Journal of Veterinary Internal Medicine
Open Access
ACVIM American College of Veterinary Internal Medicine

J Vet Intern Med 2016;30:653–663

Effect of Age, Season, Body Condition, and Endocrine Status on Serum Free Cortisol Fraction and Insulin Concentration in Horses

K.A. Hart, D.M. Wochele, N.A. Norton, D. McFarlane, A.A. Wooldridge, and N. Frank

Background: Increased free cortisol fraction is associated with insulin dysregulation (ID) in people with Metabolic Syndrome and Cushing's Disease. Free cortisol has not been investigated in equine endocrine disorders.

Hypotheses: (1) In healthy horses, sex, age, body condition score (BCS), and season impact free cortisol; (2) free cortisol is increased in horses with Pituitary Pars Intermedia Dysfunction (PPID) or Equine Metabolic Syndrome (EMS).

Animals: Fifty-seven healthy horses; 40 horses and ponies with PPID (n = 20) or EMS (n = 20).

Methods: Prospective study. Serum collected seasonally from healthy animals and archived serum from PPID and EMS animals was analyzed for insulin, total and free cortisol concentrations, and free cortisol fraction (FCF). Linear mixed models were used to determine effects of age, sex, season, and BCS on hormones in controls. Hormone measurements were compared between disease groups and age- and season-matched controls with *t*-tests. EMS and hyperinsulinemic PPID animals were combined in an ID (hyperinsulinemia) group.

Results: Free cortisol concentrations were increased in overweight/obese controls (0.3 ± 0.1 μg/dL) compared to lean controls (0.2 ± 0.1 μg/dL; $P = .017$). Mean FCF was significantly higher in animals with PPID (8.8 ± 5.8 μg/dL, $P = .005$) or ID (8.8 ± 10.2 μg/dL, $P = .039$) than controls (5.0 ± 0.9 μg/dL), but total cortisol concentrations were similar ($P \geq .350$) (PPID: 4.2 ± 4.3 μg/dL; ID: 5.0 ± 4.5 μg/dL; controls: 4.6 ± 1.7 and 5.1 ± 2.1 μg/dL).

Conclusions and Clinical Importance: Increased FCF is associated with obesity in healthy horses and with ID (hyperinsulinemia) in horses and ponies with endocrine disease. Decreased plasma cortisol-binding capacity could be a component of these endocrine disorders in horses.

Key words: Equine; Equine Metabolic Syndrome; Insulin resistance; Obesity; Pituitary pars intermedia dysfunction; Steroid.

Pituitary Pars Intermedia Dysfunction (PPID) and Equine Metabolic Syndrome (EMS) are common equine endocrine disorders.[1–3] Insulin dysregulation (ID), typically characterized by resting or postprandial hyperinsulinemia, is a key component of EMS,[1,4] and occurs in approximately one third of horses and ponies with PPID.[2,5,6] Glucocorticoid (cortisol) excess has been theorized to contribute to the development of ID in some animals with PPID or EMS.[7–9] Both short and long-term experimental administration of synthetic glucocorticoids such as dexamethasone or triamcinolone can decrease insulin sensitivity and cause hyperinsulinemia in healthy horses.[10–13] Ponies with hyperlipidemia (common in EMS[1]) or PPID and presumptive ID also had increased urinary corticoids reflective of increased circulating cortisol.[14]

Abbreviations:

BCS	body condition score
CBG	cortisol-binding globulin
EMS	equine metabolic syndrome
FCC	serum free cortisol concentration
FCF	serum free cortisol fraction
HPA	hypothalamic-pituitary-adrenal
ID	insulin dysregulation
MR	mineralocorticoid receptor
PPID	pituitary pars intermedia dysfunction

Increased plasma total cortisol concentration is not a common finding in horses with PPID and EMS.[2,15] Recent evidence in people suggests that measurement of free rather than total cortisol might more accurately reflect systemic cortisol activity.[16,17] In healthy horses, approximately 90% of plasma cortisol is bound to cortisol-binding globulin (CBG) and albumin.[18,19] It is the remaining 10% of unbound, free cortisol that is considered biologically active, and is available to bind cytoplasmic steroid receptors to mediate the majority of cortisol's systemic effects.[20]

The conditions associated with increased free cortisol fraction (FCF) or free cortisol concentration in people include increased age, obesity, and ID, and also occur in horses with PPID and EMS.[1,2] If free cortisol is similarly increased in these equine endocrine disorders, assessment of free cortisol in affected horses might provide diagnostic and pathophysiologic information in affected animals. Free cortisol fraction has been measured in adult horses and foals,[18,19,21,22] but to our knowledge has not been assessed in horses with endo-

From the University of Georgia College of Veterinary Medicine, Athens, GA (Hart, Wochele, Norton); Oklahoma State College of Veterinary Medicine, Stillwater, OK (McFarlane); Auburn University College of Veterinary Medicine, Auburn, AL (Wooldridge); Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA (Frank). *This study was performed at the University of Georgia. Portions of these data were presented in abstract form at the 2013 American College of Veterinary Internal Medicine Forum.*

Corresponding author: Kelsey A. Hart, Department of Large Animal Medicine, University of Georgia College of Veterinary Medicine, Athens, GA 30602; e-mail: khart4@uga.edu.

Submitted July 29, 2015; Revised November 4, 2015; Accepted January 18, 2016.

DOI: 10.1111/jvim.13839

crine disease. In addition, the effect of season on HPA-axis activity and total cortisol concentrations in horses and ponies is well-described,[15,23–28] but it is not known if season also impacts equine plasma cortisol-binding dynamics and alters free cortisol availability.

Thus, the objectives of this study were to: (1) characterize the effects of sex, age, body condition, and season on free cortisol measurements in healthy horses; (2) compare total and free cortisol measurements between healthy adult horses and horses with endocrine disease (PPID, EMS, and ID); and (3) to determine if total or free cortisol measurements are significantly associated with insulin concentration in horses. We hypothesized that: (1) in healthy horses, sex, age, body condition, and season will significantly impact free but not total cortisol measurements; (2) FCF, but not total cortisol concentration, will be significantly increased in animals with PPID and EMS as compared to healthy horses, and will be significantly associated with ID, as defined by resting or postprandial hyperinsulinemia, in PPID and EMS.

Materials and Methods

Animals

Control Group. Fifty-seven healthy university- or client-owned horses were used. Inclusion criteria were: age ≥1 year, a normal physical examination, and negative screening tests for PPID and EMS at the start of the study. Specifically, horses were only included if plasma adrenocorticotropic hormone (ACTH) concentration measured during the fall (October) was <47 pg/mL[25] and serum insulin concentration was <20 μIU/mL in fasted horses or <30 μIU/mL in horses housed on pasture before sampling.[1,29] An insulin value (30 μIU/mL), well below a suspect or positive insulin responses to an oral sugar challenge (≥45–60 μIU/mL)[29] was chosen as a cutoff value for fed insulin concentrations in control horses to minimize the likelihood of including horses with subclinical ID in the control group. Control group mean plasma ACTH and serum insulin concentrations in these fall screening samples were 24.5 ± 7.3 pg/mL (range 10.0–40.4 pg/mL) and 11.7 ± 6.3 μIU/mL (range 2.4–27.2 μIU/mL), respectively. Horses with a previous diagnosis of laminitis, PPID, or EMS were also excluded from the study. Sampling protocols for and care of University-owned animals were conducted under an Animal Use Protocol approved by the University of Georgia Institutional Animal Care and Use Committee (AUP# A2011 05-010-Y1-A1). For client-owned animals, study design was approved by the University of Georgia College of Veterinary Medicine Clinical Research Committee and informed client consent was obtained before sampling.

PPID Group. Archived serum samples from 20 additional horses and ponies with a previous diagnosis of PPID were used. All animals had clinical signs consistent with mid-to-late-stage PPID including topline muscle atrophy or generalized weight loss, and prolonged, incomplete or absent shedding patterns.[2] PPID diagnosis had been made using one of the following criteria: (1) resting plasma ACTH concentration >35 pg/mL in the nonfall months[2,25] (January or April, n = 6); (2) pituitary histopathology consistent with pars intermedia hyperplasia or adenoma[30] (n = 2); or both nonfall ACTH concentration >35 pg/mL and consistent pituitary histopathology[30] (n = 12). Plasma ACTH concentration measured at the time of sample collection was available for 19/20 PPID group animals (mean 206.4 ± 223.2 pg/mL, range 33.3–719.7 pg/mL). For the 2 animals in which PPID diagnosis was made via pituitary histopathology alone, plasma ACTH concentration at the time of sample collection was unavailable in one, and was <35 pg/mL in a nonfall sample (33.3 pg/mL) from the other. Serum samples were stored frozen at −80°C after sample collection until they were shipped frozen on dry ice to the University of Georgia for study assays.

EMS Group. Archived serum samples collected from 20 horses with a previous diagnosis of obesity-associated EMS were used. Specifically, horses had been diagnosed with EMS if they met the following criteria:[29,31] overweight or obese body condition based on a body condition score (BCS)[32] ≥6/9, with or without regional adiposity (cresty neck scores or morphometric measurements were not determined); and one of the following manifestations of hyperinsulinemia: (1) a exaggerated insulin response to oral sugar challenge detected during an oral sugar test (insulin concentration 60–90 minutes post corn syrup administration >60 μIU/mL;[29] n = 10); (2) a fasted resting serum insulin concentration >20 μIU/mL[1] (n = 4); or (3) a fed resting serum insulin concentration >60 μIU/mL[29] (n = 6). Again, this fed insulin concentration was selected based on definitions for excessive insulin responses to oral sugar challenge in an oral sugar test, to help ensure that animals with suspect insulin responses to dietary sugar challenge (45–60 μIU/mL)[29] were excluded from both the control and EMS groups. Serum samples were similarly stored and shipped frozen until assays were performed.

Hyperinsulinemia Group. Horses with evidence of ID, characterized by resting or postprandial hyperinsulinemia as defined by one of the 3 criteria described above, were grouped together from the PPID (n = 7) and EMS (n = 20) groups to form a hyperinsulinemia group containing 27 animals.

Control Group Sampling Protocol

Body condition score was determined as above and 20 mL of blood collected via jugular venipuncture from control group animals once during each season for a total of 4 samples over a 9-month period. Specifically, fall samples were collected in October, winter samples in January, spring samples in April, and summer samples in July. A complete physical examination was performed at each sampling time point to ensure the animals remained healthy throughout the study period. Body condition score was determined by the same investigator (KAH) at each time point. All samples were collected between 6–9 am, and were obtained before the morning feeding for animals that received supplementary feed or hay. Animals that were housed on pasture were permitted to graze overnight but were sampled before any morning hay or grain was given. Animals were maintained in their routine environment (stall or pasture) at their home farm with their normal work routine and had not traveled or had a change in routine for at least 7 days before sampling. Blood samples were collected into glass tubes without anticoagulant and permitted to clot at ambient temperature. All samples were centrifuged within 3 hours of collection, and serum separated and stored at −80°C until analysis.

Assays

Plasma ACTH concentration and serum total cortisol concentration (hereafter referred to as total cortisol) were measured using previously validated chemiluminescent immunoassays.[a] Serum-free cortisol fraction (FCF) was determined using a radioactive ligand-binding ultrafiltration assay validated and optimized for equine samples as described previously.[19] Free cortisol fraction is expressed as % free cortisol. Estimated free cortisol concentration (FCC) was calculated by multiplying the total cortisol concentration by the FCF, as described previously.[19] Serum insulin concentration (hereafter referred to as insulin) was determined using a previously validated radioimmunoassay.[b]

Data Analysis

Data distribution and equality of variances among groups were assessed with the Shapiro–Wilk test and F test, respectively. Data are presented as mean ± standard deviation unless otherwise stated. Total cortisol, FCF, FCC, and insulin were compared among control group horses using univariable and multivariable linear mixed models with horse as a random effect to determine effects of age, sex, season, and body condition on the variables measured. Specifically, independent variables with P values <.2 in the univariable analysis were included in the multivariable analysis for each hormone. Two-way interactions among the independent variables were not assessed in this analysis as there were no specific *a priori* interactions of interest. Body condition score data were analyzed both as a continuous variable and as a dichotomous variable, with control group animals further stratified by BCS into lean (BCS ≤ 5/9) and overweight/obese (BCS ≥ 6/9) groups. Analysis results were comparable when BCS data were analyzed with either approach, so only the lean versus overweight/obese group analysis are presented in the Results section for simplicity. This BCS stratification was selected to include both overweight and obese animals in a single overweight/obese group, because data in people have demonstrated a substantially increased risk of ID in even mildly overweight adults.[33]

Because preliminary analysis revealed a significant effect of age or season on some endocrine measurements in the control group, comparisons between control animals and PPID and EMS groups were age- and season-matched, and were conducted using Student's *t*-tests with Welch's correction because group variances differed among variables and groups. For these comparisons, horses in the control group were divided into the following 3 age groups based on age at the first sample collection: age group A, 1–6 years of age; age group B, 7–14 years of age; and age group C, ≥15 years of age. Data from the larger group of EMS and PPID animals with hyperinsulinemia was frequency matched for comparison with an equivalent number of healthy animals by random selection of animals from the appropriately age- and season-matched control group using a random number generator.[c] Data from horses and ponies within the EMS or PPID groups were compared with Mann–Whitney μ tests because small sample sizes precluded accurate determination of data distribution in these animals. Because data distribution varied among variables measured, nonparametric Spearman correlation analysis was performed to assess associations between FCF and insulin in all groups. Analysis was performed using commercial software,[d,e] and statistical significance was set at $P < .05$ for all analyses.

Results

Animals

The control group consisted of 26 mares, 26 geldings, and 5 stallions aged 1–26 years (mean age 11.1 ± 7.0 years). The predominant breeds represented were Quarter Horses and related breeds (eg, Quarter Horse cross, Appaloosa, Paint; n = 41), with other breeds including warmbloods (n = 9), Thoroughbreds (n = 4), Arabians (n = 1), Paso Finos (n = 1), and 1 grade animal with an unknown breed. When control group animals were further divided by age, 17 animals were in age group A (1–6 years old; 8 males, 9 females; mean age 2.9 ± 1.3 years); 18 animals were in age group B (7–14 years old; 9 males, 9 females; mean age 9.9 ± 2.0 years), and 22 animals were in age group C (≥15 years old, 14 males, 8 females; mean age 18.4 ± 3.7 years). When further stratified by BCS, 22 control group animals were lean (mean BCS 4.3 ± 1.0; mean age 11.1 ± 8.1 years, range 2–25 years) and 35 animals were in the overweight/obese group (mean BCS 6.7 ± 0.8; mean age 11.1 ± 6.3 years, range 2–26 years).

The PPID group included 11 mares and 9 geldings aged 14–40 years (mean age 25.1 ± 6.4 years) and was comprised of the following breeds: Quarter Horse and related breeds (eg, Paint, Quarter Horse cross; n = 6), mixed-breed ponies (n = 4), Thoroughbred (n = 2), Tennessee Walking Horse (n = 2), grade/unknown breed (n = 2), Arabian (n = 1), Morgan (n = 1), Canadian Sport Horse (n = 1), and Missouri Fox Trotter cross (n = 1). The majority of PPID horses and ponies were sampled in the winter (n = 15) so winter samples from age group C control animals (≥15 years old) were used to provide age- and season-matched comparisons. BCS at the time of blood sampling in PPID horses was not available, so further stratification of the PPID group by BCS as for the control group was not possible.

The EMS group (n = 20) consisted of 4 mares and 16 geldings, aged 5–21 years (mean age 13.4 ± 5.1 years) and was comprised of the following breeds: Quarter Horse or Quarter Horse cross (n = 3), mixed-breed pony (n = 3), Hackney (n = 3), Icelandic Horse (n = 3), Saddlebred (n = 2), Haflinger (n = 2), Morgan (n = 2), Arabian (n = 1), and Gypsy Vanner (n = 1). All but 2 of the EMS horses were sampled in the fall so fall samples from age group B control animals (7–14 years old) were used to provide age- and season-matched comparisons. Mean BCS in the EMS group was 7.5 ± 0.8.

The hyperinsulinemia group was formed by pooling data from animals included in the EMS and PPID groups, using the criteria for inclusion stated above. All EMS horses (n = 20) and the 7 of 20 horses and ponies with PPID that had resting hyperinsulinemia were included in this group.

Effect of Sex, Age, Season, and Body Condition on Cortisol and Insulin Measurement in Healthy Horses

Cortisol and insulin measurements from healthy horses over the study period are shown in Figure 1. There was no effect of sex on total cortisol, FCF, FCC, or insulin ($P = .70$, $P = .47$, $P = .85$, and $P = .31$, respectively) in the univariable analysis, so males and females were pooled for subsequent multivariable analysis in controls and for control group comparisons with PPID and EMS animals.

Other factors significantly associated with each hormone in the univariable analysis and results of the multivariable analysis utilizing these factors for each hormone are shown in Table 1. There was no significant effect of age on total cortisol, FCF, or FCC, but insulin was significantly higher in older animals. Season significantly impacted total cortisol and insulin, with increased total cortisol in the winter as compared to the fall, and the highest insulin concentrations observed in the spring. There was no effect of season on FCF or FCC. Horses classified as overweight/obese had significantly higher total cortisol, FCC, and insulin as compared to lean horses, but FCF was not significantly

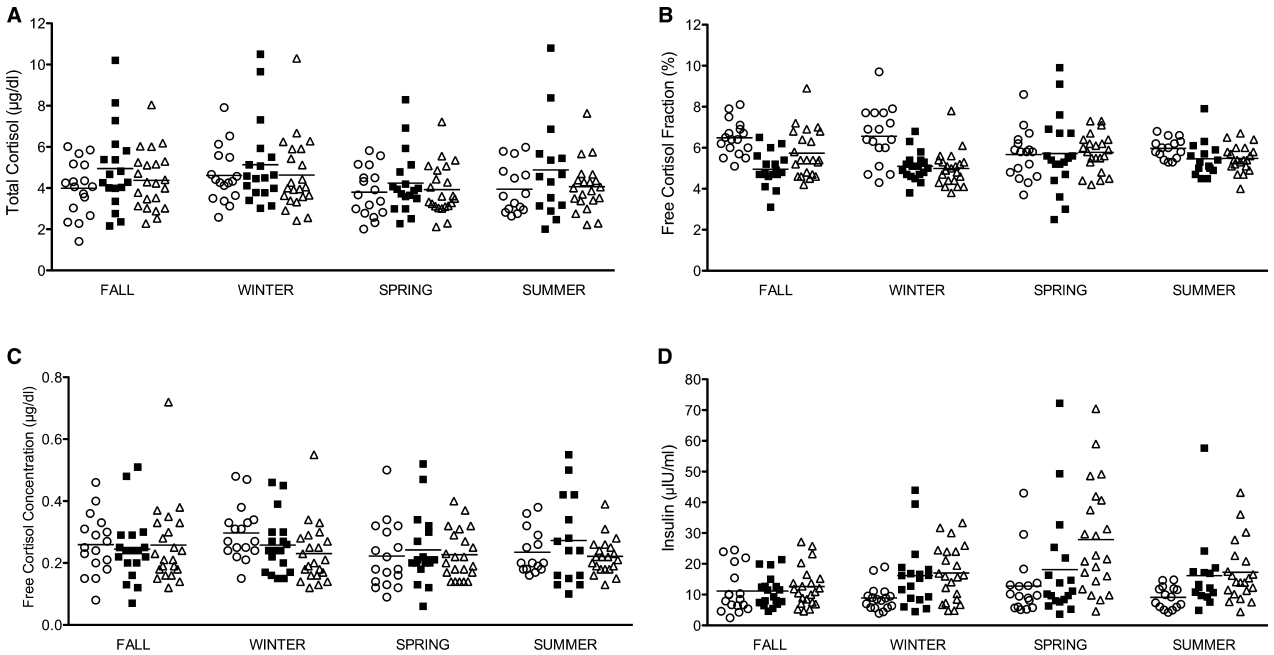

Fig 1. Serum total cortisol concentration (**A**), serum free cortisol fraction (**B**), serum free cortisol concentration (**C**), and serum insulin concentration (**D**) in 57 healthy horses over the 9-month sampling period. Each data point represents 1 animal, and animals are divided into 3 age groups: age group A, 1–6 years (open circles), age group B, 7–14 years (black squares), and age group C, ≥15 years (open triangles). The horizontal lines represent the mean for each age group during each season.

different between lean and overweight/obese horses. Mean cortisol and insulin meaurements over the study period for healthy animals, further stratified into lean and overweight/obese groups, are shown in Table 2.

Cortisol and Insulin Measurements in Horses and Ponies with PPID

Cortisol and insulin measurements in horses and ponies with PPID and age- and season-matched control horses are shown in Figure 2. Total cortisol and FCC were not different between PPID and control animals ($P = .35$ and $P = .14$, respectively), but FCF was significantly higher in the PPID group ($P = .005$). Insulin was also significantly higher in the PPID group ($P = .004$). When PPID animals were further stratified by the presence or absence of hyperinsulinemia, there was no difference in TCC, FCF, or FCC between normoinsulinemic animals (n = 13) and hyperinsulinemic animals (n = 7; $P = .19$, $P = .17$, $P = .20$, respectively).

Hormone comparisons between horses and ponies with PPID are shown in Table 3. There was no significant difference in TCC, FCF, FCC, or insulin between horses and ponies with PPID ($P = .17$, $P = .40$, $P = .24$, and $P = .081$, respectively).

Cortisol and Insulin Measurements in Horses and Ponies with EMS

Cortisol and insulin measurements in horses with EMS and age- and season-matched control animals are also presented in Figure 2. Insulin was significantly higher in horses with EMS ($P = .002$), but total cortisol, FCC and FCF were not significantly different between EMS and control horses ($P = .61$, $P = .21$, and $P = .083$, respectively).

Hormone comparisons between horses and ponies with EMS are also shown in Table 3. Insulin and TCC were not significantly different in ponies with EMS ($P = .063$ and $P = .07$), but FCF and FCC were significantly higher in ponies with EMS as compared to horses with EMS ($P = .014$ and $P = .009$).

Cortisol and Insulin Measurements in Horses and Ponies with Hyperinsulinemia

When EMS horses (defined by exaggerated insulin responses on OST or resting hyperinsulinemia as above) and PPID horses with resting hyperinsulinemia were pooled together (n = 27) and compared against 27 age- and season-matched control horses, total cortisol, and FCC were not significantly different between groups ($P = .92$ and $P = .21$, respectively), but FCF was significantly higher ($P = .039$) in horses with hyperinsulinemia than controls (Fig 2).

Correlation Between FCF and Insulin in Health and in Endocrine Disease

Correlation analysis results are shown in Table 4. In healthy horses, a significant weak to moderate negative correlation between FCF and insulin was observed in winter and summer, but not in spring or fall. Free cortisol fraction was not significantly

Table 1. Multivariable linear mixed regression model for the prediction of serum total cortisol concentration, free cortisol fraction, free cortisol concentration, or insulin concentration in 57 healthy horses sampled once during each season over a 9-month period. Horse was included in the model as a random effect to account for the correlation between repeated measurements.

Hormone	Variables[a]	Coefficient (SE)	95% Confidence Interval	*P* Value
Total cortisol (μg/dL)	Season			
	Fall	Referent	Referent	
	Winter	0.46 (0.19)	0.09–0.82	*.015*
	Spring	−0.31 (0.19)	−0.69 to 0.06	.097
	Summer	−0.01 (0.20)	−0.40 to 0.38	.95
	BCS category			
	Lean	Referent	Referent	
	Overweight/Obese	0.57 (0.18)	0.22 to 0.94	*.002*
	Constant	4.1 (0.23)	3.6–4.5	*< .001*
Free cortisol fraction (%)	Age (years)	−0.0002 (0.0001)	−0.0004 to 0.0001	.18
	BCS category			
	Lean	Referent	Referent	
	Overweight/Obese	−0.002 (0.002)	−0.006 to 0.0006	.12
	Constant	0.06 (0.0118)	0.056–0.063	*< .001*
Free cortisol (μg/dL)	Season			
	Fall	Referent	Referent	
	Winter	−0.01 (0.01)	−0.02 to 0.04	.46
	Spring	−0.02 (0.01)	−0.05 to 0.01	.28
	Summer	−0.01 (0.02)	−0.04 to 0.03	.73
	BCS category			
	Lean	Referent	Referent	
	Overweight/Obese	0.03 (0.01)	0.01–0.06	*.019*
	Constant	0.23 (0.02)	0.20–0.27	*< .001*
Insulin (μIU/mL)	Age (years)	0.36 (0.15)	0.07–0.65	*.015*
	Season			
	Fall	Referent	Referent	
	Winter	3.51 (1.61)	0.36–6.66	*.029*
	Spring	9.73 (1.62)	6.55–12.9	*< .001*
	Summer	3.84 (1.70)	0.52–7.17	*.024*
	BCS category			
	Lean	Referent		
	Overweight/Obese	4.66 (1.47)	1.78–7.54	*.002*
	Constant	4.88 (2.29)	0.39–9.37	*.033*

BCS, body condition score; SE, standard error. Italics denote statistically significant ($P < 0.05$) variables in the multivariable model.

[a]All variables (age, sex, season, BCS, BCS category as lean or overweight/obese) were tested in the univariate analysis. Variables with $P < .2$ that were tested in the multivariate analysis are presented in Table 1.

Table 2. Mean total serum cortisol concentration, serum free cortisol fraction, serum free cortisol concentration, and serum insulin concentration in lean (BCS ≤ 5/9, n = 22) and overweight/obese (BCS ≥ 6/9, n = 35) healthy horses for the entire 9-month sampling period. Data shown are mean ± standard deviation (range).

	Total Cortisol Concentration (μg/dL)	Free Cortisol Fraction (%)	Free Cortisol Concentration (μg/dL)	Insulin Concentration (μIU/mL)
Lean	3.8 ± 1.1 (2.3–6.1)	5.8 ± 1.1 (4.1–7.9)	0.2 ± 0.1 (0.1–0.4)	10.0 ± 5.3 (4.3–23.9)
Overweight/Obese	4.8 ± 1.8* (1.4–10.2)	5.7 ± 1.2 (3.1–8.9)	0.3 ± 0.1* (0.1–0.7)	12.8 ± 6.7* (2.4–27.2)

*Denotes significant ($P < .05$) differences between lean and overweight/obese animals.

correlated with insulin in healthy overweight/obese horses, horses and ponies with PPID, or horses and ponies with EMS. A significant moderate positive correlation between FCF and insulin was observed when EMS animals and hyperinsulinemic PPID animals were pooled into a larger group of animals with hyperinsulinemia.

Discussion

Total cortisol, FCF, and insulin concentrations found in healthy horses in our study were similar to those reported previously.[15,19,22] Body condition significantly impacted free cortisol as anticipated and as described in people and rodents,[16,17,34] with significantly increased

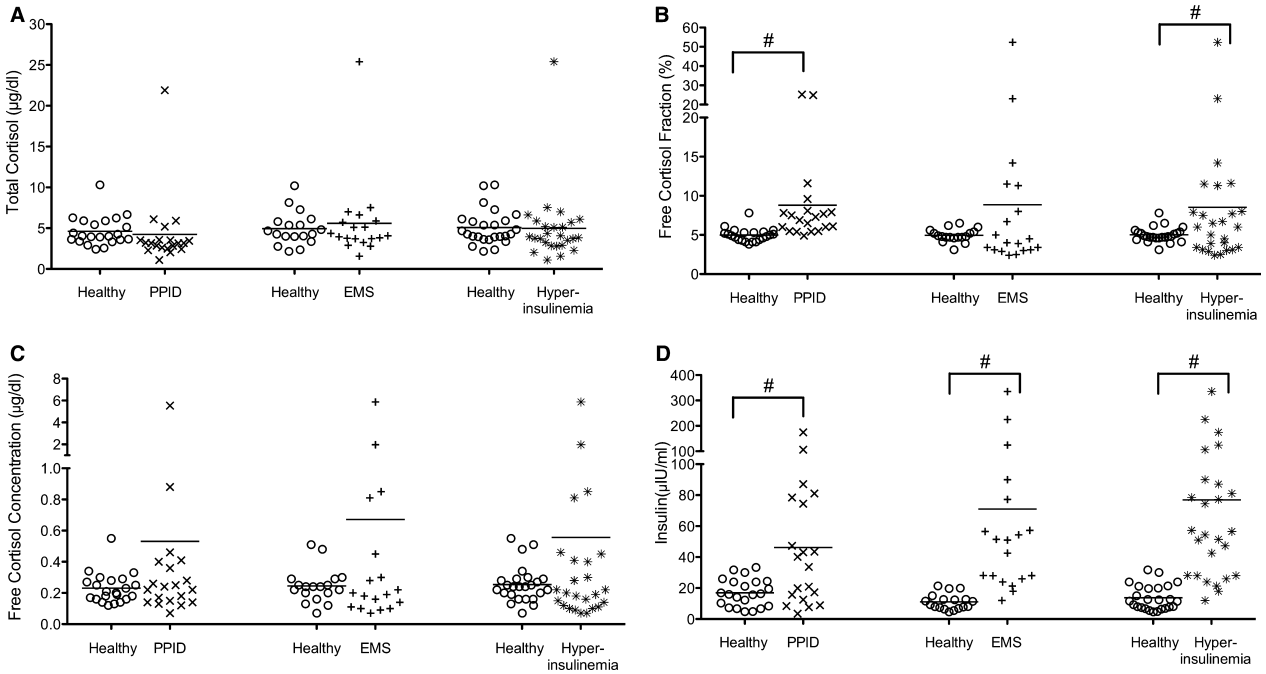

Fig 2. Serum total cortisol concentration (**A**), serum free cortisol fraction (**B**), serum free cortisol concentration (**C**), and serum insulin concentration (**D**) in horses and ponies with Pituitary Pars Intermedia Dysfunction (PPID, n = 20; X), Equine Metabolic Syndrome (EMS, n = 20, plus signs +), or PPID or EMS and concurrent hyperinsulinemia (n = 27, asterisks *) and in healthy age- and season-matched control horses (n = 18–27, open circles). #Denotes significant ($P < .05$) difference between groups. Horizontal lines represent the mean for each group.

Table 3. Mean total serum cortisol concentration, serum free cortisol fraction, serum free cortisol concentration, and basal serum insulin concentration in horses and ponies with PPID or EMS. Data shown are mean ± standard deviation (range).

	Total Cortisol Concentration (µg/dL)	Free Cortisol Fraction (%)	Free Cortisol Concentration (µg/dL)	Insulin Concentration (µIU/mL)
PPID				
Horses (n = 16)	3.1 ± 0.9 (2.0–5.9)	8.1 ± 4.8 (4.9–24.9)	0.3 ± 0.2 (0.1–0.9)	34.5 ± 27.7 (3.5–87.1)
Ponies (n = 4)	8.6 ± 9.2 (1.1–21.9)	11.6 ± 9.1 (6.0–25.3)	1.6 ± 2.6 (0.1–5.5)	93.1 ± 65.6 (17.3–174.5)
EMS				
Horses (n = 12)	4.1 ± 1.6 (1.6–7.5)	4.5 ± 2.7 (2.4–11.3)	0.2 ± 0.2 (0.1–0.9)	52.7 ± 57.5 (12.0–225.1)
Ponies (n = 8)	7.7 ± 7.3 (3.8–25.4)	16.2 ± 17.4* (3.4–52.3)	1.4 ± 2.1* (0.2–5.9)	138.9 ± 143.2 (17.9–392.5)

*Denotes significant ($P < .05$) differences between horses and ponies within each disease group.

free cortisol concentration in healthy overweight/obese control horses. The data herein also provide support for our second hypothesis that FCF is increased in horses with endocrine disease and associated with hyperinsulinemia, with a significant, almost 2-fold increase in FCF, but not total cortisol observed in animals with PPID as compared to healthy age-matched horses. Free cortisol fraction was also similarly and significantly increased in horses and ponies with endocrine disease (PPID or EMS) and concurrent ID characterized by hyperinsulinemia, and FCF and insulin were significantly and positively correlated in these hyperinsulinemic animals as anticipated and as described in people.[16,17,20,34,35]

Specific mechanisms linking increased BCS and increased circulating free cortisol are not well defined. Increased cortisol might promote generalized and regional (central) obesity, as glucocorticoids stimulate adipocyte proliferation and differentiation, and glucocorticoid receptor expression is increased in visceral adipose tissue compared to subcutaneous adipose tissue in other species.[17,36,37] Alternatively, obesity itself might be the primary stimulus for either global overactivation of the HPA axis resulting in increased circulating cortisol concentrations, or increases in cortisol availability or activity at the level of the target tissues, or both. Chronically increased glucocorticoid production and increased hair cortisol (consistent with chronic free cortisol excess) have been associated with obesity in several studies in people,[38–41] and are consistent with long-standing overactivity of the HPA axis in obese states. Tissue cortisol metabolism also appears to be altered in obesity. Many

Table 4. Results of Spearman correlation analysis between serum-free cortisol fraction and serum insulin concentration in all healthy control horses during each season (n = 57), healthy overweight/obese control horses (n = 35), and horses and ponies with PPID (n = 20), EMS (n = 20), or PPID or EMS with concurrent hyperinsulinemia (n = 27). For the healthy overweight/obese control horses (BCS ≥ 6/9), mean free cortisol fraction and insulin concentrations over the 4 seasons were used. Significant ($P < .05$) correlations between the 2 measurements are shown in italics.

	Spearman r	95% Confidence Interval	*P* Value
Healthy control horses			
Fall	−0.140	−0.393 to 0.133	.30
Winter	*−0.449*	*−0.640 to −0.206*	*< .001*
Spring	−0.197	−0.442 to 0.075	.14
Summer	*−0.291*	*−0.531 to −0.009*	*.038*
Healthy overweight/Obese control horses	−0.105	−0.432 to 0.246	.55
PPID animals	0.092	−0.378 to 0.524	.70
EMS animals	0.414	−0.064 to 0.738	.078
PPID and EMS animals with hyperinsulinemia	*0.455*	*0.070 to 0.722*	*.019*

tissues, including adipose tissue, can convert the inactive cortisol metabolite cortisone back to biologically active cortisol via the enzyme 11-β-hydroxysteroid dehydrogenase type 1 (11-β-HSD1), resulting in increased tissue and intracellular levels of cortisol.[34,42] Rodent models and human studies both demonstrate 11-β-HSD1 overexpression in adipose tissue and associations between increases in local and systemic cortisol and development of insulin dysregulation and diabetes in the Metabolic Syndrome in people.[43–47]

Because total cortisol was comparable among PPID, EMS, and healthy horses, increased FCF reflects a decrease in bound cortisol in PPID and EMS animals. There might be decreased availability or binding-affinity of CBG, the primary plasma binding protein for cortisol, in these endocrine diseases. Both obesity and aging also appear to be associated with a chronic low-grade inflammatory state in both people and animals, characterized by increased expression or activity of adipose-derived proinflammatory mediators such as interleukin-6 (IL-6) and leptin[48–55] In sepsis and other systemic inflammatory states, inflammatory mediators such as IL-6 and neutrophil elastase respectively inhibit hepatic production of CBG and perpetuate the cleavage of high-binding affinity CBG into a low-affinity confirmation.[56–58] Thus, both mechanisms could lead to increases in FCF. Cortisol-binding globulin gene polymorphisms are also described in pigs and people, and appear to influence circulating cortisol concentrations, tissue cortisol sensitivity, and ratios of fat/lean tissue.[59,60] Further studies to measure concentrations of cortisol-binding proteins (CBG and albumin) in affected animals and to define equine CBG gene polymorphisms are needed to better characterize plasma and tissue cortisol dynamics in equine endocrine disease.

Specific mechanisms relating increased free cortisol and ID in any species are poorly understood. Cortisol is an important glucose counter-regulatory hormone that increases blood glucose; specifically, corticosteroids antagonize the effects of insulin by stimulating hepatic gluconeogenesis and by inhibiting cellular glucose uptake by preventing membrane localization of GLUT-4 glucose transporters.[20,61] This peripheral tissue insulin resistance results in persistently normal to high blood glucose concentrations, which provides continued stimulus for insulin release and eventually results in hyperinsulinemia. Thus, increased biologically available free circulating cortisol or increased tissue/intracellular cortisol because of altered 11-β-HSD1 activity in PPID could result in tissue insulin resistance and hyperinsulinemia via direct antagonism of insulin. Alternatively or additionally, increased free cortisol might cause or perpetuate insulin dysregulation via cortisol's activity on the mineralocorticoid receptors (MR). Mineralocorticoid receptor binds its classical ligand, aldosterone, and cortisol with equal affinity, but in classic aldosterone target tissues such as the renal tubular cells and vascular smooth muscle, cortisol is quickly converted to the inactive metabolite cortisone through the action of 11-β-hydroxysteroid dehydrogenase 2 (11-β-HSD2).[62] Thus, excessive MR activation by cortisol, via either increased circulating cortisol caused by HPA axis overactivty or increased tissue cortisol due to 11-β-HSD1 overactivity, might contribute to insulin dysregulation in equine obesity and endocrine disease.

Total cortisol concentration was significantly higher in healthy overweight/obese control horses. This is mostly likely because of obesity-related increases in HPA axis activity or 11-β-HSD1 expression,[48–55] as discussed above in relation to FCF in animals with endocrine disease. It is possible that an increase in total circulating cortisol, which has global anti-inflammatory effects that include inhibition of pro-inflammatory mediator production and leukocyte migration into tissues,[7,20,63] is actually an appropriate response to an obesity-induced inflammatory state. Alternatively, it is possible that, as discussed for FCF, circulating total cortisol excess attributable to genetic differences, environmental stress, or undiagnosed hypothalamic–pituitary–adrenal (HPA) axis dysregulation could actually be the primary factor that stimulates adipocyte proliferation and increased body fat deposition,[37] and thus ultimately results in obesity in affected horses and ponies. These findings could suggest a continuum of alterations in plasma cortisol-binding dynamics in some horses that could predispose them to insulin dysregulation, beginning with obesity-related HPA axis overactivity that results in increased free and total cortisol, followed by inflammation-related decreases in CBG production and binding-affinity that ultimately leads to the increased FCF without concurrent increases in total cortisol, such as observed in horses with endocrine disorders in this study. Further longitudinal study is needed to assess changes in plasma cortisol-binding dynamics over time

in horses and ponies that do and do not go on to develop EMS, PPID, or ID.

Season significantly impacted total cortisol and insulin concentrations, but not free cortisol measurements, in our healthy horses. An effect of season on hypothalamic–pituitary–adrenal (HPA) axis activity is well documented in horses and ponies, resulting in autumn increases in circulating ACTH and α-melanocyte-stimulating hormone (α-MSH) concentrations and failure to suppress endogenous cortisol in response to dexamethasone in both healthy and PPID animals.[15,23–28] Previous studies have yielded conflicting results on the impact of season on cortisol concentrations in healthy animals, with some studies showing no effect of season on basal total cortisol concentrations[15,27,64,65] or 24-hour cortisol secretion,[66] and others demonstrating increased 24-hour cortisol secretion in the spring.[26] Our results differ from these previous reports because higher total cortisol concentrations were detected in this group of 57 healthy horses in the winter compared to the fall. However, the overall difference among seasons in our study, and in others, was small and might not be clinically significant. Direct comparison of results among studies is difficult given the differences in group size, animal characteristics, geographic location, time of day of sample collection, and assay methodology.

Higher insulin concentrations were also detected in healthy horses in all seasons compared to the fall, and were highest in the spring, which is consistent with a previous report demonstrating increased insulin concentrations in the spring in normal ponies in some years but not others.[64] Other studies have reported conflicting findings regarding the effect of season on insulin concentrations, ranging from no effect of season[15] to higher insulin concentrations in September[65] to lower insulin concentrations in June compared to February/March.[24] In our study, many healthy horses were maintained on pasture overnight before sample collection to minimize the effect of husbandry changes on cortisol secretion,[67] so increases in dietary carbohydrates in rapidly growing pastures likely played a role in this observed increase in serum insulin concentrations at the spring sampling time. Again, breed, sample timing, and dietary differences among animals in these studies preclude direct comparison among these studies.

There are several limitations of this study. First, it would have been ideal to use dynamic testing methods such as TRH stimulation and oral glucose tolerance testing to screen control horses for PPID and EMS, rather than resting ACTH and insulin concentrations. Thus, it is possible that some animals in the control group might have had early-stage endocrine disease that was not apparent with screening tests. However, no control group animals developed clinical signs associated with EMS or PPID during the 9-month course of the study. Serum insulin concentration was also significantly increased in horses and ponies with PPID and EMS compared to age-matched healthy controls, which would be anticipated for these disorders, and provides further evidence that the control animals did not have subclinical disease.

Potential differences in group age and breed characteristics might also have impacted results. PPID animals were older (mean age 25.05 ± 6.44 years) than the age group C control horses (mean age 18.41 ± 3.65 years); because FCF and insulin were significantly increased in older control animals, some differences might be accounted for by age rather than endocrine disease. A truly age-matched control group for the PPID horses would have been ideal for comparison, but we were unable to secure access to an adequate number of such animals that both met our inclusion criteria and had negative PPID test results. Ponies were also excluded from the control group to permit assessment of the effects of age, sex, body condition, and season on cortisol measurements using a more homogeneous control group, as endocrine differences between horses and ponies are described.[1,2,31] However, ponies were included in the endocrine disease groups in order to increase disease group size. This is a substantial limitation of this study, as some of the differences in cortisol and insulin measurements identified between control and affected animals could be related at least in part to breed differences. However, there is conflicting evidence regarding increased susceptibility to PPID in some pony breeds,[2] and there are breed differences in susceptibility to EMS,[1,31] so any breed differences in endocrine measurements in animals with these diseases might be relevant. Further study in larger numbers of animals is necessary to separate the effects of age versus PPID and of breed on equine cortisol-binding dynamics.

In addition, variation in body condition among groups could also have impacted the results. Obesity was an inclusion criteria for the EMS group in this study, but EMS manifest by ID and dyslipidemia and altered adipokine concentrations can (albeit less commonly) also occur in nonobese animals.[1,4,31] Thus, the inclusion criteria of this study selected for a subset of EMS animals that were concurrently obese; further study to assess free cortisol and insulin dynamics in equine ID in both obese and nonobese animals is needed to elucidate associations among obesity, cortisol, and ID in horses. Body condition score also was not available for PPID group animals, so determination of the impact of BCS on free cortisol measurements in this disease group was not conducted. It is also possible that some animals in the EMS group suffered from concurrent PPID, because both endocrine disorders can occur at the same time.[4] It would have been ideal to measure plasma ACTH concentrations to screen horses in the EMS group for PPID, as performed in the control group, but archived samples were only available in limited amounts for EMS animals. However, animals in the EMS group were younger than the PPID group animals, and had no clinical signs specific to PPID such as muscle wasting or hair coat abnormalities.

Finally, while horses with PPID and EMS also exhibit similar regional adiposity, dyslipidemia, and insulin dysregulation, as observed in people with Cushing's Syndrome and Metabolic Syndrome, and the results of this study demonstrate similar increases in free cortisol in PPID and insulin dysregulation (hyperinsulinemia) as in comparable human disorders, there are key differences among the human and equine corollaries of these

diseases. For instance, increased total plasma cortisol is a typical finding in people with Cushing's Disease[68,69] but is not commonly observed in horses and ponies with PPID.[2] Further, atherosclerosis and related cardiovascular disease (ie, stroke, myocardial infarction) are a key aspect of Metabolic Syndrome in people and are associated with increases in total and free cortisol,[34,70] but these clinical abnormalities are not appreciated in EMS.[1,31] In EMS, cardiovascular pathology resulting from vascular endothelial dysfunction might manifest as laminitis,[71] but other mechanisms, such as insulin-mediated overstimulation of the insulin-like growth factor 1 receptor leading to lamellar hypertrophy and weakening, might also contribute to the development of laminitis in EMS.[72] Thus, it is possible that the alterations in cortisol-binding dynamics reflected in this study develop via different mechanisms or have a different pathophysiologic significance in equine endocrine disease as in previous rodent and human studies.

In conclusion, increased free cortisol was detected in obese control horses and in horses and ponies with PPID. Serum FCF and insulin were positively correlated in hyperinsulinemic animals with EMS or PPID. Decreased availability of cortisol-binding proteins such as CBG, and resultant increases in free cortisol, might occur in PPID or hyperinsulinemia, or might develop with age or obesity. Further study is needed to determine if increased FCF plays a causal role in EMS, PPID, or ID in horses and ponies, and to characterize and clarify relationships between free cortisol and clinical abnormalities in these equine endocrinopathies.

Footnotes

[a] Immulite, Diagnostics Product Corporation, Los Angeles, CA. Assays performed at the Animal Health Diagnostic Center, Cornell University College of Veterinary Medicine, Ithaca, NY, except for ACTH assays in the PPID group, which were performed with identical methodology in one of the author's (DM) labs

[b] EMD Millipore, Billerica, MA. Assays performed at the Animal Health Diagnostic Center, Cornell University College of Veterinary Medicine, Ithaca, NY

[c] http://www.mathgoodies.com/calculators/random_number.html

[d] Stata IC/11, StataCorp LP, College Station, TX

[e] GraphPad Prism Version 5.0, La Jolla, CA

Acknowledgments

The authors acknowledge the University of Georgia Equestrian Team, the University of Georgia College of Veterinary Medicine's Equine Teaching Facility at Oconee Farm, and University of Georgia faculty, staff and students for provision of healthy horses for this study; and Dr. Roy Berghaus for assistance with data analysis. This study was funded by the Veterinary Medical Experiment Station, University of Georgia College of Veterinary Medicine.

Conflict of Interest Declaration: Authors disclose no conflict of interest.

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

References

1. Frank N, Geor R, Bailey S, et al. Equine metabolic syndrome. J Vet Intern Med 2010;24:467–475.

2. McFarlane D. Equine pituitary pars intermedia dysfunction. Vet Clin North Am Equine Pract 2011;27:93–113.

3. Wyse C, McNie K, Tannahill V, et al. Prevalence of obesity in riding horses in Scotland. Vet Rec 2008;162:590–591.

4. Frank N, Tadros EM. Insulin dysregulation. Equine Vet J 2014;46:103–112.

5. McGowan T, Pinchbeck G, McGowan C. Prevalence, risk factors and clinical signs predictive for equine pituitary pars intermedia dysfunction in aged horses. Equine Vet J 2013;45:74–79.

6. Mastro LM, Adams AA, Urschel KL. Pituitary pars intermedia dysfunction does not necessarily impair insulin sensitivity in old horses. Domest Anim Endocrinol 2015;50:14–25.

7. Bailey S. Corticosteroid-associated laminitis. Vet Clin North Am Equine Pract 2010;26:277–285.

8. McGowan C. Endocrinopathic laminitis. Vet Clin North Am Equine Pract 2010;26:233–237.

9. Bailey S, Menzies-Gow N, Harris P, et al. Effect of ordinary dietary fructans and dexamethasone administration on the insulin response of ponies predisposed to laminitis. J Am Vet Med Assoc 2007;231:1365–1373.

10. French K, Pollitt C, Pass M. Pharmacokinetics and metabolic effects of triamcinolone acetonide and their possible relationship to glucocorticoid-induced laminitis in horses. J Vet Pharmacol Ther 2000;23:287–292.

11. Haffner JC, Eiler H, Hoffman RM, et al. Effect of a single dose of dexamethasone on glucose homeostasis in healthy horses by using the combined intravenous glucose and insulin test. J Anim Sci 2009;87:131–135.

12. Tiley HA, Geor RJ, McCutcheon LJ. Effects of dexamethasone on glucose dynamics and insulin sensitivity in healthy horses. Am J Vet Res 2007;68:753–759.

13. 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.

14. van der Kolk JH, Wensing T. Urinary concentration of corticoids in ponies with hyperlipoproteinaemia or hyperadrenocorticism. Vet Q 2000;22:55–57.

15. Place N, McGowan C, Lamb S, et al. Seasonal variation in serum concentrations of selected metabolic hormones in horses. J Vet Intern Med 2010;24:650–654.

16. Fernandez-Real J, Pugeat M, Grasa M, et al. Serum corticosteroid binding globulin concentration and insulin resistance syndrome: a population study. J Clin Endocrinol Metab 2002;87:4686–4690.

17. Wang M. The role of glucocorticoid action in the pathophysiology of the metabolic syndrome. Nutr Metab 2005;2:3. doi: 10.1186/1743-7075-2-3.

18. Alexander S, Irvine C. The effect of social stress on adrenal axis activity in horses: the importance of monitoring corticosteroid-binding globulin capacity. J Endocrinol 1998;157:425–432.

19. Hart K, Barton M, Ferguson D, et al. Serum free cortisol fraction in healthy and septic neonatal foals. J Vet Intern Med 2011;25:345–355.

20. Stewart P. The adrenal cortex. In: Kronenberg H, Melmed S, Polonsky K, Larsen P, eds. Williams Textbook of Endocrinology, 11th ed. Philadelphia: Saunders Elsevier; 2008:445–503.

21. Gayrard V, Alvinerie M, Toutain P. Interspecies variations of corticosteroid-binding globulin parameters. Domest Anim Endocrinol 1996;13:35–45.

22. Irvine C, Alexander S. Measurement of free cortisol and the capacity and association constant of cortisol-binding proteins in plasma of foals and adult horses. J Reprod Fertil Suppl 1987;35:19–24.

23. Beech J, Boston R, Lindborg S, et al. Adrenocorticotropin concentration following administration of thyrotropin-releasing hormone in healthy horses and those with pituitary pars intermedia dysfunction and pituitary gland hyperplasia. J Am Vet Med Assoc 2007;231:417–426.

24. Beech J, Boston RC, McFarlane D, et al. Evaluation of plasma ACTH, alpha-melanocyte-stimulating hormone, and insulin concentrations during various photoperiods in clinically normal horses and ponies and those with pituitary pars intermedia dysfunction. J Am Vet Med Assoc 2009;235:715–722.

25. Copas V, Durham A. Circannual variation in plasma adrenocorticotropic hormone concentrations in the UK in normal horses and ponies, and those with pituitary pars intermedia dysfunction. Equine Vet J 2012;44:440–443.

26. Cordero M, Brorsen B, McFarlane D. Circadian and circannual rhythms of cortisol, ACTH and alpha-melanocyte stimulating hormon in healthy horses. Domest Anim Endocrinol 2012;43:317–324.

27. Donaldson M, McDonnell S, Schanbacher B, et al. Variation in plasma adrenocorticotropic hormone concentration and dexamethasone suppression test results with season, age, and sex in healthy ponies and horses. J Vet Intern Med 2005;19:217–222.

28. McFarlane D, Paradis MR, Zimmel D, et al. The effect of geographic location, breed, and pituitary dysfunction on seasonal adrenocorticotropin and alpha-melanocyte-stimulating hormone plasma concentrations in horses. J Vet Intern Med 2011;25:872–881.

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

30. Miller MA, Pardo ID, Jackson LP, et al. Correlation of pituitary histomorphometry with adrenocorticotrophic hormone response to domperidone administration in the diagnosis of equine pituitary pars intermedia dysfunction. Vet Pathol 2008;45:26–38.

31. Frank N. Equine metabolic syndrome. Vet Clin North Am Equine Pract 2011;27:73–92.

32. Henneke D, Potter G, Kreider J, et al. Relationship between condition score, physical measurments, and body fat percentage in mares. Equine Vet J 1983;15:371–372.

33. Wild SH, Byrne CD. ABC of obesity. Risk factors for diabetes and coronary heart disease. BMJ 2006;333:1009–1011.

34. Anagnostis P, Athyros V, Tziomalos K, et al. The pathogenic role of cortisol in the metabolic syndrome: a hypothesis. J Clin Endocrinol Metab 2009;94:2692–2701.

35. Fernandez-Real J, Grasa M, Casamitjana R, et al. The insulin resistance syndrome and the binding capacity of cortisol binding globulin (CBG) in men and women. Clin Endocrinol 2000;52:93–99.

36. Rebuffe-Scrive M, Walsh UA, McEwen B, et al. Effect of chronic stress and exogenous glucocorticoids on regional fat distribution and metabolism. Physiol Behav 1992;52:583–590.

37. Lee MJ, Pramyothin P, Karastergiou K, et al. Deconstructing the roles of glucocorticoids in adipose tissue biology and the development of central obesity. Biochim Biophys Acta 2014;1842:473–481.

38. Crowley RK, Hughes B, Gray J, et al. Longitudinal changes in glucocorticoid metabolism are associated with later development of adverse metabolic phenotype. Eur J Endocrinol 2014;171:433–442.

39. Stalder T, Kirschbaum C, Alexander N, et al. Cortisol in hair and the metabolic syndrome. J Clin Endocrinol Metab 2013;98:2573–2580.

40. Veldhorst MA, Noppe G, Jongejan MH, et al. Increased scalp hair cortisol concentrations in obese children. J Clin Endocrinol Metab 2014;99:285–290.

41. Wester VL, Staufenbiel SM, Veldhorst MA, et al. Long-term cortisol levels measured in scalp hair of obese patients. Obesity 2014;22:1956–1958.

42. Rask E, Olsson T, Soderberg S, et al. Tissue-specific dysregulation of cortisol metabolism in human obesity. J Clin Endocrinol Metab 2001;86:1418–1421.

43. Baudrand R, Vaidya A. Cortisol dysregulation in obesity-related metabolic disorders. Curr Opin Endocrinol Diabetes Obes 2015;22:143–149.

44. Masuzaki H, Paterson J, Shinyama H, et al. A transgenic model of visceral obesity and the metabolic syndrome. Science 2001;294:2166–2170.

45. Morgan SA, McCabe EL, Gathercole LL, et al. 11beta-HSD1 is the major regulator of the tissue-specific effects of circulating glucocorticoid excess. Proc Natl Acad Sci USA 2014;111:E2482–E2491.

46. Morton NM. Obesity and corticosteroids: 11beta-hydroxysteroid type 1 as a cause and therapeutic target in metabolic disease. Mol Cell Endocrinol 2010;316:154–164.

47. Morton NM, Paterson JM, Masuzaki H, et al. Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11 beta-hydroxysteroid dehydrogenase type 1-deficient mice. Diabetes 2004;53:931–938.

48. Aguilar-Valles A, Inoue W, Rummel C, et al. Obesity, adipokines and neuroinflammation. Neuropharmacology 2015;96:124–134.

49. Baylis D, Bartlett DB, Patel HP, et al. Understanding how we age: insights into inflammaging. Longev Healthspan 2013;2:8.

50. De Martinis M, Franceschi C, Monti D, et al. Inflammation markers predicting frailty and mortality in the elderly. Exp Mol Pathol 2006;80:219–227.

51. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci 2014;69(Suppl 1):S4–S9.

52. Vasto S, Candore G, Balistreri CR, et al. Inflammatory networks in ageing, age-related diseases and longevity. Mech Ageing Dev 2007;128:83–91.

53. Habib SA, Saad EA, Elsharkawy AA, et al. Pro-inflammatory adipocytokines, oxidative stress, insulin, Zn and Cu: interrelations with obesity in Egyptian non-diabetic obese children and adolescents. Adv Med Sci 2015;60:179–185.

54. Sindhu S, Thomas R, Shihab P, et al. Obesity is a positive modulator of IL-6R and IL-6 expression in the subcutaneous adipose tissue: significance for metabolic inflammation. PLoS One 2015;10:e0133494.

55. Dib LH, Ortega MT, Fleming SD, et al. Bone marrow leptin signaling mediates obesity-associated adipose tissue inflammation in male mice. Endocrinology 2014;155:40–46.

56. Bartalena L, Hammond GL, Farsetti A, et al. Interleukin-6 inhibits corticosteroid-binding globulin synthesis by human hepatoblastoma-derived (Hep G2) cells. Endocrinology 1993;133:291–296.

57. Bernier J, Jobin N, Emptoz-Bonneton A, et al. Decreased corticosteroid-binding globulin in burn patients: relationship with interleukin-6 and fat in nutritional support. Crit Care Med 1998;26:452–460.

58. Nenke MA, Rankin W, Chapman MJ, et al. Depletion of high-affinity corticosteroid-binding globulin corresponds to illness severity in sepsis and septic shock; clinical implications. Clin Endocrinol 2015;82:801–807.

59. Barat P, Duclos M, Gatta B, et al. Corticosteroid binding globulin gene polymorphism influences cortisol driven fat distribution in obese women. Obes Res 2005;13:1485–1490.

60. Ousova O, Guyonnet-Duperat V, Iannuccelli N, et al. Corticosteroid binding globulin: a new target for cortisol-driven obesity. Mol Endocrinol 2004;18:1687–1696.

61. Coderre L, Vallega GA, Pilch PF, et al. In vivo effects of dexamethasone and sucrose on glucose transport (GLUT-4) protein tissue distribution. Am J Physiol 1996;271:E643–E648.

62. Briet M, Schiffrin EL. The role of aldosterone in the metabolic syndrome. Curr Hypertens Rep 2011;13:163–172.

63. Hart K, Barton M, Vandenplas M, et al. Effects of low-dose hydrocortisone therapy on immune function in neonatal horses. Pediatr Res 2011;70:72–77.

64. Borer-Weir KE, Menzies-Gow NJ, Bailey SR, et al. Seasonal and annual influence on insulin and cortisol results from overnight dexamethasone suppression tests in normal ponies and ponies predisposed to laminitis. Equine Vet J 2013;45:688–693.

65. Schreiber C, Stewart A, Kwessi E, et al. Seasonal variation in results of diagnostic tests for pituitary pars intermedia dysfunction in older, clinically normal gelding. J Am Vet Med Assoc 2012;241:241–247.

66. Haritou S, Zylstra R, Ralli C, et al. Seasonal changes in circadian peripheral plasma concentrations of melatonin, serotonin, dopamine, and cortisol in aged horses with Cushing's disease under natural photorperiod. J Neuroendocrinol 2008;20: 988–996.

67. Irvine C, Alexander S. Factors affecting the circadian rhythm in plasma cortisol concentrations in the horse. Domest Anim Endocrinol 1994;11:227–238.

68. Newell-Price J, Bertagna X, Grossman AB, et al. Cushing's syndrome. Lancet 2006;367:1605–1617.

69. Sharma ST, Nieman LK, Feelders RA. Cushing's syndrome: epidemiology and developments in disease management. Clin Epidemiol 2015;7:281–293.

70. Di Dalmazi G, Vicennati V, Rinaldi E, et al. Progressively increased patterns of subclinical cortisol hypersecretion in adrenal incidentalomas differently predict major metabolic and cardiovascular outcomes: a large cross-sectional study. Eur J Endocrinol 2012;166:669–677.

71. Geor R. Current concepts on the pathophysiology of pasture-associated laminitis. Vet Clin North Am Equine Pract 2010; 26:265–276.

72. de Laat MA, Pollitt CC, Kyaw-Tanner MT, et al. A potential role for lamellar insulin-like growth factor-1 receptor in the pathogenesis of hyperinsulinaemic laminitis. Vet J 2013;197: 302–306.