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Clinical implications of imprecise sampling time for 10- and 30-min thyrotropin-releasing hormone stimulation tests in horses

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Abstract

Background: The thyrotropin-releasing hormone (TRH) stimulation test is used to diagnose pituitary pars intermedia dysfunction (PPID) using 10- or 30-min protocols. Imprecise sampling time for the 10-min protocol can lead to misdiagnoses.

Objectives: To determine the effect of imprecise sampling time for the 30-min protocol of the TRH stimulation test.

Study design: In vivo experiment.

Methods: Plasma immunoreactive adrenocorticotropin (ACTH) concentrations were measured 9, 10, 11, 29, 30 and 31 min after intravenous administration of 1 mg of TRH in 15 control and 12 PPID horses. Differences in ACTH concentrations between sampling times, variability in ACTH concentrations between protocols, and diagnostic classification of PPID were assessed using Friedman's test, Bland–Altman plots, and Fisher's exact test, respectively, with 95% confidence intervals reported and significance set at $p < 0.05$.

Results: Imprecise sampling time resulted in variable ACTH concentrations, but significant differences in absolute ACTH concentrations were not detected for imprecise sampling within each protocol or between protocols. Imprecise sampling changed PPID diagnostic classification for 3/27 (11 [4–28] %) horses for both protocols. Using the 30-min protocol as a reference, 1/12 (8 [1–35] %) horses returned a negative test result and 5/12 (42 [19–68] %) horses returned equivocal test results that would be considered positive in practice due to the presence of supportive clinical signs.

Main limitations: Limited sample size and inter-horse variability reduced the ability to detect small but potentially relevant differences.

Conclusions: Overall, the impact of imprecise sampling was not significantly different between the 10- and 30-min TRH stimulation test protocols. However, diagnostic classification for PPID would have varied between the 10- and 30-min protocols in this population, if clinical signs had been ignored. Precise timing during TRH stimulation tests and contextual interpretation of ACTH concentrations remain fundamental for the diagnosis of PPID.

KEYWORDS
adrenocorticotropic hormone, diagnostic test, endocrine, horse, laminitis, pituitary pars intermedia dysfunction

1 | INTRODUCTION

Pituitary pars intermedia disfunction (PPID) is a prevalent endocrine condition of older horses, which is likely under-recognised in its earlier stages due to challenges of diagnosis.[1,2] Clinical signs of advanced cases include hypertrichosis, epaxial muscle atrophy, pendulous abdomen, polyuria/polydipsia, and laminitis.[3–5] Nitration and increased alpha-synuclein expression associated with degeneration of hypothalamic dopaminergic neurons, hypothesised to be caused by oxidative stress, results in loss of inhibition of the pars intermedia and increased production of pro-opiomelanocortin-derived peptides, including adrenocorticotropin (ACTH).[6,7]

Baseline and post-thyrotropin-releasing hormone (TRH) stimulation ACTH concentrations are commonly used for the diagnosis of PPID, and show seasonal variability with significantly higher concentrations in autumn in both healthy and PPID horses.[8,9] The magnitude of ACTH fluctuation varies at different latitudes, while factors such as stress, feed status, disease, and age also influence baseline ACTH concentrations, further complicating its reliability as diagnostic tool.[10–12] To accommodate for these fluctuations, month-specific reference intervals and diagnostic cut-off values have been developed by investigators.[8,13] The progressive nature of the disease means there remains a transition period during which horses might present with vague clinical signs and have normal baseline ACTH concentrations.[2] During this phase the risk of misdiagnosis increases if only baseline ACTH is used, and TRH stimulation tests are recommended due to superior sensitivity and specificity.[8,14,15]

Adrenocorticotropin concentrations after TRH administration and timing of the peak are inconsistent.[15] Suggestions for peak concentrations include 2–6 min, 0–14 min, and 5–30 min post-TRH administration, leading to the use of two protocols in practice, one with blood sampling at 10 min post-TRH administration and one with blood sampling at 30 min post-TRH administration.[14,15] Recent investigations demonstrated that a 1-min difference in sampling time for the 10-min protocol significantly affected ACTH concentrations: in 75% of the tested horses, at least one early or late sample differed in ACTH concentration by ≥10%.[16] In practice, early or late sampling is a realistic limitation to TRH stimulation tests, with the consequence of misdiagnosing a patient. Adrenocorticotropin concentrations increase rapidly during first 15 min post-TRH administration and appear to be more stable around 30 min, warranting further investigation into the significance of a 1-min difference on post-TRH ACTH concentrations for the 30-min protocol.[15] The objective of this study was to investigate if the 30-min protocol is also impacted by sampling time, to determine which protocol is more appropriate for clinical settings.

2 | MATERIALS AND METHODS

2.1 | Animals

Twenty-seven horses ≥12 years of age from The University of Queensland institutional herd were enrolled (Table S1). The study population consisted of 11 mares and 16 geldings with ages ranging from 13 to 30 (median 20) years. Breeds included Standardbred ($n = 15$), Stock Horse ($n = 6$), Thoroughbred ($n = 4$), Warmblood ($n = 1$) and Quarter horse ($n = 1$). No ponies were included in the study. Fifteen horses showed no clinical signs of PPID, while 12 horses presented with clinical signs consistent with PPID, including delayed or decreased hair shedding ($n = 12$), persistent hypertrichosis ($n = 7$) and epaxial muscle atrophy ($n = 10$). All horses were fed hay and pasture for at least 30 days before the study started, with no grain or concentrate feed provided at any point prior to or during the study.

2.2 | Experimental design

Each horse underwent a single TRH stimulation test in early summer (December) as previously described.[17] A temporary catheter (18G; Surflo, Terumo) was aseptically placed in the left jugular vein, and a 5-mL baseline blood sample was collected and placed into a plastic vacutainer tube containing ethylenediaminetetraacetic acid (EDTA; Vacutainer, BD). Following this, 1 mL (1 mg/mL solution) of recombinant TRH (Sigma-Aldrich) was administered intravenously and flushed with 0.9% saline, with a timer started immediately upon delivery of the full dose. Blood samples (5 mL) were collected through the venous catheter at precisely 9, 10, 11, 29, 30 and 31 min after TRH administration. Samples were kept on ice and centrifuged within 4 h of collection. Plasma concentrations of ACTH were measured using chemiluminescent immunoassay (Immulite 1000, Siemens Diagnostics) with the assay quantification range of 10 to 1250 pg/mL and intra-assay variation of 4.8%.[18] Samples with ACTH concentrations >1250 pg/mL were diluted to obtain an actual concentration.

2.3 | Statistical analysis

Sample size was determined a priori based on an expected standardised difference between 10- and 30-min ACTH concentrations in a paired design with $d = 1.16$, $\alpha = 0.05$ and power $(1\text{-}\beta) = 0.8$.[19] This analysis indicated an appropriate sample size of 12 horses per group. Differences in absolute ACTH concentrations between groups, variability between early and late sampling times at 10 and 30 min after TRH administration, and categorical diagnosis of PPID at different time points were analysed

using GraphPad Prism software (version 9.4; GraphPad). Continuous data were tested for normality with a Shapiro–Wilk test, and data that did not follow a normal distribution were analysed with nonparametric tests and reported as median and 95% confidence interval (CI). One PPID horse was excluded from the figures but not from the analyses. The effect of imprecise sampling on immunoreactive ACTH concentration at 10 and 30 min was analysed with Friedman's test and Dunn's post hoc test to compare early (9 or 29 min) or late (11 or 31 min) sampling times with the standard sampling time (10 or 30 min, respectively). To evaluate variability due to early or late sampling at 10 and 30 min, percent difference was calculated as the difference between early (9 or 29 min) or late (11 or 31 min) sampling, and the standard sampling time (10 or 30 min, respectively), divided by the standard sampling time. Percentage differences were analysed with Friedman's test accounting for repeated measures of the same animals. Variability in ACTH concentrations were visualised with Bland–Altman plots; however, repeated measures were not accounted for to determine the limits of agreement in the Bland–Altman analysis. The critical value for bias was determined to be 5%, as values below this threshold could be attributed to the intra-assay variability of the chemiluminescent assay. For all comparisons, analysis was performed on the control group and the PPID group separately. The number of misclassifications because of imprecise sampling time was compared between protocols with a Fisher's exact test. Throughout, $p < 0.05$ was considered significant.

3 | RESULTS

3.1 | Diagnostic classification

All horses tolerated the TRH stimulation test, and no adverse effects were recorded. Horses that presented with consistent clinical signs (delayed shedding, hypertrichosis or epaxial muscle atrophy) and had ACTH concentrations >41.6 pg/mL at 30-min after TRH administration were assigned to the PPID group.[8] Horses in which clinical signs were absent and ACTH concentrations were <41.6 pg/mL were assigned to the control group. This resulted in a control group of 15 horses and a PPID group of 12 horses ($n = 11$ in the figures due to a horse with ACTH concentrations >1250 pg/mL).

3.2 | ACTH concentrations over time

Plasma ACTH concentrations after TRH administration are shown in Figure 1. Peak ACTH concentrations occurred at 9 min for 13 horses, 10 min for 9 horses, 11 min for 3 horses, and 29 min for 1 horse (Figure 1).

3.3 | Sampling time and ACTH concentrations

In the control group, ACTH concentrations differed significantly across sampling times for the 10-min protocol (9 min: 49.4 pg/mL [95% CI: 36.1–62.6] vs. 10 min: 46.8 pg/mL [95% CI: 37.8–61.5] vs. 11 min: 44.3 pg/mL [95% CI: 32.8–60.4], $p = 0.03$, Figure 2A). However, overall significance was due to differences between 9- and 11-min time points ($p = 0.03$), and post hoc tests did not reach statistical significance for early or late sampling, compared with standard sampling ($p = 0.9$ and $p = 0.1$, respectively). In the PPID group, sampling time did not have a significant effect on ACTH concentrations for the 10-min protocol (9 min: 217 pg/mL [95% CI: 150–387] vs. 10 min: 210 pg/mL [95% CI: 143–394] vs. 11 min: 267 pg/mL [95% CI: 157–341], $p = 0.3$, Figure 2B).

Plasma ACTH concentrations did not differ significantly across sampling times for the 30-min protocol among the control group (29 min: 33.2 pg/mL [95% CI: 22.2–37.0] vs. 30 min: 32.5 pg/mL [95% CI: 21.2–37.5] vs. 31 min: 33.7 pg/mL [95% CI: 23.9–38.2], $p = 0.2$, Figure 2A) or PPID group (29 min: 100 pg/mL [95% CI: 68.8–156.0] vs. 30 min: 95.1 pg/mL [95% CI: 62.6–159.0] vs. 31 min: 95.8 pg/mL [95% CI: 62.2–157.0], $p = 0.4$, Figure 2B).

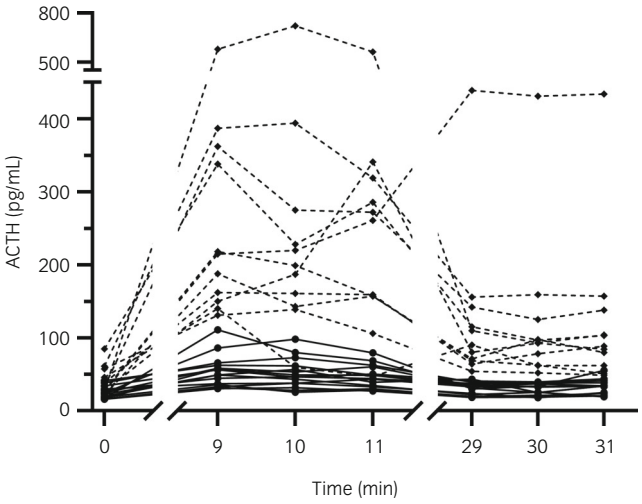

FIGURE 1 Plasma adrenocorticotropin (ACTH) concentrations of 26 horses at 0, 9, 10, 11, 29, 30 and 31 min following administration of thyrotropin releasing hormone. Control horses ($n = 15$) are indicated by solid lines and horses with PPID ($n = 11$) are indicated by dashed lines. Horse #27 was excluded from the figure but not from the analysis; its results are in Table S1.

3.4 | Sampling time and variation in ACTH concentrations

No significant differences in the magnitude of variability in ACTH concentrations for early or late sampling were detected between the 10- and 30-min protocols in either the control group (early sampling with 10-min protocol: 12.9% [95% CI: 4.7–17.3] vs. early sampling with 30-min protocol: 11.0% [95% CI: 3.2–12.9] vs. late sampling with 10-min protocol: 13.8% [95% CI: 9.5–19.9] vs. late sampling with 30-min protocol: 9.8% [95% CI: 3.8–36.5], $p = 0.6$; Figure 3A) or the PPID group (early sampling with 10-min protocol: 19.8% [95% CI: 2.3–31.6] vs. early sampling with 30-min protocol: 15.7% [95% CI: 4.4–27.1] vs. late sampling with 10-min protocol: 20.8%

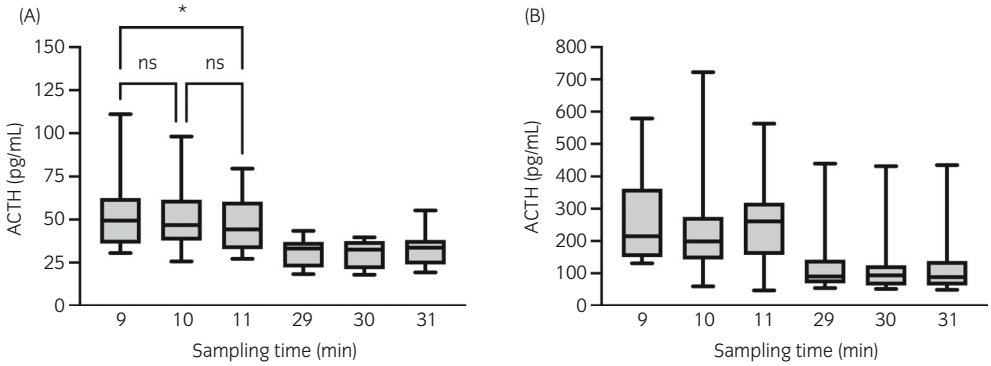

FIGURE 2 Plasma adrenocorticotropin (ACTH) concentrations of 26 horses following administration of thyrotropin releasing hormone in the (A) control horses ($n = 15$) and (B) horses with pituitary pars intermedia dysfunction ($n = 11$). Boxes represent interquartile range and whiskers represent range. Note different scale of y axes. * indicates significant difference ($p < 0.05$) between ACTH concentrations at 9 and 11 min within the 10-min protocol. Horse #27 was excluded from the figure but not from the analysis; its results are in Table S1.

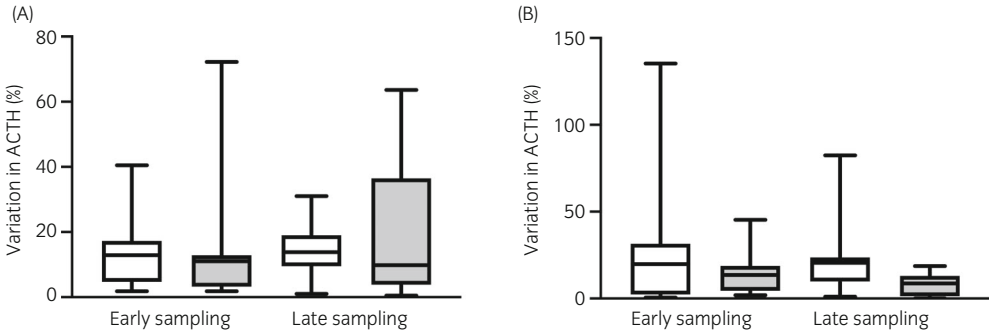

FIGURE 3 Percent variability in adrenocorticotropin (ACTH) concentrations of 26 horses due to early or late sampling for the 10-min (white bars) and 30-min (grey bars) thyrotropin stimulating hormone test protocols for the (A) control horses ($n = 15$) and (B) horses with pituitary pars intermedia dysfunction ($n = 11$). Boxes represent interquartile range and whiskers represent range. Horse #27 was excluded from the figure but not from the analysis; its results are in Table S1.

[95% CI: 9.8–25.4] vs. late sampling with 30-min protocol: 7.6% [95% CI: 1.3–13.0], $p = 0.07$; Figure 3B).

Bland–Altman plots showed broad limits of agreement and a bias that exceeded the critical values of 5% and −5% for the 10-min protocol; −8% and 5% for early and late sampling, respectively (Figure 4A,B). There were narrower limits of agreement and smaller bias which did not exceed the critical values of 5% and −5% for the 30-min protocol; 3% and −5% for early and late sampling, respectively (Figure 4C,D).

3.5 | Sampling time and diagnosis of PPID

Diagnostic cut-off values derived from previous studies were used to classify horses as PPID at 10- and 30-min after TRH administration, respectively. For the 10-min protocol, a horse was considered negative for PPID if ACTH concentration was <110 pg/mL, equivocal if ACTH concentration was between 110 and 220 pg/mL and positive if ACTH concentration was >220 pg/mL.[20] For the 30-min protocol, a horse was considered positive for PPID if ACTH concentration was >41.6 pg/mL.[8] Comparing diagnostic classification between the two protocols, 10-min samples from PPID horses classified 6/12 as positive, 5/12 as equivocal and 1/12 as negative, compared with 12/12 being positive at 30-min (by definition). For control horses, 15/15 returned negative results at both the 10- and 30-min times. Therefore, 6/12 [50 [95% CI, 25–75] %) horses that were positive for the 30-min protocol were not positive for the 10-min protocol, while all the horses that were positive or equivocal for the 10-min protocol were positive for the 30-min protocol.

Regarding the effect of imprecise sampling time on diagnostic classification, for the 10-min protocol among PPID horses, 5/12 were positive at all sampling times, 3/12 were equivocal at all sampling times, and 4/12 had mixed classifications across different sampling times, while among control horses, 14/15 were negative at all sampling times and 1/15 had mixed classifications across different sampling times. For the 30-min protocol among PPID horses, 12/12 were positive at all sampling times, while for control horses, 12/15 were negative at all sampling times and 3/15 had mixed classifications across different sampling times. Given that, as per inclusion criteria, all horses classified as PPID in this study had clinical signs consistent with the disease, an equivocal result would have been considered positive in clinical practice; therefore, imprecise

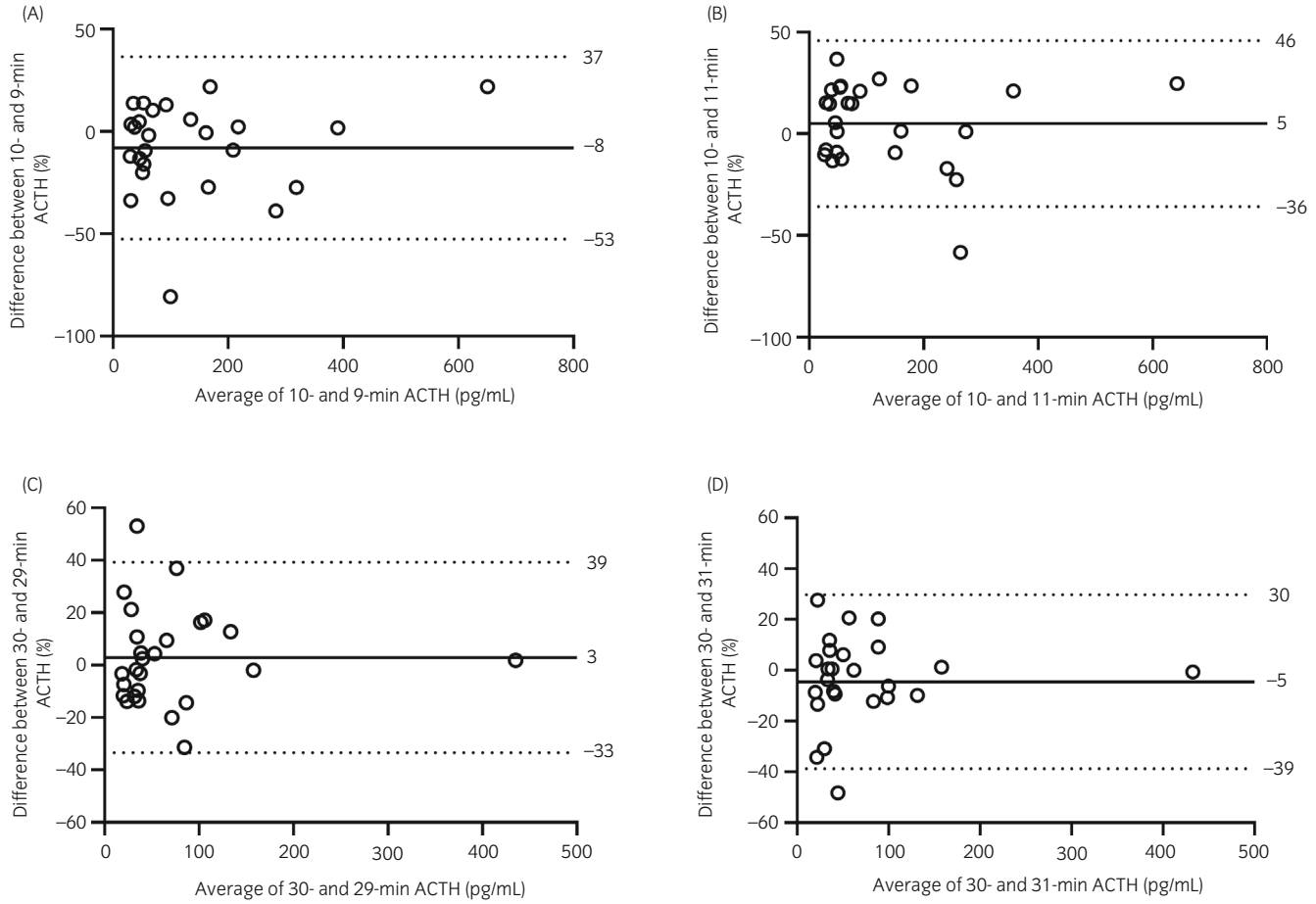

FIGURE 4 Bland–Altman plots of percentage difference in adrenocorticotropin (ACTH) concentrations of 26 horses sampled at (A) 10 versus 9 min, (B) 10 versus 11 min, (C) 30 versus 29 min and (D) 30 versus 31 min after administration of thyrotropin-releasing hormone. Solid lines represent the bias, dotted lines represent 95% limits of agreement. Horse #27 was excluded from the figure and the analysis; its results are in Table S1.

sampling during either protocol would have yielded misclassification in 3/27 (11 [4–28] %) of cases.

4 | DISCUSSION

The results of this study indicate that there are similarities and differences between 10- and 30-min TRH stimulation test protocols that might impact clinical interpretation. There was no significant effect of imprecise sampling time on absolute ACTH concentrations or Bland–Altman agreement for either the 10- or 30-min protocols, when samples were collected early or late by 1 min. Significant differences in absolute ACTH concentrations were observed between 9- and 11-min samples, but not between 29- and 31-min samples, indicating that imprecision of >1 min might be more impactful for the 10-min protocol. There was disagreement in diagnostic classification of PPID between protocols, whereby 6/12 horses classified as positive by the 30-min protocol would have been classified as not positive by the 10-min protocol; however, after consideration of clinical signs, the five horses with equivocal results would have been considered positive in practice.

One previous study by Thane et al. investigated the effect of early or late sampling on ACTH concentrations after TRH administration for the 10-min protocol.[16] That study found 75% (18/24) of horses had at least one early or late sample that differed by ≥10% from the standard 10-min ACTH concentration. Importantly, 21% (5/24) of horses would have demonstrated a change in PPID classification with early or late sampling although, in that study, clinical signs were not considered in the PPID classification. Our study found similar results for the 10-min protocol, in that 11% (3/27) horses would have demonstrated a change in PPID classification with early or late sampling, considering the presence of clinical signs consistent with PPID. We extended our investigation to include the 30-min protocol, and collected blood samples at 0, 9, 10, 11, 29, 30 and 31 min after TRH administration. We did not include paired aliquots for our analysis, as was done by Thane et al.[16] This would have enabled the analysis of intra-assay variability in the test population, and the calculation of a 10- and 30-min standard for each horse to which early and late sampling could have been compared. We measured ACTH concentrations using a chemiluminescent immunoassay with a published intra-assay variability of 4.8%, which is consistent with the

calculated intra-assay variability found by Thane et al. (median 3%, range 0%–6%) and initial assessment of the assay (mean 9.3%, range 3.2%–12.9%).[18,21] Although we expect that intra-assay variability in this study was representative, the calculation and analysis of true intra-assay variability within the test population might have been preferable to compare variability between 10- and 30-min protocols.

The diagnostic cut-off values used in this study were based on published recommendations for the diagnosis of PPID but may be open to conjecture. Current recommendations from the Equine Endocrinology Group are based on the Immulite 2000XPI analyser, but have recently been adapted for the Immulite 1000 analyser, using cut-offs of >220 pg/mL to categorise a horse as positive, 110–220 pg/mL as equivocal and <110 pg/mL as negative for PPID.[20] The use of these cut-off values could be questioned due to the generalisation of Northern Hemisphere data that is applied equally over a 6-month period. The Equine Endocrinology Group does not provide recommended cut-off values for the 30-min protocol. However, month- and geographically-specific diagnostic cut-off values for the 30-min protocol have been determined in a previous study at the same institution where this study was performed using 40 control horses, ranging from 10 to 27 years of age (median 16) and 10 PPID horses, ranging from 15 to 24 years of age (median 22). This institution primarily uses the 30-min protocol, and there are no matched cut-off values available for the 10-min protocol. To draw a more accurate comparison between the 10- and 30-min TRH stimulation test protocols, month- and geographically-specific cut-off values for the 10-min protocol would have been required.

Using the above-mentioned cut-off values, there was a difference in diagnostic classification of PPID between protocols. While 12 of 12 horses were classified as PPID using the 30-min protocol (by definition), 6 of 12 horses were classified as equivocal or negative using the 10-min protocol. The 10-min protocol is different in this regard, as an equivocal range is included rather than a binary positive or negative test result, as was determined for the 30-min protocol. When equivocal results are obtained, the observation of strong clinical signs or re-testing are recommended to confirm a diagnosis.[2,22] Given that the horses in this study with an equivocal 10-min result demonstrated supportive clinical signs, in practice these horses would have been deemed positive for the purposes of clinical management. However, 3 of 5 equivocal horses were in the lower half of the equivocal range, and that they did not clearly test positive on the 10-min protocol would suggest that further investigation of appropriate clinical cut-off values could be warranted. This is especially important, as one indication to perform the TRH stimulation test can be a clinical suspicion of PPID but when early or vague clinical signs are present.

Testing was performed in December in the Southern Hemisphere. This period has been described as part of the quiescent phase of the circannual rhythm of endogenous ACTH concentrations, and is characterised by lower ACTH concentrations that show less variability than in other periods of the year.[19] For the geographical location in which this study was performed, the quiescent period is hypothesised to range from April to December, after which the circannual rhythm transitions into its dynamic phase in which endogenous and TRH-stimulated ACTH concentrations and their variability increase.[10,23,24] Further investigation of circannual repeatability is necessary to determine if the difference between 10- and 30-min protocols in this study holds true for other phases of the pituitary pars intermedia cycle. Other factors that can influence ACTH concentrations include exercise, illness, stress, diet, age and breed.[11,12,25–28] To minimise these potential effects, the conditions of this study were controlled as much as possible. The horses used were from an institutional research herd, where exercise, feed and husbandry conditions could be standardised prior to and during testing. Time of day in which TRH stimulation tests were performed was consistent and all tests were performed within 2 weeks. Only horse breeds were enrolled, as recent work has demonstrated pony breeds to have higher ACTH concentrations, especially during autumn.[26,29] Whether there is a similar breed effect on TRH stimulation test results remains to be determined.

Finally, there are practical considerations that might be weighed up by a clinician when choosing whether to use the 10- or 30-min protocol. One advantage of the 10-min protocol is the reduced time required to perform the test. One advantage of the 30-min protocol is that it can be used for combined testing of PPID and insulin dysregulation, as is commonly considered for animals that present with laminitis. Specifically, the oral sugar test or a two-step insulin tolerance test can evaluated concurrently.[30–32] Combined use of the TRH-stimulation test and two-step insulin tolerance test has shown good agreement with independent testing.[31] Given that no fasting is required, and blood is sampled at 0 and 30 min after insulin injection, the two-step insulin tolerance test can easily be combined with the 30-min TRH-stimulation test to improve practicality and reduce the number of blood samples required.

5 | CONCLUSIONS

The effect of imprecise sampling was not significantly different between the 10- and 30-min TRH stimulation test protocols. However, imprecision of >1 min is likely to be more impactful for the 10-min protocol. There was diagnostic disagreement between the 10- and 30-min protocols, whereby 50% of horses classified as PPID positive by the 30-min protocol would have been identified as not positive by the 10-min protocol if clinical signs had not been taken into account for the PPID classification. Therefore, precise sampling time, use of correct diagnostic cut-off values and contextual interpretation of ACTH concentrations remain fundamental for the diagnosis of PPID. Further investigation into the repeatability of the 10- and 30-min TRH stimulation test protocols during other phases of the pituitary pars intermedia cycle is warranted.

AUTHOR CONTRIBUTIONS

Study conception and design: François-René Bertin and Nicholas J. Bamford. *Study execution*: François-René Bertin, Wenqing Wang and Kate L. Kemp. *Data interpretation and analysis*: Dante M. Vorster, François-René Bertin and Nicholas J. Bamford. *Draft manuscript preparation*: Dante M. Vorster. *Critical editing*: François-René Bertin and Nicholas J. Bamford. *Revision and final approval of manuscript*: All

authors. François-René Bertin takes responsibility for the integrity of the data and the accuracy of data analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/evj.13991.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICAL ANIMAL RESEARCH

The study protocol was approved by The University of Queensland Animal Ethics Unit (2022/AE000462).

INFORMED CONSENT

Not applicable.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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