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Circannual variation in plasma adrenocorticotropic hormone concentrations and dexamethasone suppression test results in Standardbred horses, Andalusian horses and mixed-breed ponies

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Running title: ACTH in different equine breeds

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Abbreviations: ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; DST, dexamethasone suppression test; PPID, pituitary *pars intermedia* dysfunction.

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Abstract

Objective: To compare circannual plasma concentrations of adrenocorticotropic hormone (ACTH) and seasonal dexamethasone suppression test (DST) results between three different equine breeds.

Methods: Six Standardbred horses, six Andalusian horses and six mixed-breed ponies were followed over a 1-year period, during which time groups were managed identically. Blood samples were collected monthly (around the autumn equinox) or every second month (other times of the year) for the determination of plasma ACTH concentrations using a chemiluminescent immunoassay. Overnight DSTs were performed quarterly, with suppression of plasma cortisol to below 27 nmol/L at 19 hours considered a normal result.

Results: Seasonal variation in plasma ACTH concentrations was present among all breed groups with, as expected, higher levels detected around the autumn equinox, from February to April ($P<0.001$). Plasma ACTH concentrations were different between breed groups in March, with higher levels in Andalusians compared with Standardbreds ($P=0.048$) and in ponies compared with Standardbreds ($P=0.010$). Suppression of cortisol during the DST was normal for all animals in winter, spring and summer, but five Andalusians and three ponies returned abnormally high results in autumn, compared with zero Standardbreds.

Conclusion: Higher plasma ACTH concentrations and more false-positive DST results were present during autumn in ponies and Andalusian horses when compared to Standardbred horses. Potential differences between breeds should be considered when interpreting test results for horses and ponies that are evaluated for pituitary *pars intermedia* dysfunction.

Further work is recommended to establish population-based reference intervals and clinical cut-off values for ACTH in different equine breeds.

Introduction

Pituitary *pars intermedia* dysfunction (PPID) is a common endocrinopathy of equids, for which no specific risk factors have been identified other than advancing age.[1] The clinical importance of PPID is primarily due to an association with insulin dysregulation and laminitis, with other notable clinical features including hypertrichosis/hirsutism, muscle atrophy, lethargy/mental obtundation and predisposition to secondary infections.[2-6] Measurement of plasma adrenocorticotropic hormone (ACTH) concentration by immunoassay is the most commonly used screening test for PPID, with affected animals expected to have higher ACTH concentrations compared with unaffected animals.[7-9] In equids, ACTH exhibits a predictable circannual rhythm, with a period of increased secretion during autumn, corresponding to a decrease in daily photoperiod.[10-13]

Factors that can influence endogenous ACTH concentrations, including pain, disease, stress, exercise and feeding state, need to be considered when interpreting test results.[14-18] In a study that sampled populations of ponies in early winter and early summer, it was noted that many healthy ponies in early winter had plasma ACTH concentrations well in excess of the laboratory reference range (median around 100 pg/mL), leading to the hypothesis that ponies may exhibit higher concentrations than horse breeds.[19] A direct breed comparison by Durham

and Shreeve, based on clinical pathology submissions, indicated that breed was likely to represent one source of variation among equids (although not all animals were healthy and the data may have included PPID cases).[20] These observations are important to consider if reference intervals for ACTH are derived from populations containing a diversity of breeds. One previous report found a difference in plasma ACTH concentrations between light-breed horses and Morgan horses (a metabolically 'thrifty' breed, prone to obesity and insulin dysregulation), but not between horse and pony breeds.[21] Other studies have also failed to detect a clear difference in plasma ACTH concentrations between horse and pony breeds.[8, 10]

The overnight dexamethasone suppression test (DST) has been used as a diagnostic test for PPID, although it can suffer from poor sensitivity in early cases, and seasonal fluctuations in specificity.[22-24] Whether there are breed-related differences in DST results at different times of the year is unknown. If there are differences in ACTH production between breeds, it would be interesting to know whether corticosteroid feedback on corticotrope cells of the pituitary *pars distalis* is also affected in a breed-specific manner, in order to better understand the physiology underlying this phenomenon.

To evaluate potential differences in pituitary gland function between ponies and horses, it should be considered whether differences are associated with body size alone, or other aspects of metabolism. Native-breed ponies, Iberian horses and Thoroughbred/Standardbred horses originated along distinct evolutionary lines.[25] This study aimed to compare circannual plasma ACTH concentrations and seasonal DST results between Standardbred horses,

Andalusian horses and mixed-breed ponies under controlled management conditions. We hypothesised that ponies and Andalusian horses (due to their metabolic phenotype) would demonstrate higher plasma ACTH concentrations, and more inconsistent cortisol suppression following dexamethasone administration, compared with Standardbred horses.

Materials and methods

Six Standardbred horses (median 9 years [range 5 – 16 years]), six Andalusian horses (9 [6 – 13] years) and six mixed-breed ponies (9 [5 – 16] years) were evaluated over a one-year study period, from July 2011 to July 2012. Details of individual horses and ponies enrolled in the study are included as supporting information. No animals exhibited clinical signs consistent with PPID at the outset. They were kept at the same location (37.9°S, 144.7°E; Werribee, Victoria, Australia) for the duration and monitored daily by a veterinarian due to their enrolment in a parallel metabolism study.[26] Horses and ponies were kept together in bare paddocks (no pasture access) with shelter available. No artificial lighting was used and they were not stabled at any time. They were provided with hay and free access to fresh water and received complementary meals that contained a vitamin and mineral supplement. The diets fed during the metabolism study did not include a starch-rich formulation, which has been previously shown to affect ACTH and cortisol concentrations in horses.[17] Regular farriery, dentistry and anthelmintic treatments were provided, as required. All procedures were approved by the University of Melbourne Animal Ethics Committee (ID 1011918).

To evaluate circannual ACTH concentrations, blood samples were collected in July, September, November, monthly from January to May (two months before and after the autumn equinox), and again in the following July. Blood was obtained by venepuncture of the jugular vein using a 20-gauge needle, at approximately 1600h, performed within 3 days of the middle of each sampling month.

Overnight DSTs were performed in each season, during July (winter), October (spring), January (summer), April (autumn) and July (winter), completed within 3 days of the middle of each season. Blood samples were collected immediately before and 19 hours after the administration of 0.04 mg/kg dexamethasone sodium phosphate, intramuscularly, at approximately 1600h. Animals were weighed on the day of testing using calibrated scales to accurately determine the dose of dexamethasone required. Suppression of cortisol below 27 nmol/L at 19 hours after dexamethasone administration was considered a normal result.[27]

Blood samples were collected directly into evacuated plastic tubes containing EDTA (for ACTH) or heparin (for cortisol) anticoagulants (BD Vacutainer). Tubes were immersed in crushed ice until centrifugation at 1000 x *g* for 10 minutes at 4°C. Plasma aliquots were stored at -80°C until analysis. Plasma ACTH concentrations were determined using a validated chemiluminescent immunoassay (Immulite 1000, Siemens Diagnostics).[28] Samples were analysed in a single batch within 1 month of the conclusion of the study period and subjected to one freeze-thaw cycle, to minimise the potential variability that can occur with multiple freeze-thaw cycles.[29] Plasma cortisol concentrations were determined using a

validated radioimmunoassay (Coat-A-Count, Siemens Diagnostics).[30, 31] Samples were analysed in two batches, the first batch within 1 month of the spring DSTs and the second batch within 1 month of the final winter DSTs. Intra-assay (4.5% and 4.7%) and interassay (6.1%) coefficients of variation were acceptable.

Data analysis was performed using commercially available software (GraphPad Prism, version 8.4). For ACTH data, the assumption of normality was not satisfied for all group combinations of breed and month, as assessed using the Shapiro-Wilk test ($P<0.05$). Data were log transformed and analysed using a two-way ANOVA for repeated measures, with Tukey's *post hoc* test for multiple comparisons. For DST data, 0h and 19h cortisol concentrations were each compared between breed groups across seasons using a two-way ANOVA for repeated measures, with Tukey's *post hoc* test for multiple comparisons. Statistical significance was accepted at $P<0.05$.

Results

All animals remained healthy throughout the study period and did not suffer from any episodes of laminitis or other painful conditions that might be expected to cause temporal changes to the hypothalamo-pituitary-adrenal axis. During the study period, one of the ponies (16-year-old gelding) exhibited a delay in shedding of their winter hair coat, which raised the suspicion of early PPID. This pony subsequently began to display ill-thrift and was euthanased the following year due to an unrelated medical condition. A diagnosis of PPID

was confirmed on histological examination of the pituitary gland, and this pony was therefore excluded from the statistical analysis. Further information relating to this pony is included as supplementary information.

Circannual plasma concentrations of ACTH are shown in Figure 1. A significant effect of time was detected, with the expected autumnal increase in ACTH observed in all breed groups during the months of February, March and April (each $P<0.001$ when compared with other months of the year). A significant effect of time x breed was detected for the month of March, with higher ACTH concentrations observed in Andalusians compared with Standardbreds ($P=0.048$) and in ponies compared with Standardbreds ($P=0.010$).

If seasonally adjusted upper limits of endogenous ACTH proposed by the Australian and New Zealand Equine Endocrine Group are considered,[32] all Standardbred horses had plasma ACTH concentrations within the 'negative' range for PPID in every month. In January, one pony was within the 'grey zone' (50–80 pg/mL), and in March, two Andalusian horses and one pony returned results within the 'grey zone' (80–120 pg/mL) and one pony was within the 'positive' range for PPID (>120 pg/mL), despite being within the 'negative' range in every other month (before and after this time).

Seasonal DST results are shown in Figure 2. Baseline cortisol values were not significantly different between breed groups at any time ($P>0.35$). There was an effect of season for one comparison, with higher plasma cortisol concentrations in spring compared with the second

winter measurement (P=0.003). For 19h plasma cortisol concentrations, a significant effect of time x breed was observed in autumn, with higher plasma cortisol concentrations in Andalusians compared with Standardbreds (P=0.002) and in ponies compared with Standardbreds (P=0.003). When dichotomous classification was considered using the diagnostic threshold for PPID (cortisol >27 nmol/L at 19h), all animals returned normal results during winter, spring and summer. However, in autumn, five Andalusians and three ponies exceeded the diagnostic threshold (range, 27.2 – 39.5 nmol/L), while all six Standardbreds returned normal results.

Except for the pony subjected to euthanasia with confirmed PPID, all animals in this study were followed by the investigators for between two and eight years, during which time none of them exhibited any clinical signs consistent with PPID (supporting information). Two animals were euthanased during this period for reasons unrelated to the study, with histopathological examination of their pituitary glands confirming the absence of PPID.

Discussion

Ponies and Andalusian horses in this study were found to have higher plasma ACTH concentrations and more inconsistent DST results (reduced cortisol suppression) during autumn, when compared to Standardbred horses. These findings provide evidence that breed has the potential to affect the interpretation of diagnostic tests that are used to screen animals for PPID. This study was conducted in a research herd that included a relatively small

number of animals, and while there are advantages to this approach, in that breed groups could be managed identically throughout the study period, the generalisability of findings beyond the sampling frame cannot be determined. Field-based studies of larger populations are warranted to investigate the broader epidemiological question of whether breed-specific reference intervals and clinical cut-off values for plasma ACTH are required.

A predictable circannual rhythm of ACTH secretion is observed in equids, with increased plasma concentrations measured during autumn,[10-13] which is also the time period during which differences between breeds were detected in the present study. One explanation is that there are differences in dopaminergic inhibition of the *pars intermedia* in 'thrifty' breeds, as a result of their evolutionary adaptation to harsh winters and seasonal scarcity of forage, which is exacerbated during autumn.[24] Pony breeds, Iberian (Andalusian) horses and Thoroughbred horses (from which Standardbred horses were derived) are known to have originated from distinct evolutionary lineages based on their mitochondrial DNA.[25]

Ponies exhibit seasonal fluctuations in appetite, feeding behaviour and metabolic rate.[33, 34] The physiological mechanisms that control these changes are not fully understood but could include regulation by hormones produced by the *pars intermedia*. Previous studies have documented tissue insulin resistance, increased post-prandial insulin and incretin levels, and regional adiposity in ponies and Andalusian horses.[35-38] Standardbreds have been used as a comparative breed that are relatively insulin sensitive.[35, 36] Whether differences between

breeds for these metabolic traits are associated with pituitary gland activity is unclear, and further work is required to elucidate any relationships.

The overnight DST, which evaluates the suppression of endogenous cortisol following dexamethasone administration, was at one time the most popular diagnostic test for PPID.[27] Currently, the DST is less commonly performed, due to the relative inconvenience of the procedure (compared with the single blood sample that is required for measuring endogenous ACTH concentration) and an anecdotal concern among some veterinarians that corticosteroids could induce laminitis in susceptible animals.[9] A known limitation of the DST is the potential for false-positive results in autumn (i.e. failure of normal animals to suppress cortisol below 27 nmol/L).[10, 22-24] We observed this phenomenon during autumn in a high proportion of the ponies and Andalusian horses, but not in any of the Standardbred horses. Additionally, a false-negative result was obtained for the PPID positive pony during winter. These findings provide further evidence that the overnight DST may not be the robust test for PPID that it was once thought.

The lack of cortisol suppression observed in autumn suggests that either dexamethasone failed to reduce the secretion of ACTH from the corticotrope cells of the pituitary *pars distalis*; and/or the response of the adrenal cortex to ACTH was altered (although questionable given the similar basal cortisol concentrations between groups). Perhaps a more likely explanation is that a significant amount of the increased ACTH produced from the pituitary gland during autumn is coming from the *pars intermedia*, whose cells are resistant

to corticosteroid negative feedback.[9] This would be consistent with the concept described by McFarlane,[24] that dopaminergic inhibition of the *pars intermedia* in 'thrifty' breeds is diminished in the autumn. It would also be consistent with the finding that the pro-opiomelanocortin-derived peptide, α-melanocyte stimulating hormone, has been shown to have higher autumnal plasma concentrations in ponies compared with horses.[7, 39]

Basal cortisol concentrations did not differ between breeds, suggesting that the additional ACTH secreted in autumn, particularly in the ponies and Andalusian horses, may not be as biologically active in terms of its effect on the adrenal cortex.[9] The chemiluminescent immunoassay used in this study (that is standard at many laboratories around the world) has been shown to be susceptible to cross-reactivity with another pro-opiomelanocortin-derived hormone produced by the *pars intermedia*, corticotropin-like intermediate lobe peptide.[40] Therefore, it is also possible that the autumnal increase in plasma ACTH concentrations could be influenced by cross-reacting hormones, especially in breeds predisposed to insulin dysregulation and metabolic syndrome.[41] Further work is required to investigate potential differences in the *pars intermedia* secretome between horses and ponies.

A further limitation of this study, as with any study that does not include post mortem examination of the pituitary gland from each animal, is the lack of a definitive ante mortem diagnostic test to exclude the possibility of early/subclinical PPID.[24] No animals exhibited any clinical signs consistent with PPID in the two to eight years following the study in which they remained known to the investigators. In fact, having one pony that could be followed

though the early stages of PPID (and subsequently confirmed by post mortem examination), alongside normal animals, provided a valuable comparison.

Conclusions

Ponies and Andalusian horses demonstrated higher plasma ACTH concentrations and more false-positive DST results, during autumn, compared with Standardbred horses. The findings suggest that breed should be considered when interpreting the results of these tests that are used to diagnose PPID in equids, especially during autumn. This study highlights the necessity for larger field-based studies to investigate whether separate reference intervals and clinical cut-off values for ACTH are required for different equine breeds.

Figure legends

Figure 1: Circannual plasma concentrations of adrenocorticotropic hormone (ACTH) in Standardbred horses (white boxes; n=6), Andalusian horses (stippled boxes; n=6) and mixed-breed ponies (solid boxes; n=5), between July 2011 and July 2012. Box and whisker plots represent median, interquartile range and range. *Significant difference between Standardbred horses and Andalusian horses (P=0.048) and between Standardbred horses and ponies (P=0.010) during March.

Figure 2: Overnight dexamethasone suppression test results for Standardbred horses (white boxes; n=6), Andalusian horses (stippled boxes; n=6) and mixed-breed ponies (solid boxes; n=5) in July (winter), October (spring), January (summer), April (autumn) and July (winter). Box and whisker plots represent median, interquartile range and range. The dashed line represents the diagnostic threshold for pituitary *pars intermedia* dysfunction (PPID) of 27 nmol/L. *Significant difference between Standardbreds and Andalusians (P=0.002) and between Standardbreds and ponies (P=0.003).

References

1. Ireland JL, McGowan CM. Epidemiology of pituitary pars intermedia dysfunction: A systematic literature review of clinical presentation, disease prevalence and risk factors. *Vet J* 2018;235:22-33. https://doi.org/10.1016/j.tvjl.2018.03.002

2. Schott HC. Pituitary pars intermedia dysfunction: equine Cushing's disease. *Vet Clin N Am-Equine Pract* 2002;18:237-270. https://doi.org/10.1016/S0749-0739(02)00018-4

3. McGowan TW, Pinchbeck GP, McGowan CM. Prevalence, risk factors and clinical signs predictive for equine pituitary pars intermedia dysfunction in aged horses. *Equine Vet J* 2013;45:74-79. https://doi.org/10.1111/j.2042-3306.2012.00578.x

4. McFarlane D. Pathophysiology and clinical features of pituitary pars intermedia dysfunction. *Equine Vet Educ* 2014;26:592-598. https://doi.org/10.1111/eve.12237

5. Horn R, Bamford NJ, Afonso T et al. Factors associated with survival, laminitis and insulin dysregulation in horses diagnosed with equine pituitary pars intermedia dysfunction. *Equine Vet J* 2019;51:440-445. https://doi.org/10.1111/evj.13041

6. Tadros EM, Fowlie JG, Refsal KR, Marteniuk J, Schott HC, II. Association between hyperinsulinaemia and laminitis severity at the time of pituitary pars intermedia dysfunction diagnosis. *Equine Vet J* 2019;51:52-56. https://doi.org/10.1111/evj.12963

7. Beech J, Boston RC, McFarlane D, Lindborg S. Evaluation of plasma ACTH, α-melanocyte-stimulating hormone, and insulin concentrations during various photoperiods in clinically normal horses and ponies and those with pituitary pars intermedia dysfunction. *J Am Vet Med Assoc* 2009;235:715-722. https://doi.org/10.2460/javma.235.6.715

8. McGowan TW, Pinchbeck GP, McGowan CM. Evaluation of basal plasma α-melanocyte-stimulating hormone and adrenocorticotrophic hormone concentrations for the diagnosis of pituitary pars intermedia dysfunction from a population of aged horses. *Equine Vet J* 2013;45:66-73. https://doi.org/10.1111/j.2042-3306.2012.00575.x

9. Durham AE, McGowan CM, Fey K, Tamzali Y, van der Kolk JH. Pituitary pars intermedia dysfunction: Diagnosis and treatment. *Equine Vet Educ* 2014;26:216-223. https://doi.org/10.1111/eve.12160

10. Donaldson MT, McDonnell SM, Schanbacher BJ et al. Variation in plasma adrenocorticotropic hormone concentration and dexamethasone suppression test results with season, age, and sex in healthy ponies and horses. *J Vet Intern Med* 2005;19:217-222. https://doi.org/10.1111/j.1939-1676.2005.tb02685.x

11. Frank N, Elliott SB, Chameroy KA et al. Association of season and pasture grazing with blood hormone and metabolite concentrations in horses with presumed pituitary pars intermedia dysfunction. *J Vet Intern Med* 2010;24:1167-1175. https://doi.org/10.1111/j.1939-1676.2010.0547.x

12. Copas VEN, Durham AE. Circannual variation in plasma adrenocorticotropic hormone concentrations in the UK in normal horses and ponies, and those with pituitary pars intermedia dysfunction. *Equine Vet J* 2012;44:440-443. https://doi.org/10.1111/j.2042-3306.2011.00444.x

13. Secombe CJ, Tan RHH, Perara DI et al. The effect of geographic location on circannual adrenocorticotropic hormone plasma concentrations in horses in Australia. *J Vet Intern Med* 2017;31:1533-1540. https://doi.org/10.1111/jvim.14782

14. Alexander SL, Irvine CHG, Ellis MJ, Donald RA. The effect of acute exercise on the secretion of corticotropin-releasing factor, arginine vasopressin, and adrenocorticotropin as measured in pituitary venous blood from the horse. *Endocrinol* 1991;128:65-72. https://doi.org/10.1210/endo-128-1-65

15. Ayala I, Martos NF, Silvan G et al. Cortisol, adrenocorticotropic hormone, serotonin, adrenaline and noradrenaline serum concentrations in relation to disease and stress in the horse. *Res Vet Sci* 2012;93:103-107. https://doi.org/10.1016/j.rvsc.2011.05.013

16. Diez de Castro E, Lopez I, Cortes B et al. Influence of feeding status, time of the day, and season on baseline adrenocorticotropic hormone and the response to thyrotropin releasing hormone-stimulation test in healthy horses. *Domest Anim Endocrinol* 2014;48:77-83. https://doi.org/10.1016/j.domaniend.2014.02.004

17. Jacob SI, Geor RJ, Weber PSD, Harris PA, McCue ME. Effect of dietary carbohydrates and time of year on ACTH and cortisol concentrations in adult and aged horses. *Domest Anim Endocrinol* 2018;63:15-22. https://doi.org/10.1016/j.domaniend.2017.10.005

18. Stewart AJ, Hackett E, Bertin F-R, Towns TJ. Cortisol and adrenocorticotropic hormone concentrations in horses with systemic inflammatory response syndrome. *J Vet Intern Med* 2019;33:2257-2266. https://doi.org/10.1111/jvim.15620

19. Bailey SR, Habershon-Butcher JL, Ransom KJ, Elliott J, Menzies-Gow NJ. Hypertension and insulin resistance in a mixed-breed population of ponies predisposed to laminitis. *Am J Vet Res* 2008;69:122-129. https://doi.org/10.2460/ajvr.69.1.122

20. Durham A, Shreeve H. Horse-factors influencing the seasonal increase in plasma ACTH secretion. Dorothy Russell Havemeyer Foundation International Equine Endocrinology Summit, Coral Gables, Florida, USA, 2017. https://sites.tufts.edu/equineendogroup/files/2017/01/2017-Equine-Endocrinology-Summit-D-Russell-Havemeyer-Foundation.pdf

21. McFarlane D, Paradis MR, Zimmel D et al. The effect of geographic location, breed, and pituitary dysfunction on seasonal adrenocorticotropin and α-melanocyte-stimulating hormone plasma concentrations in horses. *J Vet Intern Med* 2011;25:872-881. https://doi.org/10.1111/j.1939-1676.2011.0745.x

22. Frank N, Andrews FM, Sommardahl CS et al. Evaluation of the combined dexamethasone suppression/thyrotropin-releasing hormone stimulation test for detection of pars intermedia pituitary adenomas in horses. *J Vet Intern Med* 2006;20:987-993. https://doi.org/10.1892/0891-6640(2006)20[987:eotcdt]2.0.co;2

23. Beech J, Boston R, Lindborg S, Russell GE. Adrenocorticotropin concentration following administration of thyrotropin-releasing hormone in healthy horses and those with pituitary pars intermedia dysfunction and pituitary gland hyperplasia. *J Am Vet Med Assoc* 2007;231:417-426. https://doi.org/10.2460/javma.231.3.417

24. McFarlane D. Diagnostic testing for equine endocrine diseases: confirmation versus confusion. *Vet Clin N Am-Equine Pract* 2019;35:327-338. https://doi.org/10.1016/j.cveq.2019.03.005

25. Jansen T, Forster P, Levine MA et al. Mitochondrial DNA and the origins of the domestic horse. *Proc Natl Acad Sci USA* 2002;99:10905-10910. https://doi.org/10.1073/pnas.152330099

26. Bamford NJ, Potter SJ, Harris PA, Bailey SR. Effect of increased adiposity on insulin sensitivity and adipokine concentrations in horses and ponies fed a high fat diet, with or without a once daily high glycaemic meal. *Equine Vet J* 2016;48:368-373. https://doi.org/10.1111/evj.12434

27. Dybdal NO, Hargreaves KM, Madigan JE et al. Diagnostic testing for pituitary pars intermedia dysfunction in horses. *J Am Vet Med Assoc* 1994;204:627-632.

28. Perkins GA, Lamb S, Erb HN et al. Plasma adrenocorticotropin (ACTH) concentrations and clinical response in horses treated for equine Cushing's disease with cyproheptadine or pergolide. *Equine Vet J* 2002;34:679-685.

29. Hu K, Stewart AJ, Yuen KY et al. The effect of freeze-thaw cycles on determination of immunoreactive plasma adrenocorticotrophic hormone concentrations in horses. *J Vet Intern Med* 2020;34:1350-1356. https://doi.org/10.1111/jvim.15771

30. Freestone JF, Wolfsheimer KJ, Kamerling SG et al. Exercise induced hormonal and metabolic changes in Thoroughbred horses: effects of conditioning and acepromazine. *Equine Vet J* 1991;23:219-223. https://doi.org/10.1111/j.2042-3306.1991.tb02760.x

31. Golland LC, Evans DL, Stone GM et al. Plasma cortisol and β-endorphin concentrations in trained and over- trained standardbred racehorses. *Pflugers Archiv Eur J Phys* 1999;439:11-17. https://doi.org/10.1007/s004240051122

32. Secombe CJ, Bailey SR, de Laat MA et al. Equine pituitary pars intermedia dysfunction: current understanding and recommendations from the Australian and New Zealand Equine Endocrine Group. *Aust Vet J* 2018;96:233-242. https://doi.org/10.1111/avj.12716

33. Dugdale AHA, Curtis GC, Cripps PJ, Harris PA, Argo CM. Effects of season and body condition on appetite, body mass and body composition in ad libitum fed pony mares. *Vet J* 2011;190:329-337. https://doi.org/10.1016/j.tvjl.2010.11.009

34. Brinkmann L, Gerken M, Riek A. Adaptation strategies to seasonal changes in environmental conditions of a domesticated horse breed, the Shetland pony (Equus ferus caballus). *J Exp Biol* 2012;215:1061-1068. https://doi.org/10.1242/jeb.064832

35. Bamford NJ, Potter SJ, Harris PA, Bailey SR. Breed differences in insulin sensitivity and insulinemic responses to oral glucose in horses and ponies of moderate body condition score. *Domest Anim Endocrinol* 2014;47:101-107. https://doi.org/10.1016/j.domaniend.2013.11.001

36. Bamford NJ, Baskerville CL, Harris PA, Bailey SR. Postprandial glucose, insulin, and glucagon-like peptide-1 responses of different equine breeds adapted to meals containing micronized maize. *J Anim Sci* 2015;93:3377-3383. https://doi.org/10.2527/jas.2014-8736

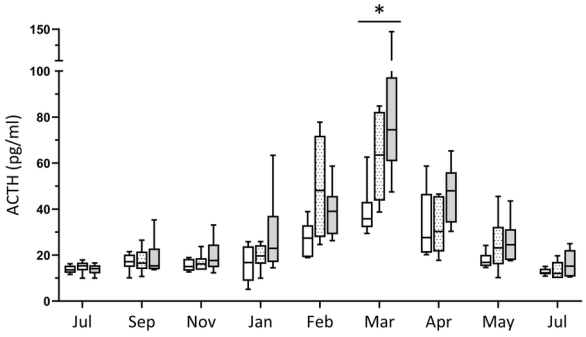
37. Riber C, Rubio MD, Marquez F et al. Haematological changes observed in Andalusian horses with laminitis. *J Vet Med Sci* 1995;57:981-984. https://doi.org/10.1292/jvms.57.981

38. Treiber KH, Kronfeld DS, Hess TM et al. Evaluation of genetic and metabolic predispositions and nutritional risk factors for pasture-associated laminitis in ponies. *J Am Vet Med Assoc* 2006;228:1538-1545. https://doi.org/10.2460/javma.228.10.1538

39. McFarlane D, Donaldson MT, McDonnell SM, Cribb AE. Effects of season and sample handling on measurement of plasma α-melanocyte-stimulating hormone concentrations in horses and ponies. *Am J Vet Res* 2004;65:1463-1468. https://doi.org/10.2460/ajvr.2004.65.1463

40. Knowles EJ, Moreton-Clack MC, Shaw S et al. Plasma adrenocorticotropic hormone (ACTH) concentrations in ponies measured by two different assays suggests seasonal cross-reactivity or interference. *Equine Vet J* 2018;50:672-677. https://doi.org/10.1111/evj.12797

41. McGilvray TA, Knowles EJ, Harris PA, Menzies-Gow NJ. Comparison of immunofluorescence and chemiluminescence assays for measuring ACTH in equine plasma. *Equine Vet J* 2020. https://doi.org/10.1111/evj.13227



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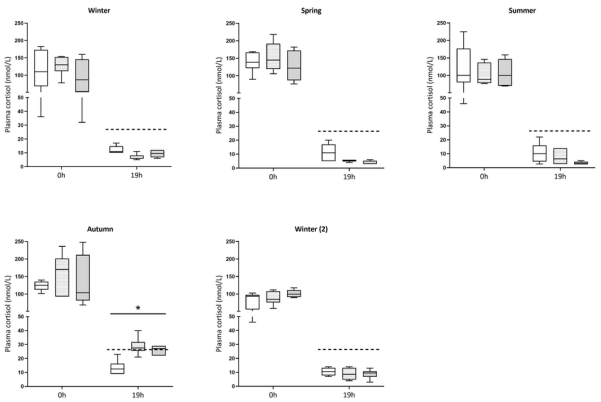

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