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Circannual variation in plasma adrenocorticotropic hormone concentrations in the UK in normal horses and ponies, and those with pituitary *pars intermedia* dysfunction

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Summary

Reasons for performing study: Pituitary *pars intermedia* dysfunction (PPID) is a common endocrinopathy, frequently diagnosed via plasma adrenocorticotropic hormone (ACTH) concentrations. Seasonal variation in plasma ACTH concentrations has been described in normal horses prompting caution in diagnosing PPID at certain times of the year. The aims of this study were to determine appropriate reference intervals for equine plasma ACTH throughout the year; and to examine the circannual variation of plasma ACTH concentrations in PPID cases.
Hypothesis: Plasma ACTH can be used as a test for PPID throughout the year with the use of appropriate reference intervals.
Methods: Data for reference interval calculations were obtained from samples collected from inpatients of Liphook Equine Hospital (non-PPID group, n = 156). Data from PPID cases (n = 941) were obtained from samples submitted to the Liphook Equine Hospital Laboratory from horses with a clinical suspicion of PPID found to have plasma ACTH concentrations greater than our upper reference interval for that time of year.
Results: Upper limits for reference interval of plasma ACTH were 29 pg/ml between November and July and 47 pg/ml between August and October. Circannual variation in plasma ACTH occurred in both non-PPID and PPID horses with the highest ACTH concentrations found between August and October in both groups (P<0.0001). The greatest difference between the 2 populations also occurred between August and October.
Conclusions: Plasma ACTH can be used for the diagnosis and monitoring of PPID throughout the year with the use of appropriate reference intervals. These findings demonstrate an increase in pituitary gland secretory activity during the late summer and autumn in both normal and PPID cases.

Keywords: horse; PPID; ACTH; seasonality; reference interval

Introduction

Pituitary *pars intermedia* dysfunction (PPID, equine Cushing's disease) is a common endocrinopathy in horses and ponies (McGowan *et al*. 2007). The condition is thought to result from a loss of dopaminergic inhibition of the *pars intermedia* (McFarlane *et al*. 2005a) and is strongly associated with an increase in plasma concentration of adrenocorticotropin hormone (ACTH) (van der Kolk *et al*. 1995). Clinical signs of PPID include hirsutism, abnormal hair shedding, laminitis, polyuria, polydipsia, muscle wasting, lethargy, recurrent infections and abnormal fat deposition (supraorbital fossae, neck crest, tail head, hindquarters) (Schott 2002).

The most commonly utilised laboratory tests for the diagnosis of PPID include the dexamethasone suppression test (DST) (Dybdal *et al*. 1994) and measurement of basal plasma ACTH concentrations (van der Kolk *et al*. 1995). However, there has been increasing recognition of seasonal variation in pituitary gland secretory activity, which may affect the interpretation of endocrine tests. Donaldson *et al*. (2005) noted an increase in plasma ACTH concentrations above reference intervals and positive DST results in clinically normal horses in Pennsylvania in September. Positive DST results have also been reported in normal horses in Michigan especially between the months of July and October (Schott *et al*. 2007).

A more recent study in the USA has confirmed seasonal variation in plasma ACTH concentrations in normal horses and ponies and those with PPID, and established that increases in plasma ACTH concentrations were associated with decreasing day length after the summer solstice (Beech *et al*. 2009). Frank *et al*. (2010) demonstrated an increase in plasma ACTH in normal (n = 9) and PPID (n = 8) cases, in the months of August, September and October. Lee *et al*. (2010) compared quarterly plasma ACTH concentrations in a small number of PPID and non-PPID cases, in the UK. They found an increase in plasma ACTH for their whole population in September and December, and suggested the use of seasonal reference intervals at different times of the year when testing for PPID.

The studies outlined above indicated that interpretation of plasma ACTH concentrations could yield false positive results at certain times of the year, with potentially expensive and unnecessary treatment for equine patients. Furthermore, seasonal variation in plasma ACTH concentrations might also affect monitoring of PPID cases and assessment of their response to therapy. Hence, seasonally adjusted reference intervals for plasma ACTH concentrations should be considered to improve the probability of an accurate diagnosis and to facilitate patient monitoring throughout the year (Toribio 2005; Frank *et al*. 2010).

Following warnings over months during which diagnostic testing for PPID should be avoided, we aimed to assess the validity of performing endocrine testing throughout the year. Thus, the objectives of the study were to describe, in detail, the circannual variation in plasma ACTH concentration in non-PPID equines throughout the year in the UK to determine appropriate reference intervals for use throughout the year. We also aimed to investigate the circannual variation of plasma ACTH in PPID cases in a far larger population than previously reported and diagnosed using properly defined circannual reference intervals.

Materials and methods

Sample collection for the calculation of reference intervals

The non-PPID group were selected from mature (age ≥4 years) horses and ponies from the in-patient population at Liphook Equine Hospital (LEH) that required blood collection for routine clinical monitoring unrelated to this study. Inclusion criteria comprised admission to the hospital >48 h previously and no clinical signs or history of PPID. Subjects were excluded if they were suffering from clinically detectable pain or systemic disease, or were overtly anxious or unsettled in their environment. Samples were taken whenever available from suitable in-patients, from 10.00–15.00 h, from 1 January 2009 to 31 December 2009. Blood was collected from the jugular vein into evacuated plastic vials containing potassium-EDTA, separated immediately via a centrifuge (5000 ***g*** for 5 min) and the supernatant plasma placed in polypropylene tubes. Plasma ACTH concentrations were measured within 2 h of sampling, using a chemiluminescent immunoassay (Immulite ACTH)[1], previously validated for equine plasma (Perkins *et al*. 2002).

Examination of circannual variation in PPID cases

The PPID group were identified from submissions to the LEH laboratory. These comprised horses and ponies with a clinical suspicion of PPID from which EDTA-plasma was received in specialised chilled packages by overnight post, with plasma ACTH concentrations found to be greater than the upper limit of the reference interval determined from the non-PPID group above. Horses were excluded if currently being treated medically for PPID.

Examination of the diagnostic usefulness of plasma ACTH throughout the year

As an indicator of comparative monthly diagnostic usefulness, data were plotted to illustrate the differences between median monthly plasma ACTH concentrations in both groups and the upper limit of the reference interval for plasma ACTH.

Data analysis

Data were checked for normality with the D'Agostino-Pearson test. Monthly data were compared within groups using the nonparametric Kruskal-Wallis with Bonferroni multiple comparisons test. The Mann-Whitney test was then used to compare data from the 2 resulting distinct time periods. Upper 97.5% limits of reference intervals were calculated from a normal approximation of data and also by Horn's robust method (Horn *et al*. 1998). Statistical analysis was performed using MedCalc 11.5.0.0 software[2]. Statistical significance was assumed when $P \leq 0.05$.

Results

Calculation of reference intervals

The non-PPID group contained 156 individual subjects with a mean ± s.d. age of 10.3 ± 4.7 years (range 4–28 years), comprising 50 mares, 104 geldings and 2 stallions. Summary data of monthly ACTH concentrations are presented in Figure 1.

Comparison of plasma ACTH concentrations between each month in the non-PPID group revealed significant differences between the months of August compared with each of the months from November to July; September compared with each of the months from November to July; and October compared with each of the months from November to July (Kruskal-Wallis P<0.0001; Bonferroni P<0.05). There were no significant differences between the months of August, September and October (Kruskal-Wallis P = 0.306). There were no significant differences between any of the months between November and July, except in the comparison between June and November (Kruskal-Wallis P = 0.005).

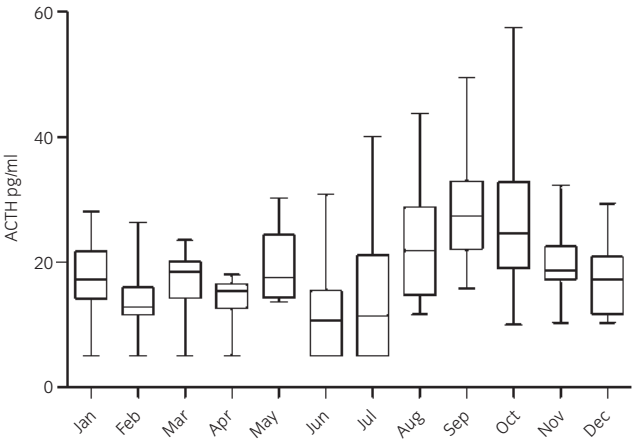

Fig 1: Box and whisker depiction of monthly plasma ACTH concentrations in the non-PPID group (boxes represent upper and lower quartiles with horizontal lines representing minimum, maximum and median values). Sample sizes: Jan n = 12, Feb n = 12, Mar n = 12, Apr n = 12, May n = 5, Jun n = 13, Jul n = 12, Aug n = 13, Sep n = 18, Oct n = 17, Nov n = 15 and Dec n = 15.

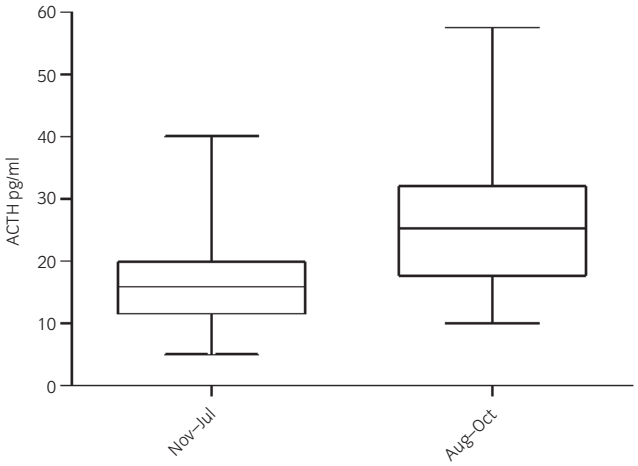

Fig 2: Box and whisker depiction of plasma ACTH concentrations for 2 time periods in the non-PPID group (boxes represent upper and lower quartiles with horizontal lines representing minimum, maximum and median values; Nov–Jul vs. Aug–Oct P<0.0001).

On the basis of these findings, data were divided into 2 distinct periods, namely August–October and November–July. The plasma ACTH concentrations for November and June were included into the period November–July rather than splitting the data into shorter time periods. Monthly data were pooled in this manner because June and November were not significantly different from any other months from the period November–July (Kruskal-Wallis: June P = 0.162; November P = 0.152), and June and November were significantly different from August, September and October (Kruskall-Wallis P<0.0001; Bonferroni P<0.05).

When data were pooled into the 2 time periods and compared, plasma ACTH concentration between August and October was significantly greater than plasma ACTH concentration between November and July (P<0.0001). Summary data for each of the 2 periods are presented in Figure 2.

Data from these 2 periods were then used to calculate 2 separate reference intervals for plasma ACTH (Table 1). When rounded to single units, the upper 95% CI of the upper limit for plasma ACTH using 2 different methods (normal distribution and Horn's robust method) produced the same results, and therefore upper limits were adopted for plasma ACTH of 47 pg/ml for August–October, and 29 pg/ml for November–July.

Examination of circannual variation in PPID cases

From a total of 1497 chilled plasma samples submitted during 2009 from untreated horses with a clinical suspicion of PPID, 556 (37%) were rejected since the plasma ACTH concentrations were within our reference interval for the time at which they were collected (as described above). A total of 941 positive PPID samples were retained in the PPID group. Comparison of

TABLE 1: A summary of the reference interval calculations for plasma ACTH concentrations for 2 distinct time periods in the non-PPID group using the parametric method and Horn's Robust method. 'Right sided' (upper limit only) analysis used for both methods determining upper limit of reference interval with 95% CI (confidence interval) of this upper limit

		Plasma ACTH concentration (pg/ml)			
		Normal distribution		Robust method	
Period	n	Upper limit	95% CI	Upper limit	95% CI
August–October	48	43.1	38.9–47.4	42.4	37.6–47.3
November–July	108	27.2	25.4–29.1	27.0	25.2–28.7

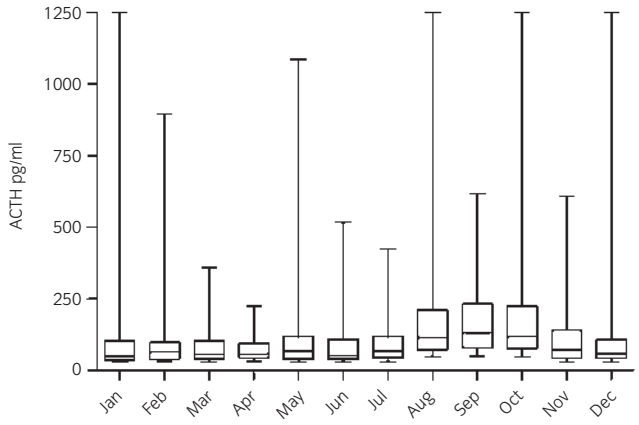

Fig 3: Box and whisker depiction of plasma ACTH concentrations (pg/ml) by month for the PPID group (boxes represent upper and lower quartiles with horizontal lines representing minimum, maximum and median values). Sample sizes: Jan n = 54, Feb n = 28, Mar n = 56, Apr n = 38, May n = 42, Jun n = 59, Jul n = 70, Aug n = 64, Sep n = 124, Oct n = 127, Nov n = 152 and Dec n = 127.

data between each month in the PPID group again revealed 2 significantly distinct periods exactly coincident with those described above for non-PPID horses (November–July and August–October; Kruskall-Wallis P<0.0001; Bonferroni P<0.05). Plasma ACTH concentration in August–October (median 120.0 pg/ml, interquartile range 76.8–223.0) was significantly greater than plasma ACTH concentration in November–July (median 61.0 pg/ml, interquartile range 40.8–118.0) (P<0.0001). A summary of the monthly plasma ACTH concentrations (including minimum, interquartile range, median and maximum) are presented in Figure 3.

Examination of the diagnostic usefulness of plasma ACTH throughout the year

A graphical illustration of the monthly median plasma ACTH concentrations in the non-PPID and PPID groups suggested that the greatest difference between the 2 groups was in the months of August, September and October (Fig 4).

Discussion

This study found that an upper limit for the reference interval for plasma ACTH of 29 pg/ml was appropriate to use between November and July, and 47 pg/ml between August and October, in the population of horses and the laboratory methodology used here (Table 1). The study also showed that circannual variation in plasma ACTH concentrations occurred in both non-PPID and PPID horses and ponies in the UK at identical times of the year (August–October) (Fig 4). To the authors' knowledge, description of the circannual plasma ACTH concentration of PPID cases has not been published previously in such a large population of horses throughout the year. The circannual variation reported here is temporally consistent with previous findings of seasonal variation in ACTH concentration in PPID and non-PPID cases (Donaldson *et al*. 2005; Beech *et al*. 2009; Frank *et al*. 2010; Lee *et al*. 2010; Place *et al*. 2010). Furthermore, our upper limits for the reference interval of plasma ACTH (29 and 47 pg/ml) are similar to previously reported (noncircannual) reference intervals of 35 and 55 pg/ml (van der Kolk *et al*. 1995; Donaldson *et al*. 2005). One limitation of this study was that the seasonal variation in plasma ACTH was determined from data collected over a single year. A longitudinal study investigating circannual variation in ACTH over several years would be interesting to establish if observed effects are consistent year by year.

In this study, increased plasma ACTH concentrations were apparent from August to October, when in the UK, day-lengths are reducing (Figs 1, 3 and 4). This supports previous evidence that increased plasma ACTH concentrations correlate with periods of decreasing daylight, rather than the absolute duration of the photoperiod *per se* (Beech *et al*. 2009). Additionally, in this study, the circannual pattern was remarkably similar in PPID and normal horses, suggesting that the photoperiodic regulation of the pituitary gland appears to be conserved in PPID cases. Donaldson *et al*. (2004) reported that laminitis was more likely to develop during the month of September than any other month of the year in PPID cases, but not those without PPID. The increase in pituitary gland secretory activity in PPID cases from August ot October is consistent with the increased incidence of clinical signs such as laminitis during this period, and might suggest consideration of the use of increased doses of dopamine agonists at this time to control seasonal pituitary hypersecretion. Further investigation is needed to understand the neuroendocrine regulation of pituitary secretion in response to photoperiodic change in clinically normal horses and those with PPID.

Previous advice is that diagnostic testing for PPID should be avoided in the autumn due to seasonal variability in plasma ACTH concentrations and a high risk of false positive diagnoses (Donaldson *et al*. 2005; Toribio 2005). Additionally, it has been suggested that fluctuations in plasma ACTH concentrations could confound the monitoring of horses with PPID and affect assessment of response to dopamine agonist therapy (Beech *et al*. 2009). In this study, circannual reference intervals were created, permitting the diagnosis and monitoring of PPID cases throughout the year. Moreover, this study demonstrated that the greatest difference in plasma ACTH concentrations between non-PPID and PPID horses was found between August and October (Fig 4). Therefore, the greatest discriminatory power of plasma ACTH concentrations for the diagnosis of PPID may well be during the period previously advised to avoid and the current study suggests that, with the application of circannual reference intervals, this period may well be the *most* appropriate time to test for PPID.

Of the wide choice of endocrine tests available for the diagnosis of PPID, plasma ACTH concentration was selected as the subject of this study for several reasons. Firstly, the result obtained from the test is a continuous numerical variable, for which circannual reference intervals can be calculated. In contrast, the DST provides a binary, qualitative result that is not readily amenable to adjustment for seasonal variation in pituitary secretory activity. Secondly, in the experience of these authors, plasma ACTH concentration is the most frequently utilised diagnostic test for PPID in the UK. Of all submissions to the Liphook Equine Hospital Laboratory for endocrine testing for PPID in 2010, 98.8% requested plasma ACTH concentration (K. Barker, unpublished data).

Interpretation of clinical laboratory data is a comparative decision-making process, which necessitates reference intervals to distinguish affected from nonaffected individuals (Horowitz *et al*. 2008). Standard methods for determining reference intervals are to define a nonaffected population and use robust nonparametric or parametric estimates of the 95% confidence interval (Horn *et al*. 1998; Horn and Pesce 2003). Additionally, some authors recommend comparing reference intervals obtained by several different approaches to ensure they are in

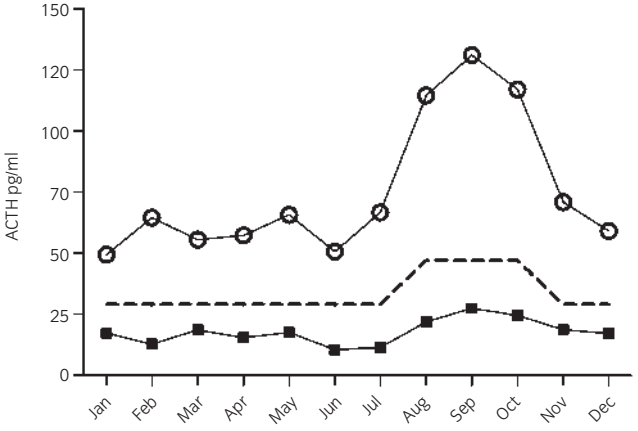

Fig 4: Graphical comparison of the median plasma ACTH concentration (pg/ml) by month for the PPID groups (circles) and non-PPID group (boxes). The dashed line represents the upper limit of the reference interval for plasma ACTH.

agreement (Geffre *et al*. 2009). In this study, both robust nonparametric and parametric methods were applied to determine reference intervals for both time periods, and the results were in fact very similar (November–July: upper limits of 28.7 and 29.1 pg/ml, respectively, and for August–October: upper limits of 47.3 and 47.4 pg/ml, respectively, Table 1).

One criticism of the non-PPID group selection in this study is that the samples were taken from in-patients at an equine hospital, rather than from horses in their home environment. Therefore, they could have been susceptible to increases in endogenous ACTH secretion caused by factors such as anxiety associated with admission to the hospital, systemic disease or pain. However, we minimised the possibility of these putative influences by excluding in-patient samples from the group if the horse was overtly anxious, in pain or suffering from systemic illness. Couetil *et al*. (1996) reported that hospitalised horses with mild systemic illnesses had plasma ACTH concentrations within their reference interval. Towns *et al*. (2010), found only moderately and severely ill horses had mean ACTH concentrations that were above our reference intervals. Furthermore, it is improbable that hospitalisation alone increased plasma ACTH concentrations, since our circannual reference intervals were in fact slightly lower than other published reference intervals (van der Kolk *et al*. 1995; Donaldson *et al*. 2005; Beech *et al*. 2009). It would have been impractical to use blood samples taken from normal horses in their home environment and submitted to the laboratory for other reasons due to the special transport and storage requirements for plasma ACTH samples.

One further possible source of error in the selection of the non-PPID group could be the inadvertent inclusion of subclinical PPID cases. Further tests that might have been applied to detect such subjects include *post mortem* examination and the DST, however, studies have shown that neither of these tests provides an entirely reliable diagnosis of PPID and were impractical given the restrictions of clinical practice (Miesner *et al*. 2003; Donaldson *et al*. 2005: McFarlane *et al*. 2005b; Schott *et al*. 2007).

Cases were selected on the basis of clinical suspicion of PPID, combined with an increase in plasma ACTH above our reference interval, which has been shown to be a reliable means of diagnosis in previous studies (van der Kolk *et al*. 1995). Clearly, further confirmatory testing (e.g. DST or *post mortem* examination) would have been preferable but was impractical within the constraints of this study. The patients in the PPID group, were not age matched against individuals in the non-PPID group, and those in the PPID group tended to be older animals. However, ageing itself is not associated with an increase in ACTH concentration, unless the patient has a dysfunctional pituitary *pars intermedia* (McGowan *et al*. 2007).

In conclusion, these results support our hypothesis that, with the use of appropriate circannual reference intervals, basal ACTH concentration can be used as a diagnostic test for PPID throughout the year and especially during the months of August, September and October.

Authors' declaration of interests

No conflicts of interest have been declared.

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Manufacturers' addresses

[1]DPC-Cirrus, Los Angeles, California, USA.
[2]MedCalc Software bvba, Mariakerke, Belgium.

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Author contributions Both authors contributed to the initiation, conception, planing, execution, statistical analysis and writing for this study.