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Dosi Miranda Carlotta Maria (Orcid ID: 0000-0002-1094-1184)
McGorum Bruce (Orcid ID: 0000-0002-6977-6101)
Keen John A (Orcid ID: 0000-0002-7862-5321)
Morgan Ruth (Orcid ID: 0000-0003-1117-2273)

The effect of season, management and endocrinopathies on vitamin D status in horses

Miranda C.M.C. Dosi*[1], Bruce C. McGorum[1], Roxane D. Kirton[2], Eugenio Cillán-García[1], Richard J. Mellanby[1,3], John A. Keen[1], Emma A. Hurst[3,4] and Ruth A. Morgan[1,5,6]

[1]Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin, Midlothian, UK; [2]Redwings Horse Sanctuary, Norwich, Norfolk, UK; [3]The Roslin Institute, University of Edinburgh Roslin, Midlothian, UK; [4]Mass Spectrometry Core, Edinburgh Clinical Research Facility, Queen's Medical Research Institute, University of Edinburgh, Edinburgh, UK; [5]Queen's Medical Research Institute, The University of Edinburgh, Little France Crescent, Edinburgh, UK and [6]Scotland's Rural College, Edinburgh, UK.

*Corresponding author: Miranda Dosi M.C.M.Dosi@sms.ed.ac.uk

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Summary

Background: Vitamin D deficiency is common in humans and is increasingly linked to the pathogenesis of a multitude of diseases including obesity and metabolic syndrome. The biology of vitamin D in horses is poorly described; the relative contribution of the diet and skin synthesis to circulating concentrations is unclear and associations with endocrine disease have not been explored.

Objectives: To determine the relationship between management, season and endocrine disease and vitamin D status in horses.

Study design: Cross-sectional cohort study.

Methods: Plasma concentrations of 25-hydroxyvitamin D2 (25(OH)D2) and D3 (25(OH)D3) were measured by LC-MS/MS in 34 healthy unsupplemented grazing ponies and 22 stabled Thoroughbreds receiving supplementary vitamin D3 in feed. A nested group of 18 grazing ponies were sampled on long and short-days (>12and<12h of light/day) to determine the effect of sunlight exposure. Additionally, the relationships between age, sex, adiposity, serum insulin, adrenocorticotropic hormone and vitamin D status were assessed in a mixed group of 107 horses using a linear regression model.

Results: All animals had measurable level of 25(OH)D2 (median 10.7nmol/L) while 25(OH)D3 was only detected in Thoroughbreds receiving D3 supplementation. Thoroughbreds had lower concentrations of 25(OH)D2 than ponies (7.4nmol/L v 12.6nmol/L $p<0.01$). In grazing ponies 25(OH)D2 concentrations were significantly higher on long-days compared to short-days (14.4nmol/L v 8.7nmol/L $p<0.01$), while 25(OH)D3 was undetectable. Measures of increased adiposity, but not basal insulin, were associated with higher 25(OH)D2 concentrations, conversely to humans. Increasing ACTH was associated with lower 25(OH)D2 ($p<0.01$).

Main limitations: Vitamin D2 concentrations were not measured in grass or forage.

Conclusions: In horses 25(OH)D2 is the predominant vitamin D metabolite, and there is an apparent lack of endogenous vitamin D3 production. The relationship between vitamin D and endocrine disorders in horses does not reflect that of other species and warrants further investigation.

Introduction

Vitamin D is implicated in many patho-physiological processes beyond its well-recognised role in skeletal health, although very little is known about the importance of this hormone in equine health and disease.[1] There are two main forms of vitamin D; vitamin D_2 and vitamin D_3. Pre-vitamin D_3 is endogenously synthesised in the skin of most mammals in response to UVB exposure, while pre-vitamin D_2 is produced by fungi growing on plants and is activated following ingestion (Figure 1).[2] In humans and other domestic herbivores vitamin D_3, synthesised in the skin, makes up most of the total vitamin D measured in plasma.[3] Horses, like other herbivores, source vitamin D_2 from their forage-based diet but it is unclear whether they can synthesise vitamin D_3 .[1] Our understanding of vitamin D biology in horses and ponies is very limited with few equine specific peer-reviewed publications in the last 30 years[1,4-14].

Vitamin D status is most commonly assessed by measuring plasma concentrations of the most abundant circulating metabolites; 25-hydroxy-vitamin D_2 ($25(OH)D_2$) and 25-hydroxy-vitamin D_3 ($25(OH)D_3$). These constitute the largest circulating pool of vitamin D, which then undergo further hydroxylations in the kidneys and other peripheral tissues to the metabolically active metabolites $1,25(OH)_2D_2$ and $1,25(OH)2D_3$. Concentrations of these metabolites are consistently reported as being lower in horses than in other domestic mammals [3] and humans; indeed the concentrations observed in healthy horses would be considered indicative of severe deficiency in humans (total 25(OH)D below 25 nmol/L).[15] Moreover, recent studies using highly sensitive assays, such as liquid chromatography tandem mass spectrometry (LC-MS/MS), showed that grazing Thoroughbreds and Standardbreds in New Zealand that were not receiving dietary vitamin D supplementation had very low to undetectable plasma concentrations of $25(OH)D_3$ throughout the year.[10, 11]

According to those studies, vitamin D_2 related metabolites were predominant in defining vitamin D status.

Seasonal changes in 25(OH)D in response to sun exposure are well documented in humans and sheep living at UK latitudes.[16,17] In humans this is generally attributed to the different amount of radiation that reaches the skin and subsequently different rates of endogenous production of 25(OH)D3. On the other hand, in herbivores seasonal changes in plasma 25(OH)D_2 are due to variations in D2 content of grass, which is also influenced by sunlight exposure. [10] There are no published data on physiological concentrations of 25(OH)D_2 or 25(OH)D_3 in UK horses or ponies measured with modern highly sensitive techniques.

Vitamin D insufficiency/deficiency is common in humans and oral supplementation is often recommended in conditions such as obesity, metabolic syndrome and type-2 diabetes, as it may improve insulin sensitivity of liver, adipose tissue and skeletal muscle.[2, 18] In contrast, Hookey et al. (2018) found no association between adiposity and vitamin D status in healthy adult dogs.[19] To date, there are no data available in horses on the relationship between vitamin D status and endocrine conditions such as insulin dysregulation (ID), obesity or pars pituitary intermedia dysfunction (PPID).

The objectives of this study were firstly to determine circulating concentrations of 25(OH)D_2 and 25(OH)D_3 using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) in two equine populations representative of the extremes of dietary management in the UK; a herd of grazing ponies not receiving any supplementary feed and a group of Thoroughbred racehorses receiving supplementary vitamin D_3. Secondly, we aimed to determine the effect of day length (UVB light exposure) on vitamin D status in non-rugged ponies kept at pasture all year. Finally, we sought to test the hypothesis that, as in humans, vitamin D status is associated with obesity, ID and PPID.

Materials and Methods

The effect of dietary management and day length on vitamin D status

Blood samples were obtained for clinical reasons from 22 Thoroughbred racehorses (group 1) stabled in Scotland (56° parallel North), and 34 Welsh and Welsh cross ponies (group 2), kept on a ryegrass pasture as a herd in England (52° parallel North).

Horses in group 1 were predominantly stabled, going outside only for training and for 40-60 mins daily turnout wearing a rug. They were fed an average of 8-10 kg of haylage and 6-8 kg of HDF Spillers Power Cubes® containing 1100IU/Kg (of dry matter) of vitamin D_3 daily. Ponies in group 2 were kept on ryegrass pasture all year round, were not rugged and were not ridden. During winter they were supplemented with hay or a mixture of hay and barley straw. Groups 1 and 2 were sampled once between March and April.

A nested sample of group 2 ponies (n=18) were also sampled between April and September (long-days, > 12 hours of light per day) and between October and March (short-days, <12 hours light per day). Cloud coverage and UV Index data for the study period (March 2018 to March 2019) were obtained from the UK Department for Environment, Food and Rural Affairs (https://uk-air.defra.gov.uk/data/uv-data).

The relationship between vitamin D status and obesity, ID and PPID

This part of the study used 107 animals (group 3), including 10 of the racehorses in group 1, all 34 ponies in group 2 and an additional 63 horses presented to the Royal (Dick) School of Veterinary Studies with and without obesity, PPID and ID. Vitamin D status was assessed as described above. Validated assays (Siemens Immulite 2000 xpi analyser) were used to measure serum insulin and plasma ACTH concentration in all 107 horses. [20,21]

Morphometric parameters were recorded by a veterinary surgeon at the time of sampling. These included Body Condition Score (BCS 1-5), Neck Crest Score (NCS 1-5), bodyweight (electronic scale) and presence or absence of regional adiposity elsewhere in the body (i.e. localised adipose accumulation on rump, lumbar region, shoulder, loin). [22,23]

Diagnosis of PPID was made if basal ACTH concentration exceeded seasonal cut-off values of 29 pg/mL between November and July and 47 pg/mL between August and October, and on the basis of clinical signs (including hirsutism, epaxial muscle wastage, lethargy).[24] In 19 horses, diagnosis of PPID was confirmed using a thyrotropin-releasing hormone stimulation test. Diagnosis of ID was made if basal serum insulin concentration exceeded 20 mIU/L. The horses were stabled the night before the sample was obtained and a haynet of soaked hay was offered. They did not receive any highly caloric meal the morning of the testing. In 25 horses, ID was confirmed using a combined glucose-insulin test.[25] The presence of laminitis, based on history, clinical examination, post-mortem observation of hoof sections and/or radiological assessment, was recorded.

Measurement of 25(OH)D_2 and 25(OH)D_3

Plasma concentrations of 25(OH)D_2 and 25(OH)D_3 (concentrations summed to provide total 25(OH)D) were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) by the Vitamin D Animal Laboratory (VitDAL) using an assay that has been certified as proficient by the international Vitamin D Quality Assessment Scheme (DEQAS) and described in detail previously. [26] Briefly, calibrators were prepared by spiking 3% bovine serum albumin with certified reference standards of 25(OH)D_2 and 25(OH)D_3 (Sigma-Aldrich, UK). Samples (200 µL serum) and 200 µL calibrators were spiked with equal amounts of isotopically labelled internal standards (deuterium-labelled 25(OH)D2 (6,19,19-d3-25(OH)D_2) and carbon-13-labelled 25(OH)D3 (23,24,25,26,27-13C5-25(OH)D_3 (Sigma-Aldrich) and prepared by automated supported liquid extraction (SLE) using 96-well plates (Biotage, UK) on the

Biotage® Extrahera™. Samples and calibrators were then subject to derivatisation with DMEQ-TAD (Abcam, UK). LC-MS/MS analysis was conducted on an Acquity ultra-high-performance liquid chromatography (UPLC) I-Class system (Waters Corporation) coupled to a Sciex QTrap 6500+ quadrupole mass spectrometer (AB Sciex, Warrington, England). Liquid chromatography separation was done using a Raptor Fluorophenyl column (2.7 µm 100 Å, LC Column 100 x 2.1 mm) (Thames Restek). 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 flow rate was 0.4 mL/min and total run time was 10 mins per sample. For mass spectrometer analysis, ionisation was performed by electrospray ionisation (ESI) in positive ion mode. Multiple reaction monitoring (MRM) was used to monitor and quantify derivatised standards and endogenous vitamin D analytes with the following m/z transitions: DMEQ-25(OH)D_2 m/z 758.4 > 740.2; DMEQ- d3-25(OH)D_2 m/z 761.3 > 743.4; DMEQ-25(OH)D_3 m/z 747.3 > 729.3; and DMEQ-13C5-25(OH)D_3 m/z 751.5 > 733.5. The results are presented as total 25(OH)D concentration which is the sum of the concentrations of 25(OH)D_2 and 25(OH)D_3. The lower limit of quantitation (LLOQ) was 0.5 nmol/L for 25(OH)D_2 and 4 nmol/L for 25(OH)D_3.

Data analysis

Retrospective power calculation indicated that sampling of 34 grazing ponies and 22 Thoroughbreds allowed adequate power (80%) for detecting an inter-group difference as small as 2.5 nmol/L of 25(OH)D. Standard deviations of 2.2 nmol/L and 3.4 nmol/L were used for Thoroughbreds and grazing ponies, respectively.

Statistical analysis was performed using SPSS 25 (IBM). When data were not normally distributed (Shapiro-Wilk test), logarithmic (Ln) transformation was performed. Student's T-test for independent and paired samples was used to compare means between groups for

normally distributed, parametric data and a Mann-Whitney U test for non-parametric data. Results are expressed as mean and standard deviation if normally distributed and as median and interquartile range (IQR) if non-parametric. One-way ANOVA was used for normally distributed variables when multiple means were compared.

To investigate the relationship between obesity, ID and PPID and vitamin D, a linear regression analysis was conducted with ln25(OH)D_2 as the outcome, as only 25(OH)D_2 was detectable in all the horses. 25(OH)D_3 was detected only in 10 horses, all of which were receiving oral supplementation, therefore these data were excluded from the linear regression analysis. The following explanatory variables were tested in a univariate model: age, sex, breed type, BCS, NCS, regional adiposity, morphometric score, diagnosis of laminitis, basal ACTH, basal insulin, diagnosis of PPID or ID. All variables that significantly contributed to a change in outcome with a $p<0.25$ were put forward to the multivariate analysis. BCS, NCS and the presence or absence of regional adiposity were strongly positively correlated to each other (NCS and BCS $r=0.563$, NCS and regional adiposity $r=0.533$, BCS and regional adiposity $r=0.676$ $p<0.001$ for all correlation coefficients); therefore, they had to be entered in the analysis individually to avoid multicollinearity. Thus, in the multivariate analysis, a composite score calculated through dimension reduction (factor analysis) has been used. Goodness of fit of the regression models was assessed using Hosmer-Lemeshow test.

Results

The effect of dietary management on vitamin D status

Group 1 comprised 22 Thoroughbreds, including 20 geldings and two mares. Group 2 comprised 34 Welsh cobs and crosses, including 24 mares and 10 geldings. Horses in group 1

were significantly younger than ponies in group 2 (median 7 years IQR 3 v 16 years IQR 10, $p<0.001$).

Total plasma 25(OH)D (i.e. $25(OH)D_2 + 25(OH)D_3$) was higher in group 1 versus group 2 (group 1 median 15.8 nmol/L, IQR 6.8 against group 2 12.6 nmol/L, IQR5.1, $p<0.01$, Figure 2a). $25(OH)D_2$ was significantly higher in Group 2 (median 12.6 nmol/L, IQR 5.1, against 7.4 nmol/L, IQR 4.5, $p<0.001$, Figure 2b), whereas $25(OH)D_3$ was only detectable above the LLOQ in the vitamin D3 supplemented Group 1 horses (median 8.5 nmol/L IQR 4.0, Figure 2c).

The effect of season on vitamin D status of grazing ponies

During each month of the long-day season there was significantly more sunny weather than the short-day season (mean 224.6±55.5 hours/month of sun against 113.4±51.7 hours/month). The mean UV Index was also significantly higher in long-day months compared to short-day months (<12 hours >12 hours of light/day). The concentration of $25(OH)D_2$ in grazing ponies was significantly higher on long-days compared to short-days (median 14.4 nmol/L IQR 4.60 v median 8.7 nmol/L IQR 4.5) respectively, $p<0.001$, Figure 3), whilst $25(OH)D_3$ remained undetectable throughout the year.

The relationship between vitamin D and obesity, ID and PPID

Data from the 107 horses in group 3 were used. The age range was 4-28 years (median 15.5, IQR 9); 54 were mares and 53 were geldings. Breeds were classified in 5 categories: Welsh cobs and crosses (WC n=58), Thoroughbreds and Thoroughbred crosses (TB n=21), Draught breeds (DB: including Percheron, Irish draught, Clydesdale and Friesian; n=6), Native Ponies (NP: including Exmoor, New forest, Shetland, Connemara, Icelandic, Highland, Dartmoor and British miniature; n=13) and a mixture of other breeds (Other: including Quarter Horse, Irish Sport Horse, Trakhener, Arab, Dutch Warmblood, American Paint; n= 9).

Of the 107 horses, 32 were diagnosed with PPID, 41 with ID, 14 with both PPID and ID. Of the 107 horses, 40 had a previous diagnosis of laminitis.

Basal insulin measurements were available for all 107 horses, while basal ACTH was available for 94/107 horses.

Plasma 25(OH)D_2 was detectable in all 107 horses (median 10.7 nmol/L, IQR 5.33), while 25(OH)D_3 was quantifiable in only 10 of the 107 horses, all of which were Thoroughbreds from group 1 that were receiving dietary supplementation. For this reason, 25(OH)D_2 was therefore the only outcome considered.

In the univariate linear regression (Table 1) BCS ($r=0.44$, $p<0.001$), NCS ($r=0.29$, $p<0.01$), presence of regional adiposity ($r=0.35$, $p<0.001$) and morphometric score ($r=0.41$, $p<0.001$) were positively correlated with 25(OH)D_2. Obese horses (BCS>3.5) had higher 25(OH)D_2 concentrations than non-obese horses (median 9.3 nmol/l IQR 4.48 against 12.5 nmol/l IQR 4.9, $p<0.001$). There were significant differences in BCS between mares and geldings (mares higher score than geldings $p<0.01$) and between horses with and without ID (ID higher score than non-ID $p<0.01$). As such, the effect of these variables on vitamin D status were confounded by BCS and we cannot draw firm conclusions about their relationship as different sex and ID groups were not matched for BCS.

Breed was put forward to the final model as it reached the predefined level of significance, although when ln25(OH)D_2 concentrations amongst breed were compared, there was a statistically significant difference between Thoroughbred and WC only ($F(4,102)=5.632$, $p<0.001$). This difference once again was considered to be affected by the significant inter-group difference in BCS ($p<0.001$). Vitamin D status did not differ in horses with or without laminitis.

Of the predictors put forward to the final model (Table 2), only basal ACTH and BCS were retained, being respectively negatively and positively associated with 25(OH)D_2 concentration.

Discussion

Vitamin D is important for calcium homeostasis but it also has a diverse range of non-skeletal actions which are poorly understood. Vitamin D deficiency is reported in approximately 50% of humans,[27] has been associated with several diseases and is an independent risk factor for mortality.[2,28] Our aim in this study was to determine normal plasma concentrations of 25(OH)D_2 and 25(OH)D_3 in horses representing the extremes of dietary management in the UK, to determine the effect of day length on vitamin D status and to investigate the relationship between vitamin D and endocrine diseases.

This study demonstrates that management factors are important determinants of a horse's vitamin D status. Consistent with previous findings, [10, 11] horses managed extensively all year round had undetectable 25(OH)D_3, and had higher 25(OH)D_2 concentrations in summer than in winter. In contrast, stabled horses supplemented with vitamin D_3 had detectable 25(OH)D_3, but concentrations of 25(OH)D_2 were lower than those of grazing ponies.

Previous work has often been hampered by a lack of a suitable assay to accurately measure vitamin D in horses. In this study, 25(OH)D_2 and 25(OH)D_3 were detected at concentrations as low as 0.5 and 4 nmol/L respectively using a DEQAS accredited LC-MS/MS which is considered the gold-standard method for vitamin D analysis.[29] Our findings confirmed that horses' total 25(OH)D concentrations are exceptionally low compared with those of other species and were similar to those reported by other equine studies which used UHPLC-MS/MS.[11,14]

Median total vitamin 25(OH)D concentration of all horses in this study (10.7 nmol/l) was considerably below the threshold indicative of deficiency in humans (insufficiency is <70-75 nmol/L and deficiency is <25-30 nmol/L total 25(OH)D). In man these concentrations would be associated with a high risk for development of nutritional osteomalacia.[15] Similarly, total

25(OH)D concentrations below 25 nmol/L are a risk factor for developing clinical calcium/phosphorous derangement in dairy cows, while values below 10 nmol/L are associated with rickets in growing sheep, llamas and alpacas.[30] Nutritional osteomalacia has not been reported in horses, while developmental rickets has been either anecdotally reported or induced only in experimental settings.[31,32] None of the horses in our study showed any clinical evidence of osteomalacia. The reason for the inter-species difference in vitamin D biology is not known, but it has been suggested that calcium metabolism in horses is influenced to a lesser extent by vitamin D status than in other species.[7] In horses, even when hypervitaminosis D is experimentally induced, the plasma calcium concentration increases only moderately.[8] Azarpeykan et al. (2016) suggest that even low concentrations of 25(OH)D metabolites are capable of regulating vitamin D-related gene expression in the equine kidney.[12]

Domestic animals have developed different evolutionary adaptations to maintain physiological levels of vitamin D. Cats and dogs, being carnivores, obtain vitamin D from their diet and therefore do not need to synthetise endogenous vitamin D_3 in their skin. [26, 33] On the other hand both dietary vitamin D_2 and endogenous vitamin D_3 production contribute to vitamin D status in other domestic grazing animals including sheep, goats, camelids, cows, and rabbits. [34-37] The undetectable plasma concentrations of 25(OH)D_3 in non-supplemented horses suggests that endogenous cutaneous production of D_3 in the horse is either absent or minimal compared to other species, and that vitamin D status is predominantly influenced by ingestion of vitamin D_2 in herbage or supplemental dietary vitamin D_2 or D_3.

The grazing ponies in group 2 were not ridden while those in group 1 were racehorses in training that were exercised either the same day or the day before sampling. Exercise could have potentially contributed to the lower 25(OH)D_2 concentrations measured in group 1, as a reduction in plasma 25(OH)D_2 is observed after a single bout of high-intensity exercise.[14] Further investigations into the effect of exercise on vitamin D metabolism in horses is

warranted to provide more specific guidance regarding vitamin D supplementation of equine athletes.

Grazing ponies had increased serum 25(OH)D_2 concentrations during long day-length months. In contrast, there was no detectable 25(OH)D_3 even on long-days when 25(OH)D_2 concentrations were the highest. The undetectable plasma concentrations of 25(OH)D3 in non-supplemented horses suggests that vitamin D system is predominantly based on D_2 rather than D_3. The amount of UVB radiation that reaches the ground influences both the fungal activity and the rate of conversion from pro-vitamin D_2 (ergosterol) to vitamin D_2 making season and latitude important factors in determining pasture D_2 content.[38] A significant seasonal variation of ergosterol in perennial rye-grass hay has been reported.[39] The seasonal variation in plasma 25(OH)D_2 observed in grazing ponies likely reflected the seasonal variation of ergosterol content of the pasture herbage.[10,39] Further investigation into the vitamin D_2 content of the pasture grazed by these ponies is required to confirm this hypothesis. Importantly, despite ponies having a 42% reduction in 25(OH)D_2 and 13/19 of them having 25(OH)D_2 concentrations below 10 nmol/L during the winter months, none had clinical evidence of disease associated with hypovitaminosis D.

In obese humans, vitamin D deficiency is common, and low 25(OH)D concentration is associated with a higher risk of developing ID and diabetes.[40] While direct causality has not been confirmed, vitamin D supplementation is often prescribed as a complementary and preventative therapy.[41, 42]

Vitamin D is a secosteroid lipophilic hormone that is stored within adipose tissue.[43] Adipose tissue is increased in volume in obese people and theoretically, vitamin D sequestration within these large stores is thought to explain, at least in part, why deficiency is routinely observed in

obese humans.[44-46] Unexpectedly, in contrast to observations in humans, obese horses had significantly higher 25(OH)D_2 than non-obese horses, with measures of adiposity being the most predictive factor of vitamin D status. Our measures of adiposity are limited to quantification of subcutaneous adipose tissue and, despite the known correlation between BCS and total body fat, BCS may not accurately reflect the visceral adipose content of these animals.[47] Quantification of vitamin D metabolites in equine subcutaneous and visceral adipose tissue is needed to determine how these compounds are distributed throughout the body stores in obese and lean horses. One could speculate that the visceral adipose tissue inflammation and dysfunction reported in hyperinsulinaemic and obese horses could impair uptake and utilisation of vitamin D from the fat stores, which might explain the higher circulating 25(OH)D_2 found in obese horses.[48]

We found no association between serum insulin concentration or diagnosis of ID and vitamin D status in our population. In humans the association between ID and 25(OH)D is mitigated by adjusting for the amount of visceral adipose implying that it is an indirect relationship.[46]

While ACTH level was negatively associated with 25(OH)D_2 concentrations, PPID was not significantly associated with vitamin D status. This observation likely reflects the opposing seasonal changes that occur with vitamin D and ACTH concentrations, rather than reflecting a direct association between PPID and vitamin D status. In this respect, basal ACTH values tend to rise as days are shortening, while vitamin D levels fall.[49] Furthermore, 8/32 horses diagnosed with PPID were treated with pergolide which could have affected basal ACTH concentrations unpredictably. We therefore consider the relationship observed between these two hormones secondary to their physiological seasonal pattern rather than being associated with PPID.

A key question facing veterinary surgeons is whether horses require vitamin D supplementation, and, if so, at what level. The National Research Council's recommendation, first published in 1989 states that the daily requirement is around 6.6 IU/Kg but does not specify

the type of vitamin D.[50] This recommendation is based on an experimental study conducted in 1979 in which circulating 25(OH)D concentrations were not measured.[32] Our data suggest that grazing animals can remain clinically healthy despite having undetectable levels of $25(OH)D_3$, because pasture derived $25(OH)D_2$ meets their vitamin D requirements; supplementation of sedentary animals with access to pasture/forage is therefore unlikely to be necessary for optimal health. Further work is necessary however to determine if vitamin D_2 or D_3 supplementation is necessary to maintain optimal health in equine athletes with limited access to pasture or forage.

The main limitation of this study is that only 25(OH)D was measured. Despite this is the analyte most commonly used to determine vitamin D status, vitamin D system is complex, and involves a multitude of others metabolites that were not here considered.

Conclusion

This study demonstrates some marked differences in vitamin D biology in the horse compared with that of other species and advocates the need of further research in this field to provide evidence-based advice on nutritional requirements for athletic horses. Finally, the physiological role of vitamin D in horses is poorly understood, therefore 25(OH)D variation in pathological condition remains of difficult interpretation. Despite this, adiposity seems to have a significant contribution on vitamin D status.

Authors' declarations of interest

No competing interests have been declared.

Ethical animal research

This study was approved by the University of Edinburgh Veterinary Ethics and research Committee (approval 124.19).

Informed consent

Consent was obtained from owners and trainers of all horses included in the study.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Authorship

M.C.M. Dosi contributed to study execution, data analysis and preparation of the manuscript. J. Keen and B. McGorum contributed to study design and sample collection. R.J. Mellanby and E.A. Hurst contributed to sample analysis, while R. Kirton and E. Cillán-García contributed to sample collection. R. Morgan contributed to study design, sample collection, data analysis and preparation of the manuscript. All authors gave their final approval of the manuscript.

Figure legends

Figure 1: Vitamin D_2 and D_3 metabolism (Modified with the authors' permission from Hurst et al., 2020).[3] The precursor 7-dehydrocholesterol is converted to pre-vitamin D_3 following exposure to UVB in the skin of most domestic herbivores. Pre-vitamin D_3 undergoes thermoisomerisation to vitamin D_3. This is the form that is most commonly added to horses' concentrates and supplements. Pro-vitamin D_2 (ergosterol) is produced by fungi colonising pasture herbage. This is converted to pre-vitamin D_2 in response to UVB irradiation. Pre-vitamin D_2 undergoes thermoisomerisation to vitamin D_2. This is the form that is normally consumed by herbivores in grass, forage and silage. Both vitamin D2 and D3 undergo hydroxylation first in the liver and then in the kidneys, resulting in the biologically active molecules 1,25-dihydroxyvitamin D_2 and 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_2$ and $1,25(OH)_2D_3$). The hydroxylation reactions are catalysed by the enzymes CYPR1, CYP27A1 and CYP27B1. Vitamin D status is conventionally considered to be the sum of the plasma/serum concentrations of $25(OH)D_2$ and $25(OH)D_3$.

Figure 2: Boxplots showing plasma a) total 25(OH)D, b) $25(OH)D_2$ and c) $25(OH)D_3$ (nmol/L) concentrations for Thoroughbreds (group1; n=22) and grazing ponies (group 2; n=34).

Figure 3: Boxplots comparing plasma $25(OH)D_2$ concentrations measured in short-day and long-day seasons in a subset (n=18) of ponies kept at pasture. *** inter-group difference significant at $p<0.001$

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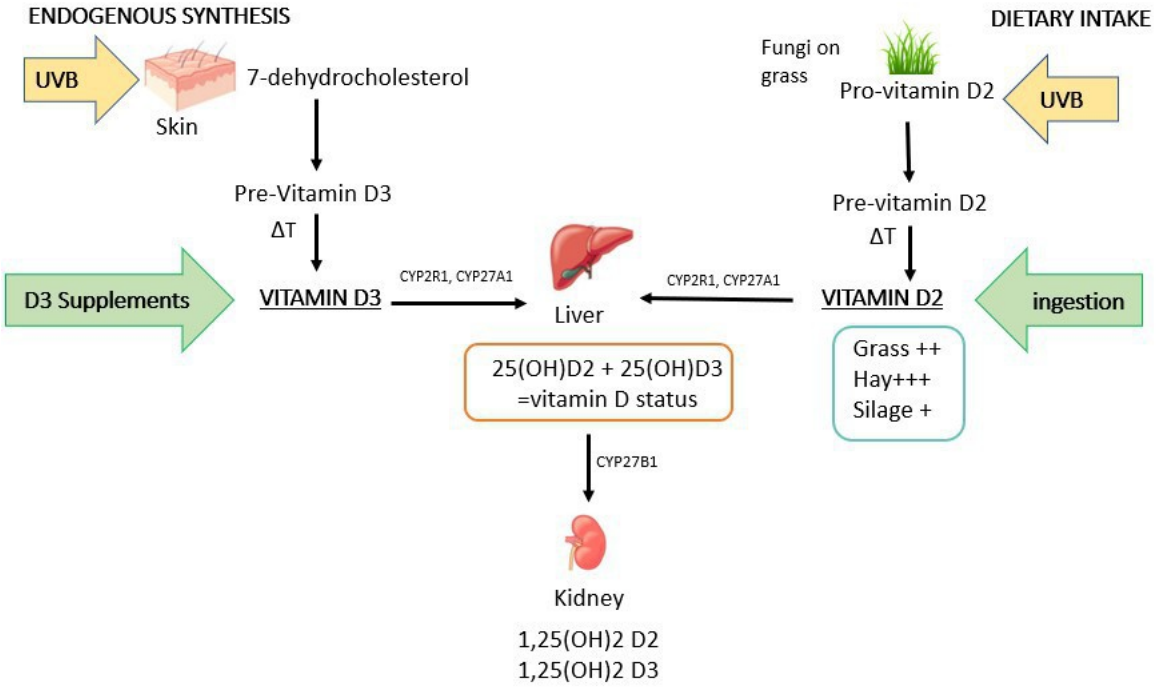
Table 1: Coefficients for BCS, regional adiposity, NCS, morphometric score, diagnosis of PPID, ln basal ACTH, diagnosis of ID, ln basal insulin, age, sex, breed and diagnosis of laminitis, calculated in the linear univariate regression having as outcome ln25(OH)D_2. †Indicates variables put forward to the multivariate logistic regression as $p<0.25$.

Univariate analysis				
Variable	**Regression Coefficient (β)**	**Standard Error**	**Pearson's Correlation Coefficient (r)**	**p-value**
BCS	0.168	0.049	0.444	$0.5x10^{-6}$ †
Regional adiposity Reference category: No	0.300	0.075	0.352	$0.1x10^{-3}$†
NCS	0.098	0.030	0.299	0.001†
Morphometric Score	0.172	0.037	0.408	$0.7x10^{-5}$†
Diagnosis of PPID Reference category: No	$0.288x10^{-3}$	0.088	$0.295x10^{-3}$	0.9
Ln basal ACTH	-0.142	0.049	0.286	0.01†
Diagnosis of ID Reference category: No	0.204	0.081	0.228	0.01†
Ln Basal Insulin	0.025	0.028	0.088	0.4
Age	0.0004	0.006	0.061	0.5
Sex **Reference category: Mare**	0.205	0.076	0.241	0.01†
Breed	-0.051	0.029	0.161	0.08†

Reference category: Welsh Cobs				
Diagnosis of laminitis Reference category: No	0.038	0.083	0.043	0.7

Table 2: Coefficients calculated for the variables retained in the multivariate linear regression having as outcome ln25(OH)D_2.

Multivariate Analysis-final model				
Variable	**Regression Coefficient**	**Standard Error**	**Pearson's Correlation Coefficient**	**p**
ln basal ACTH	-0.100	0.048	0.454	<0.001***
BCS	0.148	0.039		
Intercept	2.204	0.238		



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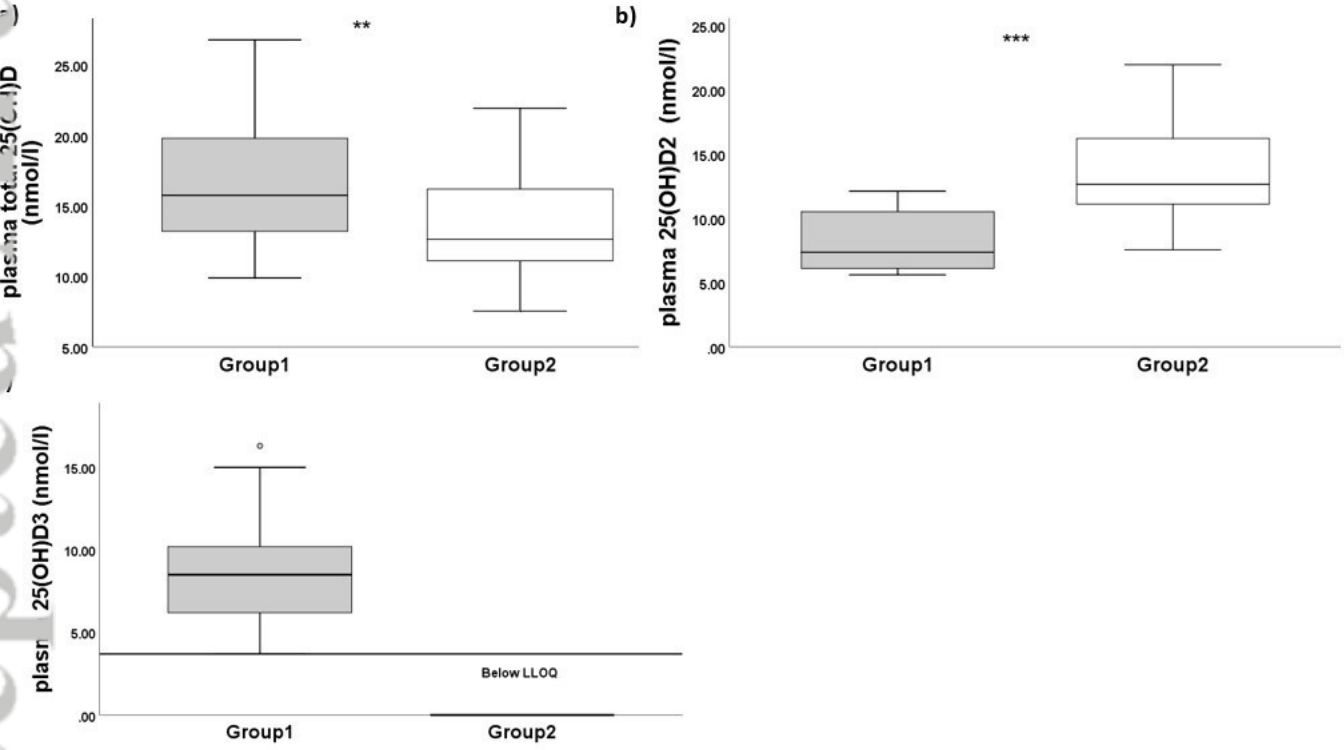

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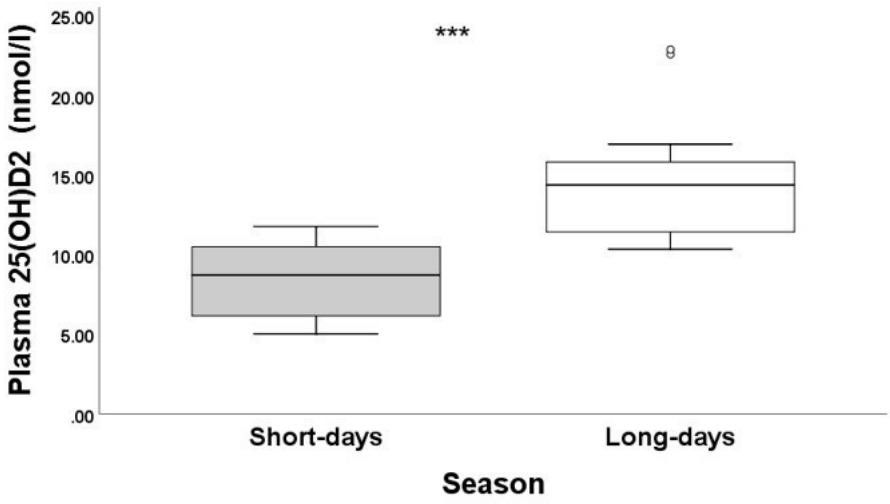

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