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Influence of feeding and other factors on adrenocorticotropin concentration and thyrotropin-releasing hormone stimulation test in horses and ponies

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Abstract

Background: The basal (bACTH) and post-thyrotropin-releasing hormone stimulation concentration of adrenocorticotropin (pACTH) are recommended for diagnosis of pituitary pars intermedia dysfunction (PPID). Many factors influence bACTH (e.g., disease, age, month) and some affect the results only in autumn (e.g., breed, colour, sex). There are discrepancies about the impact of feeding on b/pACTH.

Objectives: To determine whether feeding, month, age, breed, colour, sex and body condition score affect b/pACTH.

Study design: Prospective crossover.

Methods: Sixty-one animals were divided into groups: healthy, PPID, treated-PPID. The b/pACTH was measured three times (1 mg protirelin; blood collection after 10 min; mid-November to mid-July) after different feedings: fasting, hay, hay + grain. Friedman's test was applied to evaluate the influence of feeding on b/pACTH and linear mixed model to evaluate impact of further factors.

Results: The b/pACTH was not significantly affected by feeding ($p = 0.7/0.5$). The bACTH was lowest in healthy (29.3 pg/mL, CI 9–49.5 pg/mL) and highest in PPID-group (58.9 pg/mL, CI 39.7–78.1 pg/mL). The pACTH was significantly lower in healthy (396.7 pg/mL, CI 283.2–510.1 pg/mL) compared to PPID (588.4 pg/mL, CI 480.7–696.2 pg/mL) and treated-PPID group (683.1 pg/mL, CI 585.9–780.4 pg/mL), highest in July (881.2 pg/mL, CI 626.3–1136.3 pg/mL) and higher in grey (723.5 pg/mL, CI 577.5–869.4 pg/mL) than other colours (338.7 pg/mL, CI 324.8–452.5 pg/mL). The size of effect for those variables was >0.5.

Main limitations: Small number of animals, subsequent bACTH measurements were significantly lower in each horse.

Conclusions: There was no evidence that feeding influences the b/pACTH. There was evidence that pergolide affects the bACTH but it had little effect on pACTH. Further investigation of the impact of month and coat colour on b/pACTH is warranted to better interpret the results.

KEYWORDS
ACTH, adrenocorticotropin, endocrine, horse, PPID, TRH

1 | INTRODUCTION

Generalised hypertrichosis is considered a pathognomonic clinical sign in horses with pituitary pars intermedia dysfunction (PPID) and treatment without further testing is considered appropriate.[1,2] Unfortunately, changes in haircoat are usually manifested in the late stage of the disease and give no measure of the severity.[3] The measurement of the basal adrenocorticotropin concentration (bACTH) and thyrotropin-releasing hormone stimulation test (TST) with ACTH measurement (post-stimulation ACTH concentration: pACTH) are the most accurate diagnostic methods for PPID currently described.[4] The TST shows better than bACTH accuracy reaching 97% and is preferred for early case detection.[1,5–8] TST is recommended after feeding hay only[1] as grain was described to increase[9] and fasting to decrease the b/pACTH concentration.[10,11] Feeding was also described to increase bACTH secretion and consequently the cortisol concentration in other mammals.[12] However, studies are inconsistent and other authors showed no differences in TST results between fasted and fed horses.[11]

There are generally many factors known to affect the bACTH, but only a few have been described for the pACTH. The bACTH is influenced by the time of the year,[13,14] thus, there are season-specific reference ranges facilitating the interpretation.[1] The TST is not recommended in the late summer and autumn as the normal values have not yet been reliably studied.[1,14] Ageing, severe disease, acute pain and stress also increase the bACTH.[15–18] The significant effect of breed, colour and sex on the bACTH was also shown, with ponies, grey horses and mares having a higher bACTH in the autumn.[17,19] Studies investigating the influence of the body condition score (BCS) on the bACTH are lacking, however, it warrants further investigation as obesity and appetite correlates with dopaminergic tone in humans.[20] To the best of our knowledge, the effects of the above-mentioned factors on the pACTH have not been studied.

The aim of this study was to determine whether feeding 12 h prior to testing affects the bACTH and pACTH in endocrinologically healthy horses and ponies and those with PPID between mid-November to mid-July. Additionally, the influence of the month of testing, pony breed, grey colour, female sex, BCS and age were studied. We hypothesised that (1) feeding and age will affect the bACTH and pACTH, but (2) the month of testing, pony breed, grey colour, female sex and BCS will not affect the results of either test in the non-autumn months.

2 | MATERIALS AND METHODS

2.1 | Animals

The animals were enrolled in the study by their owners voluntarily, upon signing the consent form for the animal use in the research project. Clinically healthy horses and ponies of all breeds, older than 16 years, were included in the study. The breed, sex, age, colour, clinical signs and treatment were documented. Ponies were defined as animals with a certain pedigree (e.g., Shetland pony, Welsh A, German riding pony) or ponies of mixed breed measuring less than 148 cm at the withers (according to the International Federation of Equestrians) and weighing less than 250 kg. The weight was obtained by scale or was estimated using a measuring band (The Laminitis Clinic Weight-ape, EquiLife). The BCS (1–9) was assessed according to Henneke et al.[21]

Animals were divided into three groups based on clinical signs, treatment with pergolide and the bACTH (seasonally adjusted reference values from the Equine Endocrinology Group (2019)[22] were applied): (0) healthy, (1) PPID or (2) PPID treated with pergolide. There were horses and ponies in the healthy group without clinical signs typical of PPID (hypertrichosis and laminitis as exclusion criteria) and normal or questionable results of the bACTH (<50 pg/mL). Animals in the PPID group showed hypertrichosis and/or delayed shedding as an owner complaint or during the examination, and at least one more clinical sign described in PPID cases (e.g., polyuria/polydipsia, muscle loss, increased sweating, fat deposits, lethargy, susceptibility to infections).[2,23–25] The bACTH was increased or questionable (>30 pg/mL). Equids in the treated-group were diagnosed with PPID based on the bACTH or pACTH in the past and received pergolide mesylate (Prascend, Boehringer Ingelheim or Pergoquin, WDT) orally once daily for at least 4 weeks. The dosage was individually chosen for each case.

2.2 | TRH stimulation test

A short clinical examination (heart and respiratory rate, temperature) was performed before all tests. Each animal received 1 mg of protirelin (TRH Ferring 0.2 mg/mL, Ferring GmbH) intravenously and was observed for 30 min afterwards. All side effects were documented. The blood was collected in tubes with ethylene diamine tetraacetic acid just before and exactly 10 min after the injection. The samples were kept in a cooling box with ice packs (not refrigerated) and centrifugated within 2 h. The ACTH was measured in cooled plasma within 24 h by an external laboratory (Laboklin Laboratory for Clinical Diagnostics GmbH &Co KG) with Immulite 2000™ (Siemens Healthcare Diagnostics; solid-phase, two-site sequential chemiluminescent immunometric assay with analytic sensitivity: 5 pg/mL, intraassay coefficient of variation: 2.97, interassay coefficient of variation: 8.92%).[26] All tests were performed between mid-November and mid-July[22] in the morning hours (5–12 AM). The results were interpreted according to the recommendation of the Equine Endocrinology Group from 2019.[22]

2.3 | Study design

The study was based at an equine clinic in Germany (latitude of 52.520008°N). It had a prospective, crossover design and each animal underwent three TSTs after different overnight feeding protocols: (A) hay ad libitum, (B) hay ad libitum + grain (oat, 2% of the bodyweight, 2 h prior to testing), or (C) fasting (muzzle or box with shavings). The order was stratified randomised to obtain equal number of feeding protocol sequences in each group (blind selection of an envelope with one of the six possible feeding orders for each horse; the envelopes were reused every six horses). At least 2 weeks of washout period was provided between the tests. Animals were allowed to stay at home and perform at the usual level.

Sample size calculation was done for bACTH. The hypothesis tested was that the feeding (independent variable; fasting, hay, hay + grain) affects the bACTH (dependent factor). The examination of 20 healthy, 20 with PPID affected and 20 treated-PPID animals allows to detect the differences of 2× standard deviations with a certainty of 95% and the power of 95%.

2.4 | Data analysis

The statistical analysis was performed separately for the bACTH and pACTH using IBM® SPSS Statistics Version 29 (SPSS Inc.). The analysis of variance (related-samples Friedman's two-way analysis of variance by ranks) was applied to examine the influence of the feeding on the tests in each group. In the linear mixed models, horse, interaction horse*group and horse*feeding were included as random effects, while group, feeding, test order, month of testing, BCS, breed (pony effect), sex (mare effect), colour (grey effect), age and the interaction group*feeding were fixed effects. The b/pACTH were dependent variables. Bonferoni test was used as a post hoc analysis. The level of significance was set at $p < 0.05$. Normality and homoscedasticity of residuals were checked by visual inspection. Since the interactions of horse*group and horse*feeding were not significant, they were removed leaving horse as random effect in the final model. The effect size of variable was calculated, where appropriate.[27] The assay upper limit of detection of ACTH in the external laboratory selected was 1250 pg/mL, thus, the higher results were changed to 1251 pg/mL to facilitate the statistical analysis.

3 | RESULTS

Sixty-two examinations were performed between January 2020 and February 2022. One horse from the healthy group underwent only two out of three TSTs and was lost for the third test due to COVID-19 restrictions, therefore, it was excluded from the study. As a result, 61 examinations consisting of three TSTs each, were included (Table 1). There were 53 animals, as 8 horses were enrolled in the study twice—they started in healthy group and were included in the treated-group after the treatment with pergolide. The duration of treatment with pergolide in the treated-PPID group was recorded for 18/20 animals (two were treated for longer than a year and the owners did not provide a beginning date) and the average was 233 days (range 29–1177 days). The mean dosage of pergolide was 0.7 mg (range 0.25–2 mg). There were 33 horses and 20 ponies, 22 mares and 31 geldings, 6 grey horses and 47 of different colours. The average BCS and the average age were similar between groups (Table 1).

The TST was performed successfully in all horses. However, in two uncooperative animals there was a delay of up to 1 min in the blood collection after TST. Three animals had one pACTH result outside the detectable limit and one horse had pACTH >1250 pg/mL after all feedings, so there were six measurements above the detectable limit. All bACTH were <1250 pg/mL.

The side effects observed included sneezing (27.9%), lip smacking (27.3%), itching (rubbing the nose or rolling; 21.3%), passing faeces (18%, including 9.8% single episodes of loose faeces), flatulence (16.4%), pawing (15.3%), urination (12.0%), yawning (11.5%), coughing (10.9%), shaking out (8.7%), flehmen response (7.7%) and trembling (2.7%). Most of the observations were noticed and disappeared within 10 min. One horse showed mild colic signs 7 h after the test, which resolved after an injection of metamizole (15 mg/kg intravenously; Novaminsulfon, 500 mg/mL, bela-pharm GmBH & Co. KG).

3.1 | bACTH

The data sets were not normally distributed, thus, the data are presented as median + interquartile range (IQR). There were no differences in the bACTH after different feeding protocols in all groups applying the related-samples Friedman's two-way analysis of variance by ranks ($p = 0.7$; fasting: 24.5 pg/mL, IQR 19.0–33.6 pg/mL; hay: 24.0 pg/mL, IQR 17.8–32.5 pg/mL; hay + grain: 24.2 pg/mL, IQR 17.8–32.7 pg/mL) (Figure 1).

The residuals were normally distributed in the linear mixed model, thus, the results are presented as mean ACTH + 95% confidence interval (CI). The bACTH was significantly affected by the group ($p = 0.02$) and order of the tests ($p = 0.03$). The feeding protocol ($p = 0.4$), month of testing ($p = 0.5$), BCS ($p = 0.3$), pony breed

TABLE 1 Descriptive statistic including the number, age, BCS, sex, breed and colour of the animals included in each group in the study (healthy, PPID, treated PPID).

Group		0 (healthy)	1 (PPID)	2 (treated-PPID)
Number		20	21	20
Age in years	Mean ± SD	22.0 ± 4.5	24.2 ± 4.2	24.9 ± 5.3
	Range	16–31	17–33	16–35
BCS	Median	6	5	6
	Range	4–8	4–8	3–8
Sex	Mares	8	11	9
	Geldings	12	10	11
	Stallions	0	0	0
Breed	Ponies	10	3	7
	Horses	10	18	13
Colour	Grey	0	4	4
	Other	20	17	16

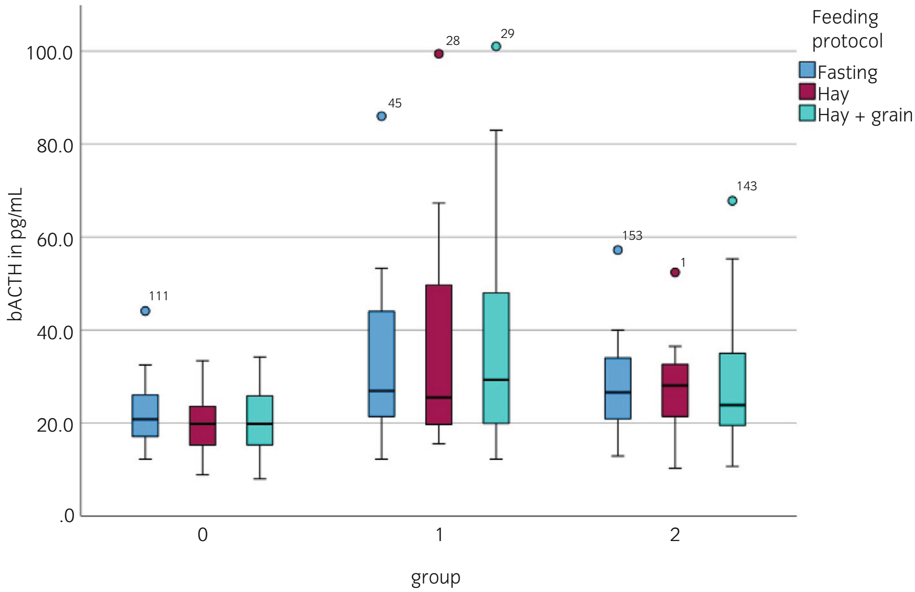

FIGURE 1 Cluster box plot of the bACTH by group (0—healthy, 1—PPID, 2—treated-PPID) and feeding protocol (fasting, hay, hay + grain).

($p = 0.8$), female sex ($p = 0.3$), grey colour ($p = 0.3$), age ($p = 0.3$) and interaction group*feeding protocol ($p = 0.3$) were not significant in the presented model.

Horses in the PPID-group (58.9 pg/mL, CI 39.7–78.1 pg/mL) had significantly higher bACTH ($p = 0.03$, Cohen's $d = 0.7$) than healthy animals (29.3 pg/mL, CI 9–49.5 pg/mL). The mean difference (mdf) was 29.7 pg/mL (1.9–57.4 pg/mL). The bACTH was not significantly ($p = 0.5$, Cohen's $d = 0.5$) higher in PPID-group than in the PPID-treated group (32.9 pg/mL, 15.6–50.1 pg/mL).

The first bACTH measurement was significantly higher than the last one in each animal ($p = 0.03$, Cohen's $d = 0.3$). The mean difference was 15.0 pg/mL (CI 1.3–28.7 pg/mL). The mean bACTH was 47.6 pg/mL (32.1–63.0 pg/mL), 40.8 pg/mL (25.4–56.4 pg/mL), 32.6 pg/mL (CI 17.2–48.0 pg/mL) in the first, second and third test respectively.

The mean bACTH was highest in July (47.3 pg/mL, CI −2.9 to 97.6 pg/mL) and lowest in May (31.6 pg/mL, CI 8.4–54.7 pg/mL). Mares (45.2 pg/mL, CI 29.7–60.7 pg/mL) had not significantly higher bACTH ($p = 0.3$, Cohen's $d = 0.4$) than geldings (35.5 pg/mL, CI 17.3–53.6 pg/mL) and grey horses (47.1 pg/mL, CI 21.2–72.9 pg/mL) higher bACTH ($p = 0.3$, Cohen's $d = 0.4$) than other coat colours (33.6 pg/mL, CI 22.1–45.2 pg/mL).

3.2 | pACTH

The data sets were not normally distributed, thus, the data are presented as median + IQR. There were no significant differences in the pACTH between feeding protocols in all groups applying the related-samples Friedman's two-way analysis of variance by ranks ($p = 0.5$; fasting: 240.0 pg/mL, IQR 94–625.5 pg/mL; hay: 233.0 pg/mL, IQR 80.5–563.0 pg/mL; hay + grain: 273.0 pg/mL, IQR 94.6–571.5 pg/mL) (Figure 2).

The residuals were normally distributed in the linear mixed model, thus, the results are presented as mean pACTH + CI. There was a significant influence of the group ($p < 0.001$), month of testing ($p < 0.001$), colour ($p < 0.001$) and age ($p = 0.002$). The pACTH was not significantly impacted by feeding ($p = 0.7$), BCS ($p = 0.9$), pony breed ($p = 0.6$), female sex ($p = 0.1$) and interaction group*feeding ($p = 0.9$) in the presented model. The pACTH was significantly lower ($p = 0.01$, Cohen's $d = 1.2$) in the healthy group (396.7 pg/mL, CI 283.2–510.1 pg/mL) compared with PPID (588.4 pg/mL, CI 480.7–696.2 pg/mL) and significantly lower ($p < 0.001$, Cohen's $d = 1.7$) compared with treated-PPID (683.1 pg/mL, CI 585.9–780.4 pg/mL). The mean pACTH was 286.5 pg/mL (CI 137.6–435.3 pg/mL) higher in PPID-treated group than in healthy animals. The pACTH was not significantly higher in treated-PPID group compared with untreated ($p = 0.4$, Cohen's $d = 0.4$; mdf 94.7 pg/mL, CI −52.0 to 241.5 pg/mL).

The pACTH was the highest in June (626.0 pg/mL, CI 486.3–765.6 pg/mL), July (881.2 pg/mL, CI 626.3–1136.3 pg/mL) and November (682.7 pg/mL, CI 539.0–826.5.9 pg/mL) and the lowest in April (408.2 pg/mL, CI 297.0–519.5 pg/mL) (Figure 3). The pACTH was significantly higher in November compared with January ($p = 0.01$, Cohen's $d = 1$; mdf 227.1 pg/mL, CI 25.4–429.0 pg/mL), in July compared with April ($p = 0.02$, Cohen's $d = 3.3$; mdf 473.1 pg/mL, CI 38.5–907.7 pg/mL), in November compared with April ($p = 0.03$, Cohen's $d = 1.4$; mdf 274.5 pg/mL, CI 14.1–534.9 pg/mL) and in November compared with December ($p = 0.01$, Cohen's $d = 1.2$; mdf 231.7, CI 23.7–439.6 pg/mL).

Furthermore, the pACTH was significantly higher ($p < 0.001$, Cohen's $d = 1.9$) in grey horses (723.5 pg/mL, CI 577.5–869.4 pg/mL) compared with other colours (338.7 pg/mL, CI 324.8–452.5 pg/mL). The mean difference was 334.8 (CI 174.6–495.0 pg/mL).

The first pACTH measurement (593.5 pg/mL, CI 508.7–678.3 pg/mL) was not significantly higher ($p = 0.1$, Cohen's $d = 0.2$) than the second (540.9 pg/mL, CI 455.9–625.9 pg/mL) and higher ($p = 0.1$, Cohen's $d = 0.2$) than the third one (533.8 pg/mL, CI 449.2–618.4 pg/mL) in each horse.

Ageing influenced the results significantly, however, the mean decrease per year was trivial and not biologically relevant. However not statistically significant ($p = 0.1$, Cohen's $d = 0.4$), mares had higher pACTH than geldings (mares: 597.2 pg/mL, CI

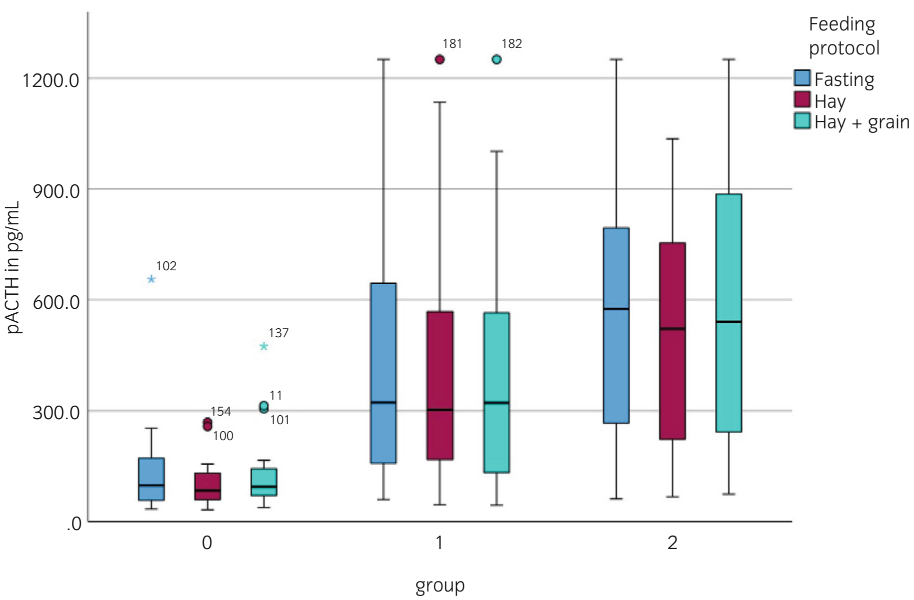

FIGURE 2 Cluster box plot of the pACTH by group (0—healthy, 1—PPID, 2—treated-PPID) and feeding protocol (fasting, hay, hay + grain).

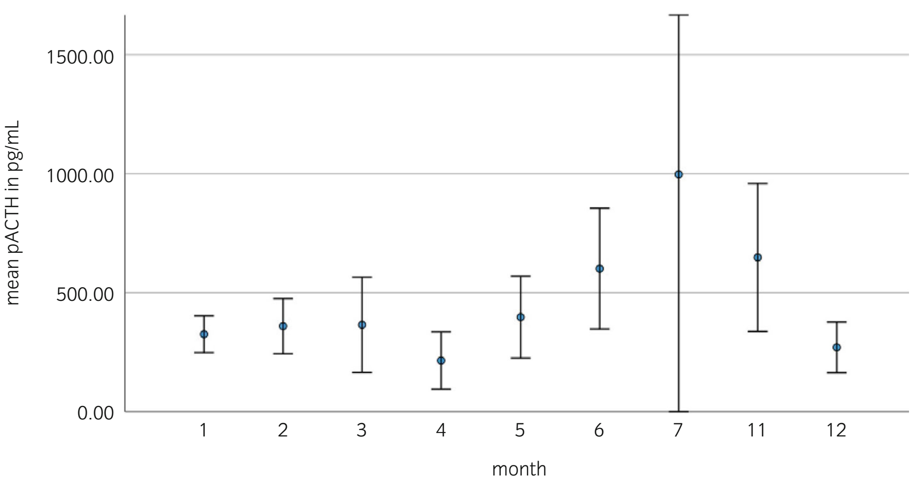

FIGURE 3 Chart with mean pACTH (pg/mL) with error bars presenting 95% confidence interval by month. The study was not conducted between August and October.

510.1–684.2 pg/mL; geldings: 515.0 pg/mL, CI 412.8–617.2 pg/mL). There was also no significant difference ($p = 0.6$, Cohen's $d = 0.3$) between ponies (568.6 pg/mL, CI 462.8–674.3 pg/mL) and horses (543.6 pg/mL, CI 459.3–627.8 pg/mL).

4 | DISCUSSION

Our hypothesis that feeding would affect the b/pACTH was not confirmed in this cohort of horses and ponies. The therapy with pergolide decreased the bACTH as horses in PPID group had higher bACTH than treated animals. However, the treatment with pergolide seemed to have little effect on pACTH as horses in treated-PPID group had highest results, thus, TST alone should be used with caution to adjust the dosage of pergolide in horses. Month of testing did not affect the bACTH in this study but had an impact on the pACTH with the highest results in June, July and November. The grey colour described to increase the bACTH in the autumn, did not affect the bACTH in other seasons, but had a significant impact on the pACTH. Furthermore, there is evidence, that re-testing horses within 2 weeks might decrease bACTH.

4.1 | Test performance

The bACTH can be performed at any time of the day[1] but it remains unclear whether the pACTH follows the circadian rhythm. Therefore, all the feeding protocols were applied at night, 12 h prior to testing in this study, and the test was conducted in the morning to avoid the impact of photoperiodicity.[28]

The protilerin dosage of 1 mg was used in all animals, including ponies, as it is proven to have the highest sensitivity and specificity.[29] However, the Equine Endocrinology Group recommends 0.5 mg for ponies weighing less than 250 kg.[1] Recent data show, that increasing the dosage to 2 mg or lowering it to 0.5 mg does not significantly alter the outcome and we assume that our findings would also remain similar with different dosages.[29,30]

The TST was safely performed in all horses and side effects were considered minor. Most of them occurred and disappeared within 10 min similarly to other studies,[10,11] therefore, longer observation after TST does not seem necessary. Yawning, lip smacking, flehmen response, coughing, sneezing or nose rubbing, pawing and head shaking were described in other studies[1,10,11]; however, urination, single episode of loose faeces, flatulence and trembling were not reported previously. They were possibly not considered as an adverse reaction by the other authors, or they are very rare. The mechanism of those reactions remains unclear, but a sudden increase of ACTH might potentially evoke sympathetic response leading to decreased production of tears, saliva and mucus in the respiratory system. The dry sensation in the head area might explain reactions, such as lip smacking and sneezing. However, the biological activity of ACTH from pars intermedia and subsequent increased adrenal secretion in PPID is questionable.[31] Stress accompanying the test/injection can also cause an episode of loose faeces.[32]

Care was taken to manage the samples according to the newest recommendations[33]—the blood samples were kept cool and processed to further shipping to an external laboratory within 2 h of collection. All measurements were performed by chemiluminescence immunometric assay.[34]

At least 2 weeks of washout period was provided between the tests as repeated TST led to decrease of subsequent measurements of pituitary hormones in horses tested every 3 or 7 days.[30,35] Furthermore, Kam et al.[36] reported that the repeatability of the tests 2 weeks apart was good in winter. Unfortunately, the subsequent measurements of bACTH (and to lesser extent also pACTH) were lower in this study. It can be caused by familiarisation with the examination and thus, less stress-related ACTH secretion in following tests. Nevertheless, the size effects of these findings were considered low and should not have considerable effects on the study results.

4.2 | Non-horse-related factors

4.2.1 | Feeding

The overnight feeding 12 h prior to the TST had little effect on the b/pACTH in described cohort of horses and ponies. These findings are similar to those of Restifo et al.,[11] who did not find any difference between fasted and fed horses. Nevertheless, we cannot exclude the influence of the feeding on b/pACTH due to low number of observations in this study. The discrepancies between studies might be due to different feeding protocols applied. The type of feed might have significant impact on TST and the influence of different hay type (e.g., grass, alfalfa) or concentrate (e.g., oat, corn, barley) remains to be investigated. Furthermore, the timing of grain feeding might alter the b/pACTH results, as there is evidence that long-term grain feeding in endocrinologically healthy horses increases bACTH,[9] and only short-term oat feeding was tested in this study. The special dietary management prior to testing[1] should therefore still be applied.

4.2.2 | Month

The month of testing did not influence the bACTH in the study time, which conflicts with the newest recommendations of the Equine Endocrinology Group, where higher normal values were described for June and November.[1] The low number of observations in these months might have been responsible for not detecting these differences.

The month had a significant impact on the pACTH, with the lowest results observed in April (Figure 3), similar to the findings of Miller et al.[37] The highest pACTH were obtained in July and November, what is in agreement with the newest recommendations of the Equine Endocrinology Group, that TST should no longer be performed to confirm PPID between July and December.[1] Miller et al.[37] also reported high pACTH and ΔACTH (= pACTH − bACTH) in November, compared with August, September and October. Further studies with more observations in each month should be conducted to confirm these findings as new reference ranges might be necessary for some months, as was suggested for the bACTH in June and November.[1,19]

4.3 | Horse-related factors

4.3.1 | PPID and treatment effect

There was no significant difference in the bACTH between horses from the healthy and treated group, suggesting that pergolide can improve the bACTH and is able to reduce it to the reference range.[25,38,39] However, some studies show that this is not always the case, and the duration of therapy is also important.[40,41] The bACTH decreased only in 6/8 horses involved in our study two times (included in PPID group and re-tested in treated group). The dosage of pergolide and the corresponding clinical improvement were out of scope of this study.

Treated-PPID animals had highest pACTH (Figure 2) because of their advanced disease and earlier diagnosis compared with PPID-group. Nevertheless, there was no significant difference in the pACTH between treated and untreated horses, suggesting that pergolide has little influence on the TST results. The pACTH improved in 5/8 horses that were tested before and after therapy, but the clinical improvement was not objectively assessed. These findings are similar to the study of Durham,[42] who described the decrease of the numerical pACTH after treatment, however, most cases did not return to the normal reference range. Furthermore, Banse and McFarlane[40] also reported no significant decrease in the pACTH in horses treated with pergolide over 12 weeks. There was also no difference in the pACTH between horses receiving pergolide and placebo. Miller et al.[37] also described that horses receiving pergolide did not reach the pACTH values of healthy horses and the authors do not recommend TST to adjust the treatment of PPID. Whether the proper individual dosage of pergolide and corresponding clinical improvement decrease the pACTH into the reference range still needs to be investigated.

4.3.2 | Grey effect

Colour appears to have an effect on the bACTH with grey horses having significantly higher values in autumn.[17] The grey colour did not affect the bACTH in non-autumn months in this study, similarly to a study of Altmeyer et al.[43] Nevertheless, grey horses had significantly higher pACTH in this study. Alpha-melanocyte-stimulating hormone (α-MSH) increases the production of melanin by melanocytes and is responsible for dark skin pigmentation.[43] It is produced alongside with ACTH by melanotropes in the pituitary gland and its concentration increases in horses with PPID.[4,44] The correlations between grey colour, α-MSH, ACTH and PPID warrant further investigation as the b/pACTH measurements in grey horses in this study was limited.[24]

4.3.3 | Other effects

To improve the practical aspect of the study and reach better comparability between the groups, all animals were older than 16 years, as the prevalence of PPID in older horses is 21%.[2] There was no association between age and b/pACTH in our study, in contrast to recent literature[2,18] and the assumption, that decreasing number of dopaminergic neurons increases ACTH secretion with age.[45] This difference might be due to the narrow age range (Table 1) and the exclusion of younger animals in this study. Furthermore, Durham and Shreeve[17] described that the differences in the bACTH in older horses were most apparent between July and November, when the study was not performed.

There were no donkeys[19,26,46] or Arabians[19] in the final group, in which the increase in the bACTH is significant all over the year. There is some evidence that thrifty breeds, such as ponies, have a higher bACTH than other equids.[26,47–49] However, this finding is inconsistent,[2,49,50] and it was proved significant almost exclusively in the autumn,[17,19,48,49,51] when our study was not performed. The significantly higher pACTH was also described in Mustangs compared with Thoroughbreds in October, but not in July.[52] We did not detect any differences in the b/pACTH between ponies and other breeds. The discrepancies between studies might be caused by collecting many pony breeds together. The significantly higher bACTH was described in Shetland, mini-Shetland and Welsh A ponies compared with other pony breeds.[17,19,51] Further studies with a higher number of certain pony breeds in each month are necessary.

4.4 | Limitations

The biggest limitation of our study was the small number of horses. The power of the test was 95% and calculated to determine the minimal number of horses in each group to prove the influence of feeding on the bACTH. The power of the test for findings in the linear mixed model was not calculated and might be much lower for findings with low number of observations. However, the effect sizes were calculated to better assess the magnitude of differences.

The second biggest limitation proved to be the decrease of the b/pACTH in subsequent tests, what could have affected our results. Nevertheless, the size effect was considered low.

There was also a discrepancy between the categorisation of few horses into groups created based on our inclusion criteria (bACTH, clinical signs and treatment) and the results of pACTH. Two horses from the healthy group had positive results of at least two TSTs and three horses from the untreated PPID group had negative results of at least two TSTs. Inclusion of horses with questionable bACTH results might be the reason for this false categorisation. Nevertheless, horses from the 'grey zone' are the target group for TST,[1,5–7] thus, they were included in our study. Furthermore, we used the reference ranges for the b/pACTH interpretation suggested by Schott et al.[22] from 2019 and new values are recommended since 2021 by Hart et al.[1] Applying the new criteria retrospectively in our study considerably increased the number of animals with equivocal bACTH (12 results were changed from unlikely to equivocal, one from likely to equivocal and three from equivocal to likely), however, the categorisation of horses did not change in any case.

A minimal delay in blood collection after TST can lead to significant differences in the ACTH results.[28] We aimed at blood collection exactly 10 min after the injection, but there was a one-minute delay in two cases, what could lead to falsely low ACTH measurements in those horses.[28] The use of a catheter in difficult horses should therefore be considered.[10] Furthermore, the blood collection after 30 min is reported to be less affected by the timing of the sample collection.[8,28,53]

Finally, the acute stress seen in a few horses might have also influenced our results,[16,54] especially in horses that were isolated from the herd for fasting.

5 | CONCLUSION

There was little evidence that feeding with oat 2 h before or fasting 12 h prior to the b/pACTH measurements have impact on both results. Horses with PPID (regardless the treatment) and grey coloured were shown to have highest pACTH in this cohort. Animals in the treated-PPID group had a lower bACTH than untreated horses, what suggests an influence of pergolide on bACTH. However, the drug seems to have little effect on pACTH as horses in the treated-PPID group had the highest results. Despite the significance and at least moderate effect size of these findings, they warrant further examinations on a larger group of animals.

AUTHOR CONTRIBUTIONS

Karolina Drozdzewska participated in the study design, collected the data, participated in the data analysis and interpretation, prepared the original manuscript and had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis. Judith Winter, Ann Kristin Barton and Heidrun Gehlen participated in the study design and the critical article revision. Roswitha Merle participated in the data analysis, interpretation and the critical article revision. All authors gave their final approval of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request: Open sharing exemption granted by editor for this clinical report.

ETHICAL ANIMAL RESEARCH

The experiment was approved by State Office for Health and Social Affairs in Berlin, Germany (G 0237/19).

INFORMED CONSENT

Owners gave consent for their animals' inclusion in the study.

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