An investigation of vitamin D metabolism in kiwi (*Apteryx mantelli*), tuatara (*Sphenodon punctatus*) and New Zealand sea lion (*Phocarctos hookeri*) and the relationship of vitamin D metabolism with their life history characteristics.

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Abstract

Vitamin D, a fat-soluble vitamin, has a wide range of functions in vertebrates. The aim of the study was to determine if the evolutionary history of different animal species affects their predominant route of vitamin D metabolism. The species chosen in this study were Brown kiwi (*Apteryx mantelli*) for their nocturnalism, tuatara (*Sphenodon punctatus*) for their diurnal sun basking nature and New Zealand sea lion (*Phocarctos hookeri*), as a marine mammal species.

A survey of plasma or serum concentrations of 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$) in kiwi, tuatara and New Zealand sea lion and analysed the ability of skin to produce vitamin D_3 in response to UV exposure from post mortem samples of these three species. Assessment of morepork (*Ninox novaseelandiae*) skin was also carried out as an additional example of a nocturnal species.

Wild kiwi had lower plasma $25(OH)D_3$ concentrations than captive kiwi and this variation was most likely of dietary origin. The low concentrations of plasma $25(OH)D_3$ in wild kiwi in their natural habitat, suggest that these minimal levels are sufficient to fulfill their vitamin D requirements in the body or they utilise calcium independent of vitamin D. Captive diets for kiwi may be over-supplemented with vitamin D. In contrast to this finding, the skin of both kiwi and morepork was able to produce small but measureable amounts of vitamin D_3 in response to UV exposure. This result was unexpected, considering their nocturnal nature and the overall pattern of vitamin D metabolism in the kiwi is still unclear.

Vitamin D metabolism in tuatara suggests that both dietary and dermal pathways are important. The survey of plasma $25(OH)D_3$ concentrations in captive tuatara showed variation between the five zoological institutions, which was correlated to the variation in the dietary vitamin D provided between captive institutions.

However, analysis of tuatara skin showed that tuatara had a strong ability to synthesise vitamin D dermally, indicating that it is an important route of vitamin D metabolism in tuatara.

New Zealand sea lion showed overall higher serum $25(OH)D_3$ concentrations than kiwi and tuatara, which might be attributed to the high UV-B radiation exposure they receive in their natural habitat. New Zealand sea lion skin also had comparatively higher vitamin D concentrations both prior to and in response to UV exposure, which shows that dermal route of vitamin D is an important route of metabolism in these marine mammals.

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CHAPTER 1 - Literature review

1.1 Introduction to vitamin D

Vitamin D belongs to a group of fat-soluble secosteroid chemicals, responsible for augmenting intestinal absorption of calcium and phosphorus (Norman, 2008). Important secosteroids in this group are vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) (Bikle, 2011; Dittmer & Thompson, 2011). Since vitamin D is biologically inert, it is classified as a pro-hormone and its conversion to 25-hydroxyvitamin D_3 ($25(OH)D_3$) in the liver, and further to the hormonal form, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in the kidney, is necessary in order to function (DeLuca, 2004; Norman, 2008). The active form, $1,25(OH)_2D_3$, performs with the help of a nuclear receptor, the Vitamin D receptor (VDR), to conduct calcaemic as well as non-calcaemic functions (Bikle, 2011; DeLuca, 2004; Norman, 2008). Calcaemic functions include intestinal calcium and phosphate absorption, mobilisation of calcium from bones, and renal calcium reabsorption (DeLuca, 2004). Apart from its calcaemic functions, $1,25(OH)_2D_3$ has also been shown to play a role in cancer cell suppression, cardiovascular disease and hypertension, and immune function (Bikle, 2009; Dittmer & Thompson, 2011). The occurrence of VDR in almost every cell in the body reflects the importance of these chemicals to biological functioning.

Vitamin D is one of the oldest hormones of animals and humans in an evolutionary context (Bikle, 2011; Holick, 2011; Kenny et al., 2004). Evidence of synthesis of this hormone in phytoplankton and zooplankton, as well as fossils from several million years ago proves its early existence in nature (Bikle, 2011; Holick, 2003b). Further discovery of the ability of different plants and animals to metabolise vitamin D from sunlight and diet, shows that different organisms have undergone evolutionary development over millions of years in order to metabolise vitamin D (Holick, 2003b).

Different animals, according to the evolutionary pressures under which they have developed, may obtain vitamin D from two sources, either exposure to ultraviolet light (predominantly UV-B) and/or from the diet. Early research showed that phytoplankton synthesise vitamin D on exposure to UV radiation from sunlight and subsequently fish obtain their vitamin D primarily by ingestion of these phytoplankton (Holick, 2003a, 2003b). Krill and brine shrimp also contain precursors for vitamin D synthesis, which on exposure to UV-B radiation results in synthesis of vitamin D (Bikle, 2011; Holick, 2003a). Plants and animals present within the ocean millions of years ago contained vitamin D precursors, and metabolism of vitamin D in animals further developed according to their environmental conditions. Therefore, it is likely that evolution plays a role in the way organisms develop their body mechanisms, and there is evidence that shows the route of vitamin D metabolism predominantly used by animals has been greatly influenced by evolutionary selection pressures (Bikle, 2011; Holick, 2003a; Holick, 2011). In order to understand the effect of evolution on the route of vitamin D metabolism predominating in animal species with different body mechanisms, a thorough knowledge about vitamin D and its routes of metabolism is necessary.

1.2 Vitamin D metabolism

One of the primary functions of vitamin D is maintenance of calcium and phosphorus homeostasis in the body (Kenny et al., 2004; McDowell, 2000). Vitamin D is required for the absorption of calcium and phosphorus from the intestine, in order to maintain blood calcium concentrations within normal limits (Dittmer & Thompson, 2011; McDowell, 2000). Calcium and phosphorus are required for mineralisation of bony structures, and thereby maintenance of a healthy skeleton (Dittmer & Thompson, 2011; McDowell, 2000). With the discovery of the important role played by vitamin D in calcium absorption and bone mineralisation, a wide range of studies were carried out to ascertain the sources and types of vitamin D (Lips, van Schoor, & de Jongh, 2014). Research has revealed that the major source of

vitamin D is that obtained from a chemical reaction occurring in the layers of the skin on exposure to sunlight (Holick, 2003b; Horst, Reinhardt, & Reddy, 2005). Vitamin D_3 (cholecalciferol) is the form of vitamin D obtained from the sun, and either vitamin D_3 or D_2 (ergocalciferol) can be obtained via the diet (Dittmer & Thompson, 2011; Kenny et al., 2004; Watson & Mitchell, 2014). Vitamin D_3 obtained from sunlight exposure is the type that is thought to be utilised by most animals, with a few exceptions (Dittmer & Thompson, 2011; Horst et al., 2005; McDowell, 2000). Consequently, differences in the route of vitamin D metabolism between different animal species occur according to body adaptions and their ability to metabolise vitamin D from either the sun or diet, or from both.

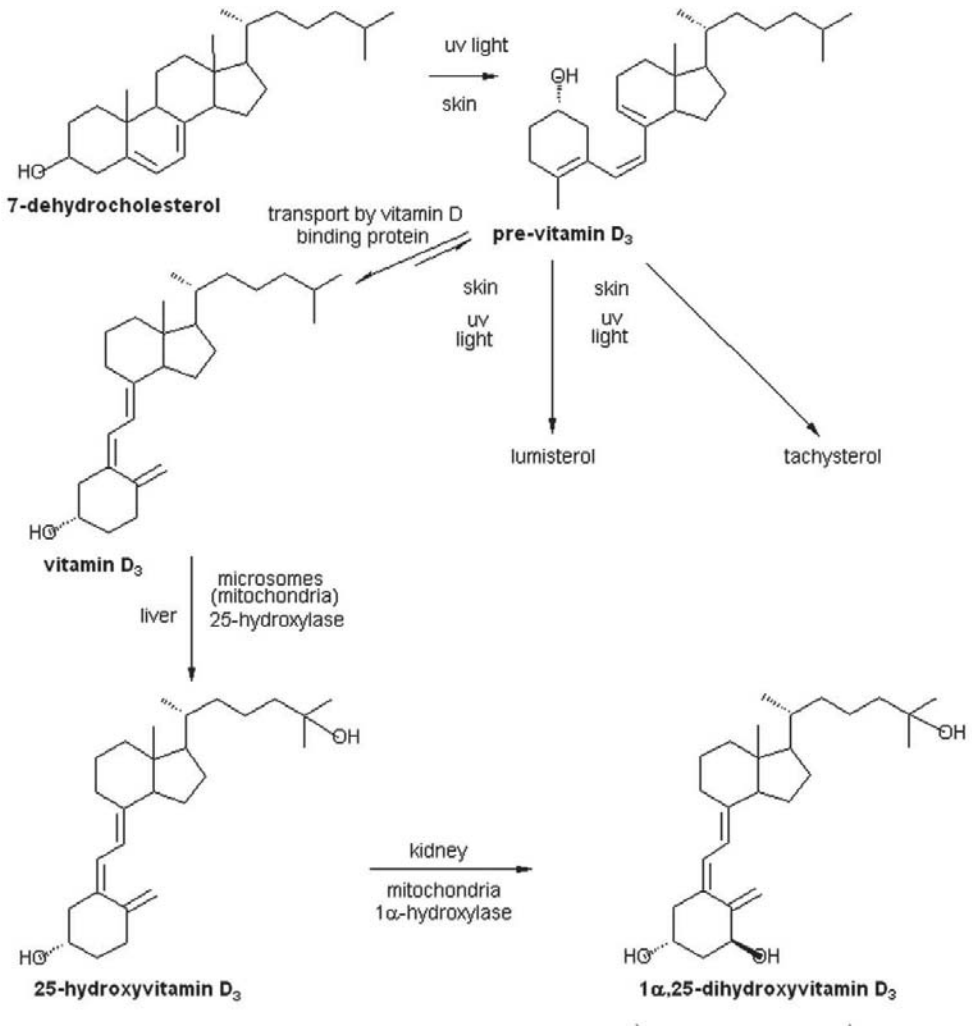

Figure 1: Synthesis of vitamin D, its activation and regulation (Dittmer & Thompson, 2011).

1.2.1 Vitamin D from sunlight

The process of photosynthesis of vitamin D_3 in the skin is the major source of this vitamin (Kenny et al., 2004). The endogenous chemical reaction involves the conversion of vitamin D precursors present in the dermal and epidermal cells when skin is exposed to ultraviolet radiation (UV-B) of 290-315 nm wavelength (Holick, 2003a; Horst et al., 2005). The precursor involved in the initial reaction of dermal

synthesis is a cholesterol like precursor known as 7-dehydrocholesterol (7-DHC) or provitamin D, which has been identified in the cell membranes of keratinocytes and fibroblasts in the epidermis and dermis (Holick, 2003a; Horst et al., 2005; Kenny et al., 2004). When UV-B radiation is absorbed by this provitamin D, it causes rearrangement and cleavage of its structure, resulting in the formation of previtamin D_3 (Figure 1) (Holick, 2003a, 2003b). The resultant previtamin D_3 is biologically inactive and unstable, and consequently undergoes a temperature dependent isomerisation to form the thermally stable compound, vitamin D_3 (Figure 1) (Holick, 2011; Holick, Chen, Lu, & Sauter, 2007; Holick, Tian, & Allen, 1995). The duration of the conversion process varies according to each animal and the conditions under which it exists, since it is temperature dependent, but occurs over a period of three days in most species (Dittmer & Thompson, 2011; Holick et al., 1995; Tian, Chen, Matsuoka, Wortsman, & Holick, 1993).

The thermal isomerisation of previtamin D_3 to vitamin D_3 occurs at a temperature of about 37°C in mammals (Holick et al., 1995; Dittmer & Thompson, 2011). The mechanism by which efficient temperature dependent conversion of previtamin D to vitamin D occurs in poikilothermic animals was not clarified until the mid 1990's (Holick et al., 1995). The early vertebrate animals on land were poikilothermic and it was assumed that the conversion process would occur at a comparatively slower pace and could subsequently result in vitamin D deficiency (Holick et al., 1995). However, it was discovered that these animals underwent evolutionary changes in the process of photosynthesis of vitamin D in the skin, particularly in the conversion step of previtamin D_3 to vitamin D_3, so as to synthesise sufficient amounts of vitamin D in their skin (Holick et al., 1995). Research carried out on iguana skin showed that previtamin D_3 was entrapped in the membranes of the dermal cells in a particular conformation, which allowed for the efficient conversion of previtamin D_3 to vitamin D_3 and this mechanism was found to be operative not only in cold blooded amphibians and reptiles, but also in warm blooded vertebrate animals (Holick et al.,

1995). This shows that the membrane entrapment of previtamin D_3 was of vital evolutionary importance.

1.2.2 Vitamin D from diet

Vitamin D_2 and D_3 may also be obtained from dietary sources (Dittmer & Thompson, 2011; Holick, 2003b; Horst et al., 2005). Some animals, presumably due to evolutionary changes, are incapable of producing vitamin D in skin (Holick, 1992; Holick, 2003a; Holick, 2011). Dogs (*Canis lupus familiaris*) and cats (*Felis catus*) provide a good example of animals that use their diet as the sole source of vitamin D (How, Hazewinkel, & Mol, 1994; Morris, 1999). One of the main dietary sources of vitamin D is fatty fish, which includes salmon (*Salmo salar*), herring (*Clupea harengus*) and mackerel (*Scomber japonicas*) (Holick, 2011; McDonnell, French, & Heaney, 2014). Cod liver oil is also known to be a rich source of vitamin D_3 (Lips et al., 2014). Studies have confirmed that the vitamin D obtained from fish is vitamin D_3 and not vitamin D_2 (Kenny et al., 2004). Egg yolks and dairy products have also been identified to contain vitamin D_3 (Dittmer & Thompson, 2011; Lips et al., 2014). Additionally, there are certain plant sources of vitamin D such as waxy leaf nightshade (*Solanum glaucophyllum*), *Cestrum diurnum, Trisetum flavescens*, where vitamin D is present in very high concentrations, primarily in the form of vitamin D_3 (Dittmer & Thompson, 2011; Holick, 2011; McDowell, 2000). Ingestion of these plants can lead to hypercalcaemia due to the toxic levels of vitamin D they contain and therefore, such plants are also called as calcinogenic plants (Buchala & Pythoud, 1988; Mello, 2003; Wasserman, 1975). In contrast, green forages have been found to be very poor sources of vitamin D_2 (McDowell, 2000; Newlander & Riddell, 1952). However, exposure of pasture species of grass to sunlight has shown formation of vitamin D within them (McDowell, 2000; Newlander & Riddell, 1952). Considering the lack of plant sources containing adequate levels of vitamin D, it has been observed that most herbivorous animals utilise sunlight in order to obtain their

vitamin D requirements (Chaudhary & Care, 1985; Dittmer & Thompson, 2011; Hidiroglou, Williams, & Proulx, 1984; How et al., 1994b).

1.2.3 Regulation of vitamin D synthesis

Vitamin D synthesis in the skin is known to be controlled via photoregulatory mechanisms, skin pigmentation, and latitude (Holick, MacLaughlin, & Doppelt, 1981). Increased skin pigmentation and latitude are effective in limiting cutaneous vitamin D synthesis during normal exposure to solar UV-B radiation, necessitating an increase in the UV-B exposure time so as to effectively synthesise vitamin D (Holick et al., 1981; Webb & Holick, 1988). During excessive UV-B exposure, photoregulatory mechanisms limit the vitamin D synthesis so as to prevent its toxicity. On receiving UV-B radiation, conversion of 7-DHC results in previtamin D_3 synthesis (Holick et al., 1981). The previtamin D_3 formed can either be synthesised via temperature dependent isomerisation to vitamin D_3 or it can undergo photoisomerisation to form lumisterol and tachysterol, which are biologically inert (Figure 1) (Holick et al., 1981; Webb & Holick, 1988). This reaction is reversible and on depletion of the previtamin D_3 stores, lumisterol and tachysterol can undergo photoisomerisation to synthesise previtamin D_3 (Holick et al., 1981). Research carried out to determine if excessive UV-B exposure can lead to hypervitaminosis D showed that there were certain feedback mechanisms present within the skin to limit the cutaneous vitamin D_3 synthesis in response to increased UV-B exposure (Holick et al., 1981). In this research it was observed that during excessive UV-B exposure, previtamin D_3 was synthesised up to a maximum of 10-15% of total 7-DHC concentrations originally present in the skin (Holick et al., 1981; How, Hazewinkel, & Mol, 1994). Upon reaching this plateau, any further UV-B radiation lead to the photosynthesis of the biologically inert sterols, lumisterol and tachysterol (Holick et al., 1981; Horst et al., 2005; How et al., 1994). Since vitamin D binding protein (DBP) has no or minimal affinity for lumisterol and tachysterol, the transfer of these compounds into blood circulation are likely insignificant and the photoisomers are

naturally sloughed off from the skin (Holick et al., 1981). Photochemical regulation of cutaneous vitamin D synthesis is the primary method of preventing hypervitaminosis D via this route.

Unlike the cutaneous route of vitamin D synthesis, there is no known feedback mechanism to prevent vitamin D toxicity via dietary oversupplementation (Adams & Lee, 1997; Wolpowitz & Gilchrest, 2006). Although, skin damage due to UV-B radiation is reported, there have been no reports of hypervitaminosis D via this route. The cases of hypervitaminosis D reported have been due to ingestion of excessive dietary vitamin D (Horst et al., 2005; Hunt, Garcia, & Hegsted, 1969; Nain, Laarveld, Wojnarowicz, & Olkowski, 2007; Olds et al., 2015). Since the accurate levels of vitamin D supplementation in diet are not exactly known, there also lies a risk of hypervitaminosis D due to oversupplementation (Nain et al., 2007). The evolutionary differences within species in the predominance of metabolic pathways of vitamin D synthesis will be further discussed in this review.

1.2.4 Metabolism of vitamin D after ingestion and sunlight exposure

Vitamin D_3 obtained endogenously through synthesis in the skin, and vitamin D_2 and D_3 obtained through the diet undergo the same process of metabolism for conversion into the active form (Dittmer & Thompson, 2011; Martini & Wood, 2006). The dermally synthesised vitamin D_3 enters the circulation by binding with DBP present in dermal capillaries; this protein has a high affinity towards vitamin D (Bikle, 2011; Kenny et al., 2004). Once the vitamin D enters the circulation after being produced in the skin or obtained from the diet, it undergoes hydroxylation reactions in the liver, and subsequently in the kidneys, to yield the biologically active form, which has the ability to carry out the various bodily functions of vitamin D (Figure 1) (Dittmer & Thompson, 2011; Haddad, Matsuoka, Hollis, Hu, & Wortsman, 1993). The first, a 25-hydroxylation reaction, results in the conversion of vitamin D to 25(OH)D (Dittmer & Thompson, 2011; Kenny et al., 2004). A number of different

hepatic enzymes can perform this reaction including CYP27A1, CYP3A4, CYP2R1, CYP2J3, and this step is unregulated (Dittmer & Thompson, 2011; Horst et al., 2005). This stable compound undergoes a second hydroxylation reaction by the renal 1α-hydroxylase enzyme (CYP27B1) resulting in the synthesis of the biologically active form, $1,25(OH)_2D$ (DeLuca, 2004; Dittmer & Thompson, 2011). Since vitamin D requires hydroxylation to be converted into a biologically active form, it is considered a prohormone, rather than a true vitamin (Tian, Chen, Lu, Shao, & Holick, 1994).

Although the process of metabolism of vitamin D does not differ according to the source, certain differences have been observed in the delivery of vitamin D metabolites obtained from the skin or diet (Haddad et al., 1993). Vitamin D produced endogenously in the skin, after undergoing the first hydroxylation reaction in the liver, results in slower and more sustained release of 25(OH)D (Haddad et al., 1993). Vitamin D obtained from ingestion delivers 25(OH)D much more rapidly, but this increase is less sustained compared to that obtained from skin synthesis (Haddad et al., 1993). This variation may be due to the mechanism by which vitamin D is transferred into circulation after entry into the body (Haddad et al., 1993). Therefore, it is considered that vitamin D synthesised in the skin has higher bioavailability than vitamin D acquired from the diet (Haddad et al., 1993).

1.3 Effect of evolution on vitamin D metabolism on different animal species

Several studies have provided information about the production of vitamin D in prehistoric times by phytoplankton and zooplankton in the ocean (Bikle, 2011; Holick, 2011). Vitamin D synthesized within the phytoplankton on exposure to sunlight became a part of the food chain and for early ocean creatures provided a route for dietary acquisition of vitamin D through phytoplankton prey (Bikle, 2011; Holick, 2008; Holick, 2011).

The effect of evolutionary pressures on organisms to procure their vitamin D may have been observed during the early evolution of animals. Complex vertebrate animals, which evolved within the ocean, required calcium to maintain their exo- and endoskeletal structures, and the high amounts of calcium and vitamin D were easily available within the ocean environment (Bikle, 2011; Holick, 2011; Holick et al., 2007). Further evolution of these animals ultimately resulted in their movement onto land, where sources of calcium were present in a variety of plant and animals sources (Bikle, 2011; Holick, 2008; Holick, 2011; Holick et al., 2007). However, these evolving individuals were unable to utilise the calcium present within the plant and animal sources on land, since an efficient physiological system for absorption of dietary calcium from the intestine had not developed in these animals (Bikle, 2011; Holick, 2008; Holick, 2011; Holick et al., 2007). The exact mechanism for the development of photosynthetic ability to produce vitamin D endogenously in vertebrate animal skin remains unknown.

Research carried out on vitamin D metabolism in mammals, reptiles, avian and invertebrate species has shown that the route of vitamin D acquisition that is predominant within animals varies between different animal species (Dittmer & Thompson, 2011; Emerson, Whittington, Allender, & Mitchell, 2014; Holick, 2003a; Rivas, Mitchell, Flower, Welle, & Whittington, 2014). Although most vertebrate species use the dermal route of vitamin D synthesis, there are a few exceptions such as dogs and cats which use dietary sources of vitamin D to fulfill their vitamin D requirement (Acierno et al., 2008; How et al., 1994; Rivas et al., 2014). In their natural environment, the dietary sources of vitamin D for dogs and cats consists of whole body of animal prey including liver, blood and body fat (Chesney & Hedberg, 2010; How et al., 1994; Scott, Greaves, & Scott, 1961). Therefore, in these carnivores, dermal synthesis of vitamin D was not essential to fulfill vitamin D requirements. Carnivores in general, are expected to have negligible amounts of vitamin D precursor 7-DHC in the skin, with extremely low concentrations in

comparison with herbivores and omnivores, which might be due to the presence of enzyme 7-dehydrocholestrol-Δ^7-reductase (How et al., 1994). Although there is no solid evidence to prove that evolution influenced the route of vitamin D metabolism that predominates in these animals, photosynthetic ability of vitamin D synthesis in the skin may have been lost, due to the fact that the natural diet of dogs and cats is rich in vitamin D (How et al., 1994).

Evolution may have also shaped the pathways of vitamin D metabolism in nocturnal animals (Acierno et al., 2008; Holick, 2003b; Horst et al., 2005). Nocturnal animals are not exposed to the same amount of sunlight as diurnal animals and hence it has been assumed that these animals might use alternative mechanisms to fulfill their vitamin D requirements (Emerson et al., 2014; Holick, 2003a; Rivas et al., 2014). Since vitamin D is vital for maintaining calcium homeostasis, some nocturnal animals such as Jamaican fruit bat *(Artibeus jamaicensis)* and naked mole rat *(Heterocephalus glaber)* have developed the ability to mobilise and control their bone and teeth mineral reserves, so as to sustain serum calcium concentrations independent of serum vitamin D concentration (Rivas et al., 2014). Both of these animals not only have very low 7-DHC concentrations in the skin but also have a negligible intake of vitamin D in their diet and hence have evolved in such a way as to be independent of vitamin D for their calcium requirements (Holick, 2003a; Kwiecinski, Zhiren, Chen, & Holick, 2001). In contrast, it was discovered that certain nocturnal animals such as Mediterranean house gecko *(Hemidactylus turcicus)* intensify the sensitivity of their skin to UV-B radiation, so as to take full advantage of poor light conditions for adequate synthesis of vitamin D (Acierno et al., 2008; Rivas et al., 2014).

A study on fish eating bat *(Myotis vivesi)* has shown evidence of high circulating levels of 25(OH)D_3 in these bats likely attributed to their piscivorous diet (Holick, 2003a; Southworth, Chen, Kunz, & Holick, unpublished work), whereas very low

levels of 25(OH)D_3 were detected in Egyptian fruit bat *(Rousettus aegyptiacus)* and Jamaican fruit bat *(Artibeus jamaicensis),* which is likely attributed to their nocturnal lifestyle and diets low in vitamin D (Cavaleros, Buffenstein, Ross, & Pettifor, 2003; Kwiecinski et al., 2001).

Camelids like llamas and alpacas are exposed to intense UV radiation, since these animals inhabit the high Andes in the natural habitat (Dittmer & Thompson, 2011; Van Saun, 2006, 2009). Consequently, these animals likely use the dermal route of vitamin D synthesis, and evolutionary pressures have resulted in them having a thicker hair coat and skin pigmentation so as to protect them against the high intensity radiation (Hill, Thompson, & Grace, 1994; Van Saun, 2006, 2009). Further, the continued dependence on UV-B radiation for vitamin D may have resulted in decreased oral bioavailability of vitamin D, making them dependent on dermal vitamin D synthesis (Hill et al., 1994; Rivas et al., 2014; Van Saun, 2006, 2009).

Another mammalian species that appears to have undergone adaptations in vitamin D metabolism as a result of their life history characteristics, is the polar bear *(Ursus maritimus)* (Kenny, Irlbeck, Chen, Lu, & Holick, 1998; Watson & Mitchell, 2014). Research carried out to determine the levels of 7-DHC in the skin revealed low levels of this precursor, which suggests that deprivation of UV-B exposure due to inhabitation of polar bears in dens and at high latitudes may have caused evolutionary changes in their vitamin D metabolism (Kenny et al., 1998). Polar bears are unable to synthesise sufficient vitamin D in the skin and hence rely on ingestion of dietary sources of vitamin D (Kenny et al., 1998; Watson & Mitchell, 2014).

A great deal of variation in the route of vitamin D metabolism has been observed in reptilian species. A wide range of lizards such as panther chameleons (*Furcifer pardalis*), iguanas (*Iguana iguana*), bearded dragons (*Pogona vitticeps*) have been shown to produce vitamin D by endogenous dermal synthesis (Hedley & Eatwell,

2013), whereas certain species like black throated monitor lizards (*Varanus albigularis*) and Hermann's tortoises (*Testudo hermanni*) may use both sunlight as well as dietary vitamin D sources to satisfy their requirements (Hedley & Eatwell, 2013; Rivas et al., 2014).

Another characteristic observed in many reptilian species, which may be as a result of evolutionary pressures in the environment, is the ability to regulate endogenous vitamin D concentrations by manipulating both UV-B exposure and dietary vitamin D intake levels (Acierno et al., 2008; Ferguson et al., 2003). A study carried out on bearded dragons (*Pogona* spp.) revealed the apparent ability of these reptilians to choose their dietary vitamin D intake levels according to the amount of UV-B exposure and level of vitamin D supplementation (Hedley & Eatwell, 2013; Oonincx, Stevens, van den Borne, van Leeuwen, & Hendriks, 2010). In the bearded dragon study, animals receiving no UV-B radiation and those receiving relatively lower vitamin D supplementation, showed stronger inclination for vitamin D rich feed items (Oonincx et al., 2010). This behavioural mechanism of vitamin D regulation in the body by assessment of the internal vitamin D status and subsequent adjustments in their UV exposure and ingestion of dietary vitamin D has also been observed in panther chameleons (*Furcifer pardalis*) (Ferguson et al., 2005). The sun basking behaviour of panther chameleons was observed to vary in accordance with their dietary intake and available UV exposure (Ferguson et al., 2003). This feature of reptiles is likely an essential adaptation of these animals to different environmental conditions.

Variation of the route of vitamin D metabolism has also been observed in snakes. For example, a comparison of vitamin D metabolism in ball pythons (*Python regius*) and corn snakes (*Pantherophis guttatus*) showed that exposure of corn snakes to UV-B radiation resulted in a corresponding increase in serum $25(OH)D_3$ concentrations, indicating this species could metabolise vitamin D_3 on exposure to

UV light (Acierno et al., 2008). In contrast, ball pythons showed no increase in serum 25(OH)D_3 concentrations on exposure to UV-B light (Hedley & Eatwell, 2013). A possible explanation for this variation within snake species is a difference in their life histories. Ball pythons, being nocturnal in nature, will be exposed to negligible UV-B light and hence the ability to photosynthesise vitamin D in the skin is not necessary (Hedley & Eatwell, 2013). Corn snakes are regularly exposed to UV-B radiation due to their diurnal nature, and have thereby retained the ability to synthesise vitamin D dermally (Acierno et al., 2008; Hedley & Eatwell, 2013). This difference in vitamin D metabolism between two species with different life histories provides evidence of the possible effect of evolutionary selection pressures on vitamin D metabolism in different species of animals.

Some species of animals have evolved in such a way that a physiological need for vitamin D in the body does not exist. Although vitamin D plays a vital role in calcium absorption, observations of animals that demonstrate low plasma concentrations of vitamin D metabolites suggest the requirement of vitamin D to maintain calcium homeostasis is negligible or absent (Horst et al., 2005). Examples of these animals include mammals such as the horse (*Equus caballus*), naked mole rat (*Heterocephalus glaber*), wildwood mouse (*Apodemus* sylvaticus) and wild wood vole (*Myodes glareolus*), but also certain aquatic species such as carp (*Cyprinus carpio*), bullfrog (*Lithobates catesbeianus*), lamprey (*Petromyozontiformes*) and halibut (*Hippoglossus*) (Horst et al., 2005). These animals appear to have highly efficient intestinal calcium absorption, in spite of having low circulating 25(OH)D_3 concentrations (Horst et al., 2005). Horst et al., (2005), questioned the requirement of vitamin D in calcium homeostasis in such animals, due to the seemingly normal health observed in these animals with no signs of vitamin D deficiency. The exact reason for this occurrence is unknown, but it may be that low availability of vitamin D in the environment resulted in the adaptation of these animals to develop other metabolic processes for maintenance of calcium homeostasis in the body.

Humans have an established photosynthetic pathway within the skin to synthesise sufficient levels of vitamin D_3, as the concentrations of vitamin D in early humans' diet is thought to have been very low (Hollis, 2005). An exception to this hypothesis of human evolution are the aboriginal arctic people, who survive in areas receiving low UV-B radiation and possibly fulfill the physiological vitamin D requirement through ingestion of vitamin D rich foods like oily fish and fat containing foods (Hollis, 2005).

Thus, life history traits that result in deprivation from sunlight, low UV intensity and exposure duration, or the presence of physical characteristics such as body fur, skin pigmentation, or low concentrations of 7-DHC levels in skin are all factors that have been shown to influence vitamin D metabolism in different species (Holick, 2003a; Norman, 1998). The evolutionary selection pressures encountered by animals over millions of years has resulted in them developing different adaptation mechanisms that result in the most suitable route of vitamin D metabolism to maintain calcium homeostasis for any species in its natural environment (Ferguson et al., 2005; Holick, 2003a).

1.4 Measurement of vitamin D

A number of different metabolites in tissues or in blood can be measured to determine the vitamin D status of an animal.

1.4.1 Measurement of vitamin D metabolites in the skin

The dermal synthesis of vitamin D includes the conversion of vitamin D precursors in the skin (Tian et al., 1994). Therefore, to assess the vitamin D activity within the dermal layers, the dermal precursor 7-DHC as well as vitamin D_3 produced on exposure to UV-B radiation can be measured in skin samples (Tian et al., 1994).

Another factor to take into consideration with regards to vitamin D production in the skin is the site of photosynthesis of vitamin D. Research carried out on chicken skin has shown variation in the photosynthetic ability of different areas of skin to produce vitamin D_3 (Tian et al., 1994; Uva, Mandich, & Vallarino, 1983). For example, a study on different areas of chicken skin such as legs, feet and back, showed a high concentration of 7-DHC in the leg and feet skin, whereas back skin had a negligible amount of 7-DHC (Tian et al., 1994). It was assumed that this reduced ability of back skin to synthesise vitamin D_3 might be due to UV absorption by feathers (Tian et al., 1994). The effect of feathers or fur on dermal synthesis of vitamin D has been observed in other studies as well, and it was therefore advised to use shaved samples to avoid any effect of feather or fur on the vitamin D levels in skin (How et al., 1994; Tian et al., 1994). However, removal of overlying fur and feathers might present an unrealistic picture of the vitamin D physiology in an animal and is therefore debatable.

Correlation of the concentrations of 7-DHC and vitamin D_3 with the plasma $25(OH)D_3$ concentrations gives a representation of plasma components of vitamin D metabolism, which includes vitamin D chemicals acquired from both dermal synthesis as well as dietary vitamin D.

1.4.2 Measurement of vitamin D metabolites in plasma/serum

Several studies, primarily in humans, have determined the best analyte for assessing vitamin D status in the body is 25(OH)D (DeLuca, 2004; Kenny et al., 2004; Norman, 2008). Firstly, 25(OH)D has higher circulating levels in blood compared to 1,25(OH)D and a longer half-life of approximately two weeks, whereas, $1,25(OH)_2D$ has a short half-life of approximately 4 hours in circulation, and the circulating $1,25(OH)_2D$ concentrations are roughly 1000 times less (Holick, 2009; Kenny et al., 2004). Secondly, renal 1α- hydroxylase enzyme, responsible for conversion of 25(OH)D to $1,25(OH)_2D$ is highly regulated, mainly by parathyroid hormone, but also negative

feedback by, $25(OH)_2D$, and plasma calcium and phosphate concentrations (Dittmer & Thompson, 2011; Horst et al., 2005; Norman, 2008). Since 25(OH)D and its formation in the liver from vitamin D is relatively unregulated, plasma 25(OH)D concentration is considered a precise reporter of vitamin D obtained from skin and/or diet (Horst et al., 2005; Martini & Wood, 2006; Norman, 2008).

Plasma $25(OH)D_3$ concentrations provide a picture of vitamin D acquired from sunlight and/or dietary sources (Rajakumar, 2003), whereas, plasma $25(OH)D_2$ represents only dietary items of plant origin (Cashman et al., 2014). Therefore, measurement of both plasma $25(OH)D_2$ and $25(OH)D_3$ concentration is necessary, to get a complete picture of the vitamin D status. Several assays have been developed over the years for measurement of these metabolites.

1.5 Consequences of vitamin D deficiency

Hypovitaminosis D can result in serious health concerns. One of the primary effects of vitamin D deficiency is on calcium homeostasis and bone mineralisation resulting in metabolic bone disease (McDowell, 2000; Watson & Mitchell, 2014). Apart from disorders in bone metabolism, vitamin D deficiency also acts as a serious risk factor in development of immune disorders, type 1 diabetes, cardiovascular disease and hypertension, multiple sclerosis, inflammatory bowel disorder, and certain types of cancers (Dittmer & Thompson, 2011; Holick, 2004; Watson & Mitchell, 2014). Vitamin D deficiency results in metabolic bone diseases such as rickets in young growing animals and osteomalacia in adults (Holick, 2003a; McDowell, 2000). Further, vitamin D deficiency may also be a risk factor for osteoporosis in humans (Holick, 2004). The calcium deficiency that occurs as a result of vitamin D deficiency may also lead to fibrous osteodystrophy, and most animals with vitamin D deficiency have a combination of rickets and fibrous osteodystrophy (Dittmer & Thompson, 2011).

1.5.1 Effect of vitamin D deficiency in captive animals

Vitamin D deficiency is rare in wild free ranging animals, due to the availability of adequate sunlight and sources of vitamin D in the diet (Phalen, Drew, Contreras, Roset, & Mora, 2005; Robbins, 2012; Tangredi & Krook, 1999). However, in the captive management of wildlife species, vitamin D deficiency is commonly encountered. One of the reasons for vitamin D deficiency among captive animals may be the lack of knowledge about the natural history and evolution of various wild animals that leads to species variation in vitamin D metabolism, making determination of the best source of vitamin D for these animals potentially problematic. Further, many zoos have indoor captive housing facilities where there is deprivation of the animals from sunlight, which may result in vitamin D deficiency (Holick, 2003a; Watson & Mitchell, 2014).

a) Mammals

Vitamin D deficiency observed in big cats housed in captivity is thought to be primarily due to the dependence of these cats on dietary sources of vitamin D (Chesney & Hedberg, 2010). Reports on captive lions (*Panthera leo*) and other big cats have described signs of metabolic bone disease including bony deformities, tooth loss, susceptibility to fractures and flexible bones, which may be related to poor diets provided to these animals in captivity (Chesney & Hedberg, 2010; Holick, 2003a; Van Rensburg & Lowry, 1988). Although the diet of these wild cats in their natural habitat fulfills their vitamin D requirement, the diet provided to these animals in captivity often lacks adequate amounts of vitamin D (Fiennes, 1974; How et al., 1994). A report describing rickets in successive lion cub litters at London zoo concluded that the deficiency was nutritional in origin and subsequently the diet was corrected by supplementing with cod liver oil and crushed bones (Chesney & Hedberg, 2010). Therefore the inclusion of vitamin D in the diet is of utmost

importance in cats kept under captive conditions in order to prevent any skeletal abnormalities (Chesney & Hedberg, 2010).

Another mammal facing the consequences of vitamin D deficiency in captive conditions is the polar bear (Kenny et al., 1998; Kenny, Irlbeck, & Eller, 1999). Dietary vitamin D deficiencies have resulted in rickets in the polar bear cubs (Kenny et al., 1999).

Skeletal abnormalities have also been observed in primates housed in glass enclosures which block UV-B radiation, thereby depriving them of the necessary sunlight required for vitamin D synthesis (Fiennes, 1974; Holick, 2003a).

b) Birds

Vitamin D deficiency is rare in wild birds, but has been described in a number of captive species (Phalen et al., 2005; Tangredi & Krook, 1999). Skeletal deformities and nutritional secondary hyperparathyroidism (NSHP) have been reported in two Northern Royal albatross chicks, which were hand reared and fed a diet containing inadequate vitamin D and calcium (Morgan, Alley, Gartrell, Thompson, & Perriman, 2011). The extensive bone remodeling, lack of bone density, fractures and fibrous osteodystrophy were indicative of vitamin D and calcium deficiency (Morgan et al., 2011). Another case report showed occurrence of rickets in double crested cormorants, which was related to inadequate exposure to sunlight as well as dietary deficiency of vitamin D (Nichols, Montali, Pickett, & Bush, 1983). Captive birds, therefore, need a suitable environment and diet in captivity to maintain their skeletal condition.

Lesions of rickets and fibrous osteodystrophy in birds

Several instances of metabolic bone disease have been observed in birds. The causes for these include deficiency of dietary calcium or phosphorus, deficiency of vitamin D_3 and an imbalance in the Ca: P ratio (Phalen et al., 2005; Thorp, 1994; Toyoda, Ochiai, Komatsu, Kimura, & Umemura, 2004). The dysfunction of bone mineralisation in rickets results in failure of endochondral ossification and thickening of the physis (Thorp, 1994). The flexibility of the long bones leads to multiple fractures in long bones, folding fractures, curving deformities and subsequent lameness (Thorp, 1994). (Gröne, Swayne, & Nagode, 1995; Thorp, 1994). In addition, deficiency of vitamin D can lead to secondary hyperparathyroidism, which causes enlargement of the parathyroid gland and irregular and vacuolated parathyroid cells (Phalen et al., 2005; Thorp, 1994). Secondary hyperparathyroidism results in excessive osteoclastic resorption of bone, and replacement with poorly mineralised woven bone and fibrous connective tissue, leading to further weakening of bone strength (Thorp, 1994; Dinev, 2012).

A consequence of NSHP is fibrous osteodystrophy (Phalen et al., 2005; Thorp, 1994). Therefore, disorders of calcium homeostasis, which can include vitamin D deficiency, can lead to a combination of metabolic bone diseases including rickets and fibrous osteodystrophy. The clinical signs of Ca:P imbalance and vitamin D deficiency are often similar and various studies of metabolic bone disorders reported are unclear in identifying which deficiency is involved in these bone metabolism disturbances.

c) Reptiles

Similar problems associated with calcium homeostasis, including vitamin D deficiency, have been observed in reptiles. Reports of embryonic death and high percentage of hatchling mortality have been observed in captive iguanian lizards deficient in vitamin D (Laing, Trube, Shea, & Fraser, 2001). Effects of vitamin D

deficiency were also observed in komodo dragons and panther chameleons who were unable to produce viable eggs under conditions of low UV-B radiation in captivity (Donoghue, 1998; Holick, 2003a). NSHP was also reported in tuatara kept in captivity, due to inadequate exposure to UV-B light (Burgess, Gartrell, & Blanchard, 2009; Twentyman, 1999).

Lesions of rickets and fibrous osteodystrophy in reptiles

Metabolic bone disease in reptiles usually presents as NSHP (Klaphake, 2010). The clinical signs and lesions of NSHP in reptiles occur as a result of hypocalcaemia (Burgess et al., 2009; Timmerman & Doolen, 1994), which causes increased activity of the parathyroid glands and subsequently increased bone calcium resorption (Klaphake, 2010). The weak bones observed in vitamin D deficiency are a result of this decreased bone calcium (Klaphake, 2010; Rivera & Lock, 2008), and predispose reptiles to fractures (pathological fractures) (Burgess et al., 2009; Dittmer & Thompson, 2011; Timmerman & Doolen, 1994). The clinical signs include anorexia, digestive problems, weakness, stunted growth and neurological signs like tetany and tremors (Rivera & Lock, 2008; Timmerman & Doolen, 1994). The fibrous osteodystrophy in reptiles is characterised by fibrous swellings and thickening of long bones and mandibles (Burgess et al., 2009; Klaphake, 2010; Timmerman & Doolen, 1994). The mandible and maxilla become rubbery and can become easily deformed on applying pressure (Rivera & Lock, 2008; Timmerman & Doolen, 1994).

1.6 Management of vitamin D deficiency

For the appropriate management and treatment of vitamin D deficiency, knowledge of the route of vitamin D metabolism used by the particular animal is necessary. Vitamin D supplementation may be carried out in deficient animals by providing UV radiation or supplementation of feed with vitamin D (Watson & Mitchell, 2014) but the importance of which method of supplementation used will depend on the predominant pathways of vitamin D metabolism that have evolved within a species.

1.6.1 Treatment

Treatment of vitamin D deficiency includes dietary supplementation of vitamin D and/or provision of UV-B radiation, depending on the major route of metabolism of vitamin D in the species concerned.

One of the treatments for vitamin D and calcium deficiency in captive reptiles is to enhance the nutritional value of insects fed to the captive reptiles (Finke, 2003). Most of the insects used in feeding of captive animals are not good sources of calcium and vitamin D (Allen & Oftedal, 1989). Research has shown that addition of vitamin D to the diet of crickets fed to panther chameleons resulted in higher reproductive efficiency in these chameleons (Ferguson, Jones, Gehrmann, Hammack, Talent, Hudson, & Holick, 1996). Dusting insects with calcium and vitamin D also helped increase the dietary content of these nutrients when fed to captive reptiles (Donoghue, 1998).

Although dietary supplementation of vitamin D is useful in some cases of deficiency, certain animals are unable to metabolise dietary vitamin D and require UV-B exposure to synthesise vitamin D (Donoghue, 1998; Oonincx et al., 2010). Research carried out on komodo dragons *(Varanus komodoensis)* has shown that supplementation of UV radiation through artificial UV light can be effective in increasing the serum $25(OH)D_3$ concentrations (Gillespie, Frye, Stockham, & Fredeking, 2000). Care should be taken to avoid glass enclosures, which prevent entry of UV radiation, resulting in lack of UV exposure in reptiles (Holick, 2003a; Watson & Mitchell, 2014). Consequently, a UV permeable roof should be provided, so as to make natural sunlight available to these animals (Donoghue, 1998).

1.7 Hypervitaminosis D

Although the supply of adequate vitamin D and prevention of vitamin D deficiency is vital in maintenance of skeletal and bone health, an excess of vitamin D can also be detrimental to the health of the animal. Observations from studies carried out on hypervitaminosis D have suggested the occurrence of vitamin D toxicity is usually the result of excess vitamin D in the diet (Watson & Mitchell, 2014).

Toxicity of vitamin D in animals manifests itself as widespread mineralisation due to deposition of the excess calcium in the organs and other soft tissues (Dittmer & Thompson, 2011; Watson & Mitchell, 2014). Studies on mammals, reptiles and birds have shown that the organs most severely affected by mineralisation are often the kidneys and the heart (Nain et al., 2007; Olds et al., 2015; Watson & Mitchell, 2014). High levels of vitamin D in the diet of chameleons can result in renal gout (Watson & Mitchell, 2014). Soft tissue calcification occurring as a result of chronic hypercalcemia as well as hyperphosphatemia produces clinical signs such as gastric disturbances, anorexia, ataxia, seizures, heart rhythm defects, and hypertension, which eventually lead to death, if left untreated (Dittmer & Thompson, 2011; Watson & Mitchell, 2014). As previously discussed, some species of animals have evolved with innately high absorption of dietary vitamin D and calcium, so hypervitaminosis D may be more common in these species if they are fed inappropriate diets. Therefore, the provision of dietary vitamin D supplementation needs to be modulated by the risk of oversupplementation and toxicity. Understanding the primary routes of vitamin D metabolism within a species can help to ensure that inadvertent hypervitaminosis D is not induced by dietary supplementation.

1.8 Aims of the study

The aim of this study is to assess the effect of evolutionary selection pressures on the route of vitamin D metabolism that predominates in different species of New Zealand animals. The species chosen for investigation have been selected to represent different life history traits that may have affected the evolution of vitamin D metabolism including nocturnalism, and basking thermal homeostasis.

Brown Kiwi (*Apteryx mantelli*)**:** This bird was chosen as a nocturnal animal, which should not be exposed to high levels of UV-B radiation in its natural habitat and daily activity pattern. Considering its life history, it is hypothesised that kiwi will rely on ingestion of vitamin D from dietary sources.

Tuatara (*Sphenodon punctatus*)**:** This reptile is known for its diurnal sun basking characteristics for thermal homeostasis. Therefore, it is hypothesised in this study that tuatara will be able to synthesise vitamin D by dermal conversion of vitamin D precursors on exposure to UV-B radiation and that this pathway will predominate over dietary routes of vitamin D acquisition.

New Zealand sea lions (*Phocarctos hookeri*)**:** This marine mammal resides in Subantarctic regions and is known to bask in the sun. The latitude in these regions result in decreased UV-B radiation in winter months, but cutaneous vitamin D synthesis possibly occurs in the summer months (Sharma et al., 2011; Norman, 2008). In addition, certain feed items consumed by New Zealand sea lions are rich in vitamin D. It is, therefore, hypothesised, that New Zealand sea lions will utilise both dietary sources and dermal synthesis in order to obtain fulfill their vitamin D requirements.

For overall assessment of vitamin D status in these proposed animals, a survey of the plasma/serum concentrations of 25(OH)D_2 and 25(OH)D_3 in captive and wild

kiwi, captive tuatara and wild New Zealand sea lions (Chapter 2). Further, an investigation of the ability of the skin to synthesise vitamin D_3 *in vivo* from all three species and morepork will be carried out, as an additional representation of nocturnalism (Chapter 3). Finally, a discussion about the implications of the findings from this on the original hypotheses and the captive management of kiwi and tuatara for conservation in New Zealand will be presented.

1.9 References

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CHAPTER 2: Survey of plasma/serum 25(OH)D_2 and 25(OH)D_3 concentration in kiwi (*Apteryx mantelli*), tuatara (*Sphenodon punctatus*) and New Zealand sea lion (*Phocarctos hookeri*)

2.1 Abstract

AIM: To determine the plasma and serum 25(OH)D_2 and 25(OH)D_3 concentrations in captive and wild kiwi (*Apteryx mantelli*), captive tuatara (*Sphenodon punctatus*) and wild New Zealand sea lions (*Phocarctos hookeri*), in order to assess the route of vitamin D utilised in these New Zealand animals.

METHODS: Plasma samples from captive kiwi and tuatara were collected from five captive institutions in New Zealand. Samples were collected from captive indoor and captive outdoor kiwi and tuatara. Wild kiwi were sampled from Rimutaka Forest park. UV-B radiation was measured in both their captive and wild surroundings. Plasma samples of kiwi and tuatara, that were patients at Wildbase hospital, Palmerston North, were also obtained in co-ordination with their veterinary diagnostic procedures. Archived serum samples were used for wild New Zealand sea lions, which were collected from Auckland Islands in 2001. Plasma/serum 25(OH)D_2 and 25(OH)D_3 concentrations were measured by isotope dilution liquid chromatography-tandem mass spectrometry.

RESULTS: The plasma 25(OH)D_2 concentrations were found to be below detectable levels of the assay for all three species. Plasma 25(OH)D_3 concentrations in captive indoor and outdoor kiwi did not differ significantly. In contrast, wild kiwi had lower plasma 25(OH)D_3 concentrations.

Plasma 25(OH)D_3 concentrations in captive tuatara varied according to different institutions. Tuatara at institute 2 had higher 25(OH)D_3 concentrations in plasma which correlates with the high levels of vitamin D in the captive diet used at this site. New Zealand sea lions had serum 25(OH)D_3 concentrations 6 times higher than that of kiwi.

CONCLUSIONS: The lower plasma 25(OH)D_3 concentrations in wild kiwi as compared to the captive ones, was due to dietary sources of vitamin D provided in captivity. The results suggest that there is a possible risk of oversupplementation in captivity and more analysis on the requirement of vitamin D in kiwi needs to be carried out. The variation in the plasma 25(OH)D_3 concentrations in captive tuatara was due to differences in the diet at the captive institutions. This shows that dietary route of vitamin D metabolism is also utilised by tuatara and more analysis needs to be done to assess the risk of dietary vitamin D oversupplementation in tuatara.
Plasma 25(OH)D_3 concentrations in New Zealand sea lions represents both dermally produced vitamin D as well as vitamin D obtained from dietary ingestion. Therefore, it is difficult to determine whether a particular vitamin D pathway predominates in New Zealand sea lions, or whether they have the ability to acquire vitamin D equally from both sources.

2.2 Introduction

Vitamin D, a fat soluble vitamin, is a prohormone, rather than a true vitamin because it must be activated in order to function (Tian, Chen, Lu, Shao, & Holick, 1994; Watson & Mitchell, 2014). To this end, vitamin D undergoes successive hydroxylation reactions in the liver and kidneys resulting in $1,25(OH)_2D$, the active form of vitamin D (Holick, 2003b; Horst, Reinhardt, & Reddy, 2005). This biologically active form has a variety of functions, most importantly maintenance of calcium homeostasis, but it also has roles in immune regulation and possibly prevention of some types of cancer (Anderson & Atkins, 2008; Dittmer & Thompson, 2011; Holick, 2011; Martini & Wood, 2006). Vitamin D stimulates absorption of calcium from the intestine, calcium mobilisation from bones, and renal calcium reabsorption (Holick, 2003a, 2003b; Horst et al., 2005; Kwiecinski, Zhiren, Chen, & Holick, 2001). Vitamin D deficiency results in the metabolic bone diseases, rickets and osteomalacia (Dittmer & Thompson, 2011).

Depending on the evolutionary characteristics of different animal species, vitamin D can be obtained through the action of sunlight on skin and/or via the diet (Holick, 2003a; Kwiecinski et al., 2001; Lips, van Schoor, & de Jongh, 2014). Vitamin D occurs in two forms. The primary source of vitamin D is vitamin D_3 (cholecalciferol), which is obtained mainly from dermal conversion of 7-dehydrocholesterol (7-DHC) on exposure to UV-B radiation, but is also found in dietary sources such as fatty fish, cod liver oil, egg yolk, and liver (DeLuca, 2004; Dittmer & Thompson, 2011; Lips et al., 2014). In contrast, vitamin D_2 (ergocalciferol) occurs only in food of plant origin (Buchala & Pythoud, 1988; DeLuca, 2004). Synthesis of vitamin D_2 has been shown to occur when roughages are cut and exposed to sunlight resulting in conversion of ergosterol to ergocalciferol (Newlander, 1948; Newlander & Riddell, 1952). Therefore, measurement of both $25(OH)D_2$ and $25(OH)D_3$ will not only provide knowledge about dermal synthesis of vitamin D but also provide insight on the importance of various dietary components as sources of vitamin D, thereby giving an

insight into vitamin D status of brown kiwi (*Apteryx mantelli*), tuatara (*Sphenodon punctatus*) and New Zealand sea lions (*Phocarctos hookeri*).
Brown kiwi, tuatara and New Zealand sea lions exhibit varied life history characteristics. Brown kiwi are nocturnal, and have been observed to be actively foraging for feed during the night (Fraser & Johnson, 2009). On the other hand, tuatara were observed to display sun basking behaviour (Besson & Cree, 2010; Gillingham, Carmichael, & Miller, 1995).

Vitamin D metabolism in kiwi, tuatara and New Zealand sea lions has not been previously studied. The main aim of this study is to determine the serum or plasma concentrations of 25(OH)D_2 and 25(OH)D_3 in these species. Further aims were to compare the plasma vitamin D concentrations of wild versus captive kiwi; and of captive tuatara and kiwi held indoors versus those kept in outdoor enclosures.

2.3 Materials and methods

2.3.1 Animals used

This study was conducted in kiwi, tuatara and New Zealand sea lions and the study protocol was approved by Massey University Animal Ethics Committee (MUAEC 15/31), Department of Conservation (DOC) (Authorisation number- 45720-FAU), kiwi and tuatara captive coordinators, the zoological institutes used for sampling of animals, and Iwi. Sea lion samples were collected with approval of the DOC Animal Ethics Committee (Approval AEC51).

For kiwi used in this study, 3 treatment groups were made according to the conditions under which animals were kept and the type of UV light they received.
Group 1: wild animals present in their natural habitat.
Group 2: captive animals housed indoors receiving artificial UVB light.
Group 3: captive animals housed outdoors receiving natural sunlight.

For tuatara used in this study, only the above mentioned groups 2 and 3 were available and no wild tuatara samples were collected.

Blood samples were collected from captive kiwi at three zoological institutions in New Zealand. Blood samples from wild kiwi were obtained from Rimutaka Forest Park (41.3600° S, 175.0000° E). Opportunistic blood samples were also obtained from kiwi that were patients in the Wildbase Hospital adjunctive to veterinary diagnostic investigations.

Tuatara samples were collected from four zoological institutions in New Zealand. Opportunistic blood samples were also obtained from captive and wild tuatara that were patients in the Wildbase Hospital adjunctive to veterinary diagnostic investigations.

Serum samples were collected from 10 wild New Zealand sea lions on the Auckland Islands in 2001 and had been archived at -80°C.

2.3.2 Housing and diet

The zoological institutions provided the necessary captive environment for housing, and the animals were under the care of keepers at the respective institutions. Captive kiwi and tuatara were provided with *ad libitum* access to water in their enclosures as well as access to their regular diet before and after sample collection. The UV exposure within the captive enclosures and in the natural habitat for wild kiwi was measured using a UV-B meter Solarmeter Model 6.2 (Solartech Inc, Harrison Township MI, USA). Measurement of UV-B light in captive indoor and captive outdoor enclosures, and wild kiwi habitat was carried out using the UV-B meter, which measured UV-B light in $\mu W/cm^2$ within the 280-320 nm wavelength. Since sampling from wild kiwi was carried out in accordance with the DOC management procedures, these animals were released immediately after sampling.

2.3.3 Blood collection and storage

Collection of blood samples was carried out under manual restraint. Kiwi were restrained by being cradled in the handlers lap with a firm grip around both legs (Fraser & Johnson, 2009; Robertson, Colbourne, Castro, Miller, Cresswell, McLennan, Dew, Miles, Pierce, & Olsen, 2003) and 0.2 - 0.4 mL of blood was collected from the medial metatarsal vein of the extended leg. Tuatara were held in a vertical position by the handler with their hands around the thoracic and pelvic girdle and 0.2 – 0.4 mL blood was collected from the ventral coccygeal vein.
Kiwi and tuatara samples obtained from captive institutes and Wildbase Hospital were centrifuged and frozen within 2-3 hours of collection. Samples from wild kiwi were centrifuged and frozen 5-6 hours after blood collection. Both wild and captive samples were kept under cold conditions by using icepacks during transportation. The blood was collected into lithium heparin tubes, which were centrifuged at a speed of 16000 G for 5 min to separate the plasma. The plasma was kept frozen at -80°C until analysis.

New Zealand sea lion samples were collected from adult female sea lions in the months of January and February that were captured and anaesthetised at Sandy Bay, Enderby Island, as part of an unrelated foraging study. Blood was collected from the coccygeal vein into a plain tube, allowed to clot at ambient temperature, and then centrifuged at 1600 G for 5 min. Serum was aspirated using a sterile Pasteur pipette, then transferred into a cryovial and placed into liquid nitrogen for transport to Massey University, where they were kept frozen at -80°C until analysis.

2.3.4 Blood analysis

The plasma and serum samples were submitted to Endolab, Canterbury Health Laboratories for measurement of both 25(OH)D_2 and 25(OH)D_3. These vitamin D

metabolite concentrations were measured by isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS).
Samples were analysed in duplicate with intra-assay coefficients of variability (CVs) of less than 12.7%, and an inter-assay CV for 25(OH)D_3 of 11.7% and 17.9% for 25(OH)D_2.

2.4 Statistical analysis

Statistical analysis of plasma/serum 25(OH)D concentrations in kiwi, tuatara and New Zealand sea lions was performed using Minitab Express for Mac (Version 1.4.0 (424835)), Minitab Ltd., UK. The results obtained are presented as mean ± standard deviation. To determine the effect of habitat, indoor, outdoor and wild, on the 25(OH)D_3 concentrations in plasma of kiwi and tuatara, and New Zealand sea lion serum, a one-way analysis of variance (ANOVA) was performed, along with Bonferroni pairwise comparisons between study groups. A stepwise general linear model (GLM) was performed to assess the effect of different variables on vitamin D. In this model, the effect of age, sex, environmental UV radiation, and the animal's origin on the plasma/serum 25(OH)D_3 concentrations were assessed. Linear regression was performed to analyse the relationship between vitamin D and environmental UV levels. For all statistical analysis performed, a $P<0.05$ significance level was assumed.

2.5 Results

2.5.1 UV

The UV-B light in wild habitat and captive enclosures of kiwi and tuatara at captive institutions is presented in Table 1.

Table 1: UV-B radiation measurement in µW/cm^2 at captive and wild habitats of kiwi and tuatara.

Captive Institute	Animal	Enclosure type	UV (µW/cm^2)
Institute 1	Kiwi	Indoor	Not recorded
		Outdoor	2-3
	Tuatara	Indoor	Light- 230-250, ground- 10-20
		Outdoor	250-270 (sunny day)
Institute 2	Kiwi	Indoor	Not recorded
		Outdoor	Partially covered, 2-6
	Tuatara	Indoor	Not recorded
		Outdoor	Partially covered, 2-6
Institute 3	Tuatara	Outdoor	Ground level- 15-40
Institute 4	Kiwi	Indoor	Nil
		Outdoor	Not recorded
	Tuatara	Indoor	Light- 141-198, ground- 9
Institute 5	Kiwi	Indoor	Not recorded
	Tuatara	Outdoor	200-250 (Sunny day)
Wild kiwi	Kiwi	Wild	200-250 (Sunny day)

2.5.2 Plasma/serum 25(OH)D_2 and 25(OH)D_3 concentrations

Considerable variation was observed in the plasma/serum 25(OH)D_3 concentrations between kiwi, tuatara and sea lions (Figure 5). Plasma/serum 25(OH)D_2 was absent in all three species.

a) Kiwi:

No significant difference was observed in the plasma 25(OH)D_3 concentrations between kiwi kept in outdoor enclosures receiving natural sunlight and those kept in nocturnal houses receiving no supplemental UV-B light (n=6, 19.83 ± 10.96 nmol/L in captive outdoor kiwi and n=8, 19.67 ± 9.47 nmol/L in captive indoor kiwi; P = 0.975) (Figure 2). However, wild caught kiwi had significantly lower plasma 25(OH)D_3 concentrations (ANOVA, n=4, df= 2,16, F=5.36, p=0.016) compared with captive kiwi (Figure 2).

There was no significant relationship (df=5,13, p=0.084, R^2=0.294) between environmental UV exposure and plasma 25(OH)D_3 concentration in kiwi.

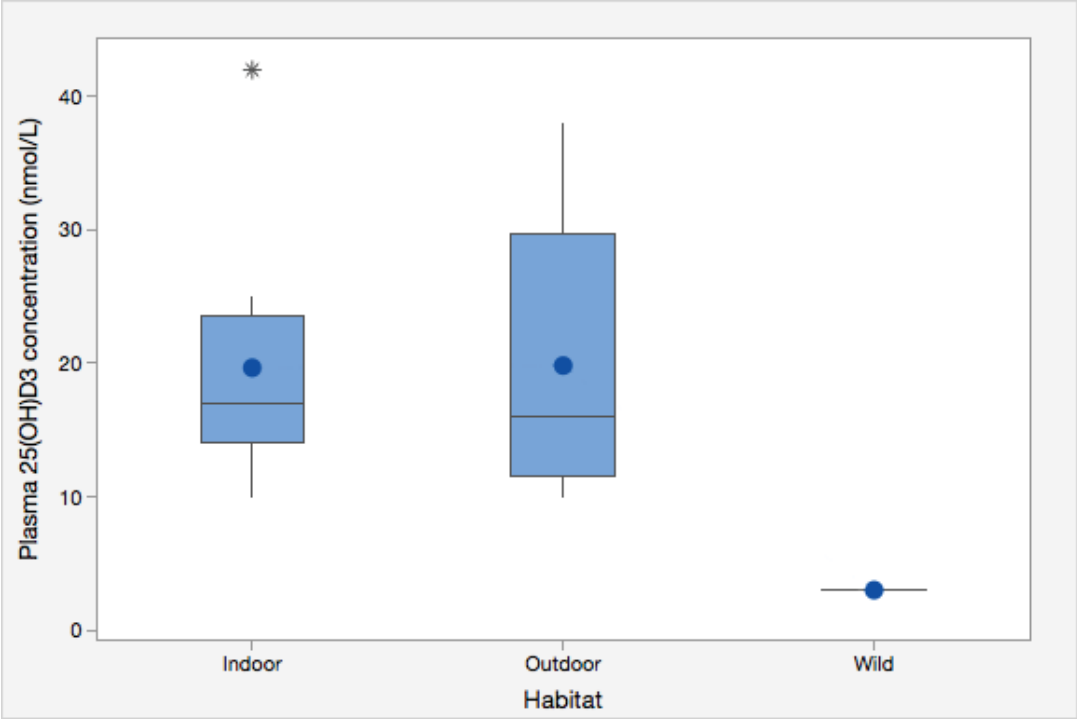

Figure 2: Boxplot of plasma 25(OH)D_3 in captive indoor, captive outdoor and wild kiwi. The boxes show interquartile interval, circles the mean, the line within the box the median and asterix the outliers.

b) Tuatara:

The mean plasma 25(OH)D_3 concentrations of captive indoor tuatara (n=3, 103.3 ± 79.4 nmol/L) and captive outdoor tuatara (n=13, 104.4± 47.0 nmol/L) were not significantly different (p=0.976).

Age and sex were not significantly related with plasma 25(OH)D_3 concentration (Age- p=0.113, Sex- p=0.092). However, there was a significant relationship between the zoological institution and plasma concentration of 25(OH)D_3 (df=4,9, p=0.007, R^2=0.702) in the tuatara (Figure 3). Within institutions, plasma 25(OH)D_3

concentrations of tuatara at Institute 2 were significantly higher (p=0.001) than in tuatara from the other zoological institutions (Figure 3).

There were significant differences (n=5, df=4,11, F=5.83, p=0.009) between institutions in the levels of UV radiation provided (Figure 4), but no significant linear relationship was present between UV and plasma 25(OH)D_3 concentrations (df=3,12, p=0.186, R^2=0.151) in the tuatara.

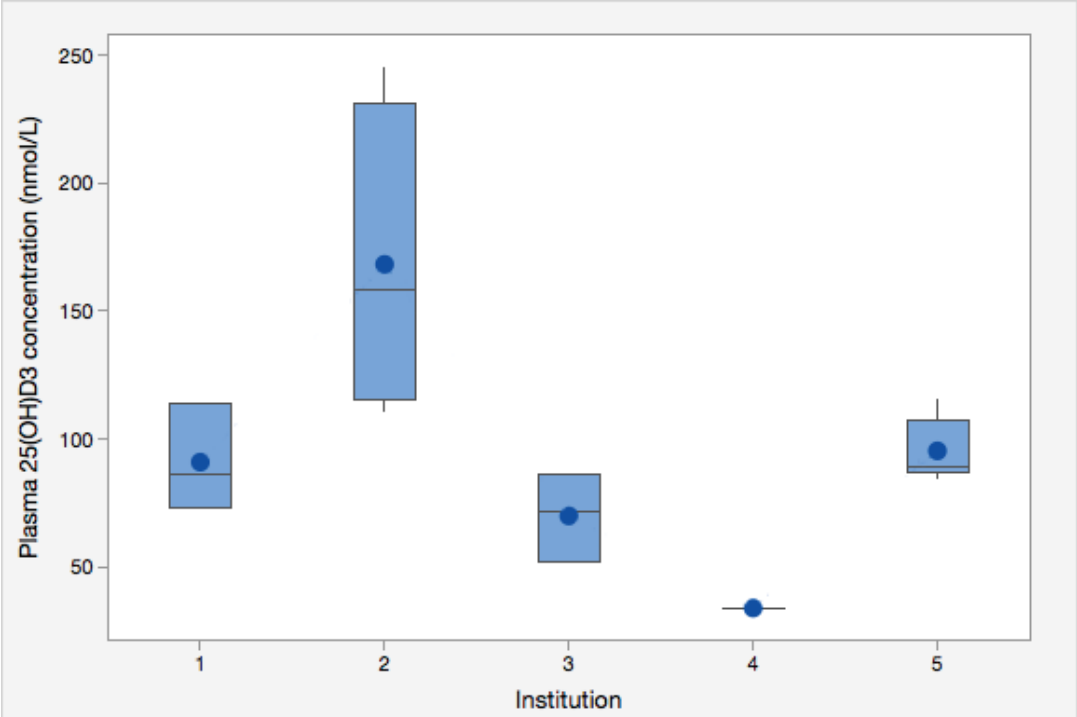

Figure 3: Boxplot of plasma 25(OH)D_3 concentrations in captive tuatara between 5 captive institutions used for sampling. The boxes show interquartile interval and circles the mean.

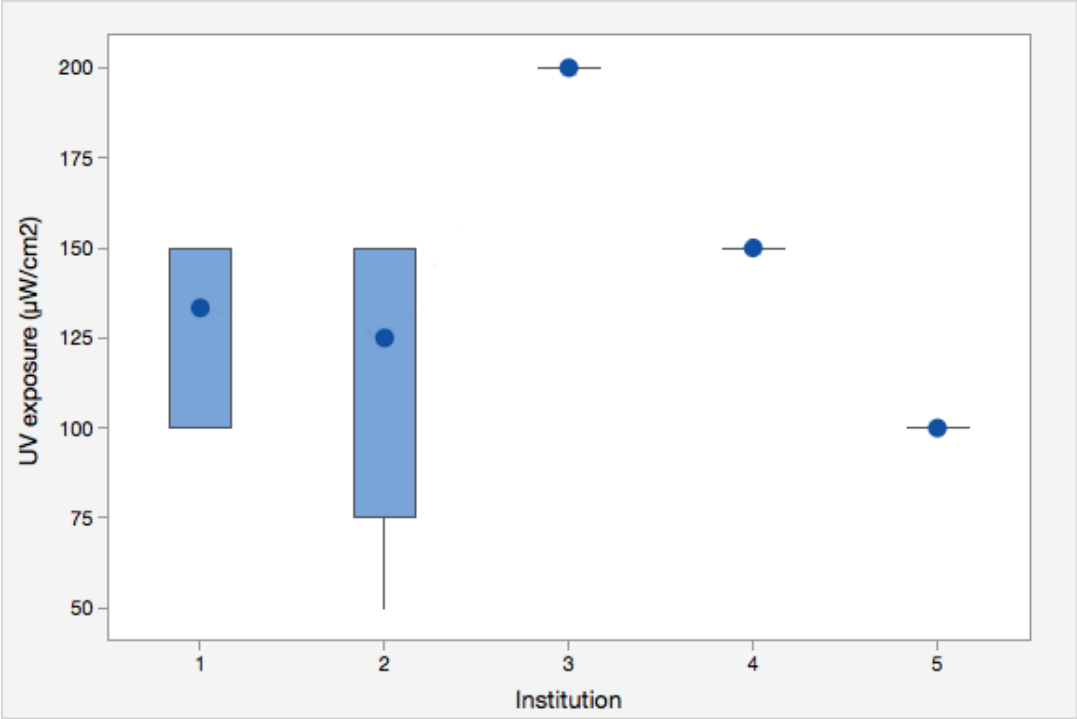

Figure 4: Variation in the UV radiation provided to tuatara at different captive institutions. The boxes show interquartile interval and circles the mean.

c) New Zealand Sea lion

The mean serum 25(OH)D_3 concentration in wild New Zealand sea lions was 111.6 ± 24.0 nmol/L.

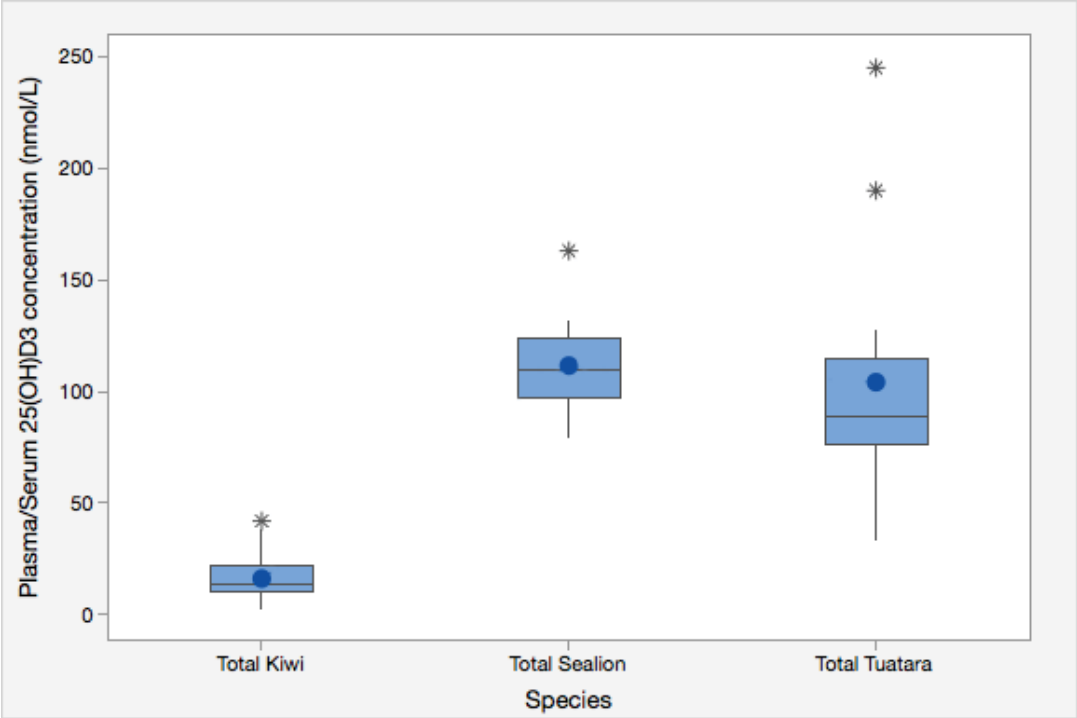

Figure 5: Boxplot of plasma/serum 25(OH)D_3 concentrations between kiwi, tuatara and New Zealand sea lion. Boxes denote interquartile interval, circles denote the mean and asterisks the outliers in the data.

2.6 Discussion

Measurement of plasma/serum 25(OH)D_3 concentration in captive indoor, captive outdoor and wild kiwi, captive indoor and outdoor tuatara, and wild New Zealand sea lions revealed considerable variation between species. However, 25(OH)D_2 was found to be below detectable levels in the plasma or serum of all 3 species. Vitamin

D_2 is found only in dietary items of plant origin and hence its absence suggests that kiwi, tuatara and New Zealand sea lion do not obtain significant amounts of vitamin D from plant sources.

Variation in plasma 25(OH)D_3 between these species may be due to the differences in the pathways of metabolism, or the diet and lifestyle of kiwi, tuatara and New Zealand sea lions. The possible causes of between species variation and within species variation of plasma 25(OH)D_3 concentrations will be discussed further according to each species.

a) Kiwi

The mean plasma concentration of 25(OH)D_3 in captive kiwi was 19.7 ± 9.7 nmol/L, which was similar to the peak plasma 25(OH)D_3 concentration of 26.2 ± 5.7 nmol/L in chickens post UV radiation exposure (Tian, Chen, Lu, Shao, & Holick, 1994).
The mean plasma 25(OH)D_3 concentrations of captive indoor and captive outdoor kiwi showed no noticeable variation. However, significant variation was observed in the plasma 25(OH)D_3 concentration between the captive and wild kiwi, and this may be of particular importance. Captive outdoor kiwi have a habitat similar to that of wild kiwi. We expected that the variation between plasma 25(OH)D_3 concentrations of captive outdoor and wild kiwi would be minimal, considering both groups receive similar amounts of sunlight. Based on the lack of supplemental light, we assume that the captive indoor kiwi sampled in this study received a negligible amount of UV-B radiation in their enclosures. Wild kiwi are also presumed to receive low amount of sunlight, due to their nocturnal foraging habits. However, the results obtained were contrary to that expected. The low plasma 25(OH)D_3 concentration in wild kiwi as compared to captive kiwi suggests that the higher concentrations of plasma 25(OH)D_3 in captive kiwi compared to wild kiwi might be due to dietary sources. The results suggest that captive birds receive excess of vitamin D in their diet compared

to the wild birds and more detailed analysis is required to assess the dietary vitamin D requirements of captive kiwi.

The diet of the kiwi in the wild and captivity is substantially different. In captivity, the institutions participating in this study provided kiwi with a captive diet of insects such as snails, earthworms, crickets, locusts and moths, mixed vegetables, fruits, minced ox heart, a vitamin premix made specifically for kiwi and in some cases grit or calcium as well as an articifial insectivore supplement (Wombaroo Insectivore Hand-rearing Mix, Wombaroo Food Products, Glen Osmond, South Australia, Australia) (Minson, 2013). Of these, the vitamin premix commonly used contains 250 IU of vitamin D_3 (Minson, 2013) and is usually added to the diet at the rate of 1g/100g food.

The diet of kiwi in the wild comprises mainly invertebrates, and occasionally vegetable matter (Reid, Ordish, & Harrison, 1982). Of the invertebrates, earthworms have been observed to be the major invertebrate consumed by kiwi and other invertebrates such as cicadas, black beetles are also present in their diet according to availability (Reid et al., 1982). A study conducted by Finke (2002), has shown that earthworms along with a few other invertebrate species contain undetectable concentrations of vitamin D_3 (Finke, 2002). This suggests that the natural diet of kiwi might not contain vitamin D rich items, and these birds may have evolved to have very low vitamin D requirements or even be independent of vitamin D for maintaining optimum calcium concentrations in their body by using alternative mechanisms. The differences between the diet of captive and wild kiwi could be estimated by measuring the intake of vitamin D_2 and vitamin D_3, however the feasibility and accuracy of this estimation cannot be ascertained.

Nocturnal mammals such as African mole rats (*Cryptomys hottentotus*) have a similar life history as that of kiwi. These mammals receive negligible sunlight and also consume a diet low in vitamin D (Bentley, 1998). In spite of the low plasma

25(OH)D_3 concentrations observed in these mammals, they do not exhibit any signs of vitamin D deficiency (Bentley, 1998; Skinner, Moodley, & Buffenstein, 1991). It has been observed instead that these mammals have a diet high in calcium and have a non-saturable calcium diffusion mechanism across the intestine (Bentley, 1998; Skinner et al., 1991). This mechanism of calcium diffusion has also been observed in chickens during their egg laying period (Bentley, 1998).

Another alternative mechanism for vitamin D independent calcium metabolism has been observed in chickens during periods of nocturnal fasting (Bentley, 1998). During these periods, calcium mobilisation from bones takes place, primarily from medullary bone (Bentley, 1998). Certain other species such as Jamaican fruit bat (*Artibeus jamaecensis*) and naked mole rat (*Heterocephalus glaber*) also utilise this mechanism (Acierno et al., 2008; How, Hazewinkel, & Mol, 1994; Kwiecinski et al., 2001; X. Tian et al., 1994).

Therefore, vitamin D and calcium metabolism in kiwi needs to be investigated further, to determine whether they obtain vitamin D through an alternate pathway or their calcium metabolism is independent of vitamin D.

b) Tuatara

There was no significant variation between the plasma 25(OH)D_3 concentrations of captive indoor and captive outdoor tuatara in this study. The average plasma 25(OH)D_3 concentration of tuatara sampled in this study was 104.2 ± 51.1 nmol/L. The plasma 25(OH)D_3 concentrations varied according to the institution from where sampling was carried out. The plasma 25(OH)D_3 concentrations of captive tuatara sampled from Institution 2 were higher compared with the other institutions. UV exposure and captive diet are some of the factors to be considered to explain this variation between institutions.

Statistical analysis showed that UV radiation varied within the 5 institutions, however the plasma 25(OH)D_3 concentrations were not associated with differences in UV exposure. The analysis from this study suggests that diet might play a role in the variation of plasma 25(OH)D_3 concentration between institutions. The diet provided to captive tuatara at zoological Institute 2 was slightly different from that of other institutions. This captive institution offered day old chickens containing a full yolk sac, whole rat rolled in a calcium and vitamin D supplement powder (Bone Gro, Veterinary remedies 2008 Ltd, Palmerston North, New Zealand) with occasional minced ox heart rolled in the same calcium and Vitamin D powder (Personal communication, Rhys Mills, 2015). The remaining diet of the tuatara consisted of invertebrates such as moths, crickets, locusts, cicadas, and snails and was similar between institutions. The supplemental powder utilised at Institute 2 contained 50 IU of vitamin D_3/g. Egg yolk is also known to contain a high amount of vitamin D_3 (Dittmer and Thompson, 2011). The results suggest that the vitamin D_3 rich diet fed to tuatara at Institute 2 is likely responsible for their elevated plasma 25(OH)D_3 concentrations as compared to tuatara at other institutions. The variation in plasma 25(OH)D_3 concentrations in captive tuatara at 5 institutions was of dietary origin, rather than as a result of variation in UV-B radiation. All tuatara sampled were receiving measurable levels of UV-B in their environment. Further, the plasma 25(OH)D_3 concentrations of all tuatara did not show hypovitaminosis D. The results suggest the UV-B exposure is adequate for vitamin D metabolism with their current diet.

An important factor to consider in vitamin D supplementation of animals kept in captivity is the relative efficacy of dietary vitamin D vs. vitamin D synthesis from sunlight. A study on bearded dragons (*Pogona* spp) has shown that the increase in plasma 25(OH)D_3 concentration after UV-B supplementation was higher as compared to that observed after dietary supplementation (Oonincx, Stevens, van den Borne, van Leeuwen, & Hendriks, 2010). More research in tuatara would be

needed to confirm whether the rise in concentration of plasma 25(OH)D_3 is higher after UV radiation or dietary vitamin D supplementation. For example, comparison of plasma 25(OH)D_3 could be done between 2 groups of tuatara; one receiving no UV radiation and the other receiving the appropriate UV radiation for vitamin D synthesis.

Also, studies could be carried out on wild tuatara to evaluate their plasma 25(OH)D_3 concentrations for further comparison with that of captive tuatara. This would provide more insight into the amount of vitamin D actually required by wild tuatara and whether there is a possibility of vitamin D over-supplementation in captive tuatara.

c) New Zealand Sea lion

The mean serum 25(OH)D_3 concentration in wild New Zealand sea lions was found to be 6 times higher than the mean kiwi plasma 25(OH)D_3 concentration but similar to that of tuatara. The hypothesis in this study was that New Zealand sea lions would utilise both pathways of vitamin D metabolism, both dietary and via dermal conversion. The results in this serum survey, in conjunction with the results obtained in my study of dermal vitamin D synthesis in New Zealand sea lion skin (see Chapter 3), suggest that this hypothesis may hold true. Plasma 25(OH)D_2 concentrations in New Zealand sea lion serum were below detectable levels, which suggests that their diet does not include plant sources of vitamin D. Since serum 25(OH)D_3 concentration can be derived from both dermal vitamin D and dietary vitamin D, it was not possible to determine whether a particular vitamin D pathway is predominant in New Zealand sea lions.

Similar to kiwi and tuatara, analysis of serum 25(OH)D_3 concentrations in New Zealand sea lions depends on their life history characteristics. Both basking in direct sunlight and the consumption of feed items containing vitamin D_3 likely contribute

to the serum 25(OH)D_3 concentrations in New Zealand sea lions. However, more research is needed to assess the vitamin D content in the New Zealand sea lion diet. The 25(OH)D_3 concentrations in New Zealand sea lion serum could vary according to certain other factors. A seasonal effect is likely to result in variation in the serum 25(OH)D_3 concentrations in New Zealand sea lions. The Subantarctic habitat of these sea lions is the primary cause of this seasonal effect, due to the greater amount of sunlight received during the summer months and decreased sunlight received during winter, the serum 25(OH)D_3 concentrations in New Zealand sea lion are likely to be higher in summer as compared to winter (Iuliano-Burns, Wang, Ayton, Jones, & Seeman, 2009; Zérath, Holy, Gaud, & Schmitt, 1999). The serum samples used in this study were collected in the months of January and February, 2001, the Austral summer. Considering the sample collection during summer months with likely higher UV radiation, the resulting serum 25(OH)D_3 concentrations may have been high due to the high UV radiation at this time of year (Iuliano-Burns et al., 2009; Zérath et al., 1999). Whether the serum 25(OH)D_3 concentrations in New Zealand sea lions vary according to the season needs further study. For example, detailed assessment of the dietary vitamin D concentrations in New Zealand sea lion prey species, the UV levels in the region of sample collection, sampling in varying seasons, along with their correlation with serum 25(OH)D_3 concentrations could be done to provide deeper understanding of the vitamin D metabolism in New Zealand sea lions.

2.7 Conclusion

Plasma and serum survey of 25(OH)D_2 and 25(OH)D_3 was carried out in three species with varying life histories. The hypothesis was that the nocturnal lifestyle of kiwi would predispose them to rely on diet for their vitamin D requirement. Results of this study show that dermal vitamin D synthesis does not predominate in kiwi and they either use dietary sources of vitamin D or survive in a vitamin D independent state.

Tuatara, a basking reptile, was assumed to primarily use dermal vitamin D synthesis. However, the plasma survey results in tuatara show that plasma $25(OH)D_3$ concentrations varied within different zoological institutions based on the diet supplied to these tuatara. Therefore, the pathway that predominates in tuatara cannot be confirmed from this study and research could be done for its determination. Based on the results of serum $25(OH)D_3$ concentration in New Zealand sea lions, determination of whether a particular vitamin D pathway predominates in these sea lions or they use both pathways equally could not be done. Although the survey of plasma and serum $25(OH)D_2$ and $25(OH)D_3$ does not entirely confirm the study hypothesis, it does show the presence of a link between the route of vitamin D metabolism utilised by different animal species and the environmental selection pressures faced by them.

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CHAPTER 3: Ability of kiwi (*Apteryx mantelli*), morepork (*Ninox novaseelandiae*), tuatara (*Sphenodon punctatus*) and New Zealand sea lion (*Phocarctos hookeri*) skin to synthesise vitamin D

3.1 Abstract

AIM: To determine the ability of kiwi (*Apteryx mantelli*), morepork (*Ninox novaseelandiae*), tuatara (*Sphenodon punctatus*) and New Zealand sea lion (*Phocarctos hookeri*) skin to synthesise vitamin D_3 by dermal conversion of vitamin D precursors on exposure to UV-B radiation.

METHODS: Samples from kiwi, morepork, tuatara and New Zealand sea lion were obtained from post mortem specimens. All four samples were divided in half and one half of each was exposed to UV-B radiation for 8 h duration (5 J/cm^2). Samples were processed and submitted to the laboratory for vitamin D_3 analysis by HPLC.

RESULTS: Species variation was observed in the concentrations of vitamin D_3 produced between kiwi, morepork, tuatara and New Zealand sea lion after UV-B radiation exposure. Kiwi and morepork skin produced small but measurable amounts of vitamin D. Tuatara skin synthesised vitamin D concentrations higher than that of kiwi and morepork. New Zealand sea lion skin produced the highest amount of vitamin D_3 between all four species studied. New Zealand sea lion differed from other species in this study, due to the presence of vitamin D_3 in their skin prior to experimental UV-B exposure.

CONCLUSIONS: The measurable vitamin D_3 concentrations in nocturnal birds, kiwi and morepork, suggest that these comparatively low vitamin D_3 concentrations are sufficient for their metabolism or they utilise alternative mechanisms for regulation of their vitamin D and calcium metabolism. The magnitude of vitamin D_3 produced in tuatara skin suggests that the dermal vitamin D synthesis route is an important route for obtaining vitamin D in these reptiles. The large amount of vitamin D_3 produced in New Zealand sea lion skin suggests that these marine mammals utilise

this route extensively and more studies need to be done to determine their overall vitamin D requirement and the vitamin D content of their diet.

3.2 Introduction

Vitamin D plays a vital role in the maintenance of calcium homeostasis and skeletal health. The synthesis of vitamin D in the skin occurs by conversion of the dermal precursor 7-dehydrocholesterol (7-DHC) to vitamin D_3 (Holick, 2003b; Holick, Chen, Lu, & Sauter, 2007). During exposure to UV-B radiation, 7-DHC in the dermal layers is converted into previtamin D_3, which further undergoes a thermal isomerisation to vitamin D_3 (Horst, Reinhardt, & Reddy, 2005; Martini & Wood, 2006; McDowell, 2000). Dermally produced vitamin D_3 binds to vitamin D binding protein and enters into the circulation, followed by activation reactions in the liver and kidneys to produce 1,25- dihydroxyvitamin D_3, the active form of vitamin D (Holick et al., 1980; Horst et al., 2005).

Variations have been observed in the vitamin D metabolism of different species of animals (Holick, 2003a, 2011), and this variation is thought to be a result of the evolutionary biology of each species (Holick, 2010, 2011). Dermal synthesis of vitamin D has been confirmed in most herbivorous species such as cows, sheep, and goats (Dittmer & Thompson, 2011), but not in cats and dogs (Dittmer & Thompson, 2011; How, Hazewinkel, & Mol, 1994).

The dermal vitamin D synthesis and resultant vitamin D status of an animal is affected by a variety of factors, including the presence of fur/feathers on skin. Research conducted on chicken skin showed that the presence of feathers affected the concentration of 7- DHC and vitamin D_3 (Tian, Chen, Lu, Shao, & Holick, 1994). The non-feathered skin of the legs and feet showed a 30 times greater concentration of 7-DHC in comparison to feathered back skin (Tian et al., 1994). Similarly, a study on sheep skin also showed increased synthesis of vitamin D in shorn sheep as compared to unshorn sheep (How et al., 1994). A probable

explanation was that the UV-B radiation is absorbed or reflected by the feathers or wool, thereby causing disruption in the penetration of UV-B radiation into the skin (Tian et al., 1994).

The uropygial gland is also known to play a role in vitamin D metabolism, with studies suggesting a link between removal of the uropygial gland and the occurrence of rickets in chickens (Hou, 1929; Knowles, Hart, & Halpin, 1935). Further, histochemical and biochemical analysis of the uropygial gland has shown the presence of the vitamin D precursor 7-DHC in uropygial gland secretions (Uva, Mandich, & Vallarino, 1983). These studies suggest that uropygial gland secretions transferred to the feathers during the process of preening might result in the synthesis of vitamin D on feathers. In addition, other factors such as skin pigmentation, quality and intensity of UV-B radiation, 7-DHC concentration in skin, seasonal changes, altitude, amount of sun protection, and behavioural characteristics can affect the dermal synthesis of vitamin D (Holick et al., 2007; Norman, 1998).

The evolutionary biology of a particular animal species plays a vital role in development of its ability to synthesise vitamin D in the skin. To assess the effect of life history characteristics on the route of vitamin D metabolism predominating in different animal species, an examination of the ability of brown kiwi (*Apteryx mantelli)*, morepork (*Ninox novaeseelandiae)*, tuatara (*Sphenodon punctatus)*, and New Zealand sea lions *(Phocarctos hookeri)* to produce vitamin D_3 in the skin was carried out. Kiwi and morepork are nocturnal birds, and the hypothesis was that, due to evolutionary selection pressures, these birds may have lost the ability to synthesise vitamin D dermally and thereby rely on dietary sources of vitamin D. In contrast, tuatara are a basking reptile, and it was hypothesised that this reptilian species would primarily be dependent on UV-B radiation from sunlight and dermal metabolism of vitamin D precursors. Marine mammals, such as the New Zealand sea

lion, not only have high vitamin D stores in their body, but also ingest prey containing high concentrations of vitamin D (Kenny et al., 2004). They also spend a large amount of time basking in the sun at high latitudes; therefore, it was hypothesised that there has been no evolutionary selection pressure on the vitamin D metabolism in these animals and that both dietary vitamin D and dermal transformation of skin precursors is likely to be present (Kenny et al., 2004).

3.3 Materials and methods

3.3.1 Animals used

Kiwi, morepork and tuatara samples were collected from post-mortem specimens submitted to the National Wildlife Diagnostic Pathology service, Massey University, Palmerston North, New Zealand. The New Zealand sea lion skin samples were obtained from post-mortem specimens archived at Massey University. These specimens are held by Wildbase pathology, Massey University under service contracts to the Department of Conservation, New Zealand. The skin samples from all four species were wrapped in aluminium foil to protect from exposure to light and frozen at – 20^{o}C until processing. All skin samples used in this study were of 10 cm^{2} area.

a) Kiwi

Two skin samples were obtained from the dorsum of the same kiwi.

b) Morepork

Skin from the entire body of two morepork was obtained. The samples were pooled and divided into two.

c) Tuatara

The skin was removed from the body and cut into two along the midline.

d) New Zealand Sea lion

The control and treatment samples obtained from the post mortem archives were from two different sea lions. Therefore, to avoid individual variation confounding the analysis, each sample was divided into two and samples were pooled for processing.

3.3.2 Procedures

As the samples were obtained opportunistically from post mortem samples, the exact information about the method by which the samples were handled prior to obtaining them and wrapping in aluminium foil was not available. During transport of skin, the samples were covered in aluminium foil to prevent exposure to light. Further processing of the skin samples was carried out in a dark room. The samples were thawed and any fur or feathers present were physically removed along with the subcutaneous fat, to avoid any effect of hair and fat on the concentration of vitamin D_3 within the skin layers (How et al., 1994; Tian et al., 1994; Tian, Chen, Matsuoka, Wortsman, & Holick, 1993).

Of the two samples collected for each species, one was used as a control and did not undergo radiation, whereas the other was exposed to UV-B radiation of 290-315 nm in wavelength for 8 hr, equivalent to 5J/cm^2 of accumulated ultraviolet light. (Holick, 1981; How et al., 1994; Tian et al., 1994; Tian et al., 1993). The skin exposure to UV-B radiation was carried out in an optimised reflector box (Width 28 cm^2, length 48 cm^2, and depth 24 cm^2) within a temperature controlled room at 37°C, and the skin was moistened with phosphate-buffered saline (PBS) at 1 h intervals (Horst et al., 2005; Martini & Wood, 2006; McDowell, 2000). Each sample was then cut into 5 mm × 5 mm pieces in a dark room and placed in a light proof bag. The skin samples were weighed, and then freeze-dried (Cuddon Freeze Dry model 0610, Blenheim, New Zealand) prior to saponification and lipid extraction.

3.3.3 Quantification of vitamin D_3

The processed samples were submitted to the Nutrition laboratory, Riddet Innovation, Massey University, Palmerston North, for measurement of vitamin D concentration using reverse phase high-performance liquid chromatography (HPLC). The skin samples underwent ethanolic saponification with potassium hydroxide (KOH) as follows. The samples were combined with 0.1 mL of sodium ascorbate (0.2g/mL), 8 mL of 1% ethanolic pyrogallol (1 g pyrogallol/100 mL ethanol) (Absolute Ethanol (200 Proof), Molecular Biology Grade, Fisher BioReagents™, Thermo Fisher Scientific, Massachusetts, USA) pyrogallol (MP Biomedicals™, Thermo Fisher Scientific, Massachusetts, USA) and 60% KOH (Analytical Reagent Grade, Thermo Fisher Scientific, Massachusetts, USA) in water in a round-bottom flask with a stirring bar and refluxed over a hot plate-stirrer. After cooling, the digest was filtered in a Buchner funnel with filter paper under partial vacuum. To perform lipid extraction, the residue on the filter paper was washed with ethyl acetate (HPLC Grade, Fisher Chemical, Thermo Fisher Scientific, Massachusetts, USA) (80:20) and then n-hexane (HPLC Grade, Fisher Chemical, Thermo Fisher Scientific, Massachusetts, USA). The combined filtrate was then transferred to a separating funnel and water was added. The filtrate was extracted two more times with hexane, and the combined hexane extracts were washed three or more times with water. Using a rotor vapour (Rotavapor® B-3, BÜCHI Labortechnik AG., Flawil, Switzerland) at 38˚C ± 2˚C, the samples were evaporated and the subsequent residue reconstituted with HPLC grade methanol (HPLC Grade, Fisher Chemical, Thermo Fisher Scientific, Massachusetts, USA), followed by sample clean-up using solid phase cartridges SPE C18 (EC 55 µm, 70A) (Phenomenex, California, USA) in order to remove subcutaneous fats. The cartridge was activated with methanol (Fisher Chemical, Thermo Fisher Scientific, Massachusetts, USA) and the sample washed with 70% methanol, followed by elution with the mobile phase (95%

methanol in milli-Q filtered water) which was collected and dried under nitrogen (Hymoller & Jensen, 2011).

Reversed-phase (HPLC) was performed using a Luna C-18 column (250 x 46 mm ID, 5 µm particle size) (Phenomenex, California, USA), with an isocratic mobile phase of methanol: H_2O (95:5), at a flow rate of 1.2 mL/min. Vitamin D_3 was detected using an ultraviolet detector at 265 nm. Controls were vitamin D_3 (Sigma-Aldrich Corporation, St. Louis, USA) in concentrations ranging from 0.05 µg/mL to 5 µg/mL (Hymoller & Jensen, 2011).

3.4 Results

Results from reverse-phase HPLC revealed the presence of vitamin D_3 in the skin of kiwi, morepork, tuatara, and New Zealand sea lion after UV-B irradiation of the skin for 8 h.

Table 2: The vitamin D_3 concentration in kiwi, morepork, tuatara and New Zealand sea lion skin samples before and after UV-B radiation of 5J/cm^2 (8hr).

Sample	Vit D_3 (µg/g)	
	0 hr	8 hr UV-B
Kiwi	<0.03	0.0383
Morepork	<0.03	0.0572
Tuatara	<0.03	0.0837
Sea lion	0.5	1.6

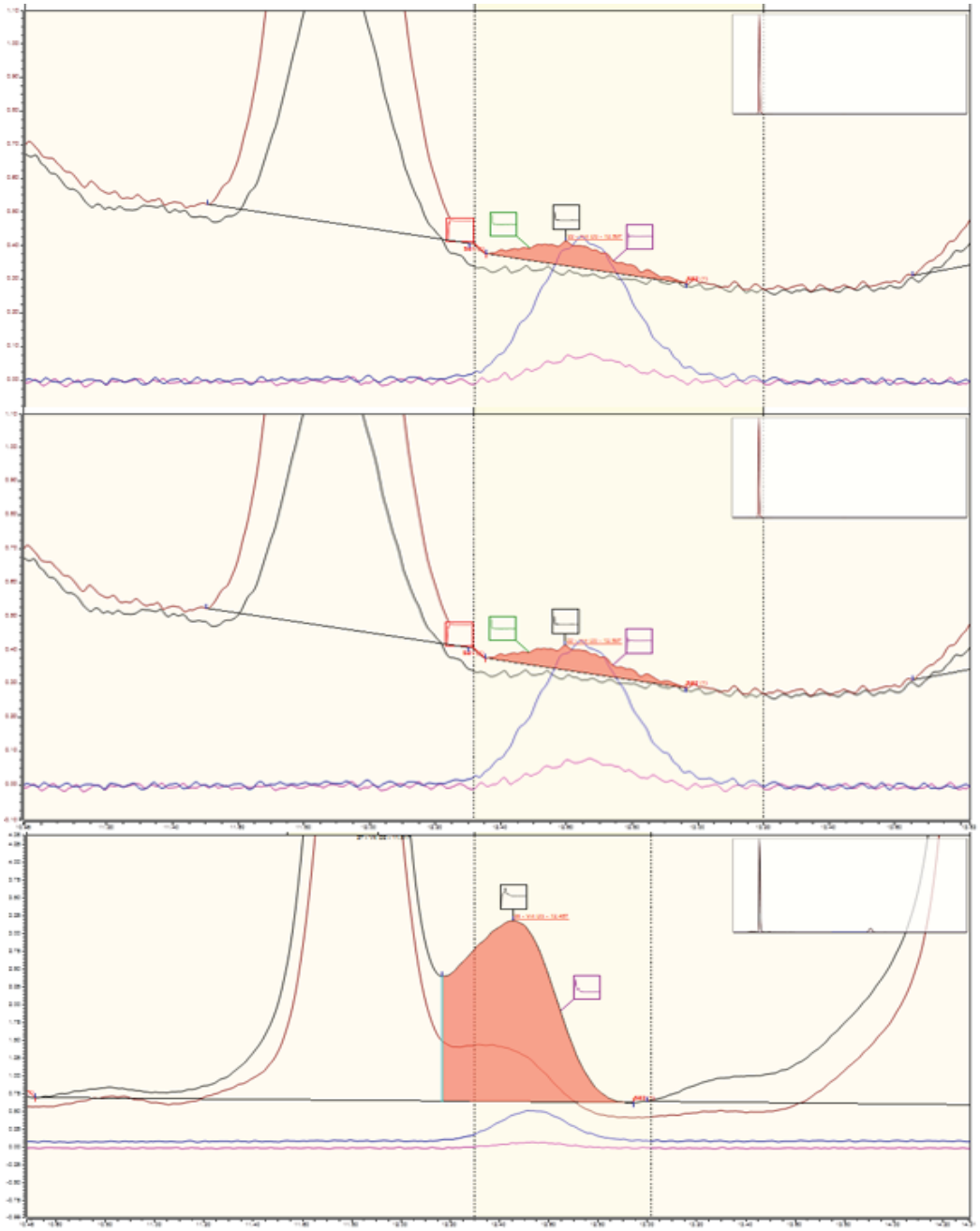

Figure 6: Vitamin D concentration in kiwi (top), tuatara (middle) and New Zealand sea lion (bottom) skin with and without irradiation with 8 hours of ultraviolet light. Black = 0 hr sample, Brown = 8 hr sample, orange highlight = amount of vitamin D produced. Pink and blue lines = Vitamin D standards of different concentrations, Pink = 0.1057 µg/mL, Blue = 0.5278 µg/mL vitamin D_3.

3.5 Discussion

Ultraviolet - B radiation resulted in the production of measurable levels of vitamin D_3 in the skin samples of kiwi, morepork, tuatara, and New Zealand sea lion. This study had very limited sample sizes so the results can't be statistically analysed between species. There was an apparent gradient from lowest to highest production of Vitamin D_3 from the skin of kiwi, morepork, tuatara to that of the New Zealand sea lion (Figure 6). The New Zealand sea lion skin produced vitamin D_3 concentrations that were two orders of magnitude higher than the other animals (Figure 6). New Zealand sea lions were also unusual in that the skin contained measurable amounts of vitamin D_3 prior to exposure to UV-B radiation.

3.5.1 Nocturnal birds

Kiwi and morepork are nocturnal birds and therefore receive negligible amounts of sunlight in the active phase of their daily activity. Considering their life history, it was hypothesised that evolutionary selection pressures would have led to their inability to synthesise vitamin D dermally, making them dependent on dietary vitamin D sources.

However, this study shows that kiwi and morepork skin have retained the ability to photosynthesise vitamin D. The vitamin D_3 concentration detected in kiwi and morepork skin after UV exposure (0.0383 and 0.0572 µg/g respectively) was greater than that produced by horse skin (<0.03 µg/g), but less than that of sheep (0.1-0.2 µg/g) (Personal communication Keren Dittmer, 2015). Further, comparison with vitamin D_3 concentrations in chickens showed that leg and feet skin in chickens had a higher concentration than kiwi and morepork skin (Tian et al., 1994; Uva et al., 1983). A study by Tian et al., 1994, showed that 7-DHC concentrations in leg and feet skin in chickens was about 30 times higher than that observed in back skin (Tian et

al., 1994). The kiwi and morepork skin used in this study was collected from the dorsum.

A study of vitamin D_3 in chicken skin showed that their concentration varies according to the site of production and presence of feathers (Uva et al., 1983; Tian et al., 1994). The authors suggested that the uropygial gland was the primary site of 7-DHC and vitamin D_3 synthesis (Uva et al., 1983). In one study, vitamin D was present in feather washing extracts, presumably from transfer of 7-DHC from the uropygial gland to feathers during preening (Uva et al., 1983). The same study found vitamin D_3 in unfeathered leg skin, which may be either of uropygial origin or from synthesis of vitamin D_3 within the dermal layers of leg skin (Uva et al., 1983). The presence of feathers is also known to affect dermal vitamin D synthesis (How et al., 1994; Tian et al., 1994; Tian et al., 1993). This can be observed from the higher vitamin D_3 concentrations in the unfeathered leg and feet skin in chickens as compared to feathered back skin (Tian et al., 1994). Higher vitamin D_3 concentrations were also observed in shorn sheep as compared to unshorn sheep after exposure to UV radiation (Chaudhary & Care, 1985; How et al., 1994; Tian et al., 1994).

In this study, skin samples devoid of feathers were used. However, this methodology only allows assessment of the skin's ability to synthesise vitamin D and does not reflect the actual vitamin D physiology in a live bird. The presence of feathers and photoreactive uropygial gland secretions coating the feathers might affect the vitamin D synthesis in these birds. The results in this study show that kiwi and morepork skin can synthesise vitamin D on exposure to UV-B radiation, but more research is necessary to assess the use of this pathway for vitamin D synthesis in live birds. Therefore, further studies on the uropygial gland and the effect of feathers on vitamin D synthesis, and vitamin D synthesis at different sites need to be performed

in order to gain a better understanding of the vitamin D metabolism in nocturnal birds.

The presence of vitamin D_3 in kiwi and morepork skin was contrary to my expected findings. The nocturnal lifestyle of kiwi and morepork predisposes them to receiving low levels of sunlight, thus there would be no evolutionary advantage in retaining the ability to synthesise vitamin D in the skin. Although the concentration of vitamin D_3 detected in kiwi and morepork skin was low, its presence shows that dermal synthesis is retained to at least some degree. This may be explained by exposure to sunlight during dawn or dusk. The comparatively lower concentrations of vitamin D observed in kiwi and morepork, suggests that other factors such as ingestion of dietary vitamin D might be involved in their vitamin D metabolism.

The diet of kiwi mainly includes earthworms, cicada nymphs, and other invertebrates (Reid, Ordish, & Harrison, 1982). Additionally, the opportunistic consumption of plant material and certain fruits and seeds has also been observed in kiwi (Reid et al., 1982). A study by Finke (2002), showed that earthworms, cicada nymphs, and mealworms contained undetectable concentrations of vitamin D_3. Similarly, morepork diet comprises mainly of invertebrates, but morepork are also known to occasionally consume vertebrate prey such as rodents and small birds (Haw & Clout, 1999; Haw, Clout, & Powlesland, 2001).

Vitamin D_3 concentrations in kiwi and morepork skin were found to be lower as compared to tuatara, sea lion, sheep (Personal communication, Keren Dittmer, 2015) as well as chickens (Uva et al.,1983). More information is necessary to assess whether the amount of vitamin D_3 obtained from dermal synthesis and diet is sufficient to fulfill the requirements of these birds or whether they use alternative pathways for regulation of their calcium metabolism. Several nocturnal animals have evolved different mechanisms to fulfil their vitamin D and calcium requirements.

These mechanisms include increased skin sensitivity to light (Mediterranean house gecko (*Hemidactylus turcicus*)), increased ability to mobilise calcium from bones and teeth (Jamaican fruit bat (*Artibeus jamaicensis*)), naked mole rat (*Heterocephalus glaber*)), and increased reliance on dietary sources of vitamin D (dogs and cats) (Acierno et al., 2008; How et al., 1994; Kwiecinski, Zhiren, Chen, & Holick, 2001; Rivas, Mitchell, Flower, Welle, & Whittington, 2014; Tian et al., 1994). Whether the nocturnal behaviour of kiwi and morepork has led to these birds being dependent on their diet for obtaining vitamin D or there exists a different metabolic pathway for vitamin D and calcium metabolism needs to be investigated further.

3.5.2 Tuatara

Ultraviolet radiation resulted in a measurable increase in the vitamin D concentration of the tuatara skin samples. The vitamin D concentration in tuatara skin after UV-B exposure (0.0837 µg/g) was greater than the concentration observed in horse skin (<0.03 µg/g) and similar to that observed in sheep skin (0.1-0.2 µg/g) (Personal communication, Keren Dittmer, 2015). These results show that tuatara have the ability to photosynthesise vitamin D in their skin.

Studies on other species of reptiles such as bearded dragons (*Pogona vitticeps*), corn snakes (*Elaphe guttata*), komodo dragons (*Varanus komodoensis*) and iguanas (*Iguana iguana*) has shown that the dermal route of vitamin D synthesis is an important route in these reptiles. Adult tuatara exhibit predominantly nocturnal foraging behaviour (Besson & Cree, 2010; Saint Girons, 1980), but are regularly recorded basking during daylight hours (Besson & Cree, 2010; Gillingham, Carmichael, & Miller, 1995). These observations in conjunction with the results obtained in this study show that dermal vitamin D synthesis during basking is an important route of vitamin D production in this species.

3.5.3 New Zealand Sea lion

Ultraviolet radiation of New Zealand sea lion skin resulted in a measurable increase in the vitamin D_3 concentration. Vitamin D_3 concentration in New Zealand sea lion skin (1.6 µg/g) was found to be higher than horse (<0.03 µg/g), kiwi (0.0383 µg/g), morepork (0.0572 µg/g), sheep (0.1-0.2 µg/g), and tuatara skin (0.0837 µg/g). The higher vitamin D_3 concentration in UV-irradiated New Zealand sea lion skin suggests that this route of vitamin D synthesis is an important vitamin D pathway in New Zealand sea lions. In addition, the vitamin D concentration of New Zealand sea lion skin prior to experimental UV-B exposure was 16 times higher than for the other species included in this study and sheep and horse (Personal communication, Keren Dittmer, 2015). There are several possible explanations for this. Firstly, high vitamin D levels could reflect previous exposure of the skin to sunlight. The UV radiation in the Subantarctic regions, where New Zealand sea lions reside, is negligible during winter but is very high during the summer months (Iuliano-Burns, Wang, Ayton, Jones, & Seeman, 2009; Zérath, Holy, Gaud, & Schmitt, 1999). In addition, UV-B radiation received in the Subantarctic is known to be very high, owing to ozone depletion in this region (Day, Ruhland, Grobe, & Xiong, 1999). It might be possible that the sea lion in this study was captured in summer months and therefore had high vitamin D_3 concentration prior to experimental UV-B exposure. However, more research is needed to determine the duration for which vitamin D persists in skin over time and whether the high vitamin D_3 concentrations prior to experimental UV radiation observed in the study is as a result of higher UV in the Subantarctic region. Alternatively, marine mammal blubber contains high concentrations of vitamin D_3 (Kenny et al., 2004), and incomplete removal of fat during skin preparation may have artificially elevated the vitamin D concentration in this study, although the sample preparation attempted to minimise this confounding factor.

Significant variations in vitamin D synthesis have been observed in humans according to skin pigmentation (Holick, MacLaughlin, & Doppelt, 1981; Norman,

1998; Sharma et al., 2011). Numerous melanocytes are present within the basal layer of the skin of both pup and sub adult New Zealand sea lions, (Khamas, Smodlaka, Leach-Robinson, & Palmer, 2012; Orr, Kirk, & Cawthorn, 1983) and the melanocytes in sub adult New Zealand sea lion skin contain more pigment than those of the skin of New Zealand sea lion pups (Orr et al., 1983). Although increased melanin concentration is known to diminish dermal vitamin D synthesis, it does not stop its production (Holick, 1981; Webb & Holick, 1988). The New Zealand sea lion skin used in this study was heavily pigmented, however, this did not seem to interfere with dermal vitamin D synthesis. However, there is lack of information about the synthesis of vitamin D in New Zealand sea lion skin devoid of pigmentation and it is not known whether vitamin D concentrations will differ according to pigmentation in New Zealand sea lions. Studies on humans have shown that plasma $25(OH)D_3$ concentrations in dark-skinned individuals were significantly lower than those of hypopigmented individuals (Harris, 2006; Harris & Dawson-Hughes, 1998). Whether vitamin D synthesis in skin increases with reduced melanin pigmentation in New Zealand sea lions needs to be investigated further.

Increased UV-B exposure time is required with increased skin pigmentation for efficient synthesis of vitamin D. UV radiation exposures of 5 J/cm^2 were used to produce maximal vitamin D synthesis in earlier human studies (Tian et al., 1994). Exposure of shaved New Zealand sea lion skin to 5 J/cm^2 UV radiation resulted in synthesis of vitamin D. Removal of the thick fur of New Zealand sea lion skin, prior to UV-B radiation, might have resulted in increased skin vitamin D concentration as compared to live New Zealand sea lions. Also, sea lion flippers are not covered by hair and more studies could be carried out to determine the vitamin D metabolism in flipper skin. A comparative study between shaved skin, non-shaved skin and flipper skin could be done to assess the effect of hair coat on dermal vitamin D synthesis.

UV radiation differs according to latitude and can, therefore, affect dermal vitamin D synthesis in different regions (Sharma et al., 2011; Webb & Holick, 1988). New Zealand sea lions are found primarily in the Auckland islands and Campbell islands in the Subantarctic regions of New Zealand (Robertson & Chilvers, 2011), at a latitude of 48°S-55°S. The availability of sunlight and UV radiation in these regions varies seasonally (Webb, 2006). Studies conducted on humans in the Subantarctic and Antarctic regions have shown higher 25(OH)D concentrations during summer months but vitamin D insufficiency during winter months (Zérath et al., 1999). Since the UV-B radiation received during summer is very high (Rowley, 1929), the New Zealand sea lions are likely receiving sufficient UV-B radiation during basking, resulting in vitamin D production in the skin.

The high vitamin D concentration in New Zealand sea lion skin observed in this study, irrespective of the heavy pigmentation and their habitat, demonstrates that dermal vitamin D synthesis is an important route of vitamin D synthesis in this species. However, it is also necessary to assess the vitamin D in the diet of New Zealand sea lions. Diet of New Zealand sea lion comprises of a wide range of fish species, with opalfish and octopus being the main prey items (Childerhouse, Dix, & Gales, 2001). Other prey items include lobster krill, hoki fish and several other species of fish (Childerhouse et al., 2001). A study on the vitamin content in marine life has shown that high vitamin D concentration was found in fish such as herring, catfish, mackerel, sardine, lamprey, salmon and tuna and to a lesser degree in other marine life such as shrimps, clams, and oysters (Sidwell, Loomis, Foncannon, & Buzzell, 1978). Determination of vitamin D content in the primary prey items of New Zealand sea lions is necessary to confirm whether vitamin D is sought from their diet and assessment of the oral bioavailability of dietary vitamin D is necessary. It might be possible that New Zealand sea lions have a higher overall requirement for vitamin D in their body metabolism and hence obtain vitamin D from both dermal synthesis as well as from ingestion of vitamin D rich items. However, the calcium and

phosphorus content of the diet needs to be studied further to ascertain whether they indeed have a higher overall requirement of vitamin D.

3.6 Conclusion

The results of this study show that the nocturnal birds, kiwi and morepork, produced measurable amounts of vitamin D in the skin. The vitamin D concentrations were lower as compared to other species of animals, which suggests that either these small amounts of vitamin D might be sufficient for their calcium metabolism or these birds might obtain vitamin D via photoisomerisation of uropygial gland secretions on feathers. Alternatively, they might either rely on dietary vitamin D, or use other mechanisms to maintain their calcium homeostasis. The basking reptile, a tuatara, produced measurable vitamin D concentrations in the skin, suggesting dermal synthesis is an important route of vitamin D synthesis. The significant increase in vitamin D concentration in New Zealand sea lion skin on UV-B exposure might be suggestive of dermal vitamin D synthesis playing a critical role in the vitamin D metabolism in these marine mammals. The evolutionary differences between these species may have influenced the route of vitamin D metabolism utilised by each species, however, more detailed studies are needed to ascertain the effect of evolution on vitamin D metabolism.

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CHAPTER 4: General discussion

4.1 Key findings

The aim of the study was to determine the route of vitamin D metabolism in animal species having diverse life histories, so as to assess whether the differences in life history play a role in route of vitamin D metabolism utilised by an animal. In this study, a survey of the plasma/serum $25(OH)D_2$ and $25(OH)D_3$ concentrations was carried out in kiwi, tuatara and New Zealand sea lions. Plasma/serum $25(OH)D_2$ concentration was below detectable levels in all three species examined. There was measurable inter-species variation in the plasma/serum $25(OH)D_3$ concentrations between these species. Analysis of the ability of skin to synthesise vitamin D in response to UV-B exposure showed considerable species variation. There was an ascending gradient in the vitamin D production from the skin of kiwi, morepork, and tuatara, with New Zealand sea lion skin having the highest vitamin D concentration amongst these species.

Within kiwi, samples from wild kiwi showed 6 times lower plasma $25(OH)D_3$ concentrations as compared to captive indoor and outdoor kiwi. These results were contrary to what was expected. The results in this study suggest that this variation was of dietary origin and raises the possibility that captive kiwi are over-supplemented with vitamin D_3. The low plasma $25(OH)D_3$ concentration in wild kiwi suggests that this species may have evolved to use a calcium metabolism mechanism that is independent of vitamin D, as occurs in Damara mole rat (*Fukomys damarensis*), naked mole rat (*Heterocephalus* glaber), chicken (*Gallus gallus domesticus*) during egg laying, and Jamaican fruit bat (*Artibeus jamaicensis*) (Buffenstein, Sergeev, & Pettifor, 1993; How, Hazewinkel, & Mol, 1994; Kwiecinski, Zhiren, Chen, & Holick, 2001; Tian, Chen, Lu, Shao, & Holick, 1994). A measurable amount of vitamin D_3 was produced in the skin of the nocturnal birds in response to UV exposure, which was unexpected given their nocturnal life history. The ability of

the skin from both nocturnal birds to produce vitamin D_3 was found to be lower as compared to the diurnal species (tuatara and New Zealand sea lion). Further, the experiments were carried out on plucked skin, which may not reflect the ability of feathered skin to synthesise vitamin D_3. The contradictory findings between the plasma and skin of kiwi require further investigation to determine the key pathways of vitamin D metabolism in kiwi.

Within tuatara, plasma 25(OH)D_3 varied between the 5 captive institutions used for sampling. In particular, plasma 25(OH)D_3 concentrations of tuatara at Institute 2 were 2-4 times higher than other institutions. The diet at institute 2 differed from that of the other institutions. Apart from the invertebrates fed to tuatara, their diet contained day old chicks with an intact yolk, which is known to be a source of vitamin D_3 (Dittmer & Thompson, 2011). These tuatara were also provided with a vitamin D and calcium supplement. This suggests that tuatara at Institute 2 were fed a diet high in vitamin D, which was possibly the reason for their comparatively higher plasma 25(OH)D_3. Tuatara skin produced vitamin D_3 on exposure to UV-B radiation, which was expected for a basking reptile. These results suggest that both dermal and dietary vitamin D metabolism occurs in tuatara.

Mean serum 25(OH)D_3 concentration in New Zealand sea lions was 6-7 times higher than mean plasma 25(OH)D_3 in kiwi and there was little variation between individuals as compared to mean plasma 25(OH)D_3 concentrations in kiwi and tuatara. New Zealand sea lion skin showed the highest vitamin D_3 concentration prior to and after UV-B exposure. Study of New Zealand sea lion vitamin D metabolism shows that dermal vitamin D synthesis is an important route of vitamin D synthesis and more detailed analysis is required to assess the role of dietary vitamin D in New Zealand sea lions. A possible explanation of the pre-exposure 25(OH)D_3 skin concentration would be that there was improper fat removal during preparation of New Zealand sea lion skin for analysis. This might have occurred

specifically in New Zealand sea lion skin and not in the skin of other species used in this study, due to the high vitamin D_3 concentration observed to be present in the blubber of marine mammals, especially those that are piscivorous feeders (Kenny, O'Hara, Chen, Lu, Tian, & Holick, 2004). In addition, this may also have occurred due to exposure to UV light prior to experimental UV-B exposure radiation. Due the latitude and ozone depletion in the Subantarctic islands, the UV-B radiation in this region can be high (Day, Ruhland, Grobe, & Xiong, 1999; Iuliano-Burns, Wang, Ayton, Jones, & Seeman, 2009; Zérath, Holy, Gaud, & Schmitt, 1999). Assuming the New Zealand sea lion skin used in this study was collected during summer, exposure to UV might have occurred during collection and transport, and the vitamin D_3 synthesised possibly persisted in the skin of the sea lion. The higher UV-B radiation in the regions of the New Zealand sea lion habitat might also be responsible for the higher vitamin D_3 concentration in sea lion skin, after experimental UV-B exposure as compared to other species used in this study.

4.2 Scope of this study

The study has two main points of significance; comparative vitamin D metabolism of birds, reptiles and marine mammals and captive husbandry of kiwi and tuatara based on their vitamin D endocrine system.

4.2.1 Comparative vitamin D metabolism

Analysis of vitamin D metabolism in kiwi, tuatara and New Zealand sea lions provides more insight into variation of endocrine profiles and their evolution between birds, reptiles and mammals. Kiwi and morepork are nocturnal birds, which might utilise vitamin D from the diet, or function via an alternative route independent of vitamin D, however this cannot be confirmed from my study. Analysis of vitamin D and calcium metabolism in chickens has shown that during periods of egg laying, their intestinal absorption of calcium is 100 times higher than

that of humans. In addition, during ovulation, the plasma $1,25(OH)_2D_3$ concentrations in chickens increase over the subsequent days (Bentley, 1998). The endocrine system of reptiles might be similar to that of birds, due to the fact that both need to produce eggs (Bentley, 1998). It is possible that both birds and reptiles might have a higher requirement for vitamin D and calcium during these periods. Based on the findings of this study, vitamin D metabolism differs between kiwi and tuatara in their ability to synthesise vitamin D dermally on exposure to UV-B light. Tuatara are capable of producing greater amounts of vitamin D_3 in their skin on exposure to UV light. Although, there may be similarities between the vitamin D and calcium endocrine systems between birds and reptiles, there definitely exists species variation in their hormonal patterns, which needs to be studied in detail further.

4.2.2 Captive husbandry of kiwi and tuatara

Vitamin D deficiency has been observed in several birds as well as reptiles in captivity (Burgess, Gartrell, & Blanchard, 2009; Morgan, Alley, Gartrell, Thompson, & Perriman, 2011; Nichols, Montali, Pickett, & Bush, 1983; Thorp, 1994; Timmerman & Doolen, 1994). A lack of specific knowledge about vitamin D metabolism in individual species can lead to improper captive management of these animals. Improper supplementation of vitamin D in diet and inadequate exposure to supplemental UV light leads to vitamin D deficiency. The findings in this study provide greater understanding of the vitamin D metabolism in kiwi and tuatara, which can be used in their captive management. For nocturnal birds such as kiwi, dermal synthesis is not the likely route of vitamin D metabolism. The results from this study show that captive kiwi fed on a diet containing vitamin D are capable of absorbing this dietary vitamin D. Therefore, supplementation of vitamin D in the diet could be the most appropriate approach for captive kiwi. However, the findings in wild kiwi show lower plasma $25(OH)D_3$ concentrations as compared to captive kiwi. Therefore, the necessity of vitamin D in the normal physiology of kiwi is not known and more research is required to assess if vitamin D is actually required by kiwi for

its metabolism and if so, what would the recommended dietary vitamin D requirements be for these birds?

The general physiology of animals evolved in their wild habitat might serve as a better indicator of the physiological requirements, as compared to a captive environment. Animals kept in captivity are subject to various artificial environmental interactions such as restricted habitat choices, artificial diet and, unfamiliar lighting, which might result in inaccurate results (Mason, 2010). It is possible that kiwi in captivity may be currently over-supplemented with vitamin D.

Previous research on tuatara has shown a link between improper captive management and presence of nutritional secondary hyperparathyroidism (NSHP) (Burgess et al., 2009). This study showed that tuatara are capable of producing vitamin D in their skin. Apart from the importance of dermal vitamin D synthesis in these reptiles, significant elevations in the plasma $25(OH)D_3$ concentrations were observed at the captive institution where tuatara were fed on a high vitamin D diet. This study, in conjunction with that of Burgess et al (2009), shows that either adequate exposure to UV-B or dietary supplementation with vitamin D are vital for prevention of metabolic bone disease in these reptiles. Captive tuatara must have access to a basking area, which is not covered, within their enclosure to obtain sunlight for vitamin D synthesis as well as for thermoregulation (Burgess et al., 2009). Burgess et al (2009) suggested that the diet of captive tuatara should be supplemented with vitamin D and calcium supplements by either gut loading of insects or dusting of insects. However, given the strong capability of tuatara skin to synthesise vitamin D_3, if tuatara are given basking opportunities, then dietary supplementation may be unnecessary or even harmful to the tuatara health.

Extrapolating from human studies, it may be easier for tuatara to regulate vitamin D metabolism from dermal synthesis than from dietary exposure (Holick, MacLaughlin, & Doppelt, 1981, Webb & Holick, 1988). For captive tuatara, the recommendation

based on the results of this research, is that dermal exposure to UV light is the preferred option to maintain vitamin D metabolism, as this allows the animal to regulate its vitamin D concentrations more effectively than through dietary supplementation. Dietary supplementation of tuatara with vitamin D should only be reserved for those animals where adequate UV exposure is not available.

Although vitamin D is essential for calcium metabolism and has other body functions, vitamin D supplementation in captivity might also have a toxic effect. Hypervitaminosis D has been observed in captive budgerigars, due to over-supplementation of vitamin D in the diet (Olds, Burrough, Madson, Ensley, Horst, Janke, Schwatrz, Stevenson, gauger, & Cooper, 2015).

Hypervitaminosis D results in the widespread deposition of excessive mineral within the soft tissues of the body (McDowell, 2000; Watson & Mitchell, 2014). The tissues primarily affected by mineralisation are renal and cardiac tissues (McDowell, 2000; Nain, Laarveld, Wojnarowicz, & Olkowski, 2007; Watson & Mitchell, 2014). Renal gout induced by renal failure due to nephrocalcinosis has been observed in reptiles as a result of dietary oversupplementation (Watson & Mitchell, 2014). Mineralisation in the myocardial tissue has been observed in birds, which can lead to cardiac arrhythmias (Nain et al., 2007; Olds et al., 2015). This dystrophic mineralisation in vitamin D toxicity causes clinical signs such as pain, ataxia, muscular weakness, twitching, polydipsia, gastrointestinal disturbances, cardiac abnormalities, organ dysfunction, anorexia and eventually, mortality (McDowell, 2000; Olds et al., 2015; Watson & Mitchell, 2014).

Based on the results in this study, the higher plasma $25(OH)D_3$ of captive tuatara at Institute 2 might suggest that there is a possible risk of over-supplementation at this zoological institution, however this cannot be confirmed from the study. In captive kiwi as well, vitamin D supplementation is provided at the captive institutions used

in my study. The lower plasma 25(OH)D_3 concentration in wild kiwi might suggest that either captive kiwi do not require vitamin D supplementation in their diet or they are provided with higher amounts of vitamin D supplementation than that required. Therefore, more nutritional studies are required to assess the exact amount of vitamin D and calcium required by captive kiwi and tuatara in order to avoid risk of over supplementation.

4.3 Speculations and suggestions

The results in from this study show that differences exist in vitamin D metabolism between the species used in this study.

4.3.1 Nocturnal birds

The nocturnal birds, kiwi and morepork, produced measurable amounts of vitamin D_3 in their skin. However, it cannot be confirmed from this study if these concentrations of vitamin D are adequate for their calcium metabolism and whether nocturnal birds exclusively use this route of vitamin D to fulfill their physiological needs. Based on previous studies (Hou, 1929; Uva, Mandich, & Vallarino, 1983), secretions of the uropygial gland may also play a role in vitamin D synthesis. To investigate this further, more skin samples could be analysed from a variety of nocturnal species, so as to determine the vitamin D metabolism in nocturnal birds in more detail. Secondly, analysis of skin samples from different sites of the body and analysis of plucked and unplucked skin could be done to assess if the vitamin D_3 synthesis differs according to the site of production and presence of feathers. Thirdly, analysis of uropygial gland secretions and feathers could be done, to assess if synthesis of vitamin D occurs on exposure to UV-B radiation at these sites.

The survey of plasma 25(OH)D_3 concentrations of captive kiwi indicated that vitamin D was of dietary origin. Wild kiwi on the other hand, showed very low 25(OH)D_3

concentrations as compared to captive kiwi. In order to assess whether the lower plasma 25(OH)D_3 concentration is sufficient for optimal calcium, analysis of plasma calcium, phosphorus, PTH and 1,25(OH)D_3 concentrations would be required.

A study on nocturnal frugivorous bats (*Rousettus aegyptiacus*) has shown that these mammals exist in a naturally vitamin D deficient state, without showing any signs of hypovitaminosis D (Cavaleros, Buffenstein, Ross, & Pettifor, 2003). These bats still possess the vitamin D binding proteins and hormone receptors in order to metabolise vitamin D efficiently (Cavaleros et al., 2003). Similarly, African mole rats (*Cryptomys hottentotus*) also survive in a vitamin D deficient state, but still possess a fully functional vitamin D system (Bentley, 1998). Based on the results of the study, there is a possibility that wild kiwi might use a vitamin D independent calcium pathway, since their plasma 25$(OH)_3$ concentrations were much lower than the captive ones. The measurable plasma 25$(OH)_3$ concentrations observed in captive kiwi suggest that these birds are absorbing dietary vitamin D, but their requirement for vitamin D remains unclear. Therefore there is a need to assess the actual vitamin D requirement in kiwi and other nocturnal species in their natural habitat so as to create guidelines for vitamin D supplementation in captivity.

4.3.2 Tuatara

The study of vitamin D metabolism in tuatara showed that these reptiles are capable of synthesising vitamin D in their skin and that this route of vitamin D metabolism is important in this species. The plasma 25(OH)D_3 survey in captive tuatara showed variation in the plasma 25(OH)D_3 concentration, which was assumed to be of dietary origin based on the results. In order to determine which pathway of vitamin D metabolism predominates in tuatara, a controlled analysis could be done, where 2 groups of tuatara could be used, one group receiving UV-B radiation and second receiving no UV-B radiation. In addition, a comparative analysis could also be done between tuatara supplemented with UV-B radiation and tuatara supplemented with

dietary vitamin D to determine the relative efficacy of the two pathways. Analysis of wild tuatara plasma 25(OH)D_3 would provide insight into the actual metabolism of vitamin D in these reptiles.

4.3.3 New Zealand sea lion

New Zealand sea lion skin not only contained vitamin D_3 after exposure to UV-B radiation but also prior to UV-B exposure. The results suggest that the dermal pathway is an important route of vitamin D metabolism in these marine mammals. In order to rule out errors in processing of the skin, more New Zealand sea lion skin samples could be analysed for the presence of vitamin D_3 prior to and after UV-B radiation.

The serum samples used in this study were all collected within the months of January and February. Studies in humans have shown that serum 25(OH)D_3 concentrations decline in winter months in Subantarctic regions (Iuliano-Burns et al., 2009; Zérath et al., 1999). There could, therefore, be possible variation in the 25(OH)D_3 concentrations in serum between summer and winter seasons. Analysis of serum samples collected all year round and at different times of the day could be done to determine the effect of varying sunshine hours on New Zealand sea lion serum 25(OH)D_3 concentrations.

The study hypothesis stated that New Zealand sea lions have not undergone any evolutionary pressures that would affect the predominant route of vitamin D metabolism. This study does show that dermal synthesis is an important route of vitamin D metabolism in these mammals; however, analysis of the concentrations of vitamin D present in their prey species would provide more insight into the ability of New Zealand sea lions to utilise dietary vitamin D.

4.3.4 Limitations and further studies

In all 4 species used in this study for skin sampling, measurement of 7-dehydrocholestrol (7-DHC) (Provitamin D) in the skin would be useful. The concentration of 7-DHC in correlation with the vitamin D_3 concentrations after UV-B radiation would provide us information about the efficiency of conversion to vitamin D_3. Manipulation of diet and UV supplementation and assessment of plasma/serum $25(OH)D_3$ concentrations could be carried out in captivity to determine whether a particular pathway predominates in a particular species. Overall, analysis of a higher number of skin and serum samples would be beneficial in ruling out any errors in the study. In addition, differences in vitamin D metabolism between sexes could be carried out in kiwi and tuatara, since both these species are oviparous species. Egg laying may result in variation in the hormonal patterns in females, therefore sampling could be done during this period to determine changes in hormone concentrations, which would provide guidelines for vitamin D supplementation in captivity. Time of sampling after dietary supplementation of vitamin D or UV-B exposure radiation, is an important factor in all 3 species used for the plasma/serum survey. A study on bearded dragons maintained on a diet low in vitamin D but receiving supplemental UV-B radiation, showed that plasma $25(OH)D_3$ concentrations remained relatively stable for 83 days after ceasing UV-B exposure (Oonincx et al., 2013). The authors hypothesised that these reptiles might be able to store vitamin D_3 in their muscles, liver tissue and adipose tissue and use these vitamin D stores on cessation of UV-B exposure (Oonincx et al., 2013). This storage of vitamin D has also been observed in certain mammals (Brouwer, Van Beek, Ferwerda, Brugman, van der Klis, van der Heiden, & Muskeit, 1998; Heaney, Armas, Shary, Bell, Binkley, & Hollis, 2008; Rivers, Frankel, Juttla, & Hay, 1979). It was observed in rats that the half life of plasma $25(OH)D_3$ was 3-4 times less than the half life of $25(OH)D_3$ in perirenal and subcutaneous adipose tissue and this stored $25(OH)D_3$ helps in maintaining $25(OH)D_3$ concentrations in circulation (Brouwer et al., 1998). However, in pigs in the growing stage, $25(OH)D_3$ was present in fat stores

for about 7-8 days, after which there was a decline in the 25(OH)D_3 concentrations (Denis, Cournot, Lacroix, Colin, Zerath, & Pointillart, 2000). In contrast to adult female bearded dragons, a similar experiment in monitor lizards resulted in a fall in their plasma 25(OH)D_3 concentrations by 25-30% after 87 days (Ferguson, Gehrmann, Peavy, Painter, Hartdegen, Chen, Holick, & Pinder, 2009). Therefore, a high degree of inter-species variation exists in the storage capacity of vitamin D, and the concentrations of 25(OH)D_3 in serum/plasma might vary according to time of sampling. As such, additional studies need to be carried out to determine the duration for which 25(OH)D_3 stays in plasma and after how much time does it decrease in kiwi, tuatara and New Zealand sea lion plasma/serum.

Vitamin D metabolism in kiwi, tuatara and New Zealand sea lion has not been studied in detail prior to this study. This present study has certain limitations. Small sample size was one of the major limitations of the study. From each species, only one skin sample was processed. The plasma samples from wild kiwi were few in number, which could have led to sampling error and lower representation of wild kiwi population in this study. Secondly, due to lack of adequate funds more skin samples could not be processed. Thirdly, due to unavailability of wild tuatara plasma samples, comparison between 25(OH)D_3 concentrations in captive and wild tuatara could not be carried out. Lastly, measurement of 7-DHC in the skin could not be carried out.

4.5 Conclusions

The results from this study show that all of the species studied retained the ability to either use dietary vitamin D or synthesise vitamin D dermally, although there was inter-species variation in the magnitude of the dermal synthesis. Wild kiwi had lower plasma concentrations of 25(OH)D_3 which suggests the birds may be over-supplemented in captivity. Dermal vitamin synthesis is an important route of metabolism in tuatara, however, the comparative efficacy of vitamin D obtained

from sunlight and diet needs to be assessed. New Zealand sea lions utilise dermal vitamin D synthesis efficiently, although the role played by diet cannot be confirmed by this study. Comparative vitamin D metabolism between nocturnal birds, a reptile and marine mammal shows that there exist differences in the vitamin D endocrine system between different species, but the study results suggest that there are more factors contributing to these pathways than might be expected from life history characteristics.

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Appendix 1: Results of serum/plasma 25(OH)D_2 and 25(OH)D_3 concentration in kiwi (*Apteryx mantelli*), Tuatara (*Sphenodon punctatus*), and New Zealand sea lion (*Phocarctos hookeri*)

Species-sample number	Habitat	Age	Sex	UV (µW/cm^2)	25(OH) D2 (nmol/L)	25(OH)D3 (nmol/L)
Kiwi- K1	Indoor	5 yr	Male	No UV		14
Kiwi- K2	Outdoor	1 yr	Male	No UV		12
Kiwi- K3	Outdoor	1 yr	Female	No UV		10
Kiwi- K4	Indoor	1 yr	Male	No UV		17
Kiwi- K5	Outdoor	3 yr	Male	No UV		20
Kiwi-K6	Outdoor	10 m	Female	002-003		27
Kiwi- K7	Outdoor	9 m	Male	002-003		38
Kiwi- K8	Wild	15 yr	Male	215-225		3
Kiwi- K9	Wild	14 yr	Female	215-225		3
Kiwi-K10	Indoor	10 yr	Male	No UV		42
Kiwi-K11	Indoor	9 yr	Male	No UV		16
Kiwi-K12	Indoor	3 yr	Male	No UV		25
Kiwi-K13	Wild	Adult	Female	148-185		3
Kiwi-K14	Wild	3 yr	Male	170-220		3
Kiwi-K15	Indoor	5 yr	Female	002-003		22
Kiwi-K16	Indoor	5 yr	Male	002-003		14
Kiwi-K17	Outdoor	3 yr	Male	Shadecloth, 002-006		12
Kiwi-K18	Indoor	4 yr	Male	No UV		10
Kiwi-K19	Indoor	4 yr	Female	No UV		17

Tuatara-T1	Indoor	Adult	Male	Light- 141-198, ground- 009		34
Tuatara-T2	Outdoor	28 yr	Male	Up- 200, ground-15-40		72
Tuatara-T3	Outdoor	28 yr	Female	Up- 200, ground-15-40		86
Tuatara-T4	Outdoor	28 yr	Female	Up- 200, ground-15-40		52
Tuatara-T5	Outdoor	14 yr	Female	Up- 150-250, ground- 100		99
Tuatara-T7	Outdoor	13 yr	Male	Up- 100-150, ground-100		85
Tuatara-T8	Outdoor	Adult	Female	Up- 100-150, ground-100		89
Tuatara-T9	Outdoor	Adult	Male	Up- 100-150, ground-100		89
Tuatara-T10	Outdoor	Adult	Female	Up- 100-150, ground-100		115
Tuatara-T11	Indoor	22-23 yr	Male?			190
Tuatara-T12	Outdoor	28 yr	Male	Shadecloth(Partially)- 150		111
Tuatara-T13	Outdoor	37 yr	Male	Shadecloth(Partially)- 150		127
Tuatara-T14	Outdoor	14 yr	Female	Shadecloth(Partially)- 150		245
Tuatara-T15	Outdoor	70-80 yr	Male	Up- 250, ground-50-100		73
Tuatara-T16	Outdoor	70-80 yr	Female	Up- 250, ground-50-100		114
Tuatara-T17	Indoor	10-11 yr	Maybe male	UV light- 230, ground- 2-10		86
Sea Lion-S1	Wild					110
Sea Lion-S2	Wild					121
Sea Lion-S3	Wild					109
Sea Lion-S4	Wild					80
Sea Lion-S5	Wild					131
Sea Lion-	Wild					163

S6						
Sea Lion-S7	Wild					103
Sea Lion-S8	Wild					111
Sea Lion-S9	Wild					108
Sea Lion-S10	Wild					80