Rhinella marina*) Blood Lactate (mmol/L) Concentrations (LS means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 119 in Human Care Consuming Two Different Diets 78

Table 9a Vitamin A, Vitamin E, and Carotenoid Analyses from Two Labratories for Two Different Diet Treatments Fed to Brown House Crickets (*Acheta domestica*) and Analysis of the Brown House Crickets Fed Those Diet Treatments 79

Table 9b Proximate Nutrient Analyses for Two Different Diet Treatments Fed to Brown House Crickets (*Acheta domestica*) and Analysis of the Brown House Crickets Fed Those Diet Treatments 80

LIST OF FIGURES

Figure 1 Whole Liver Sample Collected from Cane Toad (*Rhinella marina*) 82

Figure 2 Gastrointestinal Tract Collected from Cane Toad (*Rhinella marina*) 83

Figure 3.1 Cane Toad (*Rhinella marina*) 591 upon Arrival at the NC Zoo (July 12, 2018) 84

Figure 3.2 Cane Toad (*Rhinella marina*) 559 upon Arrival at the NC Zoo (July 12, 2018) 85

Figure 3.3 Cane Toad (*Rhinella marina*) 57A upon Arrival at the NC Zoo (July 12, 2018) 86

Figure 3.4 Cane Toad (*Rhinella marina*) 57B at Day 119 in Human Care (November 8, 2018) 87

Figure 3.5 Cane Toad (*Rhinella marina*) 57F at Day 119 in Human Care (November 8, 2018) 88

Figure 4.1 Decay Curves and Equations with Associated P-Values for Line Fit for Liver Retinol Concentrations over Time for Cane Toads (*Rhinella marina*) (CT) Fed Diet Treatment 1 (Supplemented (suppl.) Diet Fed to Brown House Crickets (*Acheta domesticus*)) and Diet Treatment 2 (Diet with No Supplementation (unsuppl.) Fed to Brown House Crickets). 89

Figure 4.2 Decay Curves and Equations with Associated P-Values for Line Fit for Liver Beta-Carotene Concentrations over Time for Cane Toads (*Rhinella marina*) (CT) Fed Diet Treatment 1 (Supplemented (suppl.) Diet Fed to Brown House Crickets (*Acheta domesticus*)) and Diet Treatment 2 (Diet with No Supplementation (unsuppl.) Fed to Brown House Crickets). 90

Figure 4.3 Decay Curves and Equations with Associated P-Values for Line Fit for Liver Beta-Carotene Ester Concentrations over Time for Cane Toads (*Rhinella marina*) (CT) Fed Diet Treatment 1 (Supplemented (suppl.) Diet Fed to Brown House Crickets (*Acheta domesticus*)) and Diet Treatment 2 (Diet with No Supplementation (unsuppl.) Fed to Brown House Crickets). 91

Figure 4.4 Decay Curves and Equations with Associated P-Values for Line Fit for Liver Zeaxanthin Concentrations over Time for Cane Toads (*Rhinella marina*) (CT) Fed Diet Treatment 1 (Supplemented (suppl.) Diet Fed to Brown House Crickets (*Acheta domesticus*)) and Diet Treatment 2 (Diet with No Supplementation (unsuppl.) Fed to Brown House Crickets) 92

CHAPTER 1: Literature Review

Introduction

Concerns about amphibian decline became more recognized worldwide after the 1989 First World Congress of Herpetology where scientists exchanged information about amphibian species dying out even within protected parks and reserves (Stuart et al., 2004). It is believed that significant amphibian declines began in the 1970s and have become more rapid with time (Stuart et al., 2004). These findings prompted the first International Union for the Conservation of Nature (IUCN)'s World Conservation Union Global Amphibian Assessment (GAA) in 2004. This assessment indicated 1856 amphibian species (32.5% of the total species) to be globally threatened, which far surpassed mammals (23%) and birds (12%) (Baillie et al., 2004). Despite some localized conservation successes, the overall global decline has not been stopped (Grant et al., 2016).

Six hypotheses have been proposed in regards to the rapid decline of amphibian species (Collins & Storfer, 2003). They are: competition from invasive species, over-exploitation (for human use such as a food source and pet trade), land changes by humans, global change via temperature warming and ultraviolet B (UV-B) radiation, harmful effects of pesticide usage, and newly introduced infectious diseases (Collins & Storfer, 2003). It is likely that multiple factors work together to cause mortality. For example, a study of amphibian declines in California demonstrated a strong positive association for mortality with elevation (more exposure to UV-B radiation), percentage of upwind agricultural use, and local urbanization (Davidson et al., 2001). These declines are consistent with the UV-B, pesticide use, and land change hypotheses (Davidson et al., 2001). Current studies are focused on attempting to understand and reduce amphibian decline. A recent study showed that tropical clawed frogs (*Xenopus tropicalis*) that were exposed

to endocrine disruptors (benzo(a)pyrene or triclosan) throughout their life cycle, at levels low enough to be considered safe for drinking water, developed a metabolic syndrome consistent with a prediabetes state (Regnault et al., 2018).

Amphibians make one of the best species indicators of overall environmental health (Roy, 2002). They live their young lives in the water and their adult lives partly or fully on land and they contact many parts of their natural environment intimately. As their skin is thin and moist enough to allow for either primary or secondary respiration, it is therefore more vulnerable to their surroundings (Roy, 2002). Many amphibians transition from an herbivorous or detritivorous diet when young to an omnivorous or carnivorous diet as adults (Whiles & Altig, 2010). Thus, they are sampling parts of their environment internally as well. Additionally, they tend to stay within their home ranges so they can be a consistent representation of changes within that particular region (Roy, 2002). However, amphibian species are so diverse in their morphometric, nutritional, behavioral, and physiological characteristics that issues that affect their mortality are not likely to be able to be pinned down to only one aspect (Blaustein & Wake, 1995).

As a result of the GAA in 2004, the IUCN/Species Survival Commission (SSC) Amphibian Conservation Summit was held in 2005 and an Amphibian Conservation Action Plan (ACAP) was created from the proceedings. The plan set forth goals to: 1) set up conservation sites; and focus on: 2) freshwater resources; 3) climate change and biodiversity; 4) infectious diseases; 5) over-harvesting; 6) evaluating the role of environmental contamination; 7) setting up human care programs; 8) reintroduction; and 9) making the GAA a continual process (Gascon, 2007).

In some cases, managed care populations of amphibians may be the only hope for species that face the threat of extinction. Currently, the Amphibian Taxonomic Advisory Group (TAG), as part of the Association of Zoos and Aquariums (AZA) has put forth a Species Survival Plan

(SSP) for six species of the anura order, an order of tailless amphibians that comprises the frogs and toads. There are SSPs for the Puerto Rican crested toad (*Peltophryne lemur*) (PRCT Recovery Program, 2019), Wyoming toad (*Anaxyrus baxteri*) (Amphibian TAG, 2019), Panamanian golden frog (*Atelopus zeteki*) (Project Golden Frog, 2019), harlequin frog (*Atelopus varius*) (Endangered Species International, 2019), dusky gopher frog (*Rana sevosa*) (Amphibian TAG, 2019), and Houston toad (*Anaxyrus houstonensis*) (Amphibian TAG, 2019). These plans include educational outreach, restoration of native habitats, breeding programs under human care, and reintroduction (AZA, 2019). The first SSP established was for the Puerto Rican crested toad, as this species became nearly extinct mostly due to human habitat alteration (Beauclerc et al., 2010; AZA, 2019). In 1984, the amphibian TAG created the SSP for the Puerto Rican crested toad that called for breeding this species under human care, along with education of island citizens, protection and restoration of the native habitat, creating new ponds, and continued research (AZA, 2019). The goal being reintroduction of this species to their native environment to become self-sustaining. There have been six successful reintroductions of this species thus far in which populations have been established in separate locations within Puerto Rico (AZA, 2019).

There exists a variety of other conservation resources and education tools for amphibians. For example, in 2011 the Amphibian Survival Alliance (ASA) was formed to help focus and lead amphibian conservation using the ACAP (Bishop et al., 2012). In addition to the species kept under human care for breeding and reintroduction purposes, there are also many non-endangered amphibians kept under human care in zoological facilities, as companion animals, and for commercial farming purposes (which includes animals for human consumption but also for research and education programs in schools) and it is important to properly care for these species as well (Neveu, 2004; Mansano et al., 2018).

Conservation plans such as those mentioned cannot be successfully carried out without having the information to properly care for amphibians nutritionally. Thus, we need to know their native diets and how to feed them properly under human care. We also need to determine their normal circulating nutrient concentrations so that we can better assess their health status.

Amphibian Classifications

Amphibians include over 8,000 species and are classified into three orders: caudata, gymnophiona, and anura (Amphibian Species of the World, 2019). Caudata are the tailed amphibians that include the salamanders. Gymnophiona are legless and include caecilians. The order anura encompasses frogs and toads in which there are over 7,000 species that make up 88% of all amphibian species (Amphibian Species of the World, 2019). The bufonidae family contains the "true toads" with over 600 species (Amphibian Species of the World, 2019). Bufonidae are native worldwide except in Antarctica, Madagascar and the Australia-New Guinea region and they inhabit very diverse environments from deserts to rainforests (Pramuk et al., 2008). Characteristics shared by bufonidae are: 1) Bidder's organ present (ovary-like structure present in both males and females); 2) no teeth; 3) no constrictor posterior muscle; 4) presence of depressor mandibulae muscle stemming only from the squamosal; 5) presence of inguinal fat bodies; and 6) highly ossified skull (Piprek et al., 2015).

Cane Toad

The cane toad (*Rhinella marina*), formerly known as *Bufo marinus*, naturally inhabits southern Texas, Mexico, Central and South America, and Trinidad (Eaststeal, 1981). The species was intentionally introduced to Hawaii in 1932 and Australia in 1935, in an attempt to control sugarcane insect pests (Phillips et al., 2007; Gruber et al., 2017). Attempts have been made elsewhere in approximately 40 countries, but the other introductions have been less documented

(Eaststeal, 1981; Hagman & Shine, 2008). In the United States, several attempts at introductions were made around the 1930s in Florida; however, the current invasive populations there are believed to have stemmed from an accidental pet trade release in 1955 at the Miami International Airport (Eaststeal, 1981).

Cane toads make for an impactful and dangerous invasive species since they can travel up to 1.8 kilometers per night and females can lay up to 35,000 eggs per clutch year-round (Phillips et al., 2006; Yeager et al., 2014). They wreak havoc to native species by either consuming them as prey or outcompeting them for resources (Shine, 2010). Finally, they are highly toxic, with a potent toxin which is secreted from their parotoid glands when localized pressure is applied or the toad is provoked (Reeves, 2004; GISD, 2005). This toxin poses a deadly threat to their predators as well as causing sickness to humans and their companion animals (Lever, 2001). Because of all of these qualities, cane toads have been classified as one of the top 100 most destructive invasive species in the world (GISD, 2005).

Due to their highly invasive species status, several methods of population control have been attempted. However, there is currently no biological control method that has been approved for environmental application (Shanmuganathan et al., 2010; The State of Queensland DAFF, 2013). Recommended control methods include exclusion fencing, trapping and physical removal, with cage-trapping being viewed as the most effective (Schwarzkoft & Alford, 2007).

Current Knowledge of Diets of Free-Ranging Anura

There have been a wide variety of gut content analysis studies in frogs and toads that have attempted to describe their free-ranging diets. In New Zealand, Archey's frogs (*Leiopelma archeyi*) and Hochstetter's frogs (*Leiopelma hochstetteri*) diet items mostly consisted of springtails, mites, ants, parasitic wasps, amphipods and isopods with the Archey's frogs also

consuming snails (Shaw et al., 2012). In China, introduced bullfrogs (*Rana catesbeiana*) had a larger number of aquatic species in their diets due to more of their time being spent in the water, with their main prey items from the classes chordates, arthropods, mollusks, and annelids (Wu et al., 2005). Juvenile frogs are also a common prey item for adult bullfrogs (Werner et. al, 1995). Strawberry poison frogs (*Dendrobates pumelo*) consume ants, mites, and other invertebrates during all months of the year (Donnelly, 1991). The common spadefoot toad (*Pelobates fuscus*) diet items are mostly snails, ants, and beetles (Cogălniceanu et al., 1998). In a larger study with six different species of anura in Brazil; Insecta, Arachnida, and plants were consumed by all with Acari, Hymenoptera, Odonata, and Coleoptera being the most popular order of prey items (Santos et al., 2004). Thus, although it depends on their home ranges, anura consume mostly invertebrates.

Free-ranging Cane Toad Diet

Cane toads are indiscriminate feeders, as concluded by the diversity of the diet items found in a study across all seasons from New Guinea (Bailey, 1976). Stomach prey items consisted of mainly ants and snails, and also included weevils, dung beetles, small toads, and skinks (Bailey, 1976). Specific to Florida, beetles were the primary prey item consumed by cane toads for both the wet and dry seasons (Meshaka & Powell, 2010). Ants also made up a large portion of the diet, with termites and flies being important items for both seasons (Meshaka & Powell, 2010).

Nutritional Challenges in Human Care

Early feeding management of animals under human care primarily focused on attempting to replicate the animal's free-ranging diet (Dierenfeld, 1997). Nutritional disorders have been the primary reason for diet modifications for animals within human care due to lack of knowledge of

nutritional requirements. The first published case of metabolic bone disease was in captive primates in 1888 with a description of reducing the disease by supplementation with bone meal and cod liver oil (Bland, 1888). Nutrition began getting more interest after this case but it was not until 1994 that the American Association of Zoos and Aquariums began classifying nutrition as one of the scientific advisory specialties (Dierenfeld, 1997). Originally, nutritional concerns were more focused on mammals; however, more attention has been placed on amphibian nutrition as a result of their significantly greater population declines and increased focus on their management within human care (Pessier, 2008).

Amphibian Nutritional Concerns

Keeping amphibians in human care has proved to be difficult, in part, due to their changing dietary needs and nutritional requirements throughout their different life stages (Whiles & Altig, 2010). Additionally, amphibians consume a diverse variety of prey in the wild; whereas, under human care they are typically only offered a few different types of human cultivated prey items (Livingston et al., 2014). There have been a variety of concerning husbandry conditions potentially due to nutritional deficiencies and toxicities reported in amphibian breeding and care programs. These include anorexia, metabolic bone disease, “short tongue syndrome” (reduced ability to capture prey using tongue), neurological and musculoskeletal abnormalities, obesity, gastric overload, renal calculi, and corneal lipidosis. Some of these issues stem from hypervitaminosis D_3, improper amounts of calcium and phosphorus, hyper and hypovitaminosis A, various B vitamin deficiencies, overfeeding, high levels of dietary cholesterol and more (Wright & Whitaker, 2001; Crawshaw, 2003; Densmore & Green, 2007; Pessier, 2013; Ferrie et al., 2014).

Calcium

One major dietary struggle for amphibians within human care facilities that primarily consume insects, is that these captive bred insects being fed often have an inverse dietary calcium to phosphorus (Ca:P) ratio (Wright, 2006). The ideal dietary insect Ca:P ratio is 1:1-2:1 based on mammalian species with Finke (2002) reporting all samples of commonly used insect prey items to be deficient in calcium when comparing to the National Research Council (NRC) nutritional requirements for rats (NRC, 1995; Livingston et al., 2014). Low calcium and high phosphorus in the diet along with improper vitamin D supplementation can lead to nutritional metabolic bone disease (Michaels et al., 2015). For example, crickets are one of the most common prey items offered to amphibians and they have been shown to be a poor source of calcium with levels ranging from 275mg/kg (nymphs) to 407mg/kg (adults) which makes up less than 30% of the requirements for rats (Allen & Oftedal, 1989; NRC, 1995; Finke, 2002). Supplementation of the insect being fed is considered necessary and is commonly conducted via powder dusting or gut-loading. Powder dusting is performed by coating the insects with a fine powder containing calcium, and gut-loading is feeding the insects a high calcium diet shortly before being fed out to the target animal (Michaels et al., 2014). However, these techniques often still do not allow the insects to reach the desired calcium concentrations for amphibian needs (Livingston et al., 2014).

Fat-soluble Vitamins

Proper concentrations of vitamin D_3 in the diet or adequate exposure to ultraviolet-B (UV-B light) radiation are necessary for proper bone development and maintenance in amphibians (Antwis & Browne, 2009). However, hypervitaminosis D_3 has been described in the ornate horned frog (*Ceratophrys ornate*) fed prey items high in vitamin D_3 (Frye, 1992).

Elevated concentrations of vitamin A can potentially disrupt the normal metabolism of vitamin D_3 (Densmore & Green, 2007). Vitamin A is a nutrient commonly supplemented through powder dusting or gut-loading insects. Similar to calcium, this supplementation is still considered to be poorly implemented (Livingston et al., 2014). In some cases, it is either too much or too little depending on the form of supplementation and variety of insect consumption (Livingston et al., 2014). Interest in the concentrations of the fat-soluble vitamins A and E in amphibian diets became of more interest once breeding programs were started and disease issues were described (Pessier et al., 2005; Pessier, 2013; Ferrie et al., 2014).

Vitamin A

Vitamin A is a fat-soluble, long-chain, unsaturated alcohol with five double bonds, existing in several isomeric forms (McDowell, 2012). It is essential to the animal body for vision, reproduction, metabolism, blood cell formation, bone development, the formation and maintenance of epithelia, and for the immune system (Ball, 2008). The all-trans form of vitamin A is most commonly found in mammalian tissues and is the most active form of vitamin A (McDowell, 2012). Plants do not contain vitamin A in this form, but rather in the form of carotenoids, in which there are several that work as precursors to vitamin A and are collectively called provitamin A (McDowell, 2012). Among these are α-, β-, and γ-carotene and cryptoxanthine which are of particular importance because in some species these carotenoids can be converted into retinal, the alcohol form of active vitamin A, by enzymes in the intestine (McDowell, 2012). β-carotene has the highest biological activity of all of these in mammals and theoretically can yield two molecules of retinal in species that are able to convert it. Conversion of carotenes into vitamin A is most efficient in mammalian herbivorous species; omnivores have

a lowered ability to covert and carnivores may not be able to do so at all (Green & Fascetti, 2016).

It is thought that many aspects of vitamin A metabolism are similar in amphibians to the mechanisms described in mammals, but more research is needed to determine the differences, if any (Clugston & Blaner, 2014). It is unknown in many amphibian species whether they have the ability to convert carotenoids into retinal (Densmore & Green, 2007). It is also unknown if they require carotenoid supplementation even when vitamin A is provided in the diet (Densmore & Green, 2007). Amphibians have been used as models in several studies regarding the importance of vitamin A signaling. These have included vision, limb regeneration and embryogenesis studies (Wald, 1935; Butler & O'Brien, 1942; Kraft et al., 1995). Despite this research, there is still a lack of knowledge of vitamin A homeostasis in amphibians and how it differs by order and/or specific species (Clugston & Blaner, 2014).

Vitamin A in Wild Anura

The dietary needs of frogs and toads change dramatically as they progress through metamorphosis. There is great variation documented on the diet of tadpoles but it is thought that most anurans transition from an herbivorous or detritivorous (more likely) to an omnivorous and then to an almost completely carnivorous diet (Altig et al., 2007). With plant materials being high in carotenoids it is possible that tadpole's vitamin A needs are being met by the conversion of a carotenoid or carotenoids. Adult anurans mostly feed on invertebrates such as arthropods. which are not likely to contain any significant amount of vitamin A (Finke, 2015). In fact, vitamin A has only been found in the heads of insects, mostly coming from the eyes (Goldsmith & Warner, 1964). Some larger species consume smaller amphibians, small birds and mammals

(Solé & Rödder, 2010), which may imply that their vitamin A needs are being met in the pre-formed state of retinol or retinyl esters.

Vitamin A Status in Wild Anura Versus Those in Human Care

The dietary vitamin A requirements of all amphibian species are currently unknown. The liver typically stores around 90% of the total vitamin A contained in the mammalian body (McDowell, 2012). Similarly, it has been demonstrated in the leopard frog (*Rana spp.*) that vitamin A is mainly stored in the liver in the form of retinyl ester (Futterman & Andrew, 1964). There is limited data on the ideal liver vitamin A concentrations of frogs and toads due to the fact that studies must be terminal in order to collect samples. In addition, literature on circulating values is very limited among the few species that have been tested (Ferrie et al., 2014). Retinol in the blood has been demonstrated in amphibians to be transported bound to retinol-binding protein similar to mammals (Shidoji & Muto, 1977). This may make values difficult to interpret, especially if the animal was under acute stress when the blood was sampled because circulating vitamin A and retinol-binding protein concentrations decrease with stress (Louw et al., 1992).

Circulating retinol levels in the African clawed frogs (*Xenopus laevis*) were reported at 0.1 nmol/ml; whereas, in Puerto Rican crested toads, values ranged from 0.91-0.98 nmol/ml (Azuma et al., 1993; McComb, 2010). The study from McComb used a RP-HPLC method as outlined by Arnaud et al. (1991) with samples run at NC State University (Raleigh, NC). Azuma et al., 1993 used the oxime method of extraction and HPLC previously described in Azuma et al. (1988 & 1990) and Irie et al. (1991) to analyze retinoids at a lab in Japan. When attempting to compare these values, caution should be used as these samples are from frog versus toad, and additionally these lab differences may further complicate the interpretation of these values.

In a study by Sullivan et al., 2014 wild caught Cuban tree frogs (*Osteopilus septentrionalis*) and cane toads were collected and their serum, liver and whole-body samples were analyzed. Cuban tree frogs had higher vitamin A status overall with 82.8 ppb in serum, 248.3 IU/g in liver, and 5474.7 IU/kg in the whole body (Sullivan et al., 2014). Cane toads, in comparison, had 60.1 ppb in serum, 105.3 IU/g in liver, and 940.7 IU/kg in whole body samples; however, differences between the species were not shown to be statistically significant (Sullivan et al., 2014). Sample size could have been a factor in these results as there was a sample size of 59 Cuban tree frogs and 20 cane toads in this study. These samples were run using HPLC methods similar to those above, but were analyzed at Michigan State Diagnostic Center for Population and Animal Health (Lansing, MI).

In a study comparing the serum and hepatic vitamin A levels between wild cane toads and those raised in human care, dramatic differences were reported in liver concentrations. Wild caught cane toad livers had an average vitamin A concentration of 61.89 µg/g; whereas, those raised under human care had an average of 0.58 µg/g (Berkvens et al., 2014). Prior to euthanasia the wild caught toads were housed and fed for 10-21 days before shipment to another zoo, with diet items including crickets, mealworms, wax-worms, and dry dog food. Thus, these diet items may have had an effect on liver concentrations of retinol. The vitamin A serum concentrations in the wild caught cane toads were significantly lower than those raised under human care (Berkvens et al., 2014). This was after being controlled for liver concentrations.

Deficiency Symptoms

Hypovitaminosis A is defined as vitamin A deficiency and is one of the most common nutritional deficiencies in all animals (Donoghue & Langenberg, 1994). Symptoms of vitamin A deficiency across species include: visual impairment, epithelial tissue defects, reproductive

issues, stunted growth, and lowered immune system function (McDowell, 2012). The first vitamin A deficiency symptom described in amphibians was “short tongue syndrome” in which the mucus-producing epithelium of the tongue was replaced by stratified squamous epithelium (Pessier et al., 2005; Pessier, 2013). Although not specifically reported in the available literature, it is likely that hypovitaminosis A is also an underlying cause to amphibian poor reproductive success while in human care, increased mortality in tadpoles and poor immune system function (Ferrie et al., 2014). Therefore, vitamin A deficiency is a major concern for amphibian breeding and reintroduction programs as well as a general animal welfare concern.

Vitamin A Toxicity and Symptoms

Because many amphibian species have evolved in neotropical regions with mineral deficient soils, they are often very efficient at digesting, absorbing and storing micronutrients. Thus, caution must be taken when supplementing their diets with vitamins and minerals. Vitamin A toxicity has been described in African clawed frogs from the feeding of rodents and the liver of mammals (Densmore & Green, 2007). Some whole prey items can be quite high in vitamin A with crawler mice having approximately 16,700 IU/kg to adult mice concentrations being up to 300,000 IU/kg (Douglas et al., 1994). Toxicity can cause multiple health concerns in anura including the development of anemia, liver damage and chronic weight loss (Crawshaw, 2003). Species differences also exist, for example, tadpoles of the Asian common toad (*Duttaphrynus melanostictus*) that were exposed to excess concentrations of vitamin A developed abnormal skin around the jaws; whereas, common skittering frog (*Euphlyctis cyanophlyctis*) tadpoles were not affected when given the same concentrations (Jangir et al., 1994).

Ability of Amphibian Species to Use Carotenoids

Presently, some amphibian diet and diet supplement manufacturers are using vitamin A precursors such as beta-carotene to supplement the diet of feeder insects which are then fed to the amphibians as a safer alternative, to avoid toxicity (van Vliet et al., 1996; Li et al., 2009). However, it is not well understood whether all amphibians have the ability to convert carotenoids to vitamin A, and it is likely that major species differences exist (Schairer et al., 1998). Several different carotenoids, including beta-carotene, lutein, xanthophyll and zeaxanthin have been identified in the liver, skin, muscle, retina and other tissues of some amphibians meaning that it may be possible for some species to absorb them from the diet (Morton & Rosen, 1949; Khachik et al., 2002). However, findings in cane toads and Cuban tree frogs support the hypothesis that some amphibians may not be able to synthesis provitamin A from beta-carotene at adequate concentrations (McComb, 2010). It has been demonstrated that false tomato frogs (*Dyscophus guineti*) have the ability to use beta-carotene and the xanthophylls lutein and zeaxanthin for vitamin A activity (Brenes-Soto & Dierenfeld, 2014). Regardless of whether they can use them for vitamin A activity, carotenoids are still thought to be an important diet component for amphibians. As demonstrated in the red-eyed tree frog (*Agalychnis callidryas*), carotenoids in the diet were shown to impact growth rate, skin coloration during a critical period, and reproductive success (Ogilvy et al., 2012).

Vitamin E

The term vitamin E encompasses a group of lipid-soluble tocopherols and tocotrienols, with α-tocopherol having the highest biological activity. Vitamin E works as an antioxidant, increases immunocompetence and thus reduces susceptibility to diseases in mammals (Brigelius-Flohe & Traber, 1999). It functions in reproduction, cellular signaling, and metabolism

(Brigelius-Flohe & Traber, 1999). Vitamin E status is measured by circulating levels of α-tocopherol as this has been demonstrated to be well-correlated with the available dietary levels in mammals (Machlin, 1980). But, in some species, such as Asian elephants (*Elephas maximus*) it has taken up to a year to see a significant increase in the circulating α-tocopherol values when diet is increased to a new level (Dierenfeld, 1989). This varies between species and it is thought because the primary storage sites for vitamin E are muscle and adipose tissue that these sites may need to be saturated before changes are seen (Dierenfeld, 1989). Little is known about the response to dietary antioxidants in amphibians, but it is thought to be a similar mechanism (Szuroczki et al., 2016). In one study using Northern leopard frogs (*Lithobates pipiens*), supplementation with antioxidants for a five-week time period resulted in tadpoles with an increased immune response capability and growth (Szuroczki et al., 2016).

Deficiency Symptoms

Vitamin E deficiency may present itself differently dependent upon species. These deficiency symptoms can include anemia, cardiomyopathy, encephalomalacia, and nerve degeneration in some animals. In reptiles, deficiency symptoms most commonly reported are steatitis, fat necrosis, and muscular degeneration (Dierenfeld, 1989). Specifically, in turtles, vitamin E deficiency may cause anorexia and subcutaneous nodules (Donoghue, 2006). There is little known about vitamin E deficiency in amphibians, specifically for toads.

Vitamin E Toxicity

While there have not been vitamin E toxicity symptoms reported in amphibians, there is still a concern based on other species. One study in chicks demonstrated that feeding excess vitamin E lowered growth rate and increased the requirement for vitamins D and K (March et al., 1973). Overall, vitamin E toxicity is considered to be much less of a concern than that of

deficiency, although understanding the kinetics of vitamin E in amphibians may prove useful for future populations housed within human care facilities.

Stress Measures

Stress is an organism's physiological response to a disruption in homeostasis such as an environmental condition, and can be activated both short-term and long-term (Mason, 1975; Selye, 1976; Sapolsky, 2002). The stress system is composed of the hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous system (ANS) which work together with the central nervous system (CNS) and organs and tissues to respond to an intrinsic or extrinsic stressor (Tsigos et al., 2016). The stress response is thought to be similar across all vertebrates; however, the way in which it is activated and responses may vary some across species (Moore & Jessop, 2003). In amphibians, slightly elevated levels of stress hormones may actually increase reproduction because energy stores become mobilized; however, marked activation of the HPA axis and high levels of stress hormones, inhibit reproduction (Moore & Jessop, 2003). Historically, stress in amphibians under human care was studied via monitoring successful reproduction and noting the growth and development of offspring while measuring hormone levels and hematology changes (Ferrie et al., 2014). These studies involved collecting blood samples via cardiac puncture, femoral, ventral abdominal or lingual vein (Wright & Whitaker, 2001). However, these potentially invasive collection methods along with capture and restraint can create an immediate stress response that confuse results (Sapolsky et al., 2000). Recently, less invasive methods such as analyzing urine metabolites have been established to measure some stress responses in amphibians (Narayan et al, 2010).

Corticosterone

Corticosterone is a glucocorticoid which is a hormone secreted by the adrenal cortex as part of the stress response (Katsu & Iguchi, 2016). In amphibians, cortisol and corticosterone metabolites can be measured using urine (Narayan et al., 2010). This method is considered non-invasive and the urinary corticosterone concentrations have been demonstrated not to increase in urine until after two hours of a stressful event, such as capture, in cane toads (Narayan et al., 2011). Urinary corticosterone in cane toads caught in the wild ranged from 13.3 to 81.6 pg/µg (Narayan et al., 2012). Urinary corticosterone concentrations can potentially be useful as a way of monitoring stress in animals under human care. This method is less invasive; however, it likely should only be used for long-term stress measurements and is not a good measure of an immediate stress response.

Lactate

Lactate is a measure of the amount of lactic acid in the blood. Lactic acid is present in the blood during anaerobic metabolism. It has been demonstrated that exercise, immobilization and emotional stress all increased the amount of lactic acid in the blood (De Bruin et al., 1990). In amphibians, the slower moving species such as the Western toad (*Bufo boreas*) produce lower amounts of lactate and have a greater stamina; additionally, the toxic or combative species such as the arboreal salamander (*Aneides lugubris*) also have smaller amounts of lactate (Bennet & Licht, 1974). Lactate production rate is directly correlated with predator avoidance, as other species rely on the use of anaerobiosis to escape quickly such as the Northern leopard frog and there seems to be a tradeoff between rapid activity or extensive anaerobiosis (Bennet & Licht, 1974). In the American bullfrog (*Rana catesbeiana*), lactate was shown to increase during a two-minute handling period producing similar effects to that of treatment with cateholamines

(MbangKollo & deRoos, 1983). In the common African toad (*Bufo regularis*), administration of cortisol demonstrated that lactate is produced and is used as a substrate in gluconeogenesis to activate cortisol hyperglycemia (Isehunwa et al., 2017). Lactate values have been reported to range from 8.3 to 28.9 mg/g of body weight in anura at rest, and from 57.8 to 165.5 mg/g of body weight in these same amphibians after ten minutes of activity (Bennet & Licht, 1974).

Rationale for Research

Research to ascertain the true metabolic vitamin A, vitamin E, and carotenoid needs for amphibian species is vital for proper care of those within human care facilities to avoid deficiencies and toxicities and to best mimic their required diet. With the decline of free-ranging amphibian species, the importance of proper human care management becomes greater. If we plan to properly house and breed them, we need to determine true nutrient needs (Kiesecker, 2011). Vitamin A, E and carotenoid kinetics over time from a free-ranging population brought into human care can provide vital data for future amphibians. Specifically, the population chosen for this study was the cane toad because these animals would have been euthanized as part of a routine population control program for invasive species at Zoo Miami (Miami, FL) regardless of our study. Additionally, determining stress measures gives indications about how these animals handle being brought into human care and potentially adapt over time after they are removed from the wild. Therefore, the objectives of this research were:

1. To determine the kinetics of vitamin A (retinol), vitamin E (alpha-tocopherol) and carotenoids (apocarotenoid, beta-carotene, beta-cryptoxanthin, lutein, and zeaxanthin) in cane toads.
2. Evaluate stress measures in cane toads when captured from the wild and brought into human care.

CHAPTER 2: Preliminary Study of Free-ranging Cane Toad (*Rhinella Marina*) Vitamin A, Vitamin E, and Carotenoid Concentrations when Transitioned to the NC Zoo Amphibian Diet

Introduction

There have been several reports of fat-soluble vitamin deficiencies and toxicities in managed care amphibians, in particular vitamin A (Pessier et al., 2002; Pessier et al., 2005; Wright, 2005; Murray, 2006). The need for dietary supplementation for these managed care animals is suspected (Li et al., 2009). Several commercial manufacturers of amphibian food supplements are presently using vitamin A precursors to address this matter. This is based upon the understanding that carotenoid precursors to vitamin A are only converted to vitamin A compounds when needed and thus provide a safer dietary source than pre-formed vitamin A (van Vliet et al., 1996). It has recently been shown in a reptile species that leopard geckos (*Eublepharis macularius*) have the ability to convert beta-carotene to vitamin A (Cojean et al., 2018). False tomato frogs (*Dyscophus guineti*) can use beta-carotene and xanthophylls like lutein and zeaxanthin for vitamin A activity (Brenes-Soto and Dierenfeld, 2014). However, findings in other amphibian species support the hypothesis that beta-carotene may not be a viable vitamin A source for some wild (and in human care) adult cane toads (*Rhinella marina*) and Cuban tree frogs (*Osteopilus septentrionalis*) (McComb et al., 2011). Despite the work published in this field, additional studies are needed to determine the nutrient requirements of vitamins A, vitamin E, and carotenoids of amphibians as well as the differences that likely exist among amphibian species (Schairer et al., 1998).

Our goal for the work presented here was to evaluate how the vitamin A, vitamin E, and carotenoid concentrations changed over time when cane toads (*Rhinella marina*) were transitioned from a free-ranging diet to a managed care amphibian diet. This was a preliminary

examination of the kinetics of vitamin A degradation in the body tissues of amphibians, with significant implications for wild-caught and rehabilitated animals. Cane toads are considered an invasive species in Florida and pose a threat to both native wildlife and animals in human care; thus, the toads used in this joint North Carolina Zoo (NC Zoo) wound healing and nutrition study would have been euthanized as part of an invasive species culling program had they not been utilized for this research (Eubig, 2001; Greenlees et al., 2006).

Materials and Methods

This trial was conducted under the approval of both the NC Zoo and Zoo Miami research committees and liver samples were collected opportunistically from animals being euthanized for an amphibian healing study that was approved by North Carolina State University (NCSU) Institutional Animal Care and Use Committee under IACUC #17-119-O. Forty-five free-ranging cane toads were collected as part of a routine population control program near Zoo Miami grounds in Miami, FL. For inclusion in the study, animals caught had to visually appear healthy. The sex of the toads captured was 32.6.7 (male, female, and unknown, respectively) with an average weight of 126.3g. Five toads were euthanized immediately from the wild and the livers were removed to serve as the day 0, free-ranging diet representative. The remaining toads had 8mm Biomark® (Boise, ID) passive integrated transponder (PIT) tags placed in the medial proximal area of either their right or left back leg subcutaneously to serve as individual identification. The liver samples were kept in a -80°C freezer until being shipped over dry ice via FedEx® to the NC Zoo along with the 40 remaining live toads.

Upon arrival at the NC Zoo, toads were immediately transitioned from their free-ranging diet to the NC Zoo amphibian diet. Toads were housed in the NC Zoo Hanes Veterinary Medical Center under established quarantine procedures for the study period of 69 days. Toads were

given a five-week acclimation period before the healing study began. Toads that did not gain weight during the acclimation period (n = 6) were treated with a single dose of Vedco Inc. (Saint Joseph, MO) ivermectin intramuscularly at 0.2 mg/kg. Additionally, during the acclimation period, a random subset of five toads were tested for *Batrachochytrium dendrobatidis* DNA via PCR of dermal swabs and these were all found to be negative. Routine fecal analysis was performed during the acclimation period and rare-to-moderate numbers of *Strongyloides* spp. ova were identified, which is not unexpected of wild-caught animals. Animals were visually examined each day and weighed once a week after being placed in human care. The NC Zoo's amphibian diet consisted of brown house crickets (*Acheta domestica*) purchased from Catawba Cricket Hatchery Inc. (Charlotte, NC) and gut-loaded with Mazuri® (St. Louis, MO) Hi Calcium Gut Loading Diet (5M38) and supplemented with small amounts of produce (carrots or sweet potatoes). Toads were offered approximately seven crickets per toad three times weekly (Monday, Wednesday, Friday). Toads were losing weight when fed solely crickets and weight was an important aspect to the healing study; therefore, toads were also offered Nestlé Purina PetCare Co. (St. Louis, MO) Cat Chow Complete commercial cat food three times per week (Tuesday, Thursday, Saturday) starting on day 30 of the study.

The toads were housed indoors and distributed between three Waterland (CA) 135-gallon tubs (70" x 32" x 14"), each with a mesh screen covering affixed to the top to prevent escape and to partially block the artificial lighting. Each tub had a dry area lined with newspaper with a hide area and a pool containing dechlorinated reverse osmosis (RO) water. In addition to artificial fluorescent lighting, a focal UV basking light was placed over each tank. The light cycle was maintained according to the working hours of the animal husbandry staff (8:00 am - 5:00 pm). The tanks were cleaned of debris daily and completely disinfected weekly. The room was kept at

ambient temperature to the outdoors throughout the study period with indoor temperatures being measured by a wireless AcuRite digital thermometer (Lake Geneva, WI) and fluctuating from 6.7-30.5°C.

Euthanasia for the toads was performed by inducing a surgical plane of anesthesia via a partial submersion in Western Chemical Inc.'s (Ferndale, WA) tricaine methanesulfonate (MS-222) at 3.3 g/L buffered on a gram per gram basis with sodium bicarbonate. This was followed by intracardiac injection of Virbac Animal Health's (Fort Worth, TX) sodium pentobarbital (Euthasol) given at 0.3-1.9 mL/kg. Death was confirmed by cessation of cardiac flow as determined via doppler probe.

As this work was in conjunction with a wound healing study, liver samples were collected from the 45 cane toads on their veterinary protocol's euthanasia dates: day 0 (n=5), day 40 (n=7), day 45 (n=6), day 48 (n=6), day 54 (n=7), day 60 (n=6) and day 69 (n=8) in human care. A small subsample of each liver was taken and all liver samples were stored in a -80°C freezer until shipped over dry ice via FedEx® to McGraw Biology Laboratory, Arizona State University, Tempe, AZ (ASU). At ASU they were analyzed for retinol, tocopherol, and carotenoids (alpha-carotene, beta-carotene, lutein, lutein ester 1, lutein ester 2, and zeaxanthin were found in the analysis) using a previously described high-performance liquid chromatography (HPLC) method (Toomey and McGraw, 2010).

Means, standard errors, and statistical differences by time within human care for vitamin A, vitamin E, and carotenoid values were evaluated by performing an ANOVA test using SAS 9.4 (Cary, NC) to compare all timepoints against each other. Differences with $P<0.05$ were considered to be statistically significant.

Results

The day 0 and day 40 retinol concentrations were higher than all other timepoints. Among euthanasia timepoints, there were various significant differences in beta-carotene, lutein, zeaxanthin, and the total carotenoids; however, these trends did not follow a clear pattern (Table 1a). Toad weights at euthanasia within this study ranged from an average of 82.4 g ± 3.71 at day 40 to 186.5 g ± 16.56 at day 69 (Table 1b) with various differences among timepoints.

Nearly all of the carotenoids measured on day 40 were significantly higher when compared to the rest of the time points (Table 1a). This may have been due to the cat food that was fed during the study period and not true toad kinetics, since the cat food contained a vitamin A and E supplement and corn (which is a known source of various carotenoids) (Perry et al., 2009). Additionally, four out of seven of the toads euthanized for liver collections during this timepoint were of concern due to their low body condition score and thus their health status may have impacted the results presented if they perhaps had concentrated the nutrients in the liver. The day 60 timepoint had toads with significantly different weights compared to other timepoints with the toads being heavier than any other group and with very high carotenoid values. Due to these research abnormalities, we ran statistical analyses without the day 40 and day 60 timepoints for nutrient comparisons (Table 1c). These results show significant differences between day 0 alpha-carotene, lutein ester 1, retinol and tocopherol, with initially higher concentrations that decreased over time in human care. There were no other significant differences observed in any of the other carotenoids.

Discussion

This was a nutrition and veterinary partner study with the NC Zoo's veterinary healing study as priority. Therefore, this project acted as a preliminary nutrition study and aligned well

with a general research goal of reducing the number of animals used in research via combining studies without increasing animal numbers. Due to the combined nature of this research, however, sample collection timepoints were not able to be selected for nutrition parameters nor were we able to control the animal groupings that were selected for euthanasia at each date. Additionally, we were notified after the study had ended that the toads were given the cat food as detailed in the materials and methods to help them gain weight during the study. Cat food is a known source of vitamins A and E and various carotenoids (including lutein and zeaxanthin) and thus this addition likely altered our vitamin and carotenoid liver results. It is interesting to note that the day 40 collection period (highest retinol and carotenoid values) took place 10 days after the cat food feeding had begun and the day after a feeding day. Yet, even with the additional supplementation of the cat food, it is clear to see that many of the nutrient concentrations were lower at the end of the study period (day 69) when compared to the free-ranging values (day 0).

With amphibian nutrient requirements currently being unknown, this preliminary study was important to evaluate a typical human care amphibian diet and compare to free-ranging diet nutrient concentrations. This study indicated that there was value to a longer, more controlled study in order to properly assess the kinetics of vitamin A, vitamin E, and carotenoids. Proper supplementation of vitamin A is important to avoid deficiency symptoms (such as “short tongue syndrome”) that have been previously documented (Pessier et al., 2002; Pessier et al., 2005; Pessier, 2013; Ferrie et al., 2014). Work done in tadpoles highlights the importance of vitamin E supplementation as well (Szuroczki et al., 2016). There have been differences demonstrated in amphibian’s abilities to use carotenoids, but various carotenoids are likely still an important diet component regardless of species ability to use them for vitamin A activity or not (for example,

they play a role in growth, skin coloration, and reproduction) (Morton & Rosen, 1949; Khachik et al., 2002; Ogilvy et al., 2012; Brenes-Soto & Dierenfeld, 2014).

It is important to note that the ASU analytical laboratory pointed out that some of the toad liver samples were much more brightly colored than others as they were analyzed via the HPLC method. Pictures were not taken of the toads and therefore we could not relate any toad coloration back to possibly higher carotenoid concentrations. We also received feedback that liver weight may be an important measure to take into account for the future study because it has been shown that organs can differ in carotenoid concentration due to size (Giraudeau et al., 2016).

Foot sores were noted in some of the toads towards the end of this study. These were likely due to the dryness of the newspaper lining their housing containers. Thus, it was suggested that changes be made to this aspect of the husbandry techniques in future studies.

Conclusion

The results from this preliminary study helped to design many aspects of the next research project discussed in this report. From the statistical results, it was decided that a longer study period should be used to get a better picture of what kinetic nutrient changes would occur if the project continued for more than a two-month time period. It was also important for the next round to distribute sex and weights as evenly as possible across euthanasia points and treatment to minimize their effects. A larger sample size was also recommended to increase statistical power and help minimize the random sample variability. Additional considerations that were taken into account for the following study included: controlling all dietary aspects, reporting total liver weights, acquiring pictures of all toads at each data collection date to be able to note if

carotenoid concentrations seemed to be at all related to skin coloration, and finally, replacing the newspaper lining in the tubs with damp towels to reduce the occurrence of foot sores.

The data presented in this research indicate that retinol decreased significantly over a two-month period of toads consuming a normal in human care amphibian diet. Table 1c also indicated that alpha-tocopherol, alpha-carotene, and lutein ester 1 significantly declined over time in human care. This is an indication that, despite the supplementation in the diet, the human care diet was not keeping up with the free-ranging diet nutrient concentrations. However, knowing the true kinetics of these nutrients over time on a human care diet is necessary to help assess how to properly feed these animals under human care.

CHAPTER 3: Cane Toad (*Rhinella marina*) Vitamin A, Vitamin E, and Carotenoid Kinetics with Potential Stress Measures

Introduction

The first International Union for the Conservation of Nature (IUCN)'s World Conservation Union Global Amphibian Assessment (GAA) was conducted in 2004 and revealed 1856 amphibian species (32.5% of the total species) to be globally threatened (Baillie et al., 2004). There have been numerous conservation actions but despite some localized successes, the overall global decline has not been stopped (Grant et al., 2016). Due to the threat of extinction of some of these species, some conservation efforts include ex-situ breeding of these animals under human care for reintroduction to their native habitats (AZA, 2019).

The nutrient requirements of all amphibians are currently unknown and there have been many reports of concerning husbandry conditions such as anorexia, metabolic bone disease, "short tongue syndrome", neurological and musculoskeletal abnormalities, obesity, renal calculi, etc. due to nutritional deficiencies and toxicities (Wright & Whitaker, 2001; Crawshaw, 2003; Densmore & Green, 2007; Pessier, 2013; Ferrie et al., 2014). Of these, vitamin A, vitamin E, and carotenoids are of particular interest because of their relation to breeding success and the symptoms associated with their deficiency (Pessier et al., 2005; Ogilvy et al., 2012; Pessier, 2013; Ferrie et al., 2014; Szuroczki et al., 2016). Thus, if we hope to manage these amphibians under human care, we need to know how to properly care for them nutritionally and we also need to determine their normal circulating bio nutrients so that we can better assess their health status.

Cane toads (*Rhinella marina*) have been classified as one of the top 100 most destructive invasive species in the world due to their toxicity, successful reproduction, and competition with native species (Lever, 2001; Reeves, 2004; GISD, 2005; Shine, 2010; Yeager et al., 2014). Due to this invasive species status, cane toads make a good model for a nutrition study such as this

one, as the toads used in this study would have been euthanized as part of a population control program whether or not included in this study.

There have been a variety of gut content analysis studies in anura that have attempted to classify their free-ranging diets (Werner et al., 1995; Santos et al., 2004; Wu et al., 2005; Shaw et al., 2012). Although it depends on their home ranges, anura consume mostly invertebrates. Cane toads are indiscriminate feeders, as concluded by the diversity of the diet items found in a study from New Guinea by Bailey, 1976. Specific to Florida, beetles were the primary prey item consumed by cane toads for both the dry and wet seasons (Meshaka & Powell, 2010). For this study it was important to perform gut content analysis on the free-ranging cane toads to ensure that they were representative of a “normal” free–ranging anuran diet.

It is important to measure stress in wild animals brought into human care to determine how adequately they adjust. In amphibians, over activation of the hypothalamic-pituitary-adrenal (HPA) axis, which is part of the stress system, and high levels of stress hormones can inhibit reproduction (Moore & Jessop, 2003). Since threatened amphibians are being brought into human care facilities for breeding, it is important to consider the stress levels of these animals to most successfully care for and breed them.

Our goals for this study were to: 1) examine the kinetics of vitamin A, vitamin E, and carotenoids in cane toads transitioned from a free-ranging diet to two different diet treatments (with and without supplementation of vitamin A and vitamin E) over time in human care; 2) evaluate stress measures over the four-month study period; and 3) collect and analyze gut contents of free-ranging cane toads.

Materials and Methods

Animals

This trial was conducted under the approval of both the North Carolina State University (NCSU) Institutional Animal Care and Use Committee (IACUC) under IACUC #18-073-O and both the North Carolina Zoo (NC Zoo) and Zoo Miami research review boards. Sixty-five adult cane toads (*Rhinella marina*) (57.8) (male and female respectively) were acquired from a free-ranging population on Zoo Miami grounds in Miami, FL during the nights of July 9-10, 2018. These toads were captured as part of an invasive species population management program that takes place annually at this facility. For inclusion in the study, toads had to appear outwardly healthy. Ten of the toads were euthanized the first night toads were collected to serve as time 0 (T0) samples, representative of free-ranging toads. Blood and urine samples were collected from the T0 toads prior to euthanasia. Liver samples were collected from the ten after euthanasia and all samples were stored at -80°C until shipping. The remaining 55 toads had urine samples collected and 8mm Biomark® (Boise, ID) passive integrated transponder (PIT) tags placed subcutaneously in the medial proximal area of either the right or left hind limb. All toads and frozen samples were shipped overnight via FedEx® to the NC Zoo in Asheboro, NC where they were housed in the Hanes Veterinary Medical Center under established protocols for amphibian quarantine for the study period of 119 days. While only 48 toads were required according to a statistical power test, additional toads were captured to ensure enough animals were available throughout the extended study period of four months in case any of the toads proved to be unhealthy despite our initial visual evaluations.

Upon arrival at the NC Zoo, the toads were briefly examined, weighed, and assigned to one of two diet treatments. Weights (range of 29 - 324g) and sex distribution was taken into

account and randomized as much as possible for all data collection dates. Sex was difficult to determine prior to euthanasia based on outward appearance; thus, animals that were thought to be females were distributed as evenly as possible across treatments. Six toads from each diet treatment were euthanized for data collection at 22 (T1), 50 (T2), 81 (T3), and 119 (T4) days after being removed from the wild. See table 2 for sample collection dates and descriptions.

Euthanasia

Euthanasia was performed for all toads by inducing a deep plane of surgical anesthesia via a partial submersion in Western Chemical Inc.'s (Ferndale, WA) tricaine methanesulfonate MS-222 at 10g/L buffered on a gram per gram basis with sodium bicarbonate, followed by each unconscious animal being pithed by an NC Zoo veterinarian, according to the NC Zoo's euthanasia protocol. Death was verified by doppler ultrasound probe.

Toad Husbandry

The 55 live toads that arrived at the NC Zoo were sorted into six Waterland (CA) 135-gallon tubs (70" x 32" x 14") with each tub containing nine to ten toads initially. The tubs all had a pool containing dechlorinated reverse osmosis (RO) water and a dry area with hide areas. The dry animal area also was lined with a damp towel in attempt to prevent pododermatitis. The tubs were fitted with polyvinyl chloride (PVC) piping so that the water could be easily drained. Corrected RO filtered water for the pools was changed every morning. The dry area damp towels were also changed every morning during routine tub cleaning. An artificial fluorescent light cycle was maintained according to the working hours of the animal husbandry staff (8:00 am - 5:00 pm). Each tub also had a mesh screen covering affixed to the top to prevent escape and to partially block the artificial lighting. The area housing the study animals was not climate controlled and indoor temperatures and humidity fluctuated daily and throughout the study

period (18.9-30.6°C; 44-99% humidity) and were recorded by a wireless AcuRite digital thermometer (Lake Geneva, WI). At each specific data collection time periods, the remaining number of toads were consolidated into a smaller number of tubs. At these time points, each toad was relocated to a new tub in an attempt to ensure that every toad was placed under the same amount of potential stress (Table 3a-c).

Toads were weighed weekly and also just before data collection periods. An Adam Equipment® (Oxford, CT) LBK 6a scale was used to weigh larger toads and an OHAUS® (Parsippany, NJ) CS200 scale for smaller toads, with all weight measurements taken to one tenth of a gram. All toads were dewormed on day 22 of the study period with 0.2mg of Vedco Inc. (Saint Joseph, MO) ivermectin per kg of body weight, injected into the left forearm. This medication was conducted as a precaution because some toads were losing or not gaining weight as predicted.

Toad Diets

Adult brown house crickets (*Acheta domestica*) were the only prey item offered to the toads. These crickets were purchased from Catawba Cricket Hatchery Inc. (Charlotte, NC) twice weekly and gut-loaded using diets by Mazuri® (St. Louis, MO). Two diet treatments were assigned; the first being the normal amphibian zoo diet consisting of crickets fed Mazuri® Hi Calcium (Hi Cal) Gut Loading Diet (5M38) plus a small amount of produce (sweet potatoes and carrots). The second diet was the study diet consisting of crickets fed Mazuri® Hi Cal diet but specially formulated with no vitamin A or vitamin E supplements with no addition of produce. Both Mazuri® diets contained the same following ingredients except where differences are noted*: ground corn, dehulled soybean meal, calcium carbonate, wheat middlings, ground soybean hulls, porcine meat and bone meal, porcine animal fat preserved with BHA and citric

acid, salt, dl-methionine, choline chloride, magnesium oxide, nicotinic acid, calcium pantothenate, riboflavin supplement, dl-alpha tocopheryl acetate (*only in diet treatment 1), thiamine mononitrate, vitamin A acetate (*only in diet treatment 1), folic acid, manganous oxide, zinc oxide, ferrous carbonate, pyridoxine hydrochloride, menadione sodium bisulfite complex, copper sulfate, zinc sulfate, calcium iodate, sodium selenite, cobalt carbonate, cholecalciferol, vitamin B_{12} supplement. Diet treatment 1 (supplemented diet) was reported to contain 12,525 IU/kg of vitamin A and 19 IU/kg of vitamin E.

Toad feedings occurred every weekday Monday–Friday. All feedings occurred in the afternoon to optimize cricket gut-load and normal feeding behavior for the toads. Initial feedings allotted for seven crickets per toad. This amount was adjusted based on overall consumption and animal weight trends over the course of the study (Appendix A and B).

Cricket Husbandry

Adult brown house crickets were housed in eighteen-gallon Rubbermaid® (Atlanta, GA) storage containers with three to four cardboard egg trays in each container to provide additional surface area and hiding space. There were four storage containers total with two being used for each diet treatment. One for each diet treatment was used for gut-loading and the other for each treatment was for the crickets that had already been gut-loaded for 48 hours and were ready to be fed out. The tops of the containers were covered with chicken wire to prevent escape. Each tub had round food and water bowls with sponge or stones placed in them to prevent drowning. Tubs were cleaned daily by removing waste and changing water, and then were completely sanitized on collection dates using Vedco Inc. chlorhexidine gluconate 2% solution diluted at 2tbsp/gal.

Urine Collection and Processing

A minimum of 0.1mL of urine was collected during each data collection time point by holding each toad over a clean collection cup. The majority of the toads urinated in the cup upon initial handling. Those that did not produce a sample immediately were placed in a clean enclosure until they urinated. If a toad did not produce an adequate sample within one and a half hours, urine collection was skipped for that data collection point. Urine was transferred from the collection cup to a Fisherbrand™ (Waltham, MA) 0.5 mL cryogenic storage vial via a plastic pipette and then centrifuged for three minutes at 1600 revolutions per minute (rpm) using either a LW Scientific (Lawrenceville, GA) or Thermo Scientific (Waltham, MA) centrifuge. Urine was then stored in a -80°C freezer until shipment over dry ice via FedEx® to Smithsonian Conservation Biology Institute (Front Royal, VA). Samples were analyzed using a corticosterone enzyme immunoassay (EIA) kit (CJM006) made by Coralie Munro at UC Davis (Davis, CA) using specific gravity and according to the Smithsonian's wildlife endocrinology manual (Graham et al., 2016; Smithsonian, 2016).

Blood Collection and Processing

Peripheral venous blood samples were obtained from an abdominal or pelvic limb by means of venipuncture using a 0.5-1 mL syringe and a 22-27 g needle depending on the toad's size. A maximum blood sample of 0.2 mL was taken, being no more than one percent of total blood volume (Wright, 2001). Blood was stored in BD Microtainer® (Franklin Lakes, NJ) heparin collection tubes. All samples were protected from light as much as possible during processing. The heparin tubes with blood were centrifuged for ten minutes at 5000 rpm using a Thermo Scientific (Waltham, MA) centrifuge. Plasma was removed from the tubes and stored in a Fisherbrand™ (Waltham, MA) 2 mL externally threaded cryogenic storage vial in a -80°C

freezer until shipped over dry ice via FedEx® to McGraw Biology Laboratory, Arizona State University, Tempe, AZ (ASU). Plasma was analyzed for vitamin A (measured as retinol), vitamin E (measured as alpha-tocopherol), and the carotenoids (apocarotenoid, beta-carotene, beta-carotene ester, beta-cryptoxanthin, lutein, lutein ester, zeaxanthin, zeaxanthin ester were found in analysis) using a previously described high-performance liquid chromatography (HPLC) method (Toomey et al., 2010).

Lactate

Lactate was measured using a single drop of whole blood with a Nova Biomedical Co. (Waltham, MA) Lactate Plus meter. Lactate readings were obtained immediately upon sample collection from each toad on data collection days.

Liver Collection

Entire livers (Figure 1) were removed after euthanasia and immediately weighed on an OHAUS® (Parsippany, NJ) C305-S Portable Plus scale. They were then wrapped in aluminum foil to protect from light, placed in Whirl-Pak® bag (Nasco, Fort Atkinson, WI), and frozen in a -80°C freezer. At the end of the trial all livers were slightly thawed to retrieve a representative subsample of each that was weighed on an OHAUS® (Parsippany, NJ) GT410 scale as close to one gram as possible and then frozen again at -80°C prior to shipment. Liver subsamples were shipped via FedEx® over dry ice to ASU. Livers were analyzed for vitamin A (measured as retinol), vitamin E (measured as alpha-tocopherol), and the carotenoids: apocarotenoid, beta-carotene, beta-carotene ester, beta-cryptoxanthin, lutein, lutein ester, zeaxanthin, zeaxanthin using a previously described high-performance liquid chromatography (HPLC) method (Toomey et al., 2010).

Cricket Feed Analysis

Fifty-gram samples of each of the cricket gut-loading diets were sent to Zooquarius and analyzed for crude protein, ADF, aNDF, WSC, starch, crude fat, crude fiber, ash, calcium, phosphorus, magnesium, potassium, sodium, sulfur, iron, zinc, copper, manganese and molybdenum. Five-gram samples of each diet treatment were also sent to both ASU and Craft Technologies, Inc. (Wilson, NC) to be analyzed for retinol, alpha-tocopherol, and carotenoids.

Cricket Analysis

Approximately 100g of gut loaded crickets from each diet treatment were euthanized using Patterson Veterinary Supply, Inc. (Saint Paul, MN) isoflurane, soaked in a cotton ball in an enclosed system which vaporized at 32%. Whole crickets were sent to Zooquarius Laboratory Services (Ithaca, NY) where they were ground and then analyzed in duplicate for crude protein, ADF, AH fat, ash, calcium, phosphorus, magnesium, potassium, sodium, sulfur, iron, zinc, copper, manganese and molybdenum. An additional 5g of gut loaded crickets from each diet treatment was sent to ASU to be analyzed for retinol, alpha-tocopherol, and carotenoids.

Stomach Content Analysis

The entire gastrointestinal tracts (Figure 2) were removed after euthanasia from the ten T0 free-ranging cane toads and frozen in a -80°C freezer. These served as our wild-type diet samples and were shipped to the NC Zoo over dry ice via FedEx® and stored in a -80°C freezer until transport to NCSU in Raleigh, NC. The stomach contents were kept in a -80°C freezer until they were analyzed for prey items under the direction of Dr. Matthew Bertone in the Department of Entomology & Plant Pathology at NCSU. The samples were partially unthawed and the stomach was cut from the remaining gastrointestinal tract. A cut was then made to the stomach, the stomach was flipped inside out, and the contents removed via a medical spatula and tweezers.

The contents were placed inside Fisherbrand™ (Waltham, MA) Falcon 50 mL conical centrifuge tubes filled with 70% ethanol for storage. Dr. Bertone then identified the consumed prey items according to the lowest taxonomic rank possible.

Photographs

A photograph of each toad was acquired for all upon arrival at the NC Zoo and for every toad at T1, T2, T3 and T4 to document the coloration of their skin. This was completed in order to make a reference to determine if there are correlations between skin color and blood or liver carotenoid values. See figures 3.1-3.5 for examples.

Experimental Design

The experimental design was a randomized block design with two treatments. Fifty-five toads were blocked by weight and sex and then randomly assigned to a diet treatment. Six tubs were used for housing and each one contained nine to ten toads initially.

Statistical Analysis

Statistical analysis was performed using ANOVA in SAS 9.4 (Cary, NC) to determine:

1) Are there significant differences between free-ranging nutrient concentrations / stress measures and all other timepoints.
2) Are there significant differences in nutrient concentrations / stress measures between timepoints.
3) Are there significant differences between weights of animals in the two different diet treatments.
4) Are there significant differences between diet treatments.
5) Is there a correlation between plasma and liver concentrations at all timepoints.

Differences were determined using Tukey-Kramer with $P<0.05$ considered to be statistically significant.

Additionally, decay curves were fit to the change in nutrient concentrations over time using Sigmaplot by Systat Software, Inc. (San Jose, CA) to determine the kinetics of vitamin A, vitamin E, and the carotenoids.

Results

Housing Data: Temperature and Humidity

The temperature and humidity of the NC Zoo quarantine room located in the Hanes Veterinary Medical Center where the toads were kept was recorded every day for the duration of the study. The average temperature for the month of July (12th -31st) was 26.1°C with an average humidity of 79.8%. During the month of August, the average temperature was 25.6°C with an average humidity of 84.0%. September had an average temperature of 25.3°C and humidity of 84.1%. November (1st-7th) temperatures averaged at 22.7°C with an average humidity of 61.6%.

Feed Intake of Toads

Initially, 7 crickets (from the appropriate diet treatment protocol) were offered per toad daily during the week (Monday-Friday). This amount was adjusted based on how many crickets were left uneaten each day in each housing tub. See Appendix A and B for daily cricket consumption data. By day 111, cricket consumption had decreased enough that the toads were switched to only being fed on Monday, Wednesday and Friday but still being offered approximately 7 crickets per toad.

Toad Body Weights and Sex

Toad body weights were recorded weekly and on euthanasia dates. There were no significant differences in body weights between diet treatments for any of the euthanasia dates.

Mean weights for each timepoint ± standard error of the mean (SEM) are presented in Table 4. Toads were primarily male (57.8). There were so few females distributed among treatment groups and euthanasia dates that statistical tests were not able to be run via sex.

Hepatic Vitamin A, Vitamin E, and Carotenoids Concentrations

Retinol, alpha-tocopherol, apocarotenoid, beta-carotene, beta-carotene ester, beta-cryptoxanthin, lutein, lutein ester, zeaxanthin, and zeaxanthin ester concentrations in the liver were all significantly higher at day 0, decreasing over time in human care ($P<0.05$) for both diet treatments 1 and 2. Least square (LS) means ± SEM are reported in table 5.

When comparing the hepatic vitamin A, E and carotenoids concentrations between diet treatment 1 and 2, treatment 1 (supplemented diet) was significantly higher ($P<0.05$) in retinol (at days 22 and 50), beta-carotene (at day 50), beta-carotene ester (at day 22), and zeaxanthin (at day 50) than diet 2 (diet with no supplementation). There were no other significant differences between diet treatments for any other nutrient. See table 5 for differences between diet treatments.

After euthanasia, each toad's liver was removed and weighed. Liver weight percentage (LWP) of each individual's total body weight at euthanasia was then calculated. Neither liver weight means or LWP differed between the two diet treatments or among euthanasia dates.

Circulating Blood Plasma Vitamin A, Vitamin E, and Carotenoid Concentrations

For diet treatment 1, beta-carotene, beta-carotene ester, beta-cryptoxanthin, lutein, lutein ester, zeaxanthin, and zeaxanthin ester concentrations in plasma were all higher at day 0, decreasing over time in human care ($P<0.05$). There were no differences in retinol or alpha-tocopherol over time for diet treatment 1. LS means ± SEM reported in table 6.

For diet treatment 2, retinol, beta-carotene, beta-carotene ester, beta-cryptoxanthin, lutein, lutein ester, zeaxanthin, and zeaxanthin concentrations in plasma were all higher at day 0, decreasing over time in human care ($P<0.05$). There were no differences in alpha-tocopherol over time. LS means ± SEM reported in table 6.

When comparing the circulating blood plasma vitamin A, E and carotenoid concentrations between diet treatment 1 and 2, diet treatment 1 was significantly higher ($P<0.05$) in beta-carotene for all euthanasia dates while in human care. Additionally, plasma retinol was significantly higher in the diet treatment 1 group at day 81. See table 6 for differences between diet treatments.

Decay Curves for Vitamin A, Vitamin E, and Carotenoids

Decay curves and equations were constructed using Sigmaplot software for the liver nutrient concentrations at each study timepoint to demonstrate the kinetics of each nutrient. Decay curves were significant for the following liver nutrients for both diet treatments except where specified: retinol, alpha-tocopherol (only for diet treatment 1), apocarotenoid, beta-carotene, beta-carotene ester, beta-cryptoxanthin, lutein, lutein ester, zeaxanthin, and zeaxanthin ester. Examples of decay curves for the nutrients with significant line fits are shown in figures 4.1-4.4.

Decay curves for plasma nutrients could not be predicted either due to non-detectable levels of the nutrient, or the decay curves did not significantly predict the data (i.e., there was not exponential decay of plasma nutrients).

Correlation between Plasma and Hepatic Concentrations of Nutrients

There were no correlations between plasma and liver concentrations during this four-month study.

Corticosterone

Corticosterone concentrations for both diet treatments were higher on day 0 and then significantly declined when measured at day 22 in human care ($P<0.05$). From there, days 50, 81, and 119 were not significantly different from any other timepoint. For diet treatment 2, despite day 119 values being lower than day 22, day 119 is not different than day 0 because of the large difference in sample sizes; the calculation for the standard error of the LS mean difference is the square root of the mean squared error divided by the sample size. When combining diet treatments to look at all individuals over time in human care, corticosterone values follow the same pattern except days 81 and 119 are also significantly lower than day 0. Corticosterone values did not differ between diet treatments. See table 7 for corticosterone values.

Lactate

There were no significant differences in lactate for diet treatment 1. Diet treatment 2 had significant differences with euthanasia day 22 being higher than days 50 and 119. When data were combined across treatments Day 22 lactate values were higher than all other days except day 81. Lactate values did not differ between diet 1 versus diet 2 treatment. See table 8 for lactate concentrations.

Cricket Feed Analyses

The concentrations of vitamin A, vitamin E, and carotenoids within treatment diets 1 and 2 are reported in table 9a. Similarly, proximate nutrient concentrations for treatment diets 1 and 2 are reported in table 9b.

Cricket Analyses

Crickets that were fed treatment diets 1 and 2 were ground and analyzed and their concentrations for vitamin A, vitamin E, and carotenoids are reported in table 9a. Similarly, crickets fed treatment diets 1 and 2 proximate nutrient concentrations are reported in table 9b.

Stomach Contents

The stomach contents from the ten T0 free-ranging toads consisted primarily of invertebrates (>92%). These invertebrates included members of the groups: Spirobolida, Hymenoptera, Araneae, Coleoptera, Odonata, Gastropoda, Chilopoda, Diptera, Dermaptera, Hemiptera, Oribatida, and Blattodea. Hymenoptera (ex. ants and bees) was the most commonly found. For the less common vertebrates, there were parts from the orders Aves and Squamata. Other items found in the gut contents were soil, shed skin from cane toads, and a piece of styrofoam.

Photographs

There was great pigment variation in color differences among the toads. There does not appear to be a clear correlation between carotenoid values and photographic color upon visual inspection. See figures 3.1-3.5 for examples of some pictures from this research study.

Discussion

The wild caught cane toads in this study were successfully managed throughout the four-month study period. There were no animal deaths during the 119 day period although two toads (3.6% of the total population) were euthanized early due to potential health concerns. These two animals were part of the additional animals and therefore not included in the experimental analyses. Toad weights increased by an average of 235.4% over the 4 months (using weights tracked from the day 119 toads starting from day 0). There were no clinical signs of nutrient

deficiency despite the liver and plasma results reported. While caring for these toads in human care is time consuming for the veterinary hospital staff due to the quarantine protocol, they are a successful model to study nutrient changes as amphibians switch from free-ranging to in human care.

Hepatic Vitamin A, Vitamin E, and Carotenoids Concentrations

Despite the supplementation of vitamin A and vitamin E in diet treatment 1 (Mazuri® Hi Calcium Gut Loading Diet) there were still significant decreases in all nutrient concentrations over the four-month study period, when compared to the free-ranging nutrient values (day 0). Liver concentrations for the toads fed diet 1 had values visually higher than those fed diet 2 (Mazuri® Hi Calcium Gut Loading Diet without vitamin A or E) in most cases; however, significant declines were seen for all euthanasia time points for toads consuming crickets fed both diet treatments when compared to free ranging toad nutrient values. While there is little evidence to prove free-ranging diets are healthier than ones fed in human care, it seems that with these nutrient values decreasing so substantially over time that there is valid reason for concern.

It is unknown what vitamin A, E, and carotenoids concentrations are required to maintain the health of toads, but knowing the nutrient concentrations of free-ranging animals could be a vital piece of information to help determine this nutrient requirement. Since 90% of vitamin A is believed to be stored in the liver, hepatic concentrations are currently thought to be the best way to evaluate vitamin A stores in amphibians (Futterman & Andrew, 1964; McDowell, 2012; Clugston & Blaner, 2014). The hepatic retinol values for the day 0 free-ranging toads in this study was an average of 87.7±16.12µg/g; compared to 168.2±52.49µg/g for the day 0 free-ranging toads in the preliminary study (Chapter 2 work). These studies had different sample sizes (n=5 for preliminary study versus n=10 for primary study) and toads were collected

approximately one year apart (2017 and 2018). The retinol values for the primary study were close to what has been previously described (61.9±63.49µg/g) in 21 free-ranging cane toads captured in Miami, FL (Berkvens et al., 2014). However, this prior value may not be the best representation of true free-ranging values as these toads were housed and offered crickets, mealworms, waxworms, and dry dog kibble for 10-21 days before liver samples were acquired (Berkvens et al., 2014). Another study in cane toads from Miami, FL caught in June of 2009 reported vitamin A liver values at 31.6±11.10µg/g for 10 toads that were euthanized immediately (Sullivan et al., 2014). In the Berkvens et al. (2014) study, the cane toads that were born and raised in human care without vitamin A supplementation for 127 days had hepatic retinol values of 0.6±0.59µg/g. The retinol values for the toads in this study were higher that the Berkvens et al. study for both diets offered (diet 1: 11.6±1.19µg/g; diet 2: 8.2±0.74µg/g) at the end of the 119 days. However, with vitamin A stores being depleted at this rate, it is likely that additional supplementation is needed if the goal is to match the free-ranging values, to successfully breed and to avoid previously documented deficiency symptoms (such as "short tongue syndrome") (Pessier et al., 2005; Pessier, 2013; Ferrie et al., 2014).

The current literature is lacking on reporting normal reference ranges for hepatic vitamin E concentrations in anura. Supplementation is thought to be important as it increases the immune capabilities and growth in tadpoles (*Lithobates pipens*) (Szuroczki et al., 2016). There are no reports of vitamin E toxicity in amphibians and it appears to be much less of a concern or less understood; however, based on other amphibian species it is still appears to be important to avoid over supplementation (March et al., 1973). Data from the current study provides information about normal hepatic alpha-tocopherol concentrations in free-ranging cane toads and how these stores depleted over time in human care. The day 0 free-ranging values were 9.2±0.54µg/g with

the day 119 concentrations being similar for diet treatment 1 (6.9±0.41µg/g) and diet treatment 2 (7.1±0.64µg/g). It is important to note that the primary storages sites for vitamin E is adipose and muscle tissue so liver samples may not give the best representation of vitamin E nutrient status (Dierenfeld, 1989).

Some amphibian species have demonstrated the ability to use carotenoids for vitamin A activity, or at least these carotenoids have been found in the tissues of some amphibians (Morton & Rosen, 1949; Khachik et al., 2002; Brenes-Soto & Dierenfeld, 2014). The ability of amphibians to utilize these carotenoids is still largely unknown for the majority of species studied (Schairer et al., 1998), but carotenoids are still thought to be an important part in amphibian diets because they can impact growth rate, skin coloration during a critical period, and reproductive success (Ogilvy et al., 2012). The results from this study show that all carotenoids significantly decreased over time when compared to the day 0 free-ranging values. This was seen for both diet treatments; thus, even the diet supplemented with carotenoids is not able to match the carotenoid values the cane toads are getting from their free-ranging diet.

When comparing the two diet treatments, hepatic retinol concentration was significantly higher at days 22 and 50 for diet 1. Additionally, beta-carotene was significantly higher at day 50 for diet 1 and beta-carotene ester significantly higher at day 22 for diet 1. It makes sense that retinol and beta-carotene liver concentrations would be higher in diet treatment 1, as it had been supplemented with both; however, this diet treatment was still unable to maintain the concentrations found in free-ranging toads. It is important to note that alpha-tocopherol values did not differ between treatments, despite diet treatment 1 being supplemented with vitamin E.

It has been shown that organs can differ in carotenoid concentration due to size and therefore we calculated liver weight means and liver weight percentage (LWP) of body weight

and tested for differences between diet treatments (Giraudeau et al., 2016). There were no significant differences between the two diet treatments or among euthanasia dates. Thus, weight of toads and LWP did not alter any nutrient concentration results and therefore this step may not be necessary for future research.

Circulating Blood Plasma Vitamin A, Vitamin E, and Carotenoid Concentrations

The plasma retinol results of the current study were consistent with the levels previously reported in free-ranging cane toads (0.1±0.01µg/g (Sullivan et al., 2014)). There were no significant differences observed in plasma retinol over time for the toads who were fed the supplemented diet 1 treatment. The plasma retinol in the toads within the diet treatment 2 slowly declined over the study period and was unmeasurable at days 81 and 119. Toads fed diet 1 had significantly higher plasma retinol at day 81 when compared to toads fed diet 2. Thus, results show that the toads on the supplemented diet had plasma retinol values that maintained the same concentrations as the free-ranging values; however, the toads fed the diet without supplementation had plasma values completely drop off to zero. Circulating retinol values are kept at homeostasis except in times of deficiency; therefore, low circulating values of retinol would likely indicate a severe vitamin A deficiency (Clugston & Blaner, 2014). However, currently "normal" values are not established and it is therefore not possible to absolutely determine "low" values. Additionally, retinol-binding protein may make values difficult to interpret, especially if the animal was under acute stress when the blood was sampled because circulating vitamin A and retinol-binding protein concentrations decrease with stress (Louw et al., 1992). Clinical deficiency was not noted in this population but future work for a longer period of time may establish values for physical deficiency symptoms.

Vitamin E plasma status has been demonstrated to be correlated with available dietary levels in mammals (Machlin, 1980). It is unknown if the same is true for amphibians and it may vary across the numerous species. Additionally, it may take up to one year for plasma concentrations to reflect diet changes in vitamin E (Dierenfeld, 1989). There are not currently reference ranges established for amphibians for alpha-tocopherol in plasma so this study contributes valuable data towards establishing normal values for cane toads. The day 0 free-ranging population of cane toads had an average plasma alpha-tocopherol value of 0.3±0.03µg/g and there were no significant changes over the 119 days in human care for toads fed either diet treatment, nor were there significant differences between the diet treatments for alpha-tocopherol. Perhaps this is because vitamin E is stored in adipose tissue and circulating plasma concentrations may not significantly change while these fat stores are being depleted (Dierenfeld, 1989).

Carotenoid concentrations were all highest in the free-ranging population and decreased over time in human care. Every carotenoid except for beta-carotene was completely depleted to a concentration of 0.0µg/g by the end of the 119 days for both diet treatments. Beta-carotene was the only carotenoid to still be present in the circulating plasma at the end of the 119 days with a concentration of 0.2±0.04µg/g, but only for the toads on diet treatment 1. Toads on diet treatment 1 had significantly higher concentrations of beta-carotene in the plasma from day 22 and on; toads on diet treatment 2 were depleted of beta-carotene in the plasma by day 22. Carotenoids are an important dietary component as they have the ability to impact development, skin coloration, and reproductive success, as demonstrated in red-eyed tree frogs (Ogilvy et al., 2012).

Decay Curves for Vitamin A, Vitamin E, and Carotenoids

Using the decay equations created by Sigmaplot to go along with the exponential curves, values can be calculated for the rate of decline of the liver concentration of nutrients over time. From this, the concentrations can be represented by percentage of the nutrient remaining compared to the initial concentration of that nutrient. For example, when using the equation created using the exponential fit line ($P<0.05$) for retinol, it is approximated that by day 6 toads on the diet supplemented with vitamin A had 25% of the initial retinol concentration stored; whereas, toads on the unsupplemented diet had concentrations of only 12% retinol remaining. This demonstrates how the supplemented diet maintained values for longer, but the decline was still rapid for both diet treatments. Similarly, when using the equation to estimate values for beta-carotene at day 9, 61% of beta-carotene remained for the supplemented diet, but only 29% remained for the unsupplemented diet. It makes sense that the diet supplemented with produce would maintain the concentration of beta-carotene for longer, as carrots and sweet potatoes are a known source of beta-carotene (Simon & Wolff, 1987; Takahata et al., 1993) Caution should be used in the interpretation of these equations because of the rapid decline between days 0 and 22. With there being no actual data points in between that time from this study, the best fit exponential line makes assumptions about the rate of decline. It is recommended that, if a similar study is conducted in the future, there should be more collection time points placed between days 0 and 22 to get a better picture of what is going on. See figures 4.1-4.4 for examples of decay curves and equations.

Correlation between Plasma and Hepatic Concentrations of Nutrients

Correlations between plasma and liver results were not detected during the four-month time period. Liver nutrient concentrations generally followed an exponential decay trend;

whereas, plasma results followed a mostly linear trend. No clinical signs of deficiency were observed in the study. It is possible that plasma may be correlated with liver concentrations in times of severe deficiency when plasma levels drop below their homeostatic concentrations (Clugston & Blaner, 2014). Therefore, if this study was extended for a longer period of time and clinical deficiency symptoms were observed, there may have been plasma and liver concentration correlations detected. However, if there was a correlation, it may not be a useful measure if it implies that liver stores are already depleted. Thus, this would be a very late indication of deficiency and possibly past the point where the animal could successfully recover.

Corticosterone

Corticosterone values showed significant differences over time in human care for both diet treatments and when the data for the diet treatments was combined. Corticosterone urinary concentrations for the free-ranging (T0) toads averaged 1.7±0.19ng/mL. Urinary corticosterone concentrations in wild cane toads caught in Australia were reported to range from 13.3 to 81.6 pg/µg (Narayan et al., 2012). The methodology used in the study by Narayan et al., 2012 was different than the testing methods used in the current study. Narayan et al., 2012 used creatine to measure against corticosterone; whereas the assay used in this study used specific gravity. The concentration of corticosterone did not increase in urine of these cane toads until two hours after a stressful event, such as capture (Narayan et al., 2011). Therefore, it is presumed that the values reported in this trial for day 0 are representative samples of non-stressed free-ranging cane toads. The corticosterone values dropped significantly for both diet treatments from day 0 to day 22. This may be due to ease of food availability or to the lack of predator presence as it has been demonstrated that amphibians have a corticosterone response to threats perceived as predators which they would have encountered frequently in their free-ranging habitat (Narayan et al.,

2013). By day 22 the toads may have realized that the humans handling them were not predators; it would have been interesting to have taken urine samples in the first couple days that the toads were brought into human care. The corticosterone concentrations increased some after day 22 but still remained lower than the initial free-ranging values. There were no significant differences in corticosterone between diet treatments. Lowered corticosterone values may be an indication that the toads are adequately handling stress in human care and that their welfare is being addressed properly. However, further research is needed and for future work we recommend recording the time it takes to gather each urine sample. While all of our urine samples were collected and frozen in less than two hours, some did take much longer than others and future work should include exact time taken to gather each sample for statistical correlations.

Lactate

When the diet treatments were analyzed individually for lactate there were significant differences in diet treatment 2 where values were highest at day 22 and lowest at day 50, but no significant differences in diet treatment 1 was noted. When data were combined for all individuals in both diet treatments, there were significant differences at day 22 which had the highest lactate values. There were no significant differences in lactate between diet treatments 1 and 2.

Lactate is a measure of the amount of lactic acid in the blood which is associated with anaerobic metabolism, and it has been shown that increased levels are associated with exercise, immobilization and emotional stress in mammals and in amphibians (Bennet & Licht, 1974; De Bruin et al., 1990). Thus, lactate is likely a good measure of short-term stress for amphibians. Day 22 was the first day that all of the toads had blood samples taken and there were multiple professionals helping process the toads that did not have extensive toad experience. Thus, day 22

was likely the day that it took longest to gather samples, although time per blood draw was not recorded. For future work, we recommend recording the time it takes to bleed each toad, as lactate is a fast, short-term stress response and timing could have altered results (Bennet & Licht, 1974).

Cricket Feed Analyses

Mazuri® Hi Calcium Gut Loading Diet (diet treatment 1) was reported by Mazuri® to contain an additional 12,525 IU/kg of vitamin A added in the form of vitamin A acetate and 19 IU/kg of vitamin E in form of dl-alpha tocopheryl acetate. It was expected for the diet without supplementation (diet treatment 2) to contain only small amounts of vitamins A and E due to the porcine meat and bone meal that was standard and included in both treatment diets. Carotenoids in both diets were provided by the corn (first ingredient in the Mazuri® diets) and supplemented for diet treatment 1 via added produce (carrots and sweet potatoes) (Simon & Wolff, 1987; Takahata et al., 1993; Perry et al., 2009).

Diet samples sent to ASU were analyzed for retinol and alpha-tocopherol and both diet treatment 1 and 2 were surprisingly almost identical. ASU reported that the acetate form of supplementation may not elute well using their HPLC method (Toomey et al., 2010) and therefore diet samples were also sent to Craft Technologies, Inc. (Wilson, NC) for analysis. Craft labs indicated no detectable results for retinol and alpha-tocopherol in both treatment diets. Detectable limits were <1mg/kg and 10mg/kg for vitamins A and E respectively. Thus, although the manufacturing facility's detailed records indicate that the diet pre-mix was made and supplemented correctly, the lab results are confounding.

There are various potential reasons for the diet analyses showing the unexpected diet treatment 1 results. There could have been a formulation or manufacturer error in which the

addition of retinol and α-tocopherol did not happen. Therefore, the only difference between diet treatments would have been whether or not the produce (sweet potatoes and carrots) was supplemented to the crickets. If this scenario was the explanation, the only nutrient alternations between the two treatment diets would have been in carotenoid concentrations, as sweet potatoes and orange carrots are known sources of high concentrations, particularly beta-carotene (Simon & Wolff, 1987; Takahata et al., 1993). Since nutrient analysis of the crickets fed diet 1 themselves were higher in four of the five detectable carotenoids but lower in retinol and α-tocopherol this explanation is plausible.

Additionally, since the diet samples were not shipped for analysis at ASU or Craft laboratories until after the completion of the second research study, there could have been vitamin loss over time due to storage conditions during the five-month research time period (Coelho, 2002). However, it should be noted that the diets were stored using all proper guidelines.

There also could have been laboratory difficulties in attempting to extract the acetate forms of supplementation. This could be due to a unique matrix composition of the sample, although this is unlikely since these are common dietary additives. Studies have indicated that there are concerns with laboratory consistencies when analyzing samples for fat-soluble vitamins (Snellman et al., 2010; Ange-van Heugten et al., 2019). These inconsistencies could relate to methodology differences, HPLC/analytical differences, technician training, etc. Thus, it is possible that diet treatment 1 did have the added vitamins and that the laboratories used were not able to find them within the diet samples provided. Retinol is higher for diet treatment 1 for various time points for toad liver and plasma throughout the study indicating that there were diet differences within the study as designed. Since the diet treatment 1 Mazuri® diet inclusion

amount of vitamin E is much lower than the inclusion amount of vitamin A, it is possible that only effects were noted for retinol within the study results. It also is possible that the diet samples sent to the analytical labs were poorly homogenized and that the vitamins were not distributed throughout adequately.

Mazuri® Hi Calcium Gut Loading Diet is reported to contain 8.2% calcium (Ca) and 0.63% phosphorus (P). The results from the analysis in this study are consistent with these projected values with diet 1 containing 8.47% Ca and 0.64% P, and diet 2 containing 8.70% Ca and 0.62% P. All the tested proximate nutrients between the two treatment diets were similar and therefore did not alter any of the parameters within this study.

Cricket Analyses

Crickets were analyzed for vitamin A, E, and carotenoids. The retinol and alpha-tocopherol values were higher in diet treatment 2 than in diet treatment 1. This was surprising at first but as previously noted, ASU reported that the acetate form of supplementation in diet 1 may not have eluted well using their HPLC method (Toomey et al., 2010).

The calcium and phosphorus concentrations in the crickets are quite concerning in both diets, considering the supplementation values for the feed was so high. Crickets fed diet 1 were composed of 0.44% Ca and 0.97% P, and those fed diet 2 were made up of 0.51% Ca and 0.98% P. This is of concern because the calcium to phosphorus ratio (Ca:P) is inverted at 1:2.2 and 1:1.9 respectively. The ideal insect Ca:P ratio is 1:1-2:1 based on mammalian species (NRC, 1995; Finke, 2002; Livingston et al., 2014). Low calcium and high phosphorus in the diet along with improper vitamin D supplementation can lead to nutritional metabolic bone disease (Michaels et al., 2015). It is recommended to further explore the Ca:P ratios in the insects fed at NC Zoo by researching the gut loading strategies and additional supplementation options.

Stomach Contents

Stomach contents of the ten day 0 free-ranging cane toads were analyzed to ensure that the population used for this study was representative of a typical cane toad diet. The results of this study showed that over 92% of the free-ranging diet consisted of invertebrates. This is consistent with what others have reported in anura (Donnelly, 1991; Cogălniceanu et al., 1998; Santos et al., 2004; Shaw et al., 2012). Specifically, the most common diet items found in the cane toads was ants (approximately 45%) which is also consistent with what has been reported before (Bailey, 1976; Meshaka & Powell, 2010). In Florida, a study showed that the most common prey item consumed by cane toads was beetles (Meshaka & Powell, 2010). While this was not the most frequently found from this study, beetles were identified in six out of the ten stomachs. Overall, the toads used in the current research appear to have consumed a natural toad diet.

Photographs

Since there did not appear to be any obvious correlation between carotenoid values upon inspection of pictures with the human eye, we advise that more research could be done with these pictures. For future experiments we would recommend using a tool such as a Minolta chromo meter to detect and analyze for true color differences. It would be expected that the most brightly colored animals are also the ones with the highest carotenoid concentrations similar to what has been demonstrated with house finches (*Carpodacus mexicanus*) in which those who ingested the most carotenoids also had the brightest carotenoid coloration (Hill et al., 2002).

Conclusion

The data presented in this thesis provides novel “normal” free-ranging concentrations and information about the kinetics of vitamin A, vitamin E, and carotenoids in the plasma and liver

of cane toads with and without dietary supplementation over a four-month period. The results of the two different cane toad diet treatments show that neither diet mimic the cane toad's free-ranging nutrient status. While this is not necessarily an indication of an unhealthy diet, it is certainly of interest due to such a rapid decline in these nutrients over a relatively short time period. It is suggested that supplementation of vitamin A and vitamin E, and carotenoids within toad and all amphibian diets need further exploration. Additionally, even with such high dietary values of calcium supplementation, Ca:P ratios were still poor in the crickets fed at the NC Zoo. Thus, it is recommended that further research is done to improve calcium supplementation methods as well.

Corticosterone values presented for the toads in this four-month study period indicate that cane toad stress while in human care is minimal and this data is therefore reconfirming that these amphibians may adjust adequately to being brought into human care. Lactate values potentially provide a picture of the animal's short-term stress levels and are less conclusive within the current study and further research is recommended.

With the current plight of amphibians in the wild, it is vital to determine their dietary requirements so that they can thrive under human care with the hope for successful breeding and reintroduction of species into their native habitats.

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TABLES

Table 1a. Cane toad (*Rhinella marina*) Liver Vitamin A, Vitamin E, and Carotenoid Concentrations (LS Means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 69 in Human Care Consuming the NC Zoo Amphibian Diet.[1]

	Day 0 (n=5)	**Day 40 (n=7)**	**Day 45 (n=6)**	**Day 48 (n=6)**	**Day 54 (n=7)**	**Day 60 (n=6)**	**Day 69 (n=8)**
Retinol (µg/g)	168.2±52.49[a,b]	406.1±164.96[a]	36.4±7.70[b]	30.0±10.19[b]	20.6±4.74[b]	39.3±16.55[b]	34.0±11.78[b]
α-tocopherol (µg/g)	10.3±3.13	3.4±1.13	3.1±0.76	5.7±1.13	3.6±1.00	7.6±3.26	2.9±0.48
α-carotene (µg/g)	2.3±0.67	12.4±6.23	0.5±0.51	1.3±0.43	1.4±0.50	10.8±7.44	0.6±0.29
β-carotene (µg/g)	15.3±7.33[a,b]	71.3±29.56[a]	4.8±2.89[b]	2.9±0.59[b]	5.9±2.38[b]	15.1±7.34[a,b]	6.4±2.42[b]
Lutein (µg/g)	40.0±12.16[a,b]	256.3±111.09[a]	22.2±4.83[b]	45.9±10.22[a,b]	33.5±5.60[b]	52.6±15.49[a,b]	89.9±47.66[a,b]
Lutein ester 1 (µg/g)	15.6±5.59	41.7±28.56	5.4±1.67	9.8±1.66	8.1±0.59	19.9±10.98	9.5±1.66
Lutein ester 2 (µg/g)	27.3±10.21	96.8±66.83	12.5±5.35	22.3±4.25	15.7±2.40	43.5±21.50	17.3±3.12
Zeaxanthin (µg/g)	20.1±9.33[a,b]	224.5±112.55[a]	10.4±3.67[b]	30.2±8.79[a,b]	17.9±4.16[a,b]	21.0±5.57[a,b]	35.6±9.44[a,b]
Total Lutein (µg/g)	82.8±24.17	394.9±202.68	40.2±9.86	78.0±15.14	57.3±8.17	116.1±47.89	116.7±50.52
Total Carotenoids (µg/g)	120.5±34.94[a,b]	703.0±340.24[a]	55.9±14.31[b]	112.3±23.12[a,b]	82.5±10.78[b]	163.0±59.82[a,b]	159.3±61.48[a,b]

[a,b]Means within the same row with these unique subscripts differ significantly ($P<0.05$).

[1]The NC Zoo amphibian diet consisted of crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet + supplemental produce (carrots and sweet potatoes).

Table 1b. NC Zoo Cane Toad (*Rhinella marina*) Mean Body Weights at Sample Collection Timepoints.

Timepoint	Weight at Euthanasia (g)
Day 40	82.4[a]
Day 45	149.5[a,b]
Day 48	198.6[b,c]
Day 54	135.2[a,b]
Day 60	283.1[c]
Day 69	186.5[b]

[a,b,c]Means within the same column with these unique subscripts differ significantly ($P<0.05$).

Table 1c. Cane toad (*Rhinella marina*) Liver Vitamin A, Vitamin E, and Carotenoid Concentrations (LS means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 69 in Human Care Consuming the NC Zoo Amphibian Diet[1], with Timepoints Day 40[2] and Day 60[3] Removed.

	Day 0 (n=5)	**Day 45 (n=6)**	**Day 48 (n=6)**	**Day 54 (n=7)**	**Day 69 (n=8)**
Retinol (µg/g)	168.2±52.49[a]	36.4±7.70[b]	30.0±10.19[b]	20.6±4.74[b]	34.0±11.78[b]
α-tocopherol (µg/g)	10.3±3.13[a]	3.1±0.76[b]	5.7±1.13[a,b]	3.6±1.00[b]	2.9±0.48[b]
α-carotene (µg/g)	2.3±0.67[a]	0.5±0.51[b]	1.3±0.43[a,b]	1.4±0.50[a,b]	0.6±0.29[b]
β-carotene (µg/g)	15.3±7.33	4.8±2.89	2.9±0.59	5.9±2.38	6.4±2.42
Lutein (µg/g)	40.0±12.16	22.2±4.83	45.9±10.22	33.5±5.60	89.9±47.66
Lutein ester 1 (µg/g)	15.6±5.59[a]	5.4±1.67[b]	9.8±1.66[a,b]	8.1±0.59[a,b]	9.5±1.66[a,b]
Lutein ester 2 (µg/g)	27.3±10.21	12.5±5.35	22.3±4.25	15.7±2.40	17.3±3.12
Zeaxanthin (µg/g)	20.1±9.33	10.4±3.67	30.2±8.79	17.9±4.16	35.6±9.44
Total Lutein (µg/g)	82.8±24.17	40.2±9.86	78.0±15.14	57.3±8.17	116.7±50.52
Total Carotenoids (µg/g)	120.5±34.94	55.9±14.31	112.3±23.12	82.5±10.78	159.3±61.48

[a,b]Means within the same row with these unique subscripts differ significantly ($P<0.05$).
[1]The NC Zoo amphibian diet consisted of crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet + supplemental produce (carrots and sweet potatoes).
[2]Day 40 had 4 of 7 animals culled due to low body condition score with another animal suspected of poor health.
[3]Day 60 was a weight anomaly with group weight being significantly higher than all others timepoints.

Table 2. Sample Collection Description for Cane Toads (*Rhinella marina*) at Each Data Collection Period During the Primary Study.

Data Collection Timepoint (T)	**Urine**	**Blood**	**Lactate**	**Liver**
T0 – Day 0 (July 9-10, 2018)	Collected from all	Collected from 10 euthanized	Collected from 10 euthanized	Collected from 10 euthanized
T1 – Day 22 (Aug 3, 2018)	Collected from all	Collected from all	Collected from 12 euthanized	Collected from 12 euthanized
T2 – Day 50 (Aug 31, 2018)	Collected from 12 euthanized	Collected from 12 euthanized	Collected from 12 euthanized	Collected from 12 euthanized
T3 – Day 81 (Oct 1, 2018)	Collected from all	Collected from all	Collected from 12 euthanized	Collected from 12 euthanized
T3 – Day 119 (Nov 8, 2018)	Collected from remaining	Collected from remaining	Collected from remaining	Collected from remaining

Table 3a. Initial Housing Setup for Cane Toads (*Rhinella marina*) at NC Zoo from July 12, 2018 – August 31, 2018.

Diet Treatment 1			**Diet Treatment 2**		
Tub 1	**Tub 2**	**Tub 3**	**Tub 4**	**Tub 5**	**Tub 6**
5A0[1]	5A3	5E3	5AC	5AF	5A5
5B2	5B3	566	5AE	5A9	5B0
5D8	5B4	57E	5A8	5B6	5E9
5FA	5EC	58B	5B9	5B7	55B
58D	55E	58C	5F4	56A	55F
5A4	559	58F	55A	585	57A
587	569	588	59C	596	576
59B	57F	62C	591	605	612
59F	581	621	595	61C	638
599					

[1]ID under tub number is the passive integrative transponder (PIT) tag code of the toad.

Table 3b. Housing Setup for Cane Toads (*Rhinella marina*) at NC Zoo from September 1, 2018 – October 1, 2018.

Diet Treatment 1		**Diet Treatment 2**	
Tub 1	**Tub 2**	**Tub 4**	**Tub 5**
5A3[1]	5A0	5AC	5A9
5B4	5FA	5A8	5B7
55E	587	5F4	596
559	59B	55A	605
57F	59F	5E9	61C
57E	5E3	612	55F
62C	58B	638	5B0

[1]ID under tub number is the passive integrative transponder (PIT) tag code of the toad.

Table 3c. Housing Setup for Cane Toads (*Rhinella marina*) at NC Zoo from October 2, 2018 – November 7, 2018.

Diet Treatment 1	Diet Treatment 2
Tub 1	**Tub 4**
5B4[1]	5AC
559	5F4
57F	55A
57E	638
587	5B7
59F	596
58B	605
	61C

[1]ID under tub number is the passive integrative transponder (PIT) tag code of the toad

Table 4. Cane Toad (*Rhinella marina*) Weight Ranges (Means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 119 in Human Care Consuming Two Different Diets.

Day 0 (n=10)	Range = 75.0–256.0g, Mean = 121.4g			
	Diet Treatment 1[1]		**Diet Treatment 2[2]**	
	Weight Range (g)	**Mean Weight (g)**	**Weight Range (g)**	**Mean Weight (g)**
Day 22	39.5-136.0 (n=6)	96.2±20.64	46.2-172.5 (n=6)	108.0±20.64
Day 50	96.0-192.0 (n=6)	137.3±20.64	75.0-186.0 (n=6)	130.3±20.64
Day 81	69.0-285.0 (n=6)	180.3±20.64	116.5-270.0 (n=6)	177.8±20.64
Day 119	174.0-284.0 (n=7)	234.0±19.11	142.0-301.0 (n=7)	212.8±19.11

[1]Diet 1 = crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet + supplemental produce (carrots and sweet potatoes).
[2]Diet 2 = crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet without vitamin A or vitamin E supplementation (no added produce).

Table 5. Cane Toad (*Rhinella marina*) Liver Vitamin A, Vitamin E, and Carotenoid Concentrations (LS means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 119 in Human Care Consuming Two Different Diets.

Nutrient	Day 0[1] (n=10)	Diet Trt[2]	Day 22[3] (n=6)	Day 50 (n=6)	Day 81 (n=6)	Day 119 (n=7)
Retinol (µg/g)	87.7±16.12^{a}	1	19.6±3.86^{b,*}	18.0±3.68^{b,*}	10.2±0.78^{b}	11.6±1.19^{b}
	87.7±16.12^{x}	2	12.7±2.16^{y,§}	11.0±1.72^{y,§}	6.5±1.07^{y}	8.2±0.74^{y}
α-tocopherol (µg/g)	9.2±0.54^{a}	1	8.8±0.98^{a,b}	7.7±0.44^{a,b}	6.4±0.44^{b}	6.9±0.41^{b}
	9.2±0.54^{x}	2	7.8±0.72^{x,y}	7.7±0.54^{x,y}	5.4±0.66^{y}	7.1±0.64^{x,y}
Apocarotenoid (µg/g)	12.3±0.84^{a}	1	11.6±2.17^{a}	9.9±1.74^{a,b}	3.3±0.88^{c}	5.5±1.33^{b,c}
	12.3±0.84^{x}	2	11.6±0.93^{x}	9.2±2.09^{x}	2.9±0.67^{y}	3.9±1.00^{y}
β-carotene (µg/g)	6.1±1.37^{a}	1	2.2±0.68^{a,b}	1.9±1.02^{b,*}	0.6±0.10^{b}	0.6±0.14^{b}
	6.1±1.37^{x}	2	1.1±0.47^{y}	0.3±0.10^{y,§}	0.0±0.01^{y}	0.0±0.02^{y}
β-carotene ester (µg/g)	4.2±1.56^{a}	1	0.8±0.24^{a,b,*}	0.4±0.07^{a,b}	0.2±0.03^{b}	0.2±0.03^{b}
	4.2±1.56^{x}	2	0.5±0.06^{x,y,§}	0.4±0.09^{x,y}	0.1±0.04^{x,y}	0.2±0.02^{y}
β-cryptoxanthin (µg/g)	1.9±0.48^{a}	1	0.3±0.16^{b}	0.3±0.12^{b}	0.0±0.00^{b}	0.0±0.02^{b}
	1.9±0.48^{x}	2	0.5±0.40^{y}	0.2±0.05^{y}	0.0±0.02^{y}	0.0±0.01^{y}
Lutein (µg/g)	17.8±6.29^{a}	1	1.1±0.27^{b}	1.5±0.66^{b}	0.2±0.03^{b}	0.6±0.20^{b}
	17.8±6.29^{x}	2	0.9±0.29^{y}	0.9±0.21^{y}	0.2±0.07^{y}	0.7±0.54^{y}
Lutein ester (µg/g)	1.8±0.49^{a}	1	0.5±0.17^{b}	0.4±0.06^{b}	0.1±0.03^{b}	0.1±0.02^{b}
	1.8±0.49^{x}	2	0.5±0.08^{y}	0.3±0.07^{y}	0.1±0.03^{y}	0.1±0.02^{y}
Zeaxanthin (µg/g)	11.6±4.62^{a}	1	0.4±0.09^{a,b}	0.7±0.38 ^{a,b,*}	0.1±0.02^{a,b}	0.2±0.09^{b}
	11.6±4.62^{x}	2	0.5±0.18^{x,y}	0.3±0.05^{x,y,§}	0.1±0.03^{x,y}	0.2±0.08^{y}
Zeaxanthin ester (µg/g)	2.1±0.57^{a}	1	0.4±0.10^{b}	0.3±0.08^{b}	0.0±0.01^{b}	0.1±0.01^{b}
	2.1±0.57^{x}	2	0.6±0.26^{x,y}	0.1±0.04^{y}	0.0±0.02^{y}	0.1±0.02^{y}

[1]The n for day 0 = 10 total animals. To best show the significant differences across the euthanasia dates for each dietary treatment, mean value for Day 0 is listed twice.
[2]Diet treatment 1 = crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet + supplemental produce (carrots and sweet potatoes); Diet treatment 2 = crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet without vitamin A or vitamin E supplementation.
[3]n for or each euthanasia day (22, 50, 81 & 119) was the same number (ex. day 22 had n=6 for diet treatment 1 and n=6 for diet treatment 2).
^{a,b,c} Diet treatment 1 means within the same row with these unique superscripts differ (P<0.05).
^{x,y} Diet treatment 2 means within the same row with these unique superscripts differ (P<0.05).
*,§ Diet treatments 1 and 2 differed between each other for a nutrient within a euthanasia timepoint if these superscripts are in that column (P<0.05).

Table 6. Cane Toad (*Rhinella marina*) Plasma Vitamin A, Vitamin E, and Carotenoid Concentrations (LS means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 119 in Human Care Consuming Two Different Diets.

Nutrient	Day 0[1] (n=10)[3]	Diet Trt[2]	Day 22 (n=6)	Day 50 (n=6)	Day 81 (n=6)	Day 119 (n=7)
Retinol (µg/g)	0.1±0.00	1	0.1±0.01	0.1±0.04	0.1±0.07*	0.1±0.01
	0.1±0.00^{x}	2	0.1±0.01^{x,y}	0.1±0.02^{x,y}	0.0±0.01^{y,§}	0.0±0.01^{y}
α-tocopherol (µg/g)	0.3±0.03	1	0.2±0.03	0.2±0.06	0.3±0.06	0.2±0.03
	0.3±0.03	2	0.2±0.02	0.3±0.06	0.3±0.02	0.2±0.04
β-carotene (µg/g)	0.5±0.10^{a}	1	0.2±0.02^{b*}	0.2±0.06^{b,*}	0.3±0.06^{b,*}	0.2±0.04^{b,*}
	0.5±0.10^{x}	2	0.0±0.01^{y,§}	0.0±0.00^{y,§}	0.0±0.00^{y,§}	0.0±0.01^{y,§}
β-carotene ester (µg/g)	0.6±0.10^{a}	1	0.0±0.01^{b}	0.0±0.01^{b}	0.0±0.01^{b}	0.0±0.00^{b}
	0.6±0.10^{x}	2	0.0±0.01^{y}	0.0±0.01^{y}	0.0±0.00^{y}	0.0±0.01^{y}
β-cryptoxanthin (µg/g)	0.4±0.14^{a}	1	0.0±0.01^{b}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}
	0.4±0.14^{x}	2	0.0±0.02^{y}	0.0±0.00^{y}	0.0±0.00^{y}	0.0±0.00^{y}
Lutein (µg/g)	1.4±0.24^{a}	1	0.1±0.02^{b}	0.1±0.05^{b}	0.0±0.01^{b}	0.0±0.01^{b}
	1.4±0.24^{x}	2	0.2±0.02^{y}	0.1±0.02^{y}	0.0±0.01^{y}	0.0±0.01^{y}
Lutein ester (µg/g)	0.3±0.05^{a}	1	0.0±0.00^{b}	0.0±0.01^{b}	0.0±0.00^{b}	0.0±0.00^{b}
	0.3±0.05^{x}	2	0.0±0.01^{y}	0.0±0.01^{y}	0.0±0.00^{y}	0.0±0.01^{y}
Zeaxanthin (µg/g)	0.7±0.15^{a}	1	0.1±0.01^{b}	0.1±0.02^{b}	0.0±0.00^{b}	0.0±0.00^{b}
	0.7±0.15^{x}	2	0.1±0.01^{y}	0.0±0.01^{y}	0.0±0.00^{y}	0.0±0.00^{y}
Zeaxanthin ester (µg/g)	0.2±0.03^{a}	1	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}
	0.2±0.03^{x}	2	0.0±0.00^{y}	0.0±0.00^{y}	0.0±0.00^{y}	0.0±0.00^{y}

[1]The n for day 0 = 10 total animals. To best show the significant differences across the euthanasia dates for each dietary treatment, mean value for Day 0 is listed twice.
[2]Diet treatment 1 = crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet + supplemental produce (carrots and sweet potatoes); Diet treatment 2 = crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet without vitamin A or vitamin E supplementation.
[3]n for or each euthanasia day (22, 50, 81 & 119) was the same number (ex. day 22 had n=6 for diet treatment 1 and n=6 for diet treatment 2).
[a,b]Diet treatment 1 means within the same row with these unique superscripts differ significantly ($P<0.05$).
[x,y]Diet treatment 2 means within the same row with these unique superscripts differ significantly ($P<0.05$).
[*,§] Diet treatments 1 and 2 differed between each other for a nutrient within a euthanasia timepoint if these superscripts are in that column ($P<0.05$).

Table 7. Cane Toad (*Rhinella marina*) Urinary Corticosterone (ng/mL) Concentrations (LS means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 119 in Human Care Consuming Two Different Diets.

	Day 0 (n=55)[1]	**Day 22 (n=48)**	**Day 50 (n=11)**	**Day 81 (n=25)**	**Day 119 (n=10)**
Diet Treatment 1[2]	1.7 ± 0.19^{a}	0.7 ± 0.12^{b}	$0.6 \pm 0.20^{a,b}$	$1.0 \pm 0.22^{a,b}$	$0.8 \pm 0.23^{a,b}$
Diet Treatment 2	1.7 ± 0.19^{x}	0.8 ± 0.20^{y}	$1.3 \pm 0.54^{x,y}$	$0.7 \pm 0.31^{x,y}$	$0.5 \pm 0.07^{x,y}$
Combined Treatments	1.7 ± 0.19^{a}	0.8 ± 0.11^{b}	$0.9 \pm 0.27^{a,b}$	0.9 ± 0.19^{b}	0.6 ± 0.09^{b}

[1]n for each day is the total number of animals from both diet treatments combined. To best show the significant differences across the euthanasia dates for each dietary treatment, mean of day 0 is listed twice.

[2]Diet 1 = crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet + supplemental produce (carrots and sweet potatoes); Diet 2 = crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet without vitamin A or vitamin E supplementation.

[a,b]Means within the same row with these unique superscripts differ significantly ($P<0.05$).

[x,y]Means within the same row with these unique superscripts differ significantly ($P<0.05$).

Table 8. Cane Toad (*Rhinella marina*) Blood Lactate (mmol/L) Concentrations (LS means ± SEM) as the Toads Adjusted from the Wild (Day 0) thru Day 119 in Human Care Consuming Two Different Diets[1].

Treatment	Day 0 (n=9)[2]	Day 22 (n=12)[3]	Day 50 (n=12)	Day 81 (n=12)	Day 119 (n=14)
Diet Treatment 1	2.8±1.25	6.7±2.08	2.9±0.95	3.1±0.83	3.6±1.47
Diet Treatment 2	2.8±1.25[a,b]	7.1±1.65[a]	1.7±0.48[b]	5.5±0.94[a,b]	2.5±0.65[b]
Combined Treatment Mean	2.8±1.25[x]	6.9±1.99[y]	2.5±0.60[x]	4.3±0.69[x,y]	3.0±0.79[x]

[1]Diet 1 = crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet + supplemental produce (carrots and sweet potatoes); Diet 2 = crickets gut-loaded with Mazuri® Hi Calcium Gut Loading Diet without vitamin A or vitamin E supplementation.
[2]The n for day 0 = 9 total animals. To best show the significant differences across the euthanasia dates for each dietary treatment, mean of day 0 is listed twice.
[3]n for or each euthanasia day (22, 50, 81 & 119) was the same number (ex. day 22 had n=6 for diet treatment 1 and n=6 for diet treatment 2).
[a,b] Means within the same row with these unique superscripts differ significantly ($P<0.05$).

Table 9a. Vitamin A, Vitamin E, and Carotenoid Analyses from Two Laboratories for Two Different Diet Treatments Fed to Brown House Crickets (*Acheta domestica*) and Analysis of the Brown House Crickets Fed Those Diet Treatments.[1]

Nutrient	Cricket Feed – ASU[2]		Cricket Feed-Craft[3]		Crickets	
	Diet 1[1]	Diet 2	Diet 1	Diet 2	Diet 1	Diet 2
Retinol (µg/g)	4.18	3.91	ND[4]	ND	5.13	6.16
α-tocopherol (µg/g)	3.90	3.74	ND	ND	3.27	5.13
Apocarotenoid (µg/g)	0.78	1.23	NT[5]	NT	1.75	3.37
β-carotene (µg/g)	0.01	0.03	0.03	0.07	2.38	0.09
β-carotene ester (µg/g)	0.03	0.05	NT	NT	0.00	0.00
β-cryptoxanthin (µg/g)	0.02	0.02	0.23	0.23	0.02	0.01
Lutein (µg/g)	0.51	0.54	1.03	1.05	0.51	0.15
Lutein Ester (µg/g)	0.03	0.05	NT	NT	0.00	0.00
Zeaxanthin (µg/g)	0.22	0.27	1.38	1.42	0.22	0.13
Zeaxanthin ester (µg/g)	0.02	0.04	NT	NT	0.00	0.00

[1]Diet 1 =Mazuri® Hi Calcium Gut Loading Diet + supplemental produce (carrots and sweet potatoes which are not included in the cricket feed analysis); Diet 2 = Mazuri® Hi Calcium Gut Loading Diet without vitamin A or vitamin E supplementation.
[2]ASU = McGraw Biology Laboratory, Arizona State University, Tempe, AZ.
[3]Craft = Craft Technologies, Inc. (Wilson, NC).
[4]ND = Non-detectable.
[5]NT = Not tested for.

Table 9b. Proximate Nutrient Analyses for Two Different Diet Treatments Fed to Brown House Crickets (*Acheta domestica*) and Analysis of the Brown House Crickets Fed Those Diet Treatments.[1]

Nutrient	Cricket Feed		Crickets	
	Diet 1	**Diet 2**	**Diet 1**	**Diet 2**
Crude Protein (%)	19.80	19.50	68.80	69.60
Acid detergent fiber (%)	9.00	9.90	15.90	18.40
Neutral Detergent Fiber (%)	18.50	18.40	ND[2]	ND[2]
Water Soluble Carbohydrates (%)	4.40	4.50	ND[2]	ND[2]
Starch (%)	21.80	21.30	ND[2]	ND[2]
Crude Fat (%)	3.90	3.90	ND[2]	ND[2]
Acid Hydrolysis Fat (%)	ND[2]	ND[2]	19.60	17.90
Crude Fiber (%)	7.20	6.90	ND[2]	ND[2]
Ash (%)	26.61	27.51	6.07	5.92
Calcium (%)	8.47	8.70	0.44	0.51
Phosphorus (%)	0.64	0.62	0.97	0.98
Magnesium (%)	0.26	0.26	0.15	0.14
Potassium (%)	0.98	0.94	1.22	1.23
Sodium (%)	0.14	0.19	0.41	0.43
Sulfur (%)	0.31	0.30	0.53	0.55
Iron (ppm)	333	328	57	63
Zinc (ppm)	82	76	184	181
Copper (ppm)	12	12	22	23
Manganese (ppm)	86	89	32	35
Molybdenum (ppm)	1.9	1.8	1.3	1.5

[1]Diet 1 = Mazuri® Hi Calcium Gut Loading Diet + supplemental produce (carrots and sweet potatoes which are not included in the cricket feed analysis); Diet 2 = Mazuri® Hi Calcium Gut Loading Diet without vitamin A or vitamin E supplementation.
[2] ND = Not determined

FIGURES

Figure 1. Whole Liver Sample Collected from Cane Toad (*Rhinella marina*).

Figure 2. Gastrointestinal Tract Collected from Cane Toad (*Rhinella marina*).

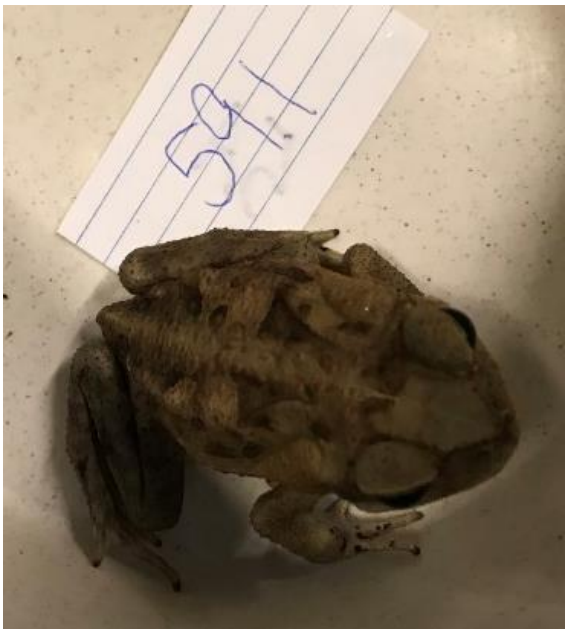

Figure 3.1: Cane Toad (*Rhinella marina*) 591 upon Arrival at the NC Zoo (July 12, 2018).

Figure 3.2: Cane Toad (*Rhinella marina*) 559 upon Arrival at the NC Zoo (July 12, 2018).

Figure 3.3: Cane Toad (*Rhinella marina*) 57A upon Arrival at the NC Zoo (July 12, 2018).

Figure 3.4: Cane Toad (*Rhinella marina*) 5B7 at Day 119 in Human Care (November 8, 2018).

Figure 3.5: Cane Toad (*Rhinella marina*) 57F at Day 119 in Human Care (November 8, 2018).

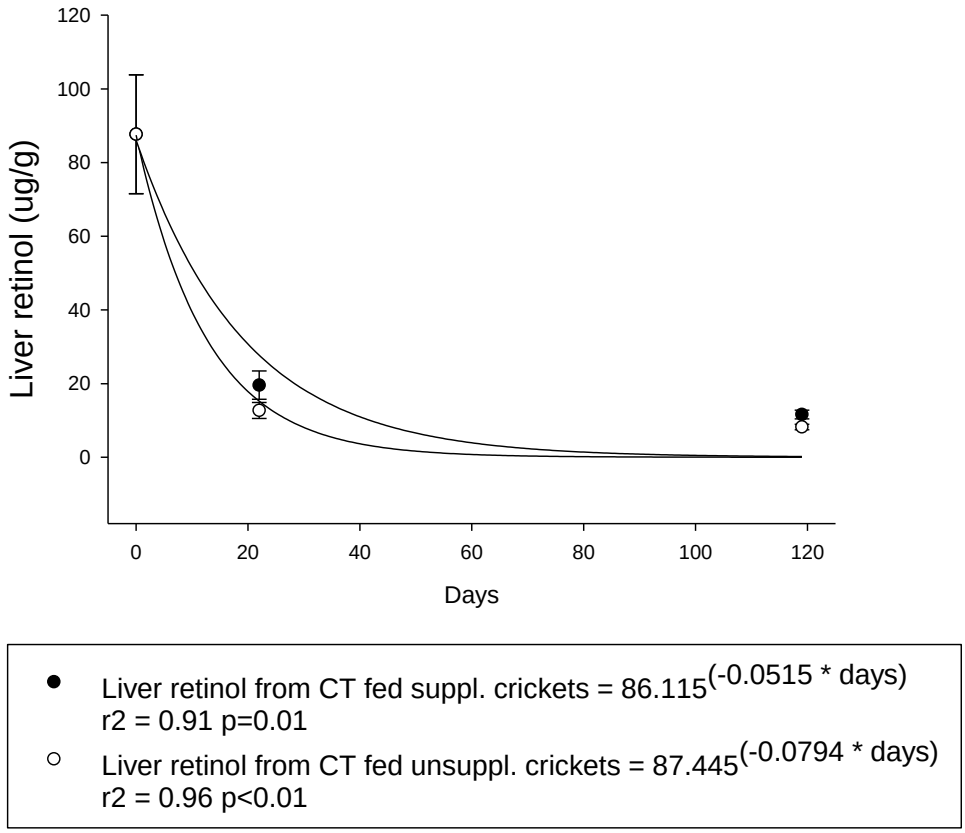

Figure 4.1. Decay Curves and Equations with Associated P-Values for Line Fit for Liver Retinol Concentrations over Time for Cane Toads (*Rhinella marina*) (CT) Fed Diet Treatment 1 (Supplemented (suppl.) Diet Fed to Brown House Crickets (*Acheta domesticus*)) and Diet Treatment 2 (Diet with No Supplementation (unsuppl.) Fed to Brown House Crickets).

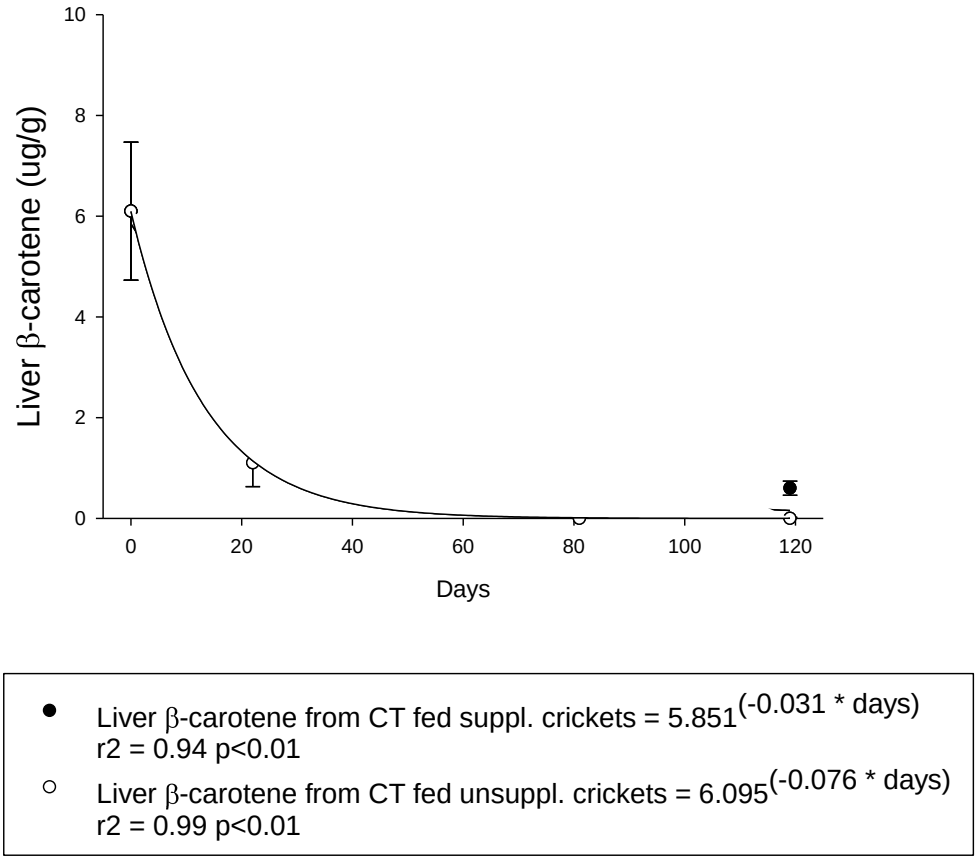

Figure 4.2. Decay Curves and Equations with Associated P-Values for Line Fit for Liver Beta-Carotene Concentrations over Time for Cane Toads (*Rhinella marina*) (CT) Fed Diet Treatment 1 (Supplemented (suppl.) Diet Fed to Brown House Crickets (*Acheta domesticus*)) and Diet Treatment 2 (Diet with No Supplementation (unsuppl.) Fed to Brown House Crickets).

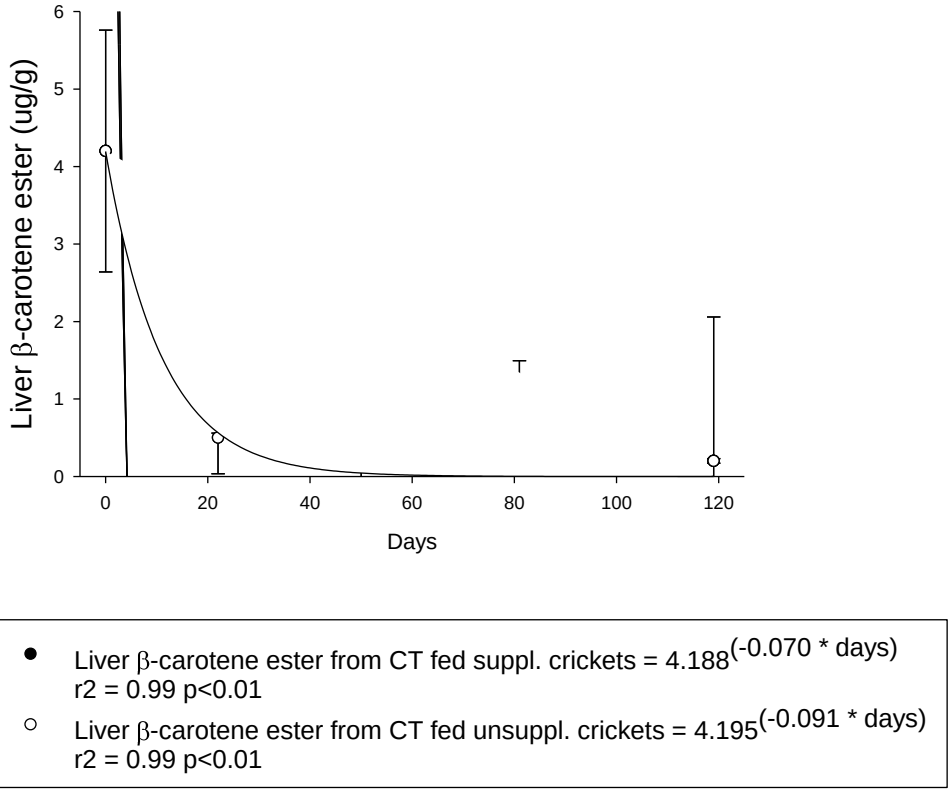

Figure 4.3. Decay Curves and Equations with Associated P-Values for Line Fit for Liver Beta-Carotene Ester Concentrations over Time for Cane Toads (*Rhinella marina*) (CT) Fed Diet Treatment 1 (Supplemented (suppl.) Diet Fed to Brown House Crickets (*Acheta domesticus*)) and Diet Treatment 2 (Diet with No Supplementation (unsuppl.) Fed to Brown House Crickets).

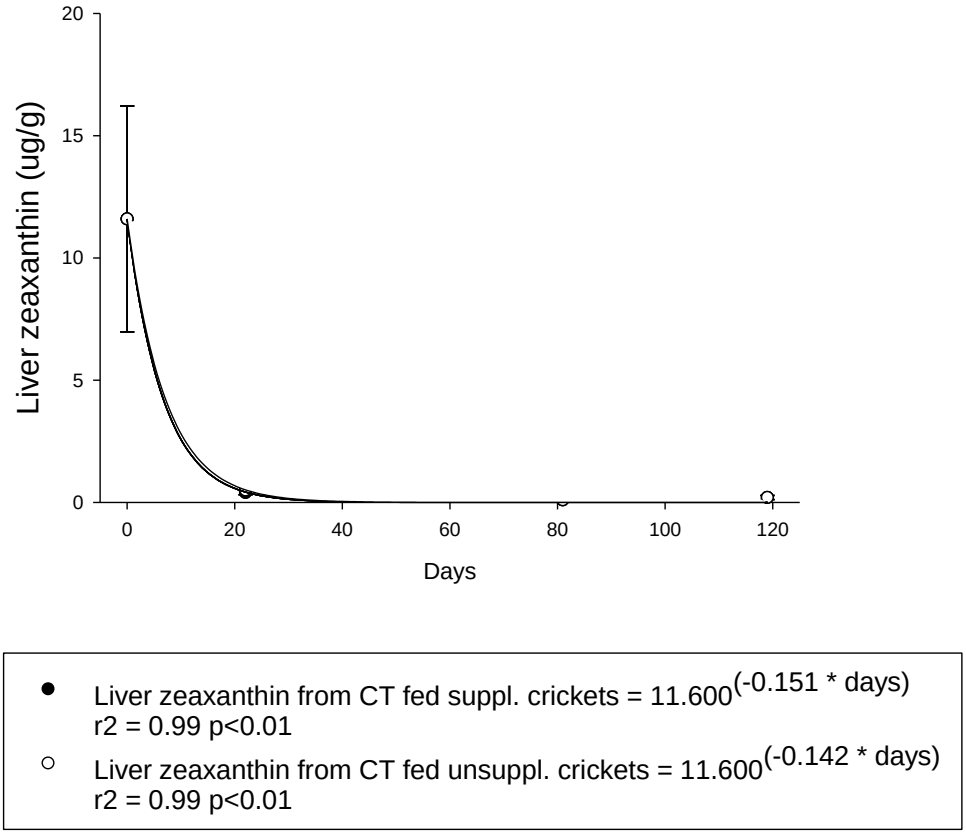

Figure 4.4. Decay Curves and Equations with Associated P-Values for Line Fit for Liver Zeaxanthin Concentrations over Time for Cane Toads (*Rhinella marina*) (CT) Fed Diet Treatment 1 (Supplemented (suppl.) Diet Fed to Brown House Crickets (*Acheta domesticus*)) and Diet Treatment 2 (Diet with No Supplementation (unsuppl.) Fed to Brown House Crickets).

APPENDICES

Appendix A

Cricket Feeding Data for Cane Toads (*Rhinella marina*) Enrolled in a Study at the NC Zoo Fed Diet Treatment 1[1].

	Housing Unit 1		**Housing Unit 2**		**Housing Unit 3**	
Date	**Toad #[2]**	**Cricket #[3]**	**Toad #**	**Cricket #**	**Toad #**	**Cricket #**
7/12/2018	10	70	9	60	9	60
7/13/2018	10	0	9	0	9	0
7/14/2018	10	0	9	0	9	0
7/15/2018	10	0	9	0	9	0
7/16/2018	10	0	9	0	9	0
7/17/2018	10	0	9	2	9	0
7/18/2018	10	0	9	0	9	0
7/19/2018	10	0	9	0	9	0
7/20/2018	10	0	9	1	9	0
7/21/2018	10	0	9	1	9	2
7/22/2018	10	0	9	0	9	0
7/23/2018	10	0	9	0	9	0
7/24/2018	10	0	9	0	9	0
7/25/2018	10	0	9	2	9	1
7/26/2018	10	0	9	0	9	0
7/27/2018	10	1	9	0	9	0
7/28/2018	10	5	9	2	9	0
7/29/2018	10	0	9	0	9	0
7/30/2018	10	0	9	0	9	0
7/31/2018	10	0	9	0	9	1
8/1/2018	10	0	9	0	9	2
8/2/2018	10	2	9	0	9	0
8/3/2018	8	0	7	0	7	0
8/4/2018	8	0	7	0	7	0
8/5/2018	8	10	7	0	7	0
8/6/2018	8	0	7	0	7	0
8/7/2018	8	1	7	0	7	0
8/8/2018	8	13	7	4	7	60
8/9/2018	8	0	7	7	7	30
8/10/2018	8	0	7	40	7	25
8/11/2018	8	0	7	2	7	3
8/12/2018	8	0	7	0	7	0
8/13/2018	8	0	7	0	7	0
8/14/2018	8	0	7	4	7	4

8/15/2018	8	0	7	2	7	16
8/16/2018	8	0	7	1	7	5
8/17/2018	8	0	7	4	7	70
8/18/2018	8	3	7	2	7	4
8/19/2018	8	1	7	1	7	30
8/20/2018	8	1	7	1	7	25
8/21/2018	8	0	7	0	7	0
8/22/2018	8	0	7	0	7	0
8/23/2018	8	0	7	0	7	0
8/24/2018	8	1	7	1	7	1
8/25/2018	8	1	7	0	7	1
8/26/2018	8	1	7	0	7	4
8/27/2018	7	6	7	25	7	4
8/28/2018	7	0	7	3	7	1
8/29/2018	7	1	7	7	7	0
8/30/2018	7	0	7	0	7	0
8/31/2018	7	0	7	0	combined into tubs 1 & 2	combined into tubs 1 & 3
9/1/2018	7	0	7	0	-	-
9/2/2018	7	1	7	2	-	-
9/3/2018	7	0	7	2	-	-
9/4/2018	7	0	7	0	-	-
9/5/2018	7	0	7	3	-	-
9/6/2018	7	0	7	30	-	-
9/7/2018	7	0	7	0	-	-
9/8/2018	7	0	6	15	-	-
9/9/2018	7	0	6	0	-	-
9/10/2018	7	0	6	0	-	-
9/11/2018	7	1	6	1	-	-
9/12/2018	7	0	6	25	-	-
9/13/2018	7	0	6	10	-	-
9/14/2018	7	0	6	0	-	-
9/15/2018	7	1	6	40	-	-
9/16/2018	7	0	6	20	-	-
9/17/2018	7	0	6	2	-	-
9/18/2018	7	0	6	4	-	-
9/19/2018	7	0	6	6	-	-
9/20/2018	7	0	6	0	-	-
9/21/2018	7	1	6	3	-	-

9/22/2018	7	1	6	1	-	-
9/23/2018	7	0	6	5	-	-
9/24/2018	7	0	6	0	-	-
9/25/2018	7	0	6	10	-	-
9/26/2018	7	4	6	20	-	-
9/27/2018	7	6	6	70	-	-
9/28/2018	7	35	6	40	-	-
9/29/2018	7	60	6	60	-	-
9/30/2018	7	4	6	50	-	-
10/1/2018	7	0	combined to tub 1	combined to tub 1	-	-
10/2/2018	7	20	-	-	-	-
10/3/2018	7	6	-	-	-	-
10/4/2018	7	3	-	-	-	-
10/5/2018	7	30	-	-	-	-
10/6/2018	7	3	-	-	-	-
10/7/2018	7	2	-	-	-	-
10/8/2018	7	6	-	-	-	-
10/9/2018	7	0	-	-	-	-
10/10/2018	7	2	-	-	-	-
10/11/2018	7	4	-	-	-	-
10/12/2018	7	0	-	-	-	-
10/13/2018	7	5	-	-	-	-
10/14/2018	7	20	-	-	-	-
10/15/2018	7	13	-	-	-	-
10/16/2018	7	30	-	-	-	-
10/17/2018	7	10	-	-	-	-
10/18/2018	7	2	-	-	-	-
10/19/2018	7	7	-	-	-	-
10/20/2018	7	40	-	-	-	-
10/21/2018	7	30	-	-	-	-
10/22/2018	7	9	-	-	-	-
10/23/2018	7	1	-	-	-	-
10/24/2018	7	1	-	-	-	-
10/25/2018	7	10	-	-	-	-
10/26/2018	7	10	-	-	-	-
10/27/2018	7	20	-	-	-	-
10/28/2018	7	0	-	-	-	-
10/29/2018	7	6	-	-	-	-
10/30/2018	7	12	-	-	-	-

10/31/2018	7	1	-	-	-	-
11/1/2018	7	40	-	-	-	-
11/2/2018	7	20	-	-	-	-
11/3/2018	7	5	-	-	-	-
11/4/2018	7	2	-	-	-	-
11/5/2018	7	1	-	-	-	-
11/6/2018	7	6	-	-	-	-
11/7/2018	7	2	-	-	-	-
11/8/2018	-	-	-	-	-	-

[1] Diet treatment 1 = Mazuri® Hi Calcium Gut Loading Diet + produce (carrots and sweet potatoes)
[2] Toad # = number of toads per tub
[3] Cricket # = number of crickets remaining after feeding

Appendix B

Cricket Feeding Data for Cane Toads (*Rhinella marina*) Enrolled in a Study at NC Zoo Fed Diet Treatment 2[1].

	Housing Unit 4		**Housing Unit 5**		**Housing Unit 6**	
Date	**Toad #[2]**	**Cricket #[3]**	**Toad #**	**Cricket #**	**Toad #**	**Cricket #**
7/12/2018	9	60	9	60	9	60
7/13/2018	9	1	9	0	9	0
7/14/2018	9	0	9	1	9	0
7/15/2018	9	0	9	0	9	0
7/16/2018	9	0	9	0	9	0
7/17/2018	8	0	9	0	9	0
7/18/2018	8	0	9	3	9	0
7/19/2018	8	0	9	0	9	0
7/20/2018	8	0	9	0	9	0
7/21/2018	8	0	9	1	9	2
7/22/2018	8	0	9	0	9	0
7/23/2018	8	0	9	0	9	0
7/24/2018	8	0	9	0	9	0
7/25/2018	8	2	9	0	9	1
7/26/2018	8	4	9	0	9	0
7/27/2018	8	0	9	0	9	0
7/28/2018	8	1	9	0	9	0
7/29/2018	8	0	9	0	9	0
7/30/2018	8	0	9	0	9	0
7/31/2018	8	0	9	1	9	1
8/1/2018	8	0	9	0	9	2
8/2/2018	8	0	9	0	9	0
8/3/2018	6	0	7	0	7	0
8/4/2018	6	0	7	0	7	0
8/5/2018	6	0	7	0	7	0
8/6/2018	6	0	7	0	7	0
8/7/2018	6	2	7	2	7	1
8/8/2018	6	4	7	12	7	60
8/9/2018	6	5	7	0	7	30
8/10/2018	6	4	7	5	7	25
8/11/2018	6	0	7	3	7	3
8/12/2018	6	0	7	0	7	0
8/13/2018	6	0	7	0	7	0
8/14/2018	6	0	7	0	7	4

8/15/2018	6	0	7	0	7	16
8/16/2018	6	1	7	0	7	5
8/17/2018	6	10	7	10	7	70
8/18/2018	6	1	7	0	7	4
8/19/2018	6	1	7	0	7	30
8/20/2018	6	7	7	0	7	25
8/21/2018	6	0	7	0	7	0
8/22/2018	6	0	7	0	7	0
8/23/2018	6	0	7	0	7	0
8/24/2018	6	1	7	2	7	2
8/25/2018	6	0	7	0	7	0
8/26/2018	6	2	7	1	7	7
8/27/2018	6	15	7	6	7	30
8/28/2018	6	1	7	1	7	4
8/29/2018	6	1	7	0	7	1
8/30/2018	6	1	7	0	7	1
8/31/2018	6	0	7	0	combined into tubs 4 & 5	combined into tubs 4 & 6
9/1/2018	6	0	7	0	-	
9/2/2018	6	2	7	1	-	
9/3/2018	6	2	7	0	-	
9/4/2018	6	1	7	0	-	
9/5/2018	7	0	7	0	-	
9/6/2018	7	1	7	0	-	
9/7/2018	7	0	7	2	-	
9/8/2018	7	0	7	0	-	
9/9/2018	7	0	7	2	-	
9/10/2018	7	0	7	1	-	
9/11/2018	7	0	7	0	-	
9/12/2018	7	30	7	15	-	
9/13/2018	7	0	7	0	-	
9/14/2018	7	0	7	0	-	
9/15/2018	7	20	7	10	-	
9/16/2018	7	10	7	6	-	
9/17/2018	7	0	7	2	-	
9/18/2018	7	0	7	3	-	
9/19/2018	7	0	7	0	-	
9/20/2018	7	0	7	0	-	
9/21/2018	7	0	7	7	-	

9/22/2018	7	0	7	3	-	
9/23/2018	7	0	7	0	-	
9/24/2018	7	0	7	0	-	
9/25/2018	7	30	7	30	-	
9/26/2018	7	7	7	9	-	
9/27/2018	7	20	7	12	-	
9/28/2018	7	31	7	7	-	
9/29/2018	7	40	7	40	-	
9/30/2018	7	1	7	5	-	
10/1/2018	7	0	combined to tub 4	combined to tub 4	-	
10/2/2018	8	5	-	-	-	
10/3/2018	8	1	-	-	-	
10/4/2018	8	1	-	-	-	
10/5/2018	8	30	-	-	-	
10/6/2018	8	0	-	-	-	
10/7/2018	8	0	-	-	-	
10/8/2018	8	1	-	-	-	
10/9/2018	8	0	-	-	-	
10/10/2018	8	1	-	-	-	
10/11/2018	8	1	-	-	-	
10/12/2018	8	3	-	-	-	
10/13/2018	8	0	-	-	-	
10/14/2018	8	0	-	-	-	
10/15/2018	8	28	-	-	-	
10/16/2018	8	0	-	-	-	
10/17/2018	8	0	-	-	-	
10/18/2018	8	3	-	-	-	
10/19/2018	8	2	-	-	-	
10/20/2018	8	50	-	-	-	
10/21/2018	8	45	-	-	-	
10/22/2018	8	20	-	-	-	
10/23/2018	8	4	-	-	-	
10/24/2018	8	5	-	-	-	
10/25/2018	8	20	-	-	-	
10/26/2018	8	26	-	-	-	
10/27/2018	8	20	-	-	-	
10/28/2018	8	0	-	-	-	
10/29/2018	8	6	-	-	-	
10/30/2018	8	12	-	-	-	

10/31/2018	8	5	-	-	-	
11/1/2018	8	50	-	-	-	
11/2/2018	8	20	-	-	-	
11/3/2018	8	10	-	-	-	
11/4/2018	8	5	-	-	-	
11/5/2018	8	0	-	-	-	
11/6/2018	8	10	-	-	-	
11/7/2018	8	4	-	-	-	
11/8/2018	-	-	-	-	-	

[1] Diet treatment 2 = Mazuri® Hi Calcium Gut Loading Diet with no vitamin A, vitamin E, or carotenoid supplementation
[2] Toad # = number of toads per tub
[3] Cricket # = number of crickets remaining after feeding