Evaluation of circulating concentrations of glucose homeostasis biomarkers, progesterone, and growth hormone in healthy Elkhounds during anestrus and diestrus

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Objective—To investigate whether circulating concentrations of biomarkers of glucose homeostasis, progesterone, and growth hormone in healthy female Elkhounds differ during diestrus and anestrus and to compare those findings with data from dogs of other breeds.

Animals—22 healthy female dogs of Elkhound breeds (known to have a high incidence of diestrus-associated diabetes mellitus) and 18 healthy female non-Elkhound dogs.

Procedures—For each dog, a blood sample (12 mL) was collected once during anestrus and once 2 to 8 weeks after cessation of estrual bleeding. Serum or whole blood samples were analyzed for glucose, growth hormone, insulin-like growth factor-1, C-peptide, fructosamine, and glycated hemoglobin A1c concentrations. Homeostasis model assessments (HOMAs) of pancreatic beta-cell function and insulin secretion were calculated.

Results—In Elkhounds, C-peptide concentration and the HOMA for beta-cell function (markers of insulin secretion) were higher in samples obtained during diestrus, compared with findings in samples obtained during anestrus. The HOMA for insulin sensitivity was lower (albeit not significantly) during diestrus than it was during anestrus in Elkhounds. Markers of insulin secretion and sensitivity were similar during anestrus and diestrus in the dogs of other breeds. Serum progesterone concentrations were greater during diestrus than during anestrus in Elkhounds and non-Elkhound dogs. All other variables did not differ between diestrus and anestrus within or between the 2 breed groupings.

Conclusions and Clinical Relevance—Results provided evidence that circulating insulin concentrations during diestrus are higher than those during anestrus in Elkhounds, which could contribute to development of diestrus-associated diabetes mellitus. (*Am J Vet Res* 2012;73:242–247)

Female dogs are nonseasonally monoestrus, undergoing estrus at 5- to 12-month intervals. The luteal phase of the ovarian cycle in nonpregnant dogs is similar in duration and hormone profile to that of pregnant dogs, with high circulating concentrations of progesterone.[1] Some dogs also secrete large amounts of GH from the mammary gland during diestrus in response to endogenous progesterone.[2,3] Both progesterone and GH induce insulin resistance and may potentially cause diabetes mellitus in the luteal phase (ie, diestrus-associated diabetes mellitus).[2,4,5] Marca and Loste[6] studied circulating glucose and HbA1c concentrations in 11 Beagle bitches in the different phases of estrus; both glucose and HbA1c concentrations in plasma and whole blood, respectively, were slightly elevated during diestrus, compared with other stages of the estrual cycle. Our group has previously reported that some breeds, such as Swedish and Norwegian Elkhounds, are predisposed to onset of diabetes mellitus during diestrus.[7,8]

Abbreviations

GH	Growth hormone
HbA1c	Glycated hemoglobin A1c
HOMA	Homeostasis model assessment
HOMA-B	Homeostasis model assessment for beta-cell function
HOMA-S	Homeostasis model assessment for insulin sensitivity
IGF	Insulin-like growth factor
IQR	Interquartile range

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In addition to dogs with overt diabetes, there may be some Elkhounds with subclinical hyperglycemia during diestrus that have aberrations in their insulin response, without overt clinical signs of diabetes.

The purpose of the study reported here was to investigate whether circulating concentrations of glucose homeostasis biomarkers, progesterone, and growth hormone in healthy female Elkhounds differ during diestrus and anestrus and to compare those findings with data from dogs of other breeds. A secondary aim was to compare glucose homeostasis biomarker concentrations during diestrus and anestrus in dogs within each breed grouping.

Materials and Methods

Dogs and blood sample collection—Blood samples were collected from privately owned healthy sexually intact female dogs > 4 years of age that were evaluated at Nordvärmlands Smådjurspraktik, Torsby, Sweden. Owners of dogs treated at the clinic for prophylactic interventions were informed about the study and asked whether they would allow participation of their dog. If they agreed, owners provided written consent for collection of blood samples from their dog, and were asked to contact the clinician the next time the dog was undergoing estrual bleeding the following year; subsequent sample collections were then scheduled. Blood sample collections for purposes of this study were approved by the Swedish Animal Ethical Committee, the Swedish Animal Welfare Agency, and the Swedish Board of Agriculture.

A blood sample was collected twice from each dog: once at 2 to 8 weeks after end of estrual bleeding (diestrus sample) and once at > 3 months after estrual bleeding (anestrus sample). For most dogs, the diestrus sample was collected first, and the anestrus sample was collected subsequently. For some dogs, the owners were certain that > 3 months had passed since the last estrual bleeding, and a blood sample was collected first during anestrus; a second sample was collected during diestrus after the next estrual episode.

Samples were collected from 2 groups of dogs. The first group was composed of purebred dogs from 2 Elkhound breeds (Swedish and Norwegian Elkhounds), and the second group was composed of purebred dogs from various other breeds. Each dog was clinically examined with standard procedures at the time of each sample collection to ensure the absence of other diseases. The attending veterinarian assessed body condition score at the time of sample collection on a 3-grade scale (1 = thin, 2 = normal, and 3 = overweight). Prior to sample collection, food was withheld from each dog for 12 hours. A blood sample (total volume, 12 mL) was obtained from a cephalic vein into tubes without anticoagulant (to provide serum samples) and into tubes with EDTA (to provide whole blood samples). Sera were separated from clotted blood within 60 minutes after collection. Sera and whole blood samples were stored at –20°C for 0 to 6 months and then transported on dry ice to the central laboratory for storage at –80°C until analysis. All blood sample collections and examinations were performed by the same veterinarian.

Analytic methods—Analyses were performed at the Clinical Pathology Laboratory, University Animal Hospital, Swedish University of Agricultural Sciences (assessment of serum C-peptide, fructosamine, progesterone, and glucose concentrations and whole blood HbA1c concentration) and the Clinical Pathology Laboratory, Utrecht University (assessment of serum GH and IGF-1 concentrations). Serum glucose concentrations were determined by use of a glucose hexokinase method.[a] Serum fructosamine concentration was measured by use of a colorimetric assay, which was based on the ability of ketoamines to reduce nitrotetrazolium blue formazans in an alkaline medium.[b] Serum progesterone concentration was determined by use of a solid-phase, competitive chemiluminescent enzyme immunoassay.[c] Serum C-peptide concentration was analyzed with a commercially available radioimmunoassay for canine C-peptide.[d] The instructions given for the kit were followed precisely, except that the protease inhibitor aprotinin[e] was added just before the analysis instead of immediately after sample collection as described by Fall et al.[9] The detection limit of C-peptide in serum was 0.05 nmol/L; when a result was less than the assay's detection limit, a value equivalent to half the detection limit (0.025 nmol/L) was assigned to that sample for purposes of statistical analysis. Serum GH concentration was analyzed with a commercially available radioimmunoassay for porcine and canine GH.[f] Serum IGF-1 concentration was measured by use of a heterologous radioimmunoassay after acid-ethanol extraction to remove interfering IGF-binding proteins as described in detail by Favier et al.[10] Glycated hemoglobin A1c concentration in whole blood was estimated with a glycosal HbA1c system[g] that uses boronate affinity chromatography to separate glycated hemoglobin from nonglycated hemoglobin. The method has been validated for use in dogs by Davison et al.[11] Samples were analyzed in duplicate for all hormones and HbA1c to increase the precision.

Statistical analysis—Because the variances for several variables differed considerably between comparison groups, it was decided to use nonparametric statistical methods. For all analyses, values of $P < 0.05$ were considered significant. Statistical software[h] was used for all analyses.

Homeostasis model assessment was used to estimate beta-cell function (HOMA-B) and insulin sensitivity (HOMA-S) from serum glucose and C-peptide concentrations determined after food withholding by use of the nonlinear method developed by Levy et al[12] and recently validated for dogs by Verkest et al.[13] The HOMA-B and HOMA-S were calculated also for dogs that had serum C-peptide concentrations less than the physiologic reference range in humans (0.2 to 3.5 nmol/L) established by Levy et al.[12]

Groupwise comparisons

The Wilcoxon rank sum test was used to assess whether serum glucose, fructosamine, C-peptide, progesterone, GH, and IGF-1 concentrations; whole blood HbA1c concentration; and HOMA-B and HOMA-S differed between the 2 breed groupings (Elkhounds and non-Elkhound dogs) during anestrus or during dies-

Table 1—Comparisons of serum and whole blood concentrations of glucose homeostasis biomarkers, progesterone, and GH measured during anestrus and diestrus in 22 Elkhounds and 18 dogs of other breeds, all of which were > 4 years old.

Variable†	Estrual cycle phase	Median (IQR‡)		Comparison (Bonferroni *P* value*)		
		Elkhounds	Other breeds	Elkhound vs other breeds	Anestrus vs diestrus in Elkhounds	Anestrus vs diestrus in other breeds
Glucose (mmol/L)	Anestrus	4.9 (4.4–5.1)	4.9 (4.5–5.2)	1	1	1
	Diestrus	4.5 (4.3–4.8)	4.8 (4.6–5.0)	1		
Fructosamine (µmol/L)	Anestrus	316 (293–330)	320 (298–376)	1	1	1
	Diestrus	307 (290–326)	327 (309–357)	0.9		
HbA1c (%)	Anestrus	4.1 (3.9–4.6)	4.6 (4.1–5.2)	1	1	1
	Diestrus	4.3 (4.1–4.6)	4.7 (4.4–4.8)	0.54		
C-peptide (nmol/L)	Anestrus	0.06 (0.03–0.11)	0.07 (0.025–0.11)	1	0.036	1
	Diestrus	0.13 (0.07–0.19)	0.08 (0.025–0.13)	1		
HOMA-B (%)	Anestrus	24.6 (21.4–34.4)	27.3 (14.9–38.8)	1	0.036	1
	Diestrus	43 (29.7–68.6)	32 (15.5–45)	0.54		
HOMA-S (%)	Anestrus	697 (423–1807)	686 (393–1714)	1	0.081	1
	Diestrus	385 (230–695)	646 (387–1726)	1		
IGF-1 (µg/L)	Anestrus	144 (91–172)	160 (132–182)	1	1	1
	Diestrus	141 (112–166)	158 (130.5–197)	1		
GH (µg/L)	Anestrus	3.8 (3.2–7.6)	4.0 (3.1–5.1)	1	1	1
	Diestrus	4.6 (3.9–5.5)	4.1 (3.6–4.9)	1		
Progesterone (nmol/L)	Anestrus	0.6 (0.6–0.6)	0.6 (0.6–0.6)	1	< 0.001	0.004
	Diestrus	37.5 (19.6–44.8)	37.9 (20.2–55.8)	1		

For each dog, a blood sample was collected once during anestrus and once 2 to 8 weeks after cessation of estrual bleeding (as reported by the owner). *A value of $P < 0.05$ was considered significant. †All variables were measured in serum samples, except for HbA1c concentration, which was measured in whole blood samples. ‡The IQR represented the 25th to 75th percentile.

trus. Values of *P* were corrected with Bonferroni correction for multiple testing (18 tests).

Pairwise comparisons

To assess whether serum glucose, fructosamine, C-peptide, progesterone, GH, and IGF-1 concentrations; whole blood HbA1c concentration; and HOMA-B and HOMA-S differed between anestrus and diestrus within each breed group, the Wilcoxon signed rank test for paired data was used. Values of *P* were corrected with Bonferroni correction for multiple testing (9 tests/breed).

Results

Dogs—Forty-two dogs were initially included in the study. Initially, there were 8 Norwegian Elkhounds, 16 Swedish Elkhounds, and 18 dogs of other breeds. Breeds of non-Elkhound dogs included Golden Retriever (n = 5), German Shepherd Dog (2), Rough-haired Collie (2), Dalmatian (1), Finnish Hound (1), Finnish Lapphund (1), Finnish Spitz (1), Hamilton Hound (1), Hovawart (1), Labrador Retriever (1), Papillon (1), and Belgian Tervuren (1). One of the Swedish Elkhounds developed diabetes mellitus during the study and was excluded from further analysis. This dog's biochemical variables were within reference range during anestrus. When performing a quality analysis of the data, another Swedish Elkhound was found to have identical results for serum glucose, fructosamine, and progesterone concentrations during anestrus and diestrus, indicating a mistake in sample handling. This dog was also excluded from further analysis. Therefore, the total number of dogs in the study was 40 (8 Norwegian Elkhounds, 14 Swedish Elkhounds, and 18 dogs of other breeds).

In addition, other discrepancies concerning serum progesterone concentration in relation to the stage of estrus were identified for 2 dogs in the Elkhound group (2 Swedish Elkhounds) and 4 dogs in the non-Elkhound group (1 Labrador Retriever, 1 Finnish Lapphund, 1 Papillon, and 1 Golden Retriever); in those dogs, anestrus serum progesterone concentrations were in the range of 0.9 to 5.3 nmol/L, which is greater than the laboratory's reference range for anestrus (< 0.6 nmol/L). For another dog (1 German Shepherd Dog) in the non-Elkhound group, the anestrus serum progesterone concentration was not available. All statistical analyses were performed both with and without the inclusion of data from these 7 aforementioned dogs.

The median age of the Elkhounds was 6.0 years (IQR [25th to 75th percentile], 5.4 to 7.7 years), and the median age of the dogs of other breeds was 6.1 years (IQR, 5.5 to 7.5 years). No dog was considered to be underweight. Among the 22 Elkhounds, 4 were considered overweight during both anestrus and diestrus; among the 18 dogs of other breeds, 3 and 4 were considered overweight during anestrus and diestrus, respectively. The median interval after estrual bleeding at which diestrus blood samples were collected in the Elkhound and non-Elkhound groups was 4.5 weeks (IQR, 3.6 to 6.0 weeks) and 4.0 weeks (IQR, 3.3 to 4.6 weeks), respectively. The median interval after estrual bleeding at which anestrus blood samples were collected in the Elkhound and non-Elkhound groups was 17.9 weeks (IQR, 16.9 to 22.8 weeks) and 19.3 weeks (IQR, 17.6 to 21.0 weeks), respectively.

Descriptive statistics and results from the group-wise and pairwise comparisons for all 40 dogs in the study were summarized (**Table 1**). After Bonferroni correction for multiple testing, no differences were found between the Elkhound and non-Elkhound groups. The pairwise comparison of data for the variables of interest during anestrus with those during diestrus within each breed grouping revealed that serum C-peptide concentration and the beta-cell function estimate (HOMA-B) were higher in diestrus samples than in anestrus sam-

ples (Bonferroni $P = 0.04$) in Elkhounds. In Elkhounds, insulin sensitivity (HOMA-S) was lower (albeit not significantly [Bonferroni $P = 0.08$]) during diestrus than during anestrus. Serum C-peptide concentration, HOMA-B, and HOMA-S during anestrus and diestrus were similar in dogs of other breeds. In both breed groupings, serum progesterone concentration was significantly (Bonferroni $P < 0.001$) increased during diestrus, compared with findings during anestrus. When the 7 dogs with anestrus serum progesterone concentrations > 0.6 nmol/L were excluded from the analyses, no large differences in the results were found (data not shown). None of the statistical test results lost significance when data from all 40 dogs were used in the analyses, although the *P* value for the anestrus-to-diestrus HOMA-S comparison in Elkhounds was lower (Bonferroni $P = 0.041$).

Discussion

In the present study, concentrations of biomarkers of glucose homeostasis in healthy female dogs and female dogs with diabetes mellitus were assessed during anestrus and diestrus. Dogs included in the study were Norwegian and Swedish Elkhounds (breeds known to be at increased risk for development of diestrus-associated diabetes mellitus) and dogs of other breeds. With regard to serum glucose, fructosamine, progesterone, GH, and IGF-1 concentrations and whole blood HbA1c concentration, no differences were found between the 2 breed groupings during anestrus or during diestrus. However, dogs in the Elkhound group had increased serum C-peptide concentration (after food withholding) and increased beta-cell function (as estimated by HOMA-B) during diestrus, compared with findings during anestrus; such differences were not evident in dogs in the non-Elkhound group. Furthermore, Elkhounds had some signs of decreased insulin sensitivity (as estimated by HOMA-S) during diestrus, compared with findings during anestrus, although this difference was not significant; a similar difference was not evident in dogs of other breeds.

C-peptide is released in equimolar concentrations with insulin when proinsulin is cleaved in the insulin-producing beta cells,[14] but because C-peptide has a negligible hepatic extraction and a much longer half-life than insulin,[15] it is a better marker of endogenous insulin secretion than insulin itself. In the present study, the analysis of beta-cell function and insulin sensitivity by use of the HOMA-B and HOMA-S estimates revealed that an increase in serum C-peptide concentration (determined after withholding of food) during diestrus in Elkhounds, compared with findings during anestrus, was indicative of both insulin resistance and increased beta-cell function (ie, decreased insulin sensitivity).

For purposes of estimating insulin secretion, assessment of stimulated C-peptide concentration is preferred over determination of serum C-peptide concentration after withholding of food,[16] and we believe that the difference in serum C-peptide concentration during anestrus and diestrus detected in Elkhounds would have been even greater if we had performed a stimulation test with food or a compound such as glucose, glucagon, or arginine. Insulin sensitivity is preferably measured by use of either clamp techniques or frequently sampled IV glucose tolerance testing. However, it was recently shown that the HOMA-B and HOMA-S calculations based on serum glucose and insulin concentrations determined after withholding of food are moderately well correlated with the results from the frequently sampled IV glucose tolerance test in dogs.[13] The results of the HOMA-B and HOMA-S are given as a percentage of the insulin secretion and insulin sensitivity of a clinically normal adult human. No reference values are available for dogs. For humans, it is not recommended to use the calculations for individuals with serum glucose and C-peptide concentrations less than the lower normal physiologic limit. Compared with human data, dogs generally have a lower range of serum C-peptide concentrations (when measured with the canine-specific assay[9]); thus, we chose to include all dogs in the HOMA calculations. According to the provider of the HOMA calculator, calculations made on observations outside the physiologic reference range should be treated with caution.[17]

Many factors are known to reduce the tissue sensitivity to insulin in humans and other animals, including visceral adiposity, inflammation, infections, glucocorticoid administration, pregnancy, and genetic factors.[18] The insulin resistance detected in Elkhounds during diestrus in the present study was probably not caused by increased obesity during diestrus because no difference in body condition score during anestrus and diestrus was found. It is more likely that some Elkhounds genetically respond to the diestrual hormones in a more insulin-resistant manner than do dogs of other breeds, which certainly could play an important role in the development of diabetes mellitus. In an animal with insulin resistance, beta cells will normally meet the augmented demands by increasing their production of insulin, which may be the explanation for the increase in beta-cell function as measured by HOMA-B in the present study. If the beta cells could not meet these demands, hyperglycemia would develop. One Elkhound developed diabetes mellitus during the study, and data from that dog were excluded from analysis; however, that dog's biochemical variables were within reference range during anestrus. Another Elkhound that was included in the study at 6 years of age was reported to have diabetes mellitus 2 years later.

It has been shown that there are small but significant differences in serum GH concentration between diabetic and nondiabetic Elkhounds during diestrus.[8] However, the causal importance of elevated serum GH concentration in the development of diabetes mellitus in Elkhounds is not yet clear, and there may be other, more important factors involved. The serum concentrations of progesterone and GH in both anestrus and diestrus samples were similar in the 2 breed groupings in the present study. In clinically normal cycling Beagles, basal GH concentration increases in the early luteal phase with some loss of the pulsatile pattern of secretion of GH.[19] We did not detect any differences in serum GH concentration during anestrus and diestrus either within each breed grouping or between the 2 breed groupings in the present study, but such differences may have been revealed if we had repeatedly measured

serum GH concentration at short intervals or had made measurements during a glucose stimulation test. There may also have been some problems with the statistical power of the present study, and an increase in the number of dogs studied would be beneficial. Growth hormone is rather sensitive to storage conditions and repeated thawing cycles, which may have lowered serum concentrations in both groups of dogs in the present study.

The results may have been different had older dogs been evaluated. The female Elkhounds used in the present study had a median age of 6 years, which was younger than the mean age of onset of diabetes mellitus in Elkhounds (7.8 years).[7] This age discrepancy may have reduced the chance to include dogs at high risk for diabetes. During aging, a progressive impairment of insulin action on glucose homeostasis develops progressively both in humans and in other animals.[20,21]

A limitation of the present study was the determination of the phase of the estrual cycle for each dog, which relied on the owner's estimation of the end of the last estrual bleeding. Confirmation of the estrual cycle phase via cytologic evaluation of vaginal smears would have been preferred over the estimated time elapsed since end of estrual bleeding, to rule out bleeding that could have been caused by vaginal or uterine pathological changes. However, we believe that any possible phase misclassification was as likely to occur within one breed grouping as within the other. Therefore, the potential misclassification should not have constituted a bias but could have interfered with the chances to find important differences between the estrual cycle phases. The number of weeks that had elapsed since the end of estrual bleeding was longer in the Elkhounds (median interval, 4.5 weeks), compared with the interval for dogs of other breeds (median interval, 4.0 weeks). However, because serum progesterone concentration (and possibly insulin resistance) is declining during this period, it is our opinion that we have not overinterpreted the difference between the 2 breed groupings; rather, the opposite is true.

Another limitation of the present study was that the sample size was too small to perform robust multivariable modeling, in which the serum concentrations of potential insulin resistance–inducing hormones could be related to the concentrations of the markers of glucose homeostasis. Studies involving larger numbers of dogs are needed. Glucose homeostasis likely differs among individuals of the Elkhound breeds, and such analysis would relate the individual laboratory results to each other and adjust for confounding factors such as body condition and age.

The present study has provided evidence of an increase in baseline insulin secretion in Elkhounds during diestrus, compared with findings during anestrus, and that the dogs in the Elkhound group were able to compensate for the diestrus-associated insulin resistance, at least after food had been withheld. In this regard, our results differ from the results of Marca and Loste,[6] who found elevations in serum glucose and whole blood HbA1c concentrations during diestrus in Beagles, compared with other stages of the estrual cycle. Elevations of glucose and HbA1c indicate relative insulin deficiency. In addition, this study has provided preliminary evidence of increased beta-cell function and decreased insulin sensitivity in Elkhounds during diestrus, compared with findings during anestrus. These findings likely reflect changes in glucose homeostasis, which may play a role in the development of diabetes mellitus in Elkhounds.

a. Glucose, Thermo Clinical Labsystems Oy, Vantaa, Finland.
b. ABX Pentra Fructosamine, ABX Diagnostics, Montpellier, France.
c. Immulite 2000 Progesterone, Siemens Healthcare Diagnostics, Deerfield, Ill.
d. Canine C-peptide radioimmunoassay, Linco Research, St Charles, Mo.
e. Trasylol, Bayer, Göteborg, Sweden.
f. PGH-46HK, Linco Research, St Charles, Mo.
g. Haemaquant/Glycosal HbA1c system, provided by Bio-Rad, Hemel Hempstead, Hertfordshire, England.
h. STATA, version 11, SAS Institute Inc, Cary, NC.

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