Evaluation of a thyrotropin-releasing hormone solution stored at room temperature for pituitary pars intermedia dysfunction testing in horses

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OBJECTIVE
To determine whether plasma ACTH concentrations vary following administration of a thyrotropin-releasing hormone (TRH) solution prepared for research purposes and stored at –20°C (rTRH) or prepared by a compounding pharmacy and stored at room temperature (approx 22°C, cTRH).

ANIMALS
34 adult horses.

PROCEDURES
The study consisted of 2 experiments. In experiment 1, each horse underwent 2 TRH stimulation tests separated by 24 hours; 10 horses were administered cTRH for the first test and rTRH for the second test (group 1), 10 horses were administered rTRH for the first test and cTRH for the second test (group 2), and 10 horses were administered rTRH for both tests (group 3). Plasma ACTH concentrations were measured at 0 (baseline) and 30 minutes after TRH administration and the delta ACTH responses (change in ACTH concentration after TRH administration) were calculated. In experiment 2, the design was the same as that for experiment 1 except there were 14 days between tests, ACTH was measured at 0 and 10 minutes after TRH administration, and 11, 9, and 10 horses were assigned to groups 1, 2, and 3, respectively.

RESULTS
Adverse effects associated with TRH administration included transient coughing and yawning. In experiment 1, the median delta ACTH response for the second test was significantly lower than that for the first test for all groups. In experiment 2, the median delta ACTH response did not differ significantly between the first and second tests for any group. ACTH concentrations after rTRH administration were positively correlated (r = 0.95) with those after cTRH administration, and the mean ± SD bias in post-TRH ACTH concentration between rTRH and cTRH was 2.9 ± 12.4 pg/mL.

CONCLUSIONS AND CLINICAL RELEVANCE
Results indicated that the TRH stimulation test should not be repeated within 24 hours, and cTRH solution stored at room temperature could be used to effectively perform TRH stimulation testing in horses. (Am J Vet Res 2015;76:437–444)
TRH stimulation test was originally developed to diagnose hypothyroidism in human patients, but its use for that purpose declined after thyroid-stimulating hormone assays became widely available. Medical-grade protirelin solution, a synthetic TRH that was stable at room temperature (approx 22°C), was commercially manufactured for TRH stimulation testing in human patients until it was withdrawn from the market in July 2002. Following withdrawal of the protirelin solution from the market, the only TRH available for TRH stimulation testing in horses was prepared from pGlu-His-Pro amide provided by a laboratory chemical supplier. This compound has the same formula and chemical structure as medical-grade protirelin, but it is only available as a powder and must be suspended in a solution and filtered within a chemical hood, and then frozen at –20°C for storage. In the study reported here, TRH prepared from pGlu-His-Pro amide is referred to as rTRH. Despite the usefulness of the TRH stimulation test for diagnosis of PPID, few practitioners use the test in the field because the rTRH required for the test has to be prepared in a laboratory and stored at –20°C. To increase the availability of the TRH stimulation test for practitioners in the field, an established compounding pharmacy began producing a TRH solution (cTRH) that can be stored at room temperature, and this product is now available to US veterinary practitioners.

Thyrotropin-releasing hormone receptors are present on melanotropes of the pars intermedia and corticotropes of the pars distalis. Stimulation of these receptors increases secretion of αMSH and ACTH. When pituitary explants obtained from healthy horses were stimulated with TRH, pars intermedia explants secreted αMSH, whereas pars distalis explants secreted ACTH. In healthy horses and horses with PPID, administration of TRH stimulates corticotropes and results in the increase in plasma ACTH concentration. In horses with PPID, this increase in ACTH concentration is markedly higher than that in healthy horses because affected melanotropes also secrete ACTH. Therefore, stimulation of the pituitary gland facilitates detection of PPID in horses, and studies must be performed to determine the sensitivity of the TRH stimulation test. These studies will be easier to perform in the field in the United States once a TRH solution is readily available to practitioners.

The purpose of the study reported here was to determine whether ACTH concentrations following IV administration of cTRH differed from the ACTH concentrations following IV administration of rTRH. We hypothesized that the ACTH concentrations following IV administration of cTRH would not differ significantly from the ACTH concentrations following IV administration of rTRH and that results of TRH stimulation tests would be repeatable as evidenced by a median within-horse CV < 20% for post-TRH ACTH concentrations between tests.

Materials and Methods

Animals

Adult horses from a university equestrian program located in Massachusetts were included in the study. The study consisted of 2 experiments, and 30 horses were included in each experiment. Twenty-six horses were included in both experiments, and 4 horses were substituted between experiments 1 and 2; therefore, a total of 34 horses were used for the study. All horses were housed in the same facility for the duration of the study and had similar feeding and turnout schedules.

The horses in experiment 1 included 8 mares and 22 geldings that ranged in age from 7 to 21 years (mean ± SD, 14.3 ± 3.6 years). Breeds represented included warmblood (n = 19), Thoroughbred (4), Irish Sport Horse (2), warmblood cross (2), and Canadian Sport Horse, Quarter Horse, and Thoroughbred-Percheron cross (1 each). The horses in experiment 2 included 8 mares and 22 geldings that ranged in age from 3 to 20 years (mean ± SD, 14.5 ± 3.6 years). Breeds represented included warmblood (n = 19), Thoroughbred (5), and Irish Sport Horse, Quarter Horse, and warmblood cross (2 each).

Prior to study initiation, PPID was diagnosed in 5 horses that were included in both experiments. Those horses were being treated with pergolide (1 mg, PO, q 24 h [n = 4]; or 1.5 mg, PO, q 12 h [1]). For each TRH stimulation test, the 4 horses that received pergolide daily were given the drug at their normal dosing time the afternoon before the test was performed, whereas the horse that received pergolide every 12 hours was given the drug during its morning feeding on the day the test was performed.

Experimental design

All study protocols were approved by the Clinical Studies Review Committee of the Cummings School of Veterinary Medicine. Experiment 1 was conducted in November 2012, and experiment 2 was conducted in April and May 2013. For each experiment, horses were randomly allocated by means of a random number table to 3 groups. Horses in group 1 received cTRH for the first TRH stimulation test and rTRH for the second TRH stimulation test. Horses in group 2 received rTRH for the first TRH stimulation test and cTRH for the second TRH stimulation test. Horses in group 3 received rTRH for both tests. Ten horses were assigned to each group during experiment 1. During experiment 2, 11, 9, and 10 horses were assigned to groups 1, 2, and 3, respectively. There was a 24-hour interval between the 2 TRH stimulation tests of experiment 1 and a 4-day interval between the 2 TRH stimulation tests of experiment 2.

For each horse immediately prior to the first TRH test of each experiment, a history was obtained and a physical examination was performed to assess its general health and identify the presence of any clinical signs potentially associated with PPID. Rec-
tial temperature, heart rate, and respiratory rate were measured before each TRH stimulation test. All tests were performed in the morning approximately 2 hours after horses were fed their normal ration of hay and grain.

**TRH solutions**

The rTRH solution (1 mg/mL) was prepared in the laboratory by mixing pGlu-His-Pro amide powder with sterile saline (0.9% NaCl) solution by the use of sterile techniques in a chemical hood. It was then filtered through a 0.2-μm filter and stored in 1-mL aliquots in 3-mL syringes at ~20°C. The fTRH solution was thawed at room temperature (approx 22°C) just prior to administration.

The cTRH solution (1 mg/mL) was prepared from protirelin and supplied by an established compounding pharmacy in sealed 1-mL vials that were stored at room temperature and capable of being shipped in the mail. The cTRH solution supplied in October (experiment 1) and April (experiment 2) was prepared from the same bulk drug. The intervals between preparation of the cTRH solution by the compounding pharmacy and its injection into the study horses were 13 and 14 days for the first and second TRH stimulation tests, respectively, of experiment 1, and 4 and 18 days for the first and second TRH stimulation tests, respectively, of experiment 2.

**TRH stimulation test**

For each horse, 1 mg of the assigned TRH (cTRH or rTRH) was administered as a 1-mL volume into a jugular vein with a 20-gauge needle and 3-mL syringe. Each horse was observed for at least 15 minutes after TRH administration, and any adverse effects were recorded.

**Sample collection and processing**

Blood samples (10 mL) were collected by jugular venipuncture with a 20-gauge needle into chilled evacuated blood collection tubes that contained EDTA to obtain plasma for determination of ACTH concentration immediately prior to (0 minutes; baseline) and 30 minutes after TRH administration during experiment 1 and at 0 and 10 minutes after TRH administration during experiment 2. Following collection, blood samples were immediately placed on ice in a cooler. All blood samples were centrifuged within 4 hours after collection. Plasma was harvested from each blood sample and stored at ~20°C until analyzed.

**Plasma ACTH concentrations**

Within 2 weeks after the second TRH stimulation test of each experiment was completed, all of the frozen plasma samples collected during that experiment were packaged with ice packs and sent by overnight mail to a diagnostic laboratory for measurement of plasma ACTH concentrations. A chemiluminescent ACTH immunoassay that was validated for use with equine plasma was used to determine the plasma ACTH concentration in each sample. All samples from each experiment were analyzed within the same batch. The plasma ACTH concentration reference range (9 to 35 pg/mL) used for both experiments was provided by the laboratory. A positive result for the TRH stimulation test was defined in accordance with recommendations established by the Equine Endocrinology Group. Briefly, a positive TRH stimulation test result was defined as an ACTH concentration > 35 pg/mL at 0 or 30 minutes after TRH administration for experiment 1 and an ACTH concentration > 35 pg/mL at 0 minutes after TRH administration or > 110 pg/mL at 10 minutes after TRH administration for experiment 2.

**Statistical analysis**

For each horse, the delta ACTH response for each TRH stimulation test was calculated as the ACTH concentration after TRH administration minus the baseline ACTH concentration. The distribution of the data was assessed for normality by visual examination of data plots and the Shapiro-Wilk test. Because the data did not have a Gaussian distribution, nonparametric tests were selected for analysis purposes. For each experiment, the plasma ACTH concentrations measured at 0 (baseline) and 10 (experiment 2) or 30 (experiment 1) minutes after TRH administration and the delta ACTH responses for each group (1, 2, and 3) were compared between TRH stimulation tests by the use Wilcoxon matched-pairs signed rank tests. Spearman correlation coefficients (r_s) were calculated and Bland-Altman plots were created to determine the bias between the 2 TRH stimulation tests performed during each experiment and between the 2 types of TRH administered. Within-horse CVs were calculated to assess the repeatability of rTRH stimulation test results for horses in group 3 of experiment 2. A statistical software program was used for all analyses, and values of P < 0.05 were considered significant.

**Results**

**Horses**

Prior to study initiation, 3 horses had clinical signs consistent with PPID such as hypertrichosis (n = 3) and loss of epaxial muscle mass (2), and all 3 horses were receiving pergolide. Prior to each TRH stimulation test, the rectal temperature, heart rate, and respiratory rate were within reference ranges for all horses. Following TRH administration, coughing, licking, yawning, and the flehmen response were observed in some horses; however, all of these responses were transient and resolved within 10 minutes after TRH administration. The most commonly observed adverse effects associated with TRH administration were licking, chewing, and lip smacking, and ≥ 1 of those effects were observed in 19 horses across both studies. Five horses developed a transient cough after TRH administration.
Experiment 1

The median delta ACTH response for the second TRH stimulation test was significantly lower than that for the first test for groups 1 (P = 0.037), 2 (P = 0.002), and 3 (P = 0.037). The median delta ACTH responses for the first and second TRH stimulation tests were 28.7 and 19.4 pg/mL, respectively, for group 1; 61.4 and 15.6 pg/mL, respectively, for group 2; and 25.5 and 11.7 pg/mL, respectively, for group 3. Thus, the median delta ACTH response for the second test was decreased by 32%, 75%, and 54% for groups 1, 2, and 3, respectively, compared with that for the first test, which was performed 24 hours earlier. Additional comparisons among groups were not performed, and positive and negative TRH stimulation test results were not interpreted further because of the significant differences in the median delta ACTH response between the first and second TRH stimulation tests that were identified within each group.

Experiment 2

The median ACTH concentration at 0 (baseline) and 10 minutes after TRH administration and the median ACTH response did not differ significantly between the first and second tests for any of the 3 groups (Table 1). The median (range) ACTH concentration was 53.3 pg/mL (22 to 558 pg/mL) and 47.0 pg/mL (21 to 527 pg/mL) 10 minutes after injection of cTRH and rTRH, respectively, and those values were positively correlated (r = 0.95; P < 0.001; Figure 1). Similarly, the median delta ACTH responses 10 minutes after injection of cTRH (34.2 pg/mL [range, 8 to 521 pg/mL]) and rTRH (29.7 pg/mL [range, 8 to 468 pg/mL]) were positively correlated (r = 0.93; P < 0.001).

The mean ± SD bias between the first and second TRH stimulation tests for the horses of groups 1 and 2 was 2.9 ± 12.4 pg/mL (95% limits of agreement, −21.5 and 27.3 pg/mL) for the median ACTH concentration 10 minutes after TRH administration and 0.5 ± 15 pg/mL (95% limits of agreement, −28.7 and 29.73 pg/mL) for the delta ACTH response (Figure 2). For the horses of group 3, the repeatability of the results when rTRH was used for the TRH stimulation tests as measured by the median (range) within-horse CVs was 7.3% (0.3% to 21%), 11.8% (0.7% to 29%), and 14.2% (1.5% to 46%) for the ACTH concentration at baseline and 10 minutes after rTRH administration and the delta ACTH response, respectively.

When a plasma ACTH concentration of > 110 pg/mL at 10 minutes after TRH administration was used as the cutoff for diagnosis of PPID, 3 horses in group 1 and 2 horses in group 3 were identified as having the disease. During the time that the TRH stimulation tests were performed, 3 of those 5 horses had clinical signs of PPID and were being treated with pergolide, whereas the remaining 2 horses appeared clinically normal. The 2 clinically normal horses were both assigned to group 3 and had post-TRH ACTH concentrations that were close to the 110 pg/mL cutoff used to define a positive test result; the post-TRH ACTH concentrations for a 20-year-old horse were 113 and 95 pg/mL for the first and second tests, respectively, and those for an 18-year-old horse were 111 and 124 pg/mL for the first and second tests, respectively. Two other horses that were receiving pergolide during the testing period had negative results (ie, post-TRH ACTH concentration < 110 pg/mL) for both TRH stimulation tests. Two of the 30 horses had a baseline ACTH concentration > 35 pg/mL prior to each TRH stimulation test. Those were 2 of the horses that had clinical signs of PPID and were being treated with pergolide daily, and both had positive results (ie, post-TRH ACTH concentration > 110 pg/mL) for both TRH stimulation tests. The plasma ACTH concentrations at baseline and 10 minutes after TRH administration were 108 and 142 pg/mL, respectively, for the first test and 83 and 134 pg/mL, respectively, for the second test for a horse that was receiving pergolide (1 mg, PO) once daily and

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<tr>
<td>Delta ACTH response</td>
<td>32 (16–468)</td>
<td>37 (11–108)</td>
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Table 1—Median (range) plasma ACTH concentrations and delta ACTH response for 30 adult horses that were randomly assigned to 1 of 3 groups and underwent 2 TRH stimulation tests with a 14-day interval between tests (experiment 2).

The units for all measurements is pg/mL. Horses in group 1 (n = 11) were administered cTRH for the first TRH stimulation test and rTRH for the second test. Horses in group 2 (n = 9) were administered rTRH for the first TRH stimulation test and cTRH for the second test. Horses in group 3 (n = 10) were administered rTRH for both TRH stimulation tests. The rTRH solution (1 mg/mL) was prepared with pGlu-His-Pro amide powder and sterile saline (0.9% NaCl) solution in the research laboratory and stored frozen at −20°C until use. The cTRH solution (1 mg/mL) was obtained from an established compounding pharmacy and was stored in sterile vials at room temperature (approx 22°C). For each test, blood was obtained for measurement of plasma ACTH concentration immediately before (baseline) and 10 minutes after IV administration of 1 mL of the assigned TRH. The delta ACTH response was calculated as the ACTH concentration at 10 minutes after TRH administration minus the baseline ACTH concentration.
37 and 558 pg/mL, respectively, for the first test and 59 and 527 pg/mL, respectively, for the second test for the other horse that was receiving a higher dose of pergolide (1.5 mg, PO, q 12 h).

Discussion

In the present study, the median delta ACTH response for the second TRH stimulation test was approximately 50% lower than that for the first test for all 3 groups of horses when there was only a 24-hour interval between the tests (experiment 1); however, the median delta ACTH response did not differ significantly between the first and second TRH stimulation tests when there was a 14-day interval between tests (experiment 2). Thus, we concluded that TRH stimulation tests should not be repeated within 24 hours in horses. In experiment 2, the median delta ACTH response did not differ significantly between TRH stimulation tests that were performed with cTRH and those that were performed with rTRH; therefore, we concluded that cTRH could be substituted for rTRH to perform TRH stimulation tests. Three of the 20 horses that were administered both cTRH and rTRH during experiment 2 were receiving pergolide for treatment of PPID and had a positive result (post-TRH ACTH concentration).

Figure 1—Scatterplots of the ACTH concentration 10 minutes after rTRH injection (post-rTRH ACTH concentration) versus the ACTH concentration 10 minutes after cTRH injection (post-cTRH ACTH concentration; A) and the delta ACTH response following rTRH injection versus the delta ACTH response following cTRH injection (B) for the adult horses of groups 1 and 2 of experiment 2. In each panel, the solid line represents the regression line and the linear regression equation and coefficient of determination (r²) are provided. Each horse underwent 2 TRH stimulation tests with a 14-day interval between tests. Horses in group 1 (n = 11) were administered cTRH for the first TRH stimulation test and rTRH for the second test. Horses in group 2 (n = 9) were administered rTRH for the first TRH stimulation test and cTRH for the second test. The rTRH solution (1 mg/mL) was prepared with pGlu-His-Pro amide powder and sterile saline (0.9% NaCl) solution in the research laboratory and stored frozen at –20°C until use. The cTRH solution (1 mg/mL) was obtained from an established compounding pharmacy and was stored in sterile vials at room temperature (approx 22°C). The delta ACTH response was calculated as the ACTH concentration at 10 minutes after TRH administration minus the ACTH concentration immediately before (baseline) TRH administration.

Figure 2—Bland-Altman plots to assess the difference between the post-rTRH ACTH concentration and the post-cTRH ACTH concentration (A) and between the delta ACTH response after cTRH injection and the delta ACTH response after rTRH injection (B) for the horses of Figure 1. See Figure 1 for remainder of key.
results following injection of rTRH were repeatable, with median CVs < 20% for the horses of group 3; however, among the individual horses, those CVs ranged from 0.3% to 21% for the baseline ACTH concentration, from 0.7% to 29% for the post-TRH ACTH concentration, and from 1.5% to 46% for the delta ACTH response, and that amount of variability could substantially affect test interpretation. Hence, similar to all endocrine tests, the results of each TRH stimulation test should be interpreted within the context of the medical history and clinical signs of the patient in question.

Potential factors that can contribute to the variability among TRH stimulation test results include season, the time of day the test is performed, feeding conditions, and the dose of TRH administered. It is unlikely that season affected the variation in results between the first and second TRH stimulation tests of experiment 2 because they were performed within 2 weeks of each other. Nevertheless, season is significantly associated with basal ACTH concentrations,11–13 and post-TRH ACTH concentrations for horses tested in September are higher than those for horses tested in March and April.14 In the present study, the time of day at which the TRH stimulation test was administered was standardized; however, practitioners in the field are likely to perform the test at various times throughout the day, and further research is necessary to determine the effect that the time of day at which the TRH is administered has on the test results. In horses and other mammals, cortisol follows a circadian rhythm, with higher concentrations present in the morning than in the afternoon and evening.15 If TRH stimulation causes the release of ACTH from the pars distalis, naturally occurring alterations in the hypothalamic-pituitary-adrenal axis throughout the day could affect the magnitude of ACTH response.

In the present study, horses were fed the morning of each test day to keep them calm while blood samples were collected and the TRH was injected. Results of another study14 indicate that the baseline and post-TRH ACTH concentrations of clinically normal horses were significantly higher when the test was performed 2 hours after the horses were fed 3 kg of alfalfa hay, compared with those when food was withheld from the horses for 12 hours prior to the test. Additionally, the differences between ACTH concentrations measured 2 hours after feeding and those measured after food was withheld for 12 hours were greater when the TRH stimulation test was performed in September than when the test was performed in March and April.14 Because food was not withheld from the horses of the present study, the ACTH concentrations might have been higher than those expected if food had been withheld for a period of time prior to testing. Regardless, the feeding conditions were standardized for this study, so feeding should not have affected the test results unless an individual horse ate at a slower rate on one test day, compared with the other.
which could have affected the within-horse variability in ACTH concentrations for that horse.

The dose of TRH (1 mg) administered to the horses of the present study was selected on the basis of the TRH dose administered in other studies\(^1\,^6\,^7\) and was the same for all horses regardless of body mass. This dose is presumed to elicit the maximal ACTH response in horses, but dose-response studies are necessary to determine whether varying the TRH dose by body mass improves test performance. The horses of this study were of similar body mass and type. A different approach to TRH dosing may be required for TRH stimulation testing of small ponies or draft horses. In the present study, the pituitary gland response to TRH stimulation was assessed by measurement of plasma ACTH concentration rather than plasma cortisol concentration. Results of a study\(^1\,^6\) in which post-TRH plasma concentrations of cortisol and ACTH for clinically normal horses were assessed in combination with postmortem results indicate that ACTH concentration was more closely correlated with the presence of lesions in the pituitary gland than was cortisol concentration.

In experiment 2 of the present study, plasma ACTH concentrations were measured 10 minutes after TRH injection. Although reference intervals for post-TRH ACTH concentration can only be established by testing a large population of horses, we used an ACTH concentration \(> 110\) pg/mL at 10 minutes after TRH injection as a cutoff to diagnose PPID on the basis of a recommendation from the Equine Endocrinology Group. Given that criterion, 5 horses had positive results for the TRH stimulation test. It is challenging to determine cutoff values for diagnostic tests to identify horses in the early stages of PPID because an antemortem gold standard test to diagnose the disease is not available. Even histologic changes within the pituitary gland are difficult to interpret in the early stages of PPID,\(^1\,^7\) and owners are unlikely to euthanize horses with early or mild endocrine disease. Use of a cutoff \(> 110\) pg/mL for plasma ACTH concentration 10 minutes after TRH administration resulted in complete agreement of results between the first and second TRH stimulation tests for the 3 horses of the present study with PPID that were tested with both the cTRH and rTRH solutions. However, when that cutoff was applied to the horses with PPID in group 3 (rTRH administered for both TRH stimulation tests), 1 horse had a positive result on the first test and a negative result on the second test, which suggested that test performance requires further evaluation.

Limitations of the present study included variation in PPID status among the horses tested and the absence of a gold standard test to definitively diagnose PPID. However, those limitations were balanced by the advantages of comparing results following administration of cTRH with those following rTRH and assessing the repeatability of TRH stimulation test results under field conditions. One goal of this study was to assess the TRH stimulation test in the field with a representa-

tive population of horses that would be expected to undergo testing to diagnose PPID. Because 5 horses at the equestrian center had been diagnosed with PPID on the basis of clinical signs and basal ACTH concentrations prior to initiation of the present study, the managers of the facility were interested in pursuing additional testing.

The present study provided only limited information about the stability of the cTRH solution. Concerns have been raised about the stability of compounded pergolide,\(^1\,^6\) and the long-term stability of other compounded products including cTRH requires further investigation. In experiment 2, the cTRH solution was stored at room temperature for a maximum of only 18 days before injection, and the results obtained for the tests in which cTRH was administered did not differ significantly from those obtained for tests in which rTRH that was stored at \(-20^\circ\)C was administered.

Although assessment of the sensitivity or specificity of the TRH stimulation test was not a goal of the present study, 5 horses with PPID that were being treated with pergolide and 2 clinically normal horses had at least 1 positive test result. These findings suggested that horses treated with pergolide can have negative or positive TRH stimulation test results, and the test outcome is likely a reflection of the stage of the disease or the dose of pergolide administered. The fact that 2 apparently healthy horses had positive results suggested that the TRH stimulation test may be able to detect horses with subclinical PPID, but further investigation is necessary because the cutoff for post-TRH ACTH concentration used to define a positive test result might have been too low and yielded false-positive results.

Results of the present study indicated that TRH stimulation tests should not be repeated within 24 hours in individual horses and that the TRH stimulation test results did not differ significantly when cTRH stored at room temperature was used instead of rTRH that had to be stored at \(-20^\circ\)C. Therefore, cTRH solution stored at room temperature and used within 18 days of preparation may be convenient for practitioners to use for TRH stimulation testing in the field. Although the results were repeatable when the TRH stimulation tests were performed under standardized conditions, there was substantial interday variability in results for individual horses.

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**Footnotes**

a. Thyrel, Ferring Pharmaceuticals Inc, Parsippany, NJ.

b. pCilu-His-Pro amide ≥ 98% (HPLC) powder (P1319), Sigma-Aldrich Corp, St Louis, Mo.
c. Protirelin solution, Wedgewood Pharmacy, Swedesboro, NJ.
d. Animal Health Diagnostic Center, Cornell University, Ithaca, NY.
e. Immulite adrenocorticotropin hormone chemiluminescent assay, Siemens Medical Solutions Diagnostics, Los Angeles, Calif.
f. Equine Endocrinology Group, http://sites.tufts.edu/equineendogroup/
g. GraphPad Prism, GraphPad Software, Inc, La Jolla, Calif.

References