



The Effect of Insulin Dysregulation and Breed on HPA Axis Function and Plasma Cortisol Binding Dynamics in Ponies and Horses

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Mechanisms resulting in breed predispositions to insulin dysregulation (ID) and endocrinopathic laminitis are poorly characterized. The adrenal steroid cortisol antagonizes insulin, and free, biologically active cortisol can be increased in ID. Breed-related differences in serum free cortisol fraction (FCF) could contribute to ID in pre-disposed breeds such as ponies, but FCF has not been quantified in ID-predisposed-breeds. The objective of this study was to compare FCF between horses and ponies during health and ID. We hypothesized: 1) in health, FCF is higher in ponies than horses; and 2) FCF is further increased in ponies with ID. Serum total cortisol (TC), ACTH, FCF and insulin were measured in 36 horses (age 1-24 years) and 31 mixed-breed ponies (age 4-27 years). Animals were sampled before morning feeding in their normal routine, and ID defined as fasted insulin > 20 μ IU/ml or non-fasted insulin > 60 μ IU/ml for animals sampled on pasture. Data were compared with Mann-Whitney tests and Spearman correlation analysis ($P < 0.05$). TC and FCF were comparable in healthy horses and ponies, but ACTH and insulin concentrations were 1.3-1.6-fold higher in ponies ($P = 0.001-0.041$). In animals with ID, TC was similar but FCF and insulin were increased 1.6-fold and 3.2-fold respectively in ponies ($n = 9$) compared to horses ($n = 11$, $P = 0.01-0.049$), and FCF and insulin were positively correlated ($P = 0.04$, $r = 0.45$, $95\%C.I = 0.014-0.746$). These data demonstrate differences in hypothalamic-pituitary-adrenal axis function during health and ID between ponies and horses. Further study is needed to determine if and how such alterations impact insulin regulation and, ultimately, laminitis risk.



Pro-Hormone Processing in PPID

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PPID is characterized by proliferation of melanotropes within the pars intermedia (PI) of the pituitary gland and increased production of pro-opiomelanocortin (POMC). POMC is cleaved by pro-hormone convertases (PC1 and PC2) to produce its derivatives, including adrenocorticotrophic hormone (ACTH). Dysregulation of PC1 and PC2 occurs in the absence of dopamine inhibition and results in overabundance of ACTH, along with impairment in conversion of ACTH to α MSH and CLIP in mice. The main objective of this study was to determine whether mRNAs encoding POMC, PC1 and PC2 in the pituitary gland are altered in PPID horses.

Pituitaries of 6 PPID and 6 normal horses were collected immediately postmortem and snap frozen. Total RNA was extracted and cDNAs were synthesized. PCRs were conducted using validated primers for POMC, PC1 and PC2 obtained using GenBank predicted sequences. Nine internal control genes were tested and the most stable (18s) was used as the internal control gene. Quantification of POMC, PC1 and PC2 mRNAs were performed using RT-qPCR. Results showed that horses with PPID had a significantly increased expression of POMC, PC1 and PC2 mRNAs. There was not a significant difference between the degree of upregulation between PC1 and PC2. In conclusion, upregulation of POMC, PC1 and PC2 in equine PPID appears to contribute to the elevated ACTH concentrations in the diseased state.



The Effect of Geographic Location on Circannual Adrenocorticotrophic Hormone Plasma Concentrations in Australian Horses

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Northern hemisphere research has documented lower, quiescent, endogenous ACTH levels from mid-July to mid-November with dynamic changes in the alternate half of the year, peaking at the autumn equinox. Longitudinal evaluation of individual horses in the southern hemisphere has not been undertaken to establish if similar reference intervals (RI) and circannual rhythm occurs.

This study involved 40 normal horses at each of two locations 31°57'S, 115°52'E (Perth, southern Australia) and 19°26'S, 146°81'E (Townsville, northern Australia). ACTH was measured at approximately the same time of day/month for 12 consecutive months and monthly and grouped RI generated.

A quiescent period of ACTH was observed at both sites, with the southern Australia location having a shorter period with lower upper reference limits compared to the northern Australian location. However, upper reference limits at both locations during this period were higher than those previously reported (43pg/ml Perth, 67pg/ml Townsville). The duration of the dynamic phase differed at each location, but peaked at the autumn equinox with upper reference limits similar between both Australian locations and northern hemisphere studies (100pg/ml). During the dynamic phase, ACTH was more variable in normal horses compared to the quiescent phase and, occasionally, spurious elevations occurred.

It was concluded that, similar to the Northern Hemisphere, circannual ACTH rhythmicity is associated with changing day length. Quiescent ACTH levels in normal horses appear to be higher in Australia with smaller differences between quiescent and dynamic phases the closer the location to the equator, the latter possibly reflecting reduced circannual day length variation.



Is Equine Pars Intermedia Activity Subdued in the Spring?

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Published studies describe an increase in plasma ACTH in August, September and October. Additionally, unpublished data suggested a nadir of ACTH in April. The current study was intended to re-examine the circannual pattern of ACTH in horses with particular attention to the suspected spring nadir.

Data were examined from almost 150,000 chilled plasma samples between January 2012 and August 2016. Median monthly ACTH concentrations revealed plasma ACTH concentrations between July and November inclusive to be significantly greater than December to June (Figure 1). Median ACTH concentration in April was significantly lower than at other times of year. Closer examination of weekly data indicated the nadir to be from 9th April–6th May.

In 1,565 cases the basal plasma ACTH sample was collected as part of a TRH stimulation test. In these cases, the median ACTH concentration measured 10 minutes following TRH revealed a similar circannual pattern with the highest ACTH response in October (median 381 pg/mL) and the lowest in April (85.5 pg/mL).

Both basal plasma ACTH concentration and the response to TRH appear to be lower in April compared to other months of the year suggesting decreased secretory function at that time of year. Reference intervals using the same cutoffs for “non-autumnal months” may not be appropriate for samples collected in April.

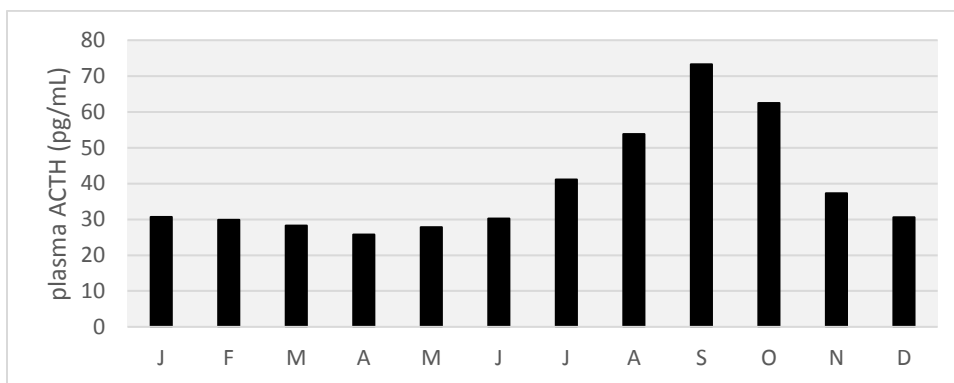


Figure 1. Median monthly plasma ACTH concentration.

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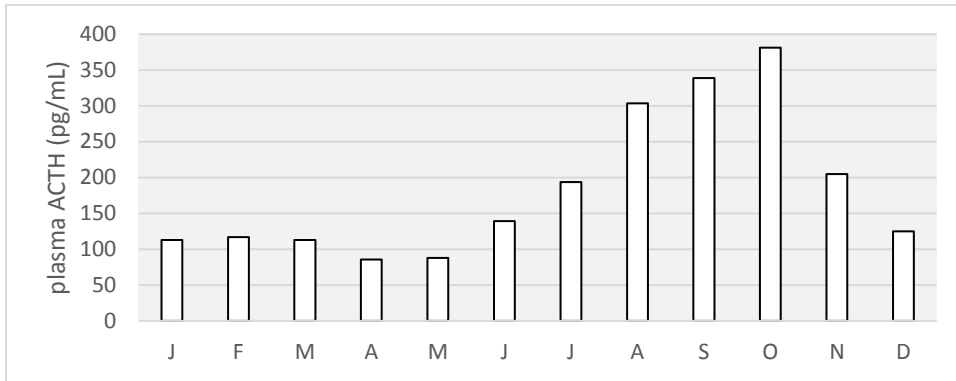


Figure 2. Median monthly plasma ACTH concentrations 10 mins following 1 mg TRH iv.



Comparison of Plasma ACTH Assays in Ponies Suggests Seasonally Dependent Assay Cross-Reactivity

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Aims: To compare plasma [ACTH] measured by validated chemiluminescent and immunofluorescent assays in spring and fall samples and to determine whether the assays provide similar binary results.

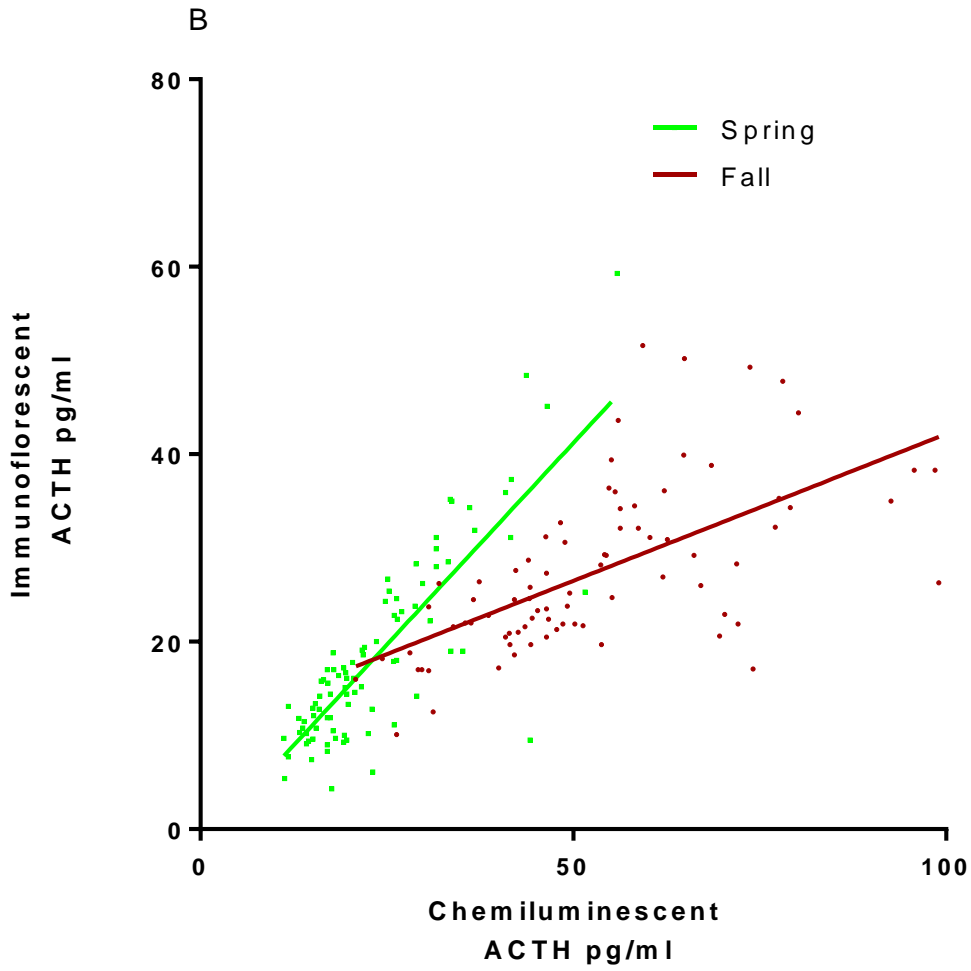
Methods: Plasma [ACTH] was measured using chemiluminescent and immunofluorescent methods in EDTA-plasma from ponies, with no known history of laminitis, in the spring and fall. Assay cross-reactivity to human synthetic corticotropin-like intermediate peptide (CLIP) was assessed by spiked recovery. Diagnostic thresholds for the immunofluorescent method were derived to correspond with common chemiluminescent thresholds for PPID diagnosis.

Results: Plasma [ACTH] differed between assays ($P < 0.001$); mean differences (chemiluminescent-immunofluorescent), (95% confidence intervals): fall ($n=99$) 38.6 (30.6-46.5)pg/ml, spring ($n=88$) 5.1 (3.9-6.3)pg/ml. The correlation between the assays differed between seasons ($P < 0.001$; figure 1). Spiked recovery demonstrated CLIP cross-reactivity of 1-2% over [CLIP] range of 548-49,000pg/ml in the chemiluminescent but not immunofluorescent assay. Good (Kappa=0.66-0.74) agreement was obtained for binary interpretation using immunofluorescent thresholds of >24 pg/ml and >27 pg/ml and chemiluminescent thresholds of >29 pg/ml and >47 pg/ml in spring and fall, respectively. In ponies with spring and fall samples ($n=88$), plasma [ACTH] exceeded a chemiluminescent fall threshold (>47 pg/ml) in 64%, but returned to normal limits without treatment in the spring in 70% of these cases. Complete data was available for 85 ponies of which 68% had no clinical or owner-reported signs of PPID.

Conclusions: The assays yielded different absolute values but had good agreement for binary classification. The hypothesis that chemiluminescent assay detection of other POMC-derived peptides, particularly in the fall, accounts for the assay differences, warrants further investigation. *Continued next page*



Figure 1: The association between ACTH concentrations measured by chemiluminescent and immunoflorescent assays in the spring and fall.





Assessment of Prolactin Concentration as a Screening Test for PPID

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Aims: To assess measurement of prolactin (PRL) concentration as a screening test for PPID.

Methods: PRL concentration was compared between 31 horses that were enrolled in and 26 horses that failed inclusion criteria for the clinical efficacy study for pergolide. Enrollment required horses to have a hypertrichosis score ≥ 1 and either an elevated plasma ACTH concentration (≥ 50 pg/mL) or abnormal overnight dexamethasone suppression test results. PRL was measured again after 3 and 6 months of treatment with pergolide in the horses enrolled in the study. PRL concentrations were compared by rank sum analysis and an ANOVA on ranks for repeated measures. A ROC curve was constructed to assess sensitivity and specificity of PRL at various cut-off values as a screening test for PPID.

Results: PRL was greater ($p=0.02$) in enrolled (7.0 ± 8.2 ng/mL, mean \pm SD) as compared to excluded (4.3 ± 2.6 ng/mL) horses. Area under the ROC curve was 0.68 (95% CI: 0.55-0.80). Using cut-off values of ≥ 3.5 and ≥ 4.0 ng/mL, sensitivity and specificity were 77 and 54% and 65 and 54%, respectively. Curiously, PRL tended to decrease after 3 months of pergolide treatment (3.8 ± 1.8 ng/mL) but the only significant difference was a higher ($p<0.01$) PRL after 180 days of pergolide treatment (9.7 ± 6.4 ng/mL) as compared to the 90 day treatment value.

Conclusion: In this cohort of horses, measurement of PRL did not appear to be a useful screening tool for PPID. Further, treatment of PPID-affected horses with pergolide did not decrease PRL.

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Development and Application of a Novel Diagnostic Test for PPID using MALDI-TOF proteomics

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Introduction: Current diagnostic tests for pituitary pars intermedia dysfunction (PPID) fail to accurately identify early cases. Physiological stimulation of the pituitary pars intermedia (PI) cannot be differentiated from pathologic loss of inhibition of the PI. As a result, horses not infrequently are being misdiagnosed and lifelong treatment of PPID ensues. A new, more discriminating diagnostic test is needed. POMC peptide production and processing has been shown to be altered in horses with PPID. Enzymes that post translationally modify POMC peptides are regulated by dopamine, thus their activity changes in PPID due to the loss of dopamine in the PI. Based on this observation we hypothesize that plasma peptide signatures might provide a sensitive assay for PPID.

Methods: Plasma samples were collected from >250 horses with accompanying clinical and histological findings. Samples were processed using extraction with organic solvents followed by purification through C18 filter tips to remove abundant proteins (e.g., albumin and globulin) to prevent masking less abundant peptides. Samples were analyzed by MALDI-TOF following extraction. An equine plasma sample spiked with β -endorphin was used to test the extraction protocol. Using 10 PPID and 10 control samples an initial peptide signature for PPID was formulated as proof of principal.

Results: A profile with 91 peptides was generated. When the 20 samples were run as blinded unknowns there was 100% recognition and an 80% cross validation using a profile generated with software without further refinement to the algorithm.

Discussion: MALDI-TOF generated plasma POMC peptide signatures might prove to be an effective diagnostic test for PPID. Additional modifications to the sample preparation protocol are underway to enhance repeatability and ease of processing. Following optimization of the sample preparation protocol, the full sample set will be tested to calculate the accuracy of this method for diagnosis of early PPID.



Evaluation of Diagnostic Tests for Pituitary Pars Intermedia Dysfunction in Donkeys

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Clinical experience indicates that pituitary pars intermedia dysfunction (PPID) is a frequent endocrine disorder in geriatric donkeys. However, endocrine testing is based on criteria for horses and ponies, but information on this species is lacking. Evaluation of other endocrine systems (thyroid, energy) in donkeys has shown that extrapolation can be misleading. The goal of this pilot study was to evaluate different accepted diagnostic methods for PPID in a group of geriatric donkeys.

Six mix-breed adult donkeys (range:13-30 years-old), 1 jack and 5 jennets from a donkey sanctuary suspect of PPID (age, hypertrichosis, laminitis, regional adiposity, lethargy, increased ACTH concentrations) were selected. Testing was carried out in August and included the dexamethasone suppression test (DST; 40 µg/kg, IM), thyrotropin-releasing hormone (TRH) stimulation test (1 mg, IV), and the combined DST-TRH performed on every donkey with 7 day washout periods.

Marked discrepancies were observed between tests. All donkeys were considered positive for PPID based on basal ACTH concentrations (range: 56-424 pg/ml) and TRH stimulation test (>100 pg/ml 10 min post-TRH), but only four animals were positive with the DST-TRH (66% cortisol increase at 195 min post-dexamethasone and 10 min post-TRH) and three with the DST (cortisol >1 µg/dl at 19 h post-dexamethasone).

Results are in agreement with PPID guidelines for horses, support the use of baseline ACTH and TRH-stimulation, but discourage the DST and DST-TRH (false negatives) for PPID diagnosis in donkeys. Evaluation of these tests in a larger population of healthy and PPID donkeys will validate these findings.



Adrenocorticotropin Hormone Response to Varying Dosages of Thyrotropin Releasing Hormone in Normal Horses

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Background: The thyrotropin-releasing hormone (TRH) response test has become the diagnostic test of choice for identifying horses and ponies with pituitary pars intermedia dysfunction (PPID). Current testing protocol recommends administration of a standard dosage of 1 mg TRH in any size equid. It is unknown whether a relationship exists between dosage of TRH and pituitary hormone response.

Objective: To evaluate the adrenocorticotropin hormone (ACTH) response to administration of 3 different doses of TRH in normal adult horses.

Methods: Using a randomized crossover trial, 12 clinically normal adult horses were assigned to receive 0.5 mg, 1 mg, or 2 mg TRH. Endogenous plasma ACTH was measured at 0, 5, 10, 15 and 30 minutes after IV administration of TRH. The test was repeated after two-week washout periods in order that individual horses received each TRH dosage during the testing period.

Results: Mean plasma ACTH concentration after TRH administration was not significantly different among the three dosages. A significant effect was noted in both endogenous and TRH-stimulated ACTH concentration by month. A difference in endogenous ACTH was noted while testing during inclement weather, but this was not significant.

Conclusions: It may be possible to perform a TRH response testing using a lower dose of 0.5 mg for economic reasons, however further studies examining horses with PPID would be necessary before making this recommendation. Season and external stressors could affect TRH response testing even when a lower dosage is administered. Therefore, environmental stress should be minimized where possible when performing this test.



Evaluating Seasonal Influences on Hormone Responses to a Diagnostic Test (Thyrotropin-Releasing Hormone Stimulation) Advocated for Early Diagnosis of Pituitary Pars Intermedia Dysfunction (PPID).

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Horses with early PPID may fail to test positive using basal endocrine diagnostic tests. Thus, the TRH stimulation test is currently recommended where early PPID is suspected. Effects of season on the hormone responses to this dynamic test have not been fully evaluated in similarly managed groups of non-PPID, subclinical and clinical PPID horses over a 12-month period. The TRH stimulation test (T0, T10 and T30 min blood collections) and clinical evaluations were performed each month for a 12-month period using the same group of horses, under the same management, characterized as non-PPID (n=17), subclinical PPID (no hirsutism and 1 abnormal endocrine test) (n=21), and clinical PPID (hirsutism and 2 or more abnormal endocrine tests) (n=25). EDTA plasma ACTH was measured by Cornell. Within non-PPID, subclinical PPID and clinical PPID groups, RM ANOVAs demonstrated basal, T10 and T30 ACTH values all varied significantly between months (P<0.001). Two-way RM ANOVA demonstrated basal, T10 and T30 ACTH values all varied significantly with both PPID status and season (P<0.001). Youden indexes were used to determine seasonal and monthly cut-off values for basal, T10 and T30 ACTH concentrations which should be considered when diagnosing PPID.



Highly Variable Autumnal TRH-Stimulation Tests in Normal Horses at two Australian Locations

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The TRH stimulation test is widely used as an additional diagnostic test for PPID. Testing is recommended at times when ACTH is quiescent, between the winter and summer solstices. Current research in the northern hemisphere recommends a cut-off of 110 pg/ml ACTH at 10 minutes and 65 pg/ml at 30 minutes post-TRH administration. It is unclear whether TRH-stimulation testing when ACTH is dynamically changing is appropriate or whether similar cut-offs are applicable in the southern hemisphere.

Thirteen normal horses at Perth and 32 at Townsville were identified by clinical parameters and normal monthly endogenous ACTH. TRH-stimulation tests were performed during the ACTH quiescent phase on the Perth cohort in September and during the dynamic phase at both sites (March, Perth; April, Townsville).

In the Perth cohort, post-TRH ACTH concentrations were significantly different between March and September ($p=0.001$). The mean ACTH concentration pre-TRH and 10 minutes post-TRH in autumn was 51 pg/ml (95% CI 46-57) and 249 pg/ml (95% CI 170-327) respectively. In the Townsville cohort, the mean ACTH concentration pre-TRH and 30 minutes post-TRH in autumn was 44 pg/ml (95% CI 38-52) and 100 pg/ml (95% CI 82-120).

The ACTH cutoff for TRH-stimulation testing during the quiescent period in Perth appears similar to the northern hemisphere. However, TRH-stimulation testing in normal horses, when ACTH is dynamically changing between summer and winter solstices, was extremely variable at both locations. During this period, there may be considerable overlap between normal and PPID-affected horses and further research is required to avoid erroneous diagnosis.



ACTH Responses after Application of two TRH Doses in Healthy Horses

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Introduction: Usually 1mg TRH per horse (2.0µg/kg in a 500kg horse) is used to stimulate ACTH secretion from the pituitary gland in order to detect PPID. The purpose of this study was to evaluate 1.0 and 2.0 µg TRH/kg bodyweight for its ability to stimulate ACTH secretion in healthy horses.

Methods: A randomised cross-over trial with horses and ponies without clinical disease and normal basal ACTH was planned. Equines ≥ 15 years were included if they showed normal DST within three weeks before the study. 1.0 and 2.0µg/kg TRH (Ferring Arzneimittel GmbH, Kiel/Germany) was given in a randomised order with a wash-out period of 6 days. Paired t-tests were used to detect significant differences between TRH doses at the different time points.

Results: 8 horses are tested so far. Statistically, no significant differences between TRH doses were detectable.

Table 1: ACTH [pg/ml] response in healthy horses [Median (1st/3rd quartile)]

min/dose	1µg TRH/kg	2 µg TRH/kg
0	15.5 (11.75/22,5)	16.0 (13.75/22.5)
5	167.5 (41.0/259,75)	142.5 (52.0/317.5)
10	94.0 (31.0/169,5)	107.0 (35.75/141.75)
15	51.0 (24.25/104,75)	67.0 (26.25/83.5)
30	32.0 (15.25/61,5)	43.0 (19.0/54.25)
60	22.5 (10.75/32,75)	26.5 (12.5/31.75)
90	17.5 (9.0/25,0)	19.5 (13.5/25.25)

Conclusion: The results suggest that half of the usual TRH dose might be sufficient for testing ACTH release.



Reliability of a Clinical Scoring System for PPID

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Aims: To assess reliability of a clinical scoring system as a predictor of PPID.

Methods: Five signs (hypertrichosis, hyperhidrosis, polyuria/polydipsia [PU/PD], abnormal fat deposition, and muscle wasting) were scored 0-3 during evaluation of horses for enrollment in the clinical efficacy study for pergolide. Enrollment required horses to have a hypertrichosis score ≥ 1 and either an elevated ACTH concentration (≥ 50 pg/mL) or abnormal overnight dexamethasone suppression test results. Differences in individual and composite (minimum 0, maximum 15) scores between 29 enrolled horses and 25 excluded horses were assessed by Wilcoxon rank sum analysis and associations between scores and PPID status were evaluated using logistic regression. In addition, several weighted scoring systems were developed and assessed.

Results: There were no significant differences in age, gender, or weight between enrolled and excluded horses. Hypertrichosis scores were different ($p < 0.01$) between enrolled (2.3 ± 1.7 , mean \pm SD) and excluded (1.7 ± 1.6) horses. However, composite clinical scores in enrolled (range 2-12, 5.9 ± 2.3) and excluded (range 3-10, 5.1 ± 1.8) horses were not different ($p = 0.21$) and there was a difference ($p < 0.01$) in composite scores between study sites, largely attributable to differences in scoring of hyperhidrosis and PU/PD. A weighted score, excluding muscle wasting and PU/PD, was a better predictor for PPID ($R^2 = 55.3\%$) than the unweighted score ($R^2 = 26.7\%$).

Conclusion: Hypertrichosis was a valid clinical parameter to differentiate PPID from non-PPID horses. A more detailed, weighted clinical scoring system, using larger numbers of PPID-positive and negative horses, warrants further investigation.



Comparison of Seasonal TRH Stimulation Test Results in Horses with PPID Treated with Prascend® or placebo

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In horses with PPID, altering pergolide dose in response to seasonal changes in ACTH concentrations (ie, increasing dose in fall) has been recommended. However, little is known about the impact of treatment on resting ACTH and TRH stimulation results across seasons. The objective of this study was to compare resting ACTH concentrations and TRH stimulation test results between PPID horses treated with pergolide or placebo for twelve weeks (May-August).

Thirteen horses with PPID were enrolled based upon an ACTH 30 minutes post-TRH of >65 pg/ml (non-fall) or a resting ACTH >50 pg/ml (non-fall) or >100 pg/ml (fall). Horses were randomly assigned to treatment (n=6) or placebo (n=7) groups. Horses were treated with Prascend (1 mg/horse) or placebo for twelve weeks. Resting ACTH and response to TRH was measured at Week 0 (prior to treatment) and at 4, 8, and 12 weeks after initiation of treatment. Treatment with Prascend was increased (by 1 mg/day) in horses at Week 5 and/or Week 9 if resting ACTH did not either 1) return to normal or 2) decrease by >75%.

Data was log transformed and analyzed using repeated measures analysis of variance. Resting ACTH and TRH response results are presented in Figures 1 and 2. There was no treatment effect on resting ACTH ($p=0.44$) or response to TRH ($p=0.48$). However, there was a time effect on TRH stimulation results ($p=0.03$) and a time x treatment interaction for both resting ACTH ($p=0.001$) and response to TRH ($p=0.005$). *Post-hoc* comparisons using a Bonferroni correction revealed a difference between treatment groups in resting ACTH concentration at week 12 (pergolide: 34 ± 10 pg/ml; placebo: 150 ± 93 pg/ml $p=0.03$). These findings suggest that higher doses of pergolide may be required to control ACTH concentrations PPID horses in summer and early fall.

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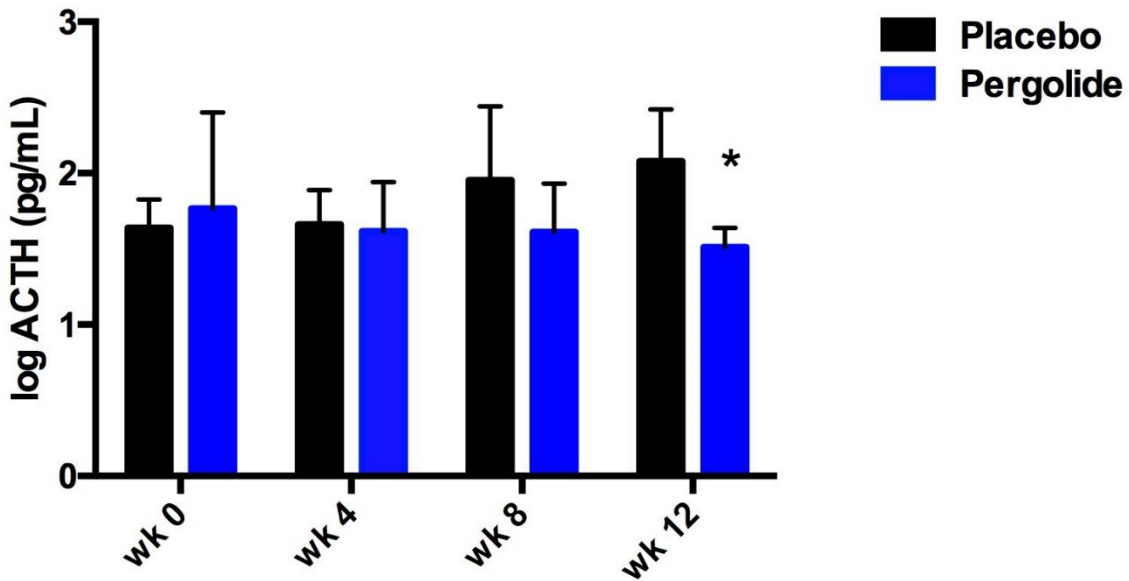


Figure 1. Comparisons of resting ACTH concentrations between pergolide and placebo treated horses from May (Week 0) to August (Week 12). Bars = mean \pm SD. * $p < 0.05$ between groups

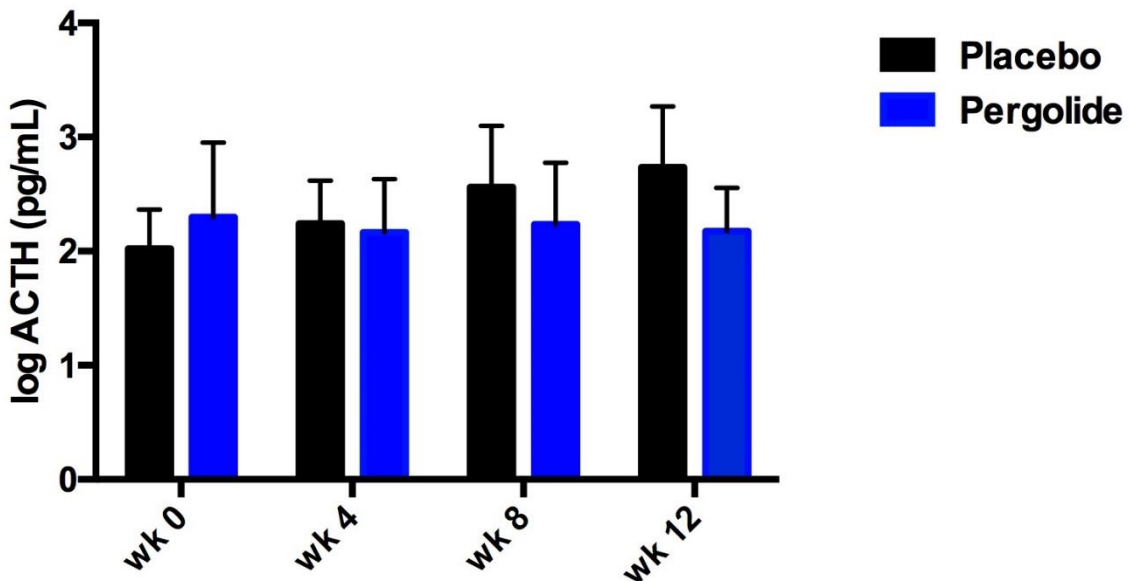


Figure 2. Comparisons of ACTH concentrations in response to TRH stimulation between pergolide and placebo treated horses from May (Week 0) to August (Week 12). Bars = mean \pm SD



Establishment of a Reference Interval for Plasma ACTH Concentration in Aged Horses

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Introduction and Rationale: Measurement of plasma ACTH concentration is commonly used in practice to diagnosis PPID. The population at greatest risk for PPID is aged horses or ponies of any breed. Therefore, a reference interval for PPID was determined using samples from horses 20 years or older, confirmed free of PPID by postmortem examination of the pituitary.

Methods: Plasma samples were collected from 77 horses 20 years or older with normal hair coats that were scheduled for euthanasia for other reasons. All samples were collected between Nov and July. The pituitary gland was collected and a mid-sagittal section was graded 1-5, using the scale of Miller et al. Horses with a PI grade > 3 were excluded. ACTH concentration was determined in remaining samples by chemiluminescence. Mean ACTH concentration plus 2 SD was calculated.

Results: Mean ACTH concentration in horses >20 years of age was 26.4 ± 11.7 . The upper reference interval cutoff suggested by this data is 50 pg/ml.

Discussion: The upper reference interval cutoff in this study is higher than suggested in other studies. Reasons for discordance among the studies may include the animal age, breed differences, health status of the horses or geographical location of the animals sampled. Similar methods of ACTH measurements were used and therefore do not explain the differences observed.

Significance: A resting plasma ACTH concentration < 50 pg/ml would be considered normal in aged horses, according to the present study.



Plasma Concentrations of Adrenocorticotrophic Hormone in Samples Obtained from the Cavernous Sinus of Normal Horses and Horses with Pituitary Pars Intermedia Dysfunction.

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We aimed to compare central ACTH concentration and pulsatility in normal and PPID horses. Samples were collected at 5 minute intervals for one hour starting at 9 am using a catheter inserted into the pituitary intercavernous sinus in four control horse mares (7.0±4.1 [SD] years) and three PPID horses (a 19-year-old Warmblood gelding, a 25-year-old Quarterhorse gelding and a 30-year-old mixed breed mare). PPID horses had generalized hypertrichosis and positive overnight dexamethasone suppression test results in contrast to control horses. ACTH was assessed using chemiluminescence detection (Nichols). PULSE4 analysis revealed one ACTH concentration peak hourly in all horses. Mean basal central ACTH concentration was significantly greater in PPID horses as compared to controls (4577±2143 and 278±388 pg/ml, respectively). The jugular ACTH concentration measured 3-15% of the central concentration in PPID horses (without significant correlation). Deconvolution analysis showed a secretion peak in two PPID horses of 13060 (t_{1/2}=1.2s) and 7888 (t_{1/2}=4.3s) compared to 411 pg/ml (t_{1/2}=15s) in a control horse. One mg TRH IV increased central ACTH concentration from 7174 to 72049 and from 5224 to 17150 pg/ml over 10 minutes in two PPID horses with central concentrations increasing more than peripherally (10 versus 3 times). Two dosages of pergolide (3 mg) decreased central ACTH concentration by 80% (peripherally by 77%) and TRH test results by 57 and 91%, respectively in a 25-year-old QH. In conclusion, adenomas in PPID horses might show pulsatile ACTH secretion leading to very high ACTH concentrations in peripheral blood despite the theoretical concept of autonomous functioning.



Clinical Signs Associated with PPID in the Equine Athlete

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Pituitary Pars Intermedia Dysfunction (PPID) has been described as one of the most common diseases of horses and ponies 15 years of age and older. Recently, the clinical signs of PPID have been divided into early and advanced clinical signs. Establishing a diagnosis of PPID in horses with early clinical signs is currently a difficult challenge facing equine veterinarians. Particularly difficult may be the diagnosis of horses with PPID in the equine athlete. Many of the same clinical signs identified in early or advanced PPID may be recognized in the sport horse along with tendon or suspensory ligament degeneration. Suspensory ligament injuries have been considered a common cause of lameness, in the equine athlete involved in competitive events. A recent histopathological study concluded that an association exists between PPID and suspensory ligament (SL) degeneration. The objective of this study was to identify the most common clinical signs associated with PPID in the sport horse.

Sport horses >10 years of age, any breed, and sex were eligible for study enrollment as long as they were documented to be exhibiting one or more of the early or advanced clinical signs of PPID including suspensory ligament desmitis. Forty-nine horses were evaluated and included in the final data analysis with at least one clinical sign of early or advanced PPID. Demographic data, signalment, and a physical examination was conducted and each horse was tested for PPID using the TRH stimulation test measuring ACTH at 0 (T0ACTH) and 10 (T10ACTH) min. Insulin and glucose levels were also determined. Normal horses were excluded from the study. Blood samples were shipped overnight to the Animal Health Diagnostic Center, Cornell University, Ithaca, NY for analysis. The association between PPID status, based on ACTH and insulin results, and each of the demographic variables (age, sex, and breed), clinical signs, the two test result variables insulin and glucose were statistically evaluated individually using the Pearson chi-square test. Odds ratios for significant predictors of PPID status were computed using corresponding 95% confidence intervals when applying multiple logistic regression analysis.

Of the 49 horses, 19 (39%) were PPID⁺ and 8 (16%) were IR⁺. Of the 19 PPID⁺ horses, only 2 (11%) were PPID⁺ at the T0ACTH time point, whereas 19/19 (100%) were PPID⁺ at T10ACTH. Six (32%) of 19 PPID⁺ horses were hyperinsulinemic. Horses' ages ranged from 11 to 25 years of age (arithmetic mean 17 years) in the PPID⁺ group. The most common clinical signs observed in the PPID⁺ horses were
Continued next page



delayed regional shedding, loss of epaxial muscle mass, regional adiposity, skeletal muscle atrophy and suspensory desmitis. PPID⁺ was significantly ($P= 0.023$) associated with lameness (suspensory desmitis, tendon laxity, superficial digital flexor tendonitis). Of the horses that were lame, 70% were PPID⁺.

Based on the results of this study, the TRH stimulation procedure was required in 89% of enrolled horses for laboratory confirmation of PPID. In the sport horse, suspensory desmitis was significantly associated with PPID⁺ status. Veterinarians should include PPID in the list of differential diagnoses when examining sport horses with suspensory desmitis along with early and advanced clinical signs of PPID.



Adipose Tissue Morphology and Hormone-Sensitive Lipolytic Protein Expression in Lean and Obese Horses and Ponies

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Body weight management is important for the mitigation of risk for the development of insulin dysregulation and associated conditions. Lipolysis is dynamically regulated by several neuroendocrine factors, including insulin and thyroid hormone. Obesity is associated with increased adipocyte volumes and perturbations of lipolysis in other species. This study tested the hypothesis that adipocyte size is increased across specific fat storage depots in obese horses and ponies and this cellular expansion is associated with changes in the expression of key lipid-droplet proteins, perilipin-1 (PLIN1) and hormone-sensitive lipase (HSL).

Adipose tissue samples from 5 anatomically-discrete depots (ventro-abdominal retroperitoneal, epicardial, omental, nuchal crest and tailhead) were collected immediately *post-mortem* from 6 lean (BCS 3.1/9 \pm 0.50) and 6 obese (BCS 7.7/9 \pm 0.46) horses and ponies of mixed breed and gender for both histological and western blot analysis.

Adipocyte area was significantly ($p < 0.05$) greater for obese animals compared to lean animals for all adipose depots studied apart from epicardial WAT. No differences in adipocyte area between depots within lean/obese animals. Protein expression of PLIN1 and HSL was significantly ($p < 0.05$) lower in obese animals for retroperitoneal WAT in both cytosolic and lipid droplet associated protein fractions. Data confirm that equine obesity is associated with changes in the expression of lipolytic proteins in certain depots which may indicate functional differences between regional adipose depots. These data provide a foundation for future work to improve our understanding of endocrine/hormone-sensitive protein interactions and how these proteins may be functionally modified in insulin-dysregulated horses.



Plasma Insulin-Like Growth Factor-1 Concentrations in Ponies and Horses under Conditions of Weight Gain and Weight Loss

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Low levels of insulin-like growth factor-1 (IGF-1) have been associated with obesity and metabolic syndrome in humans. It has been postulated that the link between hyperinsulinaemia and laminitis in equids is mediated via the IGF-1 receptor on lamellar epithelial cells; however, the relationship between breed, diet and adiposity on plasma IGF-1 concentrations has not been fully evaluated.

Eight Standardbred horses, 8 mixed-breed ponies and 8 Andalusian-cross horses were studied (5-15 years old; all of moderate body condition). Animals received either a cereal-rich diet (4 of each breed; meals contained micronized maize) or an isocaloric fat/oil-rich diet (4 of each breed; meals contained vegetable oil). These rations, providing 200% of daily DE requirements, were fed over 20 weeks to induce obesity. Blood samples were collected before and after weight gain, and plasma IGF-1 was measured using a validated ELISA. A multivariate general linear model was used for statistical analysis. Breed differences in plasma IGF-1 were present at the outset, with ponies (149.5 ± 22.1 ng/mL) having higher levels than Standardbreds (115.0 ± 17.9) and Andalusians (88.8 ± 7.5). Plasma IGF-1 concentrations did not change significantly with weight gain. There was no difference between diet groups detected, despite the cereal-rich diet inducing insulin resistance.

Plasma IGF-1 levels appear to be relatively stable in adult equids transitioning between moderate and obese body condition. The higher levels observed in ponies may be related to their size rather than insulin sensitivity since Andalusian horses, with similar insulin responses to ponies, had IGF-1 concentrations which were not detectably different to Standardbreds.



Metabolomics of Equine Metabolic Syndrome

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There are approximately 9.2 million horses in the US, nearly half of which may be overweight and at risk for laminitis due to Equine Metabolic Syndrome (EMS). This study utilizes global metabolomics assays to characterize “metabotypes” associated with EMS. Study horses were volunteered by their owners, from the Arabian breed to control for genetic background, and were age, sex and farm of residence case/control matched whenever possible. A total of 50 animals, 26 females and 24 castrated males with an average age of 16 years, fit the study criteria. Diagnostic endocrinology parameters for these horses, including plasma insulin, leptin, ACTH, glucose and triglycerides were combined with biometric measures of obesity in a factor analysis to yield a quantitative score of EMS severity. Liquid Chromatography-Mass Spectroscopy (LC-MS) analysis of frozen plasma from study horses was conducted through the Southeast Center for Integrative Metabolomics core facilities. Stringent quality control filters yielded 2019 LC-MS peak features, out of a raw count of 6619. Nine features correlated significantly ($p < 7.8e-5$) with the composite EMS score among these 49 horses. Additional analysis including compound identification and pathway exploration is ongoing. As the first dataset of its kind in the horse, this work will reveal promising new biomarkers for veterinary diagnostic use. This discovery-based approach may also generate novel targets for the development of therapeutic interventions for obesity, insulin resistance, and laminitis in horses.



Case Control Study of Pasture and Endocrinopathy-Associated Laminitis in Horses

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Significant efforts have been made in the past decade to further our understanding of laminitis in horses; however, much research has been limited to the study of the mechanistic pathways following experimental induction of disease. The conduct of observational studies of naturally-occurring laminitis is necessary for the improvement of our knowledge and understanding of disease predisposition and the design of future investigations into the prevention and control of this debilitating disease. Thus, the objective of this study was to determine risk factors for the development of pasture- and endocrinopathy-associated laminitis (PEAL) in horses evaluated by veterinarians in North America. In this case-control study, incident cases of PEAL evaluated by veterinary practitioners in North America from 2012-2015 and horses from 2 control populations were included. Participating veterinarians provided historical data from a case of PEAL, a healthy control, and a lameness control. Conditional logistic regression analysis was used to compare data from PEAL-affected horses and each set of controls. A total of 199 horses with acute, incident PEAL, 198 healthy controls, and 153 lameness controls were included in the analysis. Horses with an obese body condition (BCS ≥ 7), generalized or regional adiposity, a historic diagnosis of an endocrinopathy, and recent glucocorticoid administration were at increased odds of developing PEAL. Elucidating the determinants and earlier recognition of obesity, adiposity, and endocrinopathies might be a strategy for reducing the burden of this form of laminitis. (233 words)

This study was approved by the Institutional Animal Care and Use Committee and the Clinical Research and Review Committee of the College of Veterinary Medicine & Biomedical Sciences at Texas A&M University



Equine Metabolic Syndrome and Laminitis: Estimation of Risk, and Induction of Laminitis in Insulin Dysregulated Ponies using a New Dietary-Challenge Model

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It is known that severe insulin dysregulation (ID) in ponies with Equine Metabolic Syndrome (EMS) places the animal at risk of laminitis, but the precise relationship between insulin concentrations and laminitis is not well understood. The present study used 37 ponies identified as having mild to severe ID using an in-feed oral glucose test (OGT, 1 g dextrose/kgBW). Within three weeks after the OGT was performed, the ponies were given a high-energy 'challenge diet' containing 12.3 g NSC/kg BW for up to 18 days. Fourteen ponies developed Obel grade 1 or 2 laminitis. On day 2 of the diet challenge, the maximum serum insulin concentration (C_{max}) was recorded for each pony during a 4 h period after feeding. The C_{max} values showed a positive association with the speed of laminitis onset ($r^2 = 0.45$, $P < 0.05$), and the mean (\pm SEM) C_{max} insulin concentration was higher ($P < 0.05$) in ponies that developed laminitis ($428 \pm 59 \mu\text{U/mL}$) than in ponies that did not ($274 \pm 52 \mu\text{U/mL}$). Laminitis incidence was also related to the pre-trial OGT results. Laminitis occurred at frequencies of 0% (0/7) if post-dextrose insulin was $<50 \mu\text{U/mL}$; 36% (8/23) if insulin was 50-195 $\mu\text{U/mL}$; and 86% (6/7) if insulin was $>195 \mu\text{U/mL}$. Although the sample size was small, these data support the hypothesis that laminitis risk can be estimated in EMS ponies by testing the insulin response to oral glucose. Furthermore, the diet induction model provides a useful platform to test treatment and preventative measures.



Aspects of Veterinary-Diagnosed Endocrinopathic and Pasture-Associated Laminitis: A Prospective, Cohort Study

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Abbreviations: ACTH; adrenocorticotrophic hormone, EMS; equine metabolic syndrome, F; female, LAM; endocrinopathic and pasture-associated laminitis, M; male, PPID; pituitary pars intermedia dysfunction

Background: Epidemiological data about endocrinopathic and pasture-associated laminitis (LAM) are limited. Increased understanding of the characteristics, risk factors and recurrence rates of LAM would aid management and prevention.

Aim: To investigate the characteristics, treatment and management of LAM and to determine risk factors and recurrence rates.

Methods: A prospective, longitudinal two-year study of veterinary-diagnosed cases of LAM. Data were acquired using online questionnaires and are reported as median [interquartile range]. Laminitis causality was assigned using history, basal serum insulin and plasma ACTH concentrations, clinical examination and phenotype.

Results: Cases (n=301; 151M/150F; 15 [11-20] years) had either endocrine-associated (PPID, EMS) or pasture-associated laminitis, or combined causality. A larger number of cases were recruited in spring (36%). Autumn ACTH concentrations (38.8 [23.9-173] pg/mL) were higher (P<0.05) than other seasons. The insulin concentration (μ IU/mL) of cases with concurrent PPID and EMS (47 [19-128]; n=57) was higher (P<0.05) than cases with PPID (5 [2-12.5]; n=25), EMS (24.5 [5-81.3]; n=120) or pasture-associated laminitis (11 [3-16.5]; n=17) alone. Insulin concentration was positively correlated (P=0.05) with Obel laminitis grade. The most common clinical signs were increased digital pulses (82%) and hoof tester sensitivity (68%). Common treatments included anti-inflammatory drugs (77%) and farriery (74%). Dietary modification was the most frequent management recommendation (74%). A subset of cases (n=30) has been followed for two years, with a laminitis recurrence rate of 46%.

Conclusions: The greater hyperinsulinism associated with concurrent PPID and EMS may increase laminitis risk and severity. The recurrence rate of LAM may be higher than anticipated.

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Repeatability of the Combined Glucose/Insulin Test in Ponies of the same Breed and Gender, across Time

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The CGIT is recommended as a dynamic test for the evaluation of insulin dysregulation (ID) and defines diagnostic thresholds for blood insulin and glucose concentrations collected 45 and 75 minutes post-infusion. This study tested the reliability and repeatability of the CGIT in diagnosing ID in native ponies maintained under common husbandry/dietary management.

Six, Welsh Mountain pony mares (5-14 years, Outset BM, 216-244kg, BCS 3.8-5.3/9) were fed hay at 2% BM as DM/day. The 30 week study (May–December) was subdivided into six, successive 5-week periods (Periods 1-6). CGIT's were repeated on 4 occasions for all ponies, at intervals ≥ 5 weeks. Ponies were randomly allocated for study across all 6 periods.

Ponies gained $8.2 \pm 2.9\%$ of BM and $0.43/9 \pm 0.67/9$ BCS points. Mixed-effects models identified no effect of period on area-under-curve (AUC) for glucose, however there was a trend for AUC insulin (using iT0, iT45, iT75, Immulite) to increase from Period 1 to Period 6. Insulin concentrations at iT45 and iT75 were tightly associated with AUCi (iT45, $r^2=0.99$, iT75= 0.84). All fasted insulin concentrations were $<20\text{mU/L}$. At T45, 4/6 ponies had insulin concentrations $>100\text{mU/L}$ on at least one occasion (2 once; 1 twice; 1 all 4 tests). On no occasion did any pony return to fasted insulin concentrations by T75. 4/6 ponies were normoinsulinaemic ($<20\text{UmU/L}$) at T75 once (all P1) and one also at P2. Glucose concentrations returned to baseline values by T45 for all bar one pony (last 2 tests). There is a requirement to redefine diagnostic 'cut-off' values for native ponies.



Immunohistochemical Expression of Insulin, Glucagon and Somatostatin in Pancreatic Islets from Insulin Sensitive and Insulin Resistant Horses

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Objective: Hyperinsulinemia and insulin resistance are associated conditions in horses and insulin secretion increases as tissue insulin sensitivity decreases. We hypothesized that insulin-resistant (IR) horses would have increased insulin staining of pancreatic islets when compared to insulin-sensitive (IS) horses.

Animals: 23 horses

Procedures: Horses were assigned to IS (n = 13) and IR (n = 10) groups on the basis of frequently-sampled intravenous glucose tolerance tests and minimal model analysis, or fasted insulin concentrations. Humane euthanasia was performed for reasons other than this study, and pancreas samples were obtained for immunohistochemical analysis. Pancreas tissues were stained for insulin, glucagon and somatostatin and digital images were analyzed to determine expression of each hormone relative to total islet area.

Results: No significant differences in insulin staining were detected between groups and our hypothesis was not supported, but IR horses had a significantly less glucagon within pancreatic islets than IS horses. Median (interquartile range) percentage of total islet area staining positive for glucagon was 12% (5%, 13%) of total islet area in IR horses, compared to 18% (16%, 22%) in IS horses ($P = 0.001$). Immunoreactive glucagon, insulin, and somatostatin were present in approximately 15%, 63%, and 8% of total islet area, respectively.

Conclusions and Clinical Relevance: Insulin expression did not differ between IR and IS groups and there was no evidence that insulin resistance increases insulin secretion within pancreatic islets. Lower glucagon expression in IR horses may be a result of compensatory down-regulation of hormone secretion in response to hyperinsulinemia or hyperglycemia.



Effect of Varying the Dose of Karo Light Syrup on the Insulin Response to the Oral Sugar Test

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The oral sugar test (OST) is used to identify equine insulin dysregulation (ID); however only 0.1 and 0.15ml/kg Karo Light syrup (KLS) doses have been evaluated previously. The study aimed to determine the effect of varying the KLS dose on the OST insulin response and the test's ability to distinguish between previously laminitic (PL) and non-laminitic (NL) ponies. Eight ponies (5 PL, 3 NL) were fasted overnight before and throughout testing. In a 3-way randomised crossover design with 7 days between tests, 0.15ml/Kg, 0.3ml/Kg or 0.45ml/Kg KLS was administered orally and blood samples obtained at 0-120 min. Serum [insulin] was measured using a previously validated radioimmunoassay. Area under insulin response curve (AUC_i) was calculated. Differences in serum insulin response between doses and between groups were assessed using mixed effects models. Cut-offs were extrapolated. Serum [insulin] following 0.15ml/Kg significantly ($p < 0.04$) differed from 0.3ml/Kg only at 120 min; whilst serum [insulin] following 0.45ml/Kg significantly ($p < 0.02$) differed from 0.15ml/Kg and 0.3ml/Kg at 30-120 min. The insulin response significantly ($p < 0.02$) differed between NL and PL only following 0.45ml/Kg at 60 (mean \pm SD 86.4 \pm 17.7 vs. 140.9 \pm 17.8 μ iu/ml), 75 (120.3 \pm 31.2 vs. 200.3 \pm 72.0 μ iu/ml) and 90 (86.5 \pm 21.1 vs. 209.7 \pm 72.6 μ iu/ml) min. AUC_i significantly ($p < 0.05$) differed between NL and PL following 0.3 and 0.45ml/Kg. Using [insulin] >68, 59 or 135 μ IU/ml between 60-90 min as cut-offs following 0.15ml/Kg, 0.3ml/Kg or 0.45ml/Kg respectively, 5/5 OSTs from PL were positive and 2/3 OSTs from NL were negative. Overall, a dose of 0.45ml/Kg Karo light syrup may be preferably to differentiate PL and NL ponies.



Androgen Excretion in Horses with Equine Metabolic Syndrome and Pituitary Pars Intermedia Dysfunction

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Androgens are steroid hormones best known for their role in the development and maintenance of sex phenotypes but also important in other organ systems including bone and metabolism. Androgen production occurs principally in reproductive tissues in males and both the adrenal glands and ovaries in females. The main adrenal androgens in the human are dehydroepiandrosterone (DHEA) and androstenedione, while androgens synthesised in extra-adrenal sites include testosterone and dihydrotestosterone (DHT). Dysregulation of androgens and androgen metabolism is associated with various disease states in humans. In polycystic ovary syndrome for example, adrenal androgen synthesis is increased in response to elevated ACTH and is thought to contribute to insulin resistance and obesity in this syndrome. Furthermore, in obesity androgen metabolising enzyme 5 α -reductase is up-regulated and androgen metabolite excretion is increased. This study aimed to investigate whether equine metabolic syndrome (EMS) and pituitary pars intermedia dysfunction (PPID) were associated with altered androgen metabolism. Blood and urine samples were collected from castrated male and female healthy horses (n=10), horses with EMS (n=6) and horses with PPID (n=6). Androgen metabolites were measured by gas chromatography- tandem mass spectrometry in urine and by ELISA in plasma. There was no effect of disease on plasma androgen metabolite levels. Horses with EMS and PPID had significantly higher urinary testosterone, aetiocholanolone and androstendiol levels compared to healthy horses. Total androgen excretion was increased in both EMS and PPID. This pilot study indicates that altered androgen metabolism may be a feature of EMS and PPID and warrants further investigation.



Dissecting Metabolic Syndrome and Insulin Dysregulation Molecular Pathophysiology using “Big Data”

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Complex, multifactorial phenotypes, such as Equine Metabolic Syndrome (EMS), can be dissected into molecular phenotypes at different levels across multiple tissues that sum together to create clinical disease. Yet, few studies have attempted to identify the metabolic derangements of EMS at a cellular or tissue level; thus our understanding of EMS pathophysiology is based on clinical assays that fail to assess the underlying molecular alterations within tissues. We have collected 11 morphometric, biochemical and hormonal phenotypes from > 900 horses/ponies. Using this cohort, we have begun to dissect EMS molecular pathophysiology. At the **genomic** level, heritability estimates for neck-circumference-to-height ratio (21%), girth-to-height ratio (11%), insulin (32%), insulin post-OST (34%), leptin (14%), adiponectin (55%) and ACTH (56%), have demonstrated that these phenotypes are influenced by genetics. Using genome wide association in Welsh Ponies and Morgans we have identified >180 chromosomal regions harboring >3,000 positional candidate genes associated with these phenotypes. At the **transcriptomic** level, preliminary analysis has identified 463 skeletal muscle and 798 adipose tissue genes differentially expressed between hyper- and normo-insulinemic horses, and 1,001 muscle and 497 adipose genes differentially expressed between individuals with high and low adiponectin concentrations. At the **metabolomic** level, preliminary analyses of serum metabolites have identified significant differences between hyper- and normo-insulinemic individuals for >130 molecules, including metabolites involved in the tricarboxylic acid cycle, fatty acid and branched-chain amino acid metabolism. Using biological networks of interactions between these three categories of molecular phenotypes, we are working to unravel the complex molecular interactions in EMS.



Identification of a Genetic Locus associated with Height and Fasting Insulin in Welsh Ponies and Morgan Horses

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Ponies have higher fasting insulin concentrations and are more insulin resistant than horses. In humans and mice, genetic alleles resulting in small skeletal size have also been associated with alterations in insulin and glucose metabolism. We hypothesized genetic that loci affecting height in ponies have pleiotropic effects on metabolic traits. Pearson's correlation coefficient identified an inverse relationship between wither height and fasting insulin in 298 Welsh ponies (WP) (-0.26; 95% CI -0.36 to -0.15; p-value: <6.4e-06). Using genome-wide association studies were performed for height and fasting insulin in 232 WP and 286 Morgan horses, a locus on chromosome 6 was significantly associated with height and fasting insulin in the WP, but not in Morgans. HMGA2 was identified as a potential candidate gene, due to a recently identified C.83G>A variant associated with height in Shetland ponies. To date, we have genotyped 250 WP and 60 Morgans for this variant and identified an allelic frequency of 0.802 and 0.375, respectively for the A allele. Correlations were identified for the additive effect of the A allele on height [0.59; 95% CI -0.66 to -0.51; p-value: <2.2e-16 (WP) and 0.69; 95% CI -0.81 to -0.59; p-value: 7.4e-10 (Morgan)] and baseline insulin values [0.22; 95% CI 0.10 to 0.34; p-value 3.5e-04.22 (WP) and 0.46; 95% CI 0.23 to 0.64; p-value: 2.1e-04 (Morgan)]. These data suggest that the variant in HMGA2 may have an effect on both height and insulin, which has important implications for equine metabolic syndrome susceptibility.



The Association between Endocrine Disrupting Chemicals and Equine Metabolic Syndrome

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Our group has demonstrated that 49% of variability in Equine Metabolic Syndrome (EMS) phenotypes is explained by shared environment; however only 4-18% of this variability is explained by diet, exercise and season, suggesting that other environmental factors play a role in EMS. Endocrine Disrupting Chemicals (EDCs) are associated with metabolic syndrome and other endocrine abnormalities in humans. Preliminary data demonstrated that horses from farms ≤ 30 miles of EDC disposal sites were more likely to have had laminitis, and had higher post oral sugar challenge insulin concentrations (OST-INS), suggesting EDC exposure is an EMS risk factor. We sought to determine if plasma EDC concentration is correlated with metabolic measurements in 301 horses from 32 farms with bioassays that measure aryl hydrocarbon receptor (AHR) and estrogen receptor (ER) activation by EDCs. Mean (range) EDC-AHR and EDC-ER concentrations were 0.223 (0.02-2.47) and 279.66 (4.35-15,000) pg/ml plasma. EDCs were below the detection limit in approximately half of the horses. AICc statistics were used to determine the best linear multivariable regression model for EDC association with 9 EMS phenotypic variables (glucose, OST-glucose, INS, OST-INS, triglycerides, leptin, adiponectin, ACTH). Month sampled was associated with EDC-AHR ($p=0.007$). Hours grazing was associated with EDC-ER ($p=0.043$). EDC-AHR interaction with plasma fat extracted ($p=0.060$), and EDC-ER ($p=0.050$) approached significance with OST-INS. The interaction between pregnancy and EDC-AHR concentration was associated with leptin ($p=0.046$). The results suggest that some of the unexplained environmental variance in individuals with EMS is due to EDC exposure mediated through the AHR and ER.



Gene Expression Differences of Adipose and Gluteal Muscle Tissues in Four Breeds of Horses with a Range of Insulin Sensitivities

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Equine metabolic syndrome (EMS) and associated insulin dysregulation (ID) has been identified as the most common cause of laminitis. Certain breeds seem susceptible to EMS, and we have identified breed differences in metabolic phenotypes. Muscle and adipose tissue have large roles in glucose and insulin regulation. Here we compare gene expression within the tailhead adipose tissue (TAT) and gluteal muscle in different breeds. Frequently sampled intravenous glucose tolerance tests (FSIGTT), TAT, and muscle biopsies were performed in 28 geldings from four breeds. Gene expression in gluteal muscle and TAT was measured using RNASeq. Differential gene expression was determined using HTSeq and Limma Voom. Functional analysis of genes was performed using Ingenuity Pathway Analysis (IPA). Each breed had a range of insulin sensitivities based on minimal model analysis of the FSIGTT and uniquely differentially expressed genes in each tissue (7-1347 in adipose, 94-691 in muscle). In TAT, top networks in Arabians and Welsh Ponies (WP) were Carbohydrate Metabolism and Developmental Disorders/Lipid Metabolism respectively. WP had upstream analysis activation of cytochrome p450 reductase. There was upstream activation of hypoxia-inducible factor 1-alpha and transforming growth factor beta 1 in Morgans and Arabians, with deactivation in Arabians and activation in Morgans of forkhead box protein 01, C-X-C Motif Chemokine Ligand 12, and growth hormone. In muscle, the top QH network was Lipid Metabolism, with upstream analysis showing deactivation of fenofibrate, pirinixic acid, and rosiglitazone. The top WP network was Energy Production/Lipid Metabolism. Breed specific patterns of differentially expressed genes may contribute to ID.



Horse-Factors Influencing the Seasonal Increase in Plasma ACTH Secretion

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Published studies describe an increase in plasma ACTH in August, September and October with PPID cases exhibiting a greater magnitude of seasonal increase than normal horses. Data were examined from almost 150,000 chilled plasma samples between January 2012 and August 2016. Data were categorised by age, gender, breed and colour to examine differences in ACTH concentration through the year.

Age had a pronounced effect on ACTH concentrations through the year but most especially in the period of seasonal increase (figure 1).

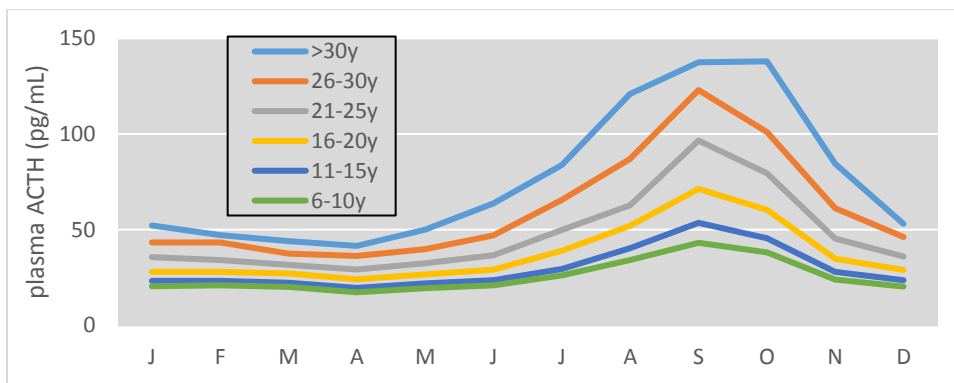


Figure 1. Median monthly plasma ACTH concentration categorised by age.

Gender appeared to have an effect on ACTH concentration with mares having a significantly ($P < 0.0001$) higher ACTH concentration in September (figure 2).

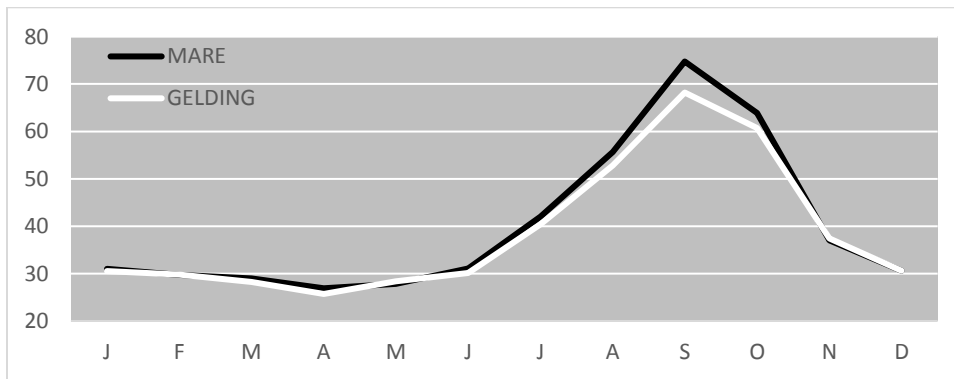


Figure 2. Median monthly plasma ACTH concentrations in mares and geldings.

Breed also had a marked effect on ACTH concentrations with Shetland ponies having significantly higher ACTH in September than other breeds ($P < 0.05$ vs Welsh



A; $P < 0.001$ for others) except for miniature Shetlands ($P > 0.05$).
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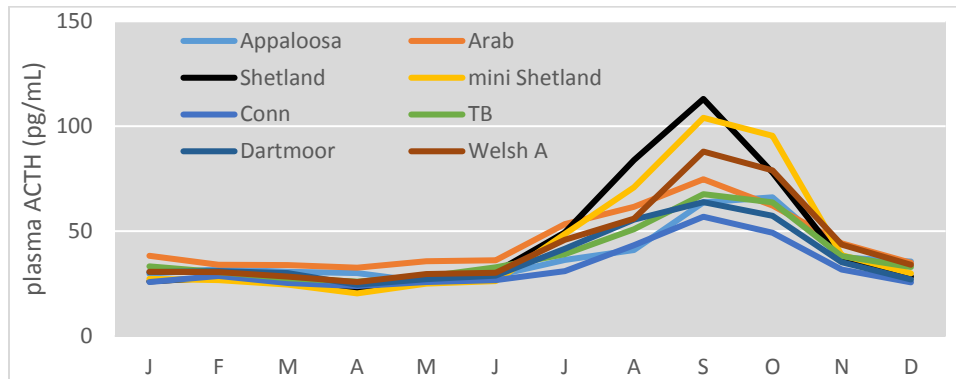


Figure 3. Median monthly plasma ACTH concentrations of selected breeds.

Additionally preliminary data indicated that grey horses had significantly higher ACTH values than non-grey horses in the autumn period.

Given previous evidence of a greater magnitude of seasonal increase in PPID vs non-PPID horses (Copas and Durham 2012), and the known age association with PPID prevalence, it was not surprising to observe the age-effect on September ACTH concentrations (figure 1). However, given closely similar ages between mares versus geldings or between animals of different breeds and colours, the observed greater September ACTH concentrations in mares, in Shetland ponies and grey horses are less easily explained.



Pharmacokinetics and Pharmacodynamics of Oral Pergolide Mesylate in Horses with Pituitary Pars Intermedia Dysfunction

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Objectives: Investigate pharmacokinetic and pharmacodynamic properties of orally administered pergolide in horses with pituitary pars intermedia dysfunction (PPID).

Study design: Cohort study

Methods: Six horses with clinical and endocrinological PPID received oral pergolide (Prascend; Boehringer Ingelheim) at 4 µg/kg for 18 days. Samples were collected 0.5 hours before and 2 and 12 hours after administration of pergolide daily for 18 days. Plasma pergolide and adrenocorticotrophic hormone (ACTH) concentrations were determined using high-performance liquid chromatography–tandem mass spectrometry and a chemiluminescent immunoassay respectively.

Results: Maximum plasma concentrations after the first oral dose of pergolide (0.104 to 0.684 ng/mL; mean 0.308 ± 0.201 ng/mL) were not significantly different to the maximum steady state concentration at day 18 (0.197 to 0.628 ng/mL; mean 0.336 ± 0.159 ng/mL). A marked difference between mean plasma peak and trough concentrations of pergolide remained throughout the study. Pergolide concentration reached steady state within 3 days and the drug did not accumulate ($R=1.09$). Plasma ACTH concentration reduced significantly within 12 hours of the first dose of pergolide with further reductions occurring over the next 10 days. Although there were parallel fluctuations in the concentrations of pergolide and ACTH, timing of ACTH measurement in relation to the administration of pergolide did not have a significant effect.

Clinical Implications: Oral pergolide rapidly suppresses the pars intermedia in horses with PPID. Follow-up testing may be possible earlier than is employed currently. More frequent dosing may reduce fluctuation in pergolide and ACTH concentrations which might have implications for patient monitoring.



Insulin Assay Choice Affects Results of the Oral Sugar Test

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Aims: Diagnostic thresholds for serum [insulin] in the oral sugar test (OST) were originally derived using a radioimmunoassay (RIA) that is discontinued. Different immunoassays may yield different results and affect interpretation of the OST. This study aimed to compare OST results using different assays for serum [insulin] analysis.

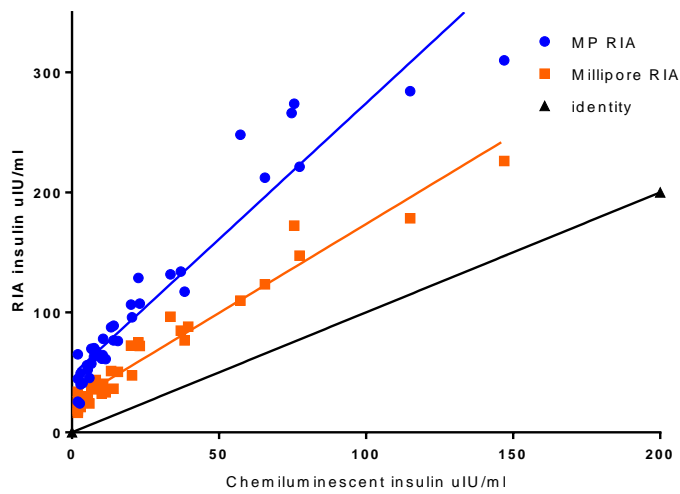
Methods: OSTs (0.3ml/kg karo-light syrup) were performed on 41 ponies with no known history of laminitis. Serum [insulin] 60 minutes after dosing was analysed concurrently using 3 insulin assays, a chemiluminescent immunoassay (Immulite) and two radioimmunoassays (MP Biomedicals^b (MP) and Millipore^c). Differences between results were compared by Bland-Altman plots and a within-subjects ANOVA of logarithmically transformed data. Values above (n=1) or below (n=4) the calibration range for each assay were assigned an arbitrary value of the range limit.

Results: Median (range) for each assay were MP 70 (24-310)µIU/ml, Millipore 40 (16-226)µIU/ml, Immulite 11 (2-147)µIU/ml. The differences between all assays were proportional to their means. Percentage difference Bland-Altman plots indicated proportionally lower results for the Immulite assay at low concentrations than at high concentrations when compared with either radioimmunoassay. All assays differed from each other (p<0.001). Mean ratios (95% confidence intervals) for the differences between assays were: MP:Immulite 6.7 (5.2-8.7), Millipore: Immulite 4.0 (3.1-5.1), MP:Millipore 1.7 (1.6-1.8). Figure 1 shows the correlation between assays.

Conclusions: The chemiluminescent assay yielded lower results than either RIA particularly at low serum [insulin] and may be unsuitable for OST interpretation at this dose of sugar. Insulin results are assay dependent. Laboratory specific diagnostic thresholds should be derived. *Continued next page*



Figure 1: Serum [insulin] measured by two radioimmunoassays compared with a chemiluminescent assay.



Manufacturers' addresses

^aImmulite 1000 insulin, Siemens, Camberley, UK

^bEMD Millipore human insulin specific RIA (HI-14K), EMD Millipore, Watford, UK

^cMP Biomedicals Insulin Coated Tube RIA Kit (07260105), MP Biomedicals EMEA, Illkrich, France



The Effect of pH on Large Intestinal Ion Transport and Transepithelial Resistance in Suspected Insulin Resistance Horses

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Aims: Hyperinsulinemia might cause alterations in large intestinal ion transport and transepithelial resistance which might lead to colitis and diarrhea. These factors might be critical when colon pH is decreased because of intake of feeds with high soluble carbohydrate content. The purpose of this study was to determine the effect of the pH on colonic mucosa in insulin resistance horses using an in-vitro Ussing chamber system.

Methods: Tissue samples of the right dorsal colon were collected in suspected insulin resistant (n=4) and normal (n=5) horses immediately after euthanasia. The colonic mucosa was dissected from the muscular layers and mounted in an Ussing chambers. Ussing chambers are used to measure ion transport and transepithelial resistance in gut mucosa, as an indicator of viability and permeability. Colonic tissue was exposed to pH 4, 6, or 7 (control) on the mucosal side and pH 7.0 on the submucosal side. Potential difference (PD) and short circuit current (Isc) were measured every 15 minutes. Tissue transepithelial resistance (TER) was calculated from the open-circuit PD and Isc, using Ohm's Law ($R=PD/Isc$).

Results: The mean Isc and TER did not change in the colonic tissues from control horses when exposed to different pH. However, mean Isc decreased rapidly in right dorsal colon tissues exposed to pH 4.0, but this was not significant. Also, mean TER decreased significantly ($P<0.015$) in colon tissues exposed to pH 4.0 from insulin resistant horses, when compared to tissues exposed to pH 6 or 7.

Conclusion: Hyperinsulinemia in horses with insulin resistance might be predisposed to alterations in mucosal ion flux and transepithelial resistance, especially when there is a drop in pH after feeding a diet high in soluble carbohydrates and lead to colitis and diarrhea. These data are preliminary and further research is indicated to confirm this trend.



Comparison and Clinical Interpretation of Three Methods for Measurement of Equine Insulin

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In clinical use, determination of equine insulin dysregulation requires exact insulin quantification. However, striking differences in values obtained from different immunoassays exist. Most assays used in veterinary medicine include antibodies directed against human insulin, although amino acid sequences between equine and human insulin differ. Therefore, the aim of this study was to test the accuracy of an equine-optimized insulin ELISA, to compare it to a frequently used radioimmunoassay (RIA), to a chemiluminescence immunoassay (CLIA), and to test their clinical applicability. Forty samples used for comparison of methods were obtained from seventeen horses and ponies of various breeds and body condition scores (6.8 ± 1.1) under fasting conditions as well as during oral glucose testing procedures. All intra- and inter-assay coefficients of variations (CVs) determined by the ELISA differed concentration-dependent, but were within the acceptable range for clinical use of immunoassays. In diluted and non-diluted samples from both, fasted and glucose-stimulated horses, insulin values obtained by CLIA were significantly ($p < 0.001$) lower when compared to values obtained by ELISA or RIA. However, values measured by ELISA and RIA were not significantly different. Calculated recovery upon dilution was 101 ± 11 % for CLIA, 160 ± 41 % for RIA, and 98 ± 4 % for ELISA. Our findings indicate that the tested equine-specific insulin ELISA appeared to be an appropriate assay to determine equine insulin concentrations. Nonetheless, comparison of insulin concentrations measured by different methods should be interpreted carefully, especially in clinical cases, where small changes in insulin levels can cause false classification of healthy or insulin dysregulated horses.

Acknowledgement: This work was financially supported by Boehringer Ingelheim.



Blood Glucose and Insulin Concentrations Following Xylazine HCl and Detomidine HCl administration in Insulin Resistant and Normal Horses

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Alpha2 sedatives cause decreases in insulin secretion followed by hyperglycemia in normal horses. This study was conducted to determine how insulin resistant (IR) horses respond to alpha2 drugs.

Eight horses, 4 IR and 4 controls, were given either 1.1 mg/kg xylazine HCl or 30 µg/kg detomidine HCl I.V. Blood was collected for glucose and insulin concentrations at 0 (baseline), 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, and 5.0 h. Horses received each in random order with a 24 h washout period between drugs.

Baseline insulin was significantly higher in ID horses (49.9 ± 39.2 µIU/mL vs 16 ± 6.8 µIU/mL, $p < 0.001$); however, there was no difference in glucose baseline. Injection of xylazine resulted in significant decreases in insulin ($p < 0.001$) and increases in glucose ($p < 0.001$) in both ID and control horses with no significant effect of insulin dysregulation. Injection of detomidine also resulted in significant decreases in insulin ($p < 0.001$) and increases in glucose ($p < 0.001$) in both ID and control horses; however, there was a trend for a milder effect in ID horses ($p = 0.086$). Regardless of the drug used, alpha2-agonists caused a decrease in insulin after 15 min and an increase in glucose maximal at 45 min; however, in control horses, detomidine injection resulted in a significantly lower insulin at 1.5 and 2.0h (11.94 ± 4.07 vs. 34.60 ± 25.33 , $p < 0.01$ and 17.86 ± 6.00 vs. 37.48 ± 15.00 , $p < 0.05$, respectively).



Sensitivity and Specificity of Fasted Basal Insulin compared to Dynamic Testing in Horses and Ponies with suspected Equine Endocrine Disease

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Basal insulin testing is popular in practice yet limited data exist comparing it to dynamic testing for diagnosis of insulin dysregulation in clinical cases. We hypothesised that basal insulin would have a low sensitivity compared to the combined glucose-insulin test (CGIT) and this would be more pronounced in horses on a low NSC diet. 129 CGIT tests from 67 fasted horses were evaluated. Basal insulin was correlated ($P < 0.001$) to insulin at 45min ($r_s = 0.66$) and 75min ($r_s = 0.72$), AUCinsulin ($r_s = 0.67$), glucose at 45min ($r_s = 0.53$) and AUCglucose ($r_s = 0.50$). There were 82/129 (64%) CGIT and 12/129 (9%) basal insulin positive tests using conventional criteria (basal insulin < 20 mIU; 45min insulin > 100 mIU/L and/or glucose $>$ baseline and > 6 mmol/L at 45min). Specificity and sensitivity of basal insulin were 100% and 16.5% respectively; ROC analysis revealed maximum sensitivity (77.8%) and specificity (68.8%) occurred at ≥ 3.03 mIU/L. Horses with current (59/129) or current and historical laminitis (95/129) were more likely to have a positive CGIT test ($P < 0.05$), but there was no effect of obesity or baseline diet. There was a trend for horses on a low NSC diet to have a false negative basal insulin ($P = 0.07$) based on conventional CGIT cutoffs, especially for glucose at 45min ($P = 0.05$), but no effect of current laminitis or obesity. Basal insulin is strongly correlated to tests of dynamic insulin sensitivity but has a poor sensitivity at conventional cut-offs in fasted horses. Basal insulin cut-off values should be revised and further work comparing unfasted to fasted horses is warranted.



Postprandial Gastric Inhibitory Polypeptide Responses of Different Equine Breeds adapted to meals containing Micronized Maize

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The pancreatic insulin response to dietary non-structural carbohydrates (NSC) is partly due to the release of incretin hormones (gastric inhibitory polypeptide [GIP] and glucagon-like peptide-1 [GLP-1]) from specialized enteroendocrine cells. There are breed differences in the GLP-1 response to dietary NSC in equids, but GIP levels have not been similarly determined.

Four Standardbred horses (5 to 14 yr, BCS 5.2 ± 0.2), 4 mixed-breed ponies (5 to 10 yr, BCS 5.3 ± 0.3) and 4 Andalusian-cross horses (4 to 11 yr, BCS 5.7 ± 0.3) were studied. Animals were adapted to twice-daily meals containing micronized maize over a 12-week period, ultimately providing 1.1 g/kg BW of starch in each meal. Serial blood samples were obtained following the first meal at 8am and the second meal at 4pm. Plasma concentrations of total GIP were determined using an ELISA (Merck Millipore, Darmstadt, Germany) previously validated for equine plasma. The area under the plasma GIP curves (AUC) were compared between breeds by Kruskal-Wallis test with Dunn's *post hoc* test.

Plasma GIP peaked approximately 2 hours after the grain-containing meals. Ponies exhibited significantly greater peak GIP and AUC values compared to the other two breeds ($P < 0.05$). This study further demonstrates breed differences in the enteroinsular endocrine axis that may contribute to hyperinsulinemia in ponies. An improved understanding of the factors that contribute to equine hyperinsulinemia is of critical importance in reducing the prevalence of laminitis in domestic horse populations.



Associations between Body Fat and Indicators of Insulin Dysregulation

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Obesity and age are known risk factors for insulin dysregulation (ID) in the horse. This study investigated associations between markers of adiposity and indicators of insulin dynamics as evaluated by the CGIT.

Eighteen mature individually housed Welsh Mountain pony mares were fed hay at an estimated maintenance level (2% of BM as daily DMI). Animals were divided into 3 groups: Control (n=6; 5-15 years, BCS 3.8-5.3/9, BM 216-244kg), obese (n=6; 5-15 years, BCS >7/9, BM 276-323kg), geriatric (n=6; 19-27 years, BCS 4-7.4/9, BM 212-288kg). The 30 week study (May–December) was subdivided into six, successive 5-week periods (Periods 1-6). CGIT's were repeated on 2 occasions for all ponies, at intervals of ≤ 5 weeks. Ponies were randomly allocated for study across all 6 periods.

Mixed-effects models identified with area-under-curve for insulin (using T0, 45 and 75; Immulite) or iT0 as outcome variables revealed a strong positive association between age and AUCi ($p < 0.05$), with a weaker positive association between AUCi and BCS and no associations for ultrasound measures of fat (rump, ribeye, ventro-abdominal retroperitoneal) or percentage body fat (deuterium dilution) and AUCi. Conversely, there was no association for age and ultrasound or deuterium dilution measures of fat with iT0, with moderate positive associations between BCS and iT0. Together, these data suggest that ultrasound and deuterium dilution measures of body fat are not predictive of insulin dynamics and that in this study, whilst geriatric ponies showed no difference in baseline insulin, they demonstrated greater AUCi than their younger counterparts.



Sensitivity or Resistance to Diet-Induced Weight Loss is associated with Insulin/Glucose Dynamics

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For obese animals, weight loss (WL) management requires the imposition of negative energy balance. Physiological responses to under-nutrition are poorly understood. Starved horses switch to tissue catabolism with corresponding decreases in plasma insulin.

We used the CGIT to evaluate pre- and post-diet insulin/glucose dynamics in 8 obese (BCS>7/9), native pony mares undergoing controlled WL management in winter. All ponies were fed hay at 2% BM as daily DMI (~maintenance), before hay intake was halved (diet) for 7 weeks.

Ponies lost $1.15 \pm 0.20\%$ of BM weekly. Published CGIT 'cut-offs' classified 1/8, 8/8 and 7/8 ponies as insulin-dysregulated pre-diet on the basis of insulin concentrations (Immulite) at T0 (11.3 ± 7.2 mU/L), T45 (217.6 ± 90.1 mU/L) and T75 (152 ± 184 mU/L). Post-diet, insulin concentrations were decreased at T0 ($-33 \pm 20\%$) but increased in 7/8 animals at T45 ($+47 \pm 50\%$) and T75 ($+168 \pm 191\%$). The magnitude of individual WL was associated with the percentage change in insulin concentrations pre- and post-diet (r^2 's, T0, 0.38; T45, 0.88; T75, 0.47; AUCi, 0.86). Pre-diet insulin concentrations were useful predictors of WL sensitivity/resistance (r^2 's, T0, 0.55; T45, 0.78; T75, 0.47; AUCi, 0.79). AUCi and iT45 were strongly associated ($r^2=0.98$). All ponies were normoglycaemic (T0, 5.1 ± 0.2 mmol/L) pre- and post-diet but the time taken to return to baseline glucose (RBG) concentrations post-infusion, increased post-diet (78.1 ± 37 to 120 ± 34 minutes). Pre-diet RBG was a useful predictor of WL sensitivity ($r^2=0.76$).

WL resistant animals had lower insulin responses but more rapid glucose clearance rates pre-diet. Although these animals had relatively greater increases in insulin concentrations post-diet, glucose clearance rates did not increase proportionately.



Diet-Induced Obesity does not similarly affect Glucose Tolerance in Shetland Pony Mares

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Background: Under field circumstances not every obese pony in a herd develops hyperinsulinemia. Is experimentally-induced obesity able to overcome these individual differences?

Objectives: To examine the repeatability of the effects of an experimentally dietary-induced obesity on oral glucose tolerance in three different groups of Shetland pony mares in three different years.

Methods: In three following years different groups of pony mares were divided in a control (C) and test group (T). (Group1: 7T/4C; Group2: 3T/3C; Group3: 2T/3C). Test group was fed 200% of maintenance netto energy (NE) requirements to induce weight gain for about 20-28 weeks. Control group was fed 100% NE requirements. Each pony underwent oral glucose tolerance testing before the start, at the end and during the dietary period (2-3 times). Ponies were weighed weekly. Statistical analyses were performed using a general linear mixed model and Bonferroni test. Differences were considered significant when $P < 0.05$.

Results: Bodyweight increased in all test ponies (bodyweight range 123-202 kg; increase range 30-65 kg; median 45.5 kg). On the diet all test ponies significantly decreased the area under the curve (AUC) for plasma glucose concentration compared to the control group. On the diet group 1 and 3 test ponies increased the AUC insulin in contrast with group 2 test ponies. The control group did not show significant changes in AUC glucose or AUC insulin in all three years.

Conclusion: Dietary-induced obesity is an important factor in the occurrence of insulin dysregulation however does not overrule individually based factors involved in regulating the response.



Long-Term and Short-Term Dopaminergic (Cabergoline) and Antidopaminergic (Sulpiride) Effects on Indices of Insulin Sensitivity in Horses

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Dopaminergic drugs used to treat pituitary pars intermedia dysfunction (PPID) in horses have been shown to improve insulin sensitivity in humans and rodents. Moreover, D2 receptor knockout mice have revealed that dopamine is needed for normal insulin secretion and normal glucose tolerance. Other reports show that dopaminergic agents may not have any effect on insulin sensitivity in horses. High serum insulin concentrations, insulin resistance and laminitis are typical symptoms of horses with equine metabolic syndrome and in some instances, but not always, in horses affected with PPID. There is speculation whether the loss of dopaminergic control to the pars intermedia leads to higher insulin secretion. Four experiments were conducted to determine the effect of dopaminergic inhibition or stimulation on insulin secretion and insulin sensitivity in horses. The first experiment tested the long-term effects of cabergoline in insulin insensitive mares, while the second tested the short-term effects of cabergoline on insulin sensitive and insensitive mares. Experiments three and four tested the long-term effects of sulpiride in insulin sensitive and insensitive geldings and the short-term effects of sulpiride in insulin sensitive and insensitive mares, respectively. Results gathered from these experiments concluded that neither increased nor decreased dopaminergic activity, in the long or short-term, had any impact on insulin secretion after glucose infusion or insulin sensitivity in horses ($P > 0.1$), regardless if starting insulin sensitivity status. These results support the model in which dopaminergic agents have little efficacy on insulin sensitivity and that insulin resistance may develop independently of dopaminergic alterations.



Incretin Receptor Antagonists Decrease the Equine Insulin Response to Incretin Hormones *In Vitro*

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Abbreviations: EC₅₀; half-maximal effective concentration, GIP; glucose-dependent insulinotropic peptide, GLP-1; glucagon-like peptide-1

Background: The incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) augment the insulin response to oral glucose in insulin-dysregulated ponies. If antagonists of the human GLP-1 and GIP receptors can attenuate the effects of incretins in horses they may be useful for treating hyperinsulinaemia.

Aim: To develop an *in vitro* model for investigating equine incretin action and to determine whether commercially-available GLP-1 and GIP antagonists inhibit incretin-stimulated insulin release.

Methods: Pancreatic islets were isolated from fresh, abattoir-sourced horse pancreas (n=5) using collagenase digestion and layering through a density gradient. Islet viability was confirmed microscopically and by demonstrating insulin release in response to glucose stimulation. Insulin secretion was stimulated for 60 min with increasing concentrations of GIP or GLP-1 to determine the half-maximal effective concentration (EC₅₀) for each hormone. Insulin secretion was then measured at the EC₅₀ for GLP-1 and GIP, in the presence and absence of their respective antagonists (Exendin 9-39 and pro³GIP).

Results: Viable pancreatic islets were isolated successfully from horses and their insulin secretion was concentration-dependent with glucose. Insulin release was stimulated by both hormones in a concentration-dependent manner with a maximal insulin concentration of 484 μ IU/mg of protein at 1 μ M GLP-1 and 299 μ IU/mg of protein at 0.1 μ M GIP. The antagonists achieved a similar decrease (30%) in the insulin response to GLP-1 (P=0.06) and GIP (P=0.04).

Conclusions: Incretin-stimulated insulin secretion in horses can be partially inhibited with antagonists. Further investigation of these compounds *in vivo* may identify new therapeutic strategies for equine insulin dysregulation.

Acknowledgement: This study was funded by the Australian Research Council



Metformin for the Treatment of Equine Metabolic Syndrome: A Prospective Field Study

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Metformin is used to treat Metabolic Syndrome in humans, owing to its anti-hyperglycemic properties, which improve insulin sensitivity and reduce hepatic gluconeogenesis. Despite an unfavorable pharmacokinetic in this species, veterinarians and clients have anecdotally reported that metformin improves the clinical signs associated with Equine Metabolic Syndrome (EMS). The aim of this study was to assess the owner appreciation, the clinical signs and the insulinemic response of horses with EMS following an enteral administration of metformin in a placebo controlled blinded prospective study.

Sixteen horses were recruited by equine veterinarians from Quebec. Enrollment criteria included clinical signs of EMS including being an easy keeper, exercise intolerance, regional/general obesity and history of laminitis. Horses with laminitis requiring medications or having concurrent medical conditions were excluded. Horses were randomly administered either metformin (n=10) or placebo (n=6) orally twice daily for 30 days. A standardized physical examination and an oral glucose stimulation test (OGST) were performed by attending veterinarians prior to and after either treatment. Owners were also asked to fill out a questionnaire, before, during and after the completion of the study.

Metformin improved the body weight ($p=0.04$) and the owner's assessment of the overall condition of the horses (current activity acceptance, overweight) and their subjective assessment of the body weight ($p=0.01$). There were no significant changes in the OGST and the baseline insulin levels decreased only after placebo treatment. Taken together, these results support a beneficial effect of metformin in the clinical signs of EMS, but the insulin response was unaltered.

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Use of an IGF-1 Receptor Antagonist in Hyperinsulinemic Laminitis

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Introduction: Endocrinopathic laminitis has been induced in normal horses by sustained administration of high dose insulin, resulting in clinical and histological signs similar to naturally occurring disease. Preliminary studies investigating the expression of receptors and glucose transporters in the equine foot along with the proliferative and elongated appearance of the secondary lamellae in endocrinopathic laminitis have led to the hypothesis that hyperinsulinemia may cause endocrinopathic laminitis through activation of the IGF-1 receptor (IGF-1R) in the laminae rather than a direct action at the insulin receptor.

Hypothesis: Regional perfusion of an IGF-1R antagonist will prevent induction of laminitis secondary to hyperinsulinemic clamp

Methods and Materials:

- Horses (n=5) were exposed to high insulin concentrations (HEC) for 72 hrs
- One limb injected with PBS, one with an IGF-R antagonist once daily by regional perfusion for 3 doses
- Clinical response was monitored
- Horses were euthanized, hoof sections collected and assessed by a blinded pathologist (JE)

Results:

Clinical signs:

- 2/5 horses were lame at 72 hrs
- 4/5 horses had heat in feet, increased digital pulses or weight shifting during the last 24 hrs; all worse in control limb
- 1/5 horses no clinical evidence of laminitis

Histological signs:

- 5/5 horses showed bilateral evidence of laminitis
- 3/5 horses had marginally less severe signs in treated foot

Summary:

- The hyperinsulinemic clamp caused clinical lameness in only 2/5 horses
- Clinical signs were worse in the control foot, typically at 48 hours of clamp, in 4/5 horses
- IGF-1R antagonist was associated with a mild improved histology in only 3/5 cases
- Reason for "failure" may include timing of sample collection, inappropriate IGF-1 receptor antagonist dose or drug delivery to the tissue, or lack of drug specificity to equine receptor
- Alternatively, IGF-1 R activation may not be the mechanism of endocrinopathic laminitis



Insulin Dysregulation and Inflammation

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Abstract

Insulin dysregulation (ID) in horses is associated with many negative health conditions. Particularly concerning is its association with the inflammatory disease of the hoof tissue, laminitis. However, the link between ID and systemic inflammation in the horse remains unclear. In order to better understand potential relationships between ID and inflammation, inflammatory and insulinemic responses to an oral sugar test (OST) were determined. To accomplish this, 5 horses with ID and 5 normal controls were selected. All horses were administered an OST at 0.15ml*bw (kg), with jugular venipuncture blood samples taken prior to as well as 30, 60, 90, and 240 minutes post oral sugar administration. Serum samples were used to measure insulin concentrations and heparinized plasma to isolate peripheral blood mononuclear cells (PBMCs). PBMCs were used to measure inflammatory cytokine protein concentrations using flow cytometry. Results were analyzed with a repeated measures ANOVA procedure in SIGMAPLOT version 12.3 (Systat Software Inc.) and results considered statically significant when $p < 0.05$. Horses with ID had significantly higher serum insulin concentrations compared to controls 60 minutes following oral sugar administration. Furthermore, ID horses had a significantly higher percentage of cells producing inflammation at this same time point compared to control horses. These data provide evidence for a link between insulin dysregulation and inflammation, and may indicate potential mechanisms relating to the predisposition to and pathogenesis of laminitis.



The Effect of Docosahexaenoic Acid (DHA) Supplementation on Metabolic and Inflammatory Parameters of Horses with Equine Metabolic Syndrome (EMS)

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Abstract

There is a high incidence of obesity in the equine population. Obesity in horses is associated with health concerns such as equine metabolic syndrome (EMS). Omega-3 fatty acid supplementation has been suggested for humans with metabolic dysfunction, as it has been reported to improve insulin sensitivity and reduce inflammation in these individuals. However, no research has been conducted to determine the effects of DHA supplementation on metabolic and inflammatory responses in EMS horses. To investigate its effects in horses with EMS, 10 mixed-sex and mixed-breed adult EMS horses were selected. Treated horses (n=6) were supplemented with 16 g DHA per day for 46 days while controls (n=4) received no supplementation. Pre (Day 0) and Post (Day 46) supplementation samples were collected via jugular venipuncture and insulinemic responses measured via oral sugar test (OST). Inflammatory cytokines were measured by serologic ELISA and analysis of peripheral blood mononuclear cells (PBMCs) via flow cytometry and RT-PCR. Circulating fatty acids, triglyceride, leptin, and adiponectin concentrations were also measured. Post supplementation, treated horses had a significant increase in many circulating fatty acids compared to controls. These horses also had lower serum triglycerides post supplementation. In addition, supplemented horses had a reduction in TNF- α mean florescent intensity per lymphocyte. Interestingly, control horses had a rise in insulin concentrations 60 minutes post oral sugar administration 46 days post supplementation whereas DHA supplemented horses did not. These data indicate that algal DHA supplementation modulated circulating fatty acids, reduced inflammation, and influenced insulinemic responses in EMS horses.



HMW Adiponectin Concentrations and Association with Critical Illness in Horses

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Adiponectin is an adipocyte-derived hormone with insulin sensitizing, anti-inflammatory, and vascular protective properties. The metabolically active form of adiponectin, high molecular weight (HMW), is decreased in humans with obesity, insulin resistance, and inflammatory disease. However, higher adiponectin gene expression in adipose tissue is associated with increased mortality in horses undergoing emergency laparotomy. The purpose of this study was to examine HMW adiponectin concentrations in horses with critical illness. Serum HMW adiponectin and insulin concentrations were measured in archived clinical samples using a validated ELISA. Insulin concentrations were measured using a validated radioimmunoassay. Severity of illness was scored using weighted parameters (white blood cell count, band neutrophils, platelets, heart rate, temperature, increased digital pulse, mucous membrane color, etc.), and horses were designated into the following groups (Score 0, 1-4, 5-9, >10). Higher scores indicated worse severity of illness. Data were compared between groups using Kruskal-Wallis nonparametric analysis with Dunn's multiple comparison test and $P < 0.05$. HMW adiponectin concentrations in each group were as follows: score 0 (n=27): 4.0 (2.0-7.4) $\mu\text{g/ml}$; score 1-4 (n= 39): 3.9 (2.0-6.7) $\mu\text{g/mL}$; score 5-9 (n= 20): 4.9 (2.1-7.7) $\mu\text{g/mL}$; score >10 (n=13): 8.4 (2.4-11.5) $\mu\text{g/mL}$. Insulin concentrations were as follows: score 0 (n=24): 8.4 (2.9-12.5) $\mu\text{U/ml}$; score 1-4 (n= 31): 7.3 (3.2-15.5) $\mu\text{U/mL}$; score 5-9 (n= 14): 17.1 (4.2-32.6) $\mu\text{U/mL}$; score >10 (n=12): 2.7 (2.3-12.3) $\mu\text{U/mL}$. There were no significant differences between groups in HMW adiponectin concentrations or insulin concentrations. There was a trend toward higher HMW adiponectin concentrations in horses with the worst severity of illness score (>10) and a trend toward higher insulin concentrations in horses with scores 5-9. HMW adiponectin is not decreased in horses with critical illness. Studies specifically measuring markers of inflammation and examining correlation with adiponectin concentrations are needed.



Osteocalcin, CTX-1, Insulin, Vitamin D and PTH in Hospitalized Foals

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Hypovitaminosis D and elevated parathyroid hormone (PTH) concentrations are frequent in critically ill foals and linked to disease severity and mortality. However, information on markers of bone turnover, including osteocalcin (OCN: formation) and C-terminal telopeptide-1 (CTX-1; resorption) concentrations and their association with 25-hydroxyvitamin D₃ [25(OH)D₃], 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], PTH, and insulin concentrations is unknown. In other species, insulin increases OCN production (bone anabolic) and OCN promotes insulin secretion (bone-insular axis). The goals of this study were to assess bone remodeling biomarkers (OCN, CTX-1) and determine their association with vitamin D metabolites, PTH, and insulin concentrations, disease severity and mortality in hospitalized foals.

Foals (≤ 72 -hour-old; n=98) divided into hospitalized (n=82) and healthy (n=16) groups were included. Samples were collected on admission. Factors were measured by immunoassays. CTX-1, insulin, and PTH were higher while OCN, 25(OH)D₃ and 1,25(OH)₂D₃ concentrations lower in hospitalized than healthy foals (P<0.05). PTH was positively associated with CTX-1, and OCN was positively correlated with 1,25(OH)₂D₃ concentrations (P<0.05). CTX-1, OCN, and 1,25(OH)₂D₃ concentrations were also associated with disease severity. Non-survivors had higher CTX-1 and insulin but lower OCN, 25(OH)D₃, and 1,25(OH)₂D₃ concentrations than survivors (P<0.05). Hospitalized foals with low insulin concentrations were more likely to die.

Reduced OCN with elevated insulin concentrations suggests that osteoblast insulin signaling may be impaired (deregulated bone-insular axis), possibly reflecting sepsis-mediated insulin resistance. Elevated CTX-1 with reduced OCN together with low 1,25(OH)₂D₃, but increased PTH concentrations in sick foals indicates increased bone turnover and decreased bone formation.

