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STANDARD ARTICLE

Effect of early or late blood sampling on thyrotropin releasing hormone stimulation test results in horses

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Abstract

Background: Diagnosis of pituitary pars intermedia dysfunction (PPID) using the thyrotropin-releasing hormone (TRH) stimulation test requires blood collection 10 minutes after TRH injection; it is unknown if small differences in timing affect test results.

Objective: To determine whether early or late sampling results in a significant (≥10%) difference in plasma adrenocorticotropic hormone (ACTH) concentration compared to standard 10-minute sampling.

Animals: Twenty-four healthy adult horses with unknown PPID status.

Methods: In this prospective study, subjects underwent a single TRH stimulation test, with blood collected exactly 9 minutes (early), 10 minutes (standard), and 11 minutes (late) after injection. ACTH was measured by chemiluminescent immunoassay. Two aliquots of the 10-minute plasma sample were analyzed separately to assess intra-assay variability. Data were reported descriptively and bias was calculated using Bland-Altman plots. Significance was set at $P = .05$.

Results: Minor variability was observed between the paired 10-minute sample aliquots (range, 0%-6%; median 3%). Overall variability of early or late samples compared to the corresponding paired (average) 10-minute standard concentration ranged from 0% to 92% (median 10%). Seventy-five percent of horses (18/24) tested had at least 1 early or late reading that differed by ≥10% from its corresponding 10-minute standard concentration, and 21% of horses (5/24) would have a different interpretation of testing result with either early or late sampling. Incidence of ≥10% variability was independent of PPID status ($P = .59$).

Conclusions and Clinical Importance: Precise timing of sample collection is critical to ensure accurate assessment of PPID status given the observation of significant variability associated with minor alterations in timing of sample collection.

KEYWORDS
ACTH, diagnostic testing, endocrinology, equine Cushing's, pituitary pars intermedia dysfunction, PPID

Abbreviations: ACTH, adrenocorticotropic hormone; CLIP, corticotropin-like intermediate lobe peptide; EDTA, ethylenediaminetetraacetic acid; PPID, pituitary pars intermedia dysfunction; TRH, thyrotropin-releasing hormone; UMASS, University of Massachusetts.

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1 | INTRODUCTION

Pituitary pars intermedia dysfunction (PPID, previously known as Equine Cushing's Disease) is a common endocrinopathy of older horses that is associated with a variety of serious sequelae, including laminitis, poor wound healing, endoparasitism, chronic infections, and behavioral change.[1-4] One method commonly used to diagnose PPID utilizes intravenous administration of thyrotropin-releasing hormone (TRH) to stimulate release of adrenocorticotropic hormone (ACTH). This dynamic test can offer the possibility of earlier diagnosis or confirmation of PPID in horses with suggestive clinical signs but equivocal or normal basal ACTH concentrations. Over the last decade, many refinements to TRH stimulation testing have been investigated to improve sensitivity of PPID diagnosis and augment practical clinical utility.[5-10] The Equine Endocrinology Group recommends the TRH stimulation test and provides interpretation criteria for diagnosis of PPID based on the ACTH concentration of a plasma sample obtained 10 minutes after intravenous administration of TRH.[11]

TRH stimulation testing produces an exaggerated ACTH release in horses with PPID because of presence of TRH receptors located within hyperplastic tissue of the pars intermedia of the pituitary.[12] Plasma ACTH concentrations change quickly in response to TRH administration in both normal and PPID-affected horses, reaching peak levels between 2 and 6 minutes after TRH injection in 1 study,[13] before 14 minutes in a second study,[14] and between 5 and 30 minutes in a third study,[15] implying that precise timing of sample collection could be critical to establishing an accurate diagnosis of PPID. Clinicians might be unaware of the rapidity with which the plasma ACTH concentration can change and thus underestimate the importance of precise timing in sample collection. Minor differences in accuracy of sample timing could be anticipated in an environment in which other tasks are being simultaneously performed or in a situation in which the person administering the TRH delegates the blood collection task to a different party. To date, there have been no studies evaluating whether a reasonably small difference of 1 minute in the timing of sample collection causes a significant effect on the plasma ACTH concentration, and subsequently the overall interpretation of TRH stimulation test results and determination of PPID status.

The objective of this study was to determine whether altering the timing of sample collection significantly affected TRH stimulation test results. We hypothesized that small differences in timing (1 minute early or late) would result in ≥10% differences in plasma ACTH concentrations compared to the standard 10-minute sampling protocol, and further, that this could have a clinically relevant effect on test interpretation and diagnosis of PPID.

2 | MATERIALS AND METHODS

2.1 | Animals

Twenty-four clinically healthy adult horses from the University of Massachusetts (UMASS) Amherst research program were enrolled in the study. Horses were determined to be healthy on the basis of medical records and physical examinations before the study starting. All horses had been housed on the farm under the care of UMASS personnel for a minimum of 1 year. Horses were housed in groups on pasture or dry lots with access to forage. All enrolled horses were of unknown PPID status (no prior testing had been performed), though 6/24 (25%) exhibited 1 or more clinical signs that could be attributed to PPID (including hypertrichosis, muscle wastage, and history of a laminitis episode occurring 2 or more years before the study date). None of the enrolled horses were being treated for PPID at the time of the study. Horses ranged in age from 3 to 28 years (median 10.5 years) and included 9 mares, 14 geldings, and 1 stallion. None of the mares were pregnant or lactating at the time of the study. Multiple breeds of horse were represented including Appaloosa (1), Draft cross (3), Hanoverian (2) Morgan (6), Quarter Horse (3), Standardbred (3), Thoroughbred (4), and Thoroughbred cross (2). Horses enrolled in the study were used for various university-related activities, including teaching, research, lesson activity, and mounted police patrol of the UMASS Amherst campus.

2.2 | Experimental design

Study protocols were approved by the Institutional Animal Care and Use Committee at UMASS Amherst. All testing was conducted in 2 periods occurring in December 2019 and January 2020 (16 horses) and December 2020 (8 horses). The gap in sample collection was because of COVID-19 pandemic restrictions. All samples were stored frozen (see below) and analyzed within 40 days from the time of collection. Testing was performed between 8 AM and 10 AM on testing days, with the full study procedure (physical examination, TRH administration, and blood sample collection) completed over a 20-minute period for a single enrolled horse before starting the procedure on the next subject. TRH stimulation testing was performed in accordance with published guidelines.[11] All grain and concentrate feed was withheld for a minimum of 10 hours before testing and normal hay feeding was continued before and during the testing period. A dose of 1 mg (1 mL) of commercially available synthetic TRH (Protirelin Injection Solution, Wedgewood Pharmacy, Swedesboro, NJ) was administered IV in the right jugular vein in all subjects and timing of subsequent blood collections was measured using a digital stopwatch. Blood was collected via jugular venipuncture and transferred into 6 mL vacuum tubes (BD Vacutainer© EDTA Tubes, BD-367863, Becton Dickinson, Franklin Lakes, NJ) containing ethylenediaminetetraacetic acid (EDTA) before TRH injection (0 minutes/pre) and at 9 minutes (early), 10 minutes (standard), and 11 minutes (late) after TRH injection. All blood collection was performed using an 18-gauge needle and the 9-, 10-, and 11-minute samples were sequentially drawn from a single venipuncture site in the left jugular vein to minimize vascular trauma. All horses were monitored for adverse reactions for the duration of the testing protocol.

2.3 | Sample processing and analysis

All blood samples were stored on ice until processing, which occurred within 4 hours of collection. Plasma was separated via centrifugation

at 720*g* at 4°C for 15 minutes and stored in 1 mL aliquots at −20°C until batch analysis. Frozen samples were stored for up to 40 days for 4/20 horses' samples and less than 14 days for the remaining 20/24 horses' samples per published guidelines.[16] No hemolysis or other gross sample abnormalities were observed. One aliquot from each 0-, 9-, and 11-minute sample and 2 aliquots from each 10-minute sample were shipped overnight on dry ice to the Animal Health Diagnostic Center Laboratory at Cornell University for analysis. Plasma ACTH concentration was measured by chemiluminescent immunoassay (Immulite 1000, Siemens Medical Solutions Diagnostics, Los Angeles, CA) on an automated analyzer, which had been previously validated for equine samples.[17] Single plasma aliquots were analyzed for each subject for the 0-, 9-, and 11-minute samples. Separate analyses on 2 individual plasma aliquots from the 10-minute samples for each subject were performed to assess intra-assay variability.

2.4 | Data and statistical analyses

Median and range are reported for all data analyses. For each horse, 5 ACTH concentrations were recorded (at 0, 9, 10, 10, and 11 minutes). The 2 10-minute samples (individually analyzed aliquots taken from a single 10-minute blood draw) were averaged to create a reference value to which the early (9-minute), late (11-minute), and individual 10-minute samples could be compared to calculate variability. Hereafter, this averaged reference value is referred to as the 10-minute standard. Linear regression was performed to assess the correlation between the 2 10-minute samples to assess intra-assay variability. Timing-associated variability was calculated as percent variance using the difference between the early (9-minute), late (11-minute), or individual 10-minute sample ACTH concentration and the 10-minute standard. A ≥10% difference in ACTH concentrations was considered sufficiently different to ensure that differences were greater in magnitude than would be expected from intra-assay variability alone. Bland-Altman plots were used to evaluate the effects of collection timing on variability across all data. These were repeated for data excluding horses with marked ACTH response (>250 pg/mL after TRH administration) to determine a more conservative assessment of bias.

For analysis of effect of sample timing on variability, samples were divided into early (9-minute) and late (11-minute) groups. For analysis of the effect of PPID status on variability of early or late sampling, horses were divided into PPID and non-PPID groups based on an established ACTH concentration threshold to determine PPID status of >50 pg/mL for the time 0 sample and/or >200 pg/mL at 10 minutes after TRH injection.[11] Chi-square testing was used to compare groups. Wilcoxon Signed-Rank testing was used to compare differences in magnitude of variability for early and late sampling. A *P*-value of .05 was considered significant. Excel (Microsoft Corporation, Redmond, WA) and GraphPad Prism (GraphPad 9.2.0 for Windows, GraphPad Software, San Diego, CA) were used for all statistical analyses.

3 | RESULTS

3.1 | Animals

A physical examination at the time of TRH testing confirmed that the heart rate, respiratory rate, and rectal temperature were normal for all horses and no health problems were reported by the staff caring for the horses at the facility. All subjects tolerated the TRH testing protocol well. After intravenous TRH injection, many subjects exhibited transient minor physical responses, all lasting 2 minutes or less. Most horses (22/24) demonstrated "mouthing" consisting of chewing, licking, or lip smacking; additional responses after injection included coughing in 4 horses, minor pawing behavior in 2 horses, flehmen response in 2 horses, and muscle fasciculations in 1 horse.

3.2 | Plasma ACTH concentrations and determination of PPID status

All horses had an increase in plasma ACTH concentration after intravenous administration of TRH, with most horses (88%, 21/24) exhibiting maximum ACTH concentrations at 9 or 10 minutes. (Figure 1). Seventeen percent of subjects (4/24) experienced marked increases in ACTH concentration (reaching a maximum of 500-1000 pg/mL), including 2 horses with baseline ACTH concentrations that were positive (>50 pg/mL) or equivocal (30-50 pg/mL) for PPID diagnosis. Using the 10-minute standard value, 6 horses (25%) had ACTH concentrations consistent with PPID (>200 pg/mL), and 1 horse (4%) had an equivocal ACTH value (110-200 pg/mL). Based on these established diagnostic thresholds, 5 horses (21%) would have a change in PPID status interpretation with early or late sampling. These PPID status changes include: 1 PPID horse appearing equivocal with later sampling, 1 equivocal PPID horse appearing positive with earlier sampling, and 3 normal horses appearing equivocal with early sampling; Figure 1. In total, including all measured values (early, 10-minute standard, and late), 42% of horses (10/24) had at least 1 ACTH measurement consistent with equivocal or positive PPID status. PPID status was significantly associated with age; the median age of subjects with at least 1 (early, standard 10-minute, or late) ACTH concentration consistent with equivocal or positive PPID status was 18 years (range, 4-28), while the median age for horses with normal ACTH concentration was 7 years (range, 3-19). Eighty percent of subjects (8/10) with at least 1 ACTH concentration consistent with equivocal or positive PPID status were over the age of 10 years, whereas only 29% of subjects (4/14) with normal ACTH concentrations were over 10 years old ($P = .01$).

3.3 | Variability of plasma ACTH concentrations and the effect of sample collection timing

Plasma ACTH concentrations from the paired 10-minute sample aliquots were highly repeatable, demonstrating excellent correlation ($P < .0001$; Figure S1). Minor variance was observed between the individual 10-minute aliquots and their respective 10-minute standard

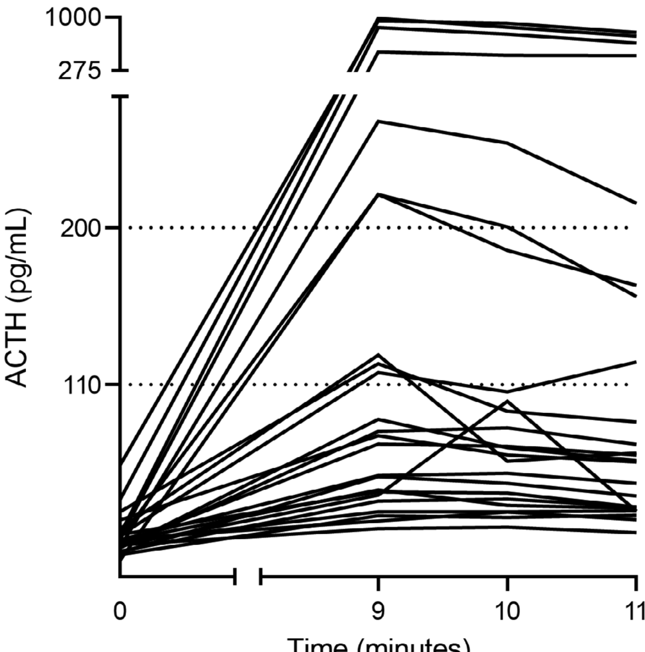
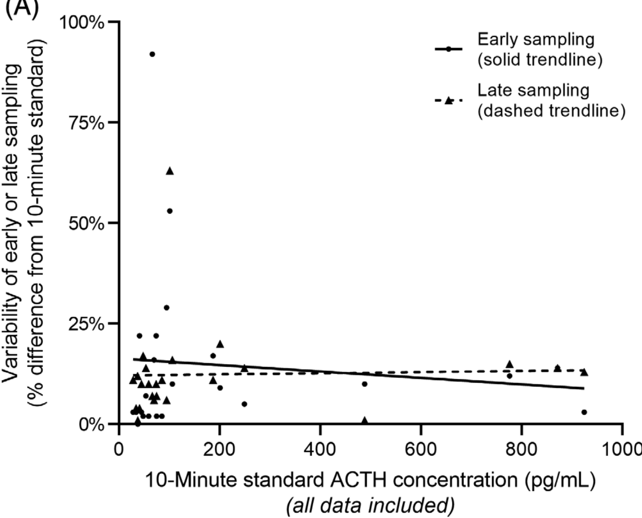


FIGURE 1 Plasma ACTH concentrations at 0, 9, 10, and 11 minutes after intravenous administration of TRH to 24 adult horses. Plasma ACTH (pg/mL) in samples collected before TRH administration (0, pre), and at 9 minutes (early), 10 minutes (standard), and 11 minutes (late) after TRH injection. The 10-minute value shown is the average of the paired aliquots taken from the same sample (10-minute standard). The diagnostic interpretation thresholds are represented by dotted lines, corresponding with equivocal PPID status (110-200 pg/mL) and positive PPID status (>200 pg/mL). The Y axis is broken to include the maximum values measured in a subpopulation of tested horses



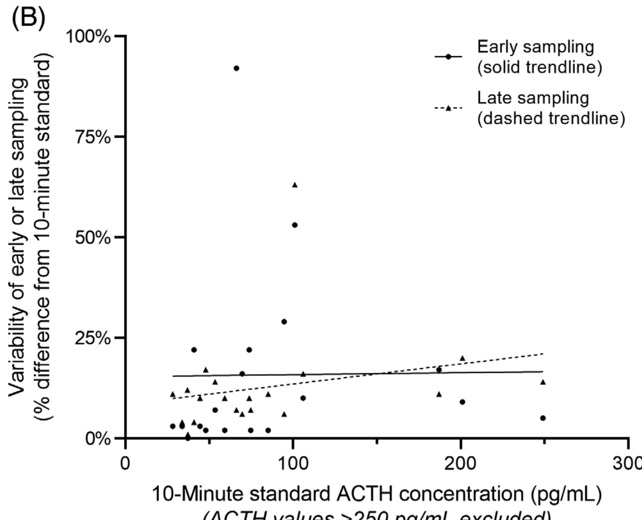
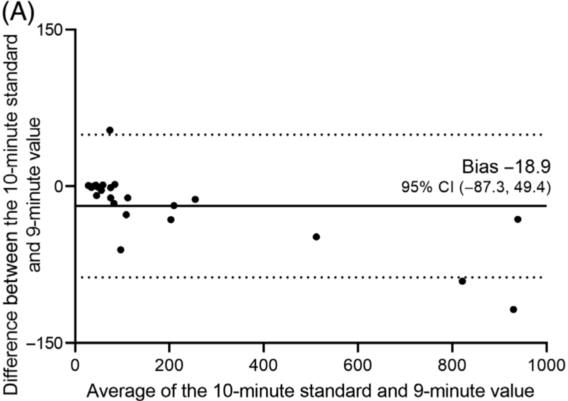
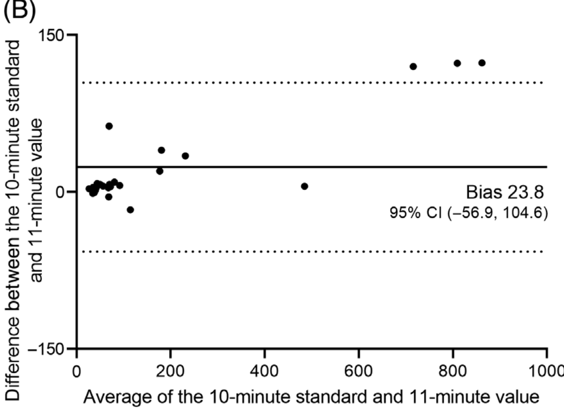


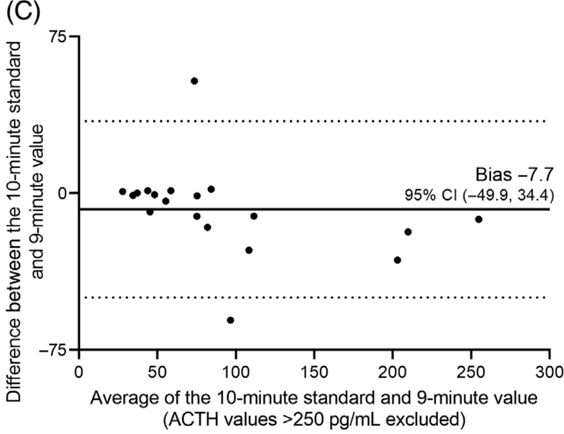
FIGURE 2 Correlation between 10-minute standard ACTH concentration and magnitude of variability observed with early or late sampling during a TRH stimulation test in 24 adult horses. Variability is reported as percentage difference in the measured value compared to the paired 10-minute standard value for each collected sample. Early sampling values are represented by circles (solid trendline); late sampling values are represented by triangles (dashed trendline). Analysis of all data is included in (A); results from horses with 10-minute standard ACTH concentration >250 are excluded in (B)

(median 3%; range, 0%-6%). Greater variability was noted when comparing values from 9 or 11 minutes to the 10-minute standard. Early sampling yielded a median variability of 9% (range, 0%-92%) and late sampling yielded a median variability of 11% (range, 1%-63%). The likelihood of observing ≥10% variation from the 10-minute standard was not statistically different for early or late sampling ($P = .24$; 1 horse excluded as only 10- and 11-minute samples were obtained). A Wilcoxon Signed-Ranks test indicated that the magnitude of variability did not differ significantly between 9- and 11-minute sampling times ($P = .31$; 1 horse excluded as only 10- and 11-minute samples were obtained). A majority of horses (75%, 18/24) had at least 1 early or late value that differed by ≥10% from the 10-minute standard and 25% of horses (6/24) had both early and late values that were ≥10% different from the 10-minute standard. Over half of individual early or late samples (51%, 24/47) differed by ≥10% from their respective 10-minute standard.

Assessment of the effect of horses' PPID status on the frequency of variability with early or late sampling indicated that incidence of at least 1 episode of ≥10% variability with early or late sampling was independent of PPID status ($P = .59$). For the 18 horses with at least 1 episode of ≥10% variability with early or late sampling, the 10-minute standard ACTH concentration was positive for PPID (>200 pg/mL) in 5 (28%), equivocal (110-200 pg/mL) in 1 (6%) and negative (<110 pg/mL) in 12 (67%). Of the 6 horses with <10% variability with both early and late sampling, the 10-minute standard ACTH concentration was positive for PPID status in 1 (17%) and negative in 5 (83%). No association was observed between subject age and frequency of ≥10% variability with early or late sampling; horses were equally divided into cohorts below and above the age of 10 years (12 horses in each group), and within each group, an equal fraction (9/12, 75%) of subjects exhibited 1 or more instances of ≥10% variability with early or late sampling ($P = 1$). Similarly, neither month of the year (December vs January) nor sampling period







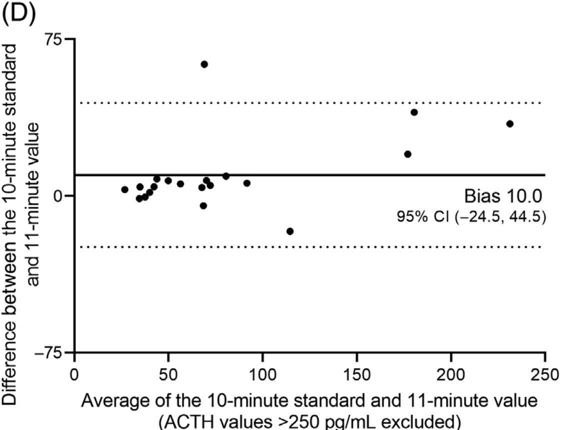


FIGURE 3 Legend on next page.

(December 2019-January 2020 vs December 2020) was associated with frequency of ≥10% variability with early or late sampling ($P = 1$).

There was no significant correlation between the 10-minute standard ACTH concentration and the magnitude of variability associated with early or late sampling when evaluating either the full data set (early sampling, $P = .62$; late sampling, $P = .88$); Figure 2A), or when excluding horses with ACTH concentration exceeding 250 pg/mL (early sampling, $P = .96$; late sampling, $P = .31$); Figure 2B).

Bland-Altman plots were used to demonstrate that early sampling yields a higher ACTH concentration (bias −18.9 pg/mL) whereas late sampling yields a lower ACTH concentration (bias +23.8 pg/mL) when evaluating all data (Figure 3A,B). The direction, though not magnitude, of these observed biases is maintained when limiting analysis to horses with 10-minute standard ACTH concentration <250 pg/mL (Figure 3C,D). For the limited data subset, the bias for early sampling was −7.72 pg/mL and the bias for late sampling was +10.0 pg/mL.

4 | DISCUSSION

The present study demonstrates that small alterations in sample collection timing (1 minute early or late) significantly alters plasma ACTH concentration in a majority of horses undergoing a TRH stimulation test. Seventy-five percent of horses in this study had at least 1 early or late sample result that differed by ≥10% from the 10-minute standard, and in 25% of horses, both early and late values differed by ≥10% from the 10-minute standard. Additionally, 21% of horses (5/24) in this study with ACTH concentrations near the established threshold between equivocal and PPID status would have different clinical test interpretation associated with early or late sample collection.

Rapid change in plasma ACTH concentration after TRH administration has been observed in all previous studies,[13-15] and we have demonstrated here that a difference of 1 minute in sampling time can have a significant effect on the measured ACTH concentration. Natural variation in basal ACTH concentration has been reported for samples taken minutes apart;[18] however, TRH stimulation is intended to induce a maximal pituitary response and is thereby expected to overwhelm any small-scale variations in measured ACTH concentration.[19]

The precise timing of the peak ACTH concentration differs among studies, and varies by individual horse and disease state, but is

FIGURE 3 Bland-Altman plots showing of the effect of sample collection timing on plasma ACTH concentration during a TRH stimulation test in 24 adult horses. Calculated bias is shown on all graphs as a solid line; the dotted lines mark the limits of each 95% confidence interval (CI). Comparison of 10-minute standard plasma ACTH value to early (A) and late (B) sampling values with all data included. Data from horses with 10-minute standard ACTH concentration >250 pg/mL were excluded and analysis repeated for comparison of the 10-minute standard to early (C) and late (D) sampling to provide a more conservative estimate of bias

consistently reported to occur <15 minutes after TRH administration.[9,13,20] Increased frequency of sampling does not improve diagnostic accuracy when evaluating baseline ACTH concentration[18,21] or repeated TRH stimulation tests in previous studies. Establishing universal timing of peak ACTH concentration in response to TRH stimulation is likely impossible because of differences in drug metabolism and individual horse responses, but minimization of the effects of confounders such as the timing of sample collection is critical for accurate test interpretation.

Effects of single-minute differences in sample collection times on test interpretation have not been evaluated in previous studies; results presented here demonstrate that minor changes in the timing of blood collection can have significant effects on test results and diagnostic interpretation. In this study, observed intra-assay variability was minor (median 3%, ≤6% overall) and this compares to the intra-assay variability ranging from 3.2% to 12.9% (mean 9.3%) reported in the initial validation of the chemiluminescent immunoassay for equine ACTH.[17] In our study, the paired analyses were performed on replicate aliquots taken from a single blood sample to minimize variability associated with blood collection, yet small differences in aliquot handling before analysis could have contributed to this minor variability. Reported effects of sample handling on measured plasma ACTH concentrations are inconsistent, ranging from no significant effect[22] to marked variability[23] in ACTH concentration after prolonged time at room temperature or alteration of centrifugation methods. Additionally, minimal change in ACTH from baseline concentration (<6%) is reported with appropriate long-term sample storage conditions (frozen at −20 or −80°C) for 30 to 60 days.[16]

In our study, all sample processing was performed by the same investigator using a standardized method, and the 2 10-minute sample aliquots were processed simultaneously to minimize variability arising from differences in sample handling. However, blood samples collected early during the testing window experienced a 1 to 2 hour delay in centrifugation compared to samples collected at the end of the testing window for each day. All samples were kept chilled or refrigerated before and during processing, which occurred within 6 hours of collection. Samples were stored frozen at −20°C before they were shipped on dry ice to the laboratory, undergoing a single freeze-thaw cycle before analysis, thus minimizing the effect of storage and handling conditions on ACTH concentration variability.

Lack of concordance has been reported between the different assay types used for measuring ACTH concentrations.[24-27] All samples for this study were analyzed using a validated chemiluminescent immunometric assay[17] performed by a commercial veterinary laboratory utilizing routine quality control assessments. Though no significant differences have been noted in equine ACTH concentrations from glass sample tubes rather than plastic ones,[17] all samples in this study were collected into plastic tubes containing EDTA from the same manufacturing lot to eliminate this potential source of error.

A commercially available, shelf-stable, compounded TRH was used, reflecting the TRH stimulation test performed by field practitioners. The TRH dose used in this study (1 mg/horse) follows the current recommendations[11] as well as the historic development of this testing modality.[5,9,13] Horses were maintained in their normal social groups to minimize stress, and had their usual access to forage-based feed throughout the duration of the study, as fasted vs fed state has been shown to have an effect on baseline and post-TRH-stimulation ACTH concentration.[28,29] Much work has been done to describe the significant effects of season, time of day, and photoperiod on ACTH concentration in horses across the globe.[15,28,30-35] Accordingly, all sampling was performed during the morning hours (8 AM-11 AM) of the winter months (December and January) to align with current testing recommendations and minimize seasonal effects.[11] Testing was performed in 2 groups of horses from the same facility approximately 12 months apart because of limitations placed on the study by the COVID-19 pandemic, but results were comparable at the different sampling periods.

Bland-Altman plots illustrated that ACTH concentration decreased with increasing time after TRH stimulation, identifying a bias of −18.9 pg/mL with early sampling (9 min), and a bias of +23.8 pg/mL with late sampling (11 min). Thus, sampling 1 minute early yielded an average ACTH concentration approximately 19 pg/mL higher than the value obtained at 10 minutes, while sampling 1 minute late resulted in an average ACTH concentration approximately 24 pg/mL lower than the 10-minute value. To further clarify this bias, data from horses with markedly increased ACTH concentrations as a result of TRH stimulation (>250 pg/mL) were excluded and the Bland-Altman analyses were repeated, which revealed the same directionality, but smaller magnitudes of bias (−7.72 pg/mL with early sampling and +10.0 pg/mL with late sampling). These biases would not be expected to affect test interpretation in horses with advanced PPID and markedly increased ACTH concentrations, but this is not the population of horses that typically undergoes TRH stimulation testing. This test is typically selected for horses with early PPID and the bias we report is more concerning when interpreting test results that approach the established thresholds or fall within equivocal test status.

Most horses evaluated in this study had ACTH values that were consistently positive or negative for PPID status despite significant variability associated with sample collection timing. However, 5 (21%) of the subjects had ACTH concentrations close to the threshold for equivocal (110 pg/mL) or positive PPID status (200 pg/mL). Even the more conservative bias values approach 10% of the lower diagnostic threshold value for this test, and in the aforementioned 5 horses, early or late sampling would have caused a change in test interpretation between equivocal and positive results or normal and equivocal results. Sixty percent (3/5) of these horses were among the group that exhibited ≥10% variability in both early and late sampling values. In a previous study evaluating effects of sample processing on ACTH concentrations, there was sufficient variability to change the PPID diagnosis in 30% of horses.[36] Veterinarians must be cognizant of these sources of variability, which could affect accurate diagnosis of PPID and impact clinical decision making surrounding cost of care and quality of life associated with lifetime management of this disease in the horse.

The main limitation of this study is a small sample size and the limited number of horses within this group with early PPID. The age

of the study cohort does not fully reflect the most typical age of horses that would undergo this type of diagnostic testing, though 41% of subjects (10/24) had at least 1 ACTH measurement consistent with equivocal or positive PPID status. Furthermore, we had no histopathologic confirmation of disease state because this can only be obtained through postmortem examination. No horses in the study had a history of PPID or obvious clinical signs of the disease; however, analysis of baseline ACTH concentration alone would have been equivocal or positive for PPID in a small subset of study participants (8%, 2/24). A TRH stimulation test would not typically be performed on those horses, and indeed the TRH response in these 2 horses was profound (>800 pg/mL).

Later blood sample collection at 30 minutes after TRH injection, as described in other protocols,[1,10,37] could attenuate the effect of very small (single-minute) alterations in sample collection timing as ACTH concentrations might be changing at a slower rate at this time. However, this cannot be assumed, and additional research is needed to determine the effects of differences in sample collection timing on ACTH concentrations measured at the 30-minute sampling point. It might also be possible to identify alternate sampling points that balance consistent test performance, test practicality for practitioners, and minimization of variability associated with minor alterations in sample collection timing.

The TRH stimulation test is recommended to elicit a diagnosis in horses with early PPID, when clinical signs can be absent or subtle and baseline ACTH concentration is within reference interval.[34,38] Recent work suggests that performing a TRH stimulation test on horses with an equivocal basal ACTH concentration is merited to enhance diagnosis of PPID.[10] We have demonstrated here that the timing of sample collection affects TRH stimulation test results, and values that fall close to diagnostic thresholds should be viewed critically and in conjunction with the patient's risk factors, medical history, and clinical signs. Development of season-specific test interpretation addresses 1 significant area of known variation of basal ACTH concentration, and the recommendations should extend to interpretation of TRH stimulation testing results to optimize testing utility.[11,37] The effect of early or late sample collection during the TRH stimulation test can be expected to compound the known areas of testing variability (season, photoperiod, assay, and horse-specific factors) and hinder accurate diagnosis of PPID.

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CONFLICT OF INTEREST DECLARATION

Dr Frank consults on study design and the diagnosis and treatment of pituitary pars intermedia dysfunction in horses for Boehringer Ingelheim, which provided funds to support this study.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the University of Massachusetts IACUC, protocol #2019-0033).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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REFERENCES

1. Spelta CW. Equine pituitary pars intermedia dysfunction: current perspectives on diagnosis and management. *Vet Med (Auckl)*. 2015;6:293-300.
2. Schott HC 2nd. Pituitary pars intermedia dysfunction: equine Cushing's disease. *Vet Clin North Am Equine Pract*. 2002;18:237-270.
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.
4. 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.
5. Frank N, Andrews FM, Sommardahl CS, Eiler H, Rohrbach BW, Donnell RL. 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.
6. Goodale L, Frank N, Hermida P, D'Oench S. Evaluation of a thyrotropin-releasing hormone solution stored at room temperature for pituitary pars intermedia dysfunction testing in horses. *Am J Vet Res*. 2015;76:437-444.
7. Hodge E, Kowalski A, Torcivia C, et al. Effect of thyrotropin-releasing hormone stimulation testing on the oral sugar test in horses when performed as a combined protocol. *J Vet Intern Med*. 2019;33:2272-2279.
8. Horn R, Bertin FR. Evaluation of combined testing to simultaneously diagnose pituitary pars intermedia dysfunction and insulin dysregulation in horses. *J Vet Intern Med*. 2019;33:2249-2256.
9. Beech J, Boston R, Lindborg S. Comparison of cortisol and ACTH responses after administration of thyrotropin releasing hormone in normal horses and those with pituitary pars intermedia dysfunction. *J Vet Intern Med*. 2011;25:1431-1438.
10. Horn R, Stewart AJ, Jackson KV, Dryburgh EL, Medina-Torres CE, Bertin FR. Clinical implications of using adrenocorticotropic hormone diagnostic cutoffs or reference intervals to diagnose pituitary pars intermedia dysfunction in mature horses. *J Vet Intern Med*. 2021;35:560-570.
11. Schott HC, 2nd, Andrews FM, Durham AE, et al. *Recommendations for the Diagnosis and Treatment of Pituitary Pars Intermedia Dysfunction (PPID)* [Internet]. Equine Endocrinology Group; 2019 [cited 2021]. Available from: https://sites.tufts.edu/equineendogroup/
12. McFarlane D, Beech J, Cribb A. Alpha-melanocyte stimulating hormone release in response to thyrotropin releasing hormone in healthy horses, horses with pituitary pars intermedia dysfunction and equine pars intermedia explants. *Domest Anim Endocrinol*. 2006;30:276-288.
13. 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.

14. Beech J, McFarlane D, Lindborg S, Sojka JE, Boston RC. α-Melanocyte—stimulating hormone and adrenocorticotropin concentrations in response to thyrotropin-releasing hormone and comparison with adrenocorticotropin concentration after domperidone administration in healthy horses and horses with pituitary pars intermedia dysfunction. *J Am Vet Med Assoc*. 2011;238:1305-1315.
15. Funk RA, Stewart AJ, Wooldridge AA, et al. Seasonal changes in plasma adrenocorticotropic hormone and alpha-melanocyte-stimulating hormone in response to thyrotropin-releasing hormone in normal, aged horses. *J Vet Intern Med*. 2011;25:579-585.
16. Haffner JC, Neal DL, Hoffman RM, Grubbs ST. Plasma adrenocorticotropic hormone concentration in horses decreases after freezing for 60 days. *J Vet Diagn Invest*. 2019;31:856-858.
17. Perkins GA, Lamb S, Erb HN, Schanbacher B, Nydam DV, Divers TJ. 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.
18. Rendle DI, Duz M, Beech J, Parkin T, Durham AE. Investigation of single and paired measurements of adrenocorticotropic hormone for the diagnosis of pituitary pars intermedia dysfunction in horses. *J Vet Intern Med*. 2015;29:355-361.
19. Rendle DI. Laboratory diagnosis of the endocrine causes of laminitis. *Livestock*. 2017;22(4):216-220. https://doi.org/10.12968/live.2017.22.4.216
20. Beech J, Boston RC, McFarlane D, Lindborg S. Evaluation of plasma ACTH, alpha-melanocyte-stimulating hormone, and insulin concentrations during various photoperiods in clinically normal horses and ponies and those with pituitary pars intermedia dysfunction. *J Am Vet Med Assoc*. 2009;235:715-722.
21. Rendle DI, Litchfield E, Heller J, Hughes KJ. Investigation of rhythms of secretion and repeatability of plasma adrenocorticotropic hormone concentrations in healthy horses and horses with pituitary pars intermedia dysfunction. *Equine Vet J*. 2014;46:113-117.
22. Shepard KN, Haffner JC, Neal DL, Grubbs ST, Pearce GL. Effect of delayed plasma centrifugation on equine adrenocorticotropic hormone concentration. *J Vet Diagn Invest*. 2019;31:585-587.
23. Rendle DI, Litchfield E, Gough S, Cowling A, Hughes KJ. The effects of sample handling and N-phenylmaleimide on concentration of adrenocorticotrophic hormone in equine plasma. *Equine Vet J*. 2015;47:587-591.
24. Irvine KL, Burt K, Hill AJ, Shaw S, Papasouliotis K. Initial analytic quality assessment and method comparison of an immunoassay for adrenocorticotropic hormone measurement in equine samples. *Vet Clin Pathol*. 2016;45:154-163.
25. Banse HE, Schultz N, McCue M, Geor R, McFarlane D. Comparison of two methods for measurement of equine adrenocorticotropin. *J Vet Diagn Invest*. 2018;30:233-237.
26. McGilvray TA, Knowles EJ, Harris PA, et al. Comparison of immunofluorescence and chemiluminescence assays for measuring ACTH in equine plasma. *Equine Vet J*. 2020;52:709-714.
27. Knowles EJ, Moreton-Clack MC, Shaw S, Harris PA, Elliott J, Menzies-Gow NJ. 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.
28. Diez de Castro E, Lopez I, Cortes B, Pineda C, Garfia B, Aguilera-Tejero E. 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.
29. Restifo MM, Frank N, Hermida P, Sanchez-Londoño A. Effects of withholding feed on thyrotropin-releasing hormone stimulation test results and effects of combined testing on oral sugar test and thyrotropin-releasing hormone stimulation test results in horses. *Am J Vet Res*. 2016;77:738-748.
30. Copas VE, 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.
31. 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.
32. Frank N, Elliott SB, Chameroy KA, Tóth F, Chumbler NS, McClamroch R. 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.
33. 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.
34. McFarlane D. Diagnostic testing for equine endocrine diseases: confirmation versus confusion. *Vet Clin North Am Equine Pract*. 2019;35:327-338.
35. Secombe CJ, Tan RHH, Perara DI, Byrne DP, Watts SP, Wearn JG. The effect of geographic location on circannual adrenocorticotropic hormone plasma concentrations in horses in Australia. *J Vet Intern Med*. 2017;31:1533-1540.
36. Prutton JS, Kass PH, Watson JL, et al. Pre-analytical stability of adrenocorticotrophic hormone from healthy horses in whole blood, plasma and frozen plasma samples. *Vet J*. 2015;204:123-124.
37. Kam YN, McKenzie K, Coyle M, Bertin FR. Repeatability of a thyrotropin-releasing hormone stimulation test for diagnosis of pituitary pars intermedia dysfunction in mature horses. *J Vet Intern Med*. 2021;35:2885-2890.
38. Frank N. Endocrine disorders of the equine athlete. *Vet Clin North Am Equine Pract*. 2018;34:299-312.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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