Repeatability of Oral Sugar Test Results, Glucagon-Like Peptide-1 Measurements, and Serum High-Molecular-Weight Adiponectin Concentrations in Horses

N. Frank (iD), and D.M. Walsh

Background: Repeatability of the oral sugar test (OST) has not been evaluated.

Objectives: We hypothesized that OST glucose, insulin, active (aGLP-1) and total (tGLP-1) glucagon-like peptide 1, and high-molecular-weight (HMW) adiponectin results would be repeatable.

Animals: Fifty-three horses from a Tennessee research facility (n = 23) and private practice in Missouri (n = 30), including animals with medical histories of equine metabolic syndrome.

Methods: Two OSTs were performed 7–14 days apart and plasma glucose and insulin concentrations were measured at 0, 60, and 75 minutes; a positive result was defined as detection of an insulin concentration >45 μU/mL at 60 or 75 minutes. Plasma aGLP-1 and serum tGLP-1 concentrations at 75 minutes and serum HMW adiponectin concentrations at 0 minute were measured in the Missouri group. Bland-Altman analyses were performed.

Results: No adverse events were reported. Bland-Altman analysis indicated mean ± SD bias of 1.5 ± 14.8 μU/mL (95% confidence interval [CI], −27.6 to 30.5 μU/mL) and 1.2 ± 16.1 μU/mL (95% CI, −30.4 to 32.8 μU/mL) for insulin concentrations at 60 and 75 minutes, respectively. There was 91 and 83% agreement in test interpretation between test days for OST insulin results for all horses in the Tennessee and Missouri groups, respectively.

Conclusions and Clinical Importance: Repeatability of the OST was acceptable when values obtained from Bland-Altman analyses were evaluated, and there was good agreement in binary (negative/positive) test interpretation for insulin concentrations. However, wide 95% CIs were detected for insulin concentrations.

Key words: Endocrinology; Equine metabolic syndrome; Insulin; Insulin dysregulation; Laminitis.

An oral sugar test (OST) has been developed to diagnose insulin dysregulation (ID) in horses and ponies, and this test is commonly performed in the field.[1] Higher than normal insulin concentrations are detected after administration of corn syrup and it is assumed that positive OST results indicate a higher risk of laminitis because of the established association between hyperinsulinemia and laminitis.[2,3] Detection of a higher than normal insulin response to a measured dose of corn syrup may serve as an early indicator that persistent hyperinsulinemia and laminitis will develop in the same animal if it is allowed to graze on pasture. Insulin dysregulation, regional or generalized adiposity, and hypertriglyceridemia are components of equine

Abbreviations:

aGLP-1	active glucagon-like peptide-1
CV	coefficient of variation
ID	insulin dysregulation
OST	oral sugar test
tGLP-1	total glucagon-like peptide-1

metabolic syndrome (EMS), a clinical syndrome associated with laminitis.[4]

Postprandial hyperinsulinemia can be detected by performing an OST or oral glucose tolerance test (OGT), or by measuring insulin concentrations after a test meal, and each of these tests has advantages and disadvantages. The OST is easily performed in the field and does not require nasogastric intubation, but corn syrup is a mixture of sugars and its composition cannot be guaranteed. In contrast, the dextrose powder used in the OGT has a predictable composition, but nasogastric intubation may be required, which induces stress. Sugars bypass the oral cavity when delivered directly into the stomach and physiological responses such as gastric emptying time may differ accordingly. An alternative approach to nasogastric intubation is to feed a test meal containing dextrose and measure insulin concentrations,[5] but results of this in-feed OGT are affected by different rates of feed consumption and variation in feed composition.

When repeatability of oral glucose tests has been examined, the in-feed OGT was found to have reasonable repeatability, with median (range) coefficient of variation (CV) values of 23% (16–44%), 19% (31–43%), and 45% (23–75%) detected at 90, 120, and 180 minutes, respectively, for 8 healthy ponies undergoing 3 consecutive tests.[6] Repeatability of the OST also

has been assessed in healthy ponies, with results compared between tests performed twice, under fed or fasted conditions.[7] The OST had good repeatability ($\kappa = 0.7$) when binary (positive/negative) results were examined using an insulin cutoff concentration of 60 μU/mL at 60 and 90 minutes, and higher insulin concentrations were detected when ponies were fasted before testing. When binary (negative/positive) results for the in-feed OGT and OST were compared, 85% agreement was detected in the 8 ponies and 5 horses that were tested.[5]

As mechanisms to explain ID in horses have been explored, attention has turned to incretin hormones and their role in stimulating insulin secretion from the pancreas. Glucose-dependent insulinotropic peptide and glucagon-like peptide-1 (GLP-1) are released from K and L cells of the intestine, respectively, and stimulate insulin secretion from beta cells in response to sugars and amino acids arriving in the intestine. Results from 2 previous studies provide evidence that active glucagon-like peptide-1 (aGLP-1) concentrations are higher in hyperinsulinemic ponies and horses, when compared with normoinsulinemic controls, which suggests that incretin hormones play a role in ID.[8,9] Incretin hormone concentrations are measured in the blood and might therefore serve as biomarkers for ID. Adiponectin may also be a biomarker for ID because this 30-kDa protein is secreted by adipocytes and circulates as high-molecular-weight (HMW) complexes in the blood. High-molecular-weight adiponectin concentrations have been shown to be lower in obese horses, when compared with lean controls, and are negatively correlated with insulin concentrations.[10]

Our study was performed to test the hypothesis that the OST yields repeatable results when performed under field conditions in normal horses and in those with medical histories of EMS. It also was hypothesized that GLP1 and HMW adiponectin concentrations are highly repeatable, as evidenced by narrow 95% CIs when Bland-Altman analysis is performed and that these variables are significantly correlated with OST insulin concentrations.

Materials and Methods

Animals

Fifty-three horses were evaluated, including 23 horses from a research facility in Tennessee (TN) and 30 horses from a private practice population (multiple farms) in Missouri (MO). Both populations were known to contain horses with medical histories of EMS, but less information was available for individual horses in the TN population. For horses in the MO group, EMS was suspected if the medical history of the horse included ≥1 of the following elements: a propensity to gain weight ("easy keeper"), clinical signs of increased adiposity ("cresty neck", fat pads, or generalized obesity) or evidence of previous laminitis (divergent hoof rings or expanded white line), or diagnostic test results consistent with ID, including increased basal insulin concentrations or positive OST results. For the MO group, 15 horses with medical histories consistent with EMS were selected, as well as 15 horses without evidence of EMS in their medical histories (controls) from the same practice, and where possible from the same farm. Six horses with medical histories of pituitary pars intermedia dysfunction (PPID) were included in the group of MO horses with histories of EMS. Medical histories of PPID included reports of hypertrichosis, muscle wasting, polyuria and polydipsia, or previous positive PPID diagnostic test results, and all horses were receiving pergolide on a daily basis. Although horses with histories of EMS were identified and included in the TN and MO populations at the start of the study to increase the likelihood of detecting positive OST results, horses subsequently were allocated to normal (N) or ID groups on the basis of results of OSTs performed in our study.

Experimental Design

Testing was performed between November 2012 and May 2013. Morphometric measurements were collected and then 2 OSTs were performed 7–14 days apart, with testing performed in the morning after feed was withheld overnight. Feed was removed from stalls, pens, or paddocks at 10:00 PM the night before and withheld until testing was completed the next day. Owners were asked to observe their horses for the development or worsening of lameness associated with laminitis after OSTs were performed. Glucose and insulin concentrations were measured in all samples collected. Active GLP-1, tGLP-1, and HMW adiponectin measurements were limited to a subset of samples collected from MO horses because of financial limitations. The Clinical Studies Review Committee of the Cummings School of Veterinary Medicine approved the study protocol.

Morphometric Measurements

Body mass was measured using weight scale (TN group) or calculated using girth at the withers and body length, as previously described[11] (MO group). Body condition score was assessed by a single investigator using a previously described system.[12] Cresty neck score was assigned using a previously described method,[13] and mid-neck circumference was measured using a measuring tape.[14]

Oral Sugar Test

A blood sample was collected, and then, corn syrup[a] was administered PO using 60-mL catheter-tip syringes at a dosage of 0.15 mL/kg body weight. Blood (20 mL) then was collected 60 and 75 minutes after administration of corn syrup. Blood tubes were immediately placed in a cooler containing ice and centrifugation was performed within 4 hours of collection. Plasma samples were stored at −20°C. A positive glucose result was defined as a glucose concentration >125 mg/dL at 60 or 75 minutes and a positive insulin result was defined by a plasma insulin concentration >45 μU/mL at the same time points. Fasting hyperinsulinemia was defined by a resting plasma insulin concentration >20 μU/mL in blood collected after withholding feed overnight.

Plasma Glucose and Insulin Concentrations

Frozen plasma samples were packaged with ice packs and sent by overnight mail to the Animal Health Diagnostic Center at Cornell University[b] for measurement of plasma glucose and insulin concentrations.

Plasma Active and Total Glucagon-like Peptide-1 Concentrations

Blood was collected into tubes without anticoagulant for measurement of serum total GLP-1 concentrations and into tubes

containing EDTA and dipeptidyl peptidase 4 (DPP4) inhibitor[c] for measurement of active GLP-1 concentrations. Commercially available enzyme-linked immunosorbent assay (ELISA) kits developed for use with human serum and plasma were used to measure active GLP-1[d] and total GLP-1[e] in DPP4-treated EDTA plasma or serum, respectively. Samples were analyzed in duplicate at Michigan State University[f] on MO group samples collected at time = 75 minutes on both test days.

Serum High-Molecular-Weight Adiponectin Concentrations

Blood was collected into tubes without anticoagulant and left at room temperature to clot. Samples then were centrifuged and serum harvested and stored at −80°C until further analyzed. A previously validated ELISA[g],[10] was used to measure resting HMW adiponectin concentrations in time 0 blood samples collected on each test day from EMS suspect cases in the MO group (n = 15). Samples were analyzed in duplicate by a referral laboratory.[h]

Statistical Analysis

Data were assessed for normality by visually examining plotted results and performing Shapiro-Wilk tests, and nonparametric tests were selected. Median (range) values are reported. Groups were compared using Mann-Whitney U-tests. Spearman correlation coefficients were calculated to compare results among different variables. Agreement was first calculated by taking the number of horses with consistent results (positive or negative) when the OST was repeated and dividing by the total number in the group, and expressing this result as a percentage. Bland-Altman analyses (difference versus average) also were performed to determine the agreement in blood variable concentrations between test days in all horses. Horses with positive insulin results (insulin concentration >45 μU/mL) then were separated from those with negative results (insulin concentration ≤45 μU/mL) at 60 or 75 minutes and Bland-Altman analyses were repeated. Horses with medical histories of PPID in the MO-ID group also were separated and compared to other groups. The kappa statistic was calculated for glucose and insulin concentrations at 0, 60, and 75 minutes, and for positive results, as defined by a glucose concentration >125 mg/dL or insulin concentration >45 μU/mL at either 60 or 75 minutes, to assess agreement between the diagnosis on the first and second tests.[15] A statistical software program[i] was used to perform these analyses and statistical significance was defined at a value of $P < .05$.

Results

No adverse events were reported when OSTs were performed. Twelve of 23 horses in the TN group had positive (>45 μU/mL) insulin results consistent with ID on 1 or both OSTs (TN-ID group), and 11 horses had normal results (TN-N group). Sixteen of 30 horses in the MO group had positive insulin results (MO-ID group) 14 of 15 horses with medical histories of EMS and 2 of 16 horses from the control group. The 2 control group horses only tested positive once (insulin concentrations of 47 and 52 μU/mL at 60 minutes), and these were different horses. Fourteen horses had normal OST results (MO-N group). Data for the 4 groups are summarized in Table 1. Significant differences were detected between N and ID groups for insulin concentrations at 60 and 75 minutes, as expected because

groups were defined according to these results. Additionally, groups differed with respect to baseline (0 minutes) insulin concentrations. Missouri N and ID groups also differed significantly in age and cresty neck score. Having a medical history of PPID did not affect results significantly.

Glucose and Insulin Concentrations

Median (range) plasma glucose and insulin concentrations measured during the OST on 2 test days are shown in Figure 1. Bland-Altman analysis results are presented in Table 2. At time = 60 minutes, mean ± SD bias was 3.5 ± 17.0 mg/dL (95% confidence interval [CI], −30.0 to 36.8 mg/dL) for glucose concentrations and 1.5 ± 14.8 μU/mL (95% CI, −27.6 to 30.5 μU/mL) for insulin concentrations in 53 horses for 2 tests repeated within 14 days (Fig 2). At 75 minutes, mean ± SD bias was 1.7 ± 16.9 mg/dL (95% CI, −31.4 to 34.7 mg/dL) for glucose concentrations and 1.2 ± 16.1 μU/mL (95% CI, −30.4 to 32.8 μU/mL) for insulin concentrations (Fig 3). Confidence intervals around mean bias values for insulin concentrations at 60 minutes were −15.6 to 16.5 μU/mL for horses with negative insulin results, compared with −35.4 to 40.2 μU/mL for those with positive results. At 75 minutes, CIs were −17.5 to 20.6 μU/mL and −41.0 to 42.6 μU/mL, respectively.

Agreement in Diagnostic Test Results

Resting (fasted) hyperinsulinemia was detected at 0 minute on 21 occasions in 13 of 53 horses, with concentrations ranging from 21 to 57 μU/mL (median, 23 μU/mL) 4 horses from the TN group and 9 horses from the MO group. All horses with fasting hyperinsulinemia had medical histories consistent with EMS.

Five of 23 horses in TN group had at least 1 positive glucose result on the OST, and there was 83% (19/23) agreement in test interpretation (positive versus negative) for glucose results between the two test days (Table 3). There was 91% (21/23) agreement in test interpretation between the 2 test days for the TN group when insulin results were examined.

Seven of 30 horses in MO group had at least 1 positive glucose result for the OST and there was 83% (25/30) agreement between glucose results (positive versus negative) on different test days. There also was 83% (25/30) agreement between insulin results. Five horses had positive insulin results on only 1 OST.

Within-subject (n = 53) agreement in binary test outcomes was good for insulin results at 0 minutes (κ = 0.75), 60 minutes (κ = 0.65), 75 minutes (κ = 0.73), and a positive result at either 60 or 75 minutes (κ = 0.77). Agreement could not be calculated for glucose results at 0 minutes, and there was only moderate (κ = 0.46) and fair (κ = 0.30) agreement for glucose results at 60 and 75 minutes, respectively. Agreement was fair when a positive test result was defined by a glucose concentration >125 mg/dL at 60 or 75 minutes (κ = 0.21).

Table 1. Comparison of normal (N) and insulin dysregulation (ID) groups identified within two populations of horses after they underwent 2 oral sugar tests within 14 days of one another. Median (range) values are displayed.

	Tennessee Population (n = 23)		Missouri Population (n = 30)	
	TN-N (n = 11)	TN-ID (n = 12)	MO-N (n = 14)	MO-ID (n = 16)
Age	10 (5–13)	8 (6–11)	8 (4–24)	20.5 (9–30)*
Breed	1 App, 1 Paint, 6 QH, 3 TWH	2 Grade, 4 Paint, 4 QH, 2 TWH	1 App, 2 Arab, 1 Morg, 3 Paint, 1 Pony, 4 QH, 1 TB, 1 WB	2 App, 1 Andal, 1 Arab, 1 Miss Fox, 1 Paint, 1 Paso, 5 Pony, 1 QH, 3 TWH
Sex	3G; 8 M	1G; 11M	11G; 3M	10G; 6M
Body weight (kg)	493 (430–532)	466.5 (442–540)	488.5 (394–608)	416 (267–733)
Body condition score (1–9)	8 (7–8)	8 (7–9)	7 (6–8)	7.25 (5–8)
Cresty neck score (1–5)	3 (2–4)	3.5 (2–5)	1 (1–3)	3 (2–4)*
Mid-neck circumference (cm)	98 (84–102)	99 (88–106)	98 (89–104)	97 (84–117)
OST glucose (mg/dL)				
0 minute	70 (40–84)	73.5 (55–259)	76.5 (70–88)	79 (68–98)
60 minutes	97.5 (58–117)	104 (49–143)	105.5 (80–136)	110 (83–145)
75 minutes	93 (50–146)	64.5 (57–125)	102 (77–138)	109 (82–146)
OST insulin (μU/mL)				
0 minute	9 (5–15)	16.5 (7–26)*	6 (2–19)	20 (6–57)*
60 minutes	29 (11–42)	55 (40–94)*	20 (6–39)	59.5 (21–171)*
75 minutes	26.5 (6–43)	51 (29–90)*	18.5 (3–44)	58 (21–143)*

App, Appaloosa; Andal, Andalusian; Arab, Arabian; G, Gelding; M, Mare; Miss Fox, Missouri Foxtrotter; Morg, Morgan horse; QH, Quarter Horse; TB, Thoroughbred; TWH, Tenesssee Walking Horse; WB, Warmblood.

*Indicates a significant difference ($P < .05$) between N and ID groups, within the TN and MO populations.

In TN horses, 11 of 23 horses had negative insulin results across all time points for both tests and 1 horse had positive insulin results across all time points for both tests. Four of 23 horses had normal basal insulin concentrations and positive insulin results at 60 and 75 minutes on both OSTs. Three other horses had normal basal insulin concentrations and positive insulin results at 60 and 75 minutes on 1 test and normal basal insulin concentrations and positive insulin results at 60 or 75 minutes on the other test. One horse had a normal basal insulin concentration and positive 60- and 75-minute insulin results on OST1 and positive insulin results at all time points on OST2. One horse had a high insulin concentration at 0 minutes, but negative insulin results at 60 and 75 minutes on OST1 and positive insulin results at all time points on OST2. One horse was positive across all time points on OST1 and only had a positive insulin result at 75 minutes on OST2 and another horse was only positive at 60 minutes on OST1.

In MO horses, 14 of 30 horses had negative insulin results across all time points for both tests. Five of 30 horses had positive insulin results across all time points for both tests, and 4 horses had normal basal insulin concentrations and positive insulin results at 60 and 75 minutes on both OSTs. Two other horses had normal basal insulin concentrations and positive insulin results at 60 and 75 minutes on 1 test and normal basal insulin concentrations and positive insulin results at 60 or 75 minutes on the other test. One horse had a high insulin concentration at 0 minutes, but negative insulin results at 60 and 75 minutes on OST1 and then positive insulin results at all time points on OST2. One horse was positive across all time points on OST1 and

negative across all time points on OST2, and another horse was negative across all time points on OST1 and positive at 0 and 60 minutes on OST2. One horse had a positive insulin result (47 μU/mL) at 60 minutes on OST1 and otherwise normal results. Another horse had only 1 abnormality: a positive insulin result (52 μU/mL) at 75 minutes on OST2.

Glucagon-like Peptide 1 Concentrations

Intra-assay CV values for duplicate measurements of active GLP-1 in pooled equine plasma, Quality Control (QC) 1 (4.5–9.4 pM), and QC 2 (25–54 pM) were 4.9, 2.4, and 3.2%, respectively, and interassay CV values were 8.0, 3.5, and 9.3%, respectively. Total GLP-1 intra-assay CV values for the pooled equine serum, QC 1 (21.4–44.0 pM), and QC 2 (134–277 pM) were 4.2, 5.5, and 2.2%, respectively, and interassay CV values were 11.7, 16.1, and 8.1%, respectively.

Plasma aGLP-1 concentrations measured at 75 minutes during the OST in horses from the MO group (n = 30) ranged from 1.63 to 32.15 (median, 5.8) pM and mean $\pm$ SD bias of -0.41 ± 4.22 pM (95% CI, -8.68 to 7.86 pM) was detected by Bland-Altman analysis. Active GLP-1 concentrations ranged from 3.6 to 21.5 (median, 8.1) pM for the MO-N group (n = 14), compared with 1.6–32.2 (median, 4.0) pM for the MO-ID group (n = 16) and groups differed significantly ($P = .029$). Insulin concentrations at 75 minutes were positively correlated with glucose concentrations ($r_s = 0.39$, $P = .002$) and negatively correlated with a GLP-1 concentrations ($r_s = -0.28$, $P = .033$) in horses from the MO group.

Serum tGLP-1 concentrations ranged from 0 to 39.4 (median, 9.6) pM and mean ± SD bias of −0.88 ± 3.90 pM (95% CI, −8.52 to 6.77 pM) was detected by Bland-Altman analysis. Concentrations ranged from 0 to 17.7 (median, 10.7) pM in MO-N horses (n = 14), compared with 0–39.4 (median, 9.0) pM in MO-ID horses (n = 16), and groups did not differ

significantly ($P = .678$). Total GLP-1 was not detected in 3 samples. Total GLP-1 concentrations at 75 minutes were not significantly correlated with insulin concentrations measured at the same time point ($r_s = −0.14$; $P = .272$).

High-Molecular-Weight Adiponectin

Median (range) CV was 5.4% (0.5–12.2%) for duplicate measures of HMW adiponectin in serum samples from 30 horses. Resting (0 minutes) serum HMW adiponectin concentrations measured in 15 horses with histories of EMS in the MO group (1 N; 14 ID) ranged
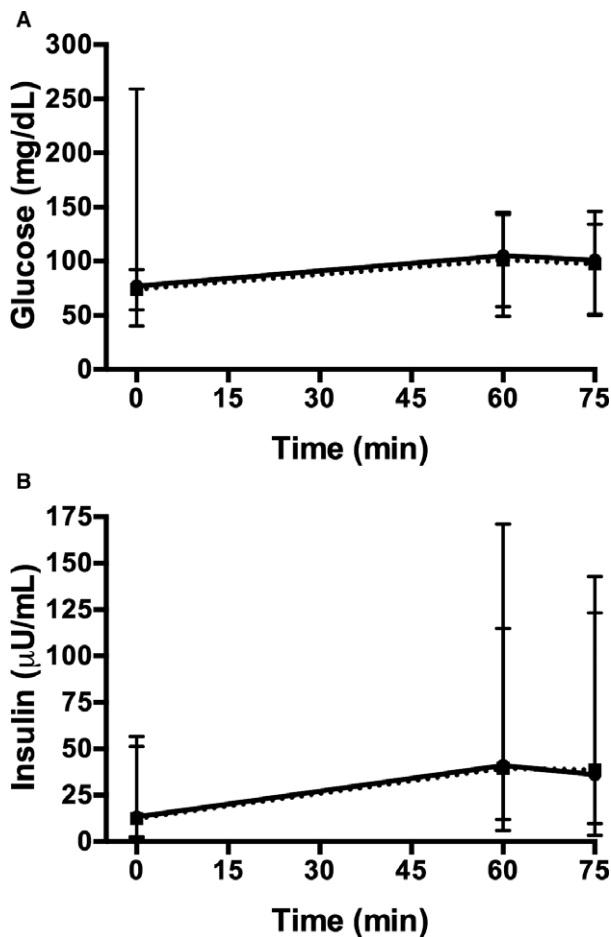

Fig 1. Median (range) plasma glucose (**A**) and insulin (**B**) concentrations measured during two oral sugar tests repeated within 14 days of one another in 53 horses. Data from the first (circles; solid line) and second (squares; dotted line) test days are displayed.
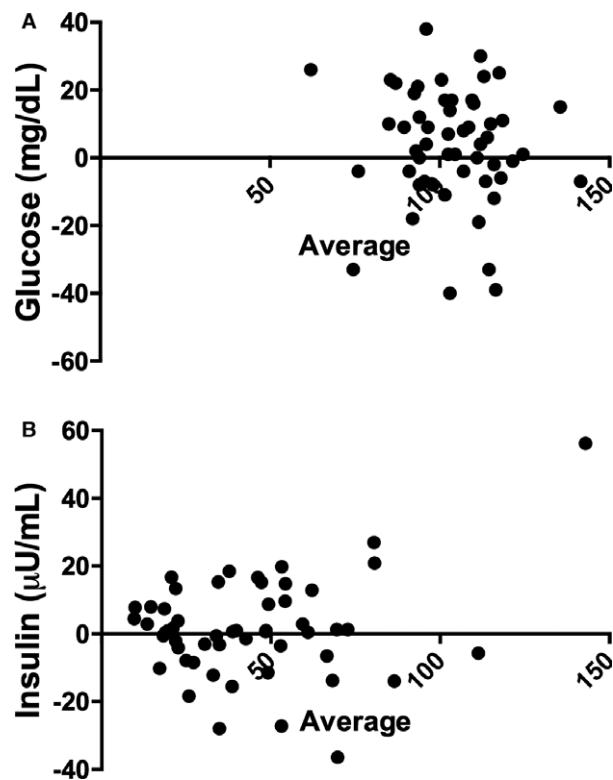

Fig 2. Bland-Altman plots (difference versus average) of plasma glucose (**A**) and insulin (**B**) concentrations measured at 60 minutes during oral sugar tests repeated within 14 days of one another in 53 horses.

Table 2. Bland-Altman analysis (difference versus average) of plasma glucose and insulin concentrations measured during 2 oral sugar tests performed within 14 days of one another. Data from all 53 horses are displayed and then subdivided according to detection of an insulin concentration >45 μU/mL at 60 or 75 minutes.

	ALL			Negative Insulin Result			Positive Insulin Result		
	Bias	SD	95% CI	Bias	SD	95% CI	Bias	SD	95% CI
Glu 0 minute	5.5	26.6	−46.7 to 57.7						
Glu 60 minutes	3.5	17.0	−30.0 to 36.8	2.2	19.7	−36.5 to 40.9	4.9	14.0	−22.7 to 32.4
Glu 75 minutes	1.7	16.9	−31.4 to 34.7	1.1	17.5	−33.2 to 35.5	2.3	16.4	−30.0 to 34.4
Ins 0 minute	0.6	4.3	−7.8 to 8.9						
Ins 60 minutes	1.5	14.8	−27.6 to 30.5	0.5	8.2	−15.6 to 16.5	2.4	19.3	−35.4 to 40.2
Ins 75 minutes	1.2	16.1	−30.4 to 32.8	1.5	9.7	−17.5 to 20.6	0.8	21.3	−41.0 to 42.6

from 0.20 to 6.97 µg/mL (median, 2.19 µg/mL) and mean ± SD bias of 0.08 ± 0.27 µg/mL (95% CI, −0.45 to 0.60 µg/mL) was detected by Bland-Altman analysis. Resting HMW adiponectin concentrations were negatively correlated with 75-minute insulin concentrations ($r_s = -0.53$, $P = .002$) and positively correlated with 75-minute aGLP-1 concentrations ($r_s = 0.45$, $P = .013$) in this subset of 15 horses.

Discussion

Repeatability of the OST was acceptable when Bland-Altman analysis results were evaluated, and there was 91 and 83% agreement in test interpretation

Fig 3. Bland-Altman plots (difference versus average) of plasma glucose (**A**) and insulin (**B**) concentrations measured at 75 minutes during oral sugar tests repeated within 14 days of one another in 53 horses.

between days for OST insulin results for horses in the TN and MO groups, respectively, and thus, our hypothesis was supported. However, glucose and insulin results falling close to diagnostic cutoff concentrations should be interpreted with caution when the OST is used as a diagnostic test for ID because wide 95% CIs were detected around mean bias values in our study.

Results of our study indicate that there is good agreement in binary results for insulin concentrations (κ = 0.65 and 0.73 at 60 and 75 minutes, respectively) when the OST is repeated in the same horse. Our results compare favorably with another study[7] that found that the OST had good repeatability (κ = 0.7) when binary (positive/negative) results were examined using an insulin cutoff concentration of 60 µU/mL at 60 and 90 minutes in fasted ponies. Bland-Altman analysis did not identify any systemic difference in the results obtained from the 2 OSTs, because mean bias values were close to zero for all of the variables evaluated. However, we also demonstrated that absolute insulin results vary considerably when testing is repeated in horses, and this may limit the usefulness of the OST as a monitoring tool. Repeatability can be assessed by examining 95% CIs obtained from Bland-Altman analyses. For example, the 95% CI for insulin concentrations measured at 60 minutes in horses testing positive for ID group was −35.4 to 40.2 µU/mL in our study, and thus, a clinician can assume with 95% confidence that a horse with an insulin concentration of 60 µIU/mL will be within approximately 35 µIU/mL of this result if tested again within 2 weeks. Accordingly, the clinician should not assume that insulin concentrations have changed significantly in an individual horse unless insulin concentrations increase or decrease by more than approximately 35 µIU/mL at 60 minutes.

Further refinements to the OST may be required to improve test performance, such as increasing the amount of corn syrup administered or measuring glucose and insulin concentrations at an earlier time point. Another study found that maximum insulin concentrations were detected more frequently at 30 minutes, rather than 60 or 90 minutes in healthy ponies,[7] and this time point warrants further investigation in horses. In our study, 60- and 75-minute time points were selected, instead of 60 and 90 minutes as described when the OST was introduced.[1] This decision was made
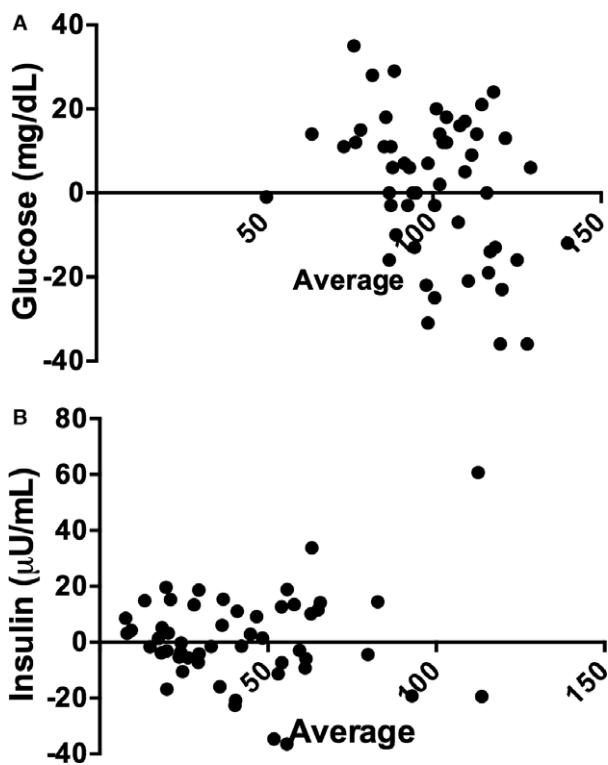
Table 3. Oral sugar test (OST) insulin results from two tests repeated within 14 days in 53 horses: 23 horses from a research facility in Tennessee and 30 horses from a private practice population (multiple farms) in Missouri.

	Insulin 0 Minute		Insulin 60 Minutes		Insulin 75 Minutes		Overall Result
	Negative ≤20 µU/mL	Positive >20 µU/mL	Negative ≤45 µU/mL	Positive >45 µU/mL	Negative ≤45 µU/mL	Positive >45 µU/mL	Agreement in Interpretation
Tennessee (n = 23)							
OST1	20	3	12	11	13	10	
OST2	20	3	14	9	14	9	21/23 (91%)
Missouri (n = 30)							
OST1	23	7	18	12	19	11	
OST2	22	8	17	13	17	13	25/30 (83%)

to compare glucose and insulin concentrations between groups at a new time point that had not been evaluated previously, and to decrease the time required to complete the OST procedure. When horses in our study were grouped according to negative or positive insulin results at 60 or 75 minutes, CIs around mean bias values were wider for horses with positive insulin results. This greater variability in results should be considered when using the OST to monitor responses to management changes or medical treatments in affected animals.

One horse had a baseline glucose concentration of 259 mg/dL on the first test and 73 mg/dL on the second test. Such a large difference in resting glucose concentrations is difficult to explain and may represent laboratory error. Test agreement was 83% when glucose results were interpreted using a cutoff concentration of 125 mg/dL. This is reasonable agreement for a test performed in the field, but may indicate that the cutoff concentration selected for glucose concentrations is too low and does not accommodate variability in test conditions. A cutoff glucose concentration of 125 mg/dL was arbitrarily selected on the basis of OST results from a small population of horses tested by this research group to establish the OST,[1] and larger studies are now required to validate glucose and insulin cutoff concentrations.

An insulin concentration of 45 μU/mL was selected here as the cutoff concentration for diagnosing ID, instead of the 60 μU/mL cutoff concentration recommended when the OST was first developed.[1] Test sensitivity increases when a lower cutoff concentration is selected, and this is advantageous when performing a screening test. A recent study reported test sensitivity of 0 and 14% for the OST when 60 μU/mL and 45 μU/mL cutoff concentrations for insulin were selected, respectively.[16] In that study, OST results were compared with sensitivity to insulin (SI) values obtained by minimal model analysis of frequent-sample intravenous glucose tolerance test results. Although the previously reported findings[16] suggest that the OST has low sensitivity, the gold standard selected by the investigators must be questioned. It has not been established that a SI value $<1.0 \times 10^{-4}$ L/mU/min defines insulin resistance in horses and the frequently sampled intravenous glucose tolerance test procedure itself can induce stress and lower tissue insulin sensitivity.[17] Recent studies also provide evidence that insulin status should be assessed after feeding to account for the effects of incretin hormones on insulin concentrations.[8,9] Sensitivity and specificity values are not reported here because a gold standard has not been established for oral glucose tests and intravenous glucose tolerance tests do not account for the enteroinsulinar axis.

Potential causes of within-horse variability in our OST results include improper administration of corn syrup, sample handling or laboratory processing errors, and water consumption during testing. Most horses readily accept corn syrup when it is administered PO, but individual animals may resist insertion of a syringe into the mouth and syrup can be lost in the process. Events of this sort were not reported in our study, but

it is conceivable that small amounts of syrup were lost from the mouth during the procedure. Samples were handled appropriately and it is unlikely that incomplete mixing with anticoagulants or warming affected glucose and insulin concentrations in our study. Assay variability also must be accounted for when using the insulin radioimmunoassay or glucose oxidase colorimetric assay. Intra-assay CVs usually are higher for the insulin assay than for glucose measurements and can approach 20%.[18] Unfortunately, the diagnostic laboratory used for this study did not report intra-assay CV values for insulin measurements. It is also conceivable that water consumption increased within-horse variability in our study because horses were given free access to water during the OST, and this could have affected clearance of sugars from the stomach. Repeatability of the OST may be improved by withholding water during testing. Feed was withheld from horses before testing in our study, and this was accomplished by instructing owners to remove feed after 10:00 PM the night before. Testing was performed the following morning, but start times varied among horses and this may have affected results. Insulin concentrations were higher when the OST was performed under fasting conditions in healthy ponies,[7] whereas others were not able to detect an effect of fasting on OST results in horses.[19]

Fasting insulin concentrations often are measured to screen horses for ID and a cutoff concentration of 20 μU/mL was recommended in the 2010 American College of Veterinary Internal Medicine consensus statement on EMS.[4] However, introduction of the OST in 2013 led to the identification of horses with normal fasting insulin concentrations, but higher than normal insulin concentrations during the test.[1] These observations suggest that the OST detects postprandial hyperinsulinemia before fasting hyperinsulinemia develops, and the role of incretin hormones must therefore be considered when comparing postprandial and fasting insulin concentrations. The incretin hormones, GLP-1 and glucose-dependent insulinotropic polypeptide, stimulate insulin secretion from beta cells of the islets of Langerhans and increase insulin concentrations after feeding.[20] The impact of incretin hormones on insulin concentrations cannot be assessed when insulin concentrations are measured after fasting or when intravenous glucose tolerance tests are performed. Established tests for insulin resistance including the euglycemic hyperinsulinemic clamp, frequently sampled intravenous glucose tolerance test, and combined glucose-insulin test do not account for the effects of incretin hormones on insulin concentrations.[21] They do not therefore assess the full impact of dietary sugars and amino acids on insulin status.

To evaluate the OST under field conditions, we elected to test horses at a research farm in Tennessee because animals were housed together in the same barn, which is similar to housing conditions for horses kept on larger horse farms. The Missouri population was made up of client-owned horses kept under different conditions and testing was performed in the field. Several different horse breeds also were represented, and this was both a strength and limitation of the study. It

can be concluded that the OST yields repeatable results in horses of different breeds, but breed groups were too small to make comparisons with adequate statistical power. Breed differences in glucose and insulin dynamics are becoming more important as evidence mounts that genetic predispositions exist for EMS.[22]

Season is an important consideration when performing endocrine testing in horses and future studies should examine the repeatability of the OST at different times of the year. Plasma adrenocorticotropic hormone concentrations are significantly impacted by season in horses, with higher concentrations detected in the late summer and fall.[23,24] Seasonal variation was not the focus of the study reported here, and all horses were tested between November and May in an attempt to avoid effects of season on results. Effects of age also should be examined in the future, and it would be useful to conduct a longitudinal study over several years to assess the impact of aging on OST results in the same cohort of horses.

Tests for ID are still being optimized and the OST recently has been compared with an in-feed OGT commonly used in the United Kingdom. Both tests were performed in 8 ponies and 5 horses and the OGT identified 7/13 animals as insulin resistant, compared to 5/13 when the OST was performed (insulin cutoff concentration: 60 μU/mL).[5] Interestingly, mean time to maximum insulin concentration differed between ponies (60 ± 10 minutes) and horses (69 ± 23 minutes), and this suggests that different sampling times may be required if ponies are being evaluated instead of horses. The approach taken to performing the OST in clinical practice is to collect blood samples at 60, and 75 or 90 minutes, and this allows for measurement of glucose and insulin at times when concentrations are peaking. Further refinements to the OST must be considered, including the selection of optimal times for blood sampling and diagnostic cutoff concentrations based upon breed.

The OST also has been compared with the insulin tolerance test (ITT), which directly measures tissue insulin sensitivity. The 90-minute time point for the OST most closely correlated ($r = -0.62$; $P = .010$) with ITT results, but test interpretation differed between tests in some of the EMS horses studied.[j] A study was conducted to compare the OST with 2 tests of insulin sensitivity, the insulin response to dexamethasone test and the hyperinsulinemic euglycemic clamp, in 15 light-breed horses.[25] Oral sugar test and hyperinsulinemic euglycemic clamp results did not correlate well, and this suggests that insulin concentrations measured after PO administration of sugar are affected by factors other than tissue insulin sensitivity, including rates of glucose absorption from the intestine and incretin hormones.

Both aGLP-1 and tGLP-1 concentrations had wide 95% CIs (−8.68 to 7.86 pM and −8.52 to 6.77 pM) when Bland-Altman analyses were performed. Lower plasma aGLP-1 concentrations were detected in the MO-ID group, compared to the MO-N group, which was surprising because aGLP-1 stimulates insulin secretion and higher concentrations were expected in horses

and ponies with ID.[8,9,26] In a study previously performed by our research group, aGLP-1 concentrations were higher (but not significantly) during the OST in EMS horses, when compared with normal horses.[8] Another study also detected higher plasma aGLP-1 concentrations in hyperinsulinemic ponies when oral glucose tests were performed.[9] Breed of horse has been shown to affect aGLP-1 concentrations, with ponies and Andalusian horses reaching higher concentrations during oral glucose tests than Standardbred horses.[26] A strong positive correlation ($r_s = 0.752$; $P < .001$) between insulin and aGLP-1 concentrations was reported in the same study.

One explanation for the lower aGLP-1 concentrations detected in our study is the timing of blood collection relative to peak aGLP-1 concentrations. Blood was only collected at 60 and 75 minutes, and this is a weakness of our study design that can be addressed by collecting multiple blood samples over several hours to identify peak concentrations. In other studies, blood samples were collected for 14 hours as 2 meals were fed 8 hours apart[26] and when ponies were sampled for 3 hours after glucose (1 g/kg) was provided in a meal.[9] Only 2 blood samples were collected after administering corn syrup in our study because testing was performed in the field.

High-molecular-weight adiponectin measurements were limited to 0-minute blood samples from 15 horses in the MO group with medical histories of EMS because of the expense of testing. Additional studies are required to assess the usefulness of this biomarker in clinical practice, but our results suggest that HMW adiponectin concentrations are highly repeatable within the same horse (95% CI, −0.45 to 0.60 μg/mL) and negatively correlated with 75-minute OST insulin concentrations. An inverse relationship between HMW adiponectin and insulin sensitivity also is reported in humans.[27] Insulin-sensitizing effects of HMW adiponectin are mediated through multiple pathways, including increased adenosine monophosphate-activated protein kinase activity in the liver, improved insulin signaling, and decreased tissue macrophage inflammatory cytokine production.[28]

Plasma HMW adiponectin concentrations detected in our study compare favorably with previously reported concentrations when serum HMW adiponectin concentrations were measured in 26 lean horses and 18 obese horses.[10] Lower HMW adiponectin concentrations were detected in obese horses (mean ± SD, 3.6 ± 3.9 μg/mL) compared with lean horses (8.0 ± 4.6 μg/mL), and negative correlations existed between serum HMW adiponectin and insulin concentrations ($r_s = -0.425$; $P = .004$) and body condition score ($r_s = -0.450$; $P = .005$). Concentrations ranged from 0.2 to 6.7 μg/mL in the 15 horses tested here, and body condition score ranged from 4 to 8.

Adiponectin complexes can be separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and identified by Western blot analysis, and a previous study detected bands in the >250 kDa (high molecular weight), 120–250 kDa (lower molecular weight), and 90–120 kDa (trimer) ranges in equine serum and

adipose tissues.[10] Dimers (60 kDa), monomers (30 kDa), and the globular head region (28 kDa) also were identified. The same study validated the commercially available human sandwich ELISA kit used in our study and recommended modifications were followed precisely.[10] The same assay was used in another study to compare the effects of high-fat, high-glucose, or control diets on HMW adiponectin concentrations in horses and ponies, and interestingly, differences were detected.[29] Total adiponectin concentrations also have been measured in equine plasma by radioimmunoassay, and significantly lower concentrations were detected in previously laminitic ponies, compared to normal ponies.[30,31] Adiponectin concentrations also may serve as a biomarker for laminitis risk because serum total adiponectin concentrations were associated with laminitis occurrence in a prospective cohort study of ponies.[3]

Fasting and postprandial adiponectin profiles have been measured in healthy and obese cats using sucrose gradient centrifugation[32,33] or size-exclusion gel chromatography[34] followed by an adiponectin-specific ELISA. Lower HMW adiponectin concentrations were detected after feeding in healthy cats that received a high carbohydrate meal (from 3.6 ± 0.6 to 3.0 ± 0.7 l μg/mL) and fasting HMW adiponectin concentrations increased when cats were switched from a moderate (1.9 ± 0.8) to low (3.1 ± 0.7 μg/mL) carbohydrate diet.[32] Lower plasma HMW adiponectin concentrations were detected in obese cats compared to lean cats, and males had lower concentrations than females.[33] In a recently reported study of cats undergoing weight gain or loss, HMW adiponectin was negatively correlated with body fat mass and positively correlated with glucose:insulin ratio and glucose effectiveness, a value obtained by minimal model analysis of frequently sampled intravenous glucose tolerance test data.[34]

A canine-specific ELISA for total adiponectin is available for use and a negative correlation with body condition score ($r = -0.446$, $P < .01$; n = 100) has been reported in dogs.[35] There was no difference in total adiponectin concentrations when overweight dogs with and without pituitary-dependent hyperadrenocorticism were compared, but lower total adiponectin concentrations were detected in dogs with newly diagnosed diabetes mellitus, compared with healthy dogs in horses.[36,37] Additional research is required to determine whether HMW adiponectin concentrations are altered by management changes and medical treatments for ID.

We conclude that the OST has acceptable repeatability when 95% CI obtained from Bland-Altman analyses were evaluated, and HMW adiponectin warrants further study as a biomarker for ID in horses.

Footnotes

Acknowledgments

References

1. Schuver A, Frank N, Chameroy KA, et al. Assessment of insulin and glucose dynamics by using an oral sugar test in horses. J Equine Vet Sci 2014;34:465–470.

2. Treiber KH, Kronfeld DS, Hess TM, et al. Evaluation of genetic and metabolic predispositions and nutritional risk factors for pasture-associated laminitis in ponies. J Am Vet Med Assoc 2006;228:1538–1545.

3. Menzies-Gow NJ, Harris PA, Elliott J. Prospective cohort study evaluating risk factors for the development of pasture-associated laminitis in the UK. Equine Vet J 2017;49:300–306.

4. Frank N, Geor RJ, Bailey SR, et al. Equine metabolic syndrome. J Vet Intern Med 2010a;24:467–475.

5. Smith S, Harris PA, Menzies-Gow NJ. Comparison of the in-feed glucose test and the oral sugar test. Equine Vet J 2016;48:224–227.

6. de Laat MA, Sillence MN. The repeatability of an oral glucose test in ponies. Equine Vet J 2017;49:238–243.

7. Knowles EJ, Harris PA, Elliott J, et al. Use of the oral sugar test in ponies when performed with or without prior fasting. Equine Vet J 2016 Jul 6. doi: 10.1111/evj.12607. [Epub ahead of print].

8. Chameroy KA, Frank N, Elliott SB, et al. Comparison of plasma active glucagon-like peptide 1 concentrations in normal horses and those with equine metabolic syndrome and in horses placed on a high-grain diet. J Equine Vet Sci 2016;40:16–25.

9. de Laat MA, McGree JM, Sillence MN. Equine hyperinsulinemia: Investigation of the enteroinsular axis during insulin dysregulation. Am J Physiol Endocrinol Metab 2016;310:E61–E72.

10. Wooldridge AA, Edwards HG, Plaisance EP, et al. Evaluation of high-molecular weight adiponectin in horses. Am J Vet Res 2012;73:1230–1240.

11. Ellis JM, Hollands T. Accuracy of different methods of estimating the weight of horses. Vet Rec 1998;143:335–336.

12. Henneke DR, Potter GD, Kreider JL, et al. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet J 1983;15:371–372.

13. Carter RA, Geor RJ, Burton Staniar W, et al. Apparent adiposity assessed by standardised scoring systems and morphometric measurements in horses and ponies. Vet J 2009;179:204–210.

14. Frank N, Elliott SB, Brandt LE, et al. Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. J Am Vet Med Assoc 2006;228:1383–1390.

15. McHugh ML. Interrater reliability: The kappa statistic. Biochem Med (Zagreb) 2012;22:276–282.

16. Dunbar LK, Mielnicki KA, Dembek KA, et al. Evaluation of four diagnostic tests for insulin dysregulation in adult light-breed horses. J Vet Intern Med 2016;30:885–891.

17. Toth F, Frank N, Geor RJ, et al. Effects of pretreatment with dexamethasone or levothyroxine sodium on endotoxin-induced alterations in glucose and insulin dynamics in horses. Am J Vet Res 2010;71:60–68.

18. Borer-Weir KE, Bailey SR, Menzies-Gow NJ, et al. Evaluation of a commercially available radioimmunoassay and species-specific ELISAs for measurement of high concentrations of insulin in equine serum. Am J Vet Res 2012;73:1596–1602.

19. Bertin FR, Taylor SD, Bianco AW, et al. The effect of fasting duration on baseline blood glucose concentration, blood insulin concentration, glucose/insulin ratio, oral sugar test, and insulin response test results in horses. J Vet Intern Med 2016;30:1726–1731.

20. de Graaf-Roelfsema E. Glucose homeostasis and the enteroinsular axis in the horse: A possible role in equine metabolic syndrome. Vet J 2014;199:11–18.

21. Firshman AM, Valberg SJ. Factors affecting clinical assessment of insulin sensitivity in horses. Equine Vet J 2007;39:567–575.

22. McCue ME, Geor RJ, Schultz N. Equine metabolic syndrome: A complex disease influenced by genetics and the environment. J Equine Vet Sci 2015;35:367–375.

23. Frank N, Elliott SB, Chameroy KA, et al. Association of season and pasture grazing with blood hormone and metabolite concentrations in horses with presumed pituitary pars intermedia dysfunction. J Vet Intern Med 2010b;24:1167–1175.

24. Donaldson MT, McDonnell SM, Schanbacher BJ, et al. Variation in plasma ACTH concentration and dexamethasone suppression test results in association with season, age, and sex in healthy ponies and horses. J Vet Intern Med 2004;18:414 (abstract).

25. Banse HE, McFarlane D. Comparison of three methods for evaluation of equine insulin regulation in horses of varied body condition score. J Equine Vet Sci 2014;34:742–748.

26. Bamford NJ, Baskerville CL, Harris PA, et al. Postprandial glucose, insulin, and glucagon-like peptide-1 responses of different equine breeds adapted to meals containing micronized maize. J Anim Sci 2015a;93:3377–3383.

27. Almeda-Valdes P, Cuevas-Ramos D, Mehta R, et al. Total and high molecular weight adiponectin have similar utility for the identification of insulin resistance. Cardiovasc Diabetol 2010;9:26.

28. Ruan H, Dong LQ. Adiponectin signaling and function in insulin target tissues. J Mol Cell Biol 2016;8:101–109.

29. Bamford NJ, Potter SJ, Harris PA, et al. Effect of increased adiposity on insulin sensitivity and adipokine concentrations in horses and ponies fed a high fat diet, with or without a once daily high glycaemic meal. Equine Vet J 2016;48:368–373.

30. Wray H, Elliott J, Bailey SR, et al. Plasma concentrations of inflammatory markers in previously laminitic ponies. Equine Vet J 2013;45:546–551.

31. Menzies-Gow NJ, Wray H, Bailey SR, et al. The effect of exercise on plasma concentrations of inflammatory markers in normal and previously laminitic ponies. Equine Vet J 2014;46:317–321.

32. Tan HY, Rand JS, Morton JM, et al. Adiponectin profiles are affected by chronic and acute changes in carbohydrate intake in healthy cats. Gen Comp Endocrinol 2011;172:468–474.

33. Bjornvad CR, Rand JS, Tan HY, et al. Obesity and sex influence insulin resistance and total and multimer adiponectin levels in adult neutered domestic shorthair client-owned cats. Domest Anim Endocrinol 2014;47:55–64.

34. Witzel AL, Kirk CA, Kania SA, et al. Relationship of adiponectin and its multimers to metabolic indices in cats during weight change. Domest Anim Endocrinol 2015;53:70–77.

35. Park HJ, Lee SE, Oh JH, et al. Leptin, adiponectin and serotonin levels in lean and obese dogs. BMC Vet Res 2014;10:113.

36. Cho KD, Paek J, Kang JH, et al. Serum adipokine concentrations in dogs with naturally occurring pituitary-dependent hyperadrenocorticism. J Vet Intern Med 2014;28:429–436.

37. Kim AY, Kim HS, Kang JH, et al. Serum adipokine concentrations in dogs with diabetes mellitus: A pilot study. J Vet Sci 2015;16:333–340.