Vitamin D status is seasonally stable in northern European dogs

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Vitamin D status is seasonally stable in northern European dogs

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Abstract

Background: Numerous studies in veterinary species have recently linked vitamin D status with nonskeletal health disorders. Previous studies have indicated that dogs cannot produce endogenous vitamin D via cutaneous production and rely solely on dietary intake of vitamin D. The seasonal variation of vitamin D seen in humans due to changes in ultraviolet (UV) exposure, therefore, is unlikely to be replicated in these animals.

Objectives: The objective of this study was to investigate the natural variation in 25-hydroxyvitamin-D concentrations in dogs subject to seasonal UV exposure.

Methods: This longitudinal study followed 18 healthy dogs fed a standardized diet over 1 year, with blood samples obtained monthly. Two key vitamin D metabolites, 25-hydroxyvitamin-D_2 and 25-hydroxyvitamin-D_3, were assessed by liquid chromatography-tandem mass spectrometry in serum samples. Various other biochemical parameters were also measured. Seasonality was assessed using cosinor statistical analysis.

Results: Although the dogs were subject to seasonally varying UV radiation, 25-hydroxyvitamin-D and related biomarkers (including calcium and parathyroid hormone) remained stable over time and did not follow a seasonal pattern. 25-hydroxyvitamin-D was not positively correlated with exposure to UV radiation. Nonetheless, variation in 25-hydroxyvitamin-D concentrations between individual dogs was detected.

Conclusions: Given the standardization of diet, we concluded that the seasonal stability of 25-hydroxyvitamin-D concentration (vitamin D status) was likely a direct result of lack of cutaneous vitamin D production in this species and highlights the importance of dietary intake. The variation in 25-hydroxyvitamin-D concentration between animals warrants further investigation.

KEYWORDS

25-hydroxyvitamin-D, LC-MS, season, ultraviolet B

Abbreviations: 1,25α(OH)2D, 1,25α-dihydroxyvitamin-D; 25(OH)D, 25-hydroxyvitamin-D; AAFCO, American Association of Feed Control Officials; BSA, bovine serum albumin; CV, coefficient of variation; ESI, electrospray ionization; FP, fluorophenyl; GWAS, genome-wide association study; HfSA, hospital for small animals; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOD, lower limit of detection; LLOQ, lower limit of quantitation; LOD, limit of detection; LOQ, limit of quantitation; MRM, multiple reaction monitoring; MSG, Meteosat second generation; MTBE, methyl tert-butyl ether; PTH, parathyroid hormone; QC, quality control; R(D)SVS, Royal (Dick) School of Veterinary Science; SLE, supported liquid extraction; UV, ultraviolet; UVB, ultraviolet B; VDR, vitamin D receptor.

1 | INTRODUCTION

The vital role vitamin D plays in maintaining skeletal health, particularly via its function in calcium homeostasis, has been well established for over a century.[1,2] Vitamin D's principal role is to maintain ionized calcium and phosphate concentrations within a physiologically appropriate range.[3] Vitamin D_2 and D_3 can be obtained through the diet, and many species, including humans, sheep, and cattle, can produce vitamin D_3 in the skin. This occurs by the photoisomerization of 7-dehydrocholesterol into previtamin D_3 by ultraviolet B radiation, then the subsequent conversion of previtamin D_3 to vitamin D_3 by heat-dependent isomerization. Once absorbed or produced, vitamin D is bound to the vitamin D-binding protein and either stored in fat or transported to the liver. Vitamin D is hydroxylated to 25-hydroxyvitamin-D (25(OH)D), which is widely used to assess vitamin D status in the liver before undergoing a second hydroxylation step in the kidney to 1,25α-dihydroxy vitamin-D (1,25α$(OH)_2$D).[2,4]

Recently, many studies have investigated the role of vitamin D beyond the skeleton.[4-10] There is increasing evidence that low vitamin D status is predictive of outcome in many human diseases, including all-cause mortality.[11] Furthermore, it has been established that vitamin D can have a profound impact on the differentiation and phenotype of many nonskeletal cell types, particularly various types of immune cells.[6-8] The situation is similar in companion animal medicine, with low vitamin D status linked to several health disorders in cats and dogs.[5,12]

Currently, very few studies have examined the natural variation of vitamin D in companion animals.[13,14] Ex vivo studies have indicated that dogs and cats cannot produce vitamin D cutaneously due to the inadequate abundance and conversion of 7-dehydrocholesterol.[15-18] On this evidence, seasonal variation in vitamin D status observed in humans due to changes in UV exposure would not occur in dogs. However, there has only been one reported study investigating seasonal vitamin D status in dogs, which focused primarily on Greyhounds and was conducted in Australia.[13]

In this study, we aimed to establish whether there was a seasonal variation of vitamin D status in dogs. We followed 18 dogs across a 1-year period; all were fed a standardized diet and lived within 15 miles of the study center, and were, therefore, subject to approximately the same intensity of vitamin D-producing UV radiation. We established whether 25(OH)D concentrations in dogs were correlated with seasonal variations in UV exposure by measuring serum concentrations of 25(OH)D by liquid chromatography-tandem mass spectrometry (LC-MS/MS), and comparing this to the seasonal variation of UV intensity. Seasonal variation of vitamin D associated physiologic biomarkers, including parathyroid hormone (PTH), phosphorus, and calcium, were also investigated.

2 | MATERIALS AND METHODS

2.1 | Study population

This study assessed vitamin D in 18 healthy dogs over a 1-year period at the Hospital for Small Animals (HfSA), Royal (Dick) School of Veterinary Science (R(D)SVS), University of Edinburgh. All dogs lived within 15 miles of the HfSA and were household pets with daily access to the outdoors. The experiments were approved by the University of Edinburgh Animal Welfare and Ethics Review Board. All dogs were free of any clinical diseases (assessed via patient history and clinical exam) and were not on any chronic (more than 2 weeks) medication. The dogs were fed a standardized diet during the study period consisting of Hill's Science Plan Advanced Fitness Medium breed with chicken adult dry food (Hill's Pet Nutrition, Inc) (food composition is outlined in Table S1). All dogs were fed maintenance energy requirement ranging from 1.4 to 1.8× resting energy requirement based on the manufacturer's recommended amount per kg of bodyweight. No additional treats were fed. All dogs had body condition score (BCS) assessed monthly at the time of blood sampling, and a recommendation to increase or decrease the daily food allowance to maintain each dog at BCS 5/9 was made. All dogs remained at BCS 5/9 throughout the 12-month study period. Blood samples were collected monthly from each animal beginning July 2015 until June 2016 and placed into plain, ethylenediaminetetraacetic acid (EDTA), and lithium heparin blood collection tubes (as per manufacturer's instructions), which were refrigerated immediately after collection. Serum and plasma were separated by centrifugation within 4 hours of collection and aliquoted. Samples were either stored frozen at −80°C until analysis or used on the day of collection.

2.2 | Biochemical analysis

Albumin, creatinine, phosphate, and total calcium were measured in serum samples, and ionized calcium was measured on lithium heparin plasma. All biochemical parameters were measured on the day of blood collection. Parathyroid hormone concentrations were measured in EDTA plasma in a single batch analysis. Albumin, total calcium, creatinine, and inorganic phosphate were measured using an Au480 Chemistry Analyser (Beckman Coulter (UK) Ltd) by the Veterinary Pathology Unit R(D)SVS, University of Edinburgh. Ionized calcium was analyzed on a GEM Premier 3500 system (Instrumentation Laboratory) by the Veterinary Pathology Unit. Plasma PTH concentrations were measured using a two-site immunoradiometric assay by NationWide Specialist Laboratories. For all biochemical analyses, there were 18 dog samples per month, with the exception of June, for which there were only 16 dog samples available.

2.3 | Vitamin D analysis by LC-MS

2.3.1 | Chemicals and reagents

Reference standards of 25(OH)D_2 and 25(OH)D_3 and isotopically labeled internal standards (deuterium-labeled 25(OH)D_2 (6,19,19-d_3-25(OH)D_2) and carbon-13-labeled 25(OH)D_3 (23,24,25,26,27-$^{13}C_5$-25(OH)D_3)) were purchased from Sigma-Aldrich. High-performance liquid chromatography (HPLC)-grade water, HPLC-grade methyl

tert-butyl ether (MTBE), HPLC-grade ethyl acetate, and an ammonia solution (35%) were purchased from Fisher Scientific. Liquid chromatography-tandem mass spectrometry grade water, LC-MS grade methanol, and HPLC-grade formic acid were purchased from VWR Chemicals. Bovine serum albumin (BSA) and ammonium formate were purchased from Sigma-Aldrich. ISOLUTE supported liquid extraction (SLE) 96-well plates were purchased from Biotage. The derivatization reagent DMEQ-TAD (4-[2-(3,4-Dihydro-6,7-dimethoxy-4-methyl-3-oxo-2-quinoxalinyl)ethyl]-3H-1,2,4-triazole-3,5(4H)-dione) was purchased from Abcam. The Raptor Fluorophenyl (FP) reversed phase liquid chromatography column (2.7 µm 100 Å, LC Column 100 × 2.1 mm) was purchased from Thames Restek UK Ltd.

2.3.2 | Sample preparation

A calibration curve was produced by spiking aqueous 1% BSA with 25(OH)D_2 and 25(OH)D_3 standards at concentrations ranging from 0.2-25 ng/mL for 25(OH)D_2 and 1.6-200 ng/mL for 25(OH)D_3. Each canine serum sample and standard (both 200 µL) was spiked with an equal concentration of labeled internal standards (10 ng/mL of d_3-25(OH)D_2 and 20 ng/mL of $^{13}C_5$-25(OH)D_3) and subject to SLE. Briefly, 200 µL of 0.5 mM ammonium hydroxide was added to each serum sample and standard, mixed by aspiration, and loaded on to the SLE 96-well plate. A vacuum was applied until the samples were fully loaded onto the SLE columns and then left to adsorb to the SLE material for 10 minutes (without vacuum). Vitamin D analytes were eluted from the SLE column with 750 µL of MTBE: ethyl acetate (90:10, v:v) under gravity. This was repeated twice (750 µL MTBE: ethyl acetate), followed by a final (third) elution with a lower volume of MTBE: ethyl acetate (400 µL). A vacuum was applied for 2 minutes to ensure complete elution. The eluate was dried under nitrogen at 45°C. Dried samples were subject to derivatization by DMEQ-TAD. Briefly, 25 µL of 0.1 mg/mL DMEQ-TAD was added to each sample, mixed on a plate shaker, and incubated at room temperature in darkness for 30 minutes. An additional 25 µL of DMEQ-TAD was added to each sample and incubated for a further 1 hour. Ethanol was added (40 µL) to each sample to quench the reaction before drying under nitrogen at 45°C. Each sample was reconstituted in 70 µL of LC-MS grade water: methanol (70:30, v:v) ready for the LC-MS/MS analysis.

2.3.3 | LC-MS/MS analysis

The LC-MS system used was a Shimadzu Nexera ultra-high-performance liquid chromatography system (Shimadzu Corporation) coupled to a Sciex QTrap 6500 quadrupole mass spectrometer (AB Sciex). Liquid chromatography separation was carried out using a Raptor FP column (2.7 µm 100 Å, LC Column 100 × 2.1 mm) (Thames Restek), which was maintained at 30°C in a CT-20 column oven (Shimadzu). The mobile phase was 2 mM ammonium formate in water with 0.1% formic acid (A) and 2 mM ammonium formate in methanol with 0.1% formic acid (B). The mobile phase gradient is described in Figure 1.

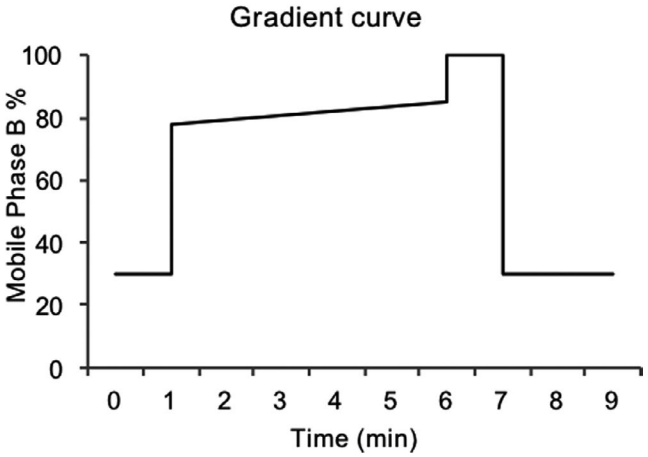

FIGURE 1 The liquid chromatography mobile phase gradient for the analysis of vitamin D analytes. Visual representation of mobile phase gradient. Each sample run is 9 min long. Mobile phase B begins at 30%; increases sharply to 78% at 1.2 min; increases gradually from 78% to 85% B from 1.2 to 6 min; increases to 100% B at 6 min; is held at 100% B from 6.2 to 7 min; returns to 30% B and is held for the final 1.4 min

The flow rate was set at 0.6 mL/minute, and the total run time was 9 minutes per sample. For mass spectrometer analysis, ionization was performed by electrospray ionization in positive ion mode. A multiple reaction monitoring mode was used to monitor and quantify derivatized standards, endogenous vitamin D analytes 25(OH)D_2 and 25(OH)D_3, and internal standards, d_3-25(OH)D_2 and $^{13}C_5$-25(OH)D_3. Full mass spectrometry settings can be viewed in Table 1. Liquid chromatography-tandem mass spectrometry/MS data were analyzed using Analyst software (version 1.6.3, AB Sciex). Representative chromatograms of 25(OH)D_2 and 25(OH)D_3 analyte standards and corresponding internal standards in 1% BSA are shown in Figure 2.

2.3.4 | Method validation and quality control

Validations were performed to assess linearity and repeatability of data. A total of five runs were conducted to analyze the 214 samples. Calibration curves were produced for each run by spiking 1% BSA with known concentrations of analyte standards. Calibration curve correlation coefficients ranged from 0.9906-0.9993 for 25(OH)D_2, and 0.9914-0.9986 for 25(OH)D_3 over the five runs. The limit of detection (LOD) and limit of quantitation (LOQ) were determined by assessing signal: noise ratios of the standard curve. The lower LOD (LLOD) was determined by a signal: noise ratio of >3 and the lower LOQ (LLOQ) by a signal:noise ratio of >10. The LLOQ for each analyte was assessed in each run, and calculated concentrations of each endogenous analyte were only accepted if above this value. For 25(OH)D_2, the LLOD of the assay was 0.5 nmol/L, with the LLOQ ranging from 0.5 to 4 nmol/L. For 25(OH)D_3, the LLOD of the assay was 4 nmol/L, with the LLOQ ranging from 4 to 7.7 nmol/L.

The recovery rate of the sample preparation method was assessed by comparing the chromatographic peak area of 1% BSA spiked with

TABLE 1 Mass spectrometer settings for the analysis of derivatized $25(OH)D_2$ and $25(OH)D_3$ and internal standards

	Analyte (derivatized)			
Mass spectrometer parameters	**$25(OH)D_2$**	**d_3-$25(OH)D_2$**	**$25(OH)D_3$**	**$^{13}C_5$-$25(OH)D_3$**
Q1 mass (Da)	758.4	761.3	747.3	751.5
Q3 mass (Da)	740.2	743.4	729.3	733.5
Scan time (msec)	100.0	100.0	100.0	100.0
Declustering potential (V)	145.0	120.0	145.0	145.0
Entrance potential (V)	8.0	6.0	8.0	6.0
Collision energy (V)	30.0	30.0	33.0	32.0
Collision cell exit potential (V)	35.0	12.0	35.0	35.0
Curtain gas	40.0	40.0	40.0	40.0
Collision gas	Medium	Medium	Medium	Medium
IonSpray voltage	5500.0	5500.0	5500.0	5500.0
Temperature (°C)	350.0	350.0	350.0	350.0
Ion source gas 1	60.0	60.0	60.0	60.0
Ion source gas 2	40.0	40.0	40.0	40.0

Abbreviations: °C, celsius; $^{13}C_5$-$25(OH)D_3$, carbon 13 labeled $25(OH)D_3$; $25(OH)D_2$, 25-hydroxyvitamin-D_2; $25(OH)D_3$, 25-hydroxyvitamin-D_3; d_3-$25(OH)D_2$, deuterium-labeled $25(OH)D_2$; Da, dalton; msec, milliseconds; V, volt.

$25(OH)D_2$ and $25(OH)D_3$ standards pre- and postextraction. The recovery rate for $25(OH)D_2$ was 70.4%, and for $25(OH)D_3$ was 73.2%. Matrix effects were assessed by comparing the chromatography of unextracted standards with 1% BSA spiked standards. BSA (1%) had a matrix effect of 0.1% and 0.6% for $25(OH)D_2$ and $25(OH)D_3$, respectively. Ion suppression was assessed by comparing the average peak area of internal standards in the 1% BSA spiked standard curve samples and all of the experimental canine serum samples. Ion suppression was variable between runs, ranging from 86% ion enhancement to 84% ion suppression for $25(OH)D_2$, and 146% ion enhancement to 78% ion suppression for $25(OH)D_3$. Although ion suppression was variable, the internal standards used to normalize this and the peak area ratio of the analyte to the internal standard are included in the calculation of analyte concentration.

Intra- and inter-assay accuracy was assessed by the inclusion of a quality control (QC) sample in each run. Canine serum samples (n = 20) were pooled, mixed, and aliquoted before freezing. One pooled QC serum sample was thawed and included in each run and subject to the same sample preparation procedures as experimental samples. The QC sample was analyzed at the beginning and end of every run to assess intra-assay accuracy. Intra-assay accuracy was calculated as 16.5% for $25(OH)D_2$, and 3.8% for $25(OH)D_3$. Data for QC samples were compared across runs to assess inter-assay accuracy, as well as comparing data from 24 other samples that have been repeatedly assessed by this assay (in two or more runs). Inter-assay accuracy was 17.5% for $25(OH)D_2$, and 12.1% for $25(OH)D_3$.

2.3.5 | Assay application

To assess vitamin D status in 12 monthly serum samples from 18 dogs (17 for November and June), $25(OH)D_2$ and $25(OH)D_3$ were analyzed by LC-MS following the protocol outlined above. A representative chromatogram of $25(OH)D_3$ and $^{13}C_5$-$25(OH)D_3$ internal standards from a canine serum sample is shown in Figure 3.

2.4 | Assessment of UV radiation

To determine the levels of UV radiation that the dogs in this study were exposed to, data was extracted from the TEMIS UV version 2.0 datasets [19] for the period January 2011-December 2016. UV dose was computed from satellite observations of ozone and a parametrization of UV reaching the earth's surface as a function of ozone and the solar zenith angle. The parametrization accounts for weighting of the UV radiation within the vitamin D action spectrum[20] (describing the wavelength of UV that is required for vitamin D synthesis) and will be referred to from here on as "vitamin D UV dose." Attenuation of the UV radiation by clouds was determined from Meteosat second generation satellite observations, and this is displayed as "cloud modified" data. The vitamin D UV dose over Edinburgh was calculated using the daily averages of vitamin D UV dose from 2011 to 2016 (inclusive of the study sampling time) from the TEMIS UV version 2.0 datasets.[19]

2.5 | Statistical analyses

Cosinor analysis was used to evaluate seasonal variation in 25(OH)D concentrations and related biochemical variables. Regarding patterns in health and disease, a season is defined as "a pattern in a health outcome or exposure that increases then decreases with some regularity".[21] It is well established that vitamin D status follows a sinusoidal-like seasonal pattern in humans,[22-24] and cosinor

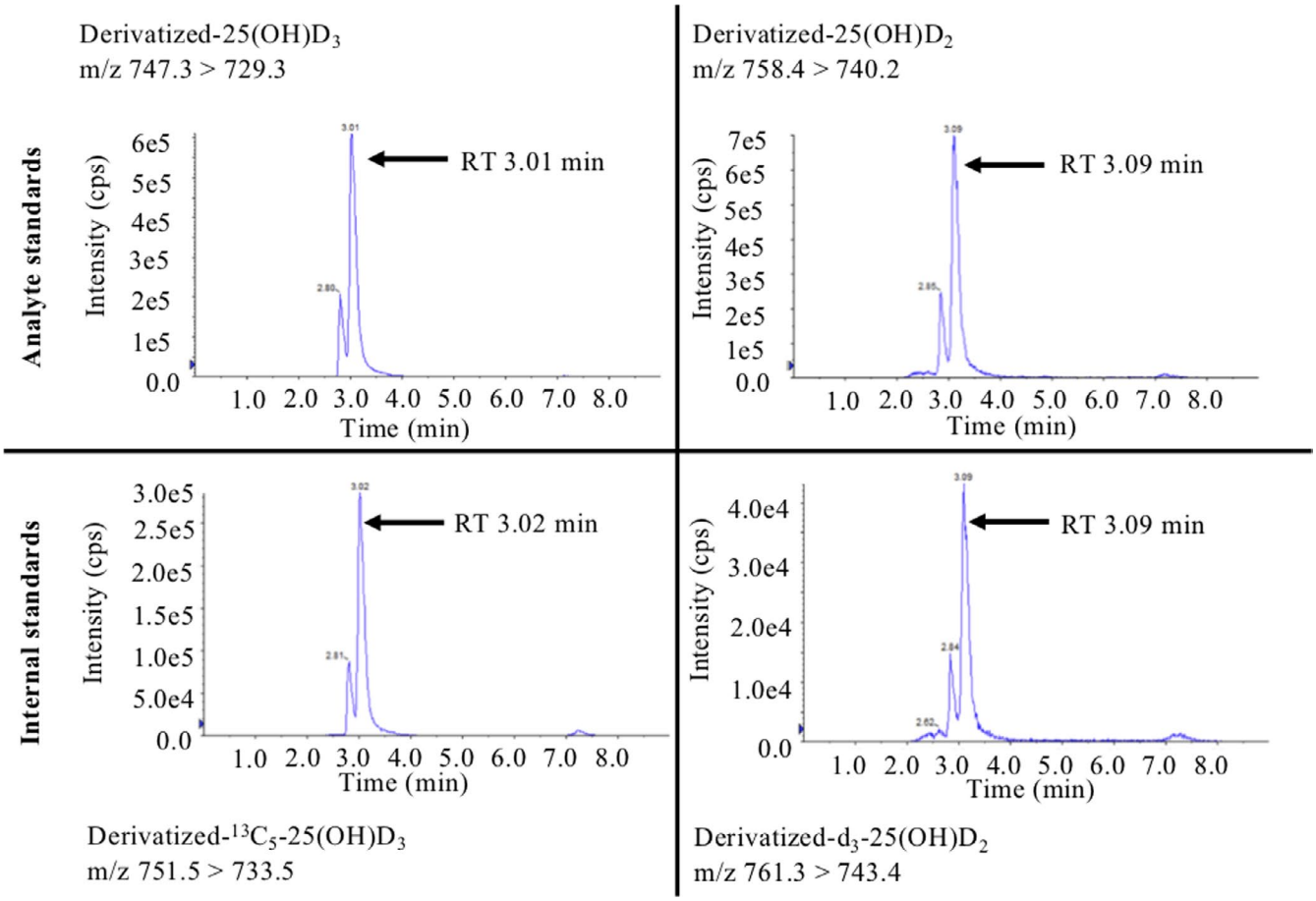

FIGURE 2 Representative chromatograms of 25(OH)D_2 and 25(OH)D_3 standards from calibration curve. Representative chromatogram of BSA spiked with derivatized 25(OH)D_2 and 25(OH)D_3 standards and labeled internal standards highlighting the retention time (RT) and mass/charge (m/z) transition of each analyte. 25(OH)D_2, 25-hydroxyvitamin-D_2; 25(OH)D_3, 25-hydroxyvitamin-D_3; BSA, bovine serum albumin

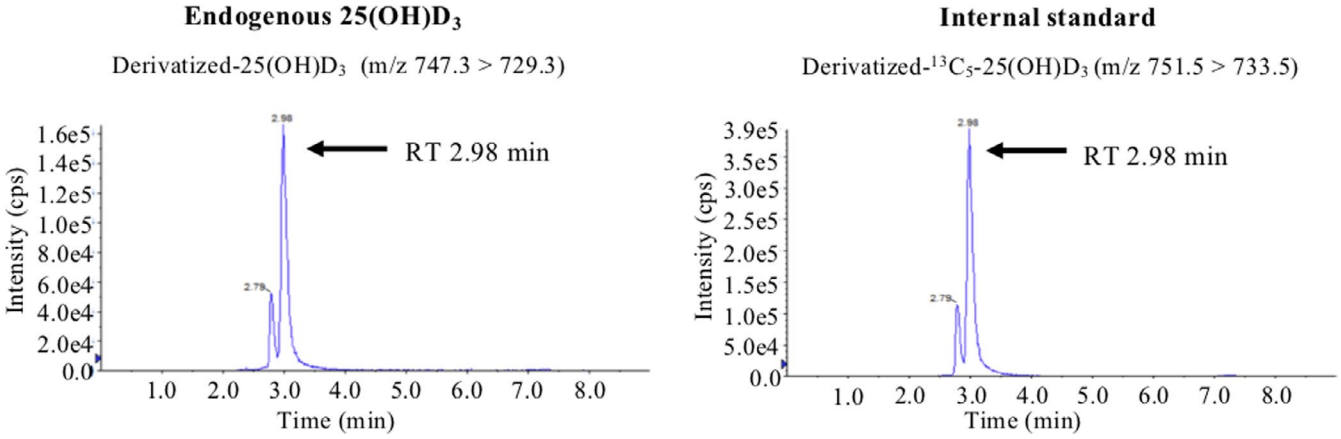

FIGURE 3 A representative chromatogram of 25(OH)D_3 in canine serum. A representative chromatogram of derivatized endogenous 25(OH)D_3 concentrations and a spiked labeled $^{13}C_5$-25(OH)D_3 internal standard in a canine serum sample highlighting the retention time and m/z transition of each analyte. 25(OH)D_3, 25-hydroxyvitamin-D_3; m/z, mass/charge; RT, retention time

analysis has been successfully used to explore seasonal variation in vitamin D status.[22,25,26] To analyze seasonal variation of 25(OH)D concentrations in this study, cosinor analysis was used to model the 25(OH)D concentration (the dependent variable) as a time-varying sine wave (with month of sampling representing time) using the R Statistical System[27] fitted with the "cosinor" function from the

"season" package.[28] The time variable, *t* (month), is transformed as cos(*t*) and sin(*t*) onto which the 25(OH)D measurements are regressed. The resulting terms give a linear representation of a sine curve, with the regression coefficients of the cos(*t*) and sin(*t*) predictors transformed to give the amplitude (distance from mean to the location of highest [peak] or lowest [trough]), which provides the magnitude of seasonal variation, and the phase shift (location of the peak and trough along the *x*-axis), which details the months where 25(OH)D concentrations are at the lowest and highest. This model was used to determine whether 25(OH)D concentration follows a one, two, or three-cycle sinusoidal seasonal pattern.[21] Cosinor models were considered to demonstrate significant seasonality if the sum of the regression coefficient *P*-values for the cos(*t*) and sin(*t*) components was less than $\alpha/2$ where α is the chosen critical test size (.05).[28] All other biochemical variables were assessed for seasonal variation by cosinor, as described above.

Pearson's correlation coefficient was used to describe the association between 25(OH)D concentration and vitamin D UV dose exposure. As measurements were repeated for each dog, a mixed effects (linear) model, with dog ID as a random effect, was used to test for statistical significance of the relationship between cloud corrected vitamin D UV dose records and 25(OH)D concentration. All statistical analysis was completed using the R Statistical System.[27] A critical *P*-value (α) of .05 was used to determine statistical significance.

3 | RESULTS

3.1 | UV intensity fluctuates in a seasonal cycle over Edinburgh

As expected, vitamin D UV dose in Edinburgh, UK, displayed a seasonal pattern, increasing in spring months, peaking in summer months at around 7 kJ/m^2, declining in autumn, and was lowest during the winter months (Figure 4).

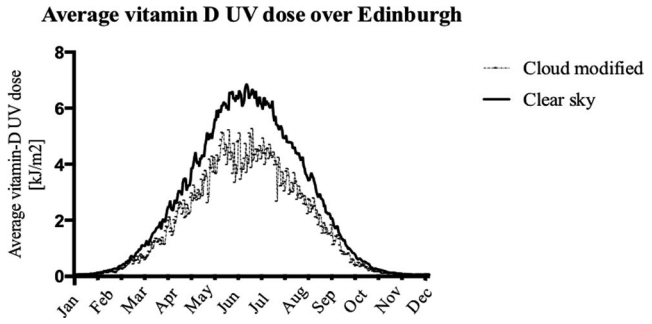

FIGURE 4 The average vitamin D ultraviolet (UV) dose over Edinburgh, UK. Vitamin D UV dose was calculated over Edinburgh, UK every day for 5 y (2011-2016), and the average over that time is presented here. Data are shown as "clear sky" assumes a cloud free dose and data shown as "cloud modified" assumes a modified dose to account for cloud coverage. "Cloud modified" data were not available from December to January as Edinburgh is too far north at this time of year to assess cloud coverage

3.2 | Vitamin D status of 18 healthy northern European dogs

3.2.1 | Signalment

The study population consisted of a range of ages and breeds. The median age of the dogs at the start of the study was 6.1 years (range 1.1-11.7 years). Of the 18 dogs, one was an unneutered male, two were unneutered females, seven were neutered males, and eight were neutered females. There were eight different breeds included in the study, including crossbreed (n = 8), Labrador Retriever (n = 3), Lurcher (n = 2), and one each of the following breeds: Cocker Spaniel, Collie, Greyhound, Hungarian Vizsla, and Jack Russell Terrier.

3.2.2 | Vitamin D status of 18 healthy dogs

The vitamin D status of 18 healthy dogs was assessed over a 1-year period by measuring 25(OH)D_2 and 25(OH)D_3 by LC-MS/MS. The mean concentration (calculated directly from each month's data) ± SD of 25(OH)D_3 ranged from 75.0 ± 23.38 nmol/L in June to 83.0 ± 37.32 nmol/L in December (Table 2). The concentration of 25(OH)D_2 in dogs was very low, resulting in many samples not meeting the lower LOQ of the assay and, therefore, could not be quantified. This resulted in much lower numbers of calculated 25(OH)D_2 concentrations per month, ranging from n = 3 in February to n = 9 in August (Table 2). Of the samples that 25(OH)D_2 was quantifiable in, the mean ± SD 25(OH)D_2 concentration ranged from 1.97 ± 1.656 nmol/L in April to 4.43 ± 3.915 nmol/L in February (Table 2).

The variation in 25(OH)D_2 and 25(OH)D_3 concentrations between dogs was calculated each month as the inter-dog coefficient of variation (CV). The inter-dog CV for 25(OH)D_3 concentrations ranged from 23.8% to 45%, depending on the month. Greater variation was detected between individuals 25(OH)D_2 concentrations with CVs ranging from 64.2% to 97.6%. The variation in 25(OH)D_2 and 25(OH)D_3 concentrations over time within an individual animal was also calculated as the intra-dog CV. The intra-dog CV for 25(OH)D_2 was 2.3-38.3%, and for 25(OH)D_3 was 5.0-30.3%.

3.3 | 25(OH)D concentrations do not exhibit seasonal variation in dogs and are not positively correlated with UV intensity

The cosinor model was used to assess whether 25(OH)D concentrations in dogs exhibit seasonal variation. Analysis of 25(OH)D_3 (Figure 5B) and total 25(OH)D (Figure 5C) concentrations by cosinor both yielded nonsignificant results ($P > .05$), demonstrating that 25(OH)D concentrations do not follow significant sinusoidal seasonal patterns in dogs. This demonstrates that vitamin D status in these animals remains stable over a 1-year period. 25(OH)D_2 was not analyzed in this way due to low sample numbers of measurable

TABLE 2 Summary data of all biochemical variables measured

Sample time (month-year)	25(OH)D_2 (nmol/L)		25(OH)D_3 (nmol/L)		PTH (ng/L)		iCa (nmol/L)		Total Ca (nmol/L)		Albumin (g/L)		Creatinine (μmol/L)		Inorganic phosphate (mmol/L)	
	n=	M ± SD/MED (IQT)	n=	M ± SD/MED (IQT)	n=	M ± SD/MED (IQT)	n=	M ± SD/MED (IQT)	n=	M ± SD/MED (IQT)	n=	M ± SD/MED (IQT)	n=	M ± SD/MED (IQT)	n=	M ± SD/MED (IQT)
Jul-2015	7	3.87 ± 2.535	17	81.6 ± 22.78	18	69.5 (45.75-109.0)	18	1.35 ± 0.04929	18	2.46 (2.428-2.588)	18	33.61 ± 2.037	18	100.7 ± 12.65	18	1.57 (1.365-1.773)
Aug-2015	9	2.90 ± 2.267	18	79.81 ± 20.73	18	77.8 ± 54.13	18	1.36 ± 0.05711	18	2.57 ± 0.1741	18	33.62 ± 2.312	18	100.0 (93-107.8)	18	1.42 (1.308-1.728)
Sep-2015	8	2.96 ± 2.634	18	78.87 ± 18.75	18	61.0 (39.25-95.0)	18	1.31 (1.26-1.353)	18	2.49 (2.38-2.56)	18	34.38 ± 2.043	18	108.0 ± 18.65	18	1.57 ± 0.2518
Oct-2015	8	3.06 ± 3.004	18	82.09 ± 22.95	18	68.5 (34.75-105.0)	18	1.35 ± 0.03968	18	2.44 (2.418-2.585)	18	35.07 ± 2.323	18	105.8 ± 13.82	18	1.46 (1.208-1.673)
Nov-2015	6	3.18 ± 2.345	17	82.47 ± 25.35	18	72.0 ± 44.03	18	1.33 ± 0.04934	18	2.52 ± 0.1171	18	34.95 (33.08-36.5)	18	100.0 (94-108.5)	18	1.44 ± 0.349
Dec-2015	6	3.70 ± 3.129	18	79.6 (56.13-100.9)	18	53.0 (37.25-87.0)	18	1.30 ± 0.04863	18	2.49 ± 0.1152	18	33.26 ± 1.84	18	109.0 (100.5-121.3)	18	1.56 ± 0.3475
Jan-2016	4	3.38 ± 2.641	18	82.27 ± 27.38	18	77.5 (49.0-113.8)	18	1.30 (1.278-1.33)	18	2.44 ± 0.07615	18	33.65 ± 1.65	18	110.5 (101-119.3)	18	1.53 ± 0.2873
Feb-2016	3	4.43 ± 3.915	18	75.4 (58.48-96.68)	18	101.0 (46.5-120.0)	18	1.42 ± 0.0624	18	2.49 (2.423-2.613)	18	33.15 (32.38-35.83)	18	114.0 (110-126.5)	18	1.46 ± 0.2693
Mar-2016	6	2.13 ± 1.435	17	76.72 ± 25.16	18	73.0 (51.0-91.25)	18	1.39 ± 0.05605	18	2.50 (2.438-2.578)	18	32.56 ± 2.264	18	118.5 (112-122)	18	1.48 (1.178-1.78)
Apr-2016	3	1.97 ± 1.656	17	80.21 ± 26.82	18	96.7 ± 66.02	18	1.42 ± 0.07496	18	2.55 ± 0.1427	18	33.04 ± 2.442	18	115.9 ± 14.65	18	1.49 (1.373-1.893)
May-2016	5	3.04 ± 1.958	18	80.52 ± 25.84	18	63.0 (45.0-89.75)	18	1.44 ± 0.05456	18	2.51 (2.423-2.54)	18	32.92 ± 2.197	18	121.5 (114.5-128.8)	18	1.33 (1.245-1.605)
Jun-2016	7	3.26 ± 2.883	16	73.15 (62.38-85.03)	16	80.9 ± 45.59	16	1.41 ± 0.07904	16	2.53 ± 0.1035	16	33.48 ± 2.565	16	128.1 ± 19.41	16	1.32 ± 0.15

Note: Number of samples biochemical variables measured (n=).

Abbreviations: 25(OH)D_2, 25-hydroxyvitamin-D_2; 25(OH)D_3, 25-hydroxyvitamin-D_3; IQT, interquartile range; M, mean; MED, median; SD, standard deviation.

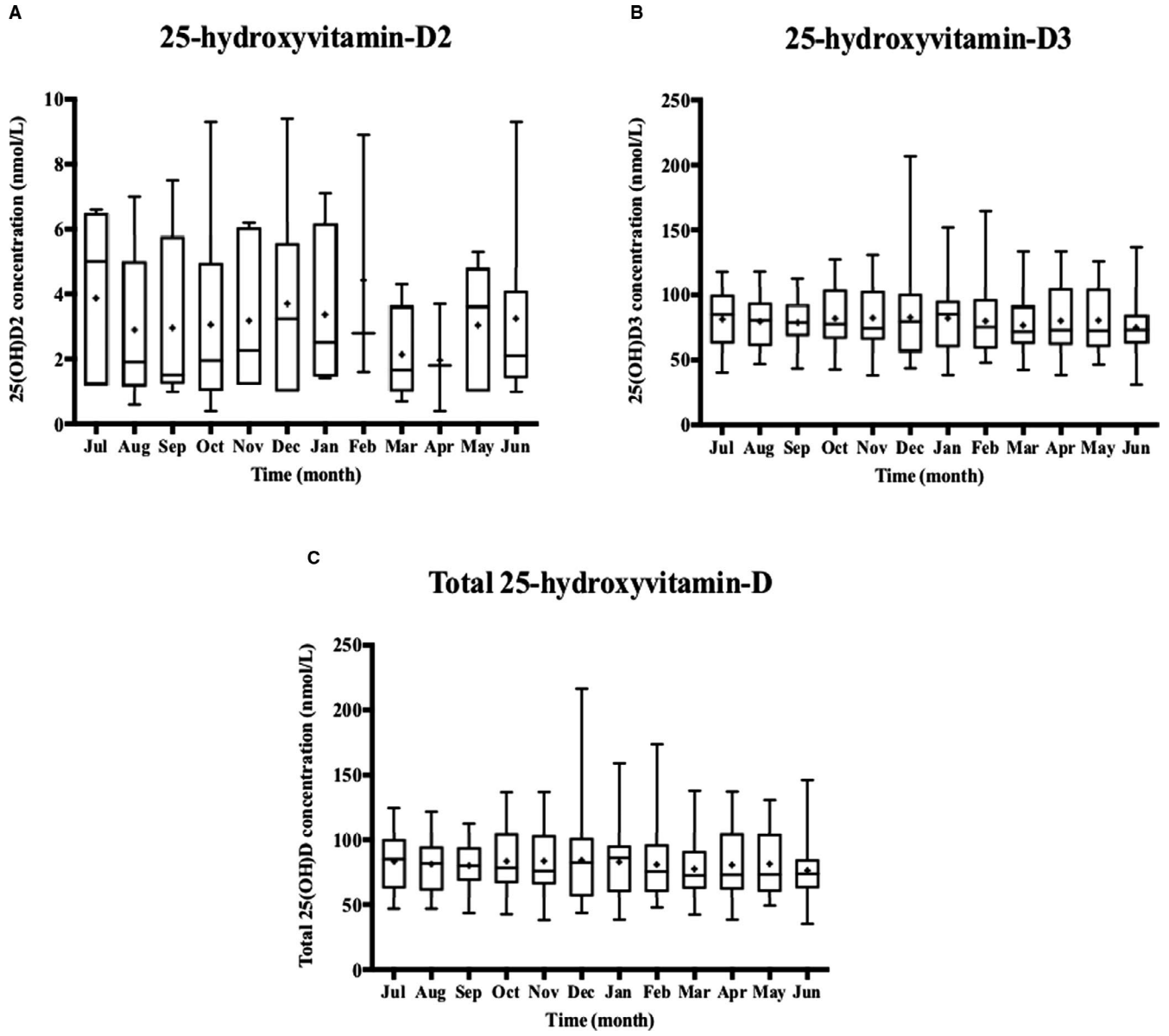

FIGURE 5 Vitamin D is stable in dogs over a 1-y period. Vitamin D status was assessed in 18 healthy dogs over a 1-y period by measuring $25(OH)D_2$ (A) and $25(OH)D_3$ (B) by liquid chromatography-tandem mass spectrometry in serum samples taken at monthly intervals. Total 25(OH)D (C) is the sum of $25(OH)D_2$ and $25(OH)D_3$ concentrations. Data displayed as box and whisker plots where boxes represent the 5-95th percentile, whiskers represent minimum-maximum data points and dots represent the mean. Cosinor analysis determined that vitamin D status does not follow a sinusoidal seasonal pattern. $25(OH)D_2$, 25-hydroxyvitamin-D_2; $25(OH)D_3$, 25-hydroxyvitamin-D_3

$25(OH)D_2$ concentrations; however, the graphical representation of the data is shown in Figure 5A.

Pearson's correlation coefficient determined that there was no correlation between either the $25(OH)D_3$ or total 25(OH)D concentration and the cloud modified vitamin D UV dose ($25(OH)D_3$ $r = -.082$, $P = .228$, 95% confidence interval −0.214-0.052 and total 25(OH)D $r = -.075$, $P = .271$, 95% confidence interval −0.207-0.059). This non-significant slightly negative relationship was confirmed using a linear mixed effect model for both $25(OH)D_3$ and total 25(OH)D, which established that for every 1 unit increase in UV intensity, $25(OH)D_3$ concentration decreased by 1.44 (SE = 0.762, correlation of fixed effects (r) = −.242 and $P = .058$) and total 25(OH)D concentration decreased by 1.37 (SE = 0.77, correlation of fixed effects (r) = −.234 and $P = .078$). Taken together, these results demonstrate that 25(OH)D concentration and vitamin D status does not exhibit seasonal variation in dogs and is not positively correlated with vitamin D UV dose intensity.

3.4 | Serum calcium, plasma PTH, and serum inorganic phosphate concentrations do not follow a seasonal cycle in dogs

A panel of biochemical variables involved in calcium metabolism or as biomarkers of kidney function was also assessed in the 18 dogs over

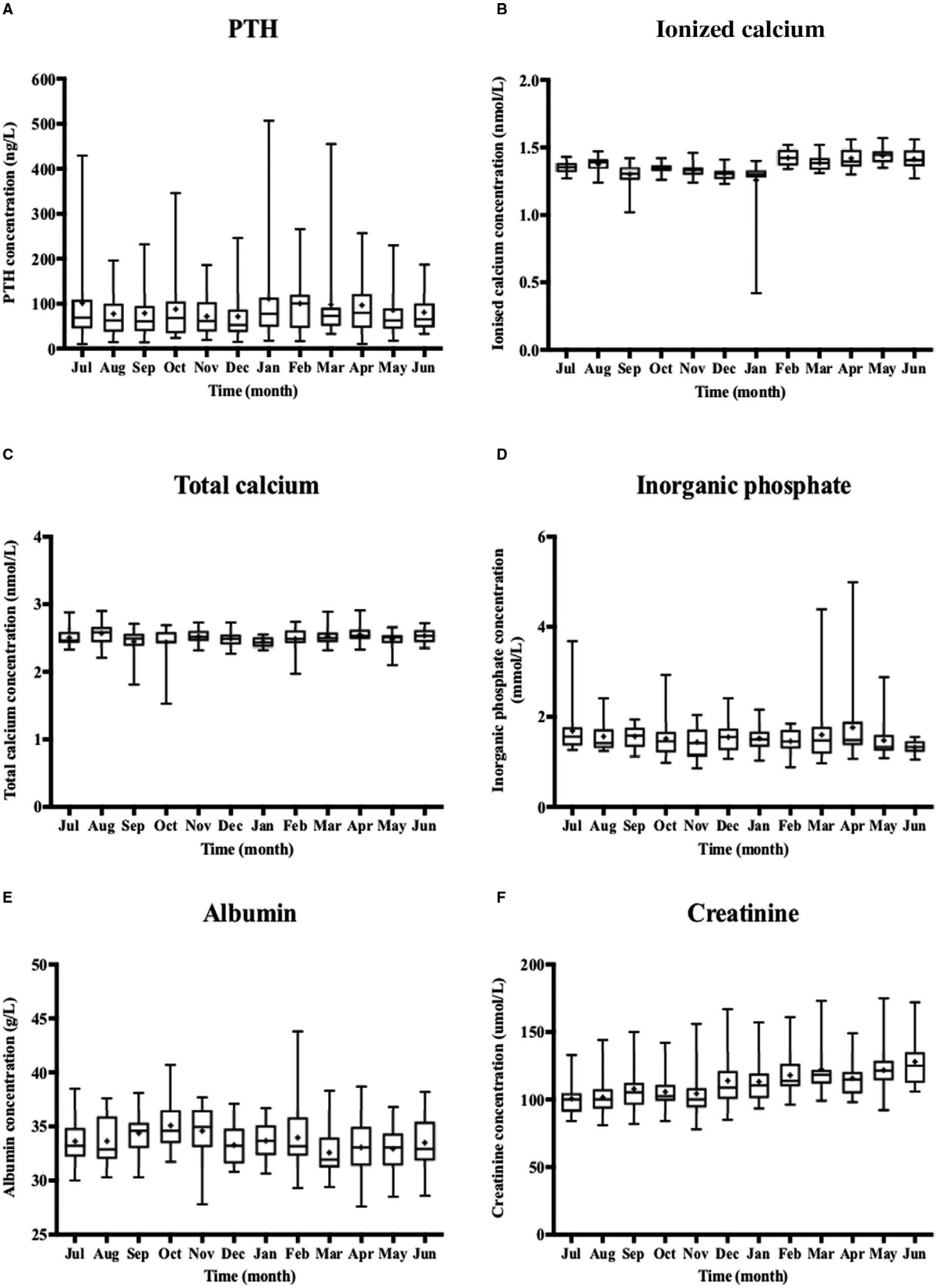

FIGURE 6 The panel of biochemical variables and hormones involved in kidney function and calcium metabolism assessed for seasonal variation over a 1-y period in dogs. A, Parathyroid hormone (PTH), (B) ionized and (C) total calcium, (D) inorganic phosphate, (E) albumin, and (F) creatinine were measured in 18 healthy dogs at monthly intervals over a 1-y period. Data displayed as box and whisker plots where boxes represent the 5-95th percentile, whiskers represent minimum-maximum data points and dots represent the mean. Cosinor analysis determined that albumin and creatinine concentrations follow a seasonal 1, 2, and 3 cycle pattern, whereas PTH, ionized, total calcium, and inorganic phosphate are stable over time

the same time period. The monthly mean concentrations ± SDs of predominately normally distributed biochemistry variables were calculated as follows: albumin ranged from 32.56 ± 2.264 g/L in March to 35.07 ± 2.323 g/L in October, and ionized calcium ranged from 1.30 ± 0.04863 mmol/L in December to 1.44 ± 0.05605 mmol/L in May. The monthly median concentrations (and interquartile ranges) of predominately not normally distributed biochemistry variables were calculated as follows; total calcium ranged from 2.44 (2.418-2.585) mmol/L in October to 2.59 (2.438-2.668) mmol/L in August, creatinine ranged from 100.0 (93-107.8) μmol/L in July to 125 (112.5-135) μmol/L in June, inorganic phosphate ranged from 1.33 (1.245-1.605) mmol/L in May to 1.57 (1.365-1.773) mmol/L in July, and PTH ranged from 53 (37.25-87) ng/L in December to 101.0 (46.5-120) ng/L in January (Table 2).

The concentrations of biochemical variables over the 1-year period were also analyzed using cosinor analyses to determine if there is seasonal variation in vitamin D-related biomarkers. Cosinor analyses established nonsignificant results ($P > .05$) for ionized and total calcium, PTH, and inorganic phosphate, demonstrating that these biomarkers do not follow a seasonal pattern and were stable for 1 year (Figure 6A-D). However, albumin and creatinine yielded significant results ($P < .05$) from the cosinor analysis, suggesting that these biomarkers did follow a seasonal pattern in these dogs over 1 year (Figure 6E-F). Albumin concentrations peaked in autumn months (October-November) and declined through the winter and spring months (December-April) (Figure 6E; Table 2). Creatinine concentrations peaked in late spring/early summer (May - June) (Figure 6F and Table 2).

4 | DISCUSSION

The central finding of this study was the demonstration that although the vitamin D UV dose follows a seasonal cycle in Edinburgh, the 25(OH)D concentration and, therefore, vitamin D status of dogs living in this area did not follow a seasonal pattern and remained stable for 1 year. Furthermore, other members of the vitamin D pathway, including calcium, phosphate, and PTH remained stable over the same period.

The mean 25(OH)D concentration of dogs each month was similar to the mean reported value for healthy dogs (77 nmol/L) in a previous studies[29,30] confirming that the dogs in this study had a vitamin D status similar to other reported populations. The 25(OH)D_3 analyte was detected at higher concentrations (averaging 75-83 nmol/L) than those of 25(OH)D_2 (averaging 1.9-4.4 nmol/L) in all dogs. Lower concentrations of 25(OH)D_2 were to be expected given that the Hill's Science Plan Advanced Fitness diet fed to these dogs was primarily supplemented with vitamin D_3. There was no seasonal variation detected for the individual analytes or total 25(OH)D concentrations (Figure 5). If the main source of vitamin D was endogenous production in skin exposed to UV radiation, dogs subjected to seasonally fluctuating UV radiation would be expected to demonstrate seasonal 25(OH)D variation. The lack of seasonal 25(OH)D concentration variation supports previous evidence that dogs do not produce vitamin D cutaneously.[15-17] In contrast, endogenous vitamin D production in humans has been shown to be highly efficient, with as little as 15 minutes of UV exposure three times a week sufficient to maintain a normal vitamin D status.[31] Therefore, even though real-time UV exposure was not assessed in these dogs, it could be assumed that even with minimal outside exposure, any seasonal variation in 25(OH)D would be detected if vitamin D was produced cutaneously. Results shown in this study are consistent with the observations of Laing et al, who demonstrated that there was no season-dependent variation in the vitamin D status of Greyhounds in Australia.[13] However, unlike Laing et al, here we describe a longitudinal study whereby serum samples were obtained from the same individual dogs over a 1-year period under a controlled, standardized diet, and 25(OH)D was quantified by LC-MS/MS, the gold-standard approach for analyzing vitamin D metabolites.

In this study, every dog was fed a standardized diet of Hill's Science Plan Advanced Fitness Medium breed with chicken, an adult dry food that meets the American Association of Feed Control Officials (AAFCO) nutritional standards. This food contained 861 IU/kg (231 IU/Mcal) of vitamin D (Table S1). Implementing this standardized diet allowed us to control the most influential factor in canine vitamin D status so that we could investigate seasonal variation. It is unlikely that the level of vitamin D supplemented in this diet was sufficient enough to mask any seasonal effects, given that this commercial food meets AAFCO standards and that all dogs measured within previously reported reference ranges for 25(OH)D concentration and normal vitamin D status.[29,30]

Even with this standardized diet in place, variation between the dogs 25(OH)D concentration was observed by calculating the inter-dog CV for 25(OH)D_2 and 25(OH)D_3 concentrations each month. The greater variation between dogs 25(OH)D_2 than 25(OH)D_3 concentration could be the result of a lower number of samples that this metabolite was able to be measured in, with the lower concentration resulting in greater variability in the measurement. Given that these dogs were fed a standardized diet, and as we concluded, that exposure to UV radiation does not influence vitamin D status in these animals, we now must consider host factors that might influence the relationship between vitamin D intake and vitamin D status in the dog. Genome-wide association studies in humans have demonstrated modest genetic heritability in 25(OH)D status (approximately 7.5%) and conclude that vitamin D status (defined by 25(OH)D concentration) is mainly determined by modifiable environmental factors.[32-34] Factors including age,[35,36] region,[35-37] season,[35,37] activity levels,[35,37] and weight[35,37,38] have all been shown to affect vitamin D status. Currently, to the authors' knowledge, there have been no studies investigating potential genetic variants, and limited studies have investigated the impact of factors such as age, breed, BCS, and activity levels on the vitamin D status of the dog.[39] One study investigating the effect of diet on canine vitamin D status demonstrated significant differences in vitamin D status between German Shepherd dogs and Golden Retrievers, suggesting that breed influences vitamin D status.[40] They also demonstrated that sex and neuter status influences

vitamin D status, showing that intact males had significantly increased vitamin D concentrations compared with neutered males and both intact and neutered females; however, there was no significant difference in vitamin D concentrations when both sexes were neutered.[40] The author suggested that sex hormones might be involved in regulating vitamin D absorption; however, further studies where the diet is standardized, and the breed is controlled would be required to elucidate this question further.[40] A limitation of this study is that dogs were not matched by breed, age, sex, or neuter status. Limited sample numbers precluded the sensible incorporation into the multivariable analysis, but these factors could play a potential role in the metabolism of vitamin D from dietary sources to 25(OH)D in these dogs.

Understanding the relationship between vitamin D intake and serum 25(OH)D concentration will be vital in translating these results into real-world applications, including vital basic information, such as defining vitamin D requirements for dog foods. Currently, the AAFCO recommendations state that, for both growth and maintenance, dogs should receive 500 IU/Kg (based on dry matter). However, dogs consuming AAFCO compliant dog food may receive anywhere between 500 and 5,000 IU vitamin D/kg dry matter depending on the level the manufacturer has chosen to include, resulting in a large variation in vitamin D intake among dogs.[40,41] Furthermore, the results of this study have shown that even when consuming the same food with the same vitamin D content, 25(OH)D concentrations vary between dogs. Several studies attempting to define vitamin D requirements have done so by studying vitamin D supplementation in puppies[42,43]; however, thus far, no definitive conclusions have been drawn. In one study, it was found that supplementing healthy adult dogs with five times the recommended safe upper limit did not significantly increase vitamin D concentrations.[44] Although a limitation of this study was that the study population was not matched by breed, age, or sex/neuter status due to the low numbers, previous work has shown differences in 25(OH)D status between breeds and sex/neuter status in dogs.[40] Factors that affect vitamin D status and could impact vitamin D requirements might include life stage (adult maintenance, reproduction, and geriatric), breed, or sex/neuter status. Therefore, the variation of 25(OH)D concentrations among dogs consuming the same amount of vitamin D warrants further investigation. Given the evidence produced in the last decade for the multiple roles that vitamin D plays in canine health, it is essential to know as much as possible about vitamin D requirements at every stage of a dog's life. The lack of seasonal variation detected here suggests that, unlike other species, there is no requirement for seasonal reference ranges for vitamin D status in dogs.

To understand 25(OH)D status variation and its clinical impact fully, other members of the vitamin D pathway must be examined in this species. Few studies have revealed that $1,25(OH)_2D$ concentration, the biologically active metabolite, is lower in disease than in healthy control dogs,[30,45] and still, fewer studies have examined $24,25(OH)_2D$ concentrations in dogs.[14,46] Other vitamin D metabolites, including $1,25(OH)_2D$, $24,25(OH)_2D$, unbound 25(OH)D, the vitamin D receptor, and vitamin D-binding protein, should be examined in dogs with hypo-, normo-, and hypervitaminosis D to fully understand the variation of vitamin metabolism in this species. Not only would this provide clinically and scientifically important information but would allow for the elucidation of 25(OH)D as an appropriate canine vitamin D status marker.

Albumin and creatinine are biomarkers of kidney function and are routinely included in the diagnosis of chronic kidney disease. In humans, seasonal variation of kidney biomarkers has been identified, with albumin concentrations tending to be highest in winter months[47-49] and creatinine concentrations highest in summer months.[50,51] In this study, cosinor analysis revealed statistically significant seasonal variation in both albumin and creatinine, with albumin concentrations peaking in autumn months (October and November) and creatinine concentrations peaking in late spring/early summer (May and June) in dogs. Limited information on the seasonal variation of these biomarkers in dogs is available. One study investigating seasonal variation in blood constituents of German Shepherd dogs revealed statistically significant seasonal variation in albumin and urea but did not assess creatinine.[52] In agreement with this study, Mohammed et al[52] reported the highest levels of albumin in autumn. Although not fully understood, factors including dietary changes, hydration, and environmental temperature (and, therefore, thermoregulation) are thought to impact this seasonal change in humans. Although outside the scope of this study, further investigations into the seasonal variation of kidney biomarkers in dogs could impact the diagnosis and treatment of kidney disease.

5 | CONCLUSIONS

In this study, we have demonstrated that, although subject to seasonally fluctuating vitamin D UV dose, vitamin D status in dogs remains stable and does not follow a sinusoidal seasonal pattern as detected in other species. Given the standardization of the diet, we conclude that this seasonal stability is likely to be a direct result of a lack of cutaneous vitamin D production in this species. This study also showed that 25(OH)D concentration varied between individual dogs, even with a standardized diet in place, indicating that other host factors likely influence vitamin D status. A further understanding of these factors could help develop personalized approaches to ensure all animals are vitamin D replete.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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