Effects of season and sample handling on measurement of plasma α-melanocyte-stimulating hormone concentrations in horses and ponies

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Objective—To investigate effects of sample handling, storage, and collection time and season on plasma α-melanocyte-stimulating hormone (α-MSH) concentration in healthy equids.

Animals—11 healthy Standardbreds and 13 healthy semiferal ponies.

Procedure—Plasma α-MSH concentration was measured by use of radioimmunoassay. Effects of delayed processing were accessed by comparing α-MSH concentrations in plasma immediately separated with that of plasma obtained from blood samples that were stored at 4°C for 8 or 48 hours before plasma was separated. Effects of suboptimal handling were accessed by comparing α-MSH concentrations in plasma immediately stored at -80°C with plasma that was stored at 25°C for 24 hours, 4°C for 48 hours or 7 days, and –20°C for 30 days prior to freezing at –80°C. Plasma α-MSH concentrations were compared among blood samples collected at 8:00 AM, 12 noon, and 4:00 PM. Plasma α-MSH concentrations were compared among blood samples collected in January, March, April, June, September, and November from horses and in September and May from ponies.

Results—Storage of blood samples at 4°C for 48 hours before plasma was separated and storage of plasma samples at 4°C for 7 days prior to freezing at –80°C resulted in significant decreases in plasma α-MSH concentrations. A significantly greater plasma α-MSH concentration was found in September in ponies (11-fold) and horses (2-fold), compared with plasma α-MSH concentrations in spring.

Conclusions and Clinical Relevance—Handling and storage conditions minimally affected plasma α-MSH concentrations. Seasonal variation in plasma α-MSH concentrations must be considered when evaluating pituitary pars intermedia dysfunction in equids. (*Am J Vet Res* 2004;65:1463–1468)

Pituitary pars intermedia dysfunction (PPID) in equids is a spontaneously occurring progressive disease primarily affecting aged ponies and horses. Despite recognition of this condition for more than 70 years, the cause and pathogenesis of PPID remain poorly defined.[1] Although the clinical signs of this disease are well recognized, antemortem diagnosis of PPID is problematic, with all available diagnostic tests having limitations. In aged horses with advanced disease, the presence of clinical signs, including hirsutism, laminitis, muscle atrophy, abnormal fat distribution, polydipsia and polyuria, hyperhidrosis, and secondary infections, may be sufficient for diagnosis.[2] However, if treatment is to be pursued, testing is still warranted as a tool to monitor response to treatment and allow individualized titration of drug dose.[3,4] In horses with early disease, the clinical signs often overlap with phenotypic changes observed with normal aging. Testing is therefore necessary to confirm the diagnosis.

Overnight dexamethasone suppression testing is considered the gold standard method for antemortem PPID diagnosis. Originally, this test was reported to have a sensitivity and specificity of 100%.[5] However, it was suggested in a recent report[a] that the reliability of the test has been over estimated. When the dexamethasone suppression test was repeated 3 times at 30-day intervals in 7 horses with clinical signs of PPID, only 1 of 7 horses had positive test results for disease on all 3 days.[a] Following an initial positive result, 5 of 7 horses had normal suppression on each subsequent test date, indicating either false-positive results at the initial test period or false-negative results during subsequent testing. Although it has not been critically assessed, a loss of feedback inhibition by glucocorticoids may be a late event in the disease progression, and the high sensitivity originally reported may reflect a case selection bias toward horses with advanced disease. Other limitations of the dexamethasone suppression test include the need for multiple days for blood sample collection and the potential exacerbation of laminitis in horses with active or historical laminitis.[6,7]

A 50% increase in serum cortisol concentration 15 to 90 minutes following thyrotropin-releasing hormone administration has also been proposed as a diagnostic test.[8] The performance of this test has not been critically evaluated in a large number of horses, particularly those with early disease. Further, this test is currently impractical as a result of the lack of approved thyrotropin-releasing hormone for use in horses and the high price of human-approved products.

The endogenous concentration of ACTH is also

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high in horses with PPID.[9,10,b] Plasma ACTH concentration measured by radioimmunoassay was reported to have a sensitivity of 82% to 90% and a specificity of 90% to 100% in the diagnosis of PPID when overnight dexamethasone suppression testing was used as the reference test.[9,b] Again, the usefulness of endgenous ACTH concentrations in the diagnosis of early PPID is unclear. Limitations of measurement of endogenous ACTH concentrations include the need to process samples within 3 hours of blood sample collection and to ship samples frozen, ideally on dry ice.[9]

Recently, the endogenous concentration of **α-melanocyte-stimulating hormone (α-MSH)** was investigated as a diagnostic test for PPID.[b] α-Melanocyte-stimulating hormone is a primary product of **proopiomelanocortin (POMC)** produced in the pars intermedia. Orth et al[10] showed the increase in concentration of α-MSH in the blood of horses with PPID to be of a greater magnitude than the increase in ACTH concentration. Therefore, a detectable increase in the plasma α-MSH concentration might precede a measurable increase in ACTH concentration. A sensitivity and specificity of 88% and 85%, respectively, were reported when using the dexamethasone suppression test results to define affected horses.[b]

In addition to its potential usefulness as a diagnostic test, accurate measurement of α-MSH concentrations has application as a research tool. Because α-MSH is primarily a product of the pars intermedia, whereas ACTH is primarily a product of the pars distalis, concurrent measurement of these POMC peptides can be used to differentiate a pars intermedia response to an environmental or pharmacologic challenge from a pars distalis response.[11] A better understanding of the physiologic role of the pars intermedia in healthy horses may provide insight into the pathogenesis of the dysfunction that occurs in horses with PPID. For measurements of plasma α-MSH concentration to be a more useful diagnostic or research tool, a more complete understanding of factors that may influence its measurement is required. Therefore, we evaluated the effect of sample handling, season, and time of day on plasma α-MSH concentrations in healthy horses and ponies.

Materials and Methods

Animals—All samples were collected in accordance with the guidelines of the Canadian Council on Animal Care. Group 1 consisted of 11 healthy adult Standardbreds (2 geldings and 9 nonpregnant mares) housed together indoors at the Atlantic Veterinary College, Prince Edward Island, Canada. Horses were kept in individual stalls and fed grass hay twice daily and water ad libitum. Group 2 consisted of 13 semiferal ponies (6 stallions and 7 breeding mares) maintained on 50 acres of natural grass pasture in Chester County, Pa. Mineral and salt blocks were provided, and supplemental hay was provided in the winter. Water was available from natural ponds and streams. Natural brush and light forest were available for shelter. All ponies were healthy and had overnight dexamethasone suppression test results that were within reference range.[5]

Measurement of plasma α-MSH concentration—Plasma α-MSH concentration in ponies and horses was measured by use of a commercially available radioimmunoassay designed for use with human plasma.[c] α-Melanocyte-stimulating hormone is a 13-amino acid peptide, with an identical sequence found in horses and humans.[11] The lack of interfering substances in equine plasma and α-MSH homology between humans and horses makes this assay suitable for use with equine plasma. The absence of interfering substances in equine plasma was confirmed by demonstrating a similar predicted and observed concentration when a known quantity of α-MSH was added to pooled equine plasma. Further, serial dilution of provided standards and equine plasma spiked with α-MSH resulted in the expected α-MSH concentrations. Sensitivity of the assay, as reported by the manufacturer and confirmed in our laboratory, was 3 pmol/L. Intra- and interassay variation reported by the manufacturer was 12% and 13% at 16 pmol/L, respectively, and 3% and 4% at 78 pmol/L, respectively. In our laboratory, both intra- and interassay variation was 5% for both high and low concentrations. Data provided by the manufacturer indicated low cross-reactivity with related peptides (< 0.002%). Specific control sera provided with each kit were included as positive controls for each assay. All samples were assayed in duplicate.

Sample handling—Sample handling effects were assessed in samples collected from horses in group 1. All conditions were assessed in groups of 6 randomly selected horses. Blood was collected by jugular venipuncture into evacuated glass tubes containing EDTA as an anticoagulant. Plasma was separated by centrifugation and transferred to polypropylene containers. The effect of freezing was assessed by comparing plasma that was separated within 1 hour of blood sample collection and assayed immediately with plasma that was frozen and stored at –80°C for 1 week. Samples were also stored for 9 months at –80°C to determine whether α-MSH was stable under long-term storage. To assess the effects of delayed processing, plasma was either immediately separated from blood samples or blood samples were stored in EDTA-containing evacuated tubes at 4°C for 8 or 48 hours before plasma was separated. Plasma was then separated and frozen as before. The effects of suboptimal handling of plasma were determined by immediately storing plasma after separation at 25°C for 24 hours, 4°C for 48 hours or 7 days, and –20°C for 30 days prior to freezing at –80°C for batch analysis.

The effect of hemolysis was investigated by spiking pooled equine plasma with known concentrations of equine hemoglobin. Fresh lysate was prepared by mixing equine packed RBCs with ice-cold distilled water at a 1:9 dilution. The lysate was centrifuged at 800 × *g* at 4°C for 20 minutes. Lysate was added to unaltered pooled equine plasma, pooled equine plasma spiked with α-MSH (37.5 pmol/L), and pooled equine plasma from horses with plasma α-MSH concentrations of > 100 pmol/L to reach final hemoglobin concentrations of 0.5, 1.0, 2.0, and 4.0 g/L.

Time of blood sample collection—The effect of blood sample collection time on plasma α-MSH concentration was assessed in 6 group 1 horses. Blood was collected by jugular venipuncture at 8:00 AM, 12 noon, and 4:00 PM. Plasma was separated within 1 hour and stored at –80°C until analyzed.

Effect of season on plasma α-MSH concentration—The effect of season on plasma α-MSH concentration was assessed by use of all group 1 horses and group 2 ponies. Blood was collected by jugular venipuncture, and plasma was separated and stored at –80°C until analyzed. All samples from group 1 horses were collected between 7:30 AM and 9:00 AM, and samples from group 2 ponies were collected between 9:00 AM and 3:00 PM. Samples from group 1 horses were collected in January, March, April, June, September, and

November. Samples from group 2 ponies were collected in September and the following May.

Statistical analysis—All results are presented as mean (± SD) values. All comparisons were made among samples from repeated blood sample collections from the same horses. For multipoint comparisons, a repeated measures ANOVA was used, with a Bonferroni correction for multiple comparisons for post hoc analysis. For 2-point comparisons, a paired *t* test was used. Values of $P < 0.05$ were considered significant. Commercially available software was used to perform analyses.[d,e]

Results

Sample handling—The effect of storage of samples at –80°C on measured plasma α-MSH concentration was assessed. No significant ($P = 0.17$) difference in plasma α-MSH concentrations was found between measurements made immediately and measurements made after storage for 1 week (13.1 ± 2.2 pmol/L vs 11.9 ± 2.8 pmol/L, respectively). Further, storage for 9 months at –80°C did not result in a significant ($P = 0.22$) decrease in measured plasma α-MSH concentration, compared with measurements made immediately (14.1 ± 2.6 pmol/L vs 13.1 ± 2.2 pmol/L, respectively). Therefore, in all following stability studies, plasma samples were frozen and stored at –80°C for batch analysis.

The effect of delayed separation of plasma on measured α-MSH concentration was assessed for various periods (**Figure 1**). Although the storage of blood samples at 4°C for 8 hours before plasma was separated did not significantly change measured concentrations, compared with that of plasma that was immediately separated (12.0 ± 2.7 pmol/L vs 11.1 ± 3.1 pmol/L, respectively), the storage of blood samples at 4°C for 48 hours before plasma was separated did result in a significant decrease in plasma α-MSH concentration (11.1 ± 3.1 pmol/L vs 8.3 ± 2.1 pmol/L, respectively).

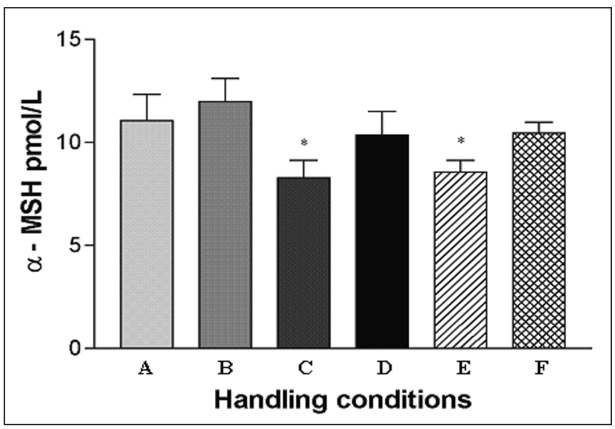

Figure 1—Effect of blood sample handling conditions (A through F) on mean (± SD) equine plasma α-melanocyte-stimulating hormone (α-MSH) concentrations. A = Plasma was separated immediately and stored at –80°C. B = Blood samples were stored at 4°C for 8 hours before plasma was separated and stored at –80°C. C = Blood samples were stored at 4°C for 2 days before plasma was separated and stored at –80°C. D = Plasma was separated and stored at 4°C for 2 days. E = Plasma was separated and stored at 4°C for 7 days. F = Plasma was separated and stored at –80°C for 9 months. *Significantly ($P < 0.05$) different plasma α-MSH concentrations (C and E), compared with samples processed immediately and stored at –80°C (A).

Storage of plasma in polypropylene tubes at 25°C for 24 hours, 4°C for 48 hours, and –20°C for 1 month prior to freezing at –80°C did not significantly affect the α-MSH concentration (Figure 1), compared with plasma stored immediately at –80°C. However, storage of plasma at 4°C for 1 week did result in a significant decrease in measured plasma α-MSH concentration, compared with plasma stored immediately at –80°C (8.6 ± 1.4 pmol/L vs 11.1 ± 3.1 pmol/L).

Hemolysis—Addition of hemolysate to plasma samples did not significantly affect the measurement of α-MSH concentration in unaltered equine plasma or equine plasma spiked with α-MSH (**Figure 2**), although a decrease of approximately 10% was observed with the hemolysate concentration of 4 g/L. Findings for pooled plasma samples from horses with naturally high plasma α-MSH concentrations were similar to findings for equine plasma spiked with α-MSH.

Time of day and time of year—No significant ($P = 0.9$) difference in plasma α-MSH concentration was found among samples collected at 8:00 AM, noon, or 4:00 PM (8.3 ± 2.6 pmol/L, 8.5 ± 3.2 pmol/L, and 8.3 ± 3.0 pmol/L, respectively). However, in horses and ponies, significant seasonal variation in plasma α-MSH concentrations was found, with the highest hormone concentrations found in September (**Figures 3 and 4**). Comparing September to the spring months, ponies had an 11-fold greater plasma α-MSH concentration, whereas in the same comparison, horses had a 2-fold greater concentration. The range of plasma α-MSH concentrations was also greatest in the fall, with ponies having values that ranged from 40 to 185 pmol/L in September, compared with 3 to 19 pmol/L in May, and horses having values that ranged from 2.7 to 58 pmol/L in September, compared with 9.5 to 15.9 pmol/L in April. No significant difference in plasma α-MSH concentrations was found between male (n = 6) and female (7) ponies in May (7.0 ± 2.3 vs 10.75 ± 4.6, respectively; $P = 0.09$) or September (95.9 ± 42.5 vs 99.3 ± 60.7, respectively; $P = 0.9$).

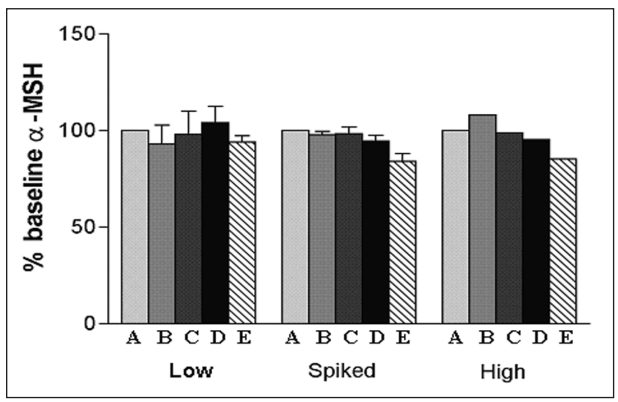

Figure 2—Effect of the addition of hemolysate on mean (± SD) measurements of equine plasma α-MSH concentrations as a percentage of baseline. Various amounts of hemolysate (A through E) were added to pooled equine plasma with low endogenous (mean, 16 pmol/L; n = 3), spiked (mean, 40 pmol/L; 3), and high endogenous (211 pmol/L; 1) concentrations of α-MSH. Equine hemolysate was added to samples to achieve final concentrations of 0, 0.5, 1.0, 2.0, and 4.0 g/L as represented by bars A, B, C, D, and E, respectively.

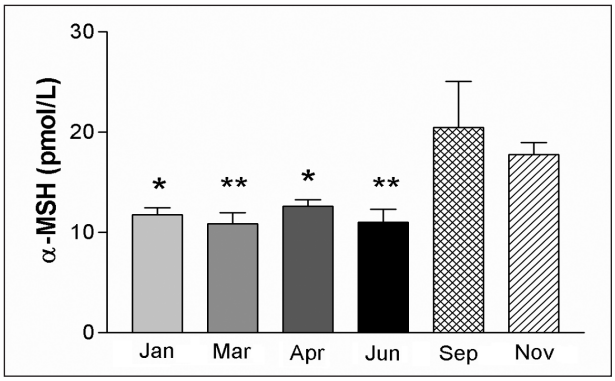

Figure 3—Effect of time of year (month) on mean (± SD) plasma α-MSH concentrations in healthy Standardbreds. *Significantly ($P < 0.05$) lower plasma α-MSH concentrations in January and April, compared with September. **Significantly ($P < 0.01$) lower plasma α-MSH concentrations in March and June, compared with September.

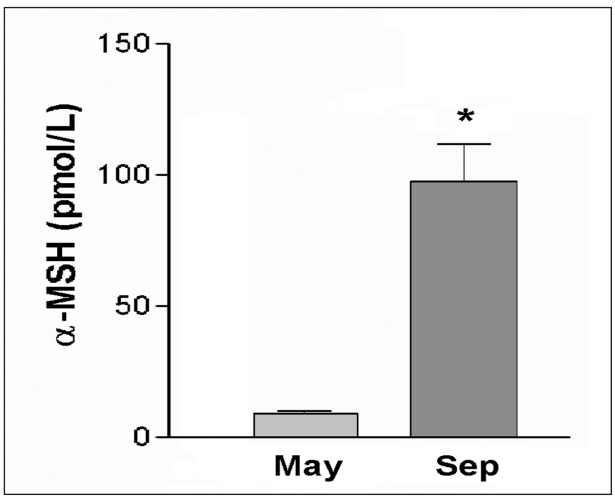

Figure 4—Effect of time of year (month) on mean (± SD) plasma α-MSH concentrations in healthy ponies. *Significantly ($P < 0.001$) lower plasma α-MSH concentrations in May, compared with September.

Discussion

As a result of the limitations of the currently available diagnostic tests for PPID, an improved testing method for this disease is needed. Pituitary pars intermedia dysfunction is a disease diagnosed primarily by practitioners in the field. Therefore, to be useful, a diagnostic test should be applicable to blood samples collected in the field. Ideally, blood samples should be able to be collected at a single farm visit, collected at any time of the day, and transported to the clinic without requiring special handling. In addition, the test should pose little to no risk to the patient. Our findings indicate that the measurement of plasma α-MSH concentration meets these requirements.

To assess the effect of delayed separation of plasma, whole blood in EDTA-containing evacuated tubes was held for 8 or 48 hours at 4°C prior to centrifugation and the separation of plasma. Although no difference was found in samples held for 8 hours, 48 hours of storage resulted in a significant decrease in plasma α-MSH concentration (Figure 1). Therefore, we suggest that plasma be separated within 8 hours of blood sample collection to avoid sample deterioration.

To assess the impact of storage conditions of plasma on hormone concentration, plasma was stored at 25°C for 24 hours, at 4°C for 48 hours or 7 days, and at –20°C for 1 month (Figure 1). When plasma was held for 1 week at 4°C, a decrease in α-MSH concentration occurred. All other conditions did not affect the hormone concentration. Therefore, plasma samples should be stored frozen until assayed, and overnight shipment of frozen plasma samples is preferable. Storage of plasma samples at –80°C did not adversely affect hormone concentration. We therefore conclude that storing of plasma samples at –80°C for later batch analysis is acceptable.

Hemolysis is known to interfere with a number of diagnostics assays.[12] The effect of hemolysis on measurement of plasma α-MSH concentration was assessed by adding a known quantity of equine hemolysate to pooled plasma samples containing low and high concentrations of hormone. No significant effect of hemolysate was found up to a hemoglobin concentration of 4 g/L. Although a decrease in measured plasma α-MSH concentration was found when hemolysate was added at a final hemoglobin concentration of 4 g/L, this difference was not significant. Nonetheless, it would be prudent to avoid severely hemolyzed samples for plasma α-MSH concentration measurements.

To determine the effect of blood sample collection time on α-MSH concentrations, we compared plasma hormone concentration in samples collected at 8:00 AM, noon, and 4:00 PM. No difference in hormone concentration was found between samples collected at any of these times throughout the day. This is consistent with the lack of circadian rhythm of α-MSH in horses reported by Horowitz et al.[6] Therefore, it does not appear that time of day is important in collecting blood samples.

In contrast to time of day, a distinct seasonal effect was found in horses and ponies. In horses and ponies, the measured plasma α-MSH concentration was considerably higher in September, compared with samples collected in the winter, spring, and early summer (Figures 3 and 4). An effect of season on α-MSH concentration has been described for humans, hamsters, sheep, and weasels.[13-16] For Siberian hamsters and short-tailed weasels, α-MSH concentration is reported to increase during the summer, at a time when coat changes color.[15,16] In humans living in Germany, plasma α-MSH concentration peaks in August, with lowest concentrations reported in January.[13] In Soay sheep, a feral breed of sheep in Scotland, plasma concentration of α-MSH is greatest in late summer and fall.[14] The functional importance of the seasonal cycle is unknown, but several physiologic events occur in parallel with the α-MSH cycle. In sheep, body weight, voluntary food intake, and condition all peak simultaneously with α-MSH concentrations, with seasonal maximums occurring in September. Soay sheep with surgically created hypothalamic-pituitary disconnection have an increase in circulating concentration of α-MSH and chronic increase in body weight.[17] These findings considered together suggest that α-MSH or other

POMC-derived peptides may play a role in metabolic preparation for winter in Soay sheep. It is possible that horses and ponies have a seasonal increase in POMC-derived peptides to metabolically prepare them for a decrease in accessible food observed in the wild in winter. If so, dysregulation of this pathway might be associated with abnormalities in body weight and fat storage. Weight loss and abnormal fat distribution are 2 clinical signs associated with equine PPID.[3,18,19]

Development of a winter coat also begins as length of day decreases in the fall. The development of hirsutism in horses with PPID leads one to speculate that the naturally occurring seasonal increase in POMC-derived peptides contributes to the development of winter coat growth. This has not been assessed in equids.

In our study, ponies had a more robust increase in plasma α-MSH concentrations in the fall, compared with horses (11-fold vs 2-fold). Several possible explanations exist for this finding. The ponies in our study were sexually intact and in natural breeding conditions under the influence of reproductive hormones, whereas the horses were nonbreeding females or geldings. When blood samples were collected in September, the female ponies were either in late pregnancy (> 8 months; n = 4) or had foaled within the last 2 weeks (3). In May, they were either nonpregnant (n = 2) or in early pregnancy (< 4 months; 5). Despite these variables, no difference in plasma α-MSH concentrations was found between the male and female ponies. Therefore, it seems unlikely that the observed difference between horses and ponies was solely the result of reproductive influences. Another possibility is the difference may reflect a metabolic and endocrinologic difference between horses and ponies. Ponies are more predisposed to obesity than Standardbreds.[20,21] In addition, they are predisposed to glucose intolerance, insulin insensitivity, and PPID.[2,20-22] A greater seasonal change in pars intermedia expression may be beneficial in preparing animals for winter, allowing survival when conditions are harsh and food is sparse. It is possible that ponies are more evolved to survive harsh winter elements, compared with horses. The difference in management conditions between the 2 groups may also have contributed to the observed difference in seasonal plasma α-MSH concentrations. The Standardbreds in our study were housed indoors, and feed intake was strictly controlled. The ponies were outside, under natural light with free access to pasture. Finally, it is possible that the seasonal hormonal peaks occur at different times of year in different geographic locations, and the actual peaks may have been missed as a result of blood sample collection intervals.

The findings of our study are important for several reasons. First, as a result of the seasonal regulation of the pars intermedia, it is important to consider the season when testing equids for PPID. In blood samples collected in September from the pony group, 6 of 13 had plasma concentrations of α-MSH considered diagnostic for PPID (> 91 pmol/L; Figure 4).[b] In all animals, concentrations from samples collected in spring and summer were within reference range. A diagnosis for PPID determined in the late winter, spring, or early summer is likely more reliable than a diagnosis made in the fall, when the pars intermedia is highly active. This would likely be true regardless of the method of testing used, as all testing is aimed at detecting dysregulation or overactivity of the pars intermedia. Further, the establishment of reference range values for diagnostic endocrine testing in equids must be done with consideration of the season. At a minimum, seasonally specific reference range values may increase sensitivity and specificity of endogenous plasma α-MSH concentrations for the diagnosis of PPID. However, the range of plasma α-MSH concentrations in September was much greater than in the spring, making differentiation of reference and diagnostic ranges more problematic. Finally, results of our work are consistent with a physiologic role of the pars intermedia of horses and ponies in metabolic preparation for winter. If seasonal variation in plasma POMC-derived peptides serves to regulate lipogenesis in horses and ponies similar to sheep, then it is possible that its dysregulation may play a role in the development of chronic obesity. The role of the POMC-derived peptides in obesity is an area of intense research in humans and warrants more critical evaluation in equids.

[a]Miesner TJ, Beard LA, Schmall SM, et al. Results of overnight dexamethasone suppression test repeated over time in horses suspected of having equine Cushing's disease (abstr). *J Vet Intern Med* 2003;17:420.

[b]Horowitz ML, Neal L, Watson JL. Characteristics of plasma adrenocorticotropin, β-endorphin and α-melanocyte stimulating hormone as diagnostic tests for pituitary pars intermedia dysfunction in the horse (abstr). *J Vet Intern Med* 2003;17:386.

[c]Euria-α-MSH RIA, American Laboratory Products Co, Windham, NH.

[d]Version 13.32, Minitab Inc, State College, Pa.

[e]Version Prism 3.03, GraphPad Software, San Diego, Calif.

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