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Clinical implications of using adrenocorticotropic hormone diagnostic cutoffs or reference intervals to diagnose pituitary pars intermedia dysfunction in mature horses

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Abstract

Background: Diagnosis of pituitary pars intermedia dysfunction (PPID) is problematic because of large variations in ACTH concentrations.

Hypothesis/Objectives: Compare the test characteristics of baseline and post–thyrotropin-releasing hormone (TRH) stimulation plasma ACTH concentrations in horses using diagnostic cutoff values (DCOVs) and reference intervals (RIs) and determine the clinical consequences of using each method.

Animals: One hundred six mature horses: 72 control cases and 34 PPID cases.

Methods: Prospective case-controlled study. Horses underwent monthly TRH stimulation tests. Diagnostic cutoff values were determined monthly by receiver operating characteristic curves using the Youden index. Reference intervals were determined monthly by a robust method. For each case age, sex and body condition score (BCS) were recorded.

Results: Baseline ACTH concentrations varied by month ($P < .001$) with significant "month × age" ($P = .003$), "month × sex" ($P = .003$), and "month × BCS" ($P = .007$) effects. Baseline ACTH concentrations were accurate to diagnose PPID (0.91 ± 0.06) with DCOVs increasing the test sensitivity (0.61 ± 0.21 to 0.87 ± 0.05, $P = .002$) and RI increasing test specificity (0.85 ± 0.12 to 0.98 ± 0.01, $P = .01$). Thyrotropin-releasing hormone stimulation improved test accuracy (0.91 ± 0.06 to 0.97 ± 0.03, $P = .004$).

Conclusions and Clinical Importance: ACTH concentrations follow a circannual rhythm and vary with physiological factors. As using DCOVs increases the ability to detect mild cases and using RI decreases the risk of unnecessary treatments, ACTH concentrations should be interpreted within a specific clinical context. The TRH stimulation test improves the diagnosis of PPID.

Abbreviations: +LR, positive likelihood ratios; α-MSH, alpha-melanocyte-stimulating hormone; ANOVA, analysis of variance; BCS, body condition score; DCOVs, diagnostic cutoff values; PPID, pituitary pars intermedia dysfunction; RI, reference interval; ROC, receiver operating characteristic; ROUT, robust regression and outlier removal; TRH, thyrotropin-releasing hormone.

KEYWORDS
diagnostic test, endocrinology, reference intervals, statistical methods, thyrotropin-releasing hormone

1 | INTRODUCTION

Pituitary pars intermedia dysfunction (PPID) is a common hormonal disease in horses, affecting 20% of mature horses.[1-3] Awareness of the disease among owners and veterinarians has considerably increased in recent years and clinical signs such as hypertrichosis, epaxial muscle wastage, abnormal fat distribution, or abnormal sweating all raise the clinical suspicion for PPID.[2,4,5] The disease is caused by the degeneration of hypothalamic dopaminergic neurons resulting in a hormone-secreting adenoma or hyperplasia of the pars intermedia of the pituitary gland.[6,7] Because ACTH is the most conveniently measured hormone originating from the pars intermedia and because its concentration has a good correlation with clinical signs, detection of an increased plasma concentration of baseline ACTH is the most common method to identify PPID cases or confirm a clinical suspicion of PPID.[4,8]

The definition of an increased plasma baseline ACTH concentration is controversial as ACTH increases with stress, exercise, season, diet, disease status, and age.[9-14] To remove some of the effects of these variables and improve the detection of subclinical PPID cases, the provocative thyrotropin-releasing hormone (TRH) stimulation test has been developed and is extensively used both clinically and in research.[15-17] However, as with measurement of plasma baseline ACTH concentration, there is similar variability with season, age, stress, and diet with the TRH stimulation test.[18,19] Although other diagnostic tools have been explored, they have limitations and are rarely used, making determination of plasma ACTH concentration the mainstay for the diagnosis of PPID.[4,5,20-25]

Several studies have determined guidelines for interpretation of plasma basal or post-TRH-stimulation ACTH concentrations to diagnose PPID; however, differences between studies in methodologies pertaining to the sampled population, the statistical analyses, and the processing of the samples have led to determination of either plasma ACTH reference intervals (RIs) or plasma ACTH diagnostic cutoff values (DCOVs).[12,26-28] The use of DCOVs or RI results in differences in the definition of an increased plasma ACTH concentration.[29] The clinical consequences of the current variations in the definition of an increased plasma ACTH concentration include the risk of missing PPID cases, exposing horses to the risk of complications associated with PPID, or on the other end of the spectrum, the risk of medicating healthy horses, limiting their athletic careers as a result of the use of controlled medications.

Therefore, the aim of our study is to compare the test characteristics and the clinical implications of determining DCOVs and RIs for plasma baseline and post-TRH stimulation ACTH concentrations for the diagnosis of PPID in the plasma of mature horses. We hypothesize that both plasma baseline and post-TRH stimulation ACTH concentrations are accurate to diagnose PPID in mature horses and that using DCOVs increases the sensitivity of the test while using RIs increases the specificity of the test.

2 | MATERIALS AND METHODS

2.1 | Study design

Control and PPID horses were recruited over a period of 24 months and followed monthly for 12 months, or until necropsy, to ensure that at least 50 horses were sampled each month. Horses of both sexes were recruited if they were ≥10 years of age, had not received pergolide in the year before the study, and had not traveled in the 24 hours before sampling. Ponies were excluded from the study.[30] Other than the signs associated with PPID in affected animals, all horses were considered healthy based on physical examination findings. Water and food were available ad libitum before and after all tests and horses were tested in a familiar environment (in the institution paddocks or on farm); however, all horses did not have access to the same diet.

The second week of each month, horses underwent a TRH stimulation test consisting of 2 jugular venipunctures for blood collection, 1 just before and 1 30 minutes after intravenous administration of 1 mg of TRH (Sigma-Aldrich Pty Ltd [subsidiary of Merck], North Ryde BC, Australia).[17] The 30-minute sampling time was elected over the 10-minute sampling time as neither time point has been shown to be superior and as multiple horses were tested simultaneously, the 30-minute sampling time is considered to be less impacted by delays of up to 30 seconds.[31] Samples were collected in plastic EDTA tubes (BD, Belliver Industrial Estate, Plymouth, UK), kept on ice, centrifuged and separated within 2 hours of collection. Plasma ACTH concentrations were analyzed within 8 hours using a chemiluminescent immunoassay (Immulite 1000 Chemiluminescent Assay, Siemens, Bayswater, Australia) with an interassay coefficient of variation of 4.8% and an intra-assay coefficient of variation of 5.4% as previously described.[32] Not all horses were tested every month. Additional data recorded included sex, age, and body condition score (BCS).[33]

A diagnosis of PPID was based on the presence of at least 2 of the following:

1. Clinical signs consistent with PPID (hypertrichosis or delayed shedding and epaxial muscle wastage, abnormal fat distribution or abnormal sweating);[4,5,7]
2. Necropsy findings confirming or excluding a pituitary adenoma or hyperplasia;[7]

3. Recurrent plasma baseline or post-TRH ACTH concentrations considered as outliers by robust regression and outlier removal (ROUT) method with Q = 1% in ≥6 of the 12 months of testing.[34]

A horse was considered as a control if it did not have clinical signs consistent with PPID during the time of the study, if deceased, necropsy findings were not consistent with a pituitary adenoma and plasma baseline or post-TRH ACTH concentrations considered as outliers by ROUT method with Q = 1% identified in ≤2 out of 12 months.[34] Horses not falling into 1 of those categories (ie, outlier by ROUT method between 3 and 5 months out of 12) were excluded as a diagnosis could neither be confirmed nor excluded.

The study was performed in the Southern hemisphere therefore December, January and February are summer months; March, April, and May are autumn months; June, July, and August are winter months; and September, October, and November are spring months. The protocol was approved by the Institutional Animal Ethics Committee.

2.2 | Data analysis

Monthly plasma baseline and post-TRH ACTH concentrations were log-transformed to meet normal distribution as per a Shapiro-Wilk test. Individual outlier values were detected as described above.[34] Horses were grouped by PPID status, sex (males or females), age (10-14 years, 15-19 years, or older than 20 years) and BCS (low [1-3], adequate [4-6], or high [7-9]) and monthly plasma baseline and post-TRH stimulation ACTH concentrations were compared using an unpaired t test, Mann-Whitney U test or 2-way repeated measure analysis of variance (ANOVA) depending on number of groups compared and distribution as determined by a Shapiro-Wilk test.

Diagnostic cutoff values were determined monthly considering data from both control and PPID horses by receiver operating characteristic (ROC) curves using the Youden index (the cut-point where "sensitivity + specificity − 1 has its maximum value) and established test accuracy (area under the curve), positive likelihood ratios (+LR), sensitivity, and specificity as previously described.[26,34,35] Reference intervals were determined monthly, only considering data from control horses by a robust method, including Box-Cox transformation and bootstrap method as previously described.[27,34] Post hoc determination of sensitivity, specificity, and +LR of RIs were then determined based on the ROC curve.[36] Test characteristics obtained by each method were then compared using a 1-way ANOVA or paired t test depending on number of groups involved.

Normally distributed data are presented as mean and SD and non-normally distributed data are presented as median and range. Statistical analysis was performed using commercially available software (GraphPad Prism 8, GraphPad Software, Inc, San Diego, California; MedCalc Software bvba, Mariakerke, Belgium) and P < .05 was considered statistically significant. When multiple comparisons between months were performed, a Bonferroni correction was used to determine differences in immunoreactive plasma ACTH.

3 | RESULTS

One hundred six horses (53 geldings, 4 stallions, and 49 mares) were recruited, resulting in a total of 1238 "horse × month" plasma ACTH concentrations. Seventy-two horses were considered as control cases (1057 "horse × month" plasma ACTH concentrations), and 34 as PPID cases (181 "horse × month" plasma ACTH concentrations). Two horses were excluded from the analysis (no clinical signs but >2 months out of 12 with plasma baseline ACTH concentrations considered as outliers). In the control group, 5 cases had outlier values as detected by the ROUT method (3 cases in January, 1 in May, and 1 in July). For those cases, only the outlier values, and not the horse, were excluded from the analysis.[34]

The median age was 17 years (range, 10-31 years) and BCS was 6 (range, 2-8). Breeds included Australian Stock Horse (n = 35), Standardbred (n = 24), Warmblood (n = 18), Thoroughbred (n = 12), Arab (n = 7), Quarter Horse (n = 4), and mixed breed (n = 6). Control horses were significantly younger than PPID horses (16 [10-27] vs 22 [11-31], P < .001); however, there was no significant difference in BCS (P = .9) nor in sex distribution (P = .1).

3.1 | Plasma baseline ACTH

In control cases, there was a significant effect of month of sampling (P < .001) with higher immunoreactive plasma baseline ACTH concentrations in autumn months. There was a significant effect of age (P = .04) and "month × age" (P = .003) on immunoreactive plasma baseline ACTH concentration with horses older than 20 years of age having significantly higher immunoreactive plasma ACTH concentrations in February (P = .003; Figure 1A). Although there was no significant effect of sex on immunoreactive plasma baseline ACTH concentrations (P = .2), there was a significant "sex × month" effect (P = .003) with mares having higher immunoreactive plasma baseline ACTH concentrations; however, no comparisons between months reached the Bonferroni-adjusted P value (Figure 1B). Similarly, although there was no significant effect of BCS on immunoreactive plasma baseline ACTH concentration (P = .7), there was a significant "BCS × month" effect (P = .007) with horses with a BCS ≤3 having higher immunoreactive plasma baseline ACTH concentrations in autumn and lower immunoreactive plasma baseline ACTH concentrations in winter but however, no comparisons between months reached the Bonferroni-adjusted P value (Figure 1C). Due to underrepresentation of some breeds, no analysis was performed on breed effect.

3.2 | Plasma post-TRH ACTH

In control cases, there was a significant effect of month (P < .001) with higher immunoreactive plasma post-TRH ACTH concentrations in autumn months. There was a significant effect of age (P = .03) but no significant "month × age" effect (P = .9) on immunoreactive plasma post-TRH ACTH concentrations (Figure 2A). Although there was no significant effect of sex on immunoreactive plasma post-TRH ACTH

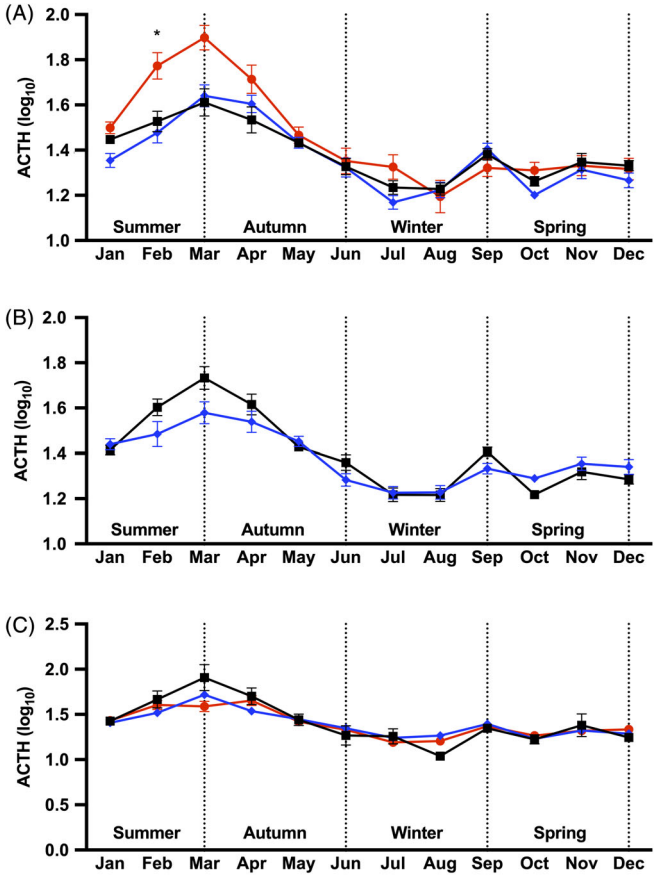

FIGURE 1 A, Monthly baseline ACTH concentrations (mean and SD of log-transformed data) in mature horses grouped by age. Age groups are 10 to 14 years in black (n = 28), 15 to 19 years in blue (n = 28), and older than 20 years in red (n = 15). The data were log-transformed for the analysis. * indicates $P < .05$ between groups. B, Monthly baseline ACTH concentrations (mean and SD of log-transformed data) in mature horses grouped by sex. Females are in black (n = 36) and males in blue (n = 36). The data were log-transformed for the analysis. * indicates $P < .05$ between groups. C, Monthly baseline ACTH concentrations (mean and SD of log-transformed data) in mature horses grouped by body condition score (BCS). Groups are low BCS (1-3) in black (n = 3), adequate BCS (4-6) in blue (n = 27), or high BCS (7-9) in red (n = 27). The data were log-transformed for the analysis. * indicates $P < .05$ between groups

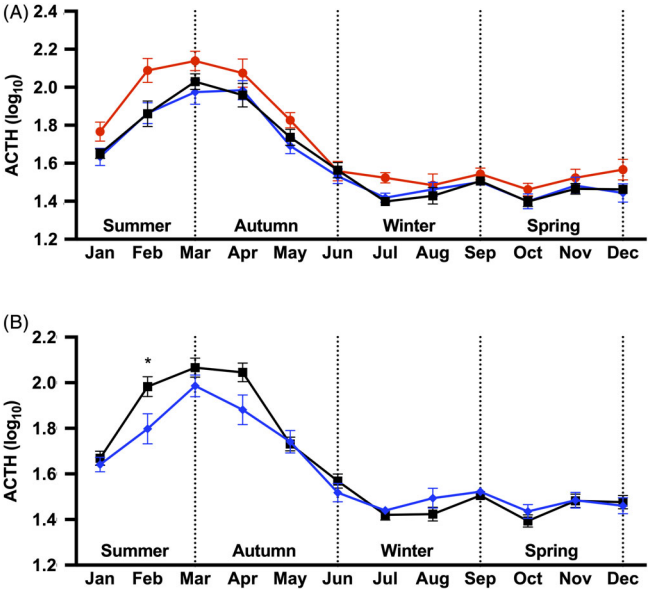

FIGURE 2 A, Monthly post–thyrotropin-releasing hormone (TRH) stimulation ACTH concentrations (mean and SD of log-transformed data) in mature horses grouped by age. Age groups are 10 to 14 years in black (n = 28), 15 to 19 years in blue (n = 27), and older than 20 years in red (n = 15). The data were log-transformed for the analysis. * indicates $P < .05$ between groups. B, Monthly post-TRH stimulation ACTH concentrations (mean and SD of log-transformed data) in mature horses grouped by sex. Females are in black (n = 36) and males in blue (n = 36). The data were log-transformed for the analysis. * indicates $P < .05$ between groups

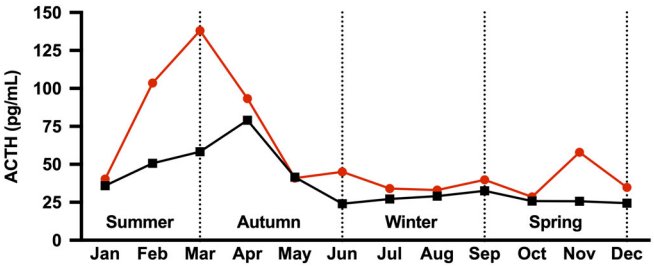

FIGURE 3 Monthly diagnostic cutoff values (in black) and upper limits of reference intervals (in red) for baseline ACTH concentrations in mature horses

concentrations ($P = .5$), there was a significant "sex × month" effect ($P = .001$) with mares having a significantly higher immunoreactive plasma post-TRH ACTH concentrations in February ($P = .002$; Figure 2B). There was no significant effect of BCS or "BCS × month" on immunoreactive plasma post-TRH ACTH concentrations ($P = .9$ and $P = .2$, respectively). Due to underrepresentation of some breeds, no analysis was performed on breed effect.

3.3 | Diagnostic cutoff values

Data from both control horses and PPID cases were included in this analysis. There were 40 to 50 immunoreactive plasma ACTH concentrations from control horses (after removal of the 5 outlying values) and 5 to 11 immunoreactive plasma ACTH concentrations from PPID horses utilized each month (Figures 3 and 4).

Based on the ROC curves, the accuracy of immunoreactive plasma baseline ACTH concentrations for a diagnosis of PPID was 0.91 ± 0.06 consistent with an "excellent" test (accuracy > 0.9); however, for the months of January, February, March, June, November, and December, the accuracy of immunoreactive plasma baseline ACTH concentrations was only consistent with a "good" test (accuracy between 0.8 and 0.9). For the months of February, March, June, July, October, November, and December, the

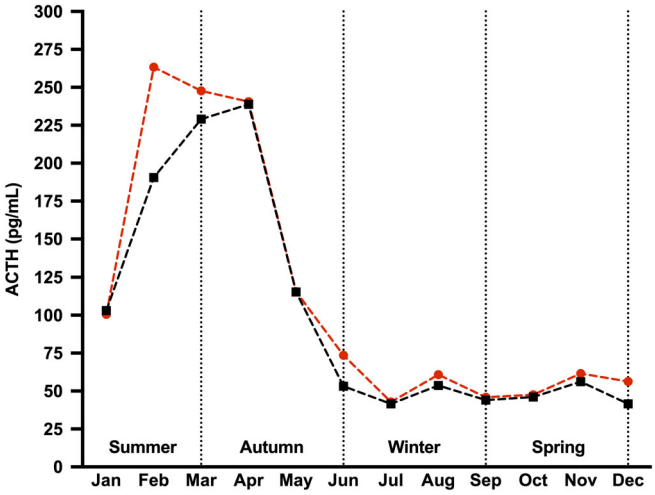

FIGURE 4 Monthly diagnostic cutoff values (in black) and upper limits of reference intervals (in red) for post–thyrotropin-releasing hormone stimulation ACTH concentrations in mature horses

immunoreactive plasma baseline ACTH concentrations had low positive likelihood ratios (+LR < 10, Table 1).[35,36] Based on the ROC curves, the accuracy of immunoreactive plasma post-TRH ACTH concentrations for a diagnosis of PPID was 0.97 ± 0.03, also consistent with an "excellent" test, with no month having an accuracy below 0.9. Accuracy for immunoreactive plasma post-TRH ACTH was significantly higher than immunoreactive plasma baseline ACTH concentrations (P = .004; Figure 5A), and also had high +LR except in February, June, and December.

Based on the DCOVs obtained using the Youden index, the sensitivity of immunoreactive plasma baseline ACTH concentrations for the diagnosis of PPID ranged from 0.83 to 1.0. The use of the TRH stimulation test did not significantly improve test sensitivity (0.8-1.0, P = .9; Figure 5B). The specificity of immunoreactive plasma baseline ACTH concentrations for the diagnosis of PPID using DCOVs ranged from 0.63 to 0.98; however, the use of a TRH stimulation test significantly improved test specificity (0.84-0.98, P = .02; Figure 5C).

3.4 | Reference intervals

Only data from control cases were included in this analysis with 40 to 50 horses utilized per month after removal of the 5 outlier values. The horse immunoreactive plasma baseline and post-TRH ACTH concentrations required to diagnose PPID using the generated RIs were significantly higher than the concentrations if DCOVs were used for diagnosis (P = .001 and P = .007, respectively; Figures 3 and 4). Diagnosis using RIs also had higher +LR (Table 2).

Based on the ROC curves, the sensitivity of the use of RIs for immunoreactive plasma baseline ACTH concentrations for a diagnosis of PPID ranged from 0.2 to 0.89, and the use of a TRH stimulation test significantly improved test sensitivity (0.67-1.00, P = .04; Figure 5B). However, when using immunoreactive plasma baseline ACTH concentrations for the diagnosis of PPID, the use of RIs had a significantly lower sensitivity compared to DCOVs (P = .002). There was no significant improvement of test sensitivity using DCOVs over RIs after a TRH stimulation test (P = .07). Based on the ROC curves, the specificity of using RIs for immunoreactive plasma baseline ACTH concentrations to diagnose PPID, ranged from 0.98 to 1.00 and the use of a TRH stimulation test did not significantly improve test specificity (0.98-0.98, P = .5; Figure 5C). However, using RIs for immunoreactive plasma baseline ACTH concentrations had a significantly higher specificity for the diagnosis of PPID compared to DCOVs (P = .01). There was no significant improvement of test specificity using DCOVs over RIs after a TRH stimulation test (P = .3).

4 | DISCUSSION

The main results of our study are that (a) plasma ACTH concentrations physiologically vary with season, age, sex, and body condition; (b) plasma baseline ACTH concentrations are accurate to diagnose PPID in mature horses with the use of DCOVs increasing the sensitivity of the test and the use of RIs increasing the specificity of the test; and (c) use of TRH stimulation increases test characteristics for the diagnosis of PPID.

Seasonal variation in plasma ACTH is well documented in horses, with higher concentrations in autumn reported worldwide; however, the reason for the increase during autumn is still unclear.[24,25,27] In horses, and other species, the plasma ACTH circannual variation is thought to be associated with a response to photoperiod change and the preparation for harsher climatic conditions with alterations in lipid metabolism.[12,25,37,38] Although the seasonal changes are mild in Queensland, the persistence of those circannual changes would be consistent with an evolutionary trait.[4] Because the changes in plasma ACTH concentrations were reportedly more severe in autumn in PPID cases, it was previously advised that the autumnal period would be the most appropriate time to test for PPID with improved test sensitivity and specificity.[12,26] This was not the case in our study as the accuracy of plasma baseline ACTH concentration was the lowest in March (equivalent of September in the Northern hemisphere) suggesting a larger overlap between ACTH concentrations in control and PPID cases in autumn.[39]

Consistent with other studies, older horses had significantly higher plasma ACTH concentrations.[13,25] The effect of aging on the hypothalamo-pituitary adrenal axis is unclear but oxidative stress is considered as a contributing factor for poor regulation.[25,40] Interestingly, oxidative changes are also considered as a potential cause of PPID by causing dopaminergic neurodegeneration.[41,42] Taken together, these data suggest either a further overlap between PPID cases and older controls or a continuum from healthy to PPID determined by the severity of hypothalamo-pituitary adrenal axis oxidative changes. Clinically, this finding could complicate the diagnosis of PPID in horses older than 20 years of age, and stresses the importance of using an age-matched control group when determining if there is an inappropriate increase in plasma ACTH concentration.[31]

TABLE 1 Diagnostic cutoff values (DCOVs) for immunoreactive plasma endogenous and post-TRH ACTH concentrations determined monthly considering data from both control horses and horses with pituitary pars intermedia dysfunction (PPID) by receiver operating characteristic curves using the Youden index

Months	n controls	n PPID	DCOVs log	DCOVs (pg/mL)	SN	95% CI	SP	95% CI	+LR	95% CI	AUC	*P* value
Plasma endogenous ACTH												
January	43	6	1.555	35.9	0.833	0.437-0.992	0.925	0.801-0.974	11.1	3.66-34.0	0.846	.007
February	48	8	1.705	50.7	0.875	0.529-0.994	0.766	0.628-0.864	3.7	2.10-6.60	0.870	<.001
March	50	7	1.766	58.3	0.857	0.487-0.993	0.633	0.493-0.753	2.3	1.45-3.75	0.819	.007
April	44	7	1.898	79.1	1.000	0.646-1.000	0.954	0.845-0.992	21.5	4.98-56.0	0.993	<.001
May	46	9	1.619	41.6	0.889	0.565-0.994	0.978	0.887-0.999	40.9	5.80-281	0.931	<.001
June	45	8	1.381	24.0	0.875	0.529–0.994	0.682	0.534-0.800	2.8	1.67-4.54	0.855	.001
July	45	11	1.435	27.2	0.818	0.523-0.968	0.907	0.784-0.963	8.8	3.39-23.0	0.962	<.001
August	42	10	1.462	29.0	0.900	0.596-0.995	0.951	0.839-0.991	18.5	4.77-71.0	0.988	<.001
September	45	8	1.512	32.5	0.875	0.529–0.994	0.911	0.793-0.965	9.8	3.72-26.0	0.972	<.001
October	42	8	1.412	25.8	0.875	0.529–0.994	0.929	0.810-0.975	12.3	4.00-38.0	0.970	<.001
November	41	10	1.410	25.7	0.800	0.490-0.965	0.725	0.572-0.839	2.9	1.62-5.20	0.838	.001
December	40	10	1.387	24.4	0.800	0.490–0.965	0.800	0.652-0.895	4.0	2.00-8.00	0.899	<.001
Plasma post-TRH ACTH												
January	40	5	2.013	103.0	0.800	0.37.55-0.990	0.975	0.87.12-0.999	32.0	4.40-233	0.990	<.001
February	47	6	2.280	190.5	0.833	0.43.65-0.992	0.913	0.79.68-0.966	9.6	3.55-26.0	0.924	<.001
March	46	7	2.360	229.1	0.857	0.48.69-0.993	0.976	0.87.40-0.999	35.1	5.52-231	0.990	<.001
April	44	5	2.379	238.8	1.000	0.56.55-1.000	0.976	0.87.40–0.999	41.0	5.56-126	1.000	<.001
May	45	7	2.062	115.3	0.857	0.48.69–0.993	0.976	0.87.68-0.999	36.0	5.41-236	0.986	<.001
June	44	6	1.726	53.2	0.833	0.43.65–0.992	0.841	0.70.63-0.921	5.2	2.43-11.0	0.936	<.001
July	43	8	1.618	41.5	0.875	0.52.91-0.994	0.976	0.87.40–0.999	35.9	5.32-250	0.994	<.001
August	42	7	1.730	53.7	0.857	0.48.69–0.993	0.952	0.84.21-0.992	18.0	4.49-71.0	0.973	<.001
September	45	5	1.643	44.0	1.000	0.56.55–1.000	0.977	0.87.94-0.999	43.0	5.69-133	1.000	<.001
October	42	6	1.663	46.0	0.833	0.43.65-0.992	0.976	0.87.68-0.999	35.0	4.88-247	0.980	<.001
November	41	8	1.750	56.2	0.875	0.52.91–0.994	0.976	0.87.40-0.999	35.9	5.09-261	0.933	<.001
December	40	9	1.619	41.6	0.889	0.56.50-0.994	0.900	0.76.95-0.960	8.9	3.41-23.0	0.950	<.001

Note: Data were log-transformed to determine DCOVs, sensitivity (SN), specificity (SP), positive likelihood ratios (+LR), and accuracy (AUC).
Abbreviation: TRH, thyrotropin-releasing hormone.

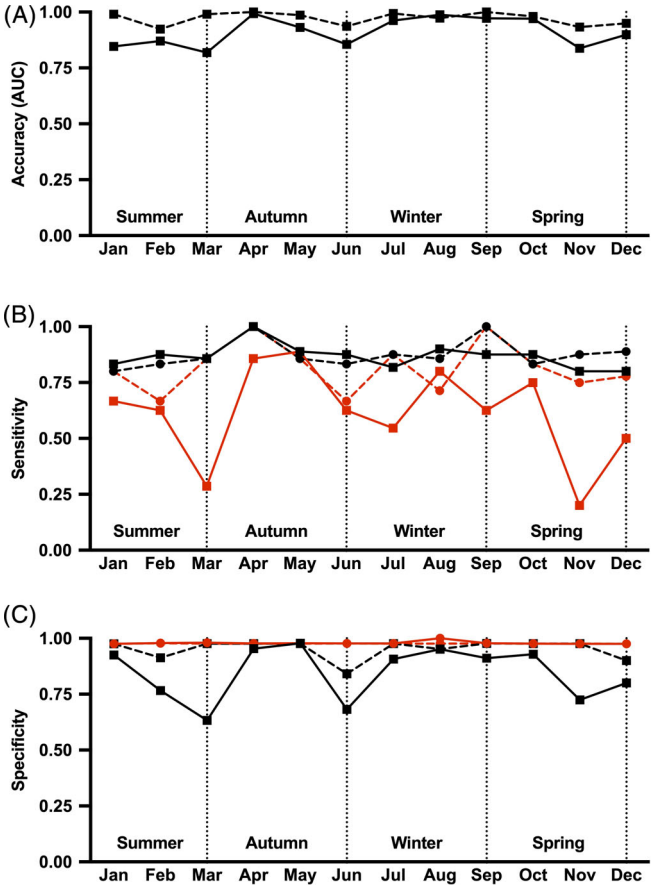

FIGURE 5 A, Monthly test accuracy for baseline (solid line) and post–thyrotropin-releasing hormone (TRH) stimulation (dotted line) ACTH concentrations in mature horses. B, Monthly test sensitivity for baseline (solid line) and post-TRH stimulation (dotted line) ACTH concentrations in mature horses using diagnostic cutoff values (in black) and upper limits of reference intervals (in red). C, Monthly test specificity for baseline (solid line) and post-TRH stimulation (dotted line) ACTH concentrations in mature horses using diagnostic cutoff values (in black) and upper limits of reference intervals (in red)

The effect of sex on plasma baseline and post-TRH ACTH concentrations has not been identified previously; however, other studies have suggested an increased hypothalamo-pituitary adrenal axis activity in females under sex hormone stimulation.[43] In several species, pregnancy is associated with increased cortisol concentrations with the placental production of corticotropin-releasing hormone, but none of the mares included in our study were pregnant and there is only limited evidence of this effect on baseline plasma ACTH concentrations in horses.[20,44-46] Determination of the estrus cycle of the sampled mares was beyond the scope of our study but would have potentially allowed the determination of an association between sex hormone and plasma ACTH concentrations.

An effect of a low BCS on plasma ACTH concentration was detected in autumn and winter. In horses older than 10 years of age, a correlation has been recorded between body mass index and alpha-melanocyte-stimulating hormone (α-MSH); however, unlike plasma ACTH in our study, horses with higher body mass index had higher α-MSH concentrations.[47] Alpha-MSH is a better marker of pars intermedia activity than plasma ACTH and its association with a high body mass index could be partially explained by how α-MSH mediates some of the effects of leptin, a marker of adiposity.[22,48,49] Although the reasons for the synchronous peaks in α-MSH and leptin concentrations are unknown, some authors have hypothesized that coexistent thrifty genes would be differently expressed for the preparation of the hypothalamo-pituitary adrenal axis and lipid metabolism to help during sparse periods.[47,50] This genetic component occurs in specific breeds, in which height and plasma ACTH concentrations are negatively correlated.[51] This could possibly explain why lower BCS horses, with possibly higher anabolic needs, could have higher plasma ACTH concentrations in early autumn. That being said, our suspicion was not confirmed with plasma post-TRH ACTH concentrations, with no significant effect identified. Although this lack of effect could be attributed to the small number of horses with a low BCS in our study, this finding should be interpreted with caution.

Plasma baseline ACTH concentrations have a good to excellent accuracy for the diagnosis of PPID; however, unlike reports of some studies, the accuracy of the test in the present study was lower in late summer and early autumn.[12,26] Regardless of the method used to make clinical decisions (DCOVs or RIs), the March +LR for plasma baseline ACTH concentrations was low indicating that during that month, the test could be of marginal diagnostic value, at least in subtropical locations in the southern hemisphere.[35] As mentioned above, this lower accuracy in March could be caused by the potential overlap between the control and the PPID populations limiting our ability to clearly separate the 2.[39] The TRH stimulation test significantly improved test accuracy regardless of the season. Although TRH receptors are found on both the pars distalis and the pars intermedia of the pituitary gland, plasma ACTH post-TRH stimulation reflects pars intermedia activity.[22] In healthy horses, ACTH post-TRH stimulation mainly originates from the pars distalis but this ACTH secretion is limited by glucocorticoid negative feedback.[52] In PPID horses, however, ACTH post-TRH stimulation mainly originates from the ACTH-secreting pars intermedia adenoma and is not limited by any loop of negative feedback leading to more dramatic increases.[53] In agreement with other studies, our data confirm the diagnostic advantage of the TRH stimulation test in the identification of PPID.[15,53]

As expected, determination of DCOVs yielded lower plasma baseline ACTH concentrations with a higher sensitivity, thus increasing the detection of positive cases. The specificities and +LR observed for several months indicate that, although similar DCOVs have been published by other groups, using those DCOVs could result in some false positive cases.[26] Nevertheless, considering the pretest probability of diagnosing PPID in a given population, lower +LR might still be of clinical relevance when interpreting data from a single animal.[54] On the other hand, determination of RIs yielded higher plasma baseline ACTH concentrations with a higher specificity and high +LR increasing the exclusion of negative cases with values similar to those found in other studies.[27,31] The poor sensitivities observed over most months, however, indicate that using those RIs will result in a large number of false negative cases. Although the use of those RIs has been advocated in

TABLE 2 Reference intervals (RIs) for immunoreactive plasma baseline and post-TRH ACTH concentrations determined monthly only considering data from control horses by a robust method, including Box-Cox transformation and bootstrap method

Months	n controls	RI log	RI (pg/mL)	90% CI				SN	95% CI	SP	95% CI	+LR	95% CI
Plasma endogenous ACTH													
January	43	1.605	40.3	1.571	37.2	1.631	42.8	0.667	0.300-0.941	0.975	0.871-0.999	26.7	3.80-188
February	48	2.015	103.5	1.892	78.0	2.121	132.1	0.625	0.306-0.863	0.979	0.889-0.999	29.4	4.01-221
March	50	2.140	138.0	2.036	108.7	2.211	162.7	0.286	0.051-0.641	0.980	0.893-0.999	14.0	1.48-138
April	44	1.970	93.3	1.887	77.1	2.042	110.1	0.857	0.487-0.993	0.977	0.879-0.999	36.9	5.31-262
May	46	1.612	40.9	1.574	37.5	1.642	43.8	0.889	0.565-0.994	0.978	0.887-0.999	40.9	5.80-281
June	45	1.654	45.1	1.592	39.0	1.724	53.0	0.625	0.306-0.863	0.977	0.882-0.999	27.5	3.76-197
July	45	1.531	34.0	1.457	28.7	1.602	40.0	0.546	0.280-0.787	0.977	0.879–0.999	23.5	3.28-172
August	42	1.520	33.1	1.459	28.8	1.588	38.7	0.800	0.490–0.965	1.000	0.914-1.000	18.5	4.15-1065
September	45	1.600	39.8	1.549	35.4	1.647	44.3	0.625	0.308-0.863	0.978	0.884-0.999	28.1	3.77-214
October	42	1.457	28.6	1.397	24.9	1.507	32.1	0.750	0.409-0.956	0.976	0.877-0.999	31.5	4.36-224
November	41	1.763	57.9	1.619	41.6	1.956	90.4	0.200	0.036-0.510	0.975	0.871–0.999	8.0	0.82-78.0
December	40	1.541	34.8	1.492	31.0	1.593	39.2	0.500	0.237-0.763	0.975	0.871–0.999	20.0	2.62-153
Plasma post-TRH ACTH													
January	40	2.002	100.5	1.903	80.0	2.117	131.1	0.800	0.376-0.990	0.975	0.871–0.999	32.0	4.40–233
February	47	2.420	263.3	2.331	214.2	2.510	323.7	0.667	0.300–0.941	0.978	0.887–0.999	30.7	4.15-221
March	46	2.394	247.7	2.327	212.2	2.450	281.7	0.857	0.487–0.993	0.976	0.874-0.999	35.1	5.52-231
April	44	2.381	240.6	2.293	196.2	2.458	287.4	1.000	0.566-1.000	0.976	0.874–0.999	41.0	5.56–126
May	45	2.063	115.6	1.992	98.1	2.133	135.9	0.857	0.487–0.993	0.976	0.877–0.999	36.0	5.41-236
June	44	1.866	73.5	1.807	64.1	1.919	82.9	0.667	0.300–0.941	0.977	0.882–0.999	29.3	3.89-216
July	43	1.631	42.8	1.589	38.8	1.673	47.1	0.875	0.529–0.994	0.976	0.874–0.999	35.9	3.90-216
August	42	1.784	60.8	1.703	50.4	1.855	71.7	0.714	0.359-0.949	0.976	0.877–0.999	30.0	4.21-210
September	45	1.662	45.9	1.631	42.7	1.692	49.2	1.000	0.566–1.000	0.977	0.880-0.999	43.0	5.55-136
October	42	1.678	47.6	1.622	41.9	1.731	53.8	0.833	0.437–0.992	0.976	0.877–0.999	35.0	4.88-247
November	41	1.789	61.6	1.714	51.8	1.852	71.1	0.750	0.409–0.956	0.976	0.874–0.999	30.8	4.26-229
December	40	1.751	56.3	1.673	47.1	1.820	66.1	0.778	0.453-0.967	0.975	0.871–0.999	31.1	4.35-222

Note: Data were log-transformed for the analysis and sensitivity (SN), specificity (SP), and positive likelihood ratios (+LR) were determined based on the receiver operating characteristic curves presented in Table 1.

Abbreviation: TRH, thyrotropin-releasing hormone.

some studies for a diagnosis of PPID, sensitivities as low as 0.2 would severely limit the screening value of such a test.[27]

Clinically, the use of DCOVs and RIs for plasma baseline ACTH concentrations could therefore serve different objectives in PPID testing. Diagnostic cutoff values would be relevant in cases in which missing a diagnosis of PPID could have detrimental effects. For example, in older horses with insulin dysregulation, where early detection of PPID and early onset of treatment would be associated with improved welfare.[4,55,56] Reference intervals, on the other hand, would be relevant when the clinical picture is inconsistent with PPID, when pergolide treatment could have some career-limiting consequences due to the controlled nature of the substance in athletic horses, or when the owner has limited financial resources or poor compliance.[57] These results emphasize the necessity of a clinical context and a diagnostic goal discussed between the veterinarian and the owner when testing a horse for PPID.

Similarly to increasing test accuracy, the TRH stimulation test increased the specificity of the DCOVs, limiting false positive cases, and the sensitivity of the RIs, limiting false negative cases.[15] As previously described, the magnitude of the ACTH response post-TRH stimulation was different between control and PPID cases allowing a better differentiation between the 2 populations.[39,53] Nevertheless, and as previously reported, there was some variation in the results of the TRH stimulation in control horses.[31] This variation was greater in autumn but was lower than previously reported and the excellent accuracy would suggest that, even in autumn, the test would perform well.[31] The difference between our results and others might be explained by the higher number of horses used in our study and the older age of our controls. This improvement of test characteristics would therefore justify the use of a TRH stimulation test in clinical practice to optimize the diagnosis of PPID, irrespective of season, always considering the clinical context in which the test is performed.

There are major limitations to our study. The first limitation is the definition of a control and a PPID case. Although necropsy confirmation of diagnosis was obtained in only 13 cases, all other horses received frequent physical examinations and endocrine testing for more than 2 years to ensure they were properly classified as either PPID positive or negative. The classification was based on the inclusion criteria of other studies and considered conservative to limit the risks of false diagnoses.[4,26,27] Nevertheless, it is possible that, in absence of an antemortem gold standard, some horses could have been misclassified. Considering that the aim of the study was to compare the test characteristics and the clinical consequences of using DCOVs and RI for a diagnosis of PPID rather than determining detailed guidelines based on specific values, the consequences of misclassified cases would have been negligible. A second limitation of the study is the number of cases included and the lack of consistency of inclusion of individual horses from month to month. Although our study regroups data from more horses than previous studies, and more than 40 control cases were included every month, the ideal number of 120 cases was not reached.[26,27] The analytical methods were therefore adapted to reduce the impact of this limitation.[34] A larger sample size could have led to stronger evidence regarding the effect of age, sex, BCS, and potentially breed on plasma ACTH concentrations. Ponies were excluded from the study as there is mounting evidence that ponies are metabolically different from horses with higher ACTH concentrations.[30] Therefore, our results should be limited to full sized horses and not be extrapolated to ponies which will require their own seasonally adjusted values. This limitation could be extended to the heterogeneity in the breeds included in this study. On 1 hand, it does reflect the general horse population but on the other hand, as some interbreed differences have been reported, it might limit the clinical value of those RIs and DCOVs.[30] The diet of horses was not documented; however, diets richer in carbohydrates can increase plasma ACTH concentrations.[13] Because some client-owned horses were included in the study, it is possible that some differences could be attributed to diet and that some cases could have been therefore misclassified. However, considering that most horses were kept on pasture at the authors' institution, the diet-induced variability was deemed limited yet possible. Another limitation of the study is that only a 30-minute post-TRH stimulation sample was obtained while a 10-minute post-TRH stimulation sample has been used previously in various studies.[31] It is our understanding that the diagnostic values of 10-minute and 30-minute samples are similar; however, our results at 30 minutes might not apply to the 10-minute timepoint. Finally, being a prospective case control study and having excluded outliers, a bias in the accuracy of the tests evaluated is possible and further research is warranted to better document the test characteristics of the plasma baseline and post-TRH ACTH concentrations to diagnose PPID.

In conclusion, our study confirms that determination of plasma ACTH concentrations is an accurate antemortem diagnostic tool for PPID. Provided that appropriate RIs and DCOVs are developed and used, interpretation of a given plasma ACTH concentration must consider the clinical context, including, but not limited to, age, sex and BCS, and the goals of the owners. Based on those goals, the use DCOVs would be recommended to improve the detection of early cases of PPID while the use RIs would be recommended to ensure no horse is treated unnecessarily. In both cases, use of the TRH stimulation test is recommended, irrespective of season, to improve diagnostic performance.

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CONFLICT OF INTEREST DECLARATION

Elizabeth L. Dryburgh is employed by Boehringer-Ingelheim Pty Ltd and François-Rene Bertin and Allison J. Stewart have consulted for Boehringer-Ingelheim Pty Ltd.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Approval from The University of Queensland IACUC, SVS/266/17.

HUMAN ETHICS APPROVAL DECLARATION

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

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