

Effect of withholding food on serum concentrations of cobalamin, folate, trypsin-like immunoreactivity, and pancreatic lipase immunoreactivity in healthy dogs

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OBJECTIVE

To evaluate the effects of withholding food on the results for measurements of serum concentrations of cobalamin, folate, canine pancreatic lipase immunoreactivity (cPLI), and canine trypsin-like immunoreactivity (cTLI) in healthy dogs.

ANIMALS

11 healthy employee- or student-owned dogs.

PROCEDURES

Food was withheld from the dogs for 12 hours, baseline blood samples were collected, then dogs were fed. Postprandial blood samples collected 1, 2, 4, and 8 hours later were assessed. A mixed-effects ANOVA model with fasting duration (time) as a fixed factor and dog as a random effect was fit for each analyte variable. Additionally, a mixed-effects ANOVA model controlling for the variable of time was fit to assess whether lipemia affected serum concentrations of the analytes.

RESULTS

The median serum cobalamin concentration was lower at 4 hours (428 ng/L) and 8 hours (429 ng/L) postprandially, compared with baseline (479 ng/L), but this difference was not clinically meaningful. Although there were no substantial differences in serum concentrations of folate, cPLI, or cTLI, postprandial changes in serum concentrations of cTLI or folate could potentially affect diagnoses in some dogs.

CONCLUSIONS AND CLINICAL RELEVANCE

Although results indicated that feedings rarely resulted in clinically important differences in the median serum concentrations of cobalamin, folate, cPLI, or cTLI in healthy dogs, given the further processing required for lipemic samples, withholding food for at least 8 hours is an appropriate recommendation when measuring these analytes. Similar research is needed in dogs with gastrointestinal disease to determine whether the withholding of food is necessary when measuring these analytes in affected dogs. (*Am J Vet Res* 2021;82:367–373)

Measurements of serum concentrations of cobalamin (vitamin B12), folate (vitamin B9), cPLI, and cTLI are widely used as minimally invasive tools to diagnose and monitor small intestinal and exocrine pancreatic disease in dogs.^{1–3} Cobalamin serves as a marker of distal small intestinal disease (owing to its ileal absorption) and EPI (owing to its unique intrinsic factor-mediated absorption).^{2,4} Folate is an analyte that informs on proximal small intestinal disease and the presence of intestinal dysbiosis.³ Measurement of cTLI is by radioimmunoassay to detect cationic trypsinogen and trypsin in serum and is used for diagnosing EPI in dogs in clinical settings.⁵ Measurement of cPLI is by an immunoassay

for pancreatic lipase in serum and serves as a sensitive and specific marker for diagnosing pancreatitis in dogs.¹

Although measuring these analytes is often performed as a standard part of the diagnostic evaluation of dogs with clinical signs of chronic gastrointestinal disease, the need to withhold food from an affected patient before collecting a blood sample to test can interfere with a streamlined diagnostic workup. Additionally, among laboratories that provide these tests for dogs, there are no standardized requirements for withholding food, with durations ranging from 6 to 12 hours.^{6,7} Therefore, withholding food for 12 hours overnight is often recommended, and although this may be performed to reduce the theoretical effects of postprandial pancreatic enzyme secretion and vitamin absorption, the practice of withholding food is arbitrary and not evidence based.

A recent retrospective study⁸ in human medicine shows that plasma concentrations of cobalamin were not affected by fasting duration (1 to > 16

ABBREVIATIONS

cPLI	Canine pancreatic lipase immunoreactivity
cTLI	Canine trypsin-like immunoreactivity
EPI	Exocrine pancreatic insufficiency
RER	Resting energy requirement
RI	Reference interval
IQR	Interquartile (25th to 75th percentile) range

hours) in women and were minimally and clinically insignificantly affected in men. To our knowledge, no studies in veterinary medicine have evaluated the relationship between serum cobalamin or folate concentrations and duration of withholding food. Small groups of healthy dogs^{9,10} and cats¹¹ have been studied to evaluate how feedings may potentially affect serum cTLI and cPLI concentrations; however, no clinically significant postprandial effects were identified. Unnecessarily withholding food from veterinary patients can lead to delayed or canceled testing, reduced patient welfare,¹² and delayed return to enteral nutrition in compromised patients.^{13,14}

The objective of the study reported here was to evaluate the effects of withholding food on the results for measurements of serum concentrations of cobalamin, folate, cPLI, and cTLI in healthy dogs. We hypothesized that there would be no clinically meaningful difference in the results of these analytes in samples obtained after withholding food 12 hours overnight (as a commonly used protocol) versus samples obtained at 1, 2, 4, and 8 hours after feeding.

Materials and Methods

Animals

Healthy, employee- and student-owned dogs were recruited at the University of Wisconsin-Madison School of Veterinary Medicine by open enrollment. For inclusion, dogs were required to be mature (1 to 6 years of age), of medium to giant breed (≥ 10 kg), healthy (as determined by history and physical examination), and amenable to the study protocol (able to have food withheld and undergo repeated venipuncture). All dogs were required to consume a diet formulated to meet the Association of American Feed Control Officials' dog food nutrient profile for adult maintenance,¹⁵ have no clinical signs of systemic disease, and have no clinically abnormal gastrointestinal signs (eg, vomiting, diarrhea, anorexia, or unexplained weight loss) in the 4 weeks prior to the study. Exclusion criteria were a history of gastrointestinal disease, administration of cobalamin or folate supplements within the 6 months before the study, and a history of pancreatitis, hyperadrenocorticism, anemia, hypovolemia, or medical conditions not easily amenable to the withholding of food or controlled feeding (eg, diabetes mellitus or dietary intolerance).

Ethics approval

The study design was approved by the Institutional Animal Care and Use Committee (Animal Use Protocol No. V006113) at the University of Wisconsin-Madison. The owners of each dog provided written consent before enrollment.

Sample collection and handling

Prior to blood sample collection, food was withheld from each dog at home for 12 hours; however, free access to water was provided at all times. No

medications were allowed during the study period. The first blood sample (4 mL) was collected at the veterinary hospital after food had been withheld for 12 ± 1 hours and was considered the baseline blood sample. Each dog was then fed its typical morning meal and kept in the hospital for the duration of the sampling period. For dogs that showed minimal interest in their typical food, a canned prescription gastrointestinal diet^a was fed. Dogs that failed to consume at least 50% of their RER (body weight^{0.75} \times 70 kcal) were excluded from the study. Subsequent blood samples (4 mL each) were collected at 1, 2, 4, and 8 hours after feeding. Additional food or treats were not permitted during the sampling period.

Blood samples were allowed to clot, then centrifuged to collect serum within 1 hour of collection. Serum samples were protected from light by the use of aluminum foil covers and were stored at temperatures between 2°C and 6°C. Within 12 hours of collection, all serum samples were frozen at -80°C, then shipped by overnight courier to the Gastrointestinal Laboratory at Texas A&M University as a single batch. This process limited the total handling duration to 1 week.

Laboratory tests

Sample lipemia was determined by gross inspection at the testing site, and samples deemed lipemic were noted and centrifuged prior to analysis. Serum concentrations of cobalamin, folate, and cTLI were measured with automated chemiluminescence assays.^{b,c} Serum concentration of cPLI¹⁶ was measured with a commercial sandwich ELISA.^d All assays were run in duplicate batches to reduce intra-assay variability, and manufacturer-provided or in-house quality control materials were used.

Owing to assay limitations, when a result for an analyte was below the lower limit of the working range of an assay (eg, cPLI concentration < 30 $\mu\text{g/L}$), the result was recorded as the next unit below that limit for the analyte (eg, cPLI concentration of 29 $\mu\text{g/L}$) for the affected time point. When a result for an analyte was above the upper limit of the working range of an assay (eg, cTLI concentration > 50 $\mu\text{g/L}$), the result was recorded as the next unit above that limit for the analyte (eg, cTLI concentration of 51 $\mu\text{g/L}$) for the affected time point. Dogs that had results outside the working range of an assay for all 5 time points were removed from that analyte-specific analysis.

Statistical analysis

Data were assessed for normality with the Shapiro-Wilk test and summarized as the mean \pm SD or the median and IQR as appropriate on the basis of statistical distribution. To assess for a statistical change in serum analyte concentrations from baseline, a mixed-effects ANOVA model with fasting duration (time) as a fixed factor and dog as a random effect was fit for each variable. Results of analysis with mixed-effects ANOVA for

repeated measures were presented as the estimated mean change from baseline and the corresponding 95% CI. In addition, a mixed-effects ANOVA model controlling for the variable of time was fit to assess whether lipemia affected serum concentrations of the analytes. Aside from the normality of data assessed with the Shapiro-Wilk test mentioned earlier, other assumptions of the model fit were assessed with the use of residual plots for heteroscedasticity and normal Q-Q plots. All statistical analyses were conducted with available software^{e,f} and a 2-sided 5% significance level. Values of $P < 0.05$ were considered statistically significant.

Results

Animals

Eleven healthy dogs were enrolled and completed the study protocol. Overall, the mean age was 3.9 years (SD, 1.7 years), and the study group consisted of 5 spayed females, 3 castrated males, 2 sexually intact males, and 1 sexually intact female. Mixed-breed dogs ($n = 7$) were most commonly represented, followed by 2 Australian Shepherds, 1 Golden Retriever, and 1 Greyhound. The mean body weight was 24.1 kg (SD, 5.7 kg). No dogs had evidence of chronic gas-

Table 1—Median (IQR) serum concentrations of cobalamin, folate, cTLI, and cPLI for 11 healthy dogs sampled after food was withheld for 12 hours (time 0; baseline) versus at 1, 2, 4, and 8 hours after feeding.

Time (h)	Cobalamin (ng/L)			Folate (μ g/L)			cTLI (μ g/L)			cPLI (μ g/L)*		
	Median (IQR)	Estimated mean of differences (95% CI) [†]	P value	Median (IQR)	Estimated mean of differences (95% CI) [†]	P value	Median (IQR)	Estimated mean of differences (95% CI) [†]	P value	Median (IQR)	Estimated mean of differences (95% CI) [†]	P value
0 (baseline)	479 (406–636)	—	Referent	10.1 (8.1–10.9)	—	Referent	32.8 (21.4–41.8)	—	Referent	39.0 (37.2–51.2)	—	Referent
1	455 (424–630)	-7 (-46 to 31)	0.702	10.2 (8.3–11.5)	-1.1 (-3.6 to 1.4)	0.367	34.6 (26.0–42.8)	2.6 (-0.9 to 6.2)	0.140	45.0 (39.2–47.0)	0.3 (-7.2 to 7.9)	0.927
2	449 (412–600)	-18 (-57 to 20)	0.340	9.8 (8.7–11.5)	-0.4 (-2.9 to 2.1)	0.743	30.8 (26.6–41.1)	1.5 (-2.1 to 5.0)	0.408	42.0 (37.8–53.0)	1.7 (-5.9 to 9.2)	0.647
4	428 (360–536)	-54 (-92 to -15)	0.008	10.1 (8.8–12.8)	-0.2 (-2.7 to 2.3)	0.861	28.1 (25.6–34.9)	-1.3 (-4.9 to 2.2)	0.459	46.0 (40.0–59.5)	2.2 (-5.4 to 9.7)	0.555
8	429 (394–552)	-59 (-97 to -20)	0.004	10.2 (8.3–12.1)	-1.6 (-4.1 to 0.8)	0.190	32.8 (28.6–37.1)	1.1 (-2.4 to 4.6)	0.533	42.0 (35.8–49.0)	-4.3 (-12 to 3.2)	0.224

*Results represent findings in 6 dogs. The remaining 5 dogs were censored for having had all results below the lower limit of the working range of the assay. [†]The estimated mean of differences was determined with mixed-effects ANOVA for repeated measures.

— = Not applicable.

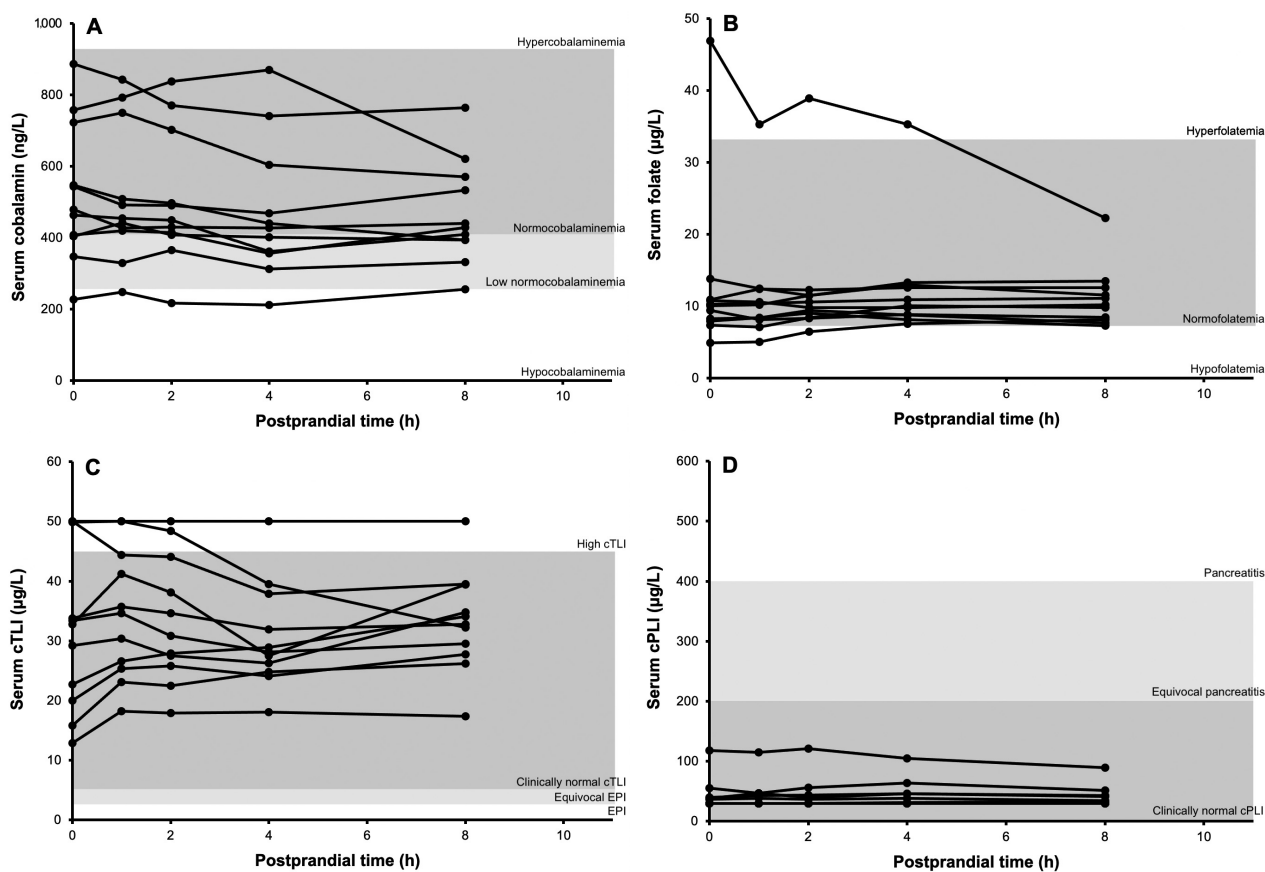


Figure 1—Serum concentrations of cobalamin (A), folate (B), cTLI (C), and cPLI (D) in 11 healthy dogs that were sampled after food but not water was withheld for 12 hours (time 0; baseline), fed their regular diet or a standardized diet, and then sampled at 1, 2, 4, and 8 hours postprandially. Each line represents an individual dog, each solid circle represents a dog's result for the given assessment time, and shading delineates various clinical interpretations on the basis of guidelines provided by the diagnostic laboratory for ranges of results for each analyte.

trointestinal disease on the basis of findings from thorough clinical history assessments and physical examination.

Feeding

Ten dogs consumed the required caloric target ($\geq 50\%$ RER), whereas 1 dog consumed slightly less (313 kcal) than its target caloric intake (326 kcal) but was included in analyses because the finding was reported to have been representative of the dog's typical long-term feeding routine. The overall mean caloric intake was 69% RER (SD, 18% RER) for all 11 dogs. Four dogs required the provision of an alternative diet (standardized study diet^a) because they lacked interest in their regular diet in the hospital. The mean dry matter protein content was 7.8 g/100 kcal (SD, 1.3 g/100 kcal), and the mean dry matter fat content was 4.4 g/100 kcal (SD, 0.5 g/100 kcal). No dogs had diarrhea, vomiting, or regurgitation during the study period.

Analytes

Skewness of data was observed for all serum analyte concentration variables. However, the assumptions of the mixed-effects ANOVA with repeated measures were sufficiently met in that the residual plots showed no sign of meaningful homoscedasticity and the Q-Q plots showed residuals that were sufficiently normally distributed.

Cobalamin—The median baseline serum cobalamin concentration was 479 ng/L (IQR, 406 to 636 ng/L; $n = 11$; **Table 1**), which was within the laboratory's reference limits (RI, 251 to 908 ng/L). Results of baseline blood samples indicated that 10 dogs were normocobalaminemic and 1 dog was hypocobalaminemic (**Figure 1**). All dogs that were normocobalaminemic at baseline had results within reference limits for all postprandial assessments. For the single dog that was hypocobalaminemic (228 ng/L) at baseline, results for the analyte fluctuated over time (lowest concentration [212 ng/L] at 4 hours postprandially) but were within reference limits for the sample obtained 8 hours postprandially (256 ng/L). Compared with results for baseline blood samples, the median serum cobalamin concentration was significantly lower at 4 hours (428; IQR, 360 to 536; estimated mean of differences, -54 ng/L; $P = 0.008$) and 8 hours (429; IQR, 394 to 552; estimated mean of differences, -59 ng/L; $P = 0.004$) postprandially.

Folate—The median baseline serum folate concentration was 10.1 $\mu\text{g/L}$ (IQR, 8.1 to 10.9 $\mu\text{g/L}$; $n = 11$; **Table 1**), which was within the laboratory's reference limits (RI, 7.7 to 24.4 $\mu\text{g/L}$). Results for baseline blood samples indicated that 8 dogs were normofolatememic, 2 dogs were hypofolatememic, and 1 dog was hyperfolatememic (**Figure 1**). One dog that was normofolatememic (8.3 $\mu\text{g/L}$) at baseline was hypofolatememic (7.3 $\mu\text{g/L}$) at the postprandial 8-hour assessment. Both dogs that were hypofolatememic at

baseline (4.9 and 7.4 $\mu\text{g/L}$) were normofolatememic at the 2-hour or 8-hour postprandial assessment. The 1 dog that was hyperfolatememic at baseline (46.9 $\mu\text{g/L}$) was normofolatememic at the 8-hour postprandial assessment. The samples from this dog were moderately hemolyzed at the baseline assessment: mildly hemolyzed at the 1-, 2-, and 4-hour postprandial assessments; and nonhemolyzed at the 8-hour postprandial assessment. Overall, compared with baseline results, there was no meaningful change in serum folate concentration detected at any postprandial time point.

cTLI—The median baseline serum cTLI concentration was 32.8 $\mu\text{g/L}$ (IQR, 21.4 to 41.8 $\mu\text{g/L}$; $n = 11$; **Table 1**), which was within the laboratory's reference limits (RI, 5.7 to 45.2 $\mu\text{g/L}$). Results for serum cTLI concentration were within reference limits for 8 dogs, above reference limits for 3 dogs, and below reference limits (consistent with a diagnosis of EPI) for no dogs (**Figure 1**). Of the 3 dogs that had high serum concentrations of cTLI at baseline (reported as $> 50 \mu\text{g/L}$, recorded for analysis as 51.0 $\mu\text{g/L}$), 1 had results consistently above reference limits across the postprandial assessments, whereas 2 had results within reference limits at the postprandial 1- or 4-hour assessment and within reference limits for subsequent assessments. All 3 of these dogs had results for ≥ 1 measurement, but not all measurements exceeded the upper limit of the working range of the assay (50.0 $\mu\text{g/L}$). The results for these 3 dogs were included in the analysis, with serum cTLI concentration recorded as 51 $\mu\text{g/L}$ for analysis purposes for any result $> 50 \mu\text{g/L}$ (the upper limit of the working range of the assay). Overall, compared with baseline results, there was no meaningful change in serum cTLI concentration detected at any postprandial time point.

cPLI—Results for serum cPLI concentration were $< 30 \mu\text{g/L}$ (the lower limit of the working range of the assay) for all 5 time points (baseline and postprandial 1-, 2-, 4-, and 8-hour assessment time points) for 5 dogs; therefore, these dogs were removed from the cPLI analysis. Of the remaining 6 dogs, 1 had serum cPLI concentrations $< 30 \mu\text{g/L}$ for 3 of 5 assessments, and this dog was included in the analysis with those 3 low results for cPLI concentration recorded as 29 $\mu\text{g/L}$. The median baseline serum concentration of cPLI was 39.0 $\mu\text{g/L}$ (IQR, 37.2 to 51.2 $\mu\text{g/L}$; $n = 6$; **Table 1**), which was within the laboratory's reference limits (RI, 0 to 200 $\mu\text{g/L}$). Compared with baseline results, there was no meaningful change in serum cPLI concentration detected at any postprandial time point.

Lipemia

Eleven of the 55 samples had a lipemic appearance when they arrived at the testing site. Of the 11 samples affected, 3 were postprandial 1-hour samples, 3 were postprandial 2-hour samples, and 5 were

postprandial 4-hour samples. None of the baseline or postprandial 8-hour samples were lipemic. When evaluated with a mixed-effects ANOVA model that controlled for sample time point, the presence of lipemia at the testing site (ie, the need to centrifuge samples once at the collection site and again at the testing site because of lipemia) had no significant impact on results for serum concentrations of cobalamin ($P = 0.808$), folate ($P = 0.871$), cTLI ($P = 0.728$), or cPLI ($P = 0.757$).

Discussion

Our findings indicated that the median serum cobalamin concentration was significantly lower in samples obtained 4 and 8 hours postprandially, compared with baseline samples (those collected after food was withheld 12 hours), for dogs of the present study. However, the differences observed would not impact the clinical interpretation of the test results; thus, we considered the differences to have been clinically insignificant.

A large study⁸ in human medicine shows that duration of fasting and results for serum cobalamin concentrations were weakly associated in males, with a decrease in cobalamin of -0.9 ng/L for each additional hour of fasting. The magnitude of this finding was considered clinically insignificant by the authors, who recommend against a fasting requirement for cobalamin measurement in people. In contrast, such an inverse relationship between duration of food withholding and results for serum concentration of cobalamin was not detected for dogs in the present study in that the median serum cobalamin concentration was higher for samples obtained 12 hours versus 4 or 8 hours postprandially but did not meaningfully differ for samples obtained 12 hours versus 1 or 2 hours postprandially. Our findings also differed from those of experimental trials that show peak serum cobalamin concentrations following oral ingestion of supraphysiologic doses of cobalamin occurred at 4 hours in dogs¹⁷ and 1 to 7 hours in humans.^{18,19} Of note, the dogs in our study only received physiologic doses of cobalamin in their diets, whereas dogs that received oral supplementation of cobalamin at the time of evaluation could have had more substantial changes in serum cobalamin concentrations between blood samples obtained after withholding food versus postprandially.

In the present study, 10 of the 11 dogs had baseline serum cobalamin concentrations within reference limits. This finding indicated adequate provision of dietary cobalamin and appropriate cobalamin absorption. Cobalamin intake was controlled in the present study by feeding dogs their regular maintenance diet or a standardized diet that contained the National Research Council's recommended¹⁵ 35 μ g of cobalamin/kg of dry matter. The fluctuations in results observed over the study period were therefore likely attributable to changes in absorption, distribution, or metabolism of cobalamin or changes in the compo-

sition of the gastrointestinal microbiota.²⁰ Given the ileal site of cobalamin absorption,⁴ it was possible that delayed gastric emptying²¹ might have prevented the expected postprandial peak in serum cobalamin concentration in dogs of the present study. A study²¹ of healthy, client-owned dogs kept in a hospital setting (as performed in the present study) shows that gastric emptying could be delayed 3-fold, presumably because of stress and anxiety. In addition, serum cobalamin and transcobalamin concentrations in individual people vary by 10% over a 24-hour period and are not associated with mealtimes.²² Instead, this variation in people correlates with changes in serum albumin concentration.^{22,23} The concept of biological variation within an individual over periods of days to months is well demonstrated in human medicine, with variability in serum cobalamin concentration results within individuals previously reported to be between 6% and 16%.²⁴⁻²⁸ Although variability of serum cobalamin concentration within individual dogs has not been evaluated, biological variation in routine serum biochemical analytes^{29,30} and cPLI^{30,31} has been observed in healthy research and client-owned dogs. If biological variation is a meaningful factor in serum cobalamin concentrations in dogs, the cobalamin fluctuations identified in the present study could have been part of normal daily fluctuations attributable to factors such as serum protein concentrations and not directly related to the duration of withholding food.

Because of the low magnitude of change in serum cobalamin concentrations between baseline and postprandial results (-7 to -59 ng/L) for dogs of the present study, it was unlikely that a sample collected 1 to 8 hours postprandially, versus after 12 hours of withholding food, would yield results that would alter clinical interpretation. This is because dogs with serum cobalamin concentrations within the lower aspect (eg, 251 to 400 ng/L) of the reference limits (251 to 908 ng/L) are at risk of cobalamin deficiency on a cellular level, and supplementation is often recommended in a clinical setting.³² The recommended use of cobalamin supplementation for patients with hypcobalaminemia or low normocobalaminemia, therefore, makes the exact distinction between whether a sample is slightly above or slightly below the lower reference limit less important to clinicians. Similarly, in dogs, there is currently no known clinical significance of serum cobalamin concentrations above the upper reference limit (> 908 ng/L), making the distinction between whether a sample is within or above the upper reference limit unnecessary. In the single dog that was hypcobalaminemic in the present study, the fluctuation in postprandial results for serum cobalamin concentration failed to support a clear positive or negative linear trend. Further, because all dogs in the present study had cTLI concentrations above the diagnostic cutoff for EPI, the few hypcobalaminemic samples observed were more likely related to subclinical distal small intestinal disease than to extrinsic factor insufficiency.^{2,4}

Feeding did not significantly affect the median folate concentration in the dogs of the present study. However, the interpretation of the folate concentration results for 4 dogs varied over the sampling period. Most notably, one dog with mild hypofolatemia (7.4 µg/L) and another with moderate hypofolatemia (4.9 µg/L) at baseline were later normofolatememic at their postprandial 2- or 8-hour assessments, respectively. In people, bioavailability studies^{33,34} of folate-rich foods show that serum folate concentrations peak at 2 hours postprandially and return to baseline by 4 to 7 hours postprandially. Therefore, it was possible that the serum folate concentrations in the dogs with baseline hypofolatemia in the present study corrected as a result of postprandial absorption of dietary folate during the sampling period. The reasons that this was not replicated in the broader group could have included variations in dietary folate intake and gastric emptying times³ and biological variation within individual dogs. Although folate abnormalities have been previously documented in dogs affected by chronic enteropathy³⁵⁻³⁷ and EPI,³⁸ the prognostic significance of hypofolatemia and the necessity for supplementation of folate in hypofolatememic dogs has not been established.

Results indicated that withholding food had negligible effects on serum concentrations of cTLI or cPLI in dogs of the present study. These findings supported those from previous studies^{9,10} that show no change in serum cTLI or cPLI concentrations at any time point over a 6- to 8-hour period following feeding. Although an RI for serum cTLI concentration has been established for healthy dogs (5.7 to 45.2 µg/L), interpretation of results below the lower reference limit and diagnosis of clinical EPI is complicated by the potential spectrum of pancreatic acinar atrophy in EPI.⁵ A serum cTLI concentration < 2.5 µg/L is considered diagnostic for EPI, whereas concentrations between 2.5 and 5.7 µg/L may be associated with EPI but are more often related to factors other than pancreatic disease.⁵ Thus, the estimated mean of differences in serum cTLI concentration (-1.3 to 2.6 µg/L) observed in dogs of the present study may have prevented a correct diagnosis of EPI in some dogs, suggesting that withholding food before taking a blood sample to assess the serum cTLI concentration would be preferable. Further studies evaluating the effect of feeding in dogs with suspected EPI are indicated.

Our findings indicated that when blood samples were centrifuged adequately before testing, postprandial lipemia did not affect serum concentrations of cobalamin, folate, cTLI, or cPLI. This tolerance for lipemia was consistent with a previously reported cPLI assay validation study¹⁶ and the manufacturer's performance specifications^{39,40} for the analyzer used to measure cobalamin and folate concentrations in the present study. However, the additional centrifugation process required for lipemic postprandial sam-

ples could obstruct diagnostic laboratory workflow and result in a higher number of laboratory errors, suggesting that samples obtained after withholding food would be preferable.⁴¹

The primary limitation of the present study was the small sample size, which was exacerbated for our analysis of serum cPLI concentrations because 5 dogs were censored for having all results below the lower limit of the working range of the assay. We recognize that the small sample size increased the potential for type II error and the likelihood of mischaracterizing the effect of withholding food on these analytes. Sample size limitations may have been ameliorated by wider recruitment or replicate testing of enrolled dogs over multiple days. Another limitation was the unknown applicability of our findings in the healthy dogs of the present study to dogs with gastrointestinal disease.

Results indicated that withholding food had limited effects on serum concentrations of cobalamin, folate, cTLI, or cPLI in dogs and that such effects were limited to a clinically insignificant decrease in serum cobalamin concentrations at 4 and 8 hours postprandially and small changes in serum concentrations of folate and cTLI that did not reach statistical significance but that could potentially change the clinical interpretation of results for some dogs. Given these findings and the additional processing requirements for lipemic samples, we recommend that whenever possible food be withheld for 8 hours before measurement of serum concentrations of cobalamin, folate, cTLI, or cPLI in dogs. Furthermore, a similar evaluation should be performed in dogs with gastrointestinal disease to determine whether the withholding of food has a more meaningful diagnostic impact in that population.

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The authors declare that there were no conflicts of interest.

Footnotes

- a. Purina Pro Plan Veterinary Diets EN Gastroenteric Canine Formula, Nestlé Purina PetCare Co, St Louis, Mo.
- b. Steiner JM, Distinguished Professor, Texas A&M University, College Station, Tex: Personal communication, 2020.
- c. Immulite 2000, Siemens Healthcare Diagnostics Inc, Deerfield, Ill.
- d. Spec cPL, Idexx Laboratories, Westbrook, Me.
- e. R: a language and environment for statistical computing, version 3.3, R Foundation for Statistical Computing, Vienna, Austria. Available at: www.R-project.org. Accessed April 21, 2020.
- f. nlme: Linear and nonlinear mixed effects models, version 3.1-103, R Foundation for Statistical Computing, Vienna, Austria. Available at: CRAN.R-project.org/package=nlme. Accessed May 1, 2020.

References

1. Xenoulis PG, Steiner JM. Canine and feline pancreatic lipase immunoreactivity. *Vet Clin Pathol* 2012;41:312-324.
2. Ruau CG. Cobalamin in companion animals: diagnostic marker, deficiency states and therapeutic implications. *Vet J* 2013;196:145-152.
3. Suchodolski JS, Steiner JM. Laboratory assessment of gastrointestinal function. *Clin Tech Small Anim Pract* 2003;18:203-210.
4. Kather S, Grützner N, Kook PH, et al. Review of cobalamin status and disorders of cobalamin metabolism in dogs. *J Vet Intern Med* 2020;34:13-28.
5. Wiberg ME, Nurmi A-K, Westermarck E. Serum trypsinlike immunoreactivity measurement for the diagnosis of subclinical exocrine pancreatic insufficiency. *J Vet Intern Med* 1999;13:426-432.
6. Idexx Reference Laboratories. Directory of tests and services. Available at: idexx.com/en/veterinary/reference-laboratories/tests-and-services/. Accessed Nov 22, 2020.
7. Texas A&M University College of Veterinary Medicine and Biochemical Sciences. Gastrointestinal Laboratory sample requirements. Available at: vetmed.tamu.edu/gilab/service/sample-requirements. Accessed May 25, 2020.
8. Orton DJ, Naugler C, Sadrzadeh SMH. Fasting time and vitamin B12 levels in a community-based population. *Clin Chim Acta* 2016;458:129-132.
9. James FE, Mansfield CS, Steiner JM, et al. Pancreatic response in healthy dogs fed diets of various fat compositions. *Am J Vet Res* 2009;70:614-618.
10. Steiner JM, Ruau CG, Williams DA. Influence of feeding on serum canine pancreatic lipase immunoreactivity concentrations. *Vet Med (Auckl)* 2014;5:139-142.
11. Steiner JM, Williams DA. Influence of feeding on serum feline trypsin-like immunoreactivity. *Am J Vet Res* 1999;60:895-897.
12. Willems RA. Regulatory issues regarding the use of food and water restriction in laboratory animals. *Lab Anim (NY)* 2009;38:325-328.
13. Harris JP, Parnell NK, Griffith EH, et al. Retrospective evaluation of the impact of early enteral nutrition on clinical outcomes in dogs with pancreatitis: 34 cases (2010-2013). *J Vet Emerg Crit Care (San Antonio)* 2017;27:425-433.
14. Mohr AJ, Leisewitz AL, Jacobson LS, et al. Effect of early enteral nutrition on intestinal permeability, intestinal protein loss, and outcome in dogs with severe parvoviral enteritis. *J Vet Intern Med* 2003;17:791-798.
15. National Research Council. *Nutrient requirements of dogs and cats*. Washington, DC: National Academies Press, 2006.
16. Huth SP, Relford R, Steiner JM, et al. Analytical validation of an ELISA for measurement of canine pancreas-specific lipase. *Vet Clin Pathol* 2010;39:346-353.
17. Rosenthal HL, Hampton JK Jr. The absorption of cyanocobalamin (vitamin B12) from the gastrointestinal tract of dogs. *J Nutr* 1955;56:67-82.
18. Carkeet C, Dueker SR, Lango J, et al. Human vitamin B₁₂ absorption measurement by accelerator mass spectrometry using specifically labeled ¹⁴C-cobalamin. *Proc Natl Acad Sci* 2006;103:5694-5699.
19. Dawson EB, Evans DR, Conway ME, et al. Vitamin B12 and folate bioavailability from two prenatal multivitamin/multimineral supplements. *Am J Perinatol* 2000;17:193-199.
20. Kasiraj AC, Harmoinen J, Isaiah A, et al. The effects of feeding and withholding food on the canine small intestinal microbiota. *FEMS Microbiol Ecol* 2016;92:fiw085.
21. Warrit K, Boscan P, Ferguson LE, et al. Effect of hospitalization on gastrointestinal motility and pH in dogs. *J Am Vet Med Assoc* 2017;251:65-70.
22. Hvas AM, Gravholt CH, Nexø E. Circadian variation of holotranscobalamin (holo-TC) and related markers. *Clin Chem Lab Med* 2005;43:760-764.
23. Björkstén KS, Thorell L-H, Nexø E. Circadian variation of plasma cobalamin, transcobalamin-bound cobalamin and unsaturated binding capacity of transcobalamin and haptocorrin in healthy elderly. *J Affect Disord* 1995;36:37-42.
24. Bor MV, Nexø E, Hvas AM. Holo-transcobalamin concentration and transcobalamin saturation reflect recent vitamin B12 absorption better than does serum vitamin B12. *Clin Chem* 2004;50:1043-1049.
25. McArthur JO, Tang H, Petocz P, et al. Biological variability and impact of oral contraceptives on vitamins B₆, B₁₂ and folate status in women of reproductive age. *Nutrients* 2013;5:3634-3645.
26. Nexø E, Hvas AM, Bleie Ø, et al. Holo-transcobalamin is an early marker of changes in cobalamin homeostasis. A randomized placebo-controlled study. *Clin Chem* 2002;48:1768-1771.
27. McKinley MC, Strain JJ, McPartlin J, et al. Plasma homocysteine is not subject to seasonal variation. *Clin Chem* 2001;47:1430-1436.
28. Brokner M, Hager HB, Lindberg M. Biological variation of holotranscobalamin and cobalamin in healthy individuals. *Scand J Clin Lab Invest* 2017;77:433-436.
29. Ruau CG, Carney PC, Suchodolski JS, et al. Estimates of biological variation in routinely measured biochemical analytes in clinically healthy dogs. *Vet Clin Pathol* 2012;41:541-547.
30. Walton RM. Subject-based reference values: biological variation, individuality, and reference change values. *Vet Clin Pathol* 2012;41:175-181.
31. Carney PC, Ruau CG, Suchodolski JS, et al. Biological variability of C-reactive protein and specific canine pancreatic lipase immunoreactivity in apparently healthy dogs. *J Vet Intern Med* 2011;25:825-830.
32. Berghoff N, Suchodolski JS, Steiner JM. Association between serum cobalamin and methylmalonic acid concentrations in dogs. *Vet J* 2012;191:306-311.
33. Fenech M, Noakes M, Clifton P, et al. Aleurone flour is a rich source of bioavailable folate in humans. *J Nutr* 1999;129:1114-1119.
34. Prinz-Langenohl R, Brönstrup A, Thorand B, et al. Availability of food folate in humans. *J Nutr* 1999;129:913-916.
35. Batt RM, Morgan JO. Role of serum folate and vitamin B12 concentrations in the differentiation of small intestinal abnormalities in the dog. *Res Vet Sci* 1982;32:17-22.
36. Xu J, Verbrugghe A, Lourenço M, et al. Does canine inflammatory bowel disease influence gut microbial profile and host metabolism? *BMC Vet Res* 2016;12:114.
37. Craven M, Simpson JW, Ridyard AE, et al. Canine inflammatory bowel disease: retrospective analysis of diagnosis and outcome in 80 cases (1995-2002). *J Small Anim Pract* 2004;45:336-342.
38. Soetart N, Rochel D, Drut A, et al. Serum cobalamin and folate as prognostic factors in canine exocrine pancreatic insufficiency: an observational cohort study of 299 dogs. *Vet J* 2019;243:15-20.
39. Vitamin B12 assay [package insert]. Los Angeles: Diagnostic Products Corp, 2006.
40. Folic acid [package insert]. Los Angeles: Diagnostic Products Corp, 2006.
41. Mainali S, Davis SR, Krasowski MD. Frequency and causes of lipemia interference of clinical chemistry laboratory tests. *Pract Lab Med* 2017;8:1-9.