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Seasonal variation in serum metabolites of northern European dogs

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Abstract

Background: Metabolic profiling identifies seasonal variance of serum metabolites in humans. Despite the presence of seasonal disease patterns, no studies have assessed whether serum metabolites vary seasonally in dogs.

Hypothesis: There is seasonal variation in the serum metabolite profiles of healthy dogs.

Animals: Eighteen healthy, client-owned dogs.

Methods: A prospective cohort study. Serum metabolomic profiles were assessed monthly in 18 healthy dogs over a 12-month period. Metabolic profiling was conducted using a canine-specific proton nuclear magnetic resonance spectroscopy platform, and the effects of seasonality were studied for 98 metabolites using a cosinor model. Seasonal component was calculated, which describes the seasonal variation of each metabolite.

Results: We found no evidence of seasonal variation in 93 of 98 metabolites. Six metabolites had statistically significant seasonal variance, including cholesterol (mean 249 mg/dL [6.47 mmol/L] with a seasonal component amplitude of 9 mg/dL [0.23 mmol/L]; 95% confidence interval [CI] 6-13 mg/dL [0.14-0.33 mmol/L], $P < .008$), with a peak concentration of 264 mg/dL (6.83 mmol/L) in June and trough concentration of 236 mg/dL (6.12 mmol/L) in December. In contrast, there was a significantly lower concentration of lactate (mean 20 mg/dL [2.27 mmol/L] with a seasonal component amplitude of 4 mg/dL [0.42 mmol/L]; 95% CI 2-6 mg/dL [0.22-0.62 mmol/L], $P < .001$) during the summer months compared to the winter months, with a peak concentration of 26 mg/dL (2.9 mmol/L) in February and trough concentration of 14 mg/dL (1.57 mmol/L) in July.

Conclusions and Clinical Importance: We found no clear evidence that seasonal reference ranges need to be established for serum metabolites of dogs.

KEYWORDS
canine, cholesterol, lactate, lipid, metabolomics

Abbreviations: GC-MS, gas chromatography coupled to mass spectrometry; HDL, high-density lipoproteins; HfSA, Hospital for Small Animals; ^{1}H NMR, proton nuclear magnetic resonance; LC-MS, liquid chromatography coupled with single-stage or tandem mass spectrometry; PUFAs, polyunsaturated fatty acids; R(D)SVS, Royal (Dick) School of Veterinary Science.

1 | INTRODUCTION

Both seasonal and diurnal influences affect concentrations of serum metabolites in people. For example, lipid metabolite analysis reveals a significant increase in cholesterol concentrations in the autumn and winter months, most pronounced in regions of greater seasonal climatic variation.[1] These seasonal metabolic variances mirror changes in the manifestation of clinical diseases, such as an increased incidence of cardiovascular events in the autumn and winter months.[2] This causes speculation that the seasonal metabolic changes might be influencing disease risk.

In contrast to human medicine, there are a dearth of studies which examine whether seasonal changes occur in serum metabolite concentrations in dogs. This is particularly surprising given the seasonal fluctuation in the prevalence of important diseases. The prevalence of several non-vector borne disease states are seasonal, including immune-mediated hemolytic anemia,[3] cyclical alopecia and hypothyroidism,[4] and the prognosis of some diseases, such as parvoviral enteritis, show seasonal variance.[5]

The rapid growth of interest in metabolomics as a tool for human medicine has resulted in studies exploring the factors influencing metabolic profiles. This includes temporal influences, such as whether there are seasonal, or circadian variations in the metabolome.[6] In contrast to classical biochemical approaches which focus on a single or set of linked metabolites, metabolomics collects comprehensive data on a wide array of metabolites.[7] This approach allows clinicians to gain a holistic understanding of metabolism and metabolic shifts, resulting in widespread uptake of the technology in human medicine.[8] In human patients, metabolic profiles correlate with the presence and prognosis of neoplastic and cardiovascular diseases,[9,10] and can be further used to predict adverse effects and facilitate early discontinuation of ineffective or harmful treatments.[11] Advances in metabolic profiling are thereby paving the way for individualized medicine and therapeutic drug targeting.[12]

The expansion of metabolome studies is facilitated by increased availability of high throughput, highly reproducible analytical methods such as proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.[13] Other commonly used metabolomics techniques include gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled with single-stage or tandem mass spectrometry (LC-MS). The advantages of ^{1}H NMR spectroscopy over mass spectrometry methods include analysis speed, high throughput, easy sample preparation, high reproducibility and capability of generating quantitative results.[13]

The aim of this study was to evaluate whether seasonal variation occurred in the monthly metabolite profiles from 18 healthy dogs fed a standardized diet over a 12-month period, analyzed using a validated, canine-specific ^{1}H NMR metabolomics platform.

2 | MATERIALS AND METHODS

Metabolomic profiles were assessed in 18 healthy dogs every month over a 12-month time period. The study was undertaken at the Hospital for Small Animals (HfSA), Royal (Dick) School of Veterinary Studies (R(D)SVS), at the University of Edinburgh and was approved by the University of Edinburgh Animal Welfare and Ethics Review Board. All dogs were household pets with daily access to the outdoors and lived within 15 miles of the hospital. All dogs were considered to be clinically disease-free as assessed by history and physical examination and were not on any chronic (defined as >2 weeks) medications. At the time of enrollment, a basic biochemical profile was performed, and a hematology profile was performed on conclusion of the study, to exclude dogs with subclinical systemic disease. Throughout the study, a standard diet was fed to all dogs, with no treats or supplementary food. All dogs were fed maintenance energy requirement (MER) ranging from 1.4 to 1.8 $\times$ resting energy requirement (RER) based on the manufacturer's recommendations. All dogs had a body condition score (BCS) assessed monthly at the time of sample collection and the feeding recommendations were adjusted to maintain a body condition score of 5/9, which was maintained by all dogs throughout the study period. The study sample was previously outlined by Hurst et al.[14]

Blood samples were collected monthly from each animal beginning July 2015 until June 2016 and placed into plain, ethylenediaminetetraacetic acid (EDTA), and lithium heparin blood collection tubes (as per manufacturer's instructions), which were refrigerated after collection. Dogs were fed according to their normal schedule throughout the study period, and dogs were usually sampled in the mornings. Serum and plasma were separated by centrifugation within 4 hours of collection and aliquoted. Samples were stored frozen at -80°C until analysis, which was performed in March 2019.

All samples were analyzed by Petbiomics Ltd using a Bruker AVANCE III HD 500 ^{1}H NMR spectrometer (Bruker Biospin, Rheinstetten, Germany). The canine-specific platform has been recently validated and reference intervals for the individual metabolites determined by Ottka et al.[15] Ninety-eight individual metabolites were included in the ^{1}H NMR platform, as outlined in Figure 1. These were selected to reflect metabolite subclasses that have seasonal variance in human medicine and in other animal species.

Metabolomic data were imported into the R statistical system[16] for generation of statistical figures and analysis. Mean (and 95% confidence interval [CI]) metabolite values were calculated for each sampling month. Each metabolite was tested for presence of an annual seasonal pattern in the mean values using a "cosinor"[17] model estimated using the "cosinor" function in the R "season" package. Cosinor models fit a sinusoidal component to a time series and allow testing for the existence of this sinusoidal component at a specified significance threshold. Thresholds for significance were set at $P < .05$ with a Bonferroni correction for each metabolite group (ie, $P < .05/n$). The seasonality effect size is reported as the amplitude component of the cosinor model (calculated as the square root of the sum of the squares of the sine and cosine coefficients).[16] As the cosinor model has 2 components to seasonality we do not calculate exact P-values but determine significance compared to a critical value.[16]

3 | RESULTS

The age of the dogs at the start of the study was 6.1 years (median; range, 1.1-11.7 years). Of the 18 dogs, 1 was an unneutered male, 2 were unneutered females, 7 were neutered males, and 8 were

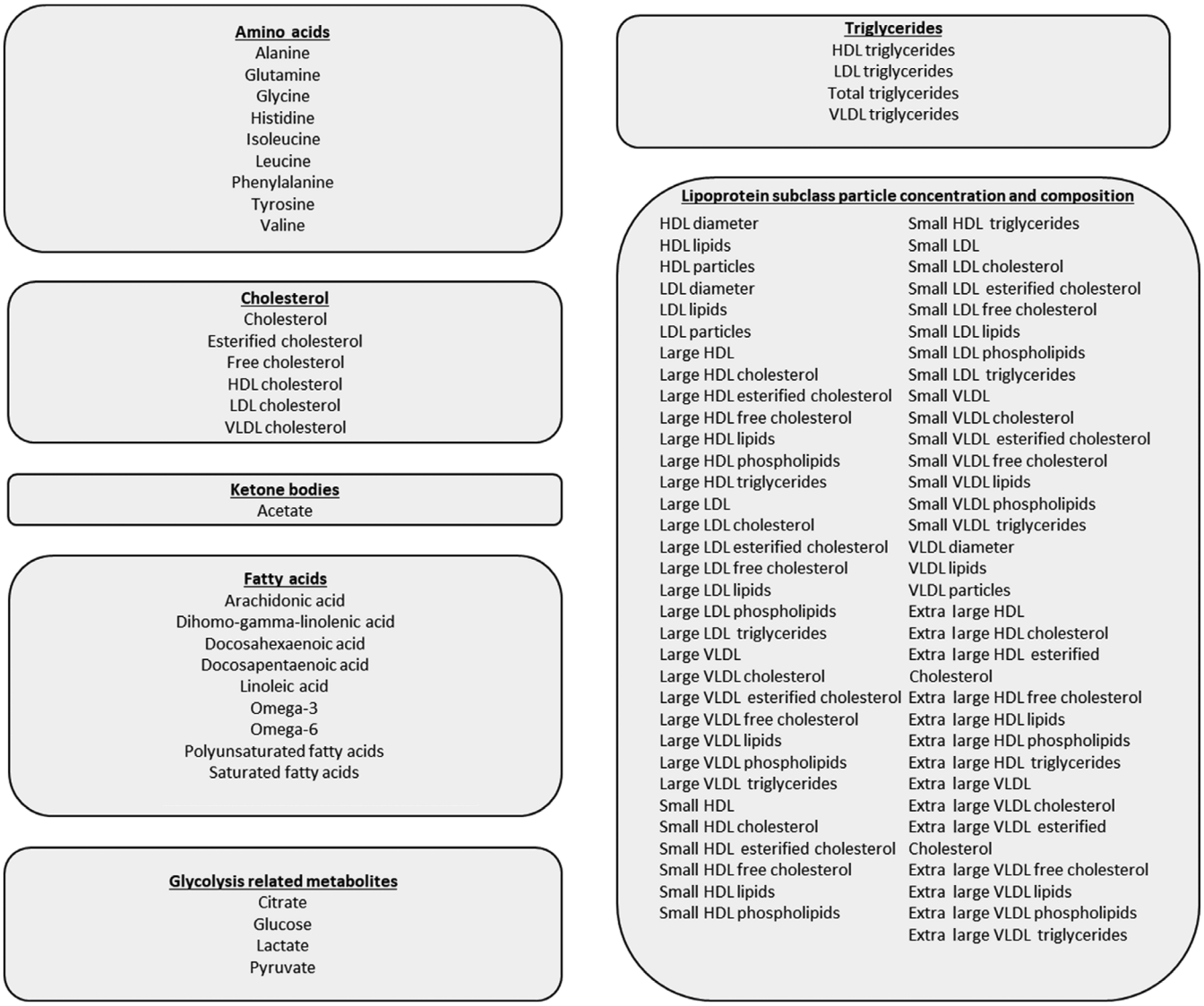

FIGURE 1 Groupings of metabolite classes (HDL, high density lipoproteins; LDL, low-density lipoproteins; VLDL, very low-density lipoproteins) analyzed from 18 healthy dogs sampled monthly over a 12-month period in a temporal climate using a canine-specific proton nuclear magnetic resonance spectroscopy platform

neutered females. There were 8 breeds included in the study, including crossbreed (n = 8), Labrador retriever (n = 3), lurcher (n = 2), and 1 each of: cocker spaniel, border collie, Greyhound, Hungarian Vizsla, and Jack Russell terrier.

The serum concentrations of 98 metabolites were measured, listed in Figure 1, which were subdivided into 7 metabolic classes: amino acids, cholesterol, ketone metabolites, fatty acids, glycolysis related metabolites, triglycerides, and lipoprotein subclass particle concentration and composition.

There were modest, but statistically significant, increases in serum lipid measurements in the summer months, in comparison to the winter (Figure 2). Of the 98 metabolites measured; cholesterol (mean 249 mg/dL [6.47 mmol/L] with a seasonal component amplitude of 9 mg/dL [0.23 mmol/L]; 95% CI 6-13 mg/dL [0.14-0.33 mmol/L], $P < .008$) a peak mean of 264 mg/dL (6.83 mmol/L) in June and trough mean of 236 mg/dL (6.12 mmol/L) in December, esterified cholesterol (mean 200 mg/dL [5.18 mmol/L] with a seasonal component amplitude of 7 mg/dL [0.18 mmol/L]; 95% CI 4-10 mg/dL [0.11-0.25 mmol/L], $P < .008$; peak mean of 211 mg/dL [5.47 mmol/L] in June and trough mean of 190 mg/dL [4.91 mmol/L] in December), free cholesterol (mean 50 mg/dL [1.3 mmol/L] with a seasonal component amplitude of 2 mg/dL [0.06 mmol/L]; 95% CI 1-3 mg/dL [0.03-0.08 mmol/L], $P < .008$; peak mean of 53 mg/dL (1.38 mmol/L) in June and trough mean of 47 mg/dL [1.22 mmol/L] in December), high density lipoprotein cholesterol (mean 214 mg/dL [5.53 mmol/L] with a seasonal component amplitude of 6 mg/dL [0.15 mmol/L]; 95% CI 3-8 mg/dL [0.09-0.21 mmol/L], $P < .008$; peak mean of 221 mg/dL [5.72 mmol/L] in June and trough mean of 205 mg/dL [5.31 mmol/L] in December), and PUFAs (mean 8.13 mmol/L with a seasonal component amplitude of 0.2; 95% CI

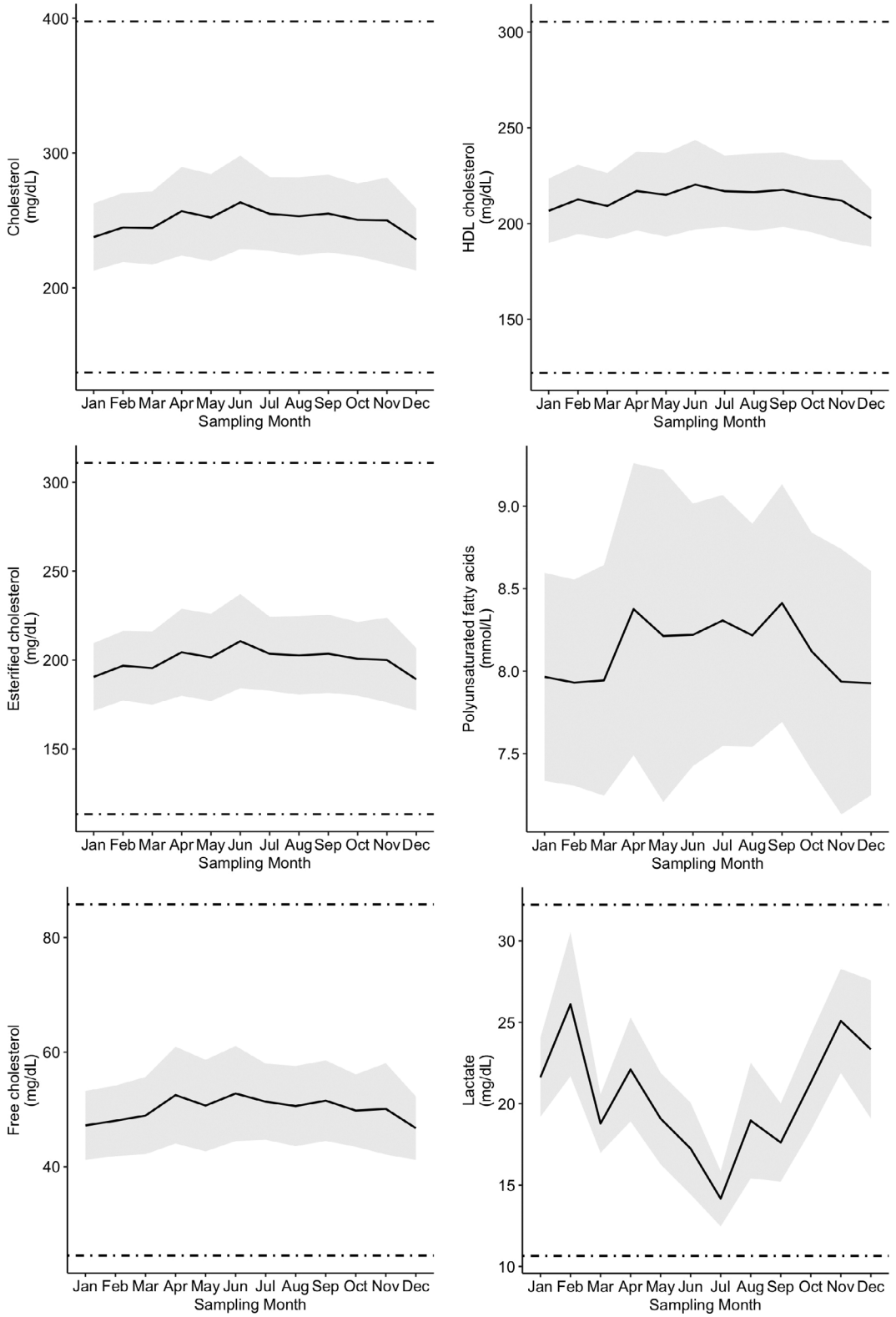

FIGURE 2 Mean monthly analyte concentrations of 6 analytes from 18 healthy dogs sampled monthly over a 12-month period in a temporal climate using a canine-specific proton nuclear magnetic resonance spectroscopy platform, which showed a significant seasonal variation in concentration. The dotted lines represent the reference interval, and shaded region the 95% confidence interval

Metabolite	Alpha	Peak mean	Trough mean
Cholesterol, mg/dL (mmol/L)	.008	264 (6.83)	236 (6.12)
Esterified cholesterol, mg/dL (mmol/L)	.008	211 (5.47)	190 (4.91)
Free cholesterol, mg/dL (mmol/L)	.008	53 (1.38)	47 (1.22)
HDL cholesterol, mg/dL (mmol/L)	.008	221 (5.72)	205 (5.31)
Polyunsaturated fatty acids, (mmol/L)	.005	(8.41)	(7.95)
Lactate, mg/dL (mmol/L)	.001	26 (2.9)	14 (1.7)

TABLE 1 Statistical analysis of the 6 metabolites showing significant seasonal variation when measured from 18 healthy dogs sampled monthly over a 12-month period in a temporal climate, using a canine-specific proton nuclear magnetic resonance spectroscopy platform

Note: Alpha = Critical *P*-value (using Bonferroni correction) used as threshold to determine statistical significance.
Abbreviation: HDL, high density lipoprotein.

0.11-0.3, $P < .005$; peak mean of 8.41 mmol/L in September and trough mean of 7.95 mmol/L in December) all showed a seasonality with significantly higher concentrations in the summer months compared to the winter months. In contrast, there was a significantly lower concentration of lactate (mean 20 mg/dL [2.27 mmol/L] with a seasonal component amplitude of 4 mg/dL [0.42 mmol/L]; 95% CI 2-6 mg/dL [0.22-0.62 mmol/L], $P < .001$) with a peak mean of 26 mg/dL (2.9 mmol/L) in February and trough mean of 14 mg/dL (1.57 mmol/L) in July during the summer months compared to the winter months (Table 1).

4 | DISCUSSION

Using a recently validated ^{1}H NMR analytical platform, this study examined whether there was seasonal variation in serum metabolites of healthy dogs. The principal observation from our study is that we found no evidence of seasonal variation in the vast majority of metabolites measured. In addition, the statistically significant variation observed in the 6 of the 98 metabolites was modest and unlikely to have a strong bearing on the clinical interpretation of results from sick dogs. Therefore, we find little evidence to support the need for season specific reference ranges for serum metabolites in dogs.

A small but statistically significant seasonal variance was identified in cholesterol, esterified cholesterol, free cholesterol, HDL cholesterol, PUFAs and lactate. Seasonal variance in cholesterol is widely documented in humans,[18] with average cholesterol concentrations being 3% to 5% higher in winter than in summer in 1 large scale study from the United Kingdom and Japan.[19] The seasonal fluctuation in cholesterol mirrors several causes of morbidity and death, such as cardiovascular disease, and has raised concerns regarding the misclassification of hypercholesterolemia with subsequent diagnostic and treatment bias. It also raises questions regarding the requirement for season specific guidelines.[1] In contrast to humans, our study found an opposite relationship between season and cholesterol, with significantly higher concentrations in summer months and lower in winter months, with average cholesterol concentrations being 11% higher in June than in December. The clinical relevance of this finding is unclear, but is unlikely to result in seasonal misclassification of hypercholesterolemia.

Thyroid hormones T3 and T4 directly affect cholesterol and fatty acid synthesis, mobilization and degradation.[20] In states of decreased thyroid activity, both the synthesis and degradation of lipids are impaired leading to increased serum concentrations.[20] Thyroid hormone signaling has also been identified as an important transducer of photoperiod information into the neuroendocrine system via melatonin secretion in sheep and birds.[21-23] There are statistically significant, although clinically unimportant reductions in thyroxine concentrations in the summer, in comparison to the winter in healthy canine subjects,[24] and thyroxine concentrations in German Shepherd dogs in Iran are lower in the summer compared with the winter.[25] Therefore, the seasonality in cholesterol metabolism observed in our study might in part be driven by fluctuations in thyroid hormones.

This study found higher lactate concentrations in the winter than the summer months, with average lactate concentrations being 84% higher in February than in July. In comparison to cholesterol, there is a paucity of data on seasonal variance in lactate concentrations in both human and animal subjects. Seasonal variance in lactate occurs in human studies, with increased concentrations during winter months and lower concentrations in summer months at a latitude of 62°.[26] This is hypothesized to be because of a greater contribution of anaerobic glycolysis to the maintenance of energy homeostasis in the autumn/winter and an increase of lipolysis during winter-spring.[26]

This study has several limitations, including the small number of dogs in the study, all from a single geographical location. A further limitation is that several breeds and adult dogs of a variety of ages were included. The inclusion of 2 entire female dogs does raise the risk of hormonal fluctuations driving some variance. Because of the low number of entire females, the influence of this is expected to be small. The timing of collection of the blood samples also showed mild variation of a few hours; therefore, the impact of diurnal influence cannot be excluded, however as they were usually sampled in the morning, diurnal variance would be expected to be small. The time of the last feed was also not standardized; however, as dogs were fed normally and samples were taken in the mornings, the variance from mealtimes would be expected to be reasonably static throughout the time period. In our current study, samples were stored at −80°C for 3 years before analysis. In a previous study validating the NMR platform, all sample metabolites were stable at −80°C for 12 months,[15] however evidence into the impact of longer storage on canine samples is lacking. Studies looking at human metabolites, however, have shown negligible effects after storage at −80°C for 2.5 years when using an NMR platform,[27] despite this, the effect of storage on the current study's samples cannot be excluded.

In conclusion, our study revealed no significant seasonal variance in 93 of 98 metabolites. We did detect statistically significant, but clinically minor, seasonal variances in cholesterol concentrations,

peaking in summer months, which follows an inverse pattern to that seen in humans and many other species. This might be driven in part by seasonal variance in thyroid hormone concentrations. In contrast, the seasonal variance in lactate mirrors that seen in human medicine and might be because of a greater contribution of anaerobic glycolysis in the colder winter months. In contrast to human counterparts, the seasonal variance in metabolites in dogs is small, and while a statistically significant variance was identified in 6 metabolites, the clinical manifestations are likely limited.

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CONFLICT OF INTEREST DECLARATION

Claudi Ottka was an employee and Hannes Lohi is a shareholder and the chairman of the board of PetBIOMICS Ltd, who developed and provides the metabolomics test. No other authors have a conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the University of Edinburgh Animal Welfare and Ethics Review Board.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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