Trailering stress creates false positive results in diagnostic testing for Pituitary Pars Intermedia Dysfunction in Horses

By

Kaylee Hayes

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Thesis Committee

Dr. Rhonda Hoffman, Chair

Dr. John Haffner

Ariel Higgins

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Abstract

Pituitary Pars Intermedia Dysfunction (PPID) occurs in over 20% of aged horses. The best diagnostic for PPID compares plasma adrenocorticotropic hormone (ACTH) before and after thyrotropic-releasing hormone (TRH) stimulation. Stress affects the pituitary–adrenal axis and may confound PPID diagnosis. This study examined if trailering stress could cause false-positive PPID diagnosis in horses, and if so, what rest time after trailering is needed before TRH-stimulation. Ten horses were hauled 40 min every 4 weeks. Blood was collected before and 0, 15, 30, 60, and 120-min post-trailering, followed by TRH-stimulation. Data were analyzed using a mixed model with repeated measures. Post-trailering ACTH was higher ($P = 0.002$) than pre-trailering ACTH and falsely positive for PPID in some horses up to 30 min post-trailering, but ACTH after TRH-stimulation was not affected. Trailering stress caused multiple false-positive results, so veterinarians should wait 30 min after trailering to test horses for PPID.

CHAPTER ONE: Literature Review

Introduction

According to the American Horse Council census report (2018), there are over 7.2 million horses and over 1.3 million horse owners in the United States. These data include those owners that lease or own horses or farms. An additional 38 million are involved as horse enthusiasts (people who participate in horse activities but do not own a horse), and 1.4 million are involved as volunteers (nonpaid people involved in the maintenance and care of horses).

Advances in veterinary care have improved the overall care of the equine population. This has increased the average lifespan for equines as they are remaining active and competing at even older ages than they have in the past. In the United States at least 63% of horses 20 years of age and older are still athletically active due to the improvements in healthcare, wellness, nutrition, and the willingness of owners to continue to use these horses into their late teens and early twenties (Reed et al., 2015). One concern for aging horses is the development of Pituitary Pars Intermedia Dysfunction (PPID), which is a prominent morbidity in more than 20% of horses 15 years of age or older (Durham et al., 2014). While PPID can be found in younger horses, it is rare.

Also known as Equine Cushing's Disease, PPID is most often found in aged horses with no predispositions to gender or breed. Cushing's Disease is an older term used commonly by horse owners, and the name traces to Dr. Harvey Cushing, who was an American neurosurgeon who first described human "Cushing's Syndrome" in 1932. Most recently, veterinarians have transformed to the use of a more technically correct

name that fits the clinical description of the disease. PPID has become a part of routine veterinary visits for most horses and owners. There are several contributing factors to PPID, many clinical signs, many diagnostic tests, and ultimately no cure (McFarlane,. 2011).

Affected Hormones

Pituitary Pars Intermedia Dysfunction affects hormone pathways that take place in the brain, predominately in the hypothalamus and pituitary glands. The pituitary glands release hormones through pathways controlled by the hypothalamus and the neurotransmitter, dopamine. When an equine has PPID the neurons that produce dopamine degenerate, resulting in the inability to regulate hormone concentrations produced by the middle lobe in the pituitary gland, also referred to as the pars intermedia. When these hormones are unregulated due to the PPID many processes throughout the body are affected (Gehlen et al., 2021).

When neurodegeneration takes place, the result is hyperplasia of the pars intermedia of the pituitary gland. The hormone-secreting hyperplastic tissue releases adrenocorticotropic hormone (ACTH) in unregulated concentrations (Horn et al., 2019). McGowan et al. (2013) examined a group of aged horses with increased levels of ACTH concentrations and positive for PPID compared with a group of aged horses without elevated ACTH levels (McGowan et al., 2013). The result showed that age is a factor in a positive diagnosis for PPID with an increased risk for every year older than 15 years of age (McGowan et al., 2013).

Clinical Signs

Several clinical signs associated with PPID can be easily noticed by even the most inexperienced horse owner or enthusiast. One of the most unique clinical signs is the development of an abnormal hair coat, also known as hirsutism or hypertrichosis, where horses have delayed or incomplete shedding, during warm seasons, with some lightening in coat color in the older horses (Innera, et al., 2013). Horses with delayed shedding are five times more likely than those unaffected by delayed shedding to generate a positive PPID test (McFarlane, 2011). Other clinical signs of PPID include muscle wasting, specifically Type 2 muscle fibers (Aleman et al., 2006), abnormal distribution of fat over the body, lethargy or docility, lowered energy level (Spelta., 2015), infertility in both mares and stallions, and an increased susceptibility to parasitic and bacterial infections. Horses with PPID are at an increased risk of infections, some of these infections include sinusitis, dermatitis, endoparasitism, and laminitis (Fortin et al., 2020). Laminitis is the most prevalent of all the infections (Spelta., 2015).

In a study on seasonal clinical signs of PPID, owners in Southeast Queensland with horses that were at least 15 years of age were given a questionnaire to determine changes in their horse's hair coat (McGowan et al., 2013). The prevalence of the owner-reported hirsutism was 16.7% of the 974 completed surveys. A second part of the study included a veterinary examination that took place on the aged horses reported in the survey. Of the 325 aged horses tested, the prevalence of PPID was 21.2%, which was determined based on basal plasma ACTH concentrations higher than the seasonally regulated cut-off values (McGowan et al., 2013).

The risk of laminitis is one of the most concerning clinical signs associated with PPID. In more severe cases of laminitis, it is not uncommon for the horses to require euthanasia (McGowan., 2005). This is not to say that laminitis is developed in every horse that is diagnosed with PPID, and many horses with laminitis do not also have PPID. In some cases, laminitis was already present in the horse for months or years prior to getting a clinical diagnosis of PPID (Durham et al., 2014). Thus, laminitis alone cannot be used to diagnosis PPID. In an Australian Study, 13% of 69 aged horses diagnosed with PPID were also diagnosed with laminitis (McGowan et al., 2013). This is a 4.65 times odds ratio for risk of laminitis in PPID-positive horses when examined on a 95% confidence interval and compared with 256 PPID-negative controls (McGowan et al., 2013).

Diagnosis

There are several tests used to determine if a horse has PPID. The most commonly used tests include basal plasma adrenocorticotropic hormone (ACTH) concentration, the dexamethasone suppression test (DST), and the thyrotropic-releasing hormone (TRH) stimulation test (Durham et al., 2014). Because PPID is a progressive disease, the diagnostic screening tests and their reference ranges are most useful with horses in the more advanced to later stages of PPID.

Adrenocorticotropic Hormone Test

Testing basal ACTH concentrations is the most commonly used diagnostic test due to the convenience it provides, as it only requires a single blood sample and may be used year-round during any season (Tatum et al., 2021). Basal plasma ACTH concentration and its associated reference ranges can produce a false-negative result

when testing horses in the earlier stages of PPID (Hodge et al., 2019). Basal ACTH concentrations are commonly affected by season, with lower and less variable ACTH in January to June, and higher with highly variable ACTH from July to December (Hart et al., 2021). Basal ACTH increases dramatically in PPID-positive horses during autumn (July to December), so it is important to use appropriate reference values when testing in Autumn vs Spring. Due to minimal differences in basal ACTH between PPID-positive and PPID-negative horses, Autumn (September, October, November) is the season most likely to produce false-positive results when testing for PPID using basal ACTH concentrations (McGowan et al., 2013).

Dexamethasone Suppression Test

The dexamethasone suppression test (DST) is considered to have the highest amount of sensitivity and specificity for the diagnosis of PPID (Spelta., 2013). A DST test is used to detect failure of suppression of cortisol after administrating dexamethasone in horses with PPID (McGowan., 2005). Performance of a DST test begins by collecting a baseline serum sample for cortisol, then injection of 0.04 mg/kg per bodyweight of dexamethasone in the muscle. Wait 18-20 hours after injection and collect another sample of cortisol, then centrifuge before shipping to the laboratory (Durham et al., 2014). Performing a DST requires veterinary assistance for two consecutive days which can be costly and time consuming for everyone involved. The DST is often the diagnostic test of choice due to cost effectiveness and simplicity of the test. Often referred to as the overnight DST, the test is not affected by the time of day and can start the day before and run overnight. When the samples are collected at least 20 hours apart, the sensitivity and specificity are almost 100 percent (Durham et al., 2014). Because the

DST may also cause abnormal insulin response (Borer-Weir et al., 2013). Its safety as a PPID-diagnostic test in laminitis-prone horses has been questioned.

Thyrotropin-Releasing Hormone Stimulation

Thyrotropin-releasing hormone (TRH) stimulation test allows for detection of PPID in the initial stages of the disease in horses that have a basal ACTH concentration within the normal reference ranges (Hodge et al., 2019). TRH stimulation tests were originally created to test cortisol levels at times of 10 minutes and 30 minutes after the administration of TRH (Spelta., 2015).

TRH stimulation tests have been widely used due to the belief that it is safer than the DST. When using DST as a diagnostic test for PPID there is a risk that the single dexamethasone dose needed to perform the test can exacerbate insulin resistance, perhaps enough to initiate a laminitic event in horses that have a predisposition for laminitis (Haffner et al., 2009). However, Durham et al. (2019), stated that there is a higher risk of laminitis with use of TRH in intravenous administration. The TRH stimulation test is most likely to create a false positive PPID diagnosis in horses that already have an increased cortisol concentrations prior to the test being performed (Durham et al., 2014). A TRH stimulation test can be especially useful in horses with early PPID that have normal basal ACTH concentrations (Hodge et al., 2019). One drawback to the TRH stimulation test is that high seasonal variation in Autumn (September, October, November) ACTH concentrations renders the TRH stimulation test ineffective, with many false positives (Hart et al., 2021). At this time, the TRH stimulation test should be used only in January to August.

Combined Testing

It is possible to combine some tests together to increase the sensitivity and specificity of the results. It is common to combine a DST test with TRH. The idea is to increase the sensitivity of a TRH stimulation test by suppressing the serum cortisol using dexamethasone before administering the TRH. Very few studies have been performed on the use of the combined test, but it has shown to establish a difference between positive and negative cases (Horn et al., 2019).

Other Tests

There are other tests that can establish if a horse is positive for PPID. These tests include basal tests, blood glucose tests, urinary corticoid-to-creatine ratio, insulin, and cortisol. Elevation of blood glucose can be used a diagnostic test as horses with a higher blood glucose concentration are believed to have PPID and are at a risk of developing insulin resistance and laminitis in the future (Spelta., 2015). However, this test has a low sensitivity as not all horses with an increase in blood glucose develop PPID (McFarlane., 2011).

Urinary corticoid-to-creatinine ratio, which is commonly used as a diagnosis for PPID in dogs (Kooistra et al., 2012), is not often used as a diagnosis for PPID in horses as not all horses with an increased ratio develop PPID (Grenager., 2010).

Basal serum insulin concentration has proven to yield a greater than 90 percent success rate in diagnosing horses with PPID (Grenager., 2010). However, serum insulin can also yield a false positive result in overweight horses and ponies that are already insulin dysregulated. Basal serum cortisol concentration has not shown to have any evidence in proving if a horse is positive for PPID or not due to the amount of circadian variation in cortisol levels over a 24-hour period (Grenager., 2010). A summary of the

types of testing available, their sensitivity, simplicity, and relative cost is summarized in Table 1.

Research On Different Testing Methods

A study at the University of Pennsylvania examined differences in plasma ACTH concentrations and DST results in relation to season, sex, and age in horses and semi feral ponies (Donaldson et al., 2005). In 38 of the 39 ponies used, plasma ACTH concentration was greater in September than in the months of January and May (Donaldson et al., 2005). The DST ranges of cortisol for these ponies was also greater in September than in January (Donaldson et al., 2005). Age and ACTH concentration had a positive correlation in both January and September. There were no differences in plasma ACTH concentration when referring to sex and breed of the ponies (Donaldson et al., 2005).

Horn et al., (2019) evaluated the characteristics of combined testing versus independent testing to diagnose PPID and insulin dysregulation (ID) in horses. Four groups of horses were used in the study: horses with neither PPID or ID, horses with ID but not PPID, horses with PPID but not ID, and horses with both PPID and ID. Each horse experienced the same series of tests in no order. These tests included a TRH stimulation test, a 2-step insulin sensitivity test, and a combined test of both 2-step insulin sensitivity and TRH stimulation. The tests were performed between the months of October and February and greater than 2 hours apart but less than 2 weeks apart. Baseline plasma ACTH concentration was significantly higher in the group with PPID compared to group without PPID. The control group showed no difference in ACTH concentration regardless of the test performed. The administration of TRH showed a significant increasing effect on ACTH concentration in both the individual and combined testing.

The ID-only, PPID-only, and ID and PPID groups all showed no significant difference in baseline plasma ACTH concentration in either the combined or individual testing (Horn et al., 2019).

Treatment and Management of PPID

Currently, there is no cure for PPID, but it can be managed with proper care and pharmaceuticals. There are currently two types of pharmaceuticals used in the treatment of PPID. The most known drug is a dopamine agonist, pergolide mesylate, commonly marketed under the trade name Prascend. The lowest maintenance dose is 0.002 mg/kg/day for a 500kg horse. The low dose is the most cost-effective route to take but if no improvement in clinical signs is noted, the dose be increased in 250 microgram increments until an adequate reduction in clinical signs is apparent (Grenager., 2010). Prascend is prescribed to most horse owners by veterinarians in the United States.

Trilostone, a cortisol inhibitor, is the other pharmaceutical used to treat PPID. It is mostly used in the United Kingdom, and there is little research regarding its use in the United States. Trilostone is a competitive inhibitor of steroid synthesis and is the only treatment examined over an extended period of time that has shown improvements in the combined DST and TRH stimulation test. The dosing rate for Trilostone is 1 mg/kg/day and should be given only once per day in the evening. It has shown to produce little to no side effects (Grenager., 2010).

Durham et al., (2014) created a protocol for the most recommended pergolide treatment of horses with PPID. This protocol included documenting clinical findings, with owners monitoring appetite, hair coat, water intake, stall habits, body condition score, laminitis or lameness, and overall demeaner. After administrating a low dose of

pergolide (0.002 mg/kg/day) daily, clinical signs are reevaluated after one month of treatment (Durham et al., 2014). Table 2 shows the recommended doses of pergolide per body weight of horse.

Horses and ponies with PPID are at higher risk of ID, and additional diagnostic testing is needed to determine if the PPID-positive animal also has ID. Dietary management of PPID horses includes higher quality protein to offset the muscle wasting, and perhaps controlled non-structural carbohydrate intake at 10% or less, but only if also diagnosed with ID. Additional PPID management includes routine fecal egg testing and an appropriate deworming program as PPID-positive horses tend to shed more eggs than an unaffected horse. Regular dental checks and vaccinations will need to be performed due to increased risk of infection (Spelta, 2015). Body clipping is also recommended for horses living in warmer climates that do not have the ability to shed their hair properly because they are at an increased risk of overheating when they do not shed their winter hair coat.

Aging Horse Population

The aging horse population across the United States is increasing at a rapid pace. In 2020, the United States had an estimated 11.4% of the equine population being 20 years of age and older (Ballou et al., 2020). The percentage of aged horses in the U.S. increased by almost 5% over the timespan of 15 years (Ballou et al., 2020). With 38 million people in the United States involved as horse enthusiast (people who participate in horse activities but do not own a horse), there is a high demand for well-trained experienced, and likely older horses used for riding lessons, trail ride rentals, summer camps, and equine assisted therapies. Aged horses are defined as those that are of at least

20 years of age and older (Ballou et al., 2020). An informal survey of collegiate equestrian programs in the Southeast indicated that approximately 15 to 30% of college-owned lesson horses are aged 20 and older, with as many as 55 % being aged 15 and older (Hayes and Hoffman, personal communication).

With proper management, aged horses are able to continue to be ridden recreationally and competitively well into their late teens and twenties. Horses already have increasingly longer lifespans than most domestic animals owned by families (Ballou et al., 2020).

Owners caring for geriatric horses face many hardships along the way. Ballou and colleagues (2020), discuss the emotional and physical care burden that these owners take on when caring for a geriatric horse, which is considered to be an aged horse that also has a chronic illness. In a survey answered by 1,448 owners of aged horses within 47 states, over half of the respondents stated their horse currently had a chronic illness. PPID was the second most common illness reported by these owners (Ballou et al., 2020). Table 3 shows the chronic illness described by the sampled horse owners. The study indicated that owners were affected by a greater emotional burden as they stated they worried more about their geriatric horse and they had a physical burden as indicated by spending 1.4 hours more per week caring for their geriatric horse causing a physical burden (Ballou et al., 2020).

Ponies and horses of all breeds and types have an equal chance of being diagnosed with PPID regardless of gender (Durham et al., 2014). The most at risk for PPID are those over the age of 15. Considering that programs and universities offering riding lessons have several horses over the age of 15 it can out strain on the program if

they are treating multiple horses for PPID. Significant portion of aged horses (up to 30% are 20 years old and 55% over 15 years old), it is likely that these programs will have several PPID horses in their barns. The number of collegiate riding programs is extensive. The Intercollegiate Horse Show Association is composed of over 400 members' colleges across 45 states and Canada, and this is representatives of over 10,000 student riders. Given the results of the informal survey if equine programs in this region, it can be assumed that programs nation-wide have a similar percentage of horses over the age of 20. The success of collegiate riding programs is testament to the fact that with proper management, it is possible for aged horses to be both functional and critically to the success of these programs by their use in regular lessons. Horses diagnosed with PPID may be more likely to be donated to these programs to remove the stress they may cause for their former owners. These horses are vital assets to lesson programs and can perform tat the same rate as an unaffected horse, with proper care, and management.

Trailering, Transport and Shipping

Trailering is a common and cost-affordable form of transportation used by most horse owners. Horses tend to be transported more frequently than any other breed of livestock (Friend., 2001). Horses are transported for many different reasons. In the past, horses were transported for war purposes, while now it is primarily for sports, recreation, and breeding (Padalino., 2015). There are several forms of transportation used in equines. For over 3,500 years horses have been transported overseas by boat. In the middle to late 1800s, it became more common for horses to be transported via train and railroad. Post-World War II, transport via truck and interstate highway became available, and the 1960s-70s were referred to as the "trailer age" of horse transport. In the late 1900s, horse

transport via aircraft became possible using large cargo containers, making this the “air age” of transport (Friend., 2001).

Transportation of equines has become even more prominent in present day as there is a greater use of horses for recreational use and sporting events. Long distance shipping is commonly used in the sport horse world as elite equine athletes are typically shipped and transported long distances for intense competitions. Long distance shipping or transportation can influence stress and behavior of equines (Padalino., 2015).

Stress and Behavioral Impact

Transportation is an important part of horse care and ownership, but it comes with its own set of risks. Transport stress is an issue along with many other problems that can be associated with equine transportation (Padalino., 2015). Even after years of research and debate the best way to manage transport stress is still under review as not all results can be considered comparable due to differences in trucks and trailers, differences in weather conditions, and variation in breeds or individual horses (Padalino., 2015). Transported horses are at risk for several different stressors that include isolation, forced proximity to unfamiliar horses, novel or threatening surroundings, exposure to exhaust pathogens, and the physical stress of standing for long periods of time in a moving trailer (Friend., 2001). In a study using 84 healthy Thoroughbred and crossbred stallions in Italy, the impact on stress and behavior from trailering was examined (Fazio et al., 2013). All horses were managed by similar methods, located on the same farm, and between the ages of 11 and 16. All horses were transported on the same commercial 6-horse vehicle for 200 km along the same route with very little traffic.

The stallions were divided into 2 groups, calm and nervous. The calm group contained 40 Thoroughbred and 24 crossbred stallions, and the nervous group contained 10 Thoroughbred and 10 crossbred stallions. Blood samples of each stallion were taken immediately before loading and immediately after transport and unloading. Cortisol concentrations of the stallions increased after transport when compared to pre transport concentrations, and an increase in stress levels in the nervous group after transport was noted. Figure 2 represents these data (Fazio et al., 2013).

Long-distance transport can be associated with stress-related changes in equine immune function and shipping related illness. However, most equines are frequently transported for short distances, but the effects of short distance transport remain unknown (Miller et al., 2021). The effects of short-term transport were examined in a study using twelve University of Kentucky owned horses, seven mares and 5 geldings, ranging between the ages of 15 and 30 years of age. The control group and the treatment group each contained 6 horses with no difference in age between the groups. All horses were housed on the farm, primarily in pasture, retired, and sometimes transported by trailer on or around the farm. Each horse had varying levels of experience with transportation, with at least half having significant experience during earlier parts of their lives (Miller et al., 2021).

Horses in the treatment group were fed a daily antioxidant supplement based on body weight recommendations provided by the supplement manufacturer. Horses were transported over 2 days in multiple sets in a 4-horse slant-load trailer. The same route was taken on each trip, with similar travel time based on traffic, and returned to their normal pastures after transportation. Serum total cortisol was collected 15 minutes pre and post

transport on days 0,1,3,7,14 and 21. Temperature and heart rate was also assessed 15 minutes pre and post transport. Total cortisol concentration at 15-minutes pre and post transportation was increased compared with the baseline values. Total cortisol levels were significantly higher at 15 minutes post transport when compared with all other time points (Miller et al., 2021).

Considering the evidence that the transportation stress of trailering increased cortisol (Fazio et al., 2013; Miller et al., 2021), and understanding that the hypothalamic-pituitary-adrenal axis is involved in the release of cortisol, it can be assumed that ACTH might also increase as an effect of trailering stress. Horses are commonly transported to and from veterinary clinics for routine and emergency care. Could trailering stress affect ACTH concentrations markedly enough to cause a false-positive PPID diagnosis in otherwise healthy horses? The focus of this research aimed to address this question.

Conclusion

The population of aged horses in the United States has been increasing with an ever-growing demand for well-trained, experienced horses for amateur horse enthusiasts, youth and collegiate riding programs, and equine-assisted activities and therapies. As many as 30% or more of horses in lesson programs are aged 20 and older. Pituitary Pars Intermedia Dysfunction is a common disease found in older horses that affects the hormone pathways within the brain. Several clinical signs that create concern for horse owners such as abnormal distribution of fat, long hair coat, lethargy, laminitis, increased risk of infection, and many others. Many tests can be performed to determine if a horse is positive for PPID. These most popular diagnostic tests include ACTH concentration, TRH stimulation, and DST. Although, there is no cure for PPID it can be managed

through pharmaceutical drugs such as pergolide, sold by the trade name Prascend, body clipping, proper diet, and maintaining routine veterinary care.

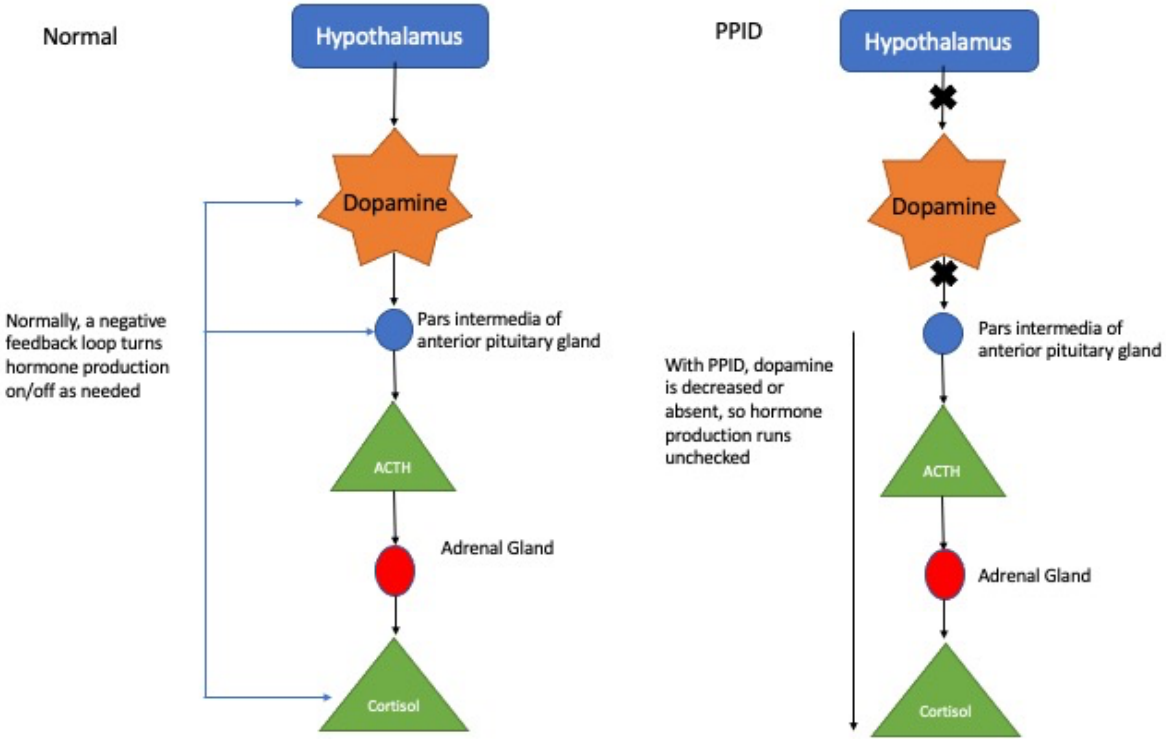

Figure 1: Normal hormone pathway of a horse that does not have PPID (left). Abnormal hormone pathway of a horse with PPID (right), as absent or reduced dopamine hormone affects the production of many other hormones (Adapted from Young., 2020).

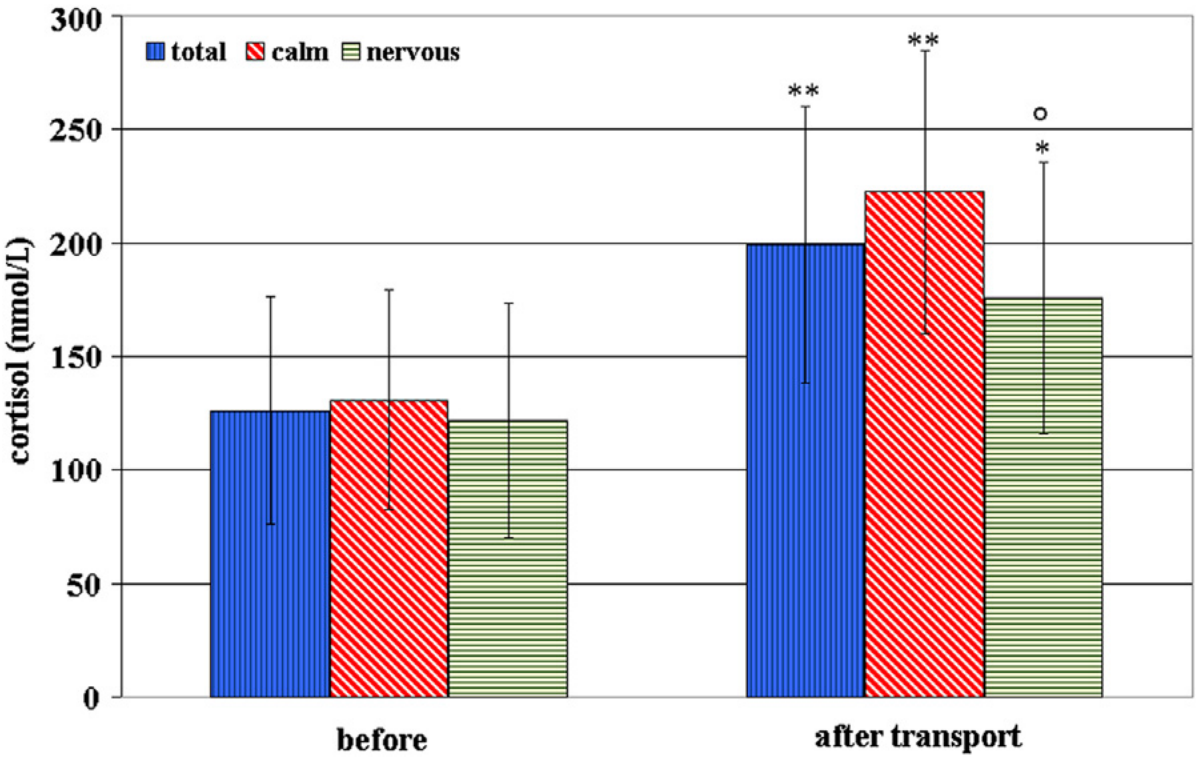

Figure 2: Cortisol concentrations in calm and nervous stallions before and after transport (Fazio et al., 2013)

Table 1: Examples of tests available in determining if a horse is positive for Pituitary Pars Intermediary Dysfunction, including sensitivity, specificity, cost, and level of difficulty (McGowan et al., 2003).

Test	Sensitivity	Specificity	Cost	Simple
Dexamethasone Suppression Test (DST)	++++	++++	Low	Yes- but need 2 visits
Basal ACTH	+++	+++	Low	No – specific handling requirements
Combined DST/TRH	++++	++++	High	No – need 2 visits and 4 samples
Clinical Signs	++	++	Low	Yes
UCCR	+++	++	Low	Yes – but requires urine collection
Basal Insulin	+++	++	Low	Yes
Glucose	++	+++	Low	Yes
TRH Stimulation	++	++	High	Medium – need 2 samples collected over 10 minutes
ACTH Stimulation	++	++	Medium	Yes – 2 samples collected over 2 hours

Table 2: Recommended starting dose of pergolide that should be given per bodyweight of the horse (Durham et al., 2014).

Suggested starting dosage of pergolide for treatment of PPID by bodyweight	
Bodyweight	Starting daily dose
200-350 kg	0.5 mg
350-600 kg	1.0 mg
601-850 kg	1.5 mg

Table 3: Chronic disease in geriatric horses from a survey performed in 2020 with 1,448 respondents (Ballou et al., 2020).

Condition	**Owner Reported**	**Veterinarian Diagnosed**
Osteoarthritis	41.4%	82.7%
Pituitary Pars Intermedia Dysfunction (PPID)	26.8%	91.6%
Dental Disease/conditions	15.1%	84.4%
Ophthalmic disease/conditions	11.1%	84.0%
Laminitis	9.8%	84.3%
Skin Cancer	9.1%	88.8%
Equine Asthma (heaves)	7.8%	87.7%
Insulin Dysregulation	7.7%	87.7%
Chronic Diarrhea	6.4%	39.3%
Heart Abnormality	4.3%	100.0%
Lyme Disease	3.6%	94.1%
Other Cancer	2.8%	96.0%
Anemia	1.5%	100.0%
Other	19.8%	N/A

CHAPTER TWO: Does trailering create false positive results when testing for Pituitary Pars Intermedia Dysfunction in Equines?

Introduction

Pituitary Pars Intermedia Dysfunction (PPID), commonly known as Equine Cushing's Disease, is a prominent morbidity in over 20% of horses 15 years of age and older. PPID affects the hormone pathways in the brain, predominately the hypothalamus and pituitary gland. When a horse has PPID, the neurons that produce dopamine degenerate, resulting in the inability to control adrenocorticotropic hormone (ACTH) production by the middle lobe in the pituitary gland, or the pars intermedia. Stress from trailering horses can increase ACTH.

The objective of this study was to explore if the stress of trailering horses to equine hospitals for PPID testing could cause false positive diagnosis of PPID. It was hypothesized that trailering horses for 40 minutes would increase cortisol levels and result in an increase in ACTH concentrations that would create a false positive result when testing for PPID.

Materials and Methods

The protocol for this study was approved by the Institutional Animal Care and Use Committee (Protocol #19-2008). Ten adult horses of various breed type and sex (5 geldings, 5 mares) housed at Middle Tennessee State University (MTSU) were used. Horses were 15.7 ± 4.1 years of age (range 7 to 20 years) and all tested to be PPID-negative prior to beginning the study.

To avoid known seasonal variation in ACTH, the animal phase start date began in January, and the end date was in June. Five horses were examined in January to June of

2020, and five more in January to June of 2021. All horses were identified by individual ID or name and description. All horses were housed, fed, and watered according to typical procedures designated by the MTSU Horse Barn. The horses were randomized using a 5x5 Latin Square Design, with horses rotating through different positions in the trailer and sample time after the trailer ride. Each group contained 5 horses hauled for 40 minutes every four weeks. The length of the 40-minute trailer ride was chosen to simulate a typical distance of transport to an equine veterinary hospital. The horses each rotated through 5 trailer positions and sampling times throughout the study, thus allowing each horse to be their own control. Blood samples were drawn at multiple time frames throughout the study. A baseline ACTH sample was taken prior to loading and TRH stimulation test was conducted at respective testing times. Each horse was unloaded at their respective post-trailering times and blood samples were collected. Times at which the blood samples were taken include Baseline (no trailer ride), and 0, 15, 30, 60, and 120-minutes post-trailer unloading.

A thyrotropin-releasing hormone (TRH) stimulation test was performed at 0, 15, 30, 60, and 120-minutes post trailering. The TRH stimulation test includes an initial blood sample (T0-ACTH) followed by 1 mg TRH administered intravenously. Exactly 10 minutes after administration of TRH, which is standard for TRH-stimulation testing, the T10-ACTH blood sample was collected. This sample was compared to the initial T0-ACTH blood sample.

Blood samples were centrifuged, and plasma was aliquoted and shipped frozen with cold packs overnight to the Animal Health Diagnostic Center of Cornell University (Ithaca, NY) for analysis. Plasma samples were batch-analyzed for ACTH concentration

by chemiluminescent immunoassay that had been previously validated for horses (Perkins et al., 2002).

Evaluation of PPID diagnosis based on the samples collected before and after trailering followed previously published cut-off values (2018) as follows: PPID-positive was diagnosed by Basal ACTH > 60 pmol/L, PPID-negative as Basal ACTH < 30 pmol/L, with an equivocal diagnosis of ACTH concentrations between 30 and 60 pmol/L. After TRH-stimulation, PPID-positive was diagnosed by T10-ACTH > 200 pmol/L, PPID-negative with T10-ACTH < 110 pmol/L, with an equivocal diagnosis of T10-ACTH concentrations between 110 and 200 pmol/L

Data were first analyzed for normality and found to be normally distributed. A mixed model with repeated measures compared ACTH pre- and post-trailering and after TRH stimulation, using horse as subject and time as repeated effect. Year of sampling had no effect ($P = 0.23$) so trailering data were combined from 2020 and 2021. Day of sampling had no effect ($P = 0.46$), so day was removed from the model.

Results

Table 4 shows results of ACTH concentrations when comparing no trailer ride or pre-trailering with post-trailering for each individual horse. Horses' names are displayed on the far left of the table to show personalization of the results. Values highlighted in orange represent ACTH concentrations over 60 pmol/L, indicating a false-positive PPID diagnosis. Values highlighted in yellow represent ACTH concentrations over 30 pmol/L, indicating an equivocal diagnosis, suggesting that additional testing would be needed at a later date to verify if PPID negative or positive. Values highlighted in green represent ACTH concentrations below 30 pmol/L, indicating a correct PPID negative diagnosis. Individual horse ACTH concentrations in response to trailering stress indicated false-positive diagnosis for PPID in 4 horses at 0 min, 4 horses at 15 min, and 3 horses at 30 min post-trailering (Table 4). Average basal ACTH was elevated ($P = 0.002$) after trailering compared to no trailer ride (Table 5). The average TRH stimulated T10-ACTH showed no difference when compared to pre-trailering T10-ACTH at 0 min or 15 min post-trailering, but was lower at 30, 60, and 120 min ($P = 0.015$). Only one TRH-stimulated horse tested false-positive for PPID at 0 min after trailering when the T10-ACTH sample was evaluated.

Table 4. Plasma ACTH (pg/mL) of individual horses before trailering and after trailering. Cells shaded in Green indicate PPID-negative diagnosis. Yellow indicates equivocal, with re-testing required and Orange indicates PPID false-positive diagnosis.

		Post-Trailering ACTH, pg/mL			
	No Trailer Ride	0 min	15 min	30 min	60 min
Chester	25.4	27	34.7	37.7	15.1
Dixie	22	21.6	27.7	17.8	13.2
Flip	17.3	62.2	51	40.4	21.5
Floyd	28.7	56.1	41.4	28.1	15.2
Lancaster	25.1	44.2	27.7	16	19.7
Penny	20.6	17.8	17.2	14	11.7
Raquel	22.3	206	66.8	41.6	11.2
Rooney	19.9	20.3	15.6	14.6	14
Sadie	7.99	9.87	10.9	3.64	10.3
Tribute	8.53	27	12	14.8	13.4

Table 5. Average plasma ACTH (Mean ± SE, pg/mL) in 10 horses before trailering (Basal ACTH), after trailering (T0-ACTH), and 10 min after the TRH-stimulation test.

	PRE-Trailering	**Post-Trailering**		
Sample Time	Basal ACTH	T0-ACTH	T10-ACTH	Row P
No Trailer	19.8 ± 2.2	19.8 ± 2.2 ^{b}	62.0 ± 8.6 ^{c}	
0 min	20.9 ± 3.5 ^{y}	49.2 ± 18.2 ^{a,x}	55.1 ± 11.9 ^{cd,x}	$^{x, y}$ P = 0.002
15 min	20.4 ± 2.1	30.5 ± 5.8 ^{b}	45.9 ± 4.5 ^{cde}	
30 min	23.8 ± 4.3	22.9 ± 4.2 ^{b}	38.9 ± 5.3 ^{de}	
60 min	20.8 ± 1.9	14.5 ± 1.1 ^{b}	38.7 ± 7.5 ^{de}	
120 min	20.8 ± 3.0	13.2 ± 1.4 ^{b}	33.1 ± 3.5 ^{e}	
Column P		^{a,b} P = 0.002	^{c,d,e} P = 0.015	

$^{a–h}$ Superscripts indicate differences within columns

$^{x, y}$ Superscripts indicate differences within the same row

Discussion

This study showed that trailering stress created several false-positive diagnoses for PPID in individual horses up to 30 min after trailering (Table 1). Horses were identified using their names to show personalization to compare the differences between each horse. Each horse on the study had different amounts of trailering experience prior to the study, but all had been previously acclimated to trailering. One horse diagnosed as false positive for PPID for up to 60 minutes after trailering, Raquel, is a horse with a significant amount of trailering experience prior to this study. It is unknown why this individual horse had persistently elevated ACTH after trailering. Previous work indicated that temperament of stallions influenced adrenocortical response to trailering stress, even in horses with significant trailering experience (Fazio et al., 2013). While temperament in the current study was not evaluated, it is possible that the individual horses with elevated ACTH post-trailering had temperaments that increased their adrenocortical response to trailering stress. On average, ACTH was elevated immediately after trailering but became non-significant as early as 15 minutes later (Table 2). While the TRH-stimulated T10-ACTH was elevated at 0 and 15 min after trailering and significantly lower at 30,60, and 120 min, the T10-ACTH concentrations still correctly indicated negative diagnosis for PPID at all times post-trailering (Table 2).

This shows that stress from trailering causes an increase in ACTH concentrations in horses that may confound accurate PPID diagnosis. This stress response should be taken into consideration when trailering horses to veterinary clinics or equine hospitals for PPID testing, especially if basal ACTH concentrations are used as the diagnosis test.

Alternately, ACTH concentrations 10 min after TRH-stimulation were less sensitive to effects of trailering stress, except in the one horse previously mentioned.

Conclusion

Stress from a 40-minute trailer ride elevated Adrenal Corticotropic Hormone concentrations and created multiple false positive diagnosis for Pituitary Pars Intermedia Dysfunction. The elevated Adrenal Corticotropic Hormone concentrations persisted up to 30 minutes post-trailering in individual horses but was resolved by 60 minutes post-trailering. Using the Thyrotropin Releasing Hormone-Stimulation test as a diagnosis for Pituitary Pars Intermedia Dysfunction did not appear to be affected by trailering stress when compared to Adrenal Corticotropic Hormone concentrations alone. Horse owners and veterinarians are recommended to wait at least 60 minutes after trailering to test for PPID when using Adrenal Corticotropic Hormone concentrations as the primary diagnostic tool, or at least 15 minutes after trailering when using TRH-stimulation test.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

APPROVAL, PROTOCOL 19-2008

IACUC
INSTITUTIONAL ANIMAL CARE and USE COMMITEE
Office of Research Compliance,
010A Sam Ingram Building,
2269 Middle Tennessee Blvd
Murfreesboro, TN 37129

MIDDLE TENNESSEE STATE UNIVERSITY

IACUCN001: PROTOCOL APPROVAL NOTICE

Tuesday, April 05, 2022

Senior Investigator	**John Haffner** (ROLE: Principal Investigator)
Co-Investigators	Jeremy Carlton, Alena Boudie and Mariah Boyl
Investigator Email(s)	*john.haffner@mtsu.edu*
Department	Agriculture
Protocol Title	***Does trailering and/or teeth floating affect adrenocorficotropic hormone levels in equine plasma samples?***
Protocol ID	**19-2008**

Dear Investigator(s),

The MTSU Institutional Animal Care and Use Committee has reviewed the animal use proposal identified above under the ***Designated Member Review (DMR) mechanism*** and has approved your protocol in accordance with PHS policy. A summary of the IACUC action(s) and other particulars of this this protocol is tabulated as below:

IACUC Action	**CONDITIONALLY APPROVED**	
Date of Expiration	**2/28/2022**	Approval Date 02.18.2019
Number of Animals	12 (TWELVE)	
Approved Species	**Equine: MTSU Herd**	
Category Subclassifications	☐ **Teaching** ☐ Classroom ☐ Laboratory	☒ **Research** ☒ Laboratory ☒ Field Research ☐ Field Study ☒ Handling/Manipulation ☐ Observation
	Comment: NONE	
Approved Site(s)	Horse Science Center barn	
Restrictions	**Satisfy DMR requirements AND annual continuing review**	
Comments	Boudie – assist horse handling, blood sampling and plasma storage; Boyl – Assist with horse handling, blodd sampling and plasma storage; Carlton – horse handling and trailering The continuing review for 02/28/2021 needs to be confirmed by the IACUC at a FCR; the protocol is administratively allowed to continue while an IACUC verdict is awaited.	

This approval is effective for three (3) years from the date of this notice. This protocol **expires on 2/28/2022** The investigator(s) MUST file a Progress Report annually regarding the status of

this study. Refer to the schedule for Continuing Review shown below; NO REMINDERS WILL BE SENT. A continuation request (progress report) must be approved by the IACUC prior to **2/28/2022** for this protocol to be active for its full term. Once a protocol has expired, it cannot be continued and the investigators must request a fresh protocol.

Continuing Review Schedule: Refer to the following table to request your CR:

Reporting Period	Requisition Deadline	IACUC Comments
First year report	1/31/2020	Continuing review was approved by FCR on 02/28/2020
Second year report	1/31/2021	CR is initiated by FCR; the protocol is administratively extended (02/01/2021) The IACUC Agenda for FCR missed this protocol due to a typographical error. The IACUC unanimously voted to conduct this CR and allow the PI to continue in the meanwhile (03/05/2021)
Final report	1/31/2022	TO BE COMPLETED

MTSU Policy defines an investigator as someone who has contact with live or dead animals for research or teaching purposes. Anyone meeting this definition must be listed on your protocol and must complete appropriate training through the CITI program. Addition of investigators requires submission of an Addendum request to the Office of Research Compliance.

The IACUC must be notified of any proposed protocol changes prior to their implementation. Unanticipated harms to subjects or adverse events must be reported within 48 hours to the Office of Compliance at (615) 494-8918 and by email – compliance@mtsu.edu.

Post-approval Protocol Amendments:

Date	*Amendment(s)*	*IRB Comments*
03/14/2019	Mariah Bovl and Kimberly White are approved co-investigators	DMR
12/16/2020	A procedural amendment was approved	FCR (12/12/2019)
02/28/2020	A procedural amendment was approved	FCR (02/28/2020)
11/25/2020	A minor amendment was approved	DMR
01/21/2021	Kaylee Hayes (CITI34863146) has been added to the protocol	An error in the student name is corrected administratively

All records pertaining to the animal care be retained by the MTSU faculty in charge for at least three (3) years AFTER the study is completed. Be advised that all IACUC approved protocols are subject to audit at any time and all animal facilities are subject to inspections at least biannually. Furthermore, IACUC reserves the right to change, revoke or modify this approval without prior notice.

Sincerely,

Compliance Office
(On behalf of IACUC)
Middle Tennessee State University
Tel: 615 494 8918
Email: iacuc_information@mtsu.edu (for questions) and
iacuc_submissions@mtsu.edu (for sending documents)