Repeatability of a thyrotropin-releasing hormone stimulation test for diagnosis of pituitary pars intermedia dysfunction in mature horses

Yan Ning Kam | Kelly McKenzie | Mitchell Coyle | François-René Bertin

School of Veterinary Science, The University of Queensland, Gatton, Queensland, Australia
Office of the Director of Gatton Campus, The University of Queensland, Gatton, Queensland, Australia

Correspondence
François-René Bertin, School of Veterinary Science, The University of Queensland, Gatton, Queensland, Australia. Email: f.bertin@uq.edu.au

Abstract

Background: Pituitary pars intermedia dysfunction (PPID) is a common endocrinopathy of horses diagnosed with a thyrotropin-releasing hormone (TRH) stimulation test.

Hypothesis/Objectives: Describe the repeatability of TRH stimulation in horses with and without PPID in winter and autumn.

Animals: Twenty adult horses; 6 controls and 6 with PPID tested in autumn, 8 controls and 6 with PPID tested in winter with 3 controls and 3 with PPID tested in both seasons.

Methods: Thyrotropin-releasing hormone stimulation was performed on 2 consecutive occasions, 1 week before and 1 week after the winter solstice and the autumn equinox. Blood was collected before and 30 minutes after IV injection of 1 mg of TRH. ACTH concentration was determined by a chemiluminescent assay. Repeatability and test-retest reliability were assessed by repeated measures analysis of variance, intraclass correlation coefficient and within-horse coefficients of variation (CV). Bland-Altman plots were generated to visualize agreement between repetitions.

Results: In winter, no week effect was detected on the results of the TRH simulation and the test had an excellent test-retest reliability. In autumn, after-TRH ACTH concentrations were significantly lower on week 2 ($P = .02$) and the test only had a good test-retest reliability. There were significantly larger within-horse CV during autumn ($P = .04$) and after TRH stimulation ($P = .04$). There were 2 misclassifications in winter and 4 in autumn.

Conclusions and Clinical Importance: The TRH stimulation test was repeatable when performed 2 weeks apart in winter; however, in autumn, more variability in after-TRH ACTH concentrations resulted in decreased repeatability.

KEYWORDS

ACTH, diagnostic test, endocrinology, equine, hormone, hypothalamo-pituitary-adrenal axis, laminitis

Abbreviations: ANOVA, analysis of variance; CI, confidence interval; CV, coefficient of variation; ICC, intraclass correlation coefficient; POMC, pro-opiomelanocortin; PPID, pituitary pars intermedia dysfunction; TRH, thyrotropin-releasing hormone.

Received: 5 January 2021 | Accepted: 1 October 2021
DOI: 10.1111/jvim.16281
1 | INTRODUCTION

Pituitary pars intermedia dysfunction (PPID) is a common, slowly progressive endocrinopathy that affects about 20% of horses aged 15 years and older.1,2 The pathophysiology of this disorder remains incompletely elucidated but the current understanding is that the disease would be attributable to an oxidative stress-induced degeneration of the dopaminergic neurons located in the hypothalamus.3 Physiologically, those neurons have an inhibitory effect on the pars intermedia of the pituitary gland; therefore, when they degenerate, hormone-secreting adenomas or hyperplasia of the pars intermedia develop.3

Horses with PPID frequently manifest overt clinical signs, including hypertrichosis, pendulous abdomen, muscle atrophy, recurrent laminitis, lethargy, increased susceptibility to infection, polyuria, polydipsia, and abnormal sweating patterns.4 As such, the diagnosis of PPID can often be made on the basis of characteristic clinical signs.4 When clinical signs are not obvious, a laboratory diagnostic test is required and measurement of ACTH concentration is often used to establish a diagnosis.5 Baseline ACTH concentration is considered as an excellent test to diagnose PPID with an overall test accuracy above 90%, a median sensitivity above 75% and a median specificity above 75%; however, in autumn, test characteristics decline reducing its diagnostic value.5-7 Considering that baseline ACTH concentrations fluctuate with seasonal changes, stress, exercise and disease status, the thyrotropin-releasing hormone (TRH) stimulation test was developed to standardize ACTH concentrations and improve diagnosis.8-14 The results of TRH stimulation tests vary with environmental factors, the test still performs better than baseline ACTH with a greater accuracy, sensitivity, and specificity.6

The relevance of the TRH stimulation test for monitoring response to treatment is unknown as there are conflicting results about an effect of pergolide on the plasma ACTH concentration after TRH administration.15,16 Season and diet are 2 factors that could confound the plasma ACTH concentration after a TRH stimulation test, with higher plasma ACTH concentrations in autumn and after starch-rich diets.17 There is no information on whether the TRH stimulation, without other variables, is a repeatable diagnostic test and would therefore be of clinical interest in the follow-up of cases suspected or treated for PPID. The objective of this study was therefore to describe the repeatability of the TRH stimulation test in horses with and without PPID in winter and in autumn.

2 | MATERIALS AND METHODS

2.1 | Study sample

A total of 20 horses from the institutional herd (located at a latitude of 27.5571° S) were selected for this study. Horses were classified as controls or PPID based on clinical signs (present and historical) and results of the first TRH stimulation test. Clinical signs consistent with PPID included hypertrichosis or delayed shedding, epiaxial muscle wastage, abnormal fat distribution or abnormal sweating.4 In winter, laboratory findings consistent with PPID were both an immunoreactive plasma ACTH concentrations above 24.0 pg/mL at baseline and above 53.2 pg/mL after TRH administration while, in autumn, laboratory findings consistent with PPID were both an immunoreactive plasma ACTH concentrations above 58.3 pg/mL at baseline and above 229.1 pg/mL after TRH administration.6 In case of discrepancy between clinical signs and ACTH concentrations (baseline or after TRH administration), the horse was excluded from the study.

Beyond the clinical signs of PPID, physical exam findings indicated that the horses were healthy. Horses were kept on the same paddock, had not traveled in the month before enrollment in the study, were fed the same hay on the same pasture and had received the same preventative medicine program. All procedures were approved by the institutional animal ethics committee.

The winter solstice group comprised of 8 control horses (14 [11-23] years; 5 geldings and 3 mares; Body condition score (BCS): 6 [3-8]/9) and 6 horses with PPID (17 [12-21] years; 5 geldings and 1 mare; BCS: 7 [4-8]/9). The winter study sample consisted of Australian Stock Horses (n = 5), Standardbreds (n = 4), Thoroughbreds (n = 3), Arabian Horse (n = 1) and Warmblood (n = 1). The autumn equinox group consisted of 6 control horses (14 [9-18] years; 4 geldings and 2 mares; BCS: 5 [4-8]/9) and 6 horses with PPID (14 [12-21] years; 6 geldings; BCS: 7 [4-8]/9). The autumn study sample comprised of Standardbreds (n = 5), Australian Stock Horses (n = 4), Warmblood (n = 1), Thoroughbred (n = 1) and Quarter Horse (n = 1). Only 6 horses were included in both the winter solstice and the autumn equinox testing times as some horses previously enrolled in the study had traveled to a different location with a different diet or had received treatment making them unavailable for the current study.

2.2 | Study design

A TRH stimulation test was performed on the horses over 2 study periods, 7 days before and 7 days after the winter solstice (winter week 1 and winter week 2) and 8 days before and 8 days after the autumn equinox (autumn week 1 and autumn week 2) resulting in 4 instances of TRH stimulation test.

2.3 | TRH stimulation test

Fifty milligrams of synthetic TRH acetate salt (Sigma-Aldrich Co, Castle Hill, Australia) were reconstituted with sterile water for injection by a sterile technique under a biosafety cabinet to produce 1 mg/mL solution. One-milliliter aliquots were stored in sterile 3-mL syringes at −20°C until use. The TRH stimulation test was conducted in the field and 10-mL blood samples were collected before and 30 minutes after IV injection of 1 mg of TRH (after TRH stimulation sample) as previously described.18 Samples were collected in chilled Ethylenediaminetetraacetic acid tubes, stored with ice packs, centrifuged and analyzed within 4 hours of collection.
2.4 | ACTH assay

Baseline and after TRH stimulation immunoreactive plasma ACTH concentrations were measured in the institution laboratory by a chemiluminescent assay (IMMULITE 1000 Immunoassay System, Siemens Healthcare Pty Ltd, Bayswater, Australia), validated for use in horses with an intra- and interassay variability of 5.4% and 4.8%, respectively.\(^19\)

2.5 | Statistical analysis

Normal distribution was evaluated with a Shapiro-Wilk test; normally distributed data are presented as mean ± SD (ACTH concentrations) and nonnormally distributed data are presented as median and 95% confidence interval (CI) (age and BCS). Immunoreactive plasma ACTH concentrations were categorized by season (winter and autumn) and the effect of TRH stimulation and time (week 1 and week 2) were analyzed by a 2-way repeated measures analysis of variance (ANOVA) in the overall study sample and then in control horses and in horses with PPID separately. Considering the variability in immunoreactive plasma ACTH concentrations and the low number of horses included in this study, the test-retest reliability of the TRH stimulation test was determined by the intraclass correlation coefficient (ICC) and 95% CI which determines the agreement between the 2 instances the TRH stimulation test was performed during the same season. The within-horse coefficient of variation (CV) was then calculated for each season between immunoreactive plasma ACTH concentrations obtained from each horse on week 1 and week 2, and the effects of TRH stimulation, season and PPID status on CV were evaluated with a 3-way repeated measures ANOVA. Finally, Bland-Altman plots were created to assess the disparity between the weeks (bias) and visualize the agreement between repetitions. A *P*-value of <.05 was considered significant. Data analysis was conducted by statistical software (Prism 8.0.1 for Mac OS X, GraphPad Software Inc, La Jolla, San Jose, California, and IBM SPSS Statistics Version 25, Sydney, NSW, Australia).

3 | RESULTS

In the winter overall sample, there was significant effect of TRH administration on immunoreactive plasma ACTH concentration \((P = .003)\), but no significant effect of week was detected \((P = .6)\), Figure 1. Two horses classified as PPID on week 1 had immunoreactive plasma ACTH concentrations below the diagnostic cut-off values on week 2. The ICC was .97 (.9-.99), indicating excellent reliability. In the autumn overall sample, there was a significant effect of both TRH stimulation \((P = .02)\) and week \((P = .004)\) on immunoreactive plasma ACTH concentrations with a significantly lower after TRH immunoreactive plasma ACTH concentration on week 2 \((P = .02)\), Figure 1. Four horses classified as PPID on week 1 had immunoreactive plasma ACTH concentrations below the diagnostic cut-off values on week 2. Those 4 horses were different from the 2 misclassified in winter. The ICC was .97 (.9-.99), indicating excellent reliability.

In the winter control sample, there was a significant effect of TRH administration on immunoreactive plasma ACTH concentration \((P = .002)\), but no significant effect of week was detected \((P = .03)\). The ICC was .92 (.62-.99), indicating excellent reliability. In the autumn control sample, a significant effect of both TRH administration \((P = .02)\) and week \((P = .04)\) was detected on immunoreactive plasma ACTH concentration; however, no post hoc comparison reached statistical significance. The ICC was .87 (.58-.98), indicating good reliability.

In the winter PPID group, there was a significant effect of TRH administration on immunoreactive plasma ACTH concentration \((P = .03)\), but no significant effect of week was detected \((P = .9)\). The ICC was .94 (.57-.99), indicating excellent reliability. In the autumn PPID group, no effect of TRH administration was detected \((P = .06)\) but a significant effect of week on immunoreactive plasma ACTH concentration was detected \((P = .02)\); however, no post hoc comparison reached statistical significance. The ICC was .97 (.8-.99), indicating excellent reliability.

The median within-horse CV in immunoreactive plasma ACTH concentrations was 15.3 (10-27.8) and within-horse CV were significantly higher in autumn \((P = .04)\) and after a TRH stimulation \((P = .04)\) but there was no significant effect of PPID status \((P = .9)\), Figure 2.

The Bland-Altman plots showed a good agreement between week 1 and week 2 in immunoreactive plasma ACTH concentrations after TRH stimulation among the overall study sample in winter; however, in autumn, the agreement was limited. The mean bias for week 1 vs week 2 immunoreactive plasma ACTH concentrations after TRH stimulation in winter was 2.6 pg/mL (95% limit of agreement: −25.2 to
30.4, Figure 3A); whereas the mean bias for week 1 vs week 2 immunoreactive plasma ACTH concentrations after TRH stimulation in autumn was 65.8 pg/mL (95% limit of agreement 91.1 to 222.6, Figure 3B).

4 | DISCUSSION

The results of our study indicate that the TRH stimulation test is repeatable test when performed 2 weeks apart in winter; however, in autumn, more variability in immunoreactive plasma ACTH concentrations after TRH administration results in clinically relevant decreased repeatability leading to misclassification with the cut-offs used in this study.

The TRH stimulation test resulted in a significant increase in immunoreactive plasma ACTH concentrations in all horses and all seasons except for PPID horses in the autumn. TRH directly stimulates TRH receptors on melanotropes in the pars intermedia of the pituitary gland to release stored pro-opiomelanocortin (POMC), which is then converted into ACTH and other peptides by prohormone convertases. In control horses, as ACTH almost exclusively originates from the pars distalis, the increase in immunoreactive plasma ACTH concentration after TRH stimulation is mild whereas in horses with PPID, as large amounts of ACTH are secreted from the hyperplastic pars intermedia, the increase in immunoreactive plasma ACTH concentration after TRH stimulation is larger. The absence of statistically significant increase in ACTH concentration in PPID cases tested in autumn could be because of the small number of PPID horses included in this study and the larger variability in ACTH concentration observed at that season (121.4 ± 66.8 vs 393.3 ± 331.4 pg/mL on week 1 and 107.4 ± 80 vs 292.7 ± 267.5 pg/mL on week 2). With this variability between cases, to achieve an α error of .05 and a power of .8, the number of horses required per group would be 3479 were there in fact a difference.

In winter, immunoreactive plasma ACTH concentrations obtained after the TRH simulation test did not significantly differ between weeks, had an excellent test-retest reliability, and had a mean bias within the intra- and interassay variability. Furthermore, the proportional bias observed in the Bland-Altman plots with smaller differences with small immunoreactive ACTH concentrations suggests that the 95% limits of agreement probably underestimate the level of agreement between tests in winter. Taken together, these results indicate that in winter, the TRH stimulation test is repeatable, and the differences observed in this study could be because of assay variability. Therefore, in a clinical context, this excellent repeatability would indicate that performing a TRH stimulation test in winter could be

FIGURE 2 Within-horse coefficient of variation (CV) for immunoreactive plasma ACTH concentrations (baseline and after thyrotropin-releasing hormone (TRH) stimulation) in winter and autumn for control and pituitary pars intermedia dysfunction (PPID). Black and gray dots represent within-horse CV for baseline and after TRH immunoreactive plasma ACTH concentrations, respectively. *P < .05 between groups

FIGURE 3 Bland–Altman plots of the differences in after thyrotropin releasing hormone (TRH) stimulation immunoreactive plasma ACTH concentrations in winter (A) and autumn (B). The solid line represents the bias whereas the dotted lines represent the upper and lower 95% limits of agreement
The potential reasons for this excellent repeatability could be that winter is considered as a quiescent phase for the hypothalamo-pituitary axis during which ACTH concentrations have less variability. Two horses classified as PPID on week 1 had immunoreactive plasma ACTH concentrations below the diagnostic cut-off values on week 2. Although those horses had clinical signs consistent with PPID (delayed shedding and hypertrichosis) and baseline immunoreactive plasma ACTH above diagnostic cut-off values, their result after TRH stimulation were only mildly above the diagnostic cut-off values on week 1 (64.9 and 53.4 pg/mL). In those cases, a small decrease in immunoreactive plasma ACTH after TRH stimulation on week 2 resulted in a misclassification (40.2 and 43.4 pg/mL, respectively). Given that diet was kept consistent between the 2 testing instances, a possible explanation for this not statistically significant yet clinically relevant misclassification is the low diagnostic cut-off values used in this study. Because the TRH stimulation test only induces the release of ACTH stored in the melanotropes rather than de novo synthesis, another explanation for this reduced response to TRH on week 2 could be the short time between the 2 tests. Repeating a TRH stimulation test within 24 hours would yield different results as ACTH stores are depleted. This explanation was also considered unlikely in winter as the tests were performed 2 weeks apart and the decreases in immunoreactive plasma ACTH concentrations after TRH stimulation not consistent between horses.

In autumn, immunoreactive plasma ACTH concentrations obtained after TRH simulation were significantly lower on week 2 in all groups, only had a good test-retest reliability and had a mean bias larger than to the intratri- and interassay variability. Also, the proportional bias observed in the Bland-Altman plots with larger differences with large immunoreactive ACTH concentrations suggests that the 95% limits of agreement probably overestimate the level of agreement between tests in autumn. Taken together, these results indicate that in autumn, the TRH stimulation test is not as repeatable as in winter, and the differences observed in this study would not be because of assay variability but to test limitations. These findings are in agreement with recent studies which consistently reported a higher variability and wider CIs for immunoreactive plasma ACTH concentrations measured 10 or 30 minutes after TRH stimulation in autumn. This variability has been explained by a dynamic phase of the hypothalamo-pituitary adrenal axis characterized by physiologically higher ACTH concentrations presumably to prepare for the harsher climatic conditions of winter. In our study, however, the magnitude of the bias and the variations were greater than previously reported. A possible explanation for this discrepancy is the difference in assays used between studies with the radio-immunoassay, commonly used in previous studies, reporting lower immunoreactive ACTH concentrations than chemiluminescent assays. Another explanation could be the physiological effect of season on our results; however, one would expect an increase in immunoreactive plasma ACTH concentrations between week 1 and week 2 rather than the decrease observed in our study. Finally, in absence of changes in diet, a likely explanation is the low number of horses enrolled in our study and the higher number of horses with PPID; however, this number is similar to previous reports. Clinically, this lower repeatability in autumn resulted in 4 horses out of 6 classified as PPID on week 1 having immunoreactive plasma ACTH concentrations below the diagnostic cut-off values on week 2. Similar explanations as above could be used; however, the high specificity of the diagnostic cut-off values of 97.6 (87.4-99.9)% used in this study for a classification as PPID after TRH stimulation test in autumn would limit the risk of false positives on week 1. This suggests that melanotrope ACTH store depletion when TRH stimulation tests are performed 2 weeks apart would be more likely. An example of this hypothesis is the result of 1 of those PPID cases with a immunoreactive plasma ACTH concentrations after TRH administration of 376 pg/mL on week 1 falling below the diagnostic cut-off value at 198 pg/mL on week 2.

The disease status of horses was not associated with higher within-horse CV. This is not consistent with a previous study that reported a high variability of immunoreactive plasma ACTH concentrations among horses with higher immunoreactive ACTH concentrations after TRH stimulation. Our inability to detect an effect of disease status on within-horses CV is likely caused by the main limitation of this study and the small sample size. The study sample consisted of a group of mature full-sized horses of various breeds with a mixture of clinically normal and abnormal horses. On 1 hand, this sample reflects a clinically relevant sample but on the other hand, it increases variability and limits statistical power. To limit other causes of variability, our study was centered on the winter solstice and autumn equinox with data collected over 2 weeks (1 week before and 1 week after the event), limiting the variation in daylight between testing times and corresponding to a comparably lower physiological variation in plasma ACTH concentrations between the start and the end of the experiment, and diets were kept the same between testing periods, to limit possible confounders. Unfortunately, this has reduced our ability to sample as many horses as initially anticipated.

In conclusion, we have demonstrated that, in this small group of horses, the TRH stimulation test is repeatable in both control and PPID horses in winter; however, in autumn, the repeatability of the test, when performed 2 weeks apart, is limited leading to misclassifications. Therefore, repeat testing in autumn might be less reliable when monitoring disease progression or response to treatment.

ACKNOWLEDGMENT
Funding provided by the John and Mary Kibble Trust Fund. This article has been presented in part as a research abstract at the 2020 European College of Equine Internal Medicine (ECEIM) online congress.

CONFLICT OF INTEREST DECLARATION
Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION
Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE OR OTHER APPROVAL DECLARATION
Approved by the University of Queensland Animal Ethics Committee (approval number: 2018/AE000494).
REFERENCES

1. 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.

2. Miller MA, Moore GE, Bertin FR, Kritchkevsy JE. What’s new in old horses? Postmortem diagnoses in mature and aged equids. *Vet Pathol.* 2016;53:390-398.

3. McFarlane D. Equine pituitary pars intermedia dysfunction. *Vet Clin North Am Equine Pract.* 2011;27:93-113.

4. Horn R, Bamford NJ, Aftonos 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. McGowan TW, Pinchbeck GP, McGowan CM. Evaluation of basal plasma alpha-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.

6. Horn R, Stewart AJ, Jackson KV, Dryburgh EL, Medina-Torres CE, Bertin FR. Clinical implications of using adrenocorticotrophic hormone diagnostic cutoffs or reference intervals to diagnose pituitary pars intermedia dysfunction in mature horses. *J Vet Intern Med.* 2021;35:560-570.

7. Tatum RC, McGowan CM, Ireland JL. Evaluation of the sensitivity and specificity of basal plasma adrenocorticotrophic hormone concentrations for diagnosing pituitary pars intermedia dysfunction in horses: a systematic review. *Vet J.* 2021;275:105695.

8. 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.

9. Alexander SL, Irvine CH, Ellis MJ, et al. 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. *Endocrinology.* 1991;128:65-72.

10. 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.

11. 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.

12. Hicks GR, Fraser NS, Bertin FR. Changes associated with the periovulatory period, age and pregnancy in ACTH, cortisol, glucose and insulin concentrations in mares. *Animals.* 2021;11:891-899.

13. Bertin FR, Pader KS, Lescun TB, Sojka-Kritchkevsy JE. Short-term effect of ovariectomy on measures of insulin sensitivity and response to dexamethasone administration in horses. *Am J Vet Res.* 2013;74:1506-1513.

14. Stewart AJ, Hackett E, Bertin FR, Towns TJ. Cortisol and adrenocorticotropic hormone concentrations in horses with systemic inflammatory response syndrome. *J Vet Intern Med.* 2019;33:2257-2266.

15. Miller AB, Loynachan AT, Bush HM, et al. Effects of pituitary pars intermedia dysfunction and Prascend ( pergolide tablets) treatment on endocrine and immune function in horses. *Domest Anim Endocrinol.* 2021;74:106531.

16. McFarlane D, Banse H, Knych HK, Maxwell LK. Pharmacokinetic and pharmacodynamic properties of pergolide mesylate following long-term administration to horses with pituitary pars intermedia dysfunction. *J Vet Pharmacol Ther.* 2017;40:158-164.

17. Jacob SI, Geor RJ, Weber PS, 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.

18. 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.

19. Hu K, Stewart AJ, Yuen KY, Hinrichsen S, Dryburgh EL, Bertin FR. The effect of freeze-thaw cycles on determination of immunoreactive plasma adrenocorticotrophic hormone concentrations in horses. *J Vet Intern Med.* 2020;34:1350-1356.

20. Secombe CJ, Tan RHH, Perera DI, Byrne DP, Watts SP, Wearn JG. The effect of geographic location on circannual adrenocorticotrophic hormone plasma concentrations in horses in Australia. *J Vet Intern Med.* 2017;31:1533-1540.

21. 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.

22. Byrne DP, Secombe CJ, Tan RHH, Perera DI, Watts SP, Wearn JG. Circannual variability in adrenocorticotrophic hormone responses to administration of thyrotropin-releasing hormone in clinically normal horses in Australia. *Vet J.* 2018;238:58-62.

23. Copas VE, Durham AE. Circannual variation in plasma adrenocorticotrophic hormone concentrations in the UK in normal horses and ponies, and those with pituitary pars intermedia dysfunction. *Equine J.* 2012;44:440-443.

24. Donaldson MT, McDonnell SM, Schanbacher BJ, et al. Variation in plasma adrenocorticotrophic 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.

25. Durham AE, Clarke BR, Potier JFN, Hammarstrand R, Malone GL. Clinically and temporarily specific diagnostic thresholds for plasma ACTH in the horse. *Equine Vet J.* 2021;53:250-260.

26. 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.

How to cite this article: Kam YN, McKenzie K, Coyle M, Bertin F-R. Repeatability of a thyrotropin-releasing hormone stimulation test for diagnosis of pituitary pars intermedia dysfunction in mature horses. *J Vet Intern Med.* 2021;35(6):2885-2890. doi:10.1111/jvim.16281