DIET IS A MAIN SOURCE OF VITAMIN D IN FINNISH PET RABBITS (*Oryctolagus cuniculus*)

Running title: Vitamin D status of Finnish pet rabbits

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ABSTRACT

During the winter time in Finland, sunlight is inadequate for vitamin D synthesis. Many pet rabbits live as house-rabbits with limited outdoor access even during summer and may therefore be dependent on dietary sources of vitamin D. The aims of this study were to report the serum 25-hydroxyvitamin D concentrations in Finnish pet rabbits, and to identify factors that influence vitamin D status. Serum 25-hydroxyvitamin D concentrations from 140 pet rabbits were determined using a vitamin D enzyme immunoassay (EIA) kit. Eleven rabbits were excluded from the statistical analysis because of unclear dietary data. The remaining 129 rabbits were divided into groups depending on outdoor access during summer (no access n=26, periodic n=57, regular n=46) as well as daily diet: little or no hay and commercial rabbit food ≤ 1/2 dl (n=12); a lot of hay and no commercial food daily (n=23); a lot of hay and commercial food <1 dl (n=59); a lot of hay and commercial food ≥ 1 dl (n=35). The range of serum 25-hydroxyvitamin D concentration was from 4.5 to 67.5 ng/ml with a mean of 26.1 ng/ml. Statistical general linear model adjusted for weight, age, and season, indicated that diet was associated with vitamin D concentrations (p=0.001), but outdoor access during summer was not (p=0.41). Mean 25-hydroxyvitamin D concentration was significantly higher in the rabbits receiving a lot of hay and commercial food ≥ 1 dl (33.9±13.2 ng/ml) than in rabbits in other diet groups (24.0±8.5 ng/ml, 21.7±8.1 ng/ml, and 22.2 ±18.0 ng/ml, respectively). This investigation showed wide variation in 25-hydroxyvitamin D concentrations among Finnish pet rabbits. Diet remains a main source since outdoor access seems to be too limited to provide adequate vitamin D synthesis for most of them and the use of vitamin D supplements is rare.

Keywords:

Rabbit, 25-hydroxyvitamin D, 25(OH)D, vitamin D deficiency, diet

INTRODUCTION

The most important function of vitamin D is to maintain skeletal calcium balance, but it is also important for many other metabolic functions, such as growth, neuromuscular activity, and immune response (DeLuca, 2004, Holick, 2004).

Vitamin D may be obtained from the diet, from vitamin supplements, or from endogenous synthesis. It is stored in the liver and in adipose tissue. In many herbivorous and omnivorous species, including rabbits, vitamin D precursor, cholecalciferol (vitamin D3) is synthesised in the skin (Hymøller & Jensen, 2010, Emerson, Whittington, Allender & Mitchell, 2014). Solar ultraviolet B radiation (wavelength 290 to 315 nm) penetrates the skin and converts 7-dehydrocholesterol to previtamin D3, which is rapidly converted to vitamin D3. In the liver D3, and another vitamin D precursors D2, are converted to 25-hydroxyvitamin D (25(OH)D, calcidiol), which is hydroxylated in the kidney to the active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH)2D, calcitriol). Parathyroid hormone (PTH) modulates this conversion in response to changes in blood calcium levels. As there are many feedback mechanisms in place controlling the circulating concentrations of 1,25(OH)2D, blood values do not always reflect vitamin D deficiency or excess properly. Instead, serum 25(OH)D levels are considered to best indicate the vitamin D status (Holick, 2002).

Vitamin D3 is found in many animal products, such as in fish liver oils, oily fish species, beef liver, and egg yolk, which serve as dietary sources of vitamin D in many animals and humans (Schmid & Walter, 2013). Plants also provide a potential source of vitamin D, which mainly stems from endophytic fungi or fungal contamination of the plant material. Cell membranes of fungi have high concentrations of precursor of vitamin D2 (ergosterol), especially after sun exposure (Jäpelt & Jakobsen, 2013). Vitamin D2 is found also in the microalgae that live in water (Jäpelt & Jakobsen 2013).

Plants provide an important exogenous source of ergosterol for rabbits, as they are found in good-quality sun-dried hay, alfalfa, and other plants at a later stage of maturity. It is recommended that more than 70% of pet rabbits' diets should comprise grass and/or dried hay ad libitum (Prebble, 2014). Leaves of some vegetables and fruits, especially the plants of Solanaceae family, such as potato, pepper, and tomato also contain vitamin D3 and its metabolites (Jäpelt & Jakobsen, 2013). Vitamin D3 is added to most commercial rabbit food. A maximum of 25 g/kg (roughly 74–90 ml/kg) of pelleted food is the recommended daily portion for pet rabbits and the rest of the daily diet (15%) should consist of green plants, vegetables, and herbs (Prebble, 2014).

In northern latitudes higher than 50° (Finland is located between 60–70°N), the amount of UVB radiation from sunlight is adequate for vitamin D synthesis only from approximately mid-March to mid-October (Kazantzidis et al., 2009). People with light skin types I-III (Caucasian, light Asian) can receive one standard vitamin D dose (1 SDD), from sunlight in 30 minutes under average atmospheric conditions in southern Finland (Kazantzidis et al. 2009). One SDD corresponds to a UV equivalent of an oral dose of approximately 1000 IU (25 μg) vitamin D when 1/4 of body surface is exposed to sunlight. Similar studies in rabbits are so far lacking and the appropriate time needed for vitamin D synthesis in these latitudes is unknown. Even during summer, many pet rabbits may be kept indoors with limited outdoor access. Window glass absorbs UVB radiation depending on the type, colour, and thickness of glass and window configuration (Tuchinda, Srivannaboon, & Lim, 2006), so exposure of pet rabbits to natural UVB radiation remains limited. Consequently, dietary sources of vitamin D and the reserves present in liver and adipose tissue may become essential. Vitamin D deficiency due to insufficient UVB exposure may be evident in Finnish pet rabbits if their dietary intake of vitamin D concentration is low.

Need for artificial UVB light, access to sunlight, and vitamin D supplements in rabbits and hypovitaminosis D in house-rabbits have been the topics of intermittent debate. The present study was thus undertaken to evaluate the serum 25(OH)D concentrations in Finnish pet rabbits, and to investigate the associations of diet and outdoor access with vitamin D status. The hypothesis is that serum 25(OH)D concentrations are lower in rabbits without outdoor access and fed on a diet containing with low vitamin levels, i.e. little or no hay and/or commercial rabbit food in comparison with those rabbits with regular outdoor access and diet comprising a lot of hay and commercial rabbit food.

2. MATERIALS AND METHODS

The study was approved by the Animal Experiment Board of Finland (5562/04.10.03/2011) and was carried out as a cross-sectional study at the Veterinary Teaching Hospital of the University of Helsinki. Owners registered their rabbits to the study by email on voluntary basis.

Animals

Serum samples from 140 rabbits (*Oryctolagus cuniculus*) participating in the Pet Rabbit Health Research Project in Finland were collected during 2012 and 2013. The owners considered their rabbits healthy and not in need of veterinary treatment at the time of the study. Nineteen breeds were represented, of which Dwarf Lop was the most common (n=39, 28.1%) followed by mixed breed rabbits (n=37, 26.6%). The mean age of the rabbits was 2.7 years (SD 2.0 years, range 0.1 to 9.3 years). Data on age was missing in 5 cases. Seventy-two (51.8%) of the rabbits were female (10 neutered, 13.9%) and 67 (48.2%) were males (31 neutered, 46.3 %). Body weight was available in 133 rabbits and mean weight of the rabbits was 2.4 kg (SD 1.2 kg, range 0.3–6.2 kg).

Questionnaire

The owners were asked about the housing, outdoor access, and diet of their rabbits using an internet-based questionnaire.

Questions on rabbits' diet included details about ingredients containing vitamin D or its precursors i.e. hay, commercial rabbit food (pellets, nuggets, muesli mixture), and vitamin supplements. The choices for the daily amount of hay were: 1) a lot, 2) quite a lot, 3) not much, 4) very little, or 5) none at all. The feeding rate was asked for commercial rabbit food and vitamin supplements with the following choices: 1) always available, 2) twice a day, 3) once a day, 4) several times a day, 5) a few times a week, 6) once a week, 7) a few times a month, 8) once a month, 9) seldom, or 10) never. The amount of commercial rabbit food portion consumed daily was: 1) one to two tablespoons, 2) half a decilitre, 3) one decilitre, or 4) more than one decilitre. The brand of the commercial food used was asked by an open-ended question.

The following choices were given for housing type: 1) house, 2) outbuilding, 3) garden, or 4) balcony. Outdoor access was assessed by asking how often the rabbit was outside during summer months. The choices were: 1) daily, 2) a few times a week, 3) a few times a month, 4) a few times during the summer, 5) the rabbit spends the whole summer outdoors, or 6) never. The questionnaire is available upon request from the corresponding author.

Blood samples

After physical examination, the rabbits were sedated by a subcutaneous injection of medetomidine 0.1 mg/kg and ketamine 5 mg/kg. A local anaesthetic cream containing lidocaine and prilocaine was applied to the skin 15 to 20 minutes before taking the blood sample. The blood samples were collected from the cephalic or lateral saphenous vein using a 23-gauge needle into a heparin syringe

and for a freely flowing sample into a plain tubes and EDTA tubes. The plain tubes containing the blood samples were immediately placed into an ice bath and centrifuged at 4000 rpm for 10 minutes. The serum from each sample was collected and frozen (at -80°C) until the 25-hydroxyvitamin D concentration was determined. Haematological and biochemical profiles as well as blood gas analysis were performed for later use. Lateral abdominal and skull radiographs were taken, and tibial bone mineral density was measured using peripheral computed tomography during the sedation. A subcutaneous injection of atipamezole 0.25 mg/kg was given to all rabbits after the procedures to reverse the effects of medetomidine. Results of the physical examination, radiographs and bone density measurements have been published previously (Mäkitaipale et al., 2015, Mäkitaipale, Sievänen, & Laitinen-Vapaavuori, 2018). None of the rabbits in this study had chronic anorexia or findings indicating severe chronic endocrinological disease that might affect to the synthesis of vitamin D.

Vitamin D analysis

Serum 25-hydroxyvitamin D concentrations were determined using an enzyme immunoassay (25-hydroxyvitamin D EIA kit AC-57SF1, Immonodiagnostic Systems Holdings PLC, Tyne & Wear, UK). The linearity of the assay was evaluated using a high vitamin D concentration rabbit serum (74.1 ng/ml) diluted 1:2, 1:4, and 1:8 with a low vitamin D concentration rabbit serum (7.8 ng/ml). The linearity was determined by comparing the observed 25(OH)D concentrations following dilution to the expected vitamin concentrations. The precision of the assay was evaluated by calculating the intra- and inter-assay coefficient of variation (CV) from three rabbit serum samples and two human controls. The intra-assay CV was calculated for serum samples with high, medium, and low vitamin D concentration from eight replicates within the same run. The inter-assay CV was calculated from two human controls on four assay runs.

In the linearity assessment, the observed mean 25-hydroxyvitamin D concentrations were 41.0 ng/ml, 24.4 ng/ml, and 16.1 ng/ml, representing 118, 117, and 122 % of the expected vitamin concentrations, respectively. The dilutions showed high linearity over the studied range (r=0.994). The intra-assay CV was 10.6%, for the low (mean 7.8 ng/ml), 7.3% for medium (mean 36.9 ng/ml), and 3.7% for high (74.1 ng/ml) concentration of vitamin D in rabbit serum. Inter-assay CV for the two human controls of the EIA kit were 4.5% for the low concentration (mean 16.7 ng/ml) and 8.0% for the high concentration (mean 59.7 ng/ml).

Statistical analysis

The rabbits were divided into four groups by their daily diet in terms of hay intake and commercial rabbit food (pellets, nuggets, muesli mixture):

1) Low hay and commercial food intake (LoHay_LoCF) group consisting of rabbits receiving not much/ very little/ no hay and commercial food 1/2 decilitre (dl) or less (n=12)

2) High hay intake and no commercial food (HiHay_NoCF) group consisting of rabbits receiving a lot of hay and no daily commercial food daily (n=23)

3) High hay and moderate commercial food intake (HiHay_ModCF) group consisting of rabbits receiving a lot/ quite a lot of hay and commercial food less than 1 dl (n=59)

4) High hay and high commercial food intake (HiHay_HiCF) group consisting of rabbits receiving a lot/ quite a lot of hay + commercial rabbit food 1 dl or more (n=35)

Rabbits eating little hay but commercial rabbit food *ad libitum* (n=5), and rabbits with unclear dietary data (n=6) were excluded from the statistical analysis. Tablespoon unit was converted to millilitres using the general conversion (1 tablespoon=15 ml).

The rabbits were also divided into three groups by their outdoors access during summer:

1) no outdoors access (rabbits living inside a house or in an outbuilding for the whole year without access to outdoors),

2) periodic outdoors access (rabbits living inside a house or in an outbuilding with access to outdoors few times a week, few times a month or seldom during the summer months),

3) regular outdoors access (rabbits living inside a house or in an outbuilding with daily access to outdoors or living whole summer outdoors and rabbits living outdoors or in a balcony).

The month of blood sampling was categorised to four seasons as follows: 1) Winter (December, January, February); 2) Spring (March, April, May); 3) Summer (June, July, August); 4) Autumn (September, October, November).

Mean, standard deviation (SD), range, and 95% confidence interval (95% CI) are given as descriptive statistics. Pearson correlation coefficients were used to estimate the strength of associations between age and weight and 25(OH)D concentrations. In order to estimate whether the outdoors access and diet were associated with 25(OH)D concentration, the general linear model (GLM) was applied using the respective categorical variables as factors and potential confounders body weight, age, and the season of sample collection as covariates. Log-transformation was performed to 25(OH)D concentration to normalise the distribution prior to the GLM analysis. P values < 0.05 were considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics, version 23 (IBM Corp, NY, US).

3.RESULTS

The mean serum 25-hydroxyvitamin D concentration in all 140 Finnish pet rabbits was 26.0 ng/ml (range 4.5-67.5 ng/ml). Weight was associated with the serum 25(OH)D concentrations ($r=0.22$, $P=0.015$), but age was not ($r=-0.11$, $P=0.12$).

Varying amounts of pellets, nuggets, or muesli mixture, representing 15 different brands, were provided daily for 111 rabbits. Vitamin D concentration of them varied between from 700 IU/kg to 2000 IU/kg. Forty-four rabbits were also fed with racing horse or beef cattle feed alone (vitamin D concentration from 1000 IU/kg to 2400 IU/kg) or mixed with rabbit feed. Ninety-three of 129 (72%) of the rabbits were in the groups HiHay_ModCF and HiHay_HiCF (Table 1). Rabbits in the HiHay_HiCF group were significantly heavier (2.9 kg) compared to the rabbits in LoHay_LoCF group (1.9 kg) ($P<0.005$), whereas the other diet groups did not differ significantly from each other.

Ten of 140 rabbits (7%) had 25(OH)D concentrations of <12 ng/ml. Of these, four were in the LoHay_LoCF group, two were in the HiHay_NoCF, and two in the HiHay_ModCF group. Dietary data was missing in the other two rabbits. One of these rabbits spent the whole summer outdoors (sample collected in May), the data about outdoor access was missing in another two rabbits (samples collected in December), and seven rabbits had periodic or no access to outdoors during summertime (samples collected between May and September).

Fourteen of 140 rabbits (10%) had 25(OH)D concentration > 40 ng/ml. Eight of these were in the HiHay_HiCF group, three in the HiHay_ModCF group, one in the LoHay_LoCF group and two had no hay/small amount of hay, and *ad libitum* pellets. These two were excluded from the statistical analysis. Seven rabbits had regular access to outdoors (samples collected in March, June, September, and November), four rabbits had periodic outdoor access (samples collected in January,

March, August, and November) and three had no access to outdoors (samples collected in February).

Vitamin supplements containing vitamin D were provided to 10 of 140 rabbits (7.1%) of which only one received it daily (serum 25(OH)D concentration 15.5 ng/ml, diet LoHay_LoCF group, vitamin D concentration in the supplement 93210 IU/kg) and nine only occasionally (seldom than once a month). Mean 25(OH)D concentration of these nine rabbits was 22.4 ng/ml (range 10.1–41.5 ng/ml). Two of them were in the diet HiHay_NoCF group and seven in the HiHay_ModCF group.

Regular access to outdoors was permitted to 46 out of 140 rabbits (33%) of which 16 rabbits lived outdoors or in a balcony for the whole year, eight house-rabbits were provided with daily outdoor access, and 22 house-rabbits lived the whole summer outdoors.

According to GLM analysis adjusted for body weight, age, and the season of sample collection, diet was significantly ($p=0.001$) associated with serum 25(OH)D concentration, but the outdoors access was not ($p=0.41$). Table 1 shows descriptive data on serum 25(OH)D concentrations in different subgroups. The adjusted mean 25(OH)D concentration of rabbits in HiHay_HiCF was 23.2 ng/ml (95% CI 9.4 to 37.0 ng/ml, $p<0,001$) higher compared to rabbits in LoHay_LoCF group, 19.0 ng/ml (95% CI 7.1 to 30.9 ng/ml, $p=0.004$) higher compared to rabbits in HiHay_NoCF group, and 13.2 ng/ml (95% CI 5.3 to 21.0 ng/ml, $p=0.007$) higher compared to rabbits in HiHay_ModCF group. The crude non-adjusted mean 25(OH)D concentrations in the 12 subgroups broken down by diet and outdoors access are illustrated in Figure 1.

DISCUSSION

The present study showed that the serum 25-hydroxyvitamin D concentrations among 140 family-owned pet rabbits varied between 5 to 68 ng/ml. This large variation is likely due to the heterogeneity of the diet and housing of the pet rabbits. The variation in 25(OH)D concentration and factors affecting pet rabbits' vitamin D status has so far been lacking, even though vitamin D deficiency is considered a possible condition in them (Harcourt-Brown 1996, Fairham & Harcourt-Brown 1999). The present study of a large sample of pet rabbits provides relevant new information to this matter. Previously, only a few experimental studies have evaluated serum 25(OH)D status among rabbits and the following ranges have been observed: from 14.0 to 81.0 nmol/l (5.6 to 32.5 ng/ml) in nine rabbits participating in an artificial UVB light study (Emerson et al., 2014), and from 133.3 to > 775.5 nmol/l (53 to >310 ng/ml) in 28 breeding rabbits (Warren, Lausen, Segre, El-Hajj, & Brown, 1989) fed on a diet high in vitamin D concentration (6420 IU/kg, respectively), which may account for the high concentrations in comparison with the results of Emerson et al. (2014) and the results of the present study.

Recommended serum 25(OH)D concentrations for optimal health of pet rabbits have not yet been established. The 25(OH)D concentration in 7 % of our rabbits was below the limit of severe vitamin D deficiency in humans (< 12 ng/ml) (Vieth, 1999, Sai, Walters, Gang, & Gallagher, 2011, Spiro & Buttriss, 2014). A minimum concentration of 30 ng/ml, or even 40 ng/ml, is likely needed in humans for optimal cellular health (Holick, 2002, Bischoff-Ferrari, Giovannucci, Willett, Dietrich, & Dawson-Hughes, 2006). In laboratory rabbits, induced vitamin D deficiency with undetectable serum 25(OH)D and 1.25(OH)D3 levels resulted in elevated PTH concentrations and, in some rabbits, hypophosphatemia, inadequate skeletal mineralization, and the classical signs of osteomalacia (Brommage et al., 1988). Vitamin D is also important for many other metabolic functions and, in humans, deficiency is associated with an increased risk of many common health disorders, such as cardiovascular diseases, cancers, asthma, allergy, and respiratory infections (Holick, 2002, Holick,

2004, Bischoff-Ferrari et al., 2006, Bozzetto, Carraro, Giordano, Boner, & Brandi, 2012). The rabbits in the present study were healthy according to the owners and not in need of veterinary treatment, although a previous study of the same group of rabbits showed that some of them did have health issues (Mäkitaipale et al., 2015). As the aim of the present study was to report serum 25-hydroxyvitamin D status and investigate selected factors influencing the concentration, the consequences of extreme low or high concentrations were not studied. The long-term consequences of hypovitaminosis D and its possible association with other health disorders in rabbits warrants for further studies.

The results of the present study showed that diet is an important source of vitamin D in Finnish pet rabbits and they are dependent on dietary source of vitamin D precursors. Rabbits consuming a lot of hay and commercial rabbit food ≥ 1 dl daily had higher 25(OH)D concentrations compared to those rabbits with lower intake of daily hay and commercial food. This makes sense as both hay and commercial food are rich in vitamin D precursors. Their mean serum 25(OH)D concentration was about 50% higher in comparison with other groups (33.9 ng/ml vs. 21.7 ng/ml, 22.2 ng/ml, and 24.0 ng/ml). The rabbits were fed on several different commercial rabbit foods with variable vitamin D concentrations. Racehorse and beef cattle feed was also common as 44 rabbits were fed with them alone or mixed with rabbit feed, which made an accurate assessment of vitamin D intake difficult. Although the daily vitamin D requirement for rabbits has not been published, a previous study demonstrated that a daily dose of 10-13 IU/kg prevented rickets in weanling Dutch rabbits (Curry, Basten, Francis, & Smith, 1974). Vitamin D recommendations for rabbits are usually given as maximum amount per kilogram of diet, which should be 800-1000 IU/kg, but not exceed 1000-1300 IU/kg, and be never higher than 2000 IU/kg (Lebas, 2000, Mateos, Rebiller, & de Blas, 2010). The direct effect of hay on serum 25(OH)D levels was not obvious from the present results. The mean concentration of vitamin D precursors in good quality hay in Finland is approximately 1000 IU/kg,

but it can vary depending on the age and storage conditions of the hay. Other plants, especially dead leaves, sun-dried plants and those at later stage of maturity are sources of vitamin D for rabbits but the vitamin D content of plant material varies highly. Vitamin D supplements are one possible source of vitamin D for rabbits. However, the estimation of the appropriate dose for supplementation may be challenging when the vitamin D content of diet is unknown. Hypervitaminosis D is a life-threatening condition so supplements should be recommended cautiously and only for those rabbits with diet low in natural sources of vitamin D precursors. Regarding the results of our study, the use of vitamin D supplements for rabbits is rare as only one rabbit received supplements daily.

Although the access to outdoors and potential exposure to UVB light did not reach statistical significance as a factor affecting serum 25(OH)D concentrations in this study of Finnish rabbits, seven out of ten rabbits with 25(OH)D concentrations below 12 ng/ml had only periodic or no access to outdoors and seven out of fourteen rabbits with 25(OH)D concentration over 40 ng/ml had regular access to outdoors. Rabbits are able to synthesise vitamin D from UVB light, but as we hypothesised, Finnish pet rabbits are more dependent on their dietary source of vitamin D precursors as the outdoor access seems to be too limited for adequate vitamin D synthesis in the majority of them. Emerson et al. (2014) reported an 11 ng/ml increase in rabbits' 25(OH)D concentration after two-weeks daily exposure to 12 hours of artificial UVB light compared to the non-UVB group. Compared to the rabbits of our study, the mean baseline serum 25(OH)D concentration of rabbits in the study of Emerson et al. were low: 11.9 ng/ml in the control group and 15.5 ng/ml in the UVB exposure group. After 2 weeks, the UVB exposure group reached the same mean concentration (26.6 ng/ml) as our heterogeneous pet rabbit population. If two-weeks daily exposure to 12 hours artificial UVB light was needed to increase the serum 25-hydroxyvitamin D concentration by 11 ng/ml, it is possible that the outdoors access was not long enough to improve vitamin D status in rabbits of our study. Information about the time and length of the rabbits' daily outdoor visits during summer

months, type of hutch or pen, or the ratio of sunny and shaded areas in the outdoor area was not established in the present study. It is common that the early summer is chilly in some parts of Finland and outdoor access is mainly limited to the late summer (July, August). Many owners walk their rabbits on a leash with a harness, while the rabbits are compelled to walk with their owners and the total time that the rabbit spent outdoors may have been too short for adequate vitamin D synthesis. Lastly, the time spent outdoors may have occurred in the afternoon, after working hours, when sunlight exposure is lower.

It is unknown whether the rabbits with sufficient dietary vitamin D need endogenous synthesis to maintain serum 25(OH) levels. The only group of rabbits that would show an effect of outdoor access in that case would be the LoHay_LowCF group with poor dietary sources. Unfortunately, there were only twelve rabbits in this group, thus the sample size was underpowered to detect a significant effect should it exist. The mean 25(OH)D concentration in this group was 22.2 ng/ml, which was comparable both to the HiHay_NoCF group (21.7 ng/ml) and the HiHay_ModCF group (24 ng/ml) (see Table 1). This suggests that endogenous synthesis of vitamin D might have taken place in the LoHay_LowCF group because exogenous sources were poor. Both homeostatic control mechanisms and baseline 25(OH)D concentrations modulate the endogenous synthesis of vitamin D in the skin (Karppinen et al., 2017) so natural UVB exposure in Finland may not be sufficient to maintain serum 25(OH)D concentration if dietary sources of vitamin D precursors simultaneously decrease. This phenomenon was observed in Danish cows living at 56° latitude (Hymøller & Jensen 2012). In Danish cows, daily outdoor access of 15 and 30 minutes was inadequate to maintain the mean 25(OH)D concentration of 18 ng/ml during 28 days trial in cows fed with a diet without added vitamin D. Instead, at least five-times longer daily access to outdoors was needed to increase the 25(OH)D concentration from 18 ng/ml to 27 ng/ml. Little is known about endogenous vitamin D synthesis in rabbits and the findings in cows may also apply to pet rabbits, especially if a lot of

fresh grass, greens, herbs, and vegetables are fed during summer instead of dry hay and commercial rabbit food rich in vitamin D precursors. Emerson et al (2014) made an interesting observation that the serum mean 25-hydroxyvitamin D concentration of 12.7 ng/ml in their small (n=4) control group did not change despite the fact that the rabbits were provided an acceptable diet of timothy hay and pellets with vitamin D supplementation (1100 IU/kg feed). This raises the question of whether dietary vitamin D concentration should be higher than 1100 IU/kg in rabbits without access to natural or artificial UVB light.

25-hydroxyvitamin D concentration can be measured using two different main methods; chromatography and immunoassay, of which the latter is currently most common (Wallace, Gibson, de la Hunty, Lamberg-Allardt, & Ashwell, 2010). The structure of 25-hydroxyvitamin D is similar in human and animal species and this particular EIA kit is widely used in different species including horses, humans, mice, pigs and tortoises (Wallace et al., 2010, Gorman et al., 2012, Selleri & Di Girolamo, 2012, Pozza, Kaewsakhorn, Trinarong, Inpanbutr, & Toribio, 2014, Lin et al., 2017), but is not validated for rabbit serum. Therefore the linearity of the 25-hydroxyvitamin D in rabbit serum was tested for potential matrix effects of rabbit serum. The measured values were 118 to 122 % of the expected concentrations and showed excellent correlation. Rabbit serum intra-assay CV varied from 3.7 to 10.6 %. The reproducibility of the assay done in different days was followed by human controls of the kit to ensure the consistency of the results between the plates. The validation parameters showed that the EIA kit was suitable for the determination of 25-hydroxyvitamin D in rabbit serum.

The major limitations of the present study pertain to the reliability of information obtained about each rabbit's diet and exposure to UV light. The information was taken from an owner-reported questionnaire and therefore the exact amount and type of consumed hay and commercial food is

subject to some uncertainty and inconsistency. This limitation also applies to categorisation of the UVB exposure that the rabbits received during the summer.

In conclusion, the present study showed a wide variation in serum 25(OH)D concentrations between 5 and 68 ng/ml in Finnish pet rabbits. Vitamin D is obtained from the diet, vitamin D supplements, or from endogenous synthesis. In Finnish pet rabbits, diet remains a main source since outdoor access seems to be too limited to provide adequate vitamin D synthesis in most of them and the use of vitamin D supplements is rare. Regular and sufficiently long secure outdoor access is beneficial for pet rabbits during the summer time as it provides them both activity and fresh grass and other wild plants. Therefore, larger studies with more specific information regarding the natural UVB exposure and diet are needed to establish the required daily sunlight exposure time for adequate vitamin D synthesis in these latitudes as well as to estimate the specific influence of low 25(OH)D concentrations on common health disorders in pet rabbits.

REFERENCES

Bischoff-Ferrari, H. A.; Giovannucci, E.; Willett, W. C.; Dietrich, T.; Dawson-Hughes, B., 2006: Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. *The American Journal of Clinical Nutrition* 84, 18-28.

Bozzetto, S.; Carraro, S.; Giordano, G.; Boner, A.; Baraldi, E., 2012: Asthma, allergy and respiratory infections: the vitamin D hypothesis. *Allergy* 67, 10-17.

Brommage, R.; Miller, S. C.; Langman, C. B., Bouillon, R.; Smith, R.; Bourdeau, J. E., 1988: The effects of chronic vitamin D deficiency on the skeleton in the adult rabbit. *Bone* 9, 131-139.

Curry, O. B.; Basten, J. F.; Francis, J. M., Smith, R., 1974: Calcium uptake by sarcoplasmic reticulum of muscle drom vitamin D-deficient rabbits. *Nature* 249, 83-84.

DeLuca, H. F., 2004: Overview of general physiologic features and functions of vitamin D. *American Journal of Clinical Nutrition* 80, (1689S-96S).

Emerson, J. A.; Whittington, J. K.; Allender, M. C.; Mitchell, M. A., 2014: Effects of ultraviolet radiation produced from artificial lights on serum 25-hydroxyvitamin D concentration in captive domestic rabbits (*Oryctolagus cuniculi*). *American Journal of Veterinary Research* 75, 380-385.

Fairham, J., Harcourt-Brown, F. M., 1999: Preliminary investigation of the vitamin D status of pet rabbits. *Veterinary Record* 145, 452-454.

Gorman, S.; Scott, N. M.; Tan, D. H. W.; Weeden, C. E.; Tuckey, R. C.; Bisley, J. L.; Grimbaldeston, M. A.; Hart, P. H., 2012: Acute erythemal ultraviolet radiation causes systemic immunosuppression in the absence of increased 25-hydroxyvitamin D_3 levels in male mice. *Plos One* 7, e46006. DOI:10.1371/journal.pone.0046006

Harcourt-Brown, F., 1996: Calcium deficiency, diet and dental disease in pet rabbits. *Veterinary Record* 139, 567-571.

Holick, M. F., 2002: Vitamin D: the under appreciated D-lightful hormone that is important for skeletal and cellular health. *Current Opinion in Endocrinology, Diabetes and Obesity* 9, 87-98.

Holick, M. F., 2004: Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers and cardiovascular disease. *American Journal of Clinical Nutrition* 80, 1678-1688.

Hymøller, L.; Jensen, S. K., 2010: Vitamin D3 synthesis in the entire skin surface of dairy cows despite hair coverage. *Journal of Dairy Science* 93, 2025–2029. DOI:10.3168/jds.2009-2991

Hymøller, L.; Jensen, S. K., 2012: 25-Hydroxycholecalciferol status in plasma is linearly correlated to daily summer pasture time in cattle at 56◦N. *British Journal of Nutrition* 108, 666–671 DOI 10.1017/S0007114511005964

Jäpelt, R. B.; Jakobsen, J., 2013: Vitamin D in plants: a review of occurrence, analysis, and biosynthesis. *Frontiers in Plant Science* 4, 136. DOI: 10.3389/fpls.2013.00136

Karppinen, T.; Ala-Houtala, M.; Ylianttila, L.; Kautiainen, H.; Lakkala, K.; Hannula, H-R.; Tu-runen, E.; Viljakainen, H.; Reunala, T.; Snellman, E., 2017: The effect of vernal solar UV radiation on serum 25-hydroxyvitamin D concentration depends on the baseline level: observations from a high latitude in Finland. *International Journal of Circumpolar Health* 76, 1272790.
DOI: 10.1080/22423982.2016.1272790

Kazantzidis, A.; Bais, A. F.; Zempila, M. M.; Kazadzis, S.; den Outer, P. N.; Koskela, T.; Slaper, H., 2009: Calculations of the human vitamin D exposure from IV spectral measurements at three European stations. *Photochemical & Photobiological Sciences* 8, 45-51.

Lebas, F., 2000: Vitamins in rabbit nutrition: literature review and recommendations. *World Rabbit Science* 8, 185-192.

Lehmann, B.; Meurer, M., 2010: Vitamin D metabolism. *Dermatologic Therapy* 23: 2-12.

Lin, Y.; Lv, G.; Dong, H-J.; Wu, D.; Tao, Z-Y.; Xu, S-Y.; Che, L-Q.; Fang, Z-F.; Bai, S-P.; Feng, B.; Li, J.; Xu, X-Y., 2017: Effects of the different levels of dietary vitamin D on boar performance and semen quality. *Livestock Science* 203: 63-68. DOI:10.1016./j.livsci.2017.07.003

Mateos, G. G.; Rebiller, P. G.; de Blas, C., (2010). Minerals, vitamins and additives: Nutrition of the rabbit. (2nd ed.) de Lebas, C.; Wiseman, J.: ed.

Mäkitaipale, J., Harcourt-Brown, F. Laitinen-Vapaavuori, O., 2015: Health survey of 167 pet rabbits (*Oryctolagus cuniculus*) in Finland. *Veterinary Record* 177, 418.

Mäkitaipale, J.; Sievänen, H.; Laitinen-Vapaavuori, O., 2018: Tibial bone density, cross-sectional geometry and strength in Finnish pet rabbits: a peripheral quantitative computed tomography study. *Veterinary Record* 183, 382. DOI:10.1136/vr.104419

Pozza, M. E.; Kaewsakhorn, T.; Trinarong, C.; Inpanbutr, N.; Toribio, R. E., 2014: Serum vitamin D, calcium, and phosphorus concentrations in ponies, horses and foals from the United States and Thailand. *The Veterinary Journal* 199, 451-456. DOI:10.1016/j.tvjl.2014.01.002

Prebble J., (2014). Nutrition and feeding: BSAVA Manual of rabbit medicine. (1st ed.) Meredith, A.; Lord, B.: ed.

Prebble, J.; Meredith, A., 2014: Food and water intake and selective feeding in rabbits on four feeding regimes. *Journal of Animal Physiology and Animal Nutrition* 98, 991-1000.

Sai, A. J.; Walters, R. W.; Fang, X.; Gallagher, J. C., 2011: Relationship between Vitamin D, Parathyroid Hormone, and Bone Health. *Journal of Clinical Endocrinology & Metabolism* 96, E436 –E46. DOI: 10.1210/jc.2010-1886

Schmid, A.; Walter, B., 2013: Natural vitamin D content in animal products. *Advances in Nutrition* 4, 453-462.

Selleri, P.; Di Girolamo, N., 2012: Plasma 25-hydroxyvitamin D_3 concetratons in Hermann's tortoises (*Testudo hermanni*) exposed to natural sunlight and two artificial ultraviolet radiation sources. *American Journal of Veterinary Research* 73, 1781-1786.

Spiro, A.; Buttriss, J. L., 2014: Vitamin D:An overview of vitamin D status and intake in Europe. *Nutrition Bulletin* 39, 322–350. DOI: 10.1111/nbu.12108

Tuchinda, C.; Srivannaboon, S.; Lim, H. W., 2006: Photoprotection by window glass, automobile glass, and sunglasses. *Journal of the American Academy of Dermatology* 54, 845-854.

Vieth, R., 1999: Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety. *American Journal of Clinical Nutrition* 69, 842–56.

Wallace, A M.; Gibson, S., de la Hunty, A.; Lamberg-Allardt, C.; Ashwell, M., 2010: Measurement of 25-hydroxyvitamin D in the clinical laboratory: Current procedures, performance characteristics and limitations. *Steroids* 75, 477-488.

Warren, H. B.; Lausen, N. C. C.; Segre, G. V.; El-Hajj, G.; Brown, E. M., 1989: Regulation of calciotropic hormones in vivo in the New Zealand White rabbit. *Endocrinology* 125, 2683-2690.

Table 1. Descriptive data on serum 25-hydroxyvitamin D concentration among pet rabbits broken down by the outdoors access and diet categories.

Variable		N†	Mean ± standard deviation SD (ng/ml)	Range (ng/ml)
Outdoor access				
	No access	27	26.8 ± 11.0	8.9 - 52.0
	Periodic access	61	25.4 ± 12.1	4.5 - 67.5
	Regular access	46	27.9 ± 12.7	7.4 - 63.9
Diet				
	Low Hay/ Low commercial Food	12	22.2 ± 18.0	4.5-67.5
	High Hay/ No commercial Food	23	21.7 ± 8.1	10.1 - 35.9
	High Hay/ Moderate Commercial Food	59	24.0 ± 8.5	11.0-48.7
	High Hay/ High Commercial Food	35	33.9 ± 13.2	13.1 - 63.9

† Number of rabbits

FIGURE LEGENDS

Figure 1. Mean serum 25-hydroxyvitamin D concentrations in Finnish pet rabbits (*Oryctolagus cuniculus*) broken down by their diet and outdoors access. Number of rabbits in each subgroup is represented as a white number in each bar. The division into groups is described in the detail in the statistical analysis.