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Plasma Vitamin D Metabolites and C-Reactive Protein in Stage-Stop Racing Endurance Sled Dogs

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Background: Dogs are a unique model for examining the effects of exercise on vitamin D status because of their lack of vitamin D synthesis by UV exposure. In addition, the inflammatory response may be associated with hypovitaminosis D.

Objectives: To investigate the effects of several days of endurance exercise on plasma vitamin D (25-(OH)D_3, 24,25-(OH)D_3 and 1,25(OH)D_3) and serum C-reactive protein (CRP) concentrations in stage-stop racing sled dogs.

Animals: 12 racing sled dogs and 8 control dogs.

Methods: Blood was collected before the race and immediately after racing on days 2 and 8. Plasma vitamin D metabolites and serum CRP concentrations were measured.

Results: Racing dogs showed a significant increase in 25(OH)D_3 on day 2 ($P = .027$) and day 8 of the race ($P < .001$), whereas no increases were observed in control dogs. The plasma concentration of 24,25(OH)D_3 showed a significant increase by day 8 ($P < .001$). There were no significant changes in 1,25(OH) D_3 concentrations across all time points and groups. Racing dogs had significantly increased CRP concentrations by day 2 (39.3 ± 30.1 μg/mL; $P < .001$).

Conclusions and Clinical Importance: Increases in vitamin D metabolites as well as increases in CRP concentrations were observed in racing sled dogs. This finding was contrary to the hypothesis that decreases in vitamin D status in athletes may be related to the acute phase inflammatory response during exercise. In addition, the increased 24,25(OH)D_3 concentrations compared to what is observed in other species suggests metabolic variations in dogs that lead to enhanced disposal of vitamin D.

Key words: Endocrinology; Metabolism; Nutrition; Physiology; Sports medicine; Vitamins and minerals.

The effects of vitamin D on human athletic performance, has been examined as early as the 1930s.[1] More recently, there has been widespread concern about vitamin D deficiency in humans.[2–4] As a result many have examined 25(OH)D_3 status in human athletes and evaluated the associated links to injury and performance,[1,5–11] as well as muscle strength and neuromuscular function in humans.[12–17]

Vitamin D, in its active form 1,25(OH)D_3, is tightly regulated by parathyroid hormone activation of renal 1-α-hydroxylase activity, is 100-fold lower in concentrations than 25(OH)D_3, and has marked influence over calcium homeostasis. Recently, it has been observed to play roles in neoplasia and muscle development, and nearly all organ systems have receptors for 1,25(OH)D_3.[2,3,18] In the dog, vitamin D concentrations have, status has been investigated and found to be decreased in protein losing enteropathy, renal disease, neoplasia, and cardiovascular disease.[19–22] Vitamin D concentrations have been found to be low in human endurance athletes[5,6,11]; it is difficult to determine if these lower concentrations are related to the exercise, shifts in dietary patterns or a lack of ultraviolet exposure.[1,11] Dogs provide an ideal model to examine endurance exercise and vitamin D status because of consistency in diet and lack of vitamin D formation after ultraviolet light exposure.[23,24]

In addition, research suggests that in inflammatory conditions, in which 25(OH)D_3 concentrations decrease, there often is a concomitant increase in C-reactive protein (CRP) concentrations.[25,26] Endurance sled dogs have been shown to mount a robust CRP response after exercise, that is 3- to 5-fold higher than that observed in human marathon runners.[27–29] This increase in CRP without chronic disease allows examination of the associations between hypovitaminosis D and increased CRP concentrations to determine if chronic inflammation drives hypovitaminosis D as a result of increased excretion, increased gastrointestinal (GI) or renal losses, or both.

Abbreviations:

1,25 (OH) D_3	1,25 dihydroxyvitamin D_3
24,25 (OH) D_3	24,25 dihydroxyvitamin D_3
25 (OH) D_3	25 hydroxyvitamin D_3
CRP	C-reactive protein
CYP24	cytochrome p450 24 hydroxylase
GI	gastrointestinal
HPLC	high pressure liquid chromatography

From the Best Care Pet Hospital, Sioux Falls, SD (Spoo); the Annamaet Petfoods, Sellersville, PA (Downey); The Traveling Vet, Loveland, CO (Griffitts); the Heartland Assays, Ames, IA (Horst); and the Department of Clinical Sciences, Cornell University College of Veterinary Medicine, Ithaca, NY (Levine, Childs, Wakshlag).

Samples were collected at the 2014 International Pedigree Stage-Stop Sled Dog Race in western Wyoming. Samples were analyzed at Cornell University in Ithaca, NY and Heartland Assays in Ames, IA.

The results were presented at the 8th International Symposium on Veterinary Rehabilitation/Physical Therapy and Sports Medicine and the 2014 Penn Working Dog Conference.

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To date, no study has investigated the vitamin D status of the canine athlete with a steady dietary intake of vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol). Results obtained could provide critical information as to the influence of exercise on serum 25(OH)D_3 concentrations in endurance athletes, both human and dog.[5–8] In addition, 24,25(OH)D_3 concentrations in dogs have not been examined and will provide valuable information related to renal 24 hydroxylase activity (CYP 24) and its relationship with vitamin D metabolism.

We assessed the complete vitamin D (25(OH)D_3, 24,25(OH) D_3, and 1,25(OH) D_3) and CRP status of control and racing sled dogs competing in a multi-day stage race on days 0, 2, and 8 of the event.

Materials and Methods

Animals

Dogs (16) from 2 teams (but the same kennel) participating in the 2014 International Pedigree Stage-Stop Sled Dog Race were enrolled in the study. The study was approved by the Cornell University Institutional Animal Care and Use Committee. The kennel owner signed a client consent form before study initiation. All dogs had full health examinations before the race, and they were deemed healthy. Dogs selected for analysis participated in 5 of the 8 days of the race with all dogs racing on days 1 and 8 of the racing schedule, with variable racing on the days between. Dogs were rested on day 3 as a scheduled travel day, and because of weather and trail conditions also were rested on day 7. The average racing time for each stage of the race was 3.5–5 hours of continuous running with no relevant resting periods, and carrying a lightweight sled with 80–95 kg including musher and supplies. Of the 12/16 racing dogs that had a complete sample set on days 0, 2, and 8; 7 were male and 5 were female, averaging 4.3 years old. Control racing dogs (n = 8) were of similar lineage, fed the same diet, trained similarly, and travelled in the same dog truck. Of the control dogs, 6 were male and 2 were female, with an average age of 3.6 years. All dogs were weighed immediately before the race on day 0 and immediately after the race on day 8 using a scale with a single investigator holding the dogs at both time points and subtracting the investigator's weight at both time points to obtain a final weight for each dog to the nearest pound, which then was converted to kilograms.

Blood Sampling and Analysis

Blood was sampled on day 0 between 12 and 1 PM, day 2 immediately after racing between 2 and 3 PM and day 8 between 2 and 3 PM. At each time-point, 6 mL of whole blood was obtained by cephalic venipuncture using a 22 gauge needle into a 5 mL lithium heparin tube. Blood samples were protected from light and immediately centrifuged at 4,000 × *g* for 10 minutes. Three aliquots of plasma were immediately stored on dry ice until transportation to the investigators' lab within 48 hours where they were immediately placed into a −80°C freezer.

25(OH)D_3 and 24,25(OH)D_3

Serum/plasma samples (100 μL) were aliquoted into 12 × 75 mm borosilicate glass in preparation for analysis of 25(OH)D_3. Samples were spiked with deuterated 25(OH)D_3 followed by protein precipitation using the $ZnSO_4$/methanol method.[30] 25(OH)D was extracted with hexane, dried and reconstituted with liquid chromatography-mass spectrometry grade methanol and water. Quantification was achieved by liquid chromatography-tandem mass spectrometry unit using an Agilent 1290 high pressure liquid chromatography (HPLC) unit coupled to an Agilent 6460 Triple-quad mass spectrometer after the procedure outlined by Agilent. Samples were prepared and analyzed for 24,25(OH)D_3 in the same manner as 25(OH)D_3 except 200 μl of sample was extracted and deuterated 24,25(OH)D_3 was used as an internal standard.[30] The inter-and intra-assay coefficients of variation were <12% for both assays.

Total 1,25(OH)$_2$D

Total 1,25(OH)$_2$D was assayed using the competitive radioimmunoassay procedure described by Hollis et al.[31] Inter- and intra-assay coefficients of variation for 1,25(OH)D_3 were 8 and 11%, respectively.

In addition, a representative food sample was taken from the day 4 feeding and was immediately frozen and shipped to the investigators' laboratory and stored at −80°C. This feed sample (150 g) then was sent to the same nationally certified laboratory for similar vitamin D analysis as performed on the plasma samples.[a] Feed samples were processed according to the methods of Phillips et al, which included saponification and solvent extraction.[32] One-hundred and fifty grams of sample was sent for proximate analysis of protein, fat, ash, fiber, and moisture at a commercial analysis laboratory.[b] Details on the constituents in the meal were obtained from the kennel owner while making the feed on site.

Vitamin D metabolite analysis was performed for the feed and serum samples after purification by a semipreparative normal-phase HPLC, and quantification with a reverse-phase HPLC/mass spectroscopy technique utilizing cholecalciferol as the internal standard for quantification.[32]

C-Reactive Protein ELISA

A canine C-reactive protein kit[c] that has been validated for use on canine serum was used. The kit was used according to the manufacturer's suggestions with all samples from the same dog being performed on the same plate in duplicate. All postexercise samples were diluted 1 : 5 as suggested by the manufacturer to ensure that concentrations fell within the linear portion of the standard curve (5–100 μg/mL).

Data and Statistical Analysis

Initial dog weights before and after the race were analyzed using a paired Student's *t*-test. Vitamin D metabolites and CRP concentrations across the 2 populations of racing and control dogs were assessed for normality utilizing the Shapiro-Wilk test. After normality was confirmed for all vitamin D metabolites (1,25(OH)D_3, 25(OH)D_3 and 24,25(OH)D_3) results were analyzed across the control and racing dogs using a 2-way analysis of variance with Tukey's posthoc analysis over time and group. Not all CRP data was normally distributed, therefore all data was transformed before analysis of variance and no residual outliers were removed. Furthermore, Pearson's correlates were examined assessing 25(OH)D_3 and CRP status across all racing dogs to establish whether there is an association between serum 25(OH)D_3 and CRP concentrations at days 2 and 8. *P* values of .05 were considered significant.

Results

Dogs and Exercise

Of the 16 dogs from which blood was initially obtained, only 12 ran in 5 of the 6 days of racing over the 8-day time period. The other 4 dogs participated in

4 or fewer days and were excluded from final analysis. The total distance each dog ran over the 8 days was between 218 and 236 miles. The average weight of the 12 dogs before the race on day 0 was 25.2 ± 2.1 kg, whereas after day 8 racing the average was 25.0 ± 2.0 kg, which was not statistically different. The control dogs' average weight on day 0 was 26.5 ± 2.5 kg and on day 8 it was 26.9 ± 2.3 kg, which was not statistically significant.

Vitamin D Metabolites

Vitamin D metabolite concentrations were different from resting during days 2 and 8 of the event (Table 1). Dogs competing in the race had a significant increase in $25(OH)D_3$ concentrations on day 2 of the race ($P = .027$) and a further increase in $25(OH)D_3$ concentration on day 8 of the race ($P < .001$). When comparing control and racing dogs, there was no difference on day 0 ($P = .09$), whereas on day 2 ($P = .006$) and day 8 ($P < .001$) there was a significant increase compared to controls. The plasma concentration of $24,25(OH)D_3$ did not change significantly in the racing dogs from the start of the race to day 2 ($P = .65$), but did significantly increase by day 8 ($P < .001$). Differences in $24,25(OH)D_3$ across days in the control group were not significant, whereas the racing group had higher $24,25(OH)D_3$ concentrations on day 8 ($P = .004$) and no significant differences on days 0 ($P = .081$) and 2 ($P = .062$) when compared to the control group. There were no significant changes in $1,25(OH)D_3$ concentration across all time points in the racing group. The $1,25(OH)D_3$ concentrations in the control dogs decreased from days 0 to 2 ($P = .014$) and 8 ($P = .016$), whereas there was only a significant difference between control and racing dogs at day 0 ($P < .001$).

Feed Analysis

Complete analysis of the feed can be found in supplemental section with tabular comparison to National Research Council established recommended daily intake values per 1000 kcals.[33] Vitamin D analysis showed ergocalciferol content of <25 IU/kg dry matter and approximately 1,919 IU/kg dry matter of cholecalciferol.

C-Reactive Protein

Plasma CRP concentrations were significantly different between control dogs and racing dogs, and there were significant changes from day-to-day within the 2 groups (Fig 1). The control group started with a concentration that was higher than that of the racing group (19.4 ± 14.8 μg/mL and 10.7 ± 4.8 μg/mL, respectively), but this difference was not statistically significant. The control group's CRP concentrations decreased over the 8 days and were significantly lower on day 8 as compared to day 0 (day 2: 11.6 ± 4.5 μg/mL: day 8: −8.3 ± 3.2 μg/mL; $P = .023$). The racing group had a significant increase in concentration from resting to day 2 (39.3 + 30.1 μg/mL; $P < .001$). The concentrations on day 8 were statistically decreased from day 2 concentrations ($P < .001$). Concentrations on day 8 (17.2 + 10.5 μg/mL), although increased from day 0, were not significantly higher.

Pearson's correlates were performed on days 2 and 8 to examine the associations between increases in CRP and $25(OH)D_3$ concentrations in racing dogs. Pearson's correlates for these associations were $R = 0.11$ on day 2 and $R = 0.10$ on day 8 and were not significantly different, hence negative associations between increasing CRP and decreasing vitamin D concentrations were not observed in this population.

Discussion

Vitamin D concentrations in the racing dogs responded in a manner contrary to the hypothesis with significant increases during exercise. Studies of human athletes have evaluated various vitamin D metabolites over the course of a competitive season, or using mean concentrations in a group of athletes, and not the effects of daily exercise.[1,6–9,11] Our results to identified significant increases in $25(OH)D_3$ concentrations.[34] Interestingly, this increase in $25(OH)D_3$ led to increases in only $24,25(OH)D_3$ by day 8 of the race suggesting increased CYP24 activity, with no increase in $1,25(OH)D_3$ concentrations. To date, $24,25(OH)D_3$ has been viewed as an inactive metabolite or excretory product of $25(OH)D_3$ metabolism.

One hypothesis for the increases in vitamin D concentrations is the aerobic nature of the exercise performed by sled dogs and the subsequent mobilization of fat stores during exercise, which may result in vitamin D release during lipolysis of adipose stores, liver fat stores, or both. Interestingly, the dogs in this study experienced no significant change in weight between the start of the race and the end of the race making lipolysis and weight loss less likely a contributing factor, but increased adipose tissue turnover still may be

Table 1. $25(OH)D_3$, $24,25(OH)_2D_3$, and $1,25(OH)_2D_3$ mean ± SD at days 0, 2, and 8 (D0 – day 0, D2 – day 2, D8 – day 8) in racing and the control subjects during an 8-day multi-stage sled canine race.

	Control D0	Control D2	Control D8	Racing D0	Racing D2	Racing D8
$25(OH)D_3$ (ng/mL)	57 ± 13	55 ± 11	57 ± 13	67 ± 9	72 ± 11[a,b]	87 ± 16[a,b]
$24,25(OH)D_3$ (ng/mL)	54 ± 12	54 ± 13	55 ± 12	66 ± 13	67 ± 15	76 ± 18[a,b]
$1,25\ (OH)D_3$ (pg/mL)	157 ± 30	127 ± 33[a]	129 + 34[a]	122 ± 34[b]	119 ± 26	121 ± 22

Significant differences are indicated as follows: [a]indicates a significant difference between racing and control groups on day 0 ($P < .05$); [b]indicates a significant difference between day 0 only ($P < .05$).

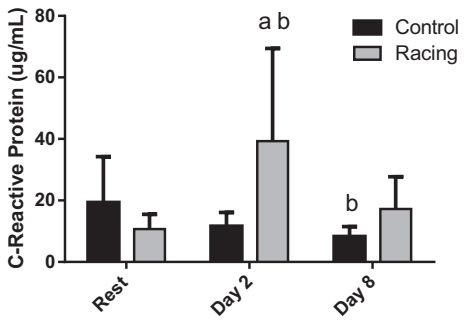

Fig 1. Racing and control canine serum C-reactive protein (CRP) concentrations. Mean ± SD of CRP concentration at Rest (days 0), day 2 and day 8 in racing sled dogs (n = 12) and the control dogs (n = 8) during an 8-day multi-stage sled dog race. [a]Indicates a significant difference between racing and control groups on day 2 ($P < .05$). [b]Indicates a significant difference between day 0 within the same group ($P < .05$).

involved. Although controversial, adipose tissue can store vitamin D, but studies in humans have shown that changes in body fat composition do not lead to an increase in serum 25(OH)D_3 concentrations during weight loss.[35]

A second possibility would be an increase in dietary intake of vitamin D_3. Here again, the dog driver verified that the approximate amount of provided feed was not changed in response to the race. Because the control dogs were fed approximately 20% less without a decrease in serum 25(OH)D_3 concentration, the likelihood that any modest increase in intake influenced the racing dogs is unlikely.

Further proposed reasoning for the increased vitamin D concentrations revolve around the endocrine responses to exercise. The anabolic hormone insulin-like growth factor- 1 (IGF-1) can increase during exercise[36] because of hepatic synthesis and may be related to vitamin D increases. Although vitamin D increases during exercise are contrary results of a human study,[37,38] younger athletes might experience an increase in IGF-1 which is directly correlated with increases in 25(OH)D_3.[39,40] Information regarding IGF-1 and IGF-1 binding proteins in working dogs is not available and maybe a fruitful area of future research related to vitamin D status.

The increases in serum 25(OH)D_3 and 24,25(OH)D_3 concentrations pose questions regarding endocrine responses, even though 1,25-(OH)D_3 concentrations were unchanged. Total calcium concentrations decrease during endurance sled dog racing by 5–10%[24,41] whereas serum phosphorous concentrations remain unchanged or decrease. Many factors affect serum calcium and phosphorous concentrations including changes in albumin serum concentration during exercise, increases in food intake, and use of meats to complement commercial canine foods. Ionized calcium, parathyroid hormone, and vitamin D metabolite concentrations and their relationship with serum calcium concentration, and fibroblast growth factor-23 (FGF-23) concentrations and phosphorous intake have not been evaluated in endurance athletes.[42] Increased phosphorous intake could lead to alterations in FGF-23, parathyroid hormone, or both that in turn could induce changes in renal hydroxylase activity. Because vitamin D is bound to vitamin D-binding protein and stored in the liver, exercise of this duration could lead to a modest increase or shift in hepatic storage that could be inconsequential to calcium and phosphorous homeostasis, particularly as there is no concomitant increase in 1,25-(OH) D_3 concentration.

In endurance racing, both GI upset with increased GI permeability, and the activities of many hepatic enzymes increase, which suggests possible alterations in GI uptake of vitamin D as well as metabolic alterations during racing that might lead to enhanced CYP24 activity and 25-hydroxylase activity causing increases in 25 (OH)D_3 concentrations and reciprocal increases in 24,25(OH)D_3 concentrations as a homeostatic mechanism in both kidney and liver.[43] The fact that 25(OH) D_3 increased on day 2, before increases in 24,25(OH) D_3 on day 8, suggests a possible homeostatic mechanism to decrease 25(OH)D_3 concentration. Increased CYP24 activity has been associated with other dietary changes and pharmacologic interventions; therefore, exercise may be the stimulus for this increase.[35,44] The changes observed warrant further exploration and the need evaluate at the role of vitamin D metabolites during exercise and potentially a need to shift away from evaluating overall vitamin D status as measured by 25 (OH)D_3 concentrations in atheletes.[1,5,6,8,9,11]

Of equal interest are 24,25(OH)D_3 concentrations found in these dogs. When compared to other species such as humans, pigs, and rodents, the concentrations are nearly 6- to 12-fold higher than observed in other species.[45,46] Our results are the first report of 24,25(OH) D_3 concentrations in a canine population. These high concentrations suggest hepatic or renal metabolism may already have enhanced CYP24 activity because of high dietary intake of cholecalciferol. The amount of vitamin D_3 in the food was 1,919 IU/kg dry matter. This amount is similar to that found in some commercial canine foods in compliance with the American Association of Feed Control Officials.[20] The meal these dogs consumed contained approximately 45% of the calories as commercial kibble[d] and approximately 55% as a mix of beef, poultry, organ meat, and fish oil. Therefore, the excessive 24,25(OH)D_3 concentrations was unlikely to have arisen from the diet. This poses the question of whether the amounts of cholecalciferol in commercial pet foods could be considered over-supplementation, because dogs in general have higher concentrations of serum 25(OH)D_3 than humans.[1,5,19–21] The dogs studied were athletes, and their vitamin D metabolism may be different than that of the average household or experimental dog. This situation warrants further examination into how cholecalciferol is metabolized in non-working companion dogs.

A decrease in vitamin D concentration may be associated with a reciprocal increase in CRP.[25,26] Such was not the case in our study in which increases in 25(OH) D_3 concentrations in the face of increases in CRP

concentrations were observed. Overtraining syndrome and chronic fatigue is common in humans[47–50] but little is known about this phenomenon in the canine athletes.[29] Acute phase proteins have been used as markers to identify these changes in human endurance athletes.[51] The CRP response in exercising sled dogs has been well established.[29,52,53] In these races, dogs ran >50 miles per day for 5–10 consecutive days. The CRP concentrations observed in previous studies of endurance sled dogs were between 100 and 200 μg/mL concentrations which are well above that observed in marathon runners, who show ranges from 15 to 30 μg/mL within 24 hours after cessation of exercise.[54–56] The dogs in this study ran ≤50 miles on any given day (average, 40 miles) which might be the reason for the lower CRP concentrations, making them more similar to a marathon runner. None of the dogs in this study (control or racing) had run in the 36 hours before the start of the race. For many of the dogs, the last run occurred 3 days before and consisted of a 20–22 mile run. On day 0, the CRP concentrations in both groups (with the exception of 1 outlier in the exercise group and 2 in the control group) were approximately 10–20 μg/mL which is higher than the average sedentary dog.[57] Previous studies in sled dogs showed that there appears to be a chronic increase in CRP concentrations at rest and that sprinting dogs tend to have lower CRP concentrations than endurance sled dogs.[58] By day 2, we saw similar increases in CRP concentrations as previously reported in sled dogs, but not to the same magnitude. In fact, the increases observed on day 2 were similar to what is observed in human marathon athletes.[51,54] We did not see the increase in CRP concentration expected on day 8, but this likely was because of day 7 of the race being canceled because of weather conditions, and all dogs were rested on day 7 and then competed on day 8. This interval presumably allowed recovery of CRP concentrations to near baseline for most dogs and we would have expected a similar increase to that observed on day 2 if we had collected blood 24 hours after the day 8 event.[29]

Conclusion

In summary, the dogs in this study showed significant increases in vitamin D concentrations as well as increases in CRP concentrations. This finding was contrary to our hypothesis that decreases in vitamin D concentrations in athletes may be related to inflammation associated with exercise. This study highlights the need for further studies of vitamin D metabolism, particularly as it relates to athletic performance. Concentrations can fluctuate in a short period of time, and this fluctuation may be related to activity and potentially the nutritional status. In addition, the pronounced increase in 24,25(OH)D_3 concentrations suggest that the metabolism of vitamin D in dogs may be different from other species or that dietary concentrations are high suggesting increased conversion for excretion from the body, which requires further study in normal sedentary canine populations. The results of this study also showed that CRP concentrations will decrease to 10–20 μg/mL 24–48 hours after peak concentration, but often remain higher than what is observed in sedentary dogs.

Footnotes

[a] Heartland Assays, Ames, IA
[b] Dairy One, Ithaca, NY
[c] Canine C-reactive protein ELISA, Tridelta Development limited, Maynooth, Ireland
[d] Redpaw 32/20, Franklin, WI

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Conflict of Interest Declaration: The authors disclose no conflict of interest.

Off-label Antimicrobial Declaration: The authors declare no off-label use of antimicrobials.

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Supporting Information

Additional Supporting Information may be found online in Supporting Information:

Table S1. Calculated nutritional analysis of total diet nutrient consumption per 1,000 kcals compared to the National Research Council (NRC) recommended daily allowance (RDA) of nutrients for dogs, based on 1000 kilocalories.