EVALUATING THE CLINICAL AND PHYSIOLOGICAL EFFECTS OF LONG TERM ULTRAVIOLET B RADIATION ON GUINEA PIGS (*CAVIA PORCELLUS*) AND RABBITS (*ORYCTOLAGUS CUNICULUS*)

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THESIS

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ABSTRACT

Vitamin D is an important hormone in vertebrates. Most animals acquire this hormone through their diet, exposure to ultraviolet B (UVB) radiation and subsequent photobiochemical synthesis, or a combination thereof. The objectives for this research were to evaluate the clinical and physiologic effects of artificial UVB light supplementation on rabbits (*Oryctolagus cuniculus*) and guinea pigs (*Cavia porcellus*) and to evaluate the long-term safety of artificial UVB light supplementation over the course of six months in these species. Twelve juvenile acromelanin albino Hartley guinea pigs and twelve juvenile New Zealand white rabbits were randomly assigned to one of two treatment groups: Group A was exposed to 12 hours of artificial UVB radiation daily and Group B received ambient fluorescent light with no UVB supplementation for 12 hours daily. Animals in both groups were offered the same diet and housed under the same conditions. Blood samples were collected every three weeks over six months to measure blood chemistry values, parathyroid hormone, ionized calcium, and serum 25-hydroxyvitamin D$_3$ (25-OHD$_3$) levels. Serial ophthalmologic examinations were performed at the beginning of the study and every two months thereafter. Computed tomography (CT) scans were performed at beginning and conclusion of the study to assess bone mineral density. Dual energy x-ray absorptiometry (DEXA) scans were performed at the conclusion of the study. At the end of the study the animals were euthanized and necropsied. Mean ± SD serum 25-OHD$_3$ concentrations differed significantly in both the guinea pigs (p < 0.0001) and rabbits (p= 0.003) between animals provided supplemental UVB radiation (Guinea pig: 101.49 ± 21.81 nmol/L; Rabbit: 83.12 ± 22.44 nmol/L) and those not provided supplemental UVB radiation (Guinea pig: 36.33 ± 24.42 nmol/L; Rabbit: 39.33± 26.07 nmol/L). No significant difference in bone mineral density was noted between groups on CT scans or the DEXA scan. An increased corneal
thickness in both eyes was found in guinea pigs supplemented with UVB compared to those provided ambient light (right eye [OD]: F=149.527, p<0.0001; left eye [OS]: F=30.525, p=<0.0001). There were no apparent negative clinical or pathologic side effects noted between the treatment and control animals. This study found that exposing guinea pigs and rabbits to UVB radiation long term significantly increased their circulating serum 25-OHD₃ levels, and that this increase was sustainable over time. In vertebrates, vitamin D is an essential hormone that regulates many different functions in the body and can be protective against various disease conditions. Providing guinea pigs, rabbits, or other diurnal rodents, exposure to UVB may be an important husbandry consideration that is not currently recommended.
# TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION ........................................................................................................ 1

CHAPTER 2: LITERATURE REVIEW ......................................................................................... 4
  2.1 History in Captivity ........................................................................................................... 4
    2.1.1 Rabbits .................................................................................................................... 4
    2.1.2 Guinea pigs .............................................................................................................. 6
  2.2 Husbandry Recommendations ...................................................................................... 7
    2.2.1 Rabbits .................................................................................................................... 7
    2.2.2 Guinea pigs .............................................................................................................. 10
  2.3 Vitamin D ...................................................................................................................... 12
    2.3.1 Acquisition of vitamin D and calcium ..................................................................... 14
    2.3.1.1 Synthesis in skin ................................................................................................. 15
    2.3.1.2 Dietary ............................................................................................................... 17
    2.3.2 Ultraviolet B radiation ............................................................................................ 19
    2.3.3 Calcium .................................................................................................................. 20
    2.3.4 Measures of vitamin D status ............................................................................... 21
    2.3.4.1 Assays ................................................................................................................ 21
    2.3.4.2 Bone density ....................................................................................................... 22
  2.4 Medical Problems in Captivity ..................................................................................... 24
    2.4.1 Hypovitaminosis D ................................................................................................. 24
    2.4.2 Rabbit and rodent specific diseases ....................................................................... 25
  2.5 Ultraviolet B Radiation ................................................................................................. 27
    2.5.1 Use in companion animals .................................................................................... 27
    2.5.2 Previously reported adverse effects ...................................................................... 31
    2.5.2.1 Ocular ............................................................................................................... 31
    2.5.2.2 Dermatologic .................................................................................................... 32
    2.5.2.3 Toxicity ............................................................................................................. 33

CHAPTER 3: EVALUATING THE CLINICAL AND PHYSIOLOGICAL EFFECTS OF
LONG TERM ULTRAVIOLET B RADIATION ON GUINEA PIGS (CAVIA PORCELLUS)
  3.1 Introduction .................................................................................................................... 37
  3.2 Materials and Methods ................................................................................................. 40
  3.3 Results ............................................................................................................................ 49
  3.4 Discussion ........................................................................................................................ 53
  3.5 Conclusions .................................................................................................................... 65

CHAPTER 4: EVALUATING THE CLINICAL AND PHYSIOLOGICAL EFFECTS OF
LONG TERM ULTRAVIOLET B RADIATION ON RABBITS (ORYCTOLAGUS
CUNICULUS)
  4.1 Introduction .................................................................................................................... 66
  4.2 Materials and Methods ................................................................................................. 69
  4.3 Results ............................................................................................................................ 77
CHAPTER 1
INTRODUCTION

Rabbits (*Oryctolagus cuniculus*) and guinea pigs (*Cavia porcellus*) are important animals to the pet, research, and production industries throughout the world. Therefore, it is important that their surroundings and environment are optimized for their overall health and well-being. Husbandry is particularly important to the overall health and longevity of many exotic species, with guinea pigs and rabbits being no exception. Many of the husbandry recommendations previously published for these species have been based on attempts to mimic or reproduce the animal’s natural environment or diet. However, when considering the life history of rabbits and guinea pigs throughout the world, and the fact that these animals are naturally diurnal, little recognition has been given to the requirements or potential benefits these animals may derive from sunlight, or more specifically ultraviolet B (UVB) radiation. Guinea pigs in particular are a high altitude species exposed to large amounts of sunlight and UVB radiation under natural conditions. It is interesting to note that while these animals have evolved to function in a diurnal setting with exposure to sunlight and natural UVB radiation as a potential source of vitamin D, in captivity we do not routinely provide these animals with access to sunlight, or even artificial UVB supplementation. When considering the many different physiological functions in vertebrates that are intimately related to vitamin D and calcium metabolism, it should be considered that disease processes or problems with these species in captivity could be related to inadequate levels or supplementation.

Vitamin D is a circulating hormone that is important to many bodily functions, including bone development, growth, neuromuscular function, reproduction, cardiovascular health, and immune function (Holick, 2007; Stumpf et al., 1979). Vitamin D deficiencies in humans and
many vertebrate species have been shown to cause rickets, osteomalacia, and reproductive failure. Not only do deficiencies lead to these specific disease processes, it is also becoming more apparent that vitamin D levels are important to overall health. Vitamin D receptors are found in numerous tissues throughout the body. In humans, adequate levels of vitamin D have been shown to decrease the risk of developing many different conditions, including diabetes, muscular dystrophy, hypertension, and inflammatory bowel disease, to name a few (Holick, 2014).

While acquisition and production of vitamin D is highly variable across vertebrate species, pilot studies have shown that rabbits and guinea pigs housed indoors have the ability to synthesize vitamin D in the epidermis secondary to exposure to artificial UVB light over a short-term period. Since many rabbits and guinea pigs are housed indoors and not exposed to natural or artificial UVB light, it is possible that these animals may experience chronic vitamin D deficiencies. Vitamin D deficiencies may be more common in many species than previously thought. It is estimated that one billion people worldwide may be at risk for vitamin D deficiency (Holick, 2007). Inappropriate levels of this essential nutrient and hormone could contribute to common disease processes in these animals such as dental disease, cardiovascular disease, or impaired immune function. Hypovitaminosis D has also been shown to be common in human medical inpatients, regardless of risk factors for disease or daily intake (Thomas et al., 1998). Many veterinary clinical patients may also be at a similar risk, and it should be considered that vitamin D deficiency could play a role in prolonged healing or recovery times.

While the benefits of UVB exposure for captive animals appear to be strong, it is not without risk. UVB radiation has been associated with the development of skin neoplasia, with the groups at greatest risk including humans with fair complexions and albino or white animals
(Dorn et al., 1971; Gallagher and Lee, 2006). Direct UVB radiation also has the potential to cause structural damage to the eye at the level of the cornea, lens, or retina. Direct UVB radiation may also cause short-term damage such as photodermatitis and erythema. For these reasons, the safety of UVB supplementation in small mammals should be investigated.

The objectives for this research were to evaluate the clinical and physiologic effects of artificial UVB light supplementation on rabbits and guinea pigs and to evaluate the long-term safety of artificial UVB light supplementation over the course of six months in these species. The biological hypotheses for this study were: 1) animals supplemented with UVB light would produce higher levels of vitamin D than those without supplementation, and that 2) there would be no significant detrimental side effects associated with UVB light supplementation.
CHAPTER 2
LITERATURE REVIEW

2.1 History in captivity

2.1.1 Rabbits

The domestic rabbit is likely a descendant of the European rabbit. The Normans, who kept them in warrens and harvested them for meat and fur, first introduced rabbits to Britain in the conquest of 1066 (Morton et al., 1993). Rabbits belong to the order Lagomorpha, distinguishing them from rodents such as rats (Rattus norvegicus) and guinea pigs. Lagomorphs have a second set of maxillary incisors caudal to the first set, otherwise known as the peg teeth (Vennen and Mitchell, 2009).

Rabbits are popular pets in the United States (U.S.), with 3.2 million animals being documented in U.S. households in a 2012 American Veterinary Medical Association survey (JAVMA, 2008). Rabbits are also appealing as project animals for kids to raise for groups such as 4H and are often shown in state fairs. The popularity of rabbits can be tied to their docile nature, unique personalities, and relatively long life span in comparison to other small exotic mammals. Typically, the life span of rabbits in captivity is 6-13 years (Vennen and Mitchell, 2009). Often, rabbit owners are very dedicated to the health and well-being of their rabbits, making them one of the most common exotic pet species presented to veterinary practices.

Rabbits have a long tradition in human and veterinary research. Their role as translational models has been essential to many of the common cures developed in human medicine. Many of the research advantages noted with rabbits are related to parallels in organ system function with humans. The immune system is one such parallel, and a common reason rabbits are used to develop immune based assays for human diseases (de Baetselier et al., 1980; Richerson, 1974).
Rabbits have also been used extensively as research subjects in numerous areas such as aging in humans, arteriosclerosis, angiogenesis, hypercholesterolemia, and ocular diseases, to name a few (Fox, 1980). Another advantage of rabbits as an animal model is that they can be purpose bred to study specific diseases and for optimizing sample collection. These rabbits are often bred for special characteristics such as minimizing adverse reaction to venipuncture, and larger, more easily accessible veins in the ears to allow for catheterization. Specific pathogen free rabbits can also be bred for the purpose of studying infectious diseases and drug administration. Recently, rabbits have been proposed as models for human growth and bone physiology, due to the fact that they reach skeletal maturity and peak bone mass after growth (Norris et al., 2001). Rabbits reach true skeletal maturity, as defined by the closure of the epiphyseal growth plates, between 24 and 32 weeks of age, with more recent data pointing to maturation at 28 weeks of age (Norris et al., 2001). Because of the importance of rabbits as research models, it is important that their basic physiologic needs are met; otherwise, deficiencies may introduce bias into the research results.

Rabbits are an important aspect of food animal production around the world due to the ease of production with small amounts of land, and the appeal across the workforce, including small land farmers and the elderly. These animals have been utilized for meat production and fur since the Middle Ages, although rabbit production did not become well established until the nineteenth century (Lebas et al., 1997). Currently, Europe accounts for 75% of the world’s production. Rabbits are useful in sustaining small farms and households in impoverished nations without ready access to other larger livestock. Backyard rabbitries are an answer to many sustainable development projects. Rabbit production is profitable and easy to integrate because these animals are highly fecund and multiparous; amenable to a simple, inexpensive cellulose
based diet; and they are easily transportable to market. For human consumption, rabbit meat is highly nutritious and low in fat (Lebas et al., 1997). In efficient food production systems, rabbits can turn 20 percent of the proteins they eat into profitable meat.

2.1.2 Guinea pigs

Guinea pigs are members of the Caviidae family of the order Rodentia. Guinea pigs were domesticated as early as 5000 B.C., and were widespread across South America in 2500 B.C. (Pigièr et al., 2012). Guinea pigs are native to the Andes and mountainous regions of South America; however, there is evidence they were brought back to Spain from the New World in the 1500s and introduced to Europe. While used predominately for food in South American countries such as Peru, Colombia, Venezuela, and Brazil, guinea pigs were kept as pets in Europe and never obtained popularity as a food source elsewhere in the world. A recent discovery of a guinea pig skeleton in Belgium showed consistencies that the guinea pig was bred for companionship vs. food consumption. Certain traits such as multicolored and white hair are associated with European domestication (Pigièr et al., 2012).

Guinea pigs are popular pets in the United States. They can be easily obtained from breeders, as well as retail pet stores. In a survey performed by the AVMA in 2012, there were over 1.3 million guinea pigs being kept as pets in the United States (JAVMA, 2008). Along with their popularity as pets, guinea pigs are also bred for competitive shows and judged based on size, hair coat, and breed. Guinea pigs are docile, responsive animals with a lively personality, and therefore make appealing pets for most households. Their life span in captivity is typically 5-7 years. Although they can scratch when cornered, they rarely bite and do not jump. Guinea pigs are unique in that they can produce many different vocalizations that are well characterized and often readily identified by many owners.
Guinea pigs have been used in research for over 400 years; hence, the familiar term or phrase “guinea pig” used to describe someone or something used as an experimental subject (Merriam-Webster, 2011). Guinea pigs are relatively easy to maintain within research facilities. Due to their smaller size, their space requirements are smaller in comparison to rabbits. They are also smaller and easier to handle. Because a guinea pig typically stands still as a defense mechanism when threatened, they lend themselves well to various research projects (Quesenberry et al., 2012).

Guinea pigs, commonly referred to as cavies in food production, are an important dietary constituent in different cultures in South and Central America. The guinea pig was first domesticated for food production in the Andes, where they are looked upon as a typical source of meat and protein. Similar to rabbits, they are an ideal meat source for households and families that may not have access to refrigeration, as a single family can finish it in one meal without waste. Also, similar to rabbits, cavy meat is low in fat content (Martin, 1998). Cavies are fairly prolific breeders, with one pair producing as many as 260 additional pairs in 2 years (Martin, 1998). A sow typically has 1-2 precocious piglets. While the original cavies used in production likely originated from a wild species, large size has been selected for over time to optimize meat production.

2.2 Husbandry recommendations

2.2.1 Rabbits

Current husbandry recommendations for pet rabbits are based on their physiologic needs and mimic conditions they would experience in a natural habitat (e.g., diet, temperature, etc.). Because of their unique gastrointestinal anatomy and physiology, diet makes up a large portion
of the recommendations for proper husbandry. Rabbits are hindgut fermenters, with a large cecum, and therefore have dietary needs that are unique in comparison to small companion animals. If given too much sugar or carbohydrates, the pH of their gastrointestinal tract can become acidic, leading to stasis and enterotoxemia. Rabbits require a large amount of fiber and roughage in their diet, specifically in the form of long-stem grass hay (e.g., timothy, prairie, or oat brome hay). Long-stem grass hay not only provides the substrate needed by the hindgut bacteria to produce volatile fatty acids, but also provides adequate substance for mastication and wearing down of the elodontic teeth. Pellets should comprise no more than 1 ounce per kilogram of body weight daily. Pellets fed should be high in fiber (>20%), low in protein (<16%), and low in fat (<2%) (Fisher, 2010). Pet rabbit diets may also be supplemented with leafy green vegetables (Vennen and Mitchell, 2009; Irlbeck, 2001). The optimal dietary vitamin D content for rabbits has been reported to be 800-1200 IU/kg (Lowe, 1998). If concentrations are greater than 2000 IU/kg, problems can develop, such as abnormal calcification of soft tissues; however, levels as low as 1000 IU/kg of vitamin D have also been shown to result in dystrophic mineralization in rabbits (Kamphues, 1991; Lebas, 2000). Rabbits have a higher water requirement than most mammals, with 80-100 mL/kg/day being required (Vella and Donnelly, 2012). Rabbits not provided sufficient water are more susceptible to gastrointestinal disorders (e.g., gastric stasis, ileus).

Cecotrophs are another unique aspect of rabbit gastrointestinal physiology. Rabbits ingest cecotrophs, or “night feces”, to gain important nutritional and microbiological constituents produced in the large intestine. These cecotrophs are formed from fermentation of non-fiber ingesta in the diet and contain volatile fatty acids, vitamins, amino acids, and microorganisms important to gastrointestinal health. Rabbits with gastrointestinal disease may not produce
Cecotrophs, leading to further compromise, and obese rabbits may not be able to acquire cecotrophs because of their inability to reach their perianal area. Because of the complexities of the gastrointestinal tract of rabbits, and their dependency on the nutritional and microbiological products produced in cecotrophs, it is essential for those working with these animals to ensure that rabbits produce and access their cecotrophs (Garcia et al., 2000; Davies and Davies, 2003).

Currently, there are no specific lighting recommendations for captive rabbits, other than a standard 12 hour photoperiod (Vennen and Mitchell, 2009), which is interesting considering the amount of effort placed on developing management plans for these animals in captivity. As crepuscular/diurnal animals, rabbits have evolved to be exposed to regular photoperiods within a circadian rhythm (24 hour cycle). Based on this, it is important that more specific lighting recommendations be developed. These lighting recommendations should not only consider photoperiod but the type of light exposure too (e.g., ultraviolet radiation exposure).

For rabbits used in research and industry, guidelines have been developed to manage the overall health and welfare of these animals, including minimum cage requirements, ideal bedding, diet, and enrichment. The Guide for the Care and Use of Laboratory Animals is produced by the National Research Council of the National Academies and contains recommendations for the care of animals commonly used in research facilities (National Research Council, 2011). Working groups are often established to reevaluate current recommendations and adapt them based on recent research in the lab animal field. The last major publication with recommendations for refinement in rabbit husbandry was in 1993 (Morton et al., 1993). Caging recommendations have changed throughout the years from singly housing animals in wooden cages, to wire, galvanized caging, and a more recent shift towards group or social housing in pens or stainless steel cages. While rabbits are recognized as social
animals and group housing is currently being recommended, many issues may make this impossible for a particular project or for veterinary care reasons. Group housing can be used to reduce stress by allowing animal interaction, and when possible the housing should be complex and allow for distraction or escape from other animals. Also, aggression issues may cause injury, and proper husbandry such as cleanliness and adequate amounts of food and water should be considered (National Research Council, 2004). Typically, intact males are more difficult to house together than intact females, particularly if there are females nearby (Morton et al., 1993). To achieve the best success with group housing, rabbits should be around the same age cohort and sex, and the group housing should be initiated shortly after weaning. Weaning typically occurs around 4-6 weeks. If group housing in pens is not possible, cage height is an important factor that should be considered. Rabbits spend a great deal of time on their hind legs with their ears perked up, inspecting their environment. Currently, based on the Guide (National Research Council, 2011), recommendations include allocating a rabbit less than 4 kg approximately 2800 cm\(^2\) of floor space and approximately 35.5 cm of vertical space. Unfortunately, this does not take into account life stage of the rabbit (National Research Council, 2004). Although standards vary by facility, all need to be approved by the institutional animal care and use committee (IACUC).

2.2.2 Guinea pigs

Current husbandry recommendations for guinea pigs are made with the goal of improving their health and longevity. There is evidence to support that food preference is established early in life, so it is important to start them on a proper diet. Guinea pigs are entirely herbivoruous and digest fiber more efficiently than rabbits. However, unlike rabbits, they do not increase their
food intake when more of their diet consists of cellulose or other fiber. This suggests that satiety in guinea pigs is dictated more by distention of the digestive tract than by pure metabolic need (Quesenberry et al., 2012).

Guinea pigs are unique from other small exotic mammals in that they have a dietary vitamin C (ascorbic acid) requirement. Guinea pigs lack the enzyme L-gulonolactone oxidase, which allows for the synthesis of ascorbic acid from glucose (Riggs, 2009). Vitamin C is important to many different system functions, including the immune system, cardiovascular system, and integument, to name a few. Because ascorbic acid is a component of collagen, deficiencies tend to result in lameness, hemorrhage, lethargy, anorexia, poor coat quality, and bruxism. While many dietary vitamin C supplements exist, such as flavored water, supplemental tablets, and fortified pellets, vitamin C can rapidly degrade in the environment, particularly if exposed to any extreme temperatures. The best sources for vitamin C are typically fresh vegetables that are high in vitamin C content, including green leafy vegetables and peppers. The vitamin C requirement of an adult, nonbreeding guinea pig is 10 mg/kg/day (Riggs, 2009).

Guinea pigs prefer a solid ground substrate, being provided with ample places to hide. Since guinea pigs are native to the high altitude, mountainous regions, they are typically more cold tolerant and heat sensitive. It should be noted that guinea pigs should not cohabitate with rabbits due to the fact that rabbits carry *Bordetella bronchiseptica*, which is pathogenic to guinea pigs.

Currently, much like rabbits, there are no specific lighting recommendations for guinea pigs other than a photoperiod of 12 hours (Riggs, 2009). This is interesting to note as these animals have evolved in a high altitude environment and would be expected to have increased exposure to ultraviolet B (UVB) radiation compared to animals at lower altitudes. Whether
exposure to UVB radiation is important for these animals is not known, but is worth further investigation.

Similar to rabbits, guidelines have been published for housing guinea pigs in a research facility in the Guide for Care and Use of Lab Animals. Guidelines are meant to optimize quality of life within limited space confinements. Guinea pigs are especially social animals, and it is recommended to group house them when possible. Recommendations on space allotments vary, but it is generally accepted that for animals housed in a group laboratory setting, the minimum required floor space is 652 cm² per adult animal weighing approximately 350 grams (National Research Council 2011; Quesenberry et al., 2012). It is also recommended that the cage walls be at least 17.8-25 cm (7-10 in) in height. Specific hygiene and environmental qualities (e.g., temperature, substrate, cleaning substances and frequency) may also be found in the previously mentioned guides for the care of laboratory animals (National Research Council, 2011).

2.3 Vitamin D

Vitamin D is a fat-soluble vitamin that is also considered an essential nutrient for many vertebrate species. The origin of this unique vitamin dates back over 750 million years, when it was produced by some of the earlier life forms. This substance was produced by phytoplankton in the ocean when exposed to natural sunlight (Holick, 2003). The name vitamin D is somewhat of a misnomer in that research has shown that it also acts as a steroid hormone (Webb and Holick, 1988). In circulation, it is bound by vitamin D binding protein. Binding prolongs the half-life of vitamin D as a hormone. Vertebrates can acquire vitamin D through their diet, photobiochemical conversion of UVB radiation, or a combination of both. Dietary sources of
vitamin D include cholecalciferol (vitamin D₃ of animal or plant origin) and ergocalciferol (vitamin D₂; and typically of plant origin) (How et al., 1994; Ferguson et al., 1996).

Calcium levels are regulated in the body by various substances or hormones, including parathyroid hormone (PTH), calcitonin, and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃ or calcitriol). The three key sites of calcium and phosphorus homeostasis include bone, kidney, and intestine. Decreasing calcium levels stimulate the secretion of PTH, which then triggers the synthesis of active vitamin D at the level of the kidney. PTH promotes the hydroxylation of 25-hydroxyvitamin D in the kidney, thereby creating the active metabolite 1,25(OH)₂D₃, also known as calcitriol (Boron and Boulpaep, 2011).

While vitamin D is an integral component to the metabolism and absorption of calcium and phosphorus, it also contributes to other physiologic processes in the body. Vitamin D directly affects the growth and development of bones, neuromuscular function, as well as being important to reproductive, immune, and cardiovascular health (Webb and Holick, 1988). Vitamin D is also responsible for mobilizing stem cells to form osteoclasts. These bone cells are responsible for remodeling and mobilizing calcium stores from the bone. In some avian and reptilian species, viability of eggs and rates of reproductive success have been correlated to optimal amounts of vitamin D₃ (Ferguson et al., 1996, Ferguson et al., 2005). In humans, vitamin D has been determined to be a selective regulator of the immune system and enhance the corneal epithelial barrier function (Yin et al., 2011). The active form of vitamin D has been shown to inhibit the development of autoimmune diseases, such as inflammatory bowel disease and Crohn’s disease (Cantorna et al., 2004). In broiler chicks, a deficiency in vitamin D₃ was shown to depress the cellular immune response (Aslam et al., 1998).
2.3.1 Acquisition of vitamin D and calcium

As shown in Figure 1, vitamin D can be acquired through two different methods: endogenous synthesis after exposure to UVB light or via direct ingestion and absorption through the gastrointestinal tract (Webb and Holick, 1988). Since vitamin D is a fat-soluble compound, fat must be present in order for vitamin D to be absorbed. In the small intestine, vitamin D is absorbed by a non-saturable passive diffusion, which then acts to assist in active transport of calcium (Cline, 2012). Dietary sources may include animal products that have already ingested or synthesized vitamin D or via the direct ingestion of plant sources (e.g., ergocalciferol). Once absorbed, vitamin D can be stored in a variety of tissues throughout the body, including fat, liver, kidneys, and small amounts in the lungs and heart (Cline, 2012). While many animals can obtain adequate amounts of vitamin D from their diet alone, there are other vertebrate species, such as humans, new world primates, and many reptiles, that require exogenous UVB light sources in order to produce an adequate amount of vitamin D in the body. New world primates do not appear to have the ability to utilize vitamin D$_2$, which is found in most plant food sources (Hunt, 1963). Animals requiring exogenous sources of vitamin D are commonly omnivorous or insectivorous vertebrates, whose natural dietary sources may not contain sufficient levels of vitamin D, or they simply may not be able to process the vitamin D that they ingest. Many animals such as sheep, cattle, rats, horses, pigs, and humans have been shown to have the ability to synthesize vitamin D in the skin via photobiochemical reaction, and more animals capable of this photochemical reaction are being discovered (Holick et al., 1995). This method of synthesizing vitamin D$_3$ follows exposure to UVB radiation in the spectrum of 290-320 nm. This spectrum is important to consider when developing lighting protocols for animals in captivity.
2.3.1.1 Synthesis in skin

The synthesis of vitamin D is the result of the photosynthetic conversion of 7-dehydrocholesterol (7-DHC, provitamin D₃) to previtamin D₃ in the skin of vertebrates after exposure to UVB (Holick et al., 1995; How et al., 1994; Webb and Holick, 1988). This occurs primarily in the epidermis, but has also been shown to occur in the cornea. The previtamin D₃ is unstable and not known to be biologically active. Previtamin D₃ undergoes a temperature dependent isomerization into vitamin D₃, which has been shown to be enhanced at higher temperatures in poikilothermic animals such as iguanas and amphibians, as well as in humans (Holick et al., 1995). Prolonged irradiation also results in a variety of photoproducts derived from previtamin D₃ including tachysterol, lumisterol, and reversion to the parent compound, provitamin D. All of these reactions are reversible (Webb and Holick, 1988). It has been shown that only radiation in the UVB wavelengths of 280-320 nm will convert 7-DHC to previtamin D₃.

Vitamin D₃ produced by the skin, as well as that absorbed by the intestine, is transported to the liver. There has been debate as to whether vitamin D₃ produced in the skin is equivalent to that found in dietary sources. Both seem to have the same biologic activity; however, the half-life of vitamin D₃ produced in the skin is much longer because it is 100% bound to vitamin D binding protein. Conversely, only about 60% of dietary vitamin D₃ is bound in circulation (Haddad and Ja Chyu, 1971). In the liver, the first step of bioactivation occurs, which is the hydroxylation to 25-hydroxyvitamin D₃, or calcidiol. The microsomal and mitochondrial factions of the liver catalyze this reaction (Dahlback and Wikvall, 1988). This is the form of vitamin D that is bound and enters into circulation. The kidneys are responsible for the final conversion to the active form, 1,25(OH)₂D₃ (calcitriol). This mechanism is regulated in part by
the parathyroid glands. When the active form, calcitriol, is synthesized in the kidneys, a feedback loop inhibits PTH transcription and secretion at the level of the parathyroid. Conversely, when calcitriol levels are low, PTH is produced to induce hydroxylation of the main circulating form of vitamin D, 25-hydroxyvitamin D (25-OHD$_3$), into the active form. A summary of the conversion of vitamin D can be seen in Figure 2.

The active form, which is hydroxylated in the kidneys, is primarily responsible for calcium absorption through the gastrointestinal tract. 1,25-(OH)$_2$D$_3$ receptors (Vitamin D receptors, or VDR) are present not only in the intestine and bone, but in a variety of other tissues including the brain, heart, stomach, pancreas, activated T and B lymphocytes, skin, and gonads, among others (Holick, 2014). Some of these receptors actually possess the enzyme responsible for conversion of 25-OHD$_3$ to 1,25(OH)$_2$D$_3$ (Holick, 2006; Holick and Garabedian, 2006). High plasma vitamin D$_3$ levels may be protective against early age-related macular degeneration and possibly protective for glaucoma (Lin et al., 2012). In humans, 1,25-(OH)$_2$D$_3$ enhances the production of insulin, and may play an important role in type II diabetes (Chiu et al., 2004). Vitamin D also plays a role in insulin secretion and glucose tolerance in the rabbit (Nyomba, 1984). The production of 1,25-(OH)$_2$D$_3$ in macrophages is important for innate immunity and may reduce the risk of developing multiple sclerosis, rheumatoid arthritis, and Crohn’s disease (Holick, 2014). Calcitriol also has the ability to increase myocardial contractility (Zitterman, 2006), inhibit renin synthesis, and controls over 200 genes throughout the body responsible for proliferation, differentiation, apoptosis, and angiogenesis (Holick, 2007). This local control of genes may be responsible for prevention of cancer at local sites such as the breast and colon, and has been hypothesized that production of 1,25(OH)$_2$D$_3$ at this sites may initiate apoptosis and prevent angiogenesis of malignant cancer cells.
2.3.1.2 Dietary

When obtained from the diet, vitamin D is metabolized and utilized in order to absorb calcium from the intestine. Interestingly, some omnivorous and carnivorous animals such as dogs (*Canis lupus familiaris*) and cats (*Felis catus*) lack the ability to synthesize vitamin D in the epidermis and must rely entirely on dietary intake, making it a required nutrient for these animals (Cline, 2012; How et al., 1994; Kenny et al., 1998). These are typically animals that have little exposure to UVB radiation, but have dietary sources rich in vitamin D₃ (How et al., 1994). Polar bears (*Ursus martimus*) have low levels of 7-DHC in their skin, suggesting that they also primarily obtain vitamin D₃ from their diet; this may be an adaption to their life history as they initially lack exposure to UVB light in the den (Kenny et al., 1998). Two different species of bats were studied to determine if different lifestyle adaptations would affect vitamin D synthesis. One species roosts in dark places during the day with minimal exposure to sunlight, while the other roosts in tree canopies with up to 12 hours of exposure to the sun. Their primary dietary sources consist of fruit and nectar, which are naturally low in vitamin D content. However, both species were found to have the ability to endogenously synthesize vitamin D (Southworth et al., 2013).

In the 1960’s, dogs were found to be deficient in vitamin D when fed a balanced diet with no supplemental vitamin D but provided regular exposure to UVB light (Hazewinkel et al., 1987). In the study, patches of hair were shaved on the back of the animals to account for possible interference of absorption of UVB radiation from their dense hair coat. Dogs may not need the ability to synthesize vitamin D; it was shown that very small quantities of 1,25-OH₂D₃ are capable of producing an adequate biologic effect (Brickman et. al, 1973). Natural cutaneous vitamin D₃ synthesis in cats has never been reported. In a study measuring the precursor 7-DHC and vitamin D₃ after exposure to UVB radiation in the skin of dogs, cats, and rats, it was
determined that dogs and cats lack the ability to increase vitamin D levels secondary to UVB radiation, whereas rats experienced a 40 fold increase of vitamin D (How et al., 1994). Morris also demonstrated that kittens exposed to sunlight, UVB light, and a vitamin D deficient diet had similar declines in all groups when comparing plasma 25-hydroxyvitamin D levels. These animals subsequently developed signs of vitamin D deficiency related to low plasma levels of vitamin D (Morris, 1999). In this study, it was shown that cats in fact have the precursor, 7-DHC, present in the skin, but it is rapidly reduced by 7-DHC delta reductase before it can be utilized for vitamin D synthesis (Morris, 1999). When administered an inhibitor to the reductase, kittens were able to synthesize 25-hydroxyvitamin D₃ and increased levels were observed in the plasma (Morris, 1999). However, conflicting information showed kittens fed a diet with adequate calcium and phosphorus and no dietary vitamin D, but exposure to UV light had normal bone growth; however, this was attributed to vitamin D stores obtained prior to weaning (Rivers et al., 1979; Cline, 2012).

It is important to know how each individual species processes and utilizes vitamin D in order to determine the best method(s) for providing this essential compound to animals maintained in captivity. Considering the animal’s life history may be important to determining the primary source of vitamin D. Assimilating all of this information, vitamin D₃ should be considered an essential nutrient, as well as functioning steroid hormone, and animals that derive it from their diet, such as dogs and cats, provided appropriate levels to meet their physiologic needs. Rich sources of dietary vitamin D include fish, egg yolk products, and poultry skin.
2.3.2 Ultraviolet B radiation

The entire range of all ultraviolet (UV) wavelengths is 100-400 nm. This includes UVA (320-400 nm), UVB (280-320 nm), and UVC (100-280 nm). The spectrum of radiation from the sun that is able to reach the earth’s atmosphere ranges from 100 to 3200 nm; however, the shorter wavelengths are absorbed at the ozone layer, removing all UVC radiation from possible exposure (Frederick et al., 1989). The optimal spectrum for UVB exposure is ~290-320 nm. In humans, both UVA and UVB are responsible for secondary effects on the skin such as erythema (sunburn) or tanning, but UVB is the only range associated with the photobiochemical synthesis of vitamin D$_3$. Like humans, many birds, reptiles, and small mammals have been shown to have the ability to synthesize vitamin D in the epidermis secondary to UVB radiation exposure. Often these are animals that have natural diets that are lower in vitamin D$_3$, and therefore rely more on endogenous sources. Rats were one of the first small mammal species that were shown to generate a large amount of vitamin D in the blood from UVB exposure, as well as detectable vitamin D levels in the skin secondary to UVB exposure. Rabbits have been shown to synthesize vitamin D metabolites in corneal epithelial cells secondary to UVB exposure (Lin et al., 2012). Current research on green iguanas suggests that the dietary route of acquiring vitamin D is not only less efficient but insufficient to maintain proper vitamin D levels without massive dietary doses likely to cause vitamin D toxicity (Allen et al., 1999).

There are two methods to provide animals with exposure to UVB radiation. The first is via natural exposure to direct sunlight and the second is via artificial supplementation with UVB producing light bulbs. The UV index of natural sunlight is highly variable based on latitude, time of day, and time of year. Studies have shown that the exposure to UVB radiation must be direct, or through air permeable membranes. Glass or acrylic materials (non-permeable) absorb
or obstruct UVB radiation, preventing the animal from gaining any exposure to the UVB radiation (Burger et al., 2007). Artificial sources of UVB light will also degrade over time due to the degradation of the phosphorus in the bulb. Even if the bulb is still emitting visual light, it may not be emitting wavelengths within the optimum spectrum for vitamin D synthesis, and for this reason, current recommendations include replacing the bulbs every 6 months to one year.

2.3.3 Calcium

In the rabbit and guinea pig, calcium metabolism is slightly different than in many non-rodent species. Rabbits absorb large amounts of calcium through the intestines, which can lead to high levels of circulating calcium and large amounts of calcium being filtered by the kidneys and deposited into the urine. The urinary excretion rate of calcium is high in rabbits compared to most laboratory animals and humans. This could be related to the high load of calcium that is filtered through the kidneys, or low tubular reabsorption (Kennedy, 1965). In inactive rabbits, high calcium levels in the urine can lead to a calcium oxalate sludge, which can settle in the urinary bladder and cause bladder distension and possible obstruction to urine outflow. Typically, total serum calcium levels in the rabbit are 30 to 50% higher than that of most mammal species (Eckermann-Ross, 2008). Intestinal absorption of calcium in most mammals involves primarily vitamin D$_3$–regulated active transport. In the rabbit, a passive absorption is the primary mechanism, which is dictated by a concentration gradient between the blood and intestinal lumen (Eckermann-Ross, 2008). Active transport does occur in rabbits, but is thought to only occur when dietary calcium is low (Jekl and Redrobe, 2013). This suggests the amount of calcium absorbed is a consequence of the amount of calcium in the diet. Inadequate calcium absorption can lead to significant abnormalities secondary to hypocalcemia.
nutritional hyperparathyroidism, often referred to as metabolic bone disease, can develop along with increased bone turnover in favor of bone resorption, with a decrease in bone mineral density in the body (Mehrotra et al., 2007). Although calcium absorption is slightly different in rabbits than other mammals, it is still dependent on PTH and vitamin D. PTH regulates calcium mobilization from the bone and excretion, while vitamin D regulates the quantity of calcium absorbed in the small intestine. In these animals, vitamin D is likely more important in the long-term maintenance of calcium homeostasis.

2.3.4 Measures of vitamin D status

2.3.4.1 Assays

When collecting samples to measure the vitamin D status of an animal, the most important thing to consider is the stage of the vitamin D cycle that is being targeted for measurement. 25-OHD₃ is the most common form in circulation. Other forms can be stored in fat and muscle, with the hydroxylation and activation of the active metabolite occurring in the liver. Vitamin D levels are highly variable based on time of day, latitude, and species of the animal, and there are various metabolites that may be measured to obtain the vitamin D status of an individual. Measuring plasma concentrations of 25-OHD₃ has been recommended as the preferred method of characterizing the vitamin D status in the body (Holick, 1990) because it is more stable than either vitamin D or calcitriol and also has a longer half-life than the other metabolites (Holick, 1990). Another advantage is that 25-OHD₃ represents the overall average concentration of dietary and sunlight induced vitamin D in the body (Holick, 2009). Vitamin D was first measured by bioassays, but realistically these were not accurate since two different bioactivation methods take place in the liver and the kidney. An assay was developed to measure
25-OHD₃ in peripheral blood based on a competitive protein binding technique, which was found to be more sensitive, precise, and applicable to small sample volumes than the previously described bioassay techniques (Haddad and Ja Chyu, 1971). Vitamin D binding protein is typically used in the assay because it will recognize both vitamin D₂ and vitamin D₃, accounting for all dietary sources. In humans, 25-OHD₃ levels less than 50 nmol/L are considered a vitamin D deficiency, with toxic effects of vitamin D seen when vitamin D levels are above 374 nmol/L (Holick, 2007).

2.3.4.2 Bone density

It has been suggested that vitamin D receptor polymorphism has a strong association with bone mineral density (Cooper, 1996). Bone mineral density is a common and accurate method to quantify bone mass. Approximately 80% of the variance in the bone strength is explained by bone mineral density. Various diagnostic tests may be used to analyze bone density, both in the clinical setting and post mortem. On the computed tomography (CT) scan, detection of Hounsfield units has been shown to characterize bone density based on a continuous, numeric measurement, also commonly referred to as quantitative CT. This modality is able to measure the true volumetric density in mg/cm³, and measurements can be taken independently of surrounding cortical bone (Stepan, 2002). Microcomputed tomography has been used to quantify density and mineralization in the mandible of Sprague Dawley rats (Donneys et al., 2012). Dual-energy X-ray absorptiometry (DEXA) scans are also used to measure bone mineral density. This imaging modality works by aiming two different energy level beams at the patient’s body, and then bone mineral density in grams per surface area (g/cm²) is determined by subtracting the absorption by the soft tissue. DEXA scans are typically used in human medicine to determine bone mineral
density in patients suffering from osteoporosis, but can also be applied to assessing other bony abnormalities such as tumors. Bone mineral density analysis via DEXA scan has been proven to be accurate and precise in small mammal models (Ammann et al., 1992). Norris et al. (2001) used DEXA scans to show an increase in bone density of the lumbar spine in rabbits fed a diet consisting of higher calcium, and proposed a mechanism of suppression of PTH, reduced skeletal remodeling, and increased apparent fractional retention of calcium (Norris et al., 2001).

The advantages of the previous imaging methods are that they can be done non-invasively and ante-mortem; however, they are limited to the readings obtained and determine bone density based on formulae derived from the modality used. Generally, validation studies of imaging methods have been compared against bone density and composition via ash content or histopathology (Jayo et al., 1991; Ammann et al., 1992); however, it should be considered that differences in bone density between species may result in unknown biases in the interpretation of the results. To minimize this and ultimately determine bone mineral content, samples of the bone must be collected using invasive antemortem methods or post-mortem methods. Post-mortem, bone may be analyzed for ash content, which can be used to determine the amount of grams of ash per grams of bone. From the ash, mineral analysis can be performed to determine the percentages of calcium and phosphorus contained in the bone, as well as other minerals present (Ammann et al., 1992). This type of measure can be used to provide insight into the mineral status of a vertebra based on physiologic stores in the bone.
2.4 Medical problems in captivity

2.4.1 Hypovitaminosis D

In humans, hypovitaminosis D can cause serious health issues, including rickets in children and osteoporosis and osteomalacia in adults. There are many different potential risk factors associated with the development of rickets, including a lack of exposure to UVB radiation in some species, diseases that cause malabsorption in the intestine (i.e., steatorrhea, inflammatory bowel disease), chronic renal failure, liver disease, unusual rapid growth, or a primary dietary deficiency. The cure for rickets was first discovered in the early 1900’s when Mellanby induced rickets in a population of dogs by feeding them a diet of oatmeal. These dogs were then supplemented with cod liver oil (Hazewinkel et al., 1991; Mellanby and Killick, 1926). Cod liver oil was determined to contain a substance that was responsible for regulating the metabolism of bones, described as a “calcifying vitamin” (McCollum et al., 1922; Mellanby, 1919), which was later determined to be vitamin D. During that time period, it was also determined that exposure to sunlight could prevent rickets in children and could also be used to treat rickets in goats. It is now accepted that chronic vitamin D deficiency may have other serious adverse consequences, including increased risk of hypertension, multiple sclerosis, cancers of the colon, prostate, breast, and ovary, and type 1 diabetes (Holick, 2014). Based on these more recent findings, it should not be surprising that hypovitaminosis D may also be an under recognized disease process in domesticated animals that are not provided access to natural sunlight, or at a minimum exposure to artificial supplementation of UVB.

In a study in rabbits inducing vitamin D deficiency via restricted diets, intestinal absorption of calcium and phosphorus was not altered; however, renal excretion of calcium was reduced (Bourdeau et al., 1985). In rabbits fed a diet that consisted of a 2:1 ratio of calcium to
phosphorus (1.0% calcium, 0.5% phosphorus), but was devoid of Vitamin D, the result was a chronic hypovitaminosis D. The hypovitaminosis D resulted in decreased intestinal phosphorus absorption, along with increased renal phosphorus conservation. Some rabbits were able to maintain normal serum phosphorus levels, while others experienced a severe decline. Those with hypophosphatemia led to inadequate skeletal mineralization or osteomalacia (Brommage et al., 1988). In another study, rabbits were fed a low calcium, high phosphorus diet, with expectations these rabbits would develop hypocalcemia and hyperphosphatemia. However, to combat these abnormalities, the parathyroid gland secreted PTH, which restored calcium and phosphorus levels to normal by increasing osteoclastic bone resorption and promoting the release of calcium and phosphate from bone (Lebas, 2000). PTH also works to stimulate calcium reabsorption and promote renal excretion of phosphorus at the level of the renal tubules, as well as stimulate renal production of 1,25-(OH)$_2$D$_3$ (Cline, 2012; Bas et al., 2005). A decrease in the number of vitamin D receptors, which impairs the ability of calcitriol to inhibit parathyroid cell proliferation, has been described in both primary hyperparathyroidism and in advanced hyperparathyroidism secondary to renal disease (Bas et al., 2005). Sufficient vitamin D in the diet will lead to appropriate absorption of calcium and prevention of diseases such as nutritional secondary hyperparathyroidism (NSHP).

2.4.2 Rabbit and rodent specific diseases

In humans, hypovitaminosis D is related to poor calcification of bones and teeth, often contributing to development of caries in children (Grant and Boucher, 2010). In the early 1900s, a rabbit model was used to demonstrate the hypothesis that a lack of vitamin D in the diet leads to dental disease, specifically caries (Mellanby and Killick, 1926). While this was not observed
directly in the rabbit, there is some thought that hypovitaminosis D and metabolic bone disease could be related to the degree of dental disease commonly observed in pet rabbits (Harcourt Brown, 2007; Jekl and Redrobe, 2013). Rabbit dentition is described as hypsodont, or open rooted teeth. Instead of a true root, there is a very long reserve crown. Guinea pig dentition is described as aradicular hypsodont, which means all teeth have a long crown and are open rooted. Their dentition is similar to rabbits, but they differ in the pattern that their teeth wear. In guinea pigs, the maxilla is slightly wider than the mandible with a marked inward angle of the teeth. Therefore, the teeth tend to grow inwards towards each other, and can cause entrapment of the tongue in severe cases of dental disease. For both rabbits and guinea pigs, manifestations of dental disease are not thought to develop in wild populations. Genetics, proper diet, and other aspects of husbandry, such as exposure to UVB radiation, may play a role in the differences between captive and wild species.

Since the constant rapid growth and continuous eruption of teeth requires a large demand for calcium, NSHP, is hypothesized to be directly linked to the development of dental disease in rabbits and rodents. NSHP has been associated with a poor diet and inadequate amounts of calcium, vitamin D, and natural sunlight, and therefore has been incriminated as a cause of malocclusion, overgrown dental roots, and mandibular abscesses (Fisher, 2010). In a study by Harcourt-Brown, as dental disease increased in severity, osteopenia was more commonly reported in the skull. This suggested that the loss of supporting alveolar bone was allowing apical elongation. Alveolar bone is particularly susceptible to metabolic bone disease, leading to displacement of the teeth (Harcourt-Brown, 2007; Jekl and Redrobe, 2013). Harcourt Brown also described that rabbits affected with dental disease exhibited decalcification of the vertebral skeleton (Harcourt-Brown, 2006). In female rabbits with experimentally induced osteoporosis,
mandibular bone mineral density was shown to decrease in relation to spinal density (Southard et al., 2000).

In other species where metabolic bone disease is also a common problem, primary dental disease is not commonly observed due to the nature of their anatomy and dentition; however, the jaw is a common site for presentation of nutritional deficiencies in reptiles and dogs. Constantly growing teeth are a unique aspect to rabbit and guinea pigs, and may be a confounding factor. Inadequate amounts of vitamin D may lead to inadequate calcium absorption by the GI tract, therefore affecting the proper health, development, and growth of teeth.

2.5 UVB radiation

2.5.1 Use in companion animals

For many species of herbivorous reptiles, such as chelonians and lizards, the use of UVB radiation in captive husbandry is both well documented and recommended in order to ensure animals can generate adequate levels of vitamin D. UVB radiation can also contribute to reproductive success of many egg-laying vertebrates, such as reptiles and birds. In chameleons, viable eggs produced by adults not exposed to UVB irradiation died at term, and it was shown that UVB irradiation significantly improved hatching success (Ferguson et al., 2002). Similar findings were found regarding hatching success in chickens (Gallus gallus domesticus) (Narbaitz, et al., 1989). If female panther chameleons (Furcifer pardalis) are provided with a sufficient light gradient, they have been observed to adjust their exposure based on dietary vitamin D intake for optimum reproductive success (Ferguson et al., 2002). Due to the extreme diversity of reptile species and various environmental adaptations, many studies have been performed to assess individual species adaptations and requirements. Two different species of
Jamaican anoles, one more sun loving and known for basking (*Anolis sagrei*) and another more shade seeking and thermal conforming (*Anolis lineotopus merope*), were studied to determine if there was a difference in dietary vitamin D intake and vitamin D synthesis in the skin related to lifestyle (Ferguson et al., 2005). The shade dwelling species appeared to be more sensitive to UVB induced vitamin D₃ photobiosynthesis than the species naturally exposed to more sunlight. This could signify a greater need for UVB screening capacity to prevent damage secondary to exposure in heliophilic species, and also suggests that diurnal and crepuscular lizards may adjust the UVB sensitivity of their skin, resulting in a balance between their ability to avoid UVB damage and their ability to photosynthesize vitamin D₃ (Ferguson et al., 2005). Panther chameleons have shown an ability to self-regulate exposure to UVB radiation and an ability to synthesize vitamin D both endogenously through the skin as well as absorption through the diet, depending on availability (Ferguson et al., 1996, Hoby et al., 2010).

As previously mentioned, it has been hypothesized that carnivorous animals, such as the cat or polar bear, do not have the capacity to synthesize vitamin D in their skin because they are able to obtain sufficient amounts from their diet. While this may be true for many carnivorous mammals, there appears to be some variability in how reptilian carnivores respond to UVB exposure. Corn snakes (*Pantherophis guttatus*) exposed to UVB radiation were able to generate significantly more 25-OHD₃ in comparison to those not exposed to UVB radiation over a 4 week fasting period (Acierno et al., 2008). Similarly, artificial UVB supplementation was also found to have a significant effect on the production of 25-OHD₃ in Komodo dragons (*Varanus komodoensis*) when compared to animals fed vitamin D in the diet alone (Nijboer et al., 2007). Contrasting findings were obtained in a recent study of ball pythons. In this study, there was no difference in 25-OHD₃ levels between groups supplemented with UVB radiation and those
without over 70 days, suggesting they may acquire vitamin D solely from their diet (Hedley and Eatwell, 2013). This suggests that there are likely a wide array of interspecies differences, possibly related to their natural environment and evolution.

A significant increase in 25-OHD₃ concentrations has also been observed in both omnivorous and herbivorous chelonians. Red-eared slider turtles (Trachemys scripta elegans) exposed to UVB were found to have significantly higher 25-OHD₃ levels compared to those exposed to non-UVB lights (Acierno et al., 2006). In the freshwater turtle Emydura stigmata, it was shown that basking behavior, conventionally thought to be driven by heat seeking, is not actually related to thermoregulation (Manning and Grigg, 1997). In these animals, as well as other basking species, it has been suggested that this behavior may also be related to the acquisition of vitamin D.

In animals housed in a group setting, dominance and other behavioral characteristics should be considered. For example, the dominant animal in the group may monopolize the prime basking spots within an enclosure. In the bearded dragon (Pogona vitticeps), which is a semi-arid, sun-loving species typically associated with high levels of exposure to natural sunlight in their natural environment, vitamin D levels were also shown to significantly increase secondary to UVB exposure (Oonincx et al., 2010). One study actually found that dietary supplementation was ineffective in raising plasma concentrations of vitamin D to the levels of UVB supplementation alone (Oonincx et al., 2010). Bearded dragons not supplemented with UVB radiation were found to select diet items higher in vitamin D levels in comparison with those that were supplemented. Green iguanas have been shown to have significantly higher vitamin D levels after exposure to UVB light versus animals not provided UVB producing light (Oftedal et al., 1997). Exposure to various forms of UVB radiation, including fluorescent bulbs,
mercury vapor bulbs, and natural sunlight, was evaluated in Hermann’s tortoises (*Testudo hermanni*), which found tortoises exposed to natural sunlight were able to generate more vitamin D3 than those exposed to artificial sources (Selleri and Di Girolamo, 2012). All of the aforementioned studies prove UVB radiation is an important source of vitamin D in a variety of species. Not only that, UVB radiation has proved to be an important regulator of behavior in many cases.

In poultry, it has been shown that dietary vitamin D and calcium must be supplied to birds to ensure reproductive success and avoid common disease issues such as hypovitaminosis D or osteoporosis. Birds have also been shown to have the ability to endogenously synthesize vitamin D in the skin (Stanford, 2006). There is a higher concentration of the precursor 7-dehydrocholesterol in featherless areas of birds than those areas covered in feathers (Tian et al., 1994b). Hypocalcemia is an issue commonly seen in African grey parrots (*Psittacus erithacus*). In a study evaluating supplementation with UVB radiation in conjunction with various diets, it was found that the plasma vitamin D levels were significantly higher in the seed fed group compared to those fed pellets, suggesting that UVB radiation may be an adequate mechanism in psittacines for acquiring vitamin D for animals fed deficient diets (Stanford, 2006). Poultry have been shown to have no dietary requirement for vitamin D if they are supplemented with UVB light, which may also be the case for African greys.

While the diversity of mammalian species is not as vast as reptiles or birds, it would make sense to consider various adaptations and requirements for different mammalian species related to their environment. For example, UVB supplementation is commonly recommended at zoological institutions that maintain callitrichids, such as tamarins and marmosets, indoors without ready exposure to direct sunlight. As previously mentioned, these New World primates
lack the ability to utilize ergocalciferol, which is the most abundant form of vitamin D in their diet (Ullrey and Bernard, 1999). In these animals, NSHP may lead to a lack of bone density, osteoporosis, and pathologic fractures. Oftentimes this can lead to weakness and significant difficulties when eating, leading to weight loss and poor body condition. Despite health concerns in humans and New World primates being directly related to hypovitaminosis D, little research has been performed characterizing vitamin D status in other omnivorous or herbivorous mammals. Surprisingly, 90 to 100% of most human being’s vitamin D requirement comes from exposure to sunlight (Holick, 2002). There are currently no other recommendations for UVB supplementation to assist in obtaining adequate vitamin D levels in other mammals.

2.5.2 Previously reported adverse effects

2.5.2.1 Ocular

When provided with UV light, the eyes are constantly exposed in a similar fashion to the skin. This has been shown to contribute to, or cause, diseases of the eye (Taylor et al., 1988; Bergmanson and Soderberg, 1995). UVB radiation has been reported to have negative effects on the cornea, possibly influencing corneal thickness and, in some cases, potentially contributing to the development of keratitis and corneal edema. The cornea provides a substantial degree of protection from UVB damage to the lens, however, the lens has been shown to be the most susceptible to damage from radiation specifically in the UVB band (Pitts et al., 1977; Taylor et al., 1988). Development of cataracts has been experimentally documented in rabbit eyes exposed to direct levels of UVB radiation via a UV laser with a power density of 600 mW/cm² (MacKeen, et al., 1973). However, this direct method of UVB exposure is much more powerful and the pathology induced had previously been difficult to demonstrate with incoherent UV
light. In humans, cataracts have been found to occur more commonly in tropical, sunny areas, and there is an association between exposure to UVB radiation and cataract formation (Taylor et al., 1988). This supports the recommendation for ocular protection in humans.

Rabbit eyes are commonly used as a model for humans to study damage from exposure to UVB radiation. Exposure to 280-nm UV radiation at a dose producing biomicroscopically significant keratitis (0.12 J/cm² for 280 nm and 0.47 J/cm² for 310 nm) has been shown to also delay corneal healing (Pitts et al., 1977). Apoptosis and oxidative impact were identified as a mechanism of corneal cell damage and death after single and repeated doses of direct (0.05 m distance) UVB irradiation (Podskochy et al., 2000; Fris et al., 2006). Various doses have been utilized in different studies including 6-W mercury arc UVB lamp (312 nm wavelength) for a total dose of irradiation being 3.12 J/cm². To put these levels of exposure previously mentioned into context, it has been surmised that in July at noon at 40 degrees latitude the amount of UVB radiation reaching the cornea would be 0.006-0.05 mW/cm² (0.022-0.187 J cm²/hr) and the amount reaching the lens even less at 0.00012-0.001 mW/cm² (0.00044-0.001 J cm²/hr). Using these environmental parameters, lens damage due to UVB exposure would take 245 hours, but the cornea could potentially be damaged in 23 minutes of direct exposure (Zigman, 1995).

2.5.2.2 Dermatologic

In humans, adverse effects of UVB radiation result from direct sun exposure or artificial UV radiation from tanning beds (approximately 97% UVA and 3% UVB). The most obvious adverse effect is primary skin damage, which in severe cases may be in the form of a sunburn or erythema. Chronic exposure to UVB radiation throughout life has been shown to be a predisposing risk factor for development of skin cancers ranging from benign changes to life
threatening malignant melanoma (Gallagher and Lee, 2006). While most mammals, including rabbits and guinea pigs, do not have as many hairless areas or as much exposed skin in comparison to humans, the pinna, nose, and skin around the eyes may still be at risk for UVB damage. In the wild, taking refuge in a shelter limits amounts of exposure, and therefore an adequate shelter or light gradient would also be recommended for a companion animal or laboratory setting. These species are crepuscular, meaning they are most active at dawn and dusk. At these times of day, UVB radiation would be less than would be expected for the times of maximum exposure in the middle of the day, and likely these animals would naturally control for that if provided proper housing.

A report of secondary photodermatitis and photokeratoconjunctivitis has been documented after exposure to UVB radiation in a ball python and a blue-tongued skink. Evaluation of a sample lamp of the type associated with these cases revealed an extremely high UV output, including very-short-wavelength UVB, neither found in natural sunlight nor emitted by several other UVB lamps not associated with photokeratoconjunctivitis (Gardiner et al., 2009). In a recent study measuring the effects of artificial UVB radiation on leopard geckos, a color change and increased ecdysis were observed and considered consistent with mild sunburn. It should be kept in mind there are various strengths and spectrums of UVB bulbs available. Distance from the bulb, strength, and any media it may have to pass through could all contribute to the potential side effects. There are also reports of skin damage caused when higher emission UVB bulbs are used.

2.5.2.3 Toxicity

Vitamin D toxicity can result in dystrophic mineralization, which occurs when excess calcium is deposited in soft tissues in the body. Deposition of mineral into soft tissues can lead to
pain as well as organ dysfunction and failure. The calcium phosphorus product (CPP) has been used previously to predict whether dystrophic mineralization will occur in humans with renal failure. This value is obtained by multiplying the serum concentrations of calcium (mg/dL) and phosphorus (mg/dL) levels. It should be considered that the CPP is largely an accepted dogma in human medicine, established as early as 1917, with limited scientific evidence demonstrating direct correlation to vascular mineralization (O’Neill, 2007). In general, it is thought animals with CPP > 55 are at risk for soft tissue mineralization, although increased concentrations likely need to be present for weeks to months (Landau, 2007).

Cases diagnosed with hypervitaminosis D are typically associated with excessive dietary vitamin D (Lebas, 2000; Lowe, 1998). Excess dietary vitamin D has been found to be associated with soft tissue calcification in rabbits, the most notable tissue being the aorta. Clinical signs associated with dystrophic mineralization include anorexia, intense thirst, ataxia, and mortality (Lebas, 2000). In reptiles, mineralization has been reported in the aorta too; and the mineralized aorta may be visible on radiographs. A similar finding of calcifying atherosclerosis is reported in birds, but at this time the inciting cause is unknown. Cases of renal gout in chameleons have been attributed to high dietary vitamin D levels (Ferguson, 1996). While most cases of hypervitaminosis D are associated with excessive dietary vitamin D and are chronic, acute vitamin D toxicity can result from the ingestion of rodenticides or topical psoriasis medication (Fan et al., 1998; Gunther et al., 1988).

The aforementioned reports of hypervitaminosis D were all secondary to dietary sources, and there have been no reports of hypervitaminosis D in any species directly related to UVB exposure. While overexposure to UVB can cause skin damage, it is highly unlikely that this can lead to the overproduction of vitamin D and systemic toxicity for a number of reasons. Studies
performed by Holick elucidated a mechanism in the skin of vertebrates to protect against the overproduction of vitamin D$_3$. After several minutes of direct exposure, the skin contains less previtamin D$_3$ and more biologically inert products, specifically lumisterol and tachysterol (Webb and Holick, 1988; Holick et al., 1981; Holick, 2003). Previtamin D$_3$ has the ability to convert to 25-OHD$_3$, lumisterol, tachysterol, or revert to provitamin D$_3$. All of these reactions are also reversible. Interestingly, in humans, the wavelengths of radiation that initiate previtamin D$_3$ synthesis also are within the waveband absorbed by melanin. This led to the hypothesis that evolution of racial distribution with latitude was due to vitamin D requirements; however, this is likely only one of the factors that control vitamin D levels in humans. Regardless of the amount of pigment in the skin, or the amount of UVB exposure, the previtamin D$_3$ produced in the skin does not increase above 15% over the original 7-DHC concentration (Webb and Holick, 1988; Fraser, 1980). Vitamin D$_3$ has also been shown to photodegrade over time, meaning prolonged exposure will not continue to increase circulating levels (Webb and Holick, 1988). Different determinants limit the cutaneous production of previtamin D$_3$ in humans, including photobiochemical regulation, pigmentation, and latitude, in order of importance (Holick et al., 1981). The isomerization of previtamin D$_3$ to 25-OHD$_3$ is also limited by the amount of exposure to UVB light. Prolonged exposure does not continuously increase the circulating levels of plasma 25-OHD$_3$. After a certain amount of exposure, previtamin D$_3$ actually begins to degrade. This suggests the body’s mechanisms of homeostasis control the ability to self-regulate the amount of 7-DHC converted and produced in the skin, reducing conversion to vitamin D precursors and instead producing biologically inert products such as lumisterol. Although not yet known, other defense mechanisms could exist to limit the amount actively transported to the liver and metabolized to the active form in the kidney or other tissues. Other studies have proven
that vitamin D metabolism in animals is very similar to that of humans (Holick et al., 1995; Webb and Holick, 1988). If these same protective mechanisms exist in animals, then UVB radiation may be the best solution for providing adequate levels of vitamin D to captive animals without fear of introducing significant adverse side effects associated with dietary toxicity.
CHAPTER 3
EVALUATING THE CLINICAL AND PHYSIOLOGICAL EFFECTS OF LONG TERM ULTRAVIOLET B RADIATION ON GUINEA PIGS (CAVIA PORCELLUS)

3.1 INTRODUCTION

Guinea pigs (Cavia porcellus) are members of the order Rodentia and family Caviidae. They were native to the Andes and other mountainous regions of South America, where they were predominately used for food in countries such as Peru, Colombia, Venezuela, and Brazil (Pigière et al., 2012). At this time they are considered extinct in the wild (Kraus And Rödel, 2004). In the United States, guinea pigs are popular pets. In a survey performed by the AVMA in 2012, there were over 1.3 million guinea pigs being kept as pets in the United States (JAVMA, 2008). Guinea pigs are docile, responsive animals with a lively personality, and therefore make appealing pets for most households. Guinea pigs have also been used in research for over 400 years (Quesenberry et al., 2012).

Current husbandry recommendations for guinea pigs are made with the goal of improving their health and longevity. However, at this time there are no specific lighting recommendations for guinea pigs other than a photoperiod of 12 hours (Riggs, 2009). This is interesting to note as these diurnal animals have evolved in a high altitude environment and would be expected to have increased exposure to ultraviolet B (UVB) radiation compared to animals at lower altitudes. Many vertebrates utilize UVB exposure as a method of generating endogenous vitamin D, an essential hormone. Whether exposure to UVB radiation is important for these animals relative to this function is not known, but is worth further investigation.

Vitamin D is a circulating hormone that is important to many bodily functions, including bone development, growth, neuromuscular function, reproduction, cardiovascular health, and
immune function (Holick, 2007; Stumpf et al., 1979). Vitamin D deficiencies in humans and other vertebrates have been shown to cause rickets, osteomalacia, and reproductive failure. Not only do deficiencies directly lead to abnormal calcium metabolism, it is also becoming more apparent that vitamin D levels are important to overall health. Vitamin D receptors are found in numerous tissues throughout the body (Holick, 2014, Nagpal et al., 2005). In humans, adequate levels of vitamin D have been shown to decrease the risk of developing many different conditions, including diabetes, muscular dystrophy, hypertension, and inflammatory bowel disease, to name a few (Holick, 2014). When considering the many different physiological functions in vertebrates that are intimately related to vitamin D and calcium metabolism, it should be considered that disease processes or problems with these species in captivity could be related to inadequate levels or supplementation.

While acquisition and production of vitamin D is highly variable across vertebrate species, pilot studies have shown that guinea pigs housed indoors have the ability to synthesize vitamin D in the epidermis secondary to exposure to artificial UVB light over the short-term (Sander et al., in review). Since many guinea pigs are housed indoors and not exposed to natural or artificial UVB light, it is possible that these animals may experience chronic vitamin D deficiencies. Inappropriate levels of this essential nutrient and hormone could contribute to common disease processes in these animals such as dental disease, cardiovascular disease, or impaired immune function.

While the benefits of UVB exposure for captive animals appear to be strong, it is not without risk. UVB radiation has been associated with the development of skin neoplasia, with the groups at greatest risk including humans with fair complexions and albino or white animals (Dorn et al., 1971; Gallagher and Lee, 2006). Direct UVB radiation also has the potential to
cause structural damage to the eye at the level of the cornea, lens, or retina. Direct UVB radiation can also cause short-term damage such as photodermatitis and erythema. For these reasons, the safety of UVB supplementation in small mammals should be investigated.

The objectives for this research were to evaluate the clinical and physiologic effects of artificial UVB light supplementation on guinea pigs and to evaluate the long-term safety of artificial UVB light supplementation over the course of six months in these species. The biological hypotheses for this study were: 1) animals supplemented with UVB light would produce higher levels of vitamin D than those without supplementation, and that 2) there would be no significant detrimental side effects associated with UVB light supplementation.
3.2 MATERIALS AND METHODS

Twelve juvenile (14-16 weeks), purpose bred, female intact Hartley guinea pigs were used in this study. Hartley guinea pigs are acromelanic albino animals. The pigmentation for acromelanic animals is genetically determined, with slightly variable amounts of pigmentation on the extremities. The animals were obtained from a commercial breeding facility (Charles River Laboratories, Wilmington, MA). Prior to their arrival at the study site, the animals received a standard commercial pelleted feed (Lab Diet, St. Louis, MO) with no additional supplemental vitamin D or UVB light. The animals were allowed to acclimate to their surroundings for 7 days prior to the onset of data collection. This project was performed in accordance with the regulations established by the Institutional Animal Care and Use Committee at the University of Illinois (protocol #:13-024).

The animals were housed in 183 cm long x 137 cm wide pens. Three animals were housed in each pen. All pens were located in the same room, were enclosed to the ceiling, and the animals were housed on the floor. Room temperature was measured weekly and was kept consistent throughout the study with an average range of 23 to 27 degrees Celsius. Paper shavings (Harlan Laboratories, Indianapolis, IN) were provided as substrate lining the floor of the pen. General room lighting was provided by non-UVB producing ambient fluorescent lighting. The animals were provided with water ad libitum through sipper bottles placed on the front of the pens. All animals were all fed the same weight based diet consisting of Oxbow timothy grass hay (Oxbow Animal Health, Murdoch, NE) and Oxbow Essentials adult guinea pig pellets (Oxbow Animal Health). The vitamin D content of this particular pelleted diet is 900 IU/kg.
After one week of acclimation, the animals were anesthetized for baseline sample collection and microchip placement. This sample collection, and all subsequent sample collections, took place between the hours of 3 and 5 pm to correct for natural circadian rhythms and variability. Anesthesia was induced via administration of 5% isoflurane gas (IsoFlo, Abbott Animal Health, North Chicago, IL) and 1 L oxygen via facemask. The animals were maintained on 2-3% isoflurane and 1 L oxygen via facemask after induction. Each guinea pig was placed in dorsal recumbency and 3 ml of blood was collected from the cranial vena cava using a 25 gauge needle attached to a 3 ml syringe. The total blood sample volume was less than 1% of the total body weight of the animal. The samples were placed in lithium heparin microtainers (Benton, Dickinson, and Company, Franklin Lakes, NJ), EDTA microtainers (Benton, Dickinson, and Company, Franklin Lakes, NJ), and glass tubes without anticoagulant (Tyco Healthcare Group LP Mansfield, MA). Avid microchips (Avid Identification Systems, Inc., Norco, CA) were placed subcutaneously in the intrascapular region. The anesthetic gas was turned off, and the guinea pigs were weighed and recovered on supplemental oxygen. Glass tubes without anticoagulant were centrifuged for 15 minutes within 90 minutes of collection. Serum was pipetted and subsequently placed into cryovials and placed in -20 degree Celsius freezer. EDTA samples were submitted to a university clinical pathology laboratory (University of Illinois, Urbana, IL) for complete blood counts and processed using a commercial analyzer (Cell-Dyn3700, Abbott Laboratories, Abbott Park, IL). Complete blood counts were only performed on samples that were not clotted. A commercial chemistry analyzer (Vetscan VS2, Abaxis Inc., Union City, CA) was used to analyze blood chemistries from plasma collected in lithium heparin microtainers within 90 minutes after collection. A mammalian comprehensive diagnostic profile rotor (Abaxis Inc.) was used to measure the biochemistries. This profile quantified levels of
albumin (Alb), alkaline phosphatase (ALP), alanine transaminase (ALT), amylase (Amy), total bilirubin (T-bil), blood urea nitrogen (BUN), total calcium (Ca), phosphorus (Phos), creatinine (Creat), glucose (Glu), ionized sodium (Na), ionized potassium (K), total protein (TP), and globulins (Glob). Plasma samples were also submitted on frozen gel packs to a veterinary diagnostic laboratory (Diagnostic Center for Population and Animal Health, East Lansing, MI) for measurement of serum 25-hydroxyvitamin D (25-OHD₃, radioimmunoassay), ionized calcium (iCa, ion-selective electrode/pH electrode at pH 7.4) and parathyroid hormone (PTH, radioimmunoassay).

Complete ophthalmologic examinations under manual restraint were also performed at the initiation of the study. One drop of Tropicamide 1% (Bausch & Lomb Inc., Tampa, FL) was administered into each eye prior to the onset of the exam. The examinations included a neuro-ophthalmic examination, fundic examination, tonometry, slit lamp biomicroscopy (Kowa-SL2, Kowa, Tokyo, Japan), and corneal pachymetry. Tonometry was performed with a rebound tonometer (Tonovet, Icare Finland Oy, Espoo, Finland). The rebound tonometer has three available calibration settings: canine, equine, and “other”: other is used for species for which a calibration table has not been established. The calibration setting for “other” was used in this study. A corneal pachymeter (Compuscan P ultrasonic pachymeter, 20MHz, Strorz Instrument Company, St. Louis, Missouri) was used in the exam as a gauge of corneal thickness. Readings were taken in triplicate for each eye during the exam. Serial ophthalmic examinations were performed at two-month intervals, and at the end of the study period, with the same study procedure repeated for each examination period. A single, blinded investigator (Dr. Amber Labelle, University of Illinois, College of Veterinary Medicine, Urbana, IL) performed all of the ophthalmologic exams.
Computed tomography (CT) scans were performed under sedation at the onset of the study. Computed tomography (CT) of the skull was performed on all subjects using a 16-slice helical CT scanner (GE Lightspeed 16 slice CT Milwaukee, WI, USA) using the following parameters: kVp of 80, mA of 180, slice width of 0.625mm, pitch of 0.5625, scan field of view 9.6 cm, 512 x 512 matrix, a rotation time of 0.8s and using a bone convolution kernel. Each guinea pig was administered a combination of 0.05 mg/kg buprenorphine hydrochloride (Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) and 0.5 mg/kg midazolam (Hospira Inc., Lake Forest, IL) via intramuscular injection. Once the animal became sedate, it was positioned in sternal recumbency and perpendicular to the gantry with forelimbs positioned caudally. After the scan was complete and the animal recovered from sedation, it was returned to laboratory housing.

Every attempt was made to ensure the head was positioned symmetrically as to not require further post processing reconstruction of the raw data for corrected image planes. The primary transverse CT images included the first two cervical vertebrae and all scans were considered to have good diagnostic quality by a board certified radiologist (SKJ). Segmentation of the skull and teeth was performed using a dedicated DICOM workstation (OsiriX version 5.8.4 64-bit, OsiriX Imaging Software, OsiriX Foundation, Geneva, Switzerland) and segmentation plug-in (Mialite, Center for Medical Image Science and Visualization (CMIV), Linköping University, Sweden) (Figures 3-8). The first step was removing the soft tissue surrounding the skull and neuro tissue within the calvarium and proximal cervical spinal cord. This was achieved by segmentation using a threshold minimum of -100HU, a maximum of 150HU and a smoothing factor of 0.1 (Wang et al., 2011). The resulting soft tissue region of interest (ROI) were removed by changing all contained pixels to a value of -2000HU. The
segmentation plugin was used again to isolate the skull using a threshold minimum of 0HU, no maximum HU constrains, smoothing factor of 0.1, and a seed (starting point) within the temporal bone. An additional blocker point was used to prevent segmentation of the cervical vertebral bodies. The parameters of the resulting three-dimensional (3D) ROI were recorded for each specimen, such as volume, average attenuation (HU), and attenuation standard deviation.

After initial sample collection, guinea pigs were assigned to two treatment groups of six animals each using a random number generator (www.random.org). Each group was then randomly divided into two pens housing three animals each. Of the treatment groups, the supplemented (treatment) group was exposed to 12 hours of artificial UVB radiation daily that was controlled by a timer; non-supplemented (control) groups received ambient fluorescent light with no UVB supplementation for 12 hours daily. To provide direct UVB radiation, nine dome lights (Fluker Farms, Port Allen, LA) were suspended above the animals at equidistant intervals, comprising three rows of three lights each. The 5.5 inch domes were anchored to a shelving unit several feet off the ground and suspended directly above the guinea pigs, with no physical barrier present between the animals and light. UVB light was provided using one 5.0, 20 W, Sun-Glow coil bulb (Fluker Farms, Port Allen, LA) in each dome. The domes were suspended 12-14 inches above the ground, in order to maximize exposure to the animals without impeding movement, as well as attempting to mimic the distance that would be used in a typical laboratory or captive cage setting. The guinea pig pens for each group are shown in Figure 9. The amount of UVB radiation and irradiance was measured in microwatts per centimeter squared (µW/cm²) by placing a digital UVB meter (Solartech, Inc., Harrison Township, MI) and an irradiance meter (Zoo Med Laboratories Inc., San Luis Obispo, CA) on the ground directly under each bulb, as well as in between the bulbs. The height of the meter was approximately at eye level of the
guinea pigs. Measurements were collected weekly, approximately 3 hours after the bulbs were turned on and approximately 3 hours prior to the bulbs being turned off. To correct for UVB degradation over time and provide consistent levels of supplementation, bulbs were replaced if at any time the UVB output from a bulb measured less than 30 µW/cm².

The study was conducted over 6 months. After the UVB light supplementation was initiated, blood samples were collected every three weeks to measure blood chemistries, serum PTH, ionized calcium, and 25-hydroxyvitamin D₃ levels, for a total of 9 sample collections. Weights were collected during each sample collection. Collection, processing and testing of the blood samples were similar to the techniques described for the samples obtained on day 0. Serial ophthalmologic examinations were performed at two month intervals, and at the end of the study period, for a total of four examinations.

At the end of the six month period, anesthesia was induced as previously described for initial sample collection. Each animal was placed in dorsal recumbency and a final 3 ml blood sample was collected from the cranial vena cava. While being maintained on 5% isoflurane and 1 L oxygen, 2-3 ml of pentobarbital (Vortech Pharmaceuticals, Dearborn, MI) was administered to the animal via intracardiac injection. Euthanasia was confirmed via auscultation and negative corneal reflex.

CT scans were performed immediately following euthanasia using the identical scanning and positioning parameters described at the beginning of the study. Segmentation of the skull, mandible and teeth was also acquired using the techniques described previously. ROI volume, mean and SD of the attenuations were recorded for each specimen.

A postmortem examination was performed on each animal. A select tissue set was collected from all animals except one animal, which experienced an anesthetic death in which a
complete set of tissues was collected. The select tissue set included lung, heart, haired skin-head, haired skin-ear, haired skin-dorsum, haired skin-ventrum, liver, pulmonary artery, aorta, adrenal gland, kidney, duodenum, pancreas, thyroid glands, spleen, esophagus, reproductive tract, trachea, skeletal muscle, eye, and femur. Tissues were fixed in 10% neutral buffered formalin. Following fixation, tissues were embedded in paraffin wax, sectioned at 4 µm, and stained with hematoxylin and eosin. Additionally histochemical staining with Verhoeff Van-Gieson satin was performed. Histologic lesions were noted and scored using the following categories: no lesions, mild lesions, moderate lesions, and severe lesions. A single, blinded pathologist (Dr. Adam Stern, University of Illinois, College of Veterinary Medicine, Urbana, IL) performed all of the microscopic examinations.

Bone mineral densities (BMD) of guinea pig skulls were measured by dual energy x-ray absorptiometry (Hologic QDR 2000, Marlborough, MA). Prior to analysis, all skulls were preserved and stored at -20 degrees Celsius. For BMD assessment, all skulls were positioned dorsoventrally, and a standardized region of interest (R1) of a constant area (cm²) was created to encompass the entire skull size of each specimen. Bone mineral density was measured and expressed as grams of bone per area (gm/cm²). The analyst of the bone concentrations was blinded to the study groups.

The sample size selected for this study (n=12; 6 per group) was based on the following assumptions: an expected mean difference in serum 25-OHD₃ concentrations of 35 nmol/L between treatment and control groups with a SD of 20 nmol/L (Emerson et al. 2014, Rivas et al. 2014), an alpha of 0.05, and a power of 0.8 (MedCalc 11.3.2.0). Distribution of the data was evaluated by use of the Shapiro-Wilk test. Mean, SD, and minimum-maximum (min-max) values were reported for data that had a normal distribution, whereas the median, 10th to 90th
percentiles (%), and min-max values were reported for data that did not have a normal distribution. Data that were not normally distributed were logarithmically transformed in order to perform parametric analysis. If the data was not normally distributed after transformation, appropriate nonparametric statistics were performed.

A linear mixed model was used to evaluate the normally distributed and correlated blood chemistry values, iCa, and 25-OHD₃ collected in the repeated measures design of the study. This statistical method was selected because one animal died over the course of the study and this type of model can account for missing data. Group and time represented the fixed and random factors, respectively, that were evaluated in the model. The -2 log likelihood was used to determine best fit of the model. Friedman’s test was performed on data that was not normally distributed. If significant, Mann-Whitney U tests were performed to determine if a difference existed between groups at each individual time point. A repeated measures ANOVA was used to evaluate differences in Hounsfield units measured on CT scans between groups over time. A repeated measures ANOVA was also used to determine if there was a difference between corneal pachymetry measurements taken in triplicate. Since there was no difference in the corneal pachymetry data, the values were averaged and a linear mixed model was used to assess average corneal pachymetry and intraocular pressure values over time. Levene’s test was used to determine if bone mineral density (gm/cm²) met the assumption of homogeneity of variance. Because it did, an independent samples t- test was used to determine if bone mineral density differed between groups. Chi square one-tailed tests with a Yates correction or Fisher exact tests were performed to determine if there was any difference in the pathologic findings documented at necropsy between groups. A value of $P \leq 0.05$ was used to determine significance.
Commercial statistical software (SPSS, version 22.0, SPSS Inc, Chicago, IL) and graphpad.com were used to analyze the data.
3.3 RESULTS

Twelve guinea pigs (6 not exposed to UVB, and 6 exposed to UVB) were used in this study. At the initiation of the study the average body weight of the guinea pigs was 497.2 grams (SD: 15.6, min-max: 465.4-520.2 grams), while at the conclusion of the study the mean body weight of the guinea pigs was 946.1 g (SD: 15.6, Min-Max 831-1070); there was no significant difference in body weight noted between groups (F = 0.347, p=0.557). The mean difference in weight gain from initiation to completion of the study was 449.38 g (SD: 82.1, Min-Max: 349-556) for all animals. Over the study period, weight gain among guinea pigs exposed to supplemental UVB lighting and those not exposed did not differ (t= 0.079, p = 0.938).

Complete blood counts were performed at the beginning and completion of the study to assess the general health of the guinea pigs. Unfortunately, a number of the samples collected at both the beginning and end of the study were clotted and could not be evaluated. Because of the small sample set, statistical comparisons were not performed. However, at least one CBC result was available for each guinea pig at either the beginning or conclusion of the study, and all results were within published reference limits (Riggs, 2009).

Serum 25-OHD$_3$ values differed significantly by group over time (F=576.126, p<0.0001) (Figures 10 and 11) with higher values seen in the UVB supplemented group. Since the animals arrived at the University of Illinois facility having been fed a different diet than the study diet, mean, SD, and min-max values including initial baseline data and excluding the baseline data are shown in Table 1. The UVB radiation levels measured in the UVB treatment groups ranged from 21-70 µwatts/cm$^2$ directly under the lights and 2-19 µwatts/cm$^2$ in between the lights. UVB radiation levels in the non-UVB groups were <1 µwatts/cm$^2$. The maximum UVB irradiance level in the pens was 3.5, which falls under IV in the UV index spectrum. This value is
considered high and equivalent to mid day baskers; however it is well within the safe region, with greater than 7 considered dangerous. There was no significant difference in the 25-OHD$_3$ concentrations between the two pens of guinea pigs exposed to the UVB lights ($F=0.022$, $p=0.884$) or between the two pens of guinea pigs not exposed to the UVB lights ($F=1.082$, $p=0.357$). This comparison was done to rule out potential differences in light exposure between treatment groups or quantity of food consumed between control groups.

When analyzing the blood chemistry values by group and over time, no significant differences were found for PTH, Ca, Phos, calcium/phosphorus ratio (Ca/Phos), K, ALP, ALT, Amy, T-bil, BUN, Creat, Glob, or Gluc values. However, significant differences by group were found for ionized calcium (iCa, $F=117.704$, $p<0.0001$), Na ($F=4448.23$, $p<0.0001$), Alb ($F=8793.077$, $p<0.0001$), and TP ($F=169258.81$, $p<0.0001$). Mean, SD, and min-max values for each group are shown in Table 2.

There was a significant difference in corneal thickness between groups for each eye (right eye [OD]: $F=149.527$, $p<0.0001$; left eye [OS]: $F=30.525$, $p<0.0001$), with the UVB supplemented group having thicker corneas (OS and OD). Average corneal pachymetry measurements by group are shown in Table 3. There was no significant difference in intraocular pressure noted between groups for either eye (right eye [OD]: $F=0.196$, $p=0.66$; left eye [OS]: $F=1.1$, $p=0.3$). No other abnormalities were noted upon examination of the eyes, with minor exceptions. One animal developed a corneal foreign body (OS) from what appeared to be organic material (i.e., bedding or food). Proparacaine was applied to the eye and the foreign body was easily removed with sterile eye wash using gentle lavage. Fluorescein stain was applied, and the cornea was then observed via slit lamp to have a mild superficial ulcer and keratitis, secondary to the corneal foreign body. The animal was administered one treatment of
triple antibiotic ointment in that eye and rechecked in 48 hours. At that time the keratitis had completely resolved and no further treatment was necessary. Another animal developed serous ocular discharge OS with no associated conjunctivitis, which resolved within 48 hours without treatment. Both of these animals were in the UVB supplementation group.

For the initial CT exams, the mean Hounsfield units were 1076.478 (SD: 32.8606, Min-Max 1015.18-1126.14); for the final exams the mean HU was 1208.003 (SD: 44.360, Min-Max 1152.64-1293.49). There was a significant increase in Hounsfield units noted on the CT images over time (F=173.414, p=<0.0001) but not between control and UVB supplemented groups (F=0.559, p=0.472). There was no evidence of dental disease on the CT scans. There also was no significant difference noted in the bone mineral density between the two groups obtained via DEXA scan (t = -0.713, p=0.494).

During the eighth (out of nine total) sample collection, a guinea pig went into respiratory arrest spontaneously upon recovery from anesthesia after four minutes of isoflurane gas administration. Respiratory arrest was followed by cardiac arrest, and cardiopulmonary resuscitation was initiated immediately. Glycopyrrolate (American Regent, Inc., Shirley, NY, 0.2 mg) and epinephrine (IMS, Limited, So. El Monte, CA, 1 mg) were administered via intracardiac injection. A spontaneous heartbeat resumed, along with voluntary breaths within 30 seconds after injection. Although heart rate and respirations returned to normal, mentation remained obtunded with inconsistent palpebral and withdrawal reflexes. Attempts were made to warm the animal, while providing 100% oxygen via facemask throughout recovery. Appropriate mentation was never regained, and the animal passed away within two hours of the anesthetic event. Necropsy was performed with no significant pathologic findings with the exception of
lesions documented in lungs and trachea that were likely consistent with anesthetic complications and resuscitation attempts.

No significant differences were found when comparing necropsy lesions between groups (all p>0.05) (Table 4). Mild dermatologic changes were noted equally across groups consisting of rare suprabasilar apoptotic keratinocytes, small numbers of lymphocytes, plasma cells, and eosinophils scattered in the superficial dermis, and minimal to mild epidermal hyperplasia on skin from the ears or head. No elastin abnormalities were noted on Verhoeff-Van Gieson staining. Multifocal and mild vacuolar hepatopathy (glycogen) were also noted equally across groups. The only mineralization documented was multifocal and mild mineralization within renal cortical tubules in a guinea pig with no UVB supplementation, and a single mineralized muscle myofiber in the skeletal muscle of a guinea pig with UVB supplementation. Specific histologic abnormalities can be seen in Appendix A.
3.4 DISCUSSION

Findings from the present study demonstrated that guinea pigs provided artificial supplementation of UVB light produced and maintained significantly higher serum 25-OHD$_3$ levels compared to those without UVB supplementation. In many vertebrates, vitamin D$_3$ (cholecalciferol) can be synthesized after exposing the skin to UVB radiation. This initiates the photobiochemical conversion of the precursor 7-dehydrocholesterol in the dermis to previtamin D$_3$, which undergoes isomerization into vitamin D$_3$ and is hydroxylated to 25-OHD$_3$ in the liver (Holick et al., 1995; How et al., 1994; Webb and Holick, 1988). 25-OHD$_3$ is the storage form of vitamin D, and therefore is considered the best measure of vitamin D status in the body (Holick, 1990; Schmidt-Gayk et al., 1997). When vitamin D is needed systemically, the kidneys are responsible for conversion of 25-OHD$_3$ to 1,25(OH)$_2$D$_3$, although conversion has been demonstrated in other tissues as well. The findings in this study, along with a previous short term pilot study (Sander et al., in review), reinforce that guinea pigs have the ability to generate significant quantities of vitamin D through UVB exposure, despite adequate levels being provided in the diet, and thus may be an important conserved evolutionary mechanism not currently utilized in captive guinea pig husbandry practices.

While a significant difference was noted between groups over time, reference ranges for optimum serum 25-OHD$_3$ in wild or captive guinea pigs have not been established. In humans, the mammal in which vitamin D deficiency is most commonly diagnosed and treated, a serum 25-OHD$_3$ concentration of <50 nmol/L is considered deficient (Holick, 2007). However, it should be noted that this minimum value is a general recommendation by most experts, and a general consensus on optimum levels of 25-OHD$_3$ in humans does not exist. Since guinea pigs and humans share similar methods of vitamin D acquisition, both utilizing UVB exposure and
diet, it might be tempting to extrapolate the guinea pig values to human reference ranges. Based on the current results, the group not supplemented with UVB light (28.33 ± 8.36 nmol/L, range 10-48) would be considered chronically deficient in vitamin D. However, such a direct comparison is difficult because of differences in physiology between our two species. Humans are omnivores that are capable of deriving vitamin D from animal and fortified sources in their diet, in addition to the vitamin D they can obtain from the plant material in their diet, as in the case of guinea pigs. On the other hand, guinea pigs have evolved in a high altitude, low latitude environment where exposure to UVB radiation would be greater than that which humans evolved. While it may appear as though both species are adapted to synthesize and maintain vitamin D levels under these different conditions, evaluating the two different methods for which they derive the vitamin D is important to consider. Dietary sources of vitamin D tend to persist in the body, while vitamin D produced via photoconversion can be turned on or off based on need. In theory, guinea pigs may be adapted to lower levels than humans, but the results of the current study suggest otherwise since animals exposed to UVB maintained 25-OHD₃ levels well over 50 nmol/L. The intent of this study was not to determine reference ranges for guinea pigs but to determine whether exposure to artificial UVB radiation would lead to increased and persistent levels of 25-OHD₃ in these animals, and whether long term (6 months) exposure led to any pathologic conditions. In order to determine a reference interval for serum 25-OHD₃ in guinea pigs, a cross-sectional study would need to be performed. The study population should include animals being maintained both indoors and outdoors. Subject recruitment must also consider diet to limit the potential heterogeneity attributed to different dietary levels of vitamin D. To establish such a reference based on the previously noted factors and others such as sex (male, female) and age (juvenile, adult) would require a minimum sample size of 250 animals. Collecting samples
from a cohort of wild *Cavia* sp. from the natural range of *C. porcellus* would also be important as this can provide insight into what reference levels would be under natural conditions. The sample size required to characterize this reference interval, because of an increased likelihood of subject homogeneity, would be 120 animals.

An important finding in this study was that, over a six-month period, prolonged exposure of guinea pigs to artificial UVB light sources led to a statistically significant increase in serum 25-OHD$_3$ levels that were sustainable over time. This ability to sustain substantially higher levels of 25-OHD$_3$ from UVB exposure compared to animals provided vitamin D in diet alone reinforces that these animals evolved with this mechanism to procure and maintain adequate levels of vitamin D. Guinea pigs are naturally a high altitude, low latitude species, with ample access to sunlight and natural UVB radiation in their home range. A recent pilot study also demonstrated that chinchillas (*Chinchilla lanigera*), which are also native to the Andes Mountains, have the ability to produce 25-OHD$_3$ through photobiochemical synthesis following exposure to artificial UVB lights (Rivas et al., 2014). Other animals native to similar environments, such as llamas (*Lama glama*) and alpacas (*Vicugna pacos*), have been shown to develop significant disease secondary to a seasonally dependent hypovitaminosis D (Van Saun, 2009). When comparing the vitamin D levels of the guinea pigs in this study to rabbits maintained in a similar study (Watson et al., *in review*), the average 25-OHD$_3$ level in guinea pigs was higher, despite similar exposure. This could suggest these animals have a higher vitamin D requirement in relation to their natural habitat being at a higher altitude and lower latitude.

An interesting observation in this study was that the baseline serum 25-OHD$_3$ values were most similar to the group supplemented with UVB light, and contrary to what was
expected, actually decreased after initial baseline collection in the group not supplemented with UVB. Unfortunately, the initial baseline sample collection after arrival at the University of Illinois took place three months prior to the first sample collection post-UVB exposure because of logistics, so it is not known how long it took for the decline in serum 25-OHD3 values to occur. During this three-month period, the guinea pigs were fed the same diet and housed under the conditions described previously. After contacting the breeding facility it was determined that, prior to acquisition at the University of Illinois, the animals were fed a pelleted guinea pig feed (LabDiet, St. Louis, MO) that contained a higher concentration of vitamin D (3400 IU/kg), in comparison to the diet fed throughout the time they were housed for the study which contained 900 IU/kg of vitamin D. The 3.8 times higher dietary concentration seen in the diet offered at the breeding facility is almost in direct agreement with the higher mean levels of serum 25-OHD3 demonstrated between groups after the initial baseline collection (3.6 times). This finding reinforces that guinea pigs indeed have the capability of increasing their serum 25-OHD3 levels via either dietary ingestion or photobiochemical conversion, and also may hint towards optimum serum 25-OHD3 maintenance levels in guinea pigs. However, how they acquire vitamin D could be important, especially as it relates to the potential development of hypervitaminosis D. The dietary vitamin D requirements for guinea pigs are not known, but it is suggested to be 1000 IU/kg diet (National Research Council, 1995). Under these guidelines, the commercial diet fed throughout the study adheres to those recommended requirements. The formula used at the breeding facility was 3.8 times higher than this and could, long term, lead to problems as dietary sources are more likely to be attributed to cases of hypervitaminosis D (see below). Although the scope of this study did not include investigations of vitamin D levels related to the diet, the diet provided to the animals was consistent between groups throughout the study, thus indicating that
the significant elevations in serum 25-OHD$_3$ were a result of the UVB supplementation only. Further studies to assess the specific role(s) of dietary vitamin D in guinea pigs are needed.

When evaluating circulating levels of 25-OHD$_3$, one important factor to consider is that systemic mineralization has been observed due to hypervitaminosis D secondary to increased levels of vitamin D in the diet via clinical or experimental report (Jensen et al., 2013). Toxicity was described in a cohort of guinea pigs fed 150 times the normal concentration of vitamin D in their diet, and subsequently they developed systemic disease with lesions consisting of renal interstitial fibrosis with tubular mineralization, soft tissue mineralization in multiple organs, hepatic lipidosis, and pneumonia (Jensen et al., 2013). Serum 25-OHD$_3$ levels were obtained in two animals (445 nmol/L and 541 nmol/L), both of which would be considered toxic if using human guidelines (Holick, 2007). There have been no reports of hypervitaminosis D secondary to photoconversion of 7-DHC. This is likely because photobiochemical conversion of 7-DHC to previtamin D$_3$ to hydroxylation in the liver to 25-OHD$_3$ is tightly regulated within the body. In humans, mechanisms have been elucidated which have shown sunlight actually destroys excess previtamin D$_3$ or vitamin D$_3$ when exposure is prolonged or excessive (Holick and Garabedian, 2006). Previtamin D$_3$ can revert to the parent compound, 7-DHC, or other biologically inert metabolites, to avoid potentially harmful accumulation of circulating vitamin D in the body (Holick et al., 1981). Since other pathways involving photoconversion in guinea pigs are similar to the human, it is highly likely that these control mechanisms exist in the guinea pig as well; however, specific investigation in guinea pigs is warranted.

Ionized calcium values were found to be significantly increased in the group supplemented with UVB radiation, while total protein and albumin were significantly higher in the non-UVB supplemented group. Vitamin D$_3$ is essential for active calcium absorption in the
intestine, as well as maintaining serum calcium levels in most vertebrates. In the blood, calcium can be free (iCa), bound to proteins, or complexed with other molecules. Ionized calcium is the most important indicator of calcium status, since it is the major active form in the body. It is also typically tightly regulated, as small increases can lead to cell dysfunction. In women, intestinal calcium transport increased by 45-65% when 25-OHD₃ levels were increased from 50 to 72 nmol/L (Heaney et al., 2003). Although biologically within normal limits, this statistically significant increase in the guinea pigs represents a trend in available active calcium, secondary to increased circulating vitamin D. In the non-UVB supplemented group, the higher albumin and total protein in combination with the lower iCa and 25-OHD₃ suggest that these animals have lower availability to active calcium. These findings are important because they reinforce that vitamin D plays an important role in calcium homeostasis for guinea pigs. While the levels obtained were within reference ranges, the dynamic nature of ionized calcium suggests that interpreting its meaning from single reference points could underestimate its true value. Follow-up longitudinal studies evaluating the relationship of iCa and 25-OHD₃ in clinically normal and diseased guinea pigs are needed.

In children, chronic vitamin D deficiency can lead to developmental abnormalities such as failure of attaining peak height or bone mineral density, pathologic fractures, poor growth, and slow development. Adults with vitamin D deficiency are at an increased risk for osteopenia, osteoporosis, and fracture (Holick, 2014). Effects of hypovitaminosis D have also been observed in other animals in experimental studies, including rabbits and dogs (Hazewinkel et al., 1987; Lebas, 2000; Brommage et al., 1988). The animals used in this study were juveniles, and thus it might be expected that those not supplemented with UVB radiation would experience decreased bone mineral density or poor growth if deficient in vitamin D during this vital growth period.
However, due to humane reasons, levels in the diet were not restricted and the amount provided should have been sufficient based on current recommendations. No significant difference in Hounsfield units measured on CT scans were observed between groups; however, a significant increase was noted over time in both groups. This is consistent with normal growth and bone development patterns. Also, there was no difference in the bone mineral density of the skulls between groups noted at the end of the study. These findings indicate that no adverse effects to the bone were noted during the growth phase of these animals, regardless of UVB exposure. Because these animals were juvenile animals that were only observed until approximately one year of age, it is not likely we would witness any effects secondary to chronic vitamin D deficiency. Dental disease, which has been hypothesized to be due to chronic vitamin D deficiency, often involves resorption of alveolar bone and also often occurs progressively over time (Harcourt-Brown, 2007; Jekl and Redrobe, 2013). The results of this study do confirm that that a dietary source of vitamin D at 900 IU/kg during development is sufficient for normal skeletal growth. Future studies that follow guinea pigs longitudinally will be important to determine whether animals exposed to UVB, and subsequently maintain higher levels of circulating 25-OHD₃, do so to minimize the potential for developing specific pathologies.

It is important to note that a change in PTH was not observed in this study, even though there was a change in 25-OHD₃ between groups. In the body, decreasing calcium levels stimulate the secretion of PTH, which then triggers the synthesis of active vitamin D at the level of the kidney. PTH promotes the hydroxylation of 25-OHD₃ in the kidney, thereby creating the active metabolite 1,25(OH)₂D₃ (calcitriol). A previous study in dogs found a synergism between PTH and the active metabolite 1,25(OH)₂D₃ (Hazewinkel and Tryfonidou, 2002). This synergism works to regulate skeletal mineralization, and should be stimulated when systemic levels of
calcium and phosphorus are low. Possible reasons that there was no change in PTH in this study could be that there was no skeletal mineralization noted between groups, and total calcium and phosphorus levels were within acceptable reference levels for both groups. Conversely, when vitamin D levels in the body are elevated, circulating PTH decreases. However, it is possible that the elevated levels of 25-OHD$_3$ recorded in the UVB group were not considered elevated by the guinea pigs, but instead still acceptable levels. Although the overall status of vitamin D in the body was increased in the UVB supplemented guinea pigs, the active metabolite 1,25(OH)$_2$D$_3$ was not measured in this study, so it is impossible to comment on how these levels were affected by active vitamin D in the guinea pig. Future studies evaluating vitamin D and PTH levels in animals with disease may provide more insight into the interaction of these hormones in guinea pigs.

Living at higher latitudes has been found to increase the risk of hypertension and cardiovascular disease in humans, and this may be directly correlated to circulating levels of vitamin D (Holick, 2007; Rostand, 1997). In a study of patients with hypertension who were exposed to UVB radiation, 25-OHD$_3$ levels increased and blood pressures decreased to within normal ranges (Krause et al., 1998). Although blood pressure was not measured in the guinea pigs used in this study, animals in the non-UVB group had significantly higher levels of sodium compared to those provided UVB exposure. Hypernatremia is a common correlate to hypertension in mammals (Freda and Nurko, 2014). Although all sodium values obtained in this study were within biologically relevant limits, this study only allowed observation over a six month period in young animals. Future studies should be pursued to determine whether sodium levels and hypertension are correlated in guinea pigs, as well as whether circulating 25-OHD$_3$ can predict the likelihood of hypertension.
Corneal thickness was found to be significantly increased in guinea pigs exposed to UVB radiation; this group also had higher circulating levels of 25-OHD$_3$. Although increased corneal thickness may be a normal aging change, a difference was identified between groups over time. The phenotype, diet, and environmental factors were all the same between groups with the exception of the UVB supplementation and there was no significant difference in weight between groups over time. Due to these factors, we can attribute the increase in corneal thickness to the UVB radiation. No corneal edema associated with UV toxicity was identified during slit lamp examinations, or on histopathology. Corneal thickness in both groups was slightly increased in comparison to another study, which found an average thickness of 227.85 ± 14.09 µm (Cafaro et al., 2009). Since no ocular pathology was identified in any of the eyes, it is possible that the increase in corneal thickness may be a protective mechanism to limit the potential for damage to internal structures of the eye such as the lens and retina. Cataracts are the most common pathology reported in the eyes of vertebrates exposed to unhealthy levels of ultraviolet radiation (UVA) (Balasubramanian, 2000). However, it has been demonstrated that the cornea provides a significant filter to the amount of UV radiation that reaches the lens (Zigman, 1995). A study that placed UV protective contacts on guinea pig eyes found increased damage to corneas without UV protective contacts (Bergbauer et al., 1991). In this high altitude species, it is possible the cornea has developed protective mechanisms over time. The cornea has also been shown to have the ability to synthesize vitamin D in a similar fashion to that of the skin (Lin et al., 2012). It has been demonstrated that increased vitamin D levels enhance the epithelial barrier function of the cornea (Yin et al., 2011). Future studies could elucidate whether the increased thickness is attributable to increased serum vitamin D concentrations, UVB
radiation, or a combination thereof. Follow up could also include investigation of corneal thickness in relation to altitude, latitude, and amount of UVB exposure.

One of the guinea pigs in the UVB group died during the study. Cardiopulmonary resuscitation was performed and the animal did regain cardiopulmonary function and could maintain a sternal position, but it did not recover mentally and went into a second fatal cardiac arrest within 60 minutes of the first event. A full necropsy was performed on this animal and the final diagnosis concluded that this animal succumbed to complications associated with anesthesia, as no pathologic conditions were found. Although no long term anesthetic studies have been performed on guinea pigs, it is documented that the risk of serious anesthetic complications increases with increased numbers of procedures in dogs (Hosgood and Scholl, 1998). The risk of anesthetic death is also higher, and likely compounded by stress, in prey species (e.g., rabbit or guinea pig) compared to dogs or cats (Gaynor et al., 1999; Brodbelt et al., 2008). In the current study the incidence of anesthetic death was 0.9% (1 death out of 107 anesthetic events). This is comparable to another study evaluating perianesthetic death in rabbits (Brodbelt et al., 2008), and lower than another report of over 2% perianesthetic mortality in guinea pigs (Carter and Story, 2013). Because these anesthetic events were performed on the same animals over time, a compounded risk might be expected. For those performing these types of serial anesthetic studies on prey species, it is important to be prepared for anesthetic complications.

No significant differences were noted in the gross necropsy or histopathologic findings between study groups. However, there were lesions recorded in individual animals that are noteworthy. The most common lesion reported was multifocal to diffuse and mild vacuolar hepatopathy (glycogen) (7/12, 58.3%). Vacuolar hepatopathy is commonly associated with
administration of corticosteroids, or excessive physiologic release of steroids (i.e. hyperadrenocorticism). Since guinea pigs are a prey species, excess corticosteroids may naturally rise as a response to stress, leading to these types of histologic changes. Additionally, the relative sizes of adrenal glands in prey species are often increased in comparison to non-prey species as a result of stress, and this study population was no exception. In humans, individuals with fair skin and fair hair are at higher risk of developing neoplasia secondary to UVB exposure (Gallagher and Lee, 2006), and the same has been shown for white cats (Dorn et al., 1971). In this study, albino animals were used because it was expected that they would be most sensitive to UVB exposure and at the greatest risk for potential adverse side effects secondary to UVB exposure. Presence of rare suprabasilar apoptotic keratinocytes were noted in three cases, with more noted in animals without UVB supplementation than with UVB. Typically, apoptotic keratinocytes are associated with UV exposure. In humans, it is well documented that UV radiation induces DNA damage, oxidative stress, and inflammatory processes in keratinocytes, which can result in photocarcinogenesis (Afaq and Mukhtar, 2001). When apoptotic keratosis is noted in small amounts, it is likely an indication of a defense mechanism network that has been developed to control potentially harmful environmental contaminants. Damage to skin collagen and elastin is a hallmark of long term exposure to solar UV radiation in humans (Kligman et al., 1986). Verhoeff-Van Gieson staining was performed to detect elastin abnormalities that may be present in conjunction with solar elastosis (Kadoya et al., 2005); however, no elastin abnormalities were noted in either group. This finding, in addition to the lack of ocular pathology, suggests that the UVB levels and irradiance used in this study are not toxic in guinea pigs over an extended period (6 months). Long term studies to determine whether pathologic conditions occur in adults over
longer periods of time are required to further elucidate the importance of UVB exposure in these animals and determine potential side effects from exposure.

It is important to note that only intact females were used in this study due to sample size limitations and a desire to decrease any potential variability associated with sex. For this reason, the effects of UVB radiation in male guinea pigs cannot be directly commented on. However, intact female guinea pigs would be expected to have the highest requirements for calcium due to their reproductive needs, and as such are likely to be the most at risk for vitamin D deficiency.

Dystrophic mineralization is another common sequellae to hypervitaminosis D in vertebrates. When multifocal and generalized, it can cause widespread damage to cells and tissues, including organ failure. Multifocal, mild mineralization of the renal cortical tubules was noted in one guinea pig in this study, but it was in an animal that was not supplemented with UVB. Additionally, a single skeletal muscle myofiber was mineralized in a guinea pig that was supplemented with UVB; however, it was likely associated with some damage to the muscle as it was focal (a single structure). In Jensen et al (2013), substantial post-mortem renal lesions were observed in all 7 of the animals that were overdosed with oral vitamin D. As previously noted, systemic dystrophic mineralization has never been reported to occur secondary to UVB exposure and subsequent photoconversion of precursors to 25-OHD₃. The findings from Jensen et al. (2013) and the current study suggest that supplementation with UVB radiation (2-70 µwatts/cm²) is a safer method of maintaining serum vitamin D levels in guinea pigs long term. Follow up studies to assess the potential for increasing 25-OHD₃ levels using different oral doses of vitamin D are also warranted, as the Jensen et al. (2013) report documented toxic levels of oral vitamin D inducing disease.
3.5 CONCLUSIONS

The results of this study indicate that guinea pigs have the ability to increase their 25-OHD$_3$ levels via photobiochemical production, and that the utilization of this pathway is sustainable over a six month period. Ionized calcium had a positive association with increased serum vitamin D levels, while albumin and total protein had an inverse relationship. Corneal thickness increased in animals exposed to UVB light; with no ocular pathology noted, this may be indicative of a protective mechanism. This study also showed that there were no significant detrimental health effects associated with artificial UVB exposure as characterized by complete blood counts, serum biochemistry analyses, ophthalmologic examinations, CT scans, a DEXA scan, and necropsy and histopathology. While it is known that serum 25-OHD$_3$ levels can also be increased via dietary supplementation alone, as evidenced by the baseline data in this study, this method of obtaining vitamin D is not necessarily benign. Hypervitaminosis D and associated lesions have been documented secondary to excess oral vitamin D supplementation. Including the findings of this study, no reports of vitamin D toxicosis have been documented secondary to UVB supplementation in vertebrates. In conclusion, the results of this study suggest that artificial UVB supplementation is a safe and effective method for increasing and maintaining vitamin D levels in guinea pigs, and that individuals responsible for managing these animals in captivity should consider providing UVB exposure to the animals in their care.
CHAPTER 4

EVALUATING THE CLINICAL AND PHYSIOLOGICAL EFFECTS OF LONG TERM ULTRAVIOLET B RADIATION ON RABBITS (ORYCTOLAGUS CUNICULUS)

4.1 INTRODUCTION

Rabbits (Oryctolagus cuniculus) belong to the order Lagomorpha, distinguishing them from rodents such as rats (Rattus norvegicus) and guinea pigs (Cavia porcellus). The domestic rabbit is likely a descendant of the European rabbit. Rabbits are popular pets in the United States (U.S.), with 3.2 million animals being documented in U.S. households in a 2012 American Veterinary Medical Association survey (JAVMA, 2008). The popularity of rabbits can be tied to their docile nature, unique personalities, and relatively long life span (12-15 years) in comparison to other small exotic mammals. Often, rabbit owners are very dedicated to the health and well-being of their rabbits, making them one of the most common exotic pet species presented to veterinary practices.

Rabbits have a long tradition in human and veterinary research. Their role as translational models has been essential to many of the common cures developed in human medicine. Recently, rabbits have been proposed as models for human growth and bone physiology, due to the fact that they reach skeletal maturity and peak bone mass after growth (Norris et al., 2001). Because of the importance of rabbits as research models, it is important that their basic physiologic needs are met; otherwise, deficiencies may introduce bias into the research results.

Current husbandry recommendations for pet rabbits are based on their physiologic needs and mimic conditions they would experience in a natural habitat (e.g., diet and temperature). However, there are no specific lighting recommendations for captive rabbits other than a standard 12 hour photoperiod (Vennen and Mitchell, 2009), which is interesting considering the
amount of effort placed on developing management plans for these animals in captivity. As crepuscular/diurnal animals, rabbits have evolved to be exposed to regular photoperiods within a circadian rhythm (24 hour cycle). Based on this, it is important that more specific lighting recommendations be developed that should not only consider photoperiod but the type of light exposure too (e.g., ultraviolet radiation exposure).

Vitamin D is a circulating hormone that is important to many bodily functions, including bone development, growth, neuromuscular function, reproduction, cardiovascular health, and immune function. Vitamin D deficiencies in humans and many vertebrate species have been shown to cause rickets, osteomalacia, and reproductive failure. Not only do deficiencies directly lead to abnormal calcium metabolism, it is also becoming more apparent that vitamin D levels are important to overall health. Vitamin D receptors are found in numerous tissues throughout the body. In humans, adequate levels of vitamin D have been shown to decrease the risk of developing many different conditions, including diabetes, muscular dystrophy, hypertension, and inflammatory bowel disease, to name a few (Holick, 2014). When considering the many different physiological functions in vertebrates that are intimately related to vitamin D and calcium metabolism, it should be considered that disease processes or problems with these species in captivity could be related to inadequate levels or supplementation.

While acquisition and production of vitamin D is highly variable across vertebrate species, pilot studies have shown that rabbits housed indoors have the ability to synthesize vitamin D in the epidermis secondary to exposure to artificial ultraviolet B (UVB) radiation over a short-term period (Emerson et al., 2014). Since many rabbits are housed indoors and not exposed to natural or artificial UVB light, it is possible that these animals may experience chronic vitamin D deficiencies. Inappropriate levels of this essential nutrient and hormone could
contribute to common disease processes in these animals such as dental disease, cardiovascular
disease, or impaired immune function.

While the benefits of UVB exposure for captive animals appear to be strong, it is not
without risk. UVB radiation has been associated with the development of skin neoplasia, with
the groups at greatest risk including humans with fair complexions and albino or white animals
(Dorn et al., 1971; Gallagher and Lee, 2006). Direct UVB radiation also has the potential to
cause structural damage to the eye at the level of the cornea, lens, or retina. Direct UVB radiation
can also cause short-term damage such as photodermatitis and erythema. For these reasons, the
safety of UVB supplementation in rabbits should be investigated.

The objectives for this research were to evaluate the clinical and physiologic effects of
artificial UVB light supplementation on rabbits and to evaluate the long-term safety of artificial
UVB light supplementation over the course of six months in this species. The biological
hypotheses for this study were: 1) animals supplemented with UVB light would produce higher
levels of vitamin D than those without supplementation, and that 2) there would be no significant
detrimental side effects associated with UVB light supplementation.
4.2 MATERIALS AND METHODS

Twelve juvenile (14-16 weeks), purpose bred, female intact New Zealand white rabbits were used in this study. The animals were obtained from a commercial breeding facility (Charles River Laboratories, Wilmington, MA). Prior to their arrival at the study site, the animals received a standard commercial pelleted feed (Lab Diet, St. Louis, MO) with no additional supplemental vitamin D or UVB light. The animals were allowed to acclimate to their surroundings for 7 days prior to the onset of data collection. This project was performed in accordance with the regulations established by the Institutional Animal Care and Use Committee at the University of Illinois (protocol #: 13-024).

The animals were housed in 183 cm long x 137 cm wide pens. Three animals were housed in each pen. All pens were located in the same room, were enclosed to the ceiling, and the animals were housed on the floor. Room temperature was measured weekly and was kept consistent throughout the study with an average range of 23 to 27 degrees Celsius. Paper shavings (Harlan Laboratories, Indianapolis, IN) were provided as substrate lining the floor of the pen. General room lighting was provided by non-UVB producing ambient fluorescent lighting. The animals were provided with water ad libitum through sipper bottles placed on the front of the pens. All animals were all fed the same weight based diet consisting of Oxbow timothy grass hay (Oxbow Animal Health, Murdoch, NE) and Oxbow Essentials Bunny Basics T adult rabbit pellets (Oxbow Animal Health). The minimum vitamin D content of this particular diet is 900 IU/kg.

After one week of acclimation, the animals were restrained for initial blood collection. This sample collection, and all subsequent sample collections, took place between the hours of 3 and 5 pm to correct for natural circadian rhythms and variability. Animals were manually
restrained in sternal recumbency, with their nose directed dorsally to allow for visualization of the neck. A 3 mL sample of blood was collected from either the right or left jugular vein using a 25 gauge needle and a 3 mL syringe. Pressure was held on the venipuncture site and checked for cessation of bleeding and hematoma formation. When neither was visualized, the animals were immediately returned to their enclosure. The total blood sample volume was less than 1% of the total body weight of the animal. The samples were placed in lithium heparin microtainers (Benton, Dickinson, and Company, Franklin Lakes, NJ), EDTA microtainers (Benton, Dickinson, and Company, Franklin Lakes, NJ), and glass tubes without anticoagulant (Tyco Healthcare Group LP Mansfield, MA). Glass tubes without anticoagulant were centrifuged for 15 minutes within 90 minutes of collection. Serum was pipetted and subsequently placed into cryovials and placed in -20 degree Celsius freezer. EDTA samples were submitted to a university clinical pathology laboratory (University of Illinois, Urbana, IL) for complete blood counts and processed using a commercial analyzer (Abbott Laboratories, Abbott Park, IL). Complete blood counts were only performed on samples that were not clotted. A commercial chemistry analyzer (Vetscan VS2, Abaxis Inc., Union City, CA) was used to analyze blood chemistries from plasma collected in lithium heparin microtainers within 90 minutes of collection. A mammalian comprehensive diagnostic profile rotor (Abaxis Inc.) was used to measure the biochemistries. This profile quantified levels of albumin (Alb), alkaline phosphatase (ALP), alanine transaminase (ALT), amylase (Amy), total bilirubin (T-bil), blood urea nitrogen (BUN), total calcium (Ca), phosphorus (Phos), creatinine (Creat), glucose (Glu), ionized sodium (Na), ionized potassium (K), total protein (TP), and globulins (Glob). Plasma samples were also submitted on frozen gel packs to a veterinary diagnostic laboratory (Diagnostic Center for Population and Animal Health, East Lansing, MI) for measurement of serum 25-hydroxyvitamin D (25-OHD₃,
radioimmunoassay), ionized calcium (iCa, ion-selective electrode/pH electrode at pH 7.4) and parathyroid hormone (PTH, radioimmunoassay).

Complete ophthalmologic examinations under manual restraint were also performed at the initiation of the study. One drop of Tropicamide 1% (Bausch & Lomb Inc., Tampa, FL) was administered into each eye prior to the onset of the exam. The examinations included a neuro-ophthalmic examination, fundic examination, tonometry, slitlamp biomicroscopy (Kowa-SL2, Kowa, Tokyo, Japan), and corneal pachymetry. Tonometry was performed with a rebound tonometer (Tonovet, Icare Finland Oy, Espoo, Finland). The rebound tonometer has three available calibration settings: canine, equine, and “other”; other is used for species for which a calibration table has not been established. The calibration setting for “other” was used in this study. A corneal pachymeter (Compuscan P ultrasonic pachymeter, 20MHz, Strorz Instrument Company, St. Louis, Missouri) was used in the exam as a gauge of corneal thickness. Readings were taken in triplicate for each eye during the exam. Serial ophthalmic examinations were performed at two-month intervals, and at the end of the study period, with the same study procedure repeated for each examination period. A single, blinded ophthalmologist (Dr. Amber Labelle, University of Illinois, College of Veterinary Medicine, Urbana, IL) performed all of the ophthalmologic exams.

Computed tomography (CT) scans were performed under sedation at the onset of the study. CT of the skull was performed on all subjects using a 16-slice helical CT scanner (GE Lightspeed 16 slice CT Milwaukee, WI, USA) using the following parameters: kVp of 80, mA of 180, slice width of 0.625mm, pitch of 0.925, scan field of view 9.6 cm, 512 x 512 matrix, a rotation time of 0.8s and using a bone convolution kernel. Each rabbit was administered a combination of 0.05 mg/kg buprenorphine hydrochloride (Reckitt Benckiser Pharmaceuticals
Inc., Richmond, VA) and 0.5 mg/kg midazolam (Hospira Inc., Lake Forest, IL) via intramuscular injection. Once the animal became sedate, it was positioned in sternal recumbency and perpendicular to the gantry with forelimbs positioned caudally. After the scan was complete and the animal recovered from sedation, it was returned to laboratory housing.

Every attempt was made to ensure the head was positioned symmetrically as to not require further post processing reconstruction of the raw data for corrected image planes. The primary transverse CT images included the first two cervical vertebrae and all scans were considered good diagnostic quality by a board certified radiologist (SKJ). Segmentation of the skull and teeth was performed using a dedicated DICOM workstation (OsiriX version 5.8.4 64-bit, OsiriX Imaging Software, OsiriX Foundation, Geneva, Switzerland) and segmentation plugin (Mialite, Center for Medical Image Science and Visualization (CMIV), Linköping University, Sweden) (Figures 3-8). The first step was removing the soft tissue surrounding the skull and neuro tissue within the calvarium and proximal cervical spinal cord. This was achieved by segmentation using a threshold minimum of -100HU, a maximum of 150HU, and a smoothing factor of 0.1. (Wang et al., 2011). The resulting soft tissue region of interest (ROI) were removed by changing all contained pixels to a value of -2000HU. The segmentation plugin was used again to isolate the skull using a threshold minimum of 0HU, no maximum HU constrains, smoothing factor of 0.1, and a seed (starting point) within the temporal bone. An additional blocker point was used to prevent segmentation of the cervical vertebral bodies. The parameters of the resulting three-dimensional (3D) ROI were recorded for each specimen, such as volume, average attenuation (HU), and attenuation standard deviation (SD).

After initial sample collection, rabbits were assigned to two treatment groups of six animals each using a random number generator (www.random.org). Each group was then
randomly divided into two pens housing three animals each. Of the treatment groups, the supplemented (treatment) group was exposed to 12 hours of artificial UVB radiation daily that was controlled by a timer; non-supplemented (control) groups received ambient fluorescent light with no UVB supplementation for 12 hours daily. To provide direct UVB radiation, nine dome lights (Fluker Farms, Port Allen, LA) were suspended above the animals at equidistant intervals, comprising three rows of three lights each. The 10 inch deep domes were anchored to a shelving unit several feet off the ground and suspended directly above the rabbits, with no physical barrier present between the animals and light. UVB light was provided using one 5.0, 20 W, Sun-Glow coil bulb (Fluker Farms, Port Allen, LA) in each dome. The domes were suspended 17-19 inches above the ground, in order to maximize exposure to the animals without impeding movement, as well as attempting to mimic the distance that would be used in a typical laboratory or captive cage setting. The pens for both groups can be seen in Figure 12. The amount of UVB radiation and irradiance was measured in microwatts per centimeter squared (µW/cm²) by placing a digital UVB meter (Solartech, Inc., Harrison Township, MI) and an irradiance meter (Zoo Med Laboratories Inc., San Luis Obispo, CA) directly under each bulb, as well as in between the bulbs. The measurements were taken by placing the UVB meter on a platform, so the level of the meter was in line with the dorsum of the rabbits. Measurements were collected weekly, approximately 3 hours after the bulbs were turned on and approximately 3 hours prior to the bulbs being turned off. To correct for UVB degradation over time and provide consistent levels of supplementation, bulbs were replaced if at any time the UVB output from a bulb measured less than 20 µW/cm².

The study was conducted over 6 months. After the UVB light supplementation was initiated, blood samples were collected every three weeks to measure blood chemistries, serum
PTH, ionized calcium, and 25-hydroxyvitamin D₃ levels, for a total of 9 sample collections. Weights were collected during each sample collection. Collection, processing and testing of the blood samples were similar to the techniques described for the samples obtained on day 0. Serial ophthalmologic examinations were performed at two month intervals, and at the end of the study period, for a total of four examinations.

At the end of the six month period, anesthesia was induced via administration of 5% isoflurane gas (IsoFlo, Abbott Animal Health, North Chicago, IL) via facemask. The animal was placed in dorsal recumbency and a final 3 ml of blood was collected from the cranial vena cava using a 22 gauge needle attached to a 3 ml syringe. While being maintained on 5% isoflurane and 1L oxygen, 2-3 ml of pentobarbital (Vortech Pharmaceuticals, Dearborn, MI) was administered to the animal via intracardiac injection. Euthanasia was confirmed via auscultation and negative corneal reflex.

CT scans were performed immediately following euthanasia using the identical scanning and positioning parameters described at the beginning of the study. Segmentation of the skull, mandible and teeth was also acquired using the identical techniques described previously. ROI volume, mean and SD of the attenuations were recorded for each specimen.

A postmortem examination was performed on each animal. A select tissue set was collected from all animals except one animal, which died as a result of an esophageal and gastric impaction and in which a complete set of tissues was collected. The select tissue set included lung, heart, haired skin- head, haired skin-ear, haired skin-dorsum, haired skin-ventrum, liver, pulmonary artery, aorta, adrenal gland, kidney, duodenum, pancreas, thyroid glands, spleen, esophagus, reproductive tract, trachea, skeletal muscle, eye, and femur. Tissues were fixed in 10% neutral buffered formalin. Following fixation, tissues were embedded in paraffin wax,
sectioned at 4 µm, and stained with hematoxylin and eosin. Additionally, histochemical staining with Verhoeff Van-Gieson satins was performed. Histologic lesions were noted and scored using the following categories: no lesions, mild lesions, moderate lesions, and severe lesions. A single, blinded pathologist (Dr. Adam Stern, University of Illinois, College of Veterinary Medicine, Urbana, IL) performed all of the microscopic examinations.

Bone mineral densities (BMD) of rabbit skulls were measured by dual energy x-ray absorptiometry (DEXA) (Hologic QDR 2000, Marlborough, MA). Prior to analysis, all skulls were preserved and stored at -20 degrees Celsius. For BMD assessment, all skulls were positioned dorsoventrally, and a standardized region of interest (R1) of a constant area (cm²) was created to encompass the entire skull size of each specimen. Bone mineral density was measured and expressed as grams of bone per area (gm/cm²). The analyst of the bone concentrations was blinded to the study groups.

The sample size selected for this study (n=12; 6 per group) was based on the following assumptions: an expected mean difference in serum 25-OHD₃ concentrations of 35 nmol/L between treatment and control groups with a SD of 20 nmol/L (Emerson et al. 2014, Rivas et al. 2014), an alpha of 0.05, and a power of 0.8 (MedCalc 11.3.2.0). Distribution of the data was evaluated by use of the Shapiro-Wilk test. Mean, SD, and minimum-maximum (min-max) values were reported for data that had a normal distribution, whereas the median, 10th to 90th percentiles (%), and min-max values were reported for data that did not have a normal distribution. Data that were not normally distributed were logarithmically transformed in order to perform for parametric analysis. If the data was not normally distributed after transformation, appropriate nonparametric statistics were performed.
A linear mixed model was used to evaluate the normally distributed and correlated blood chemistry values, iCa, and 25-OHD$_3$ collected in the repeated measures design of the study. This statistical method was selected because one animal died over the course of the study and this type of model can account for missing data. Group and time represented the fixed and random factors, respectively, that were evaluated in the model. The -2 log likelihood was used to determine best fit of the model. Friedman’s test was performed on data that was not normally distributed. If significant, Mann-Whitney U tests were performed to determine if a difference existed between groups at each individual time point. A repeated measures ANOVA was used to evaluate differences in Hounsfield units measured on CT scans between groups over time. A repeated measures ANOVA was also used to determine if there was a difference between corneal pachymetry measurements taken in triplicate. Since there was no difference in the corneal pachymetry data, the values were averaged and a linear mixed model was used to assess average corneal pachymetry and intraocular pressure values over time. Levene’s test was used to determine if bone mineral density (gm/cm$^2$) met the assumption of homogeneity of variance. Because it did, an independent samples t- test was used to determine if bone mineral density differed between groups. Chi square one-tailed tests with a Yates correction or Fisher exact tests were performed to determine if there was any difference in the pathologic findings documented at necropsy between groups. A value of $P \leq 0.05$ was used to determine significance. Commercial statistical software (SPSS, version 22.0, SPSS Inc, Chicago, IL) and graphpad.com were used to analyze the data.
4.3 RESULTS

Twelve rabbits (6 not exposed to UVB, and 6 exposed to UVB) were used in this study. At the beginning of the study the average body weight of the rabbits was 2.062 kilograms (SD: 0.113, Min-Max: 1.85-2.21 kg), while at the conclusion of the study the mean body weight of the rabbits was 3.172 kg (SD: 0.182, Min-Max: 2.94-3.47); there was no significant difference in the initial or final body weights noted between groups (F= 0.069, p= 0.793). The mean difference in weight gain for the rabbits from the beginning of the study to completion was 1.09 kg (SD: 0.119, Min-Max: 0.89-1.28). Over the study period, weight gain among rabbits exposed to supplemental UVB lighting and those not exposed did not differ (t=-1.254, p = 0.238).

Complete blood counts were performed at the beginning and completion of the study to assess the general health of the rabbits. Unfortunately, a number of the samples collected at both the beginning and end of the study were clotted and could not be evaluated. Because of the small sample set, statistical comparisons were not performed. However, at least one CBC result was available for each rabbit at either the beginning or conclusion of the study, and all results were within published reference limits (Vennen and Mitchell, 2009).

Serum 25-OHD$_3$ values differed significantly by group over time (F=10650.056, p= 0.003) (Figures 13 and 14), with higher values seen in the UVB supplemented group. Since the animals arrived at our facility having been fed a different diet than our study diet, mean, SD, and min-max values including initial baseline data and excluding the baseline data are shown in Table 5. The UVB radiation levels measured in the UVB treatment groups ranged from 12-70 µwatts/cm$^2$ directly under the lights and 1-28 µwatts/cm$^2$ in between the lights. UVB radiation levels in the non-UVB groups were <1 µwatts/cm$^2$. The maximum UVB irradiance level in the pens under the lights was 2.6, which falls under category III in the UV index spectrum. This
value is considered moderate exposure and equivalent to “mostly full sun, occasional partial sun” and is well within the index considered safe (< 7). The range of irradiance in between the lights was 0.1-0.5, which is consistent with shade or crepuscular exposure. There was no significant difference in the 25-OHD₃ concentrations between the two pens of rabbits exposed to the UVB lights (F= 1.928 p= 0.172) or between the two pens of rabbits not exposed to the UVB lights (F= 0.990, p=0.376). This was done to rule out potential differences in light exposure between treatment groups or quantity of food consumed between control groups.

When analyzing the blood chemistry values by group and over time, no significant differences were found for PTH, iCa, Ca, Phos, Na, K, ALP, ALT, Amy, T-bil, BUN, Creat, Alb, Glob, TP, or Gluc values. However, significant differences by group were found for calcium/phosphorus ratio (Ca/Phos, F=6.483, p= 0.013), with a mean of 5.058 ± 1.42 (min-max: 1.92-7.84) for the group without UVB supplementation and a mean of 4.369 ± 1.212 (min-max: 2.06-6.48) for the UVB supplemented group.

There was no significant difference in corneal thickness between groups for either the right (OD) (p= 0.467) or left eye (OS) (p= 0.497). Intraocular pressures were also found to not be significantly different between groups for either eye (right eye [OD]: p= 0.776; left eye [OS]: p= 0.769). Occasional ghost vessels on the cornea, prominent sutures, and an incipient cataract were noted in both groups during the initial examination, which are all considered normal variants. No significant ocular abnormalities were noted during any of the examinations.

The average Hounsfield units for the initial CT scans were 887.4120 (SD: 37.281; Min-Max: 851.26-982.49). The average Hounsfield units at the final CT scan were 1042.8075 (SD: 36.075; Min-Max: 999.44-1102.44). There was a significant increase in Hounsfield units noted on the CT images over time (F=152.571, p= <0.0001) but not between groups (F=1.309,
p=0.279). There was no dental disease or other abnormalities noted on the CT scans. There was no significant difference noted in the bone mineral density between the two groups obtained via DEXA scan (t = -0.318, p=0.757).

After the fifth (out of nine total) sample collection, a rabbit was acutely noted to have increased respiratory effort. No overt abnormalities were noted on physical examination and the decision was made to separate the animal from the group and provide supportive care with fluid therapy, antibiotics, and anti-inflammatories. However, before treatment could be initiated the animal passed away. Necropsy was performed immediately post mortem. Gross lesions were consistent with a marked esophageal and gastric impaction, with minimal aspiration pneumonia documented on microscopic examination. No mass or evidence of inflammation or fibrosis was identified within the pyloric outflow tract. The pathologist reported the lesions were indicative of impaction of the stomach and esophagus by feed material, which led to compression of the diaphragm, difficulty breathing and/or compression of the caudal vena cava, hypovolemic shock, and eventual death. Although this animal was in the UVB supplementation group, the necropsy and histopathologic findings did not appear to be related to husbandry practices.

No significant differences were found when comparing necropsy lesions between groups (all p>0.05; Table 6). The most common lesions identified in the study subjects were mild dermatologic changes noted equally across groups and consisting of a few apoptotic keratinocytes in the basal cell layer with associated orthokeratosis; small numbers of lymphocytes and plasma cells in the superficial dermis; and mild cytoplasmic vacuolization of keratinocytes on skin from the ears, head, or ventrum. These mild lesions were documented in the ears of 5/6 (83%) rabbits in the non UVB supplemented group and 4/6 (66.7%) rabbits in the UVB supplemented group. Mild lesions were also noted on the skin ventrum and in skin on the
head of 1/6 (16.7%) in each group for each lesion. Rare or mild elastin abnormalities were noted on Verhoeff-Van Gieson staining in 4/6 (66.7%) rabbits in the non UVB supplemented group and mild to moderate elastin abnormalities were noted in 3/6 (50%) of the UVB supplemented group. Specific histologic abnormalities can be seen in Appendix B.
4.4 DISCUSSION

Findings from the present study showed that rabbits provided artificial supplementation of UVB light produced and maintained significantly higher serum 25-OHD$_3$ levels compared to those without UVB supplementation. In many vertebrates, vitamin D$_3$ (cholecalciferol) can be synthesized after exposing the skin to UVB radiation. This initiates the photobiochemical conversion of the precursor 7-dehydrocholesterol in the dermis to previtamin D$_3$, which undergoes isomerization into vitamin D$_3$ and is further hydroxylated to 25-hydroxyvitamin D in the liver (Holick et al., 1995; How et al., 1994; Webb and Holick, 1988). 25-OHD$_3$ is the storage form of vitamin D, and therefore considered the best measure of vitamin D status in the body (Holick, 1990; Schmidt-Gayk et al., 1997). When vitamin D is needed systemically, the kidneys are responsible for converting 25-OHD$_3$ to 1,25(OH)$_2$D$_3$, although conversion has been demonstrated in other tissues as well. The findings in this current study, along with a previous short term pilot study (Emerson et al., 2014), reinforce that rabbits have the ability to generate significant quantities of vitamin D through UVB exposure, despite adequate levels being provided in the diet, and thus it may be an important conserved evolutionary mechanism not currently utilized in captive rabbit husbandry practices.

While a significant difference was noted between groups over time, reference ranges for optimum serum 25-OHD$_3$ in wild or captive rabbits have not been established. In humans, the mammal in which vitamin D deficiency is most commonly diagnosed and treated, a serum 25-OHD$_3$ concentration of <50 nmol/L is considered deficient (Holick, 2007). However, it should be noted that this minimum value is a general recommendation by most experts, and a general consensus on optimum levels of 25-OHD$_3$ in humans does not exist. Since rabbits and humans share similar methods of vitamin D acquisition, both utilizing UVB exposure and diet, it might
be tempting to extrapolate the rabbit values to human reference ranges. Based on the current results, the group not supplemented with UVB light (31.54 ± 11.6673 nmol/L, range 7.0-60) would be considered chronically deficient in vitamin D. However, such a direct comparison is difficult because of differences in physiology between our two species. Humans are omnivores that are capable of deriving vitamin D from animal and fortified sources in their diet, in addition to the vitamin D they could obtain from the plant material in their diet, as in the case of rabbits. Rabbits have evolved in a similar environment to that which humans evolved. While it may appear as though both species are adapted to synthesize and maintain vitamin D levels under these slightly different conditions, evaluating the two different methods for which they derive the vitamin D is important to consider. Dietary sources of vitamin D tend to persist in the body, while photobiochemical produced vitamin D can be turned on or off based on need. Also, the body is not as efficient at utilizing dietary sources of vitamin D when compared to vitamin D produced secondary to UVB exposure. Both have the same biologic activity; however, the half-life of vitamin D₃ produced in the skin is longer because it is 100% bound to vitamin D binding protein. Conversely, only about 60% of dietary vitamin D₃ is bound in circulation (Haddad and Ja Chyu, 1971). In theory, rabbits are likely adapted to similar levels as humans, which is suggested by the results of the study since animals exposed to UVB maintained levels well over 50 nmol/L.

The intent of this study was not to determine reference ranges for rabbits but to determine whether exposure to artificial UVB radiation would lead to increased and persistent levels of 25-OHD₃ in these animals, and whether long term (6 months) exposure led to any pathologic conditions. In order to determine a reference interval for serum 25-OHD₃ in rabbits, a cross-sectional study would need to be performed. The study population should include animals being
maintained both indoors and outdoors. Subject recruitment must also consider diet to limit the potential variability attributed to different dietary levels of vitamin D. To establish such a reference based on the previously noted factors and others such as sex (male, female) and age (juvenile, adult) would require a minimum sample size of 250 animals. Collecting and testing 25-OHD$_3$ samples from a cohort of wild animals would also be useful. This could provide insight into the natural circulating levels in animals on a natural diet and exposed to sunlight. The sample size required to characterize this reference interval, because of an increased likelihood of subject homogeneity, would be 120 animals.

An important finding in this study was that, over a six-month period, prolonged exposure of rabbits to artificial UVB light sources led to a statistically significant increase in serum 25-OHD$_3$ levels that were sustainable over time. This ability to sustain substantially higher levels of 25-OHD$_3$ from UVB exposure compared to animals provided vitamin D in their diet alone reinforces that these animals evolved with this mechanism to procure and maintain adequate levels of vitamin D. These findings also reinforce the need to pursue follow-up research to determine if there is a correlation between disease and vitamin D levels in rabbits.

When comparing the vitamin D levels of the rabbits in the current study to a similar study in guinea pigs (Watson et al., in review), the average 25-OHD$_3$ concentration in rabbits was lower, despite similar UVB exposure. However, this should not be surprising when considering the natural life histories of these two species. Rabbits may have a lower vitamin D requirement than guinea pigs because their natural habitat is at a higher latitude and lower altitude than guinea pigs, and because much of their life cycle is spent in warrens or a series of connected tunnels and burrows. Rabbits are also a crepuscular species, meaning they are most active at dawn and dusk, when natural UVB levels would not be at their peak. Additional work to
determine how different levels of vitamin D in the diet affect 25-OHD₃ levels in these two different species are needed to better characterize their needs in captivity.

An interesting observation in this study was that the baseline serum 25-OHD₃ values were the most similar to the group supplemented with UVB light, and contrary to what was expected, actually decreased after initial baseline collection in the group not supplemented with UVB. Unfortunately, the initial baseline sample collection took place three months prior to the first sample collection because of logistics, so it is not known how long it took for the decline in serum 25-OHD₃ values to occur. During this three-month period, the rabbits were fed the same diet and housed under the conditions described previously. After contacting the breeding facility it was determined that, prior to acquisition at the University of Illinois, the animals were fed a pelleted rabbit feed (LabDiet, St. Louis, MO) that contained 2200 IU/kg of vitamin D, in comparison to the diet fed throughout the time they were housed for the study which contained 900 IU/kg of vitamin D. The 2.4 times higher dietary concentration seen in the diet offered at the breeding facility is almost in direct agreement with the higher mean levels of serum 25-OHD₃ demonstrated between groups after the initial baseline collection (2.6 times). This finding reinforces that rabbits indeed have the capability of increasing their serum 25-OHD₃ levels via either dietary ingestion or photobiochemical conversion, and also may hint towards optimum serum 25-OHD₃ maintenance levels in rabbits. However, how they acquire vitamin D could be important, especially as it relates to the potential development of hypervitaminosis D.

The daily requirement for vitamin D in rabbits has not been clearly determined; however, depending on the source, it has been suggested that the optimal dietary vitamin D content should be 600-800 IU/kg (Jekl and Redrobe, 2013) to 800-1000 IU/kg but no greater than 2000 IU/kg (Lowe, 1998; Lebas, 2000). Under these guidelines, the commercial diet fed throughout the
study adheres to the recommended requirements. If concentrations are greater than 3500 IU/kg, problems have been reported, such as abnormal calcification of soft tissues; however, dietary levels as low as 1000 IU/kg of vitamin D have also been shown to result in dystrophic mineralization in the aorta in rabbits (Kamphues, 1991; Lebas, 2000). The formula used at the breeding facility was higher than the recommended maximum level, and 2.2 times higher than the recommended levels. If animals are maintained on this type of diet it could, in the long term, lead to problems as dietary sources are more likely to be attributed to cases of hypervitaminosis D (see below). Although the scope of this study did not include investigations of vitamin D levels related to the diet, the diet provided to the animals was consistent between groups throughout the study, thus indicating that the significant elevations in serum 25-OHD$_3$ were a result of the UVB supplementation only. Further studies to assess the specific role(s) of dietary vitamin D in rabbits are needed.

When evaluating circulating levels of 25-OHD$_3$, one important factor to consider is that systemic mineralization has been observed due to hypervitaminosis D secondary to increased levels of vitamin D in the diet via clinical or experimental report. A diet containing 3250 IU/kg of vitamin D produced excessive calcification in the aorta and kidneys in a matter of 4 weeks and more clearly at 8 weeks (Loliger and Vogt, 1980; Lebas, 2000). Again, significant calcification of the aorta wall was reported after administration of a feed containing 5000 IU vitamin D/kg (Kamphues et al., 1986). There have been no reports of hypervitaminosis D secondary to photoconversion of 7-DHC. This is likely because photobiochemical conversion of 7-DHC to previtamin D$_3$ and hydroxylation in the liver to 25-OHD$_3$ is tightly regulated within the body. In humans, mechanisms have been elucidated which have shown sunlight actually destroys excess previtamin D$_3$ or vitamin D$_3$ when exposure is prolonged or excessive (Holick and Garabedian,
Previtamin D₃ can revert to the parent compound, 7-DHC, or other biologically inert metabolites, to avoid potentially harmful accumulation of circulating vitamin D in the body (Holick et al., 1981). Since regulation of this pathway is dependent on vitamin D levels and not calcium absorption, and since other pathways involving photoconversion in rabbits are similar to the human, it is highly likely that these control mechanisms exist in the rabbit as well; however, specific investigation in rabbits is warranted.

Calcium/phosphorus ratios were significantly higher in the group without UVB supplementation; these animals also had lower serum 25-OHD₃ levels. While in most vertebrates vitamin D₃ is essential for active calcium absorption in the intestine and maintaining serum calcium levels, calcium metabolism differs slightly in the rabbit. Calcium is primarily absorbed via the intestine through passive diffusion and then excreted through the kidney. In the case of vitamin D deficiency, intestinal absorption of calcium and phosphorus are not modified, but renal excretion is instead reduced (Bourdeau et al., 1986). In the blood, calcium can be free (iCa), bound to proteins, or complexed with other molecules. It is important to note that total calcium, ionized calcium, and phosphorus did not differ between study groups. In a study evaluating the relationship between serum vitamin D metabolites and calcium metabolism in women, an inverse relationship to serum 1,25(OH)₂D₃ (the active metabolite) was found (Brot et al., 1999). Since it is not a good indicator of vitamin D status in the body, 1,25(OH)₂D₃ was not measured in our study; however, a possible mechanism is consistent with the findings in this study. Increased conversion to the active metabolite due to low Ca/Phos levels could result in lower 25-OHD₃, and ultimately an increase in Ca/Phos ratio, or vice versa. This finding is important because it is reinforces that vitamin D plays an important role in calcium and phosphorus homeostasis for rabbits. The dynamic nature of calcium and phosphorus suggest that
interpreting its meaning from single reference points could underestimate its true value. Follow-up longitudinal studies evaluating the relationship of Ca/Phos and 25-OHD$_3$ in clinically normal and diseased rabbits are needed.

In children, chronic vitamin D deficiency can lead to developmental abnormalities such as failure of attaining peak height or bone mineral density, pathologic fractures, poor growth, and slow development. Adults with vitamin D deficiency are at an increased risk for osteopenia, osteoporosis, and fracture (Holick, 2014). Effects of hypovitaminosis D have also been observed in animals in experimental studies, including rabbits and dogs (Hazewinkel et al., 1987; Lebas, 2000; Brommage et al., 1988). The animals used in this study were juveniles, and thus it might be expected that those not supplemented with UVB radiation would experience decreased bone mineral density or poor growth if deficient in vitamin D during this vital growth period. However, due to humane reasons, levels in the diet were not restricted and the amount provided should have been sufficient based on current recommendations. No significant difference was observed between groups in Hounsfield units measured on CT scans; however, a significant increase was noted over time. This is consistent with normal growth and bone development patterns. A recent study determined that rabbits reach skeletal maturity at 28 weeks of age (Norris et al., 2001), which was in between CT scan time points (~15 weeks of age and 1 year). Also, there was no difference in the bone mineral density of the skulls between groups noted at the end of the study. These findings indicate that no adverse effects to the bone were noted during the growth phase of these animals, regardless of UVB exposure. Southard et al., 2000 showed that in experimental osteoporotic female rabbits, mandibular bone mineral density decreased in relation to spinal density, indicating that in the current study, the skulls were an adequate model for studying bone density, in conjunction with possible dental disease.
Because these animals were juvenile animals that were only observed until approximately one year of age, it is not likely we would witness any effects secondary to chronic vitamin D deficiency. Dental disease, which has been hypothesized to be due to chronic vitamin D deficiency in rabbits, is commonly considered a progressive syndrome that affects the shape, position, and structure of the teeth, and which often develops over a significant period of time (Harcourt-Brown, 2007). Progressive loss of alveolar bone is a common feature, and it is documented that alveolar bone is particularly susceptible to metabolic bone disease, resulting in tooth displacement (Harcourt-Brown, 2006; Harcourt-Brown, 2007). In horses, alveolar socket bone loss is a common finding associated with nutritional secondary hyperparathyroidism (Krook and Lowe, 1964). Rabbits require high levels of calcium because of the continual eruption and growth of the teeth. The passive diffusion of calcium in the rabbit allows for this, however, in times of low calcium, vitamin D is necessary to instigate active transport. The results of this study do confirm that a dietary source of vitamin D at 900 IU/kg during development is sufficient for normal skeletal growth. Future studies that follow rabbits longitudinally will be important to determine whether animals exposed to UVB, and subsequently maintain higher levels of circulating 25-OHD₃, do so to minimize the potential for developing specific pathologies.

It is important to note that a change in PTH was not observed in this study, even though there was a change in 25-OHD₃ between groups. In the body, decreasing calcium levels stimulate the secretion of PTH, which then triggers the synthesis of active vitamin D at the level of the kidney. PTH promotes the hydroxylation of 25-OHD₃ in the kidney, thereby creating the active metabolite 1,25(OH)₂D₃ (calcitriol). A previous study in dogs found a synergism between PTH and the active metabolite 1,25(OH)₂D₃ (Hazewinkel and Tryfonidou, 2002). This synergism
works to regulate skeletal mineralization, and should be stimulated when systemic levels of calcium and phosphorus are low. Possible reasons that there was no change in PTH in this study could be that there was no difference in skeletal mineralization noted between groups, and total calcium and phosphorus levels were within acceptable reference levels for both groups. Conversely, when vitamin D levels in the body are elevated, circulating PTH decreases. However, it is possible that the elevated levels of 25-OHD₃ recorded in the UVB group were not considered elevated by the rabbits, but instead still acceptable levels. Although the overall status of vitamin D in the body was increased in the UVB supplemented rabbits, the active metabolite 1,25(OH)₂D₃ was not measured in this study, so it is impossible to comment on how these levels were affected by active vitamin D in the rabbits. Future studies evaluating vitamin D and PTH levels in animals with disease may provide more insight into the interaction of these hormones in rabbits.

Since no ocular pathology or significant abnormalities on ophthalmic examinations were identified in any of the rabbits, supplementation with UVB radiation at the levels provided in this long term study (over six months) appear safe. Interestingly, the cornea has been shown to have the ability to synthesize vitamin D in a similar fashion to that of the skin (Lin et al., 2012). It has also been demonstrated that the cornea provides a significant filter to the amount of UV radiation that reaches the lens (Zigman, 1995). Vitamin D may in fact have a positive impact on the eyes as it has been demonstrated that increased vitamin D levels enhance the epithelial barrier function of the cornea (Yin et al., 2011). A similar study in guinea pigs found that animals exposed to UVB radiation developed thicker corneas over time (Watson et al., in review). This may be an evolutionary trait that developed in these animals because of the higher levels of UVB exposure they would be expected to encounter at higher elevations and lower latitudes. This
active growth in the cornea may have also contributed to the higher circulating 25-OHD$_3$ levels found in these animals.

One of the rabbits in the UVB group died during the study. The animal was acutely noted to have increased respiratory effort, and died within a matter of hours despite initiating supportive care. A full necropsy was performed on this animal and the gross findings were consistent with the distention of the stomach, likely causing compression of the diaphragm and the witnessed increased respiratory effort, and also compression of the caudal vena cava resulting in hypovolemic shock and eventual death. Mild acute aspiration pneumonia was noted microscopically, however the lesion was considered too subtle to be the primary cause of death. No foreign material, masses or other causes for pyloric outflow obstruction were observed. Gastric dilatation and subsequent obstruction is commonly reported in the rabbit literature. In severe gastric distension and impaction cases, such as this one, surgery is required and cases have a grave prognosis (Lord, 2012, Harcourt-Brown, 2007). Unfortunately, there is likely nothing that could have been done to prevent this disease from developing in this rabbit. Since all rabbits in the study were provided the same environment and diet, and this animal demonstrated no pathologic conditions attributed to hypervitaminosis D, this animal’s death was considered to be unrelated to UVB supplementation.

No significant differences were noted in the gross necropsy or histopathologic findings between study groups. However, there were lesions recorded in individual animals that are noteworthy. In humans, individuals with fair skin and fair hair are at higher risk of developing neoplasia secondary to UVB exposure (Gallagher and Lee, 2006), and the same has been shown for white cats (Dorn et al., 1971). In this study, New Zealand White rabbits were used because it was expected that they would be most sensitive to UVB exposure and at the greatest risk for
potential adverse side effects secondary to UVB exposure. The most common lesion reported was apoptotic keratinocytes in the basal cell layer with associated orthokeratosis in the ear (9/12, 75%). Interestingly, apoptotic keratinocytes were noted slightly more commonly in animals without UVB supplementation than with UVB supplementation (5/6 vs. 4/6). The ears were most closely associated with the lights, and therefore likely considered to be at the highest risk for potential dermatologic side effects. Typically, apoptotic keratinocytes are associated with UV exposure. In humans, it is well documented that UV radiation induces DNA damage, oxidative stress, and inflammatory processes in keratinocytes, which can result in photocarcinogenesis (Afaq and Mukhtar, 2001). When apoptotic keratosis is noted in small amounts, it is likely an indication of a defense mechanism network that has been developed to control potentially harmful environmental contaminants. Damage to skin collagen and elastin is a hallmark of long term exposure to solar UV radiation in humans (Kligman et al., 1986). Verhoeff-Van Gieson staining was performed to detect elastin abnormalities that may be present in conjunction with solar elastosis (Kadoya et al., 2005), with mild to moderate lesions noted in 7/12 (58.3%) of the animals. Although moderate findings were reported in 2/6 (33.3%) of the rabbits with UVB supplementation, there was no statistical significance between groups. The presence of elastin is typically associated with breakdown of collagen and can be related to sunburn; however, elastin staining was also increased in rabbit cases without UVB exposure, suggesting that another mechanism is present that may be related to aging change. While lesions were present in these animals, they were not considered clinically significant to the development of disease. These findings, in addition to the lack of ocular pathology, suggest that the UVB levels and irradiance used in this study are not toxic in rabbits over an extended period (6 months). Long term studies to determine whether pathologic conditions occur in adults over longer periods of time are
required to further elucidate the importance of UVB exposure in these animals and determine potential side effects from exposure.

It is important to note that only intact females were used in this study due to sample size limitations and a desire to decrease any potential variability associated with sex. For this reason, the effects of UVB radiation in male rabbits cannot be directly commented on. However, intact female rabbits would be expected to have the highest requirements for calcium due to their reproductive needs, and as such are likely to be the most at risk for vitamin D deficiency.

As previously discussed, dystrophic mineralization is a common sequellae to hypervitaminosis D in vertebrates, and a major concern when developing diets for rabbits. The commercial diet offered to the rabbits during this study had a vitamin D content of 900 IU/kg. The findings from this study, that no mineralization was noted in any of the twelve rabbits, reinforces that this dietary level is not associated with hypervitaminosis D. However, it is also important to note that hypervitaminosis D was not observed in the animals provided UVB lights even though they had significantly higher serum 25-OHD$_3$ levels. As previously noted, systemic dystrophic mineralization has never been reported secondary to UVB exposure and subsequent photoconversion of precursors to 25-OHD$_3$. These findings suggest that supplementation with UVB radiation is a safe and effective way to increase serum vitamin D levels in rabbits long term.
4.5 CONCLUSIONS

The results of this study indicate that rabbits have the ability to generate significant amounts of 25-OHD₃ via photobiochemical production and that the utilization of this pathway is sustainable over a six month period. This study also showed that there were no significant detrimental health effects associated with long term artificial UVB exposure as characterized by complete blood counts, serum biochemistry analyses, ophthalmologic examinations, CT scans, a DEXA scan, and finally necropsy and histopathology. While it is well known that serum 25-OHD₃ levels can also be increased via dietary supplementation alone, as evidenced by the baseline data, this method of obtaining vitamin D is not necessarily benign. Hypervitaminosis D and associated severe lesions have been documented secondary to excess oral vitamin D supplementation in rabbits. Including this study, no reports of vitamin D toxicosis have ever been documented secondary to UVB supplementation. This study suggests that long term artificial UVB supplementation is safe and effectively increases 25-OHD₃ levels in rabbits.
CHAPTER 5
CONCLUSIONS

Vitamin D is a vital steroid hormone that plays diverse key roles throughout the body. With receptors in over 200 tissues in the body, the role of this hormone throughout the body has been found to extend farther than just calcium metabolism and homeostasis, as it plays a significant role in immune function, cardiovascular health, and prevention of chronic disease processes (Holick, 2014). In light of the variety of functions in the body, chronic deficiency of this compound may have significant long-term health consequences. Current guinea pig and rabbit husbandry practices often do not include lighting recommendations, and therefore most animals rely on diet as their primary source of vitamin D. In captivity, guinea pigs and rabbits experience various disease processes such as dental disease, cardiovascular disease, reproductive failure, and developmental abnormalities that are not typically prevalent in the wild. The results of this study show that rabbits and guinea pigs have the ability to generate significant amounts of vitamin D$_3$ via photobiochemical production and that the utilization of this pathway is sustainable over a six month period. In guinea pigs, iCa was increased in animals exposed to UVB radiation. In rabbits, the Ca/Phos ratio was significantly higher in animals without exposure to UVB supplementation. These findings suggest that vitamin D plays an important and likely dynamic role in calcium homeostasis in these animals. Interestingly, there was an increase in corneal thickness noted only in guinea pigs with exposure to UVB radiation, with no difference noted between rabbit groups. With no ocular pathology identified, this may suggest that increasing corneal thickness is a protective mechanism for these animals or may help produce vitamin D via photobiochemical synthesis. There were no significant detrimental health effects noted.
secondary to long term exposure of UVB radiation throughout the study as characterized by complete blood counts, serum biochemistry analyses, ophthalmologic examinations, CT scans, a DEXA scan, and finally necropsy and histopathology. While we know serum 25-OHD₃ levels can also be increased via dietary supplementation alone as evidence by the baseline data, this method of obtaining vitamin D is not necessarily benign. Hypervitaminosis D has been documented secondary to excess vitamin D oral supplementation in both guinea pigs and rabbits. Including this study, no reports of vitamin D toxicosis have been documented secondary to UVB supplementation.

Follow up studies including measuring vitamin D status in healthy and diseased individuals and different aged animals; evaluating the effect of various amounts of UVB supplementation as well as vitamin D in the diet; evaluating vitamin D levels in relation to natural and artificial UVB exposure; and also considering evaluating animals with different phenotypes are warranted. Other studies that follow guinea pigs and rabbits longitudinally will be important to determine whether animals exposed to UVB, and subsequently maintain higher levels of circulating 25-OHD₃, do so to minimize the potential for developing specific pathologies. Long-term studies to determine whether pathologic conditions occur in adults over longer periods of time are required to further elucidate the importance of UVB exposure in these animals and determine potential side effects from exposure greater than six months.

In vertebrates, vitamin D is an essential hormone that regulates many different functions in the body and can be protective against various disease conditions. The findings of this study suggest that providing artificial UVB radiation (1-70 µwatts/cm²) for a 12-hour photoperiod is safe and increased vitamin D concentrations to levels considered appropriate for other vertebrates (e.g., humans); these levels of vitamin D were also sustainable over time. Providing
guinea pigs, rabbits, or other diurnal rodents exposure to UVB is a safe practice that may be an important husbandry consideration that is not currently recommended.
CHAPTER 6
FIGURES AND TABLES

Figure 1. Summary of vitamin D metabolism through two different methods of acquisition (From Boron and Boulpaep, 2011).

Figure 2. Summary of vitamin D conversion after it is acquired via dietary ingestion or photobiochemical conversion.
CT Scan Figures

Figure 3. Transverse images at the level of the tympanic bullae showing a standard CT series in a bone window, and a transverse series showing the bone segmentation as a green region of interest (ROI).

Figure 4. Segmentation software following soft tissue removal, using a threshold range of 0HU – max (3216HU).
Figure 5. Segmentation software showing the seed position within the temporal bone of the skull, to initiate the segmentation process.

Figure 6. Segmentation software showing the initiation of the segmentation process as a small green zone (arrow).
Figure 7. Segmentation software showing the finalized process that has isolated all bony structures of the skull and mandible.

Figure 8. Final window showing the summary of 3D volume ROI parameters: Volume (mL), Mean HU, STD HU, Max HU and Min HU.
Figure 9. Guinea pigs A) with UVB light supplementation and B) without UVB supplementation.

A).

B).
Figure 10. Serum 25-OHD$_3$ in guinea pigs between groups over time.
Figure 11. A) Guinea pig serum 25-OHD3 levels with no UVB supplementation. Note the increased values for baseline (VITD-A). B) Guinea pig serum 25-OHD3 levels for UVB supplemented group.
Figure 12. Rabbits A) with UVB supplementation and B) without UVB supplementation
Figure 13. Serum 25-OHD₃ in rabbits between groups over time.
Figure 14. A). Rabbit serum 25-OHD3 levels with no UVB supplementation. Note the increased values for baseline (VITD-A). B). Rabbit serum 25-OHD3 levels for UVB supplemented group.
Table 1. Guinea pig serum 25-OHD₃ values with and without baseline values included by group.

<table>
<thead>
<tr>
<th>Serum 25-OHD₃</th>
<th>Mean (nmol/L)</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>With baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No UVB</td>
<td>36.3333</td>
<td>24.41698</td>
<td>10-114</td>
</tr>
<tr>
<td>UVB</td>
<td>101.4906</td>
<td>21.80916</td>
<td>67-165</td>
</tr>
<tr>
<td>Without baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No UVB</td>
<td>28.3333</td>
<td>8.36236</td>
<td>10-48</td>
</tr>
<tr>
<td>UVB</td>
<td>101.2553</td>
<td>22.6816</td>
<td>67-165</td>
</tr>
</tbody>
</table>

Table 2. Guinea pig biochemical values by group in which statistical significance was found (p<0.0001).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionized Calcium (mmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No UVB</td>
<td>1.52</td>
<td>0.07</td>
<td>1.35-1.65</td>
</tr>
<tr>
<td>UVB</td>
<td>1.58</td>
<td>0.09</td>
<td>1.29-1.74</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No UVB</td>
<td>144.32*</td>
<td>0.38</td>
<td>138-150</td>
</tr>
<tr>
<td>UVB</td>
<td>140.19*</td>
<td>0.35</td>
<td>137-148</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No UVB</td>
<td>4.40</td>
<td>0.28</td>
<td>4.0-5.5</td>
</tr>
<tr>
<td>UVB</td>
<td>4.22</td>
<td>0.26</td>
<td>3.7-4.8</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No UVB</td>
<td>6.08*</td>
<td>0.35</td>
<td>5.0-6.5</td>
</tr>
<tr>
<td>UVB</td>
<td>5.79*</td>
<td>0.36</td>
<td>4.6-6.3</td>
</tr>
</tbody>
</table>

*Denotes estimated marginal mean
Table 3. Guinea pig corneal pachymetry values for each eye by group (p< 0.0001).

<table>
<thead>
<tr>
<th>Eye</th>
<th>Mean (µm)</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right (OD)</td>
<td>245.72</td>
<td>14.16</td>
<td>212-269</td>
</tr>
<tr>
<td>No UVB</td>
<td>257.62</td>
<td>21.35</td>
<td>213-288</td>
</tr>
<tr>
<td>UVB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left (OS)</td>
<td>242.64</td>
<td>13.40</td>
<td>216-268</td>
</tr>
<tr>
<td>No UVB</td>
<td>262.77</td>
<td>21.95</td>
<td>213-295</td>
</tr>
<tr>
<td>UVB</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Guinea pig lesions found on necropsy by organ.

<table>
<thead>
<tr>
<th>Organ</th>
<th>No UVB</th>
<th>UVB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>percentage</td>
</tr>
<tr>
<td>Liver</td>
<td>3*</td>
<td>50%</td>
</tr>
<tr>
<td>Kidney</td>
<td>1</td>
<td>16.7%</td>
</tr>
<tr>
<td>Haired Skin- Ear</td>
<td>2</td>
<td>33.3%</td>
</tr>
<tr>
<td>Haired skin- Head</td>
<td>1*</td>
<td>16.7%</td>
</tr>
<tr>
<td>Haired skin- Ventrum</td>
<td>1</td>
<td>16.7%</td>
</tr>
<tr>
<td>Esophagus</td>
<td>1*</td>
<td>16.7%</td>
</tr>
<tr>
<td>Trachea</td>
<td>1*</td>
<td>16.7%</td>
</tr>
<tr>
<td>Femur</td>
<td>1*</td>
<td>16.7%</td>
</tr>
<tr>
<td>Lung</td>
<td>0</td>
<td>16.7%</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>16.7%</td>
</tr>
<tr>
<td>Eye</td>
<td>0</td>
<td>16.7%</td>
</tr>
<tr>
<td>Muscle</td>
<td>0</td>
<td>16.7%</td>
</tr>
</tbody>
</table>

*Denotes all lesions were documented in the same animal
#All lesions documented in the same animal. This animal died post-anesthetic recovery.
Table 5. Rabbit serum 25-OHD$_3$ values with and without baseline values included by group.

<table>
<thead>
<tr>
<th>Serum 25-OHD$_3$</th>
<th>Mean (nmol/L)</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>With baseline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No UVB</td>
<td>39.333</td>
<td>26.07464</td>
<td>7.0-134</td>
</tr>
<tr>
<td>UVB</td>
<td>83.12</td>
<td>22.44234</td>
<td>45-129</td>
</tr>
<tr>
<td><strong>Without baseline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No UVB</td>
<td>31.5417</td>
<td>11.6673</td>
<td>7.0-60</td>
</tr>
<tr>
<td>UVB</td>
<td>81.8864</td>
<td>21.75788</td>
<td>45-121</td>
</tr>
</tbody>
</table>

Table 6. Rabbit lesions found on necropsy by location.

<table>
<thead>
<tr>
<th>Organ</th>
<th>No UVB</th>
<th>UVB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>percentage</td>
<td>percentage</td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>1*</td>
</tr>
<tr>
<td>Haired Skin- Ear</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Haired skin- Head</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Haired skin- Ventrum</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Heart</td>
<td>0</td>
<td>1*</td>
</tr>
<tr>
<td>Trachea</td>
<td>0</td>
<td>1*</td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>1*</td>
</tr>
<tr>
<td>Lung</td>
<td>0</td>
<td>2*</td>
</tr>
<tr>
<td>Thyroid</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Denotes lesions all found within the same animal.
CHAPTER 7

REFERENCES


Watson, M. K., M. A. Mitchell, et al. (in review). “Evaluating the Clinical and Physiological Effects of Long Term Ultraviolet B Radiation on Guinea pigs (Cavia porcellus).”


APPENDIX

Appendix A. Guinea pig histologic lesions by organ.

*Denotes lesions found within the animal that died post-anesthetic recovery.

Lung: Multiple lymphatic vessels are dilated and contain eosinophilic and proteinaceous fluid. There is pulmonary congestion. Alveolar macrophages in small number are scatter within alveolar spaces. There is pulmonary atelectasis*. Rare focus of mineralization.

Heart: No histologic abnormalities.

Haired skin (head): Small numbers of lymphocytes, plasma cells, and eosinophils in the superficial dermis (interstitial pattern).

Liver: Multifocal to diffuse and mild vacuolar hepatopathy (Glycogen). 5 small foci of hepatocyte necrosis surrounded by macrophages and heterophils. There is a mild accumulation of lymphocytes and rare plasma cells within few portal regions.*

Aorta: No histologic abnormalities.

Haired skin (ear): Rare suprabasilar apoptotic keratinocytes. Minimal to mild epidermal hyperplasia. Within the dermis there are mild numbers of lymphocytes and plasma cells.

Pulmonary artery: No histologic abnormalities.

Adrenal gland: No histologic abnormalities.

Duodenum: No histologic abnormalities.

Pancreas: No histologic abnormalities.

Thyroid gland: No histologic abnormalities.

Spleen: There is a minimal amount of erythrophagia. There is a minimal amount of extramedullary hematopoiesis.*

Esophagus: Moderate number of granulocytes within the submucosa.

Trachea: Moderate number of granulocytes within the submucosa. There are small numbers of eosinophils within the submucosa and mucosa.*

Uterine Horn: No histologic abnormalities.

Haired skin (ventrum): Few lymphocyte and plasma cells in the superficial dermis.
Kidney: Multifocal and mild mineralization within renal cortical tubules.

Haired skin (dorsum): No histologic abnormalities.

Skeletal muscle: A single myofiber is mineralized.

Eye: Multiple lymphoid aggregates with follicles forming within the submucosal tissue of the conjunctiva*.

Bone (Femur): Myeloid:Erythroid ratio is 4-5:1. Orderly and complete maturation with increased numbers of mature granulocytes.

Verhoeff-Van Gieson stain: No abnormalities for elastin staining observed.
Appendix A. Rabbit histologic lesions by organ.

*Denotes lesions found within the animal that died of GI impaction.

Lung: Within several bronchi, bronchioles, and alveolar spaces are minimal numbers of neutrophils and macrophages, fibrin and plant material. There is mild pulmonary congestion.* Small accumulation of macrophages and multinucleated giant cells surrounding a piece of plant material (minimal aspiration pneumonia).

Heart: Minimal myocardial hemorrhage.*

Haired skin (head): Small numbers of lymphocytes and plasma cells within superficial dermis. Liver: Diffusely through the liver, hepatocytes contain 1-3, clear and distinct cytoplasmic vacuoles.*

Aorta: No histologic abnormalities.

Haired skin (ear): Few apoptotic keratinocytes (basal cell layer) with associated orthokeratosis, mild compact orthokeratosis, rare basal cell layer mitotic figures, mild cytoplasmic vacuolization of keratinocytes. Locally extensive region of pigmentary incontinence within the dermis with accumulations of both free melanin granules and melanophages.*

Pulmonary artery: No histologic abnormalities.

Adrenal gland: No histologic abnormalities.

Duodenum: No histologic abnormalities.

Pancreas: No histologic abnormalities.

Thyroid gland: Single dilated thyroid follicle, three cystic spaces lined by stratified squamous epithelium and contain few macrophages.

Spleen: No histologic abnormalities.

Esophagus: No histologic abnormalities.

Trachea: Submucosal edema and dilation of lymphatic vessels.*

Uterine Horn: No histologic abnormalities.

Haired skin (ventrum): Few lymphocyte and plasma cells in superficial dermis.

Kidney: No histologic abnormalities.

Haired skin (dorsum): No histologic abnormalities.
Skeletal muscle: No histologic abnormalities.

Eye: No histologic abnormalities.

Bone (Femur): No histologic abnormalities.

Verhoeff-Van Gieson stain: Rare and mild to moderate increased staining for elastin.