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IMPACT OF SHORT-DISTANCE ROAD TRANSPORTATION ON HORSE HEALTH

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IMPACT OF SHORT-DISTANCE ROAD TRANSPORTATION ON HORSE HEALTH

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

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ABSTRACT OF DISSERTATION

IMPACT OF SHORT-DISTANCE ROAD TRANSPORTATION ON HORSE HEALTH

Horses are regularly transported across the United States; however, the reasons for travel, trip lengths, and management practices are not well characterized. While long distance transportation has been associated with increased incidences of health-related problems, the impact of short distance transportation on horse health is less understood. Notably, aged horses (>15 years) have an altered immune response following short-distance transportation, but this has yet to be compared to young horses. It is also unknown whether transportation stress would alter endocrine responses of horses with metabolic disorders, such as insulin dysregulation (ID) or pituitary pars intermedia dysfunction (PPID).

The objectives were 1) to characterize the how and why horses are transported by road in the U.S., 2) to evaluate differences in stress and immune responses to short distance transportation between aged and young horses, 3) to determine if ID horses have altered endocrine responses following transportation and 4) to characterize the stress response to short distance transportation of horses with and without PPID.

An online nationwide survey was conducted to determine the common types of journeys horses are transported by road in the U.S and how horses are managed on trips 3 hours or less. There were 1294 survey participants with the majority being female, adult, amateurs who owned and transported their own horse for trail/leisure riding. The most common lengths of trip horses were transported was less than one hour, and the frequency of trips was greater for shorter lengths of journeys. While adult horses were transported most often, >30% of horses were over the age of 15 years. Although owners expressed concerns for horse health during transport, very few monitored vital signs before or after transportation, especially on short journeys.

Differences in short-term transportation stress responses of aged and young horses were explored with 5 aged (22 ± 1 year) and 6 young (2 ± 1 year) horses transported by road on a round trip of 1 hour and 20 minutes. Cortisol and heart rate both increased in response to transportation, with no differences due to age. Additionally aged horses had increased plasma insulin concentrations and altered gene expression of certain cytokines post-transportation compared with young horses.

The stress and insulin response of ID and non-ID horses following transportation were determined by transporting 7 ID and 7 non-ID horses using the same methodology as the previous study. ID horses had increased serum insulin concentrations compared with non-ID horses; however, even non-ID horses had post-transportation and oral sugar test (OST) insulin concentrations above the diagnostic cutoff for ID that could cause misleading diagnostic results.

Finally, certain stress hormones of the hypothalamic pituitary axis of PPID horses were explored to determine the impact of short-distance transportation and diagnostic testing. ACTH remained elevated in PPID horses following transportation; however, transportation increased cortisol in moderately PPID and non-PPID horses but remained low in severely PPID horses.

Overall, the results from this dissertation highlight the importance of understanding the endocrine, metabolic, and physiological responses of horses to the stress of being transported short durations. These studies showed differences in various physiological and endocrine responses to transportation based on age and endocrine disorder and provide justification for why it is necessary to consider these factors when managing and monitoring horses being transported by road or if performing diagnostic testing post-transportation.

KEYWORDS: Horse, transportation, stress, insulin dysregulation, PPID

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IMPACT OF SHORT-DISTANCE ROAD TRANSPORTATION ON HORSE HEALTH

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CHAPTER 1 LITERATURE REVIEW

1.1 Introduction

Transportation is a vital part of horse management as horses are frequently transported for a wide variety of reasons and using different means of transportation, with the primary way being by road [1]. Having a better understanding of how horses are transported along with characterizing their physiological responses to transportation could lead to improved horse management and welfare during transportation. Previous research has aimed to reduce transportation-related problems [2–4], but further research is needed to better classify the stress response of horses to road transportation, particularly over shorter durations. Transportation stress can be defined as the physiological and psychological stress that a horse experiences when being transported [5]. This includes physical stressors, confinement and maintaining balance; environmental stressors, temperature and air quality; social stressors, isolation or unfamiliar horses; and psychological stressors, changes in environment and noises [1]. Transportation stress in a horse can be measured by various signs such as increased heart rate, elevated cortisol, and changes in behavior, like increased restlessness and agitation [6–9]. Numerous factors can contribute to a horses stress response to transportation including previous experience or lack of experience being transported [6,10]. Additional factors such as handling, trailer type, position in the trailer, and the length of journey can all be associated with differences in stress responses [8,9,11,12]. However, less is known about how transportation stress may affect senior horses and/or those with endocrine disorders. Therefore, this dissertation aimed to evaluate components of the stress response to short-duration road transportation in horses of different ages and those with or without common endocrine disorders while

also understanding common journeys and management practices in the United States. This could provide information to help veterinarians, horse owners, and transporters to make informed monitoring and management decisions when transporting horses by road over a short duration.

1.2 Equine Road Transportation Practices

Although horses are transported by road more frequently than any other livestock species [13], there is little information about how and why they are transported, including a lack of understanding of management strategies, especially in the U.S. Additionally, it remains to be understood what types of journeys are the most common for horses being transported by road and what types of horses are being transported.

Research surveying horse owners has mainly focused on the incidence of health-related issues associated with transportation [14–20]. There is one published transportation survey in the U.S. that surveyed owners who had previous problems trailering and reported loading as the biggest cause of trailering issues; however, this survey only focused on problems and not general practices [18]. Most other surveys conducted on equine road transportation practices and management have been conducted internationally [14–17]. A study in Italy found an association between trailering related behavioral problems, such as anxiety, refusal to load, agitation in the trailer, etc. and increased occurrences of both equine and human injuries [14]. Surveys conducted in Australia looked at the relationship between horse management and transportation related health issues and found traumatic injuries were the most common problem [15,16,20]. One study from Australia found that there was an increased risk of heat stroke associated with restricted access to hay and/or

water before transit [16]. When considering professional versus amateur member of the equine community, professionals transported an increased number of horses more frequently and over longer distances [15]. While these surveys provide valuable insight into general management practices in other countries, they do not differentiate between length of journey. Additionally, while they highlight the importance of trailer-related issues they also show a gap in our understanding of the general management of horses in the time during and surrounding transportation and the need for further research to understand current practices and potentially make recommendations to mitigate health risks.

Therefore, a nationwide online survey was conducted to understand common transportation practices in the U.S. and management on trips of short durations and results are reported in Chapter 2 of this dissertation.

1.3 Road Transportation and Horse Health

While transportation is a routine event experienced by most horses multiple times throughout their life, there is still potential risk for horses to develop transportation associated health problems. It is undesirable to have problems occurring during or as a result of road transportation and is important to prevent or mitigate potentially adverse effects when possible. Various factors associated with road transportation can influence stress and have a negative impact on horse health. The trailer environment, such as ventilation and temperature, can contribute to risk of developing respiratory issues and heat stress [1]. Additionally, the confinement and isolation associated with trailering could also contribute to transportation stress [1]. These can further be exacerbated by a horse's

previous trailering experience and temperament, where less experienced and nervous or anxious horses might have heightened stress levels during travel [21].

The incidences of transportation-associated problems are impacted by the length of journey with an increased risk of health-related issues after 20 hours [1]. It can take hours for horses to adapt to conditions and stressors associated with transportation of medium to long lengths (4+ hours), as indicated by decreased indicators of stress [22,23]. This initial adaptation period can be seen in an increase in the number of movements in horses transported 1 hour versus 3 hours [9]. Additionally, there are elevated serum cortisol concentrations and stress-related behaviors during the 1st hour of transport on short journeys (3 hours or less) compared with pre-transportation [9,24]. These studies highlight a critical timepoint early in transportation where horses might experience increased stress and physiological changes while acclimating to being transported.

Transportation stress activates the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.1) [25]. During transportation, stress is perceived by the horse which sends signals to the hypothalamus in the brain that releases corticotrophin releasing hormone (CRH) that acts on the pituitary gland to secrete adrenocorticotropin releasing hormone (ACTH). ACTH then travels in circulation to the blood stream to stimulate the adrenal glands to release cortisol and adrenaline to maintain homeostasis. Cortisol plays a vital role in metabolism and immune function, while adrenaline increases heart rate and promotes breakdown of glycogen to glucose in the liver, giving the horse energy to effectively respond to a stressor [26,27]. These hormones and their downstream effects can be measured to assess stress levels associated with transportation.

Transportation of horses is associated with an increase in various circulating pituitary hormones such as ACTH and β-endorphin [12]; however, the most measured hormone to evaluate transportation stress in horses is cortisol. Circulating cortisol, in serum and/or plasma, has been well documented to increase in horses in response to transportation [9,21,28]. Recently non-invasive techniques such as salivary cortisol and fecal cortisol metabolites have also been utilized to monitor stress responses [6,7,29]. The use of saliva or feces to measure biomarkers, such as cortisol, can be beneficial because they do not require blood collection. They could also be used in situations where collecting a blood sample may not be possible or for use in horses who are averse to needles. Salivary cortisol increases in horses during transportation with highest concentrations after transport, while fecal cortisol metabolites show increases from pre-transportation approximately one day after transport [7]. In humans saliva is used as non-invasive diagnostic tool to screen for various diseases such as HIV, Cushing's, and Addison's disease [30]. In addition to cortisol, it is possible that other relevant analytes could be measured in equine saliva for use in diagnostics or monitoring. Human research has begun to evaluate salivary biomarkers associated with inflammation and insulin resistance, such as inflammatory cytokines and insulin, to use as both screening and diagnostic tools for obesity and diabetes [31–35]. Recently, insulin has also been able to be detected in equine saliva [36]. This suggest that saliva might have the potential to be a useful tool in diagnosing equine endocrine diseases and as a health monitoring tool post-transportation.

Additionally, heart rate (HR), average beats/minute, and heart rate variability (HRV), time interval between heart beats, can be used as non-invasive ways to measure stress. During a stress response adrenaline is released from the adrenal glands, causing an

increase in HR and blood pressure [25]. Adrenaline binds to beta-adrenergic receptors on cardiac muscle cells, increasing cyclic adenosine monophosphate (cAMP) and calcium levels, which enhance heart muscle contractions [37]. Additionally, adrenaline depolarizes the sinoatrial node, accelerating the electrical impulses thereby increasing HR [37]. Conversely, HRV decreases in response to stress when the sympathetic nervous system is dominant, resulting in the heart pumping faster and the time between beats being shorter [38]. Decreased HRV is associated with stressful events for horses such as changes in environment, weaning, exercise, and transportation [7,39–43]. In horses, the interval between heart beats (R-R interval) decreased with loading and at the beginning of travel the first day of transport in a 2-day long journey, with a less pronounced response on the second day of transport [6]. In another study, heart rate was increased after horses were transported 3 to 4 hours [12]. Monitoring HR and/or HRV are beneficial for evaluating stress response to transportation in horses since they can be used to monitor changes in real time. However, HRV is thought to be more specific to the stress response versus HR because it better reflects the balance between the sympathetic and parasympathetic nervous system while HR could also be reflective of energy expenditure required for loading and balancing during transport [38]. Stress can also be evaluated by monitoring changes in behavior. There is an increased frequency of stress related behaviors, including: biting their neighbor, exploratory behavior, surveying, head toss/shaking, chewing, licking, pawing, scratching, stereotypy, and turning their head, in horses transported 12 hours versus horses confined to a stall for 12 hours [44].

To complement stress-associated blood parameters, this dissertation utilizes non-invasive stress monitoring techniques, such as heart rate monitoring and salivary sampling, in Chapters 3, 4, and 5.

1.4 Transportation-associated Health Problems

The most reported health issues associated with transportation are injury, gastrointestinal problems, muscle fatigue, dehydration, and respiratory disease [15,16]. When considering gastrointestinal health, changes in fecal microbiota have been observed following transportation [45,46], there is a positive correlation between transport-stress and occurrence of gastric ulcers [47], diarrhea has been associated with transport-related stress [1], and there is a higher risk of colic in horses transported more than 6 times per year [48]. However, there is also potential to alter transportation management practices to minimize some of these risks [47,49]. In these studies, fasting horses prior to a 12-hour journey was associated with increased gastric ulcer score compared with horses who were fed 1 or 6 hours prior, suggesting that presence of feed in the stomach could decrease the potential to develop ulcers [47,49]. However, there could also be problems associated with horses having access to hay during transportation due to location of hay where the horses head is elevated and/or present of dust particles that could increase the likelihood of horses developing respiratory issues [1,50]. Additionally, it is unclear how horses are typically transported regarding access to hay and/or grain, emphasizing that more information is needed to better inform best feeding practices.

Transportation can potentially induce heat stress and dehydration, influenced by the environment inside the trailer, as well as ambient temperature and humidity. Rectal

temperatures have been shown to increase after transportation of 3-4 hours; however, they remained within a normal range [51]. Additionally, drinking is suppressed during transportation [52]. It has also been reported that there is a 6% decrease in bodyweight after a 24-hour journey, with these findings being attributed to the reduced feed/water intake and fluid loss from sweating [53]. Increased length of journey and extreme weather can also increase the risk of health problems because of the predisposition to dehydration in these conditions [1], emphasizing the importance of proper management and monitoring of horses during transportation.

Road transportation is frequently linked to the development of respiratory conditions [54], notably pleuropneumonia or "shipping fever". Pleuropneumonia is commonly associated with the bacteria *Streptococcus equi* spp. *zooepidemicus*, a secondary invader [50,55]. Development of pleuropneumonia in horses can occur due to an overload of bacterial present along with stress-induced changes that decrease pulmonary defense during transportation [56]. There is a higher risk for developing transportation-associated pleuropneumonia as the duration of journey increases [57]. Furthermore, horses being tied with an elevated head position, where they cannot lower to clear their airway, can have increased bacterial contamination of their lower respiratory tract [58], increased tracheal mucus, and increased bacteria in post-transportation tracheal washes [24]. While occurrence of shipping fever is generally low, frequent rest and regularly cleaning the trailer has been shown to decrease incidences of pyrexia in horses transported on journeys of 40 h [3]. Additionally, it is recommended to take rectal temperatures at multiple intervals after transportation to monitor any potential for development of "shipping fever" [59].

Transportation has been associated with significant health risks to the horse, having effects on various physiological systems. While most previous research evaluating potential health risks involves long distance road transportation [1], it is assumed that horses are mainly transported over short distances [1]. Thus, the objectives of Chapters 3,4, and 5 were to evaluate the impact of short-duration (<3 hours) transportation on selected stress responses of varying groups, with a focus on senior horses.

1.5 The Senior Horse

Senior horses (>15 years) make up approximately 1/3 of the world horse population [60]. In the U.S., the number of senior horses has been increasing with a large portion (61%) of senior horses still in work [61]. This is likely attributed to improved care for senior horses in the areas of nutrition, disease treatment, owner recognition, and routine veterinary care [62]. A 2022 survey found that in Kentucky alone there are 52,000 horses over the age of 15 years [63]. In the same survey, when asked what the health condition was most important to horse owners the number one answer was care for the senior horse (27.1%) [63]. Both the increasing number of senior horses and owner concern for their health highlight the importance of studying this group of horses. It is known that with increased age comes a range of health problems along with increased susceptibility to disease and decreased effectiveness of vaccination due to declining immune function [64]. As age increases so does the occurrence of different problems including lameness, osteoarthritis and laminitis; colic, impaction and strangulating lipomas; respiratory disease, heaves; and endocrine disorders, insulin dysregulation (ID) and pituitary pars intermedia dysfunction (PPID) [60]. Laminitis is one of the most common diagnoses of lameness in

senior horses with an increase associated with aging and PPID [65], while PPID is the most common endocrine disorder in senior horses [60].

Given the potential health issues related to both age and transportation, understanding how senior horses respond to being transported could help inform better management and care for senior horses during stressful conditions. Chapters 3, 4, and 5 aimed to evaluate how senior horses and those with common age-related endocrinopathies are influenced by transportation stress and how their responses compare to young horses or those without endocrine disorders.

1.6 Transportation, Immune Function, and Age

While transportation can be associated with negative impacts on horse health, less is understood about how transportation stress influences immune function and the impact of the age of horses being transported on the physiological responses to transportation. Transportation stress is influenced by several factors the horse is experiencing including loading, confinement, isolation, movement, temperature, possible withholding of food and water, and unloading [1].

Stress activates the HPA axis and sympathetic nervous system by releasing hormones that can modulate the inflammatory state and immune function. In response to stress, glucocorticoids, such as cortisol, act on the glucocorticoid receptor in the cell nucleus, interfering with nuclear factor κB (NF-κB) and activator protein 1 (AP-1) signaling pathways, to modulate gene transcription thus altering production of cytokines [66]. Catecholamines, epinephrine and norepinephrine, also alter cytokine transcription through activation of cAMP pathways [66]. Additionally, glucocorticoids mobilize

immune cells further modulating immune function [26]. Together, the increase in glucocorticoids and catecholamines in response to stress can alter immune function through changes in lymphocyte populations/proliferation and production of pro-inflammatory cytokines [67,68]. This is reflected in the neutrophil to lymphocyte ratio (N:L ratio) which increases in response to stress [69]. Specifically stress leads to an increase in neutrophils, part of the innate immune response that defend against potential pathogens, and a decrease in number of lymphocytes, which play a critical role in adaptive immune response. In the short term, acute stress (minutes to hours) activates the immune system by mobilizing immune cells and modulating cytokines to prepare for the challenges of the stressor; however, chronic stress (hours to days) can dampen immune function through decrease in number and functionality of protective immune cells [67].

Transportation stress has been associated with changes in various parameters in the immune and inflammatory response. This can be observed with an increase in the N:L ratio, following transportation of 24 hours, with a return to normal seen 24 hours post-transportation [2,28,70]. The population of lymphocytes also changed in response to transportation with an altered distribution between CD3+, CD8+ and CD4+ T-cell subpopulations and decrease in the overall number of lymphocytes [28]. Transportation not only alters immune cell populations but can also impact inflammatory response through stress signals that activate immune cells such as monocytes and macrophages to release cytokines [71]. These cytokines are produced to recruit and activate additional immune cells and/or inflammatory pathways, thus amplifying the inflammatory response [71]. They include pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNFα) that enhance inflammation, Th1 cytokines (IL-2, IFNγ, TNFα) that stimulate cellular immunity, and Th2 cytokines (Il-

4, IL-10), that are anti-inflammatory and promote humoral immunity [66]. Additionally, cytokines, IL-1, IL-6 and TNFα, signal the liver to produce acute phase proteins (APPs) that initiate a systemic response that can be characterized by fever, metabolic adaptations, and changes in behavior allowing the horse to protect against the perceived threat of stress [72]. The protein levels of inflammatory cytokines IL-6 and IL-8 increased after long-distance transportation and was higher in older mares (10-12 years) versus younger fillies [73]. Additionally, APPs have been shown to increase in response to transportation. While serum amyloid A (SAA) and haptoglobin both increased 24- and 48-hours post-transportation respectively [74], the effects on fibrinogen yielded mixed reports with increases shown after 12 hours of transportation [75], but no changes observed after 4 hours [74]. In a study transporting adult horses (6-10 years) 4 hours, the APPs, c-reactive protein (CRP), α1-, α2- and β2-globulins were increased following transportation for up to 30 minutes post-transport [76], further suggesting that there is an initial inflammatory response to transportation stress. While acute inflammation can be considered adaptive to help the body return to homeostasis, chronic inflammation disrupts this balance and is linked to numerous health problems.

Low grade chronic inflammation is associated with aging in horses, also known as "inflamm-aging". Specifically, studies have shown and increased gene expression of inflammatory cytokines IL-1β, IL-15, IL-18 and TNFα in peripheral blood and increased TNFα/IFNγ production by lymphocytes in aged horses (>20 years) [77]. Additionally, an increased mRNA expression of TNFα in stimulated peripheral blood mononuclear cells (PBMC) was associated with age [78]. Since most transportation research has utilized horses <15 years of age, there is little information regarding whether the transportation of

aged horses could further impact immune function. A previous study in our lab was the first to evaluate the impact of short distance transportation on the immune function of aged horses [79]. This study used 12 horses aged 15-20 years old and found an increased gene expression of IL-2, IL-6, and IL-10 following transportation compared to pre-transportation values. Additionally, there was a decrease in gene expression of TNFα and IFNγ as well as decreased lymphocyte production of TNFα/IFNγ [79]. These findings raise concerns as the additional stress from transportation could further impact the aged horses ability to mount an effective immune response, potentially predisposing them to transportation-associated health problems. However, it is unclear if these responses are specific to transportation alone or if they are age dependent. Therefore, in Chapter 3 we aimed to evaluate the age-related differences in the responses of various cytokines to short distance transportation of horses, comparing aged and young horses.

Most studies examining the impact of transportation stress on the horse used animals determined to be healthy prior to transportation [9,24]. However, horses with endocrinopathies could have altered stress and endocrine responses to short-term transportation. While a recent publication from our lab utilized aged horses, none of these horses had any known endocrine dysfunction (ID or PPID) and the only stress-related hormone measured was cortisol [79]. Understanding the endocrine response to short-term transportation stress of horses with endocrine disorders could lead to improved management to reduce any secondary problems because of changes in stress-related hormones and timing of diagnostic testing.

1.7 Insulin Dysregulation

Equine metabolic syndrome (EMS) is an endocrinopathy associated with obesity, ID and increased risk of developing laminitis [80,81]. Although not necessarily an age-specific disorder, EMS is commonly seen in the aged horse population [60]. Horses with EMS typically have increased adiposity, general and/or regional, and inappropriate blood insulin levels. ID is defined as abnormalities in insulin metabolism including hyperinsulinemia, excessive production of insulin, and insulin resistance (IR), decreased tissue sensitivity to insulin [81,82]. Hyperinsulinemia has the potential to induce laminitis; however, the exact mechanism is not entirely understood. Current research links ID and laminitis to changes in hormones and signaling pathways affecting the laminar endothelium [83]. Hyperinsulinemia-associated laminitis (HAL) can occur gradually and subtly then progress to becoming a chronic, recurring condition with increasing lameness [84]. However, the exact insulin levels or duration of hyperinsulinemia to induce laminitis are not defined, likely due to high individual horse variability. Managing ID horses involves dietary adjustments, such as reducing energy intake in obese horses and feeding low NSC content to minimize post-prandial insulin response [85]. Additionally, exercise can improve insulin sensitivity [86], thereby reducing the risk of HAL. However, not all obese horses are ID and conversely lean horses can be ID [83]. Therefore, diagnosis and monitoring are important for effective management of ID horses.

Diagnosis of ID in horses is based on clinical signs and measuring blood insulin levels at rest (basal) and in response to a glucose challenge through dynamic testing [84]. Basal insulin is less reliable for diagnosis due to potential influence of the horse's diet [82]. Therefore, testing postprandial hyperinsulinemia following a glucose or meal challenge

when a horse has access to forage is more representative of a horses normal insulin response. The recommended dynamic test for ID diagnosis is an oral sugar test (OST) [82]. The OST is preferred to basal testing alone because it factors in the digestion and absorption of sugars, incretin hormones, and pancreatic insulin secretion [82,84]. The OST involves collection of a basal sample of blood (T0) on animals grain fasted 3-6 hours, followed by oral administration of 0.15- or 0.45-mL corn syrup/kg via dose syringe, then a second blood collection between 60- (T60) to 90-minutes after and finally measuring serum insulin concentrations [84]. For purposes of this dissertation, OSTs were performed using 0.15 mL/kg dose, due to lack of differences when compared with the higher dose [87], and post-OST insulin was collected at T60, aligning with current recommendations [84]. Horses with basal insulin concentrations >50 μU/mL and/or OST T60 >45 μU/mL, using radioimmunoassay (RIA), are considered to be ID [84]. While accurate diagnosis is crucial for management of ID horses, there are potential factors, such as transportation, that could alter diagnostic results.

Horses are transported to veterinary clinics for a variety of routine procedures, potentially including diagnostic testing for endocrine disorders. The transportation-associated stress might impact insulin response and thus diagnostic results for ID. Previous studies have shown that plasma glucose increases in response to transportation [88,89]. This is expected due to increased gluconeogenesis and decreased glucose uptake in response to stress hormones, such as cortisol and catecholamines, to promote breakdown of glycogen stores and provide energy to the horse [90]. Additionally, it is theorized that corticosteroids antagonize the actions of insulin by inhibiting its response to target tissue [90]. Stress induced by stall confinement altered the insulin dynamics of hospitalized

horses as observed through decreased insulin sensitivity [91]. Additionally, salivary insulin has recently been detected in horses with further research needed to determine its value in diagnosis of ID horses [36].

Changes in insulin concentrations in response to transportation stress in horses are less defined. A study transporting horses to a different location to be exercised found an increase in plasma insulin concentrations after transporting horses 1.5 hours compared with insulin concentrations of horses who were not transported [89]. To the author's knowledge, there is only one study that has evaluated the insulin response of horses classified as EMS, based on pre-transportation insulin concentrations >20 μU/mL, presence of obesity, and/or history of laminitis [92]. This study transported horses with (n=13) and without (n=8) EMS a short distance of 1-3 hours and took blood samples before and after. As expected, EMS horses had increased pre-transportation serum insulin concentrations compared with horses without EMS; however, insulin concentrations were increased following transportation in all horses regardless of EMS status [92]. It should be noted that these horses did have access to hay during transportation that could have influenced insulin concentration. Additionally, 4 healthy, non-EMS horses had post-transportation insulin concentrations >20 μU/mL (cutoff value used at the time of the study) that could result in misleading diagnostic results [92]. While it seems that there is an increase in insulin following transportation, there are limitations in these studies attributed to the confounding factor of exercise, diagnosis of EMS without dynamic testing, and undefined times of collection following transportation.

Therefore, Chapter 3, 4, and 5 evaluated insulin responses to transportation stress in horses of varying ages and metabolic function. Chapter 4 evaluated the differences in

insulin responses between ID and non-ID horses, investigated if transportation could alter insulin responses, and evaluated if diagnostic results of an oral sugar test were altered if performed post-transportation. This information could be beneficial to understand the best timing of diagnostic testing for ID if a horse is being transported to a veterinary clinic.

1.8 Pituitary Pars Intermedia Dysfunction

PPID occurs in 21% of senior horses over the age of 15 and prevalence increases with age [93]. PPID is most notably characterized by hypertrichosis, long hair coat that fails to shed, or delayed shedding [94]. In addition to an abnormal hair coat, other common clinical signs of disease include muscle atrophy, decreased performance, abnormal sweating, recurrent infections, polyuria, and polydipsia, and concurrent insulin dysregulation, with variations between individual horses and severity of disease [93,95]. The equine pituitary gland plays a crucial role in hormone regulation (Figure 1.2). Pro-opiomelanocortin (POMC) is synthesizes in the pars intermedia and pars distalis as a precursor for ACTH and α-melanocyte stimulating hormone (α-MSH) [96]. ACTH acts on the adrenal cortex to stimulate release of cortisol while α-MSH plays a role in metabolism, stress, and inflammation [93]. Normally the pars intermedia is inhibited by dopamine; however, horses with PPID have one or more adenomas that cause the loss of the dopaminergic inhibition on POMC production [96]. Therefore, horses with PPID have an increase in the POMC-derived peptides such as α-MSH and ACTH. Unlike Cushing's disease in people and dogs [97,98], horses do not have elevated circulating cortisol concentrations. However, horses with PPID have increased urinary glucocorticoid and androgen metabolites, as well as, increased salivary cortisol [99,100]. The altered

endocrine response of horses with PPID could be further exacerbated by a stressor such as transportation.

In non-PPID horses, ACTH is released from the anterior pituitary in response to stress as part of the HPA axis to stimulate cortisol release from the adrenal cortex that causes metabolic, immune, and behavioral changes to cope with stress [25]; however, circulating ACTH is already elevated in horses with PPID [101,102]. Basal ACTH can used to confirm PPID diagnosis, along with clinical signs, but a shortcoming is that ACTH can be easily influenced by numerous external factors such as exercise, stress, season, sedation, and age [12,22,103–106]. Although baseline ACTH concentrations can be useful to help diagnose PPID, there can be overlap in ACTH concentrations with non-PPID horses leading to an equivocal zone. This overlap can be attributed to variations in ACTH concentrations among healthy horses and the natural increase in ACTH associated with age [107,108]. Therefore, dynamic testing using a thyrotropin-releasing hormone (TRH) stimulation test is recommended, especially in cases where basal ACTH is in an equivocal range [107,109]. TRH is produced by the hypothalamus and stimulates the pituitary to release thyroid stimulating hormone (TSH) that acts on the thyroid gland to produce various thyroid hormones that have roles in metabolism and growth. TRH also stimulates corticotrophs, and in PPID horses this leads to abnormal levels of ACTH due to a loss of receptor specificity for hypothalamic-releasing hormones in the pars intermedia [110]. The TRH stimulation test is performed by taking a basal blood collection (T0) then injecting 1.0 mg TRH intravenously and collecting a second sample 10 minutes after (T10) to analyze for ACTH concentrations and compare to diagnostic cutoff values based on the time of the year samples were collected [101,107].

Similar to diagnosing horses for ID, there might be instances where horses are transported to a veterinary clinic to test for PPID. Previous studies have evaluated the impact of short distance transportation on ACTH concentrations in horses without PPID [111–113]. One study found that collecting blood within 30 minutes of unloading horses after a 40-minute journey resulted in all horses (n=12) having elevated ACTH concentrations, with one horse's ACTH concentrations remaining elevated as long as 120 minutes post-transportation [112]. However, it was not noted what time of year this study was performed or the age of horses. Another study showed that post-transportation ACTH was increased from pre transportation with 3/10 horses having basal ACTH concentrations above the diagnostic cutoff for PPID up to 30 minutes post-transportation [111,113]. Additionally, TRH stimulation tests were performed post-transportation and only one horse had a false positive result immediately after transportation. This study used horses aged 7-20; however, it is unclear whether the age of horse had any influence on the increased ACTH results following transportation. These data suggest that some horses could have misleading diagnostic results if blood is collected too soon post-transportation to evaluate ACTH concentrations for PPID diagnosis. Some limitations of the previous research are that these studies did not investigate age difference or explain the variability in ACTH responses between studies or horses within the same study. Additionally, there are no reports in the literature regarding the transportation of horses diagnosed with PPID. While it is currently not recommended that blood be collected to measure ACTH for PPID diagnosis within 30 minutes post-transportation [107], there are still many unknown factors.

Therefore, Chapter 3 aimed to further evaluate whether transportation can alter PPID diagnostic testing by determining differences in ACTH concentrations post-transportation between aged and young horses, while Chapter 5 evaluated changes in endocrine responses of PPID horses to short distance transportation.

1.9 Dissertation objectives and hypotheses

The overall objective of this dissertation was to understand the impact of short-duration transportation on physiological parameters related to stress and immune response, focusing on aged horses and those with ID and PPID, while also examining common management practices and typical journeys of horses in the U.S. The overarching hypothesis was that short-duration transportation is more common and that even short journeys can affect aspects of the stress response in horses, with variations based on age and presence of endocrine disorders such as ID and PPID.

Although horses are frequently transported in the U.S., little is known about the specific reasons for travel, types of journeys, and management practices. Therefore, the objective outlined in Chapter 2 aimed to explain how and why horses are transported, with focus on management when transporting horses 3 hours or less. The hypothesis was that across all journeys, horses are more commonly transported shorter lengths emphasizing the importance for research transporting horses shorter durations.

While long-distance road transportation of horses has been well documented, the impact of short-distance transportation stress is less understood. Additionally, little information exists about the impact of transportation on immune function in horses following transportation and previous research has not evaluated age differences in horses. Therefore, it was the objective of Chapter 3 to evaluate age-related differences in stress

and immune responses of horses to short-distance road transportation. The hypothesis of this study was that aged horses would have similar stress responses but altered immune responses to short distance transportation when compared with young horses.

ID and PPID are common endocrinopathies and horses with these disorders might be transported to a veterinary clinic to have diagnostic procedures performed. Since these diseases are already associated with altered endocrine function, it is unclear how these horses might respond to transportation-associated stress. The objectives of Chapters 4 and 5 were to compare horses with ID and PPID, respectively, to healthy horses and determine differences in stress and endocrine responses along with the best timing for diagnostic testing. The hypotheses were that ID horses would have altered insulin responses, both baseline concentrations and in response to dynamic testing, and PPID horses would have altered ACTH responses post-transportation compared to horses without these respective disorders.

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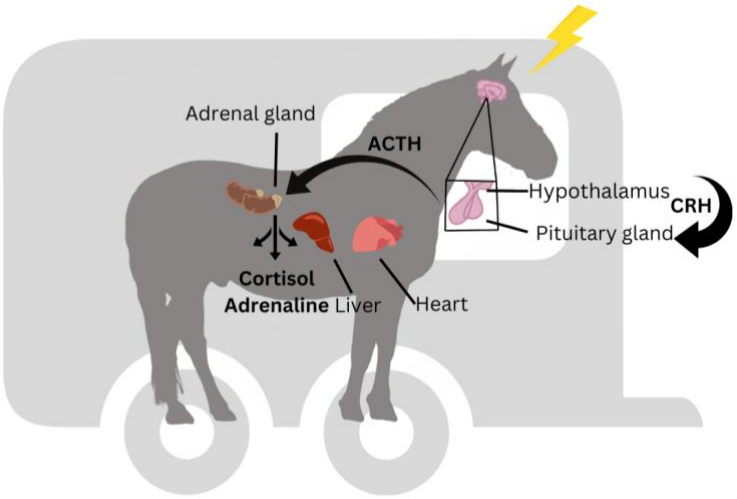

Figure 1.1 The hypothalamic-pituitary adrenal axis and stress response of horses to transportation.

Transportation stress in horses triggers the hypothalamus to release corticotropin releasing hormone (CRH), stimulating the anterior pituitary to release adrenocorticotropic hormone (ACTH) which prompts the adrenal gland to produce cortisol and adrenaline. These in turn increase blood glucose by promoting gluconeogenesis and glycogenolysis in the liver to provide energy, while also increasing heart rate and blood pressure to supply tissues with oxygen and nutrients.

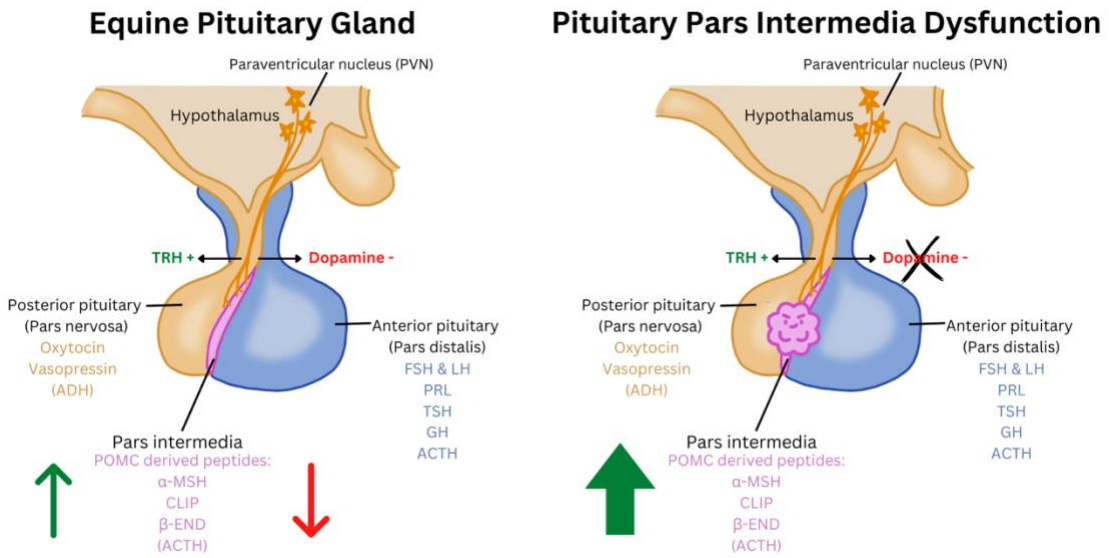

Figure 1.2 Equine pituitary gland hormone production in healthy horses and those with pituitary pars intermedia dysfunction (PPID).

The equine pituitary gland regulates hormone production through TRH and dopamine feedback to maintain homeostasis. In cases of PPID, loss of dopaminergic neurons leads to adenoma formation on the pars intermedia, resulting in excessive hormone production due to the impaired inhibitory function.
Abbreviations: TRH, thyrotropin releasing hormone; ADH, antidiuretic hormone; POMC, pro-opiomelanocortin; a-MSH, alpha melanocyte stimulating hormone; CLIP, corticotropin-like intermediate lobe peptide; b-END, beta endorphin; ACTH, adrenocorticotropin hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; PRL, prolactin; TSH, thyroid stimulating hormone; GH, growth hormone.

CHAPTER 2 A SURVEY OF GENERAL ROAD TRANSPORTATION: HOW AND WHY HORSES ARE TRANSPORTED IN THE U.S.

2.1 Abstract

Horses are regularly transported in the United States (U.S.); however, how and why horses travel by road has not been explored. Consequently, an online nationwide survey was conducted to understand 1) the most common reasons for travel; 2) the types of journeys undertaken when being transported by road in the U.S. and 3) the general management practices when transporting for 3 hours or less. Responses were collected from 1294 participants with at least one response from every state in the continental U.S. The most common survey taker was a female (93.9%), adult amateur (81.2%), horse owner (64.6%) who rode recreationally (33.1%) and transported their own horse (79.4%). The most common reasons for travel were for trail or leisure riding (34.2%) followed by showing and competition (25.3%); however, this varied by discipline. The most common trip duration was less than one hour (46.8%), with only 12.4% of the most common trip durations being 4 hours or more. The most common specific horse transported by road for 3 hours or less was an adult (age 5-15; 59.0%), Quarter Horse (21.2%), used for pleasure or trail riding (44.3%). The biggest concern when transporting was injury to the horse (26.7%), whilst the biggest factor when planning to travel was the weather (24.1%). These results provide insight into why horses are being transported by road in the U.S. and that it is more common to transport horses for shorter durations.

Keywords: horse; road; transportation; trip duration; management

Full reference: Jacquay, E.T., Harris, P.A., Stowe, C.J, McIntosh, B.J. and Adams, A.A. A survey of general road transportation: How and why horses are transported in the U.S. J Equine Vet Sci 140. https://doi.org/10.1016/j.jevs.2024.105137

2.2 Introduction

Horses are routinely transported locally and all over the world for a wide variety of reasons [16,54]; however, little information exists on what the most common journeys are for horses transported by road in the United States (U.S.). Additionally, there is only one survey about trailering problems of horses in the U.S, from 2001 [18]. Surveys from Australia, Italy, New Zealand, and the UK have investigated journey distance or duration and the frequency of transport, but they did not evaluate the specific reasons for those journey durations or how frequently different durations of trips were taken [14,16,17,19]. In one survey from the UK, there was a question about the main reason for transport, but this only consisted of three categories: 1. leisure and recreation, 2. competition and training, and 3. multiple reasons [19].

The duration of the trip can have impact on horse health, as longer journeys of 12 or more hours are associated with an increased risk of diseases such as shipping fever and gastrointestinal ulcers [47,59,114]. However, recent studies have shown that transporting horses over a short duration of 1-3 hours can impact stress and inflammatory responses even in healthy horses [76,79,115]. Therefore, while most research has focused on the effects of long-duration transportation, the impact of short-duration transportation is likely to be important, especially if horses are transported frequently for short periods.

Typical management practices of horses being transported by road in the U.S. are currently unknown. A study from Australia has evaluated management strategies when

transporting horses; however, they did not differentiate based on the duration of trip [15]. Understanding the typical management practices when transporting horses, even for short durations, would inform strategies to improve horse health.

The main objectives of this survey, therefore, were to determine the main reasons why horses are being transported by road in the U.S and how they are managed on journeys of 3 hours of less. The hypothesis was that horses would be more commonly transported on journeys lasting three hours or less, with an expected increase in trip frequency as the duration decreases.

2.3 Materials and Methods

2.3.1 Ethical Approval

Animals were not used in this study and was therefore confirmed to be IACUC exempt. The University of Kentucky's Institutional Review Board (IRB) reviewed the survey and determined it did not meet the criteria of human research and therefore did not require IRB approval as the subject matter was horses.

2.3.2 Survey Design and Distribution

The sampling population for this survey included anyone 18 years or older, who owned, leased, or was in the full care of at least one horse or pony that resided in the U.S. and had been transported by road in 2021. Informed consent to participate was required before beginning the survey, with responses limited to one survey per person.

An online questionnaire was created (Qualtrics 2022, Provo, UT) with 55 questions divided into four sections: 1. Respondent demographics; 2. Horse demographics of a selected horse that was the most frequently transported and management during a typical trip of 3 hours or less; and 4. Opinion questions. The survey consisted of multiple-choice

questions, matrix tables, and “pick, group, and rank”. When applicable, questions had “other” options with text entry for participants to specify their answer, and these responses were later sorted into the appropriate category or remained as ‘other’. The number of responses varied per question as survey participants were able to skip or omit certain questions and some questions have responses greater than the number of survey respondents due the ability to select multiple options within questions. The survey questionnaire can be found in supplemental file 2.1.

The survey was available online from February 22, 2022, to April 1, 2022. The University of Kentucky’s College of Agriculture, Food and Environment published a press release (Supplemental file 2.2) on February 22, 2022, that was distributed by e-mail to various equine organizations/groups and shared by national equine media sources (TheHorse.com, Paulick Report) and posted on social media on the University of Kentucky Maxwell H. Gluck Equine Research Center’s Facebook page. Following completion of the survey, participants had the option to enter a drawing and to maintain anonymity, emails were recorded separately from survey responses.

Pilot testing was completed by 20 participants in the equine industry to identify any problems with survey flow, understanding of questions, and general format. A power calculation (Qualtrics sample size calculator) determined that 385 participants would provide a representative sample for the estimated 7.2 million horses [116] in the U.S. with a 95% confidence level and 5% margin of error, and estimated proportion of 50%.

2.3.3 Data collection and statistical analysis

StatIQ (Qualtrics 2022, Provo, UT) was used to calculate descriptive statistics. Data are presented as frequency counts and/or a percentage of responses. Using GraphPad Prism

version 10.2.3, differences in proportions were analyzed using Chi-square or Fischer's exact test with Bonferroni adjustments made for multiple comparisons. Additionally, the Wilson/Brown method was used to calculate 95% confidence intervals (CI) of proportions based on number of responses (n) for each question using. Non-overlapping confidence intervals were considered different at a significance level of $P<0.05$ [117].

2.4 Results

The survey was completed by 1294 participants with at least one participant from each of the 48 states in the continental U.S. (excluding Hawaii and Alaska).

2.4.1 Respondent Demographics

The most common survey taker was a female (93.9%), aged 55-74 (45.5%), who owned 1 to 3 horses (55.6%) and transported their own horse (79.4%, Table 2.1). When asked about their involvement in the equine industry, most identified as being a horse owner (64.6%) whose income did not come from horses (63.7%). Most respondents identified as amateur (81.2%) versus professional participants in equestrian competitions (18.8%) who previously or currently compete at the state/regional (26.4%) and national level (25.8%) of equestrian sport in their respective disciplines/activities.

2.4.2 General Road Transportation

The most common reasons for transporting a horse by road in 2021 were trail/leisure riding (34.2%), shows/competition (25.3%) and lessons/schooling (17.4%, Figure 2.1). When asked to rank the top three reasons for transporting their horse, trail or leisure riding remained the first reason for transport; however, the most common second and third ranked reason for transport was for veterinary services (Figure 2.2).

The duration of the most common trip length taken by road in 2021 was <1 h (46.8%), followed by trip durations of 1-3 h (40.8%), with trips 4 hours or more only representing 12.4% of the most common trips (Figure 2.3). There was a significant difference in the distribution of counts between durations of trip ($\chi 2$=1156.9, df=4, P<0.05), and between each pair of trip durations (P<0.05) except <1 h vs. 1-3 h and 9-24 h vs. >24 h. When considering the duration of all trips taken, the most frequent range was 1-3 h, which was also the second most frequently traveled trip duration overall (Figure 2.4).

The typical frequency of trip durations decreased as the trip length increased from weekly for trips <1 h, to 1-2 times a month for 1-3 h trips, and once or twice a year especially for those over 24 hours (Figure 2.5). There was a significant difference between frequency and duration of trip (P<0.05, Fischer's exact test), with horses being transported on longer trips less frequently. Additionally, the duration of time between trips increases as the duration of trip increases (Figure 2.6), with shorter trip durations having a significantly shorter time between trips versus longer trip durations ($\chi 2$=499.6, df=42, P<0.05).

2.4.3 Reason for Transportation

While recreational/trail riding was the most common discipline (33.1%), when comparing the reason for transport by discipline, trail/leisure riding was more commonly undertaken by those who rode recreationally (77.6%) or were endurance riders (55.5%, Figure 2.7). Conversely, those competing in eventing (46.2%) and dressage (42.6%) transported more often for schooling and lessons. Hunter/jumpers (58.7%) and western

discipline horses (43.2-65.9%), in contrast, were transported more often for shows/competition (Figure 2.7).

The reasons for transport also varied by trip duration with horses being transported most often for trail and leisure riding on journeys of less than one hour (44.6%); however, when being transported for showing and competitions the most frequent trip duration traveled was 1-3 hours (51.0%, Figure 2.8).

2.4.4 Road Types

The most common roads traveled for any type of trip were highway (60.7%), mostly straight (41.7%) and mostly flat (46.9%, Figure 2.9).

2.4.5 Transport Information for a Specific Horse Transported on Journeys of Three Hours or Less

2.4.5.1 Trailer Layout

The trailer types most used on journeys of 3 hours or less were gooseneck (52.2%) or bumper pull (46.7%), slant (48.4%) or straight load (35.1%), with a step up (52.2%) or ramp (38.7%). The trailers usually had roof vents (71.9%) and side windows (81.9%) for ventilation with rubber mats (86.8%) and sawdust/shavings (61.4%) for flooring or bedding, respectively.

2.4.5.2 Horse Details

The horse usually faced forwards (88.7%) and were tied (76.6%) at or above eye level (69.2%). While most horses wore a halter when being trailered, 26.5% said they also used some leg/hoof protection on their horse. Most survey respondents reported that their horses did not have any problems loading on the trailer (76.7%), with some having

occasional problems (19.1%). They were usually transported alone (40.2%) or with one (40.0%), two (10.4%), or three (4.8%) additional horses in the trailer.

2.4.6 Horse Demographics

The most common horse transported by road for 3 hours of less was an adult age 5-15 years (59.0%), Quarter Horse (21.2%, Table 2.2), used for pleasure or trail riding (44.3%), which resided in the Southeast (41.6%). While most horses were 15 years of age or younger (65.5%), 34.5% of horses transported were over the age of 15.

2.4.7 Management Before, During, and After Transport of Three Hours or Less

2.4.7.1 Management in the hour before

Before transporting horses by road on journeys of 3 hours of less, most horses did not receive any administration of medications or supplements (82.4%). The use of ulcer prevention/ treatment (17.6%), electrolytes (8.3%), pre/probiotics (3.9%) and an over the counter, non-prescribed "calming" supplement (2.7%) were occasionally used (Table 2.3).

2.4.7.2 Access to feed, hay, and water

Most survey respondents reported that their horses had access to hay/pasture before (90.8%), during (81.8%), and after (83.9%) transportation (Table 2.3). And while water was commonly provided in the hour before (93.0%) and after (92.4%) transport, it was less likely to be provided during transport (12.8%) of 3 hours or less. While some horses had access to feed/concentrate in the hour before being transported by road for 3 hours or less (30.1%), it was less common during (2.17%) and after (14.6%).

2.4.7.3 Monitoring of TPR

When asked about recording temperature, pulse, and respiration (TPR) before and after transporting their horse, most said they did not record TPR (>90%) on journeys of

three hours or less (Table 2.3). When asked at what duration of trip they would take a TPR, 14.4% said they would take pulse, 16.2% would take respiration, and 20.6% said they would take their horses temperature on trip durations of 4 hours or more.

2.4.7.4 Observations in the hour after

After transport, some reported that horses had increased frequency of defecation (13.2%), vocalization (10.9%), and sweating (9.8%) following the journey of 3 hours or less (Table 2.3).

2.4.8 Travel opinions

Although there were still travel restrictions and cancellation of equine events due to the COVID-19 pandemic in 2021, most respondents reported either no change in their travel from the previous years (51.8%) or that their horses were being transported less frequently (32.9%).

The biggest health concern reported by the participants when transporting horses by road was physical injury to the horse (26.7%), followed by stress (23.1%), dehydration/overheating (15.6%) and gastrointestinal issues such as colic, diarrhea, and ulcers (11.5%, Figure 2.10). The main factors considered when planning to transport horses by road were season/weather (24.2%), trip duration/length (21.9%), horse health (16.8%), traffic (15.5%), and types of roads (11.3%, Figure 2.11).

2.5 Discussion

Results from this survey provide valuable insight into the reasons why horses were transported by road and the way they were managed on shorter journeys in the U.S. in 2021.

The demographic of horses being transported matched the population of horses in the U.S. [116] with most horses being used for recreational riding and showing in both the 2023 American Horse Council (AHC) Economic Impact Study and the results of this survey. However, in the AHC survey, racing was ranked third in the reported uses and therefore was underrepresented in our survey. Additionally, in the AHC study, Texas, California, and Florida were considered to have the most horses whereas in the current survey the states where the most responses originated from were Kentucky, Virginia, Texas, and Ohio. This discrepancy is likely due to the origin of the survey being in Kentucky, which could lead to geographical bias in horse management and trip durations due to the numerous equine recreational and competition venues in proximity in this state and therefore potential transportation opportunities in this state.

There were various factors that were associated with the duration of trip taken. When evaluating trip length, there was an increase in the frequency of transporting horses over journeys of 3 hours or less compared to 4 hours or more. Additionally, the time between trips increased as the trip duration increased. This suggests that trip lengths between 3 and 4 hours is a threshold duration of journey where those who transport horses by road perceive differences in how horses need to be managed based on trip duration. However, it is unknown if this would have changed if asked about the differences between other trip duration intervals (i.e. <2 hours or trips of 1-2 hours). Trips of 3 hours or less were the most common in this U.S. survey, which is similar to previous studies from the U.K., New Zealand, and Australia, where most trip durations were less than 2 hours, or a distance of 40-80 km [15,17,19]. This is perhaps surprising as the U.S. is a much larger country than the U.K. and New Zealand, and it might have been thought that longer

journeys would be more commonly traveled. Again, this may in part be due to the location of many of the respondents as discussed above, but this pattern was consistent across all regions. Also, in agreement with our results, a survey from Australia found that only 13.4% of trips were 4 hours or longer [15]. Taken together, the results further emphasize the importance for a better understanding of horse management and health on short journeys.

Senior horses (>15 years) make up approximately one-third of the equine population in developed countries, and there in increasing interest in how to best care for this population of horses [60,61]. Consistent with the demographics of the U.S. horse population, greater than one-third of the horses being transported in this study were over the age of 15. Recent research has shown that older horses can have altered physiological responses to short distance transportation stress [79,115]. This includes increases in gene expression of inflammatory cytokines and increases in hormones such as ACTH and insulin following transportation when compared to younger horses [79,115]. As older horses are still being regularly transported, it is important to closely monitor these horses for any changes that could potentially impact their health. However, other stressors outside of transportation can cause hormonal changes and it is important to recognize that not all these changes in hormones may be reflective of development of disease, as some hormone fluctuations, such as increased ACTH post-transportation, can be increased above suggested diagnostic cut off ranges in aged horses that do not have pituitary pars intermedia dysfunction (PPID) [115]. Therefore, alongside monitoring, it is important to consider the effects of short-term transportation on diagnostic testing.

Very few survey respondents monitored vital signs (TPR) to monitor their horses health before or after transportation of three hours or less by road. Similar results were

found in the Australian survey, where rectal temperature was taken by fewer that 30% of respondents for any duration of trip [15]. However, results from this survey showed that horse owners reported that stress and dehydration were the major health concerns when transporting horses by road; however, it should be noted that this question did not differentiate concern according to trip duration. Monitoring temperature for incidences of pyrexia can be early indicator of disease when transporting horses long-distance, but more research is needed on how temperature can be impacted when transporting short distances, especially under adverse environmental conditions [59,115]. This study does, however, highlight a potential disconnect between having health concerns and monitoring of TPR on shorter trips. Taking vital signs could be seen as tedious or unnecessary when transporting short distances or that other more general assessments of physical health were evaluated prior to transportation that this survey did not address. Additionally, while not asked in this survey, knowing how many horse owners know how to take TPR could also help with understanding the lack of monitoring vital signs when transporting horses by road. It is possible that providing advice to owners on how these can be obtained and why they could be valuable in monitoring horse health could increase utilization.

It was encouraging to report that >90% of respondents indicated that horses had access to hay and water in the hour before being transported by road 3 hours or less. It has been shown that there is an increased risk of heat stroke when water and hay are restricted prior to transport, under certain environmental conditions [20]. Transportation stress and changes in feeding practices, such as fasting prior to transportation, have been associated with the risk of developing ulcers [47,49]. In this survey, 81.8% reported that their horses had access to forage during transport, which has been shown to correlate to a decreased

chance of developing gastric ulcers [47]. However, it is important to be aware of potential concerns for respiratory health when feeding forages during transportation. Soaking or steaming hay and ensuring proper ventilation could potentially reduce associated problems with forage access during transportation.

Research has evaluated how best to position and tie horses when being transported by road [8,24,44]. It has been shown, for example, that horses preferred to face backwards in a trailer on journeys of 3 hours and 12 hours [8,44]. It was also found that tying horses without the ability to lower their head below chest level decreased the ability of a horse to clear its airway, which increased tracheal mucus and bacteria in tracheal washes taken after transport of 8 hours [24]. The findings of the current study were similar to those in Australia in which the authors found only 9% of horses were untied and most horses faced forwards [15]. While the positioning and tying of horses during transportation has been shown to impact comfort, respiratory health, and well-being, at least for the longer journeys it seems that such research findings have not been adopted by those who regularly transport horses in the U.S. over a variety of distances. More work is required to explore the possible benefits of different methods of transportation especially for the shorter journeys.

Understanding how horses are managed in the U.S. allows us to know which practices are being used and if the public is aware of possible ways to reduce stress and incidence of disease or problems when transporting horses by road. Future research should continue to evaluate how current management practices impact horse health and determine where improvements can be made. Evaluations should be made to existing management practices with particular attention given to evaluating the transportation of aged horses,

monitoring vital signs during short term transportation, and assessing current management practices before and during transport to optimize horse welfare during transportation.

Some limitations of this survey were the potential for skewed results due to a high percentage of respondents from the state of Kentucky (16% of responses), which is the state of origin of the survey and its release, as previously mentioned. Additionally, as with all surveys there is recall bias that could lead to inaccuracies due to not being able to remember past transportation events. Sampling bias could be due to the sample of individuals surveyed not being a true reflection of the of those who transport their horse by road in the U.S. For example, there were low responses from commercial drivers (<1%), and the racing industry (<1%), which means that input from these sectors would have been missed. It is assumed that these populations might be traveling longer distances more frequently; however, further research is warranted specific to these groups to determine if there would be any differences with the results found in this survey and to compare management practices across different groups. Still, approximately 20% of the respondents said that they were professional riders and around 80% described themselves as being competitive riders which suggests that the survey did receive input beyond the purely leisure rider.

2.6 Conclusion

This survey determined that short trips of 3 hours or less were the most common trips undertaken by the respondents and that the frequency of travel decreased as the duration of the trip increased and the time between trips increased. Additionally, the reason for travel changes based on trip duration and discipline. Aged horses (>15 years) were commonly transported, and more attention should be given in how to monitor and

manage this population of horses. Understanding transportation management patterns can lead to improvements in management and horse well-being as well as highlight potential areas for improvement in equine road transportation.

Table 2.1 Demographics of respondents to a survey on equine road transportation in the U.S.

Demographics	Number of Responses	Percentage (%)	95% Confidence Interval
Age (n=1294)			
18-24	53	4.1	[3.1, 5.3]
25-34	202	15.6	[13.7, 17.7]
35-44	178	13.8	[12.0, 15.7]
45-54	236	18.2	[16.2, 20.4]
55-64	328	25.3	[23.1, 27.8]
65-74	261	20.2	[18.1, 22.4]
75-84	34	2.6	[1.9, 3.7]
Gender (n=1288)			
Female	1210	93.9	[92.5, 95.1]
Male	78	6.1	[4.9, 7.5]
Income from Industry (n=1249)			
None	795	63.7	[60.9, 66.3]
1-25%	233	18.7	[16.6, 20.9]
26-50%	43	3.4	[2.6, 4.6]
51-75%	30	2.4	[1.7, 3.4]
76-100%	148	11.8	[10.2, 13.8]
Riding status (n=1244)			
Amateur	1010	81.2	[78.9, 83.3]
Professional	234	18.8	[16.7, 21.1]
Highest level competed (n=1248)			
Does not compete	265	21.2	[19.1, 23.6]
Local	271	21.7	[19.5, 24.1]
State/Regional	330	26.4	[24.1, 29.0]
National	322	25.8	[23.5, 28.3]
International	60	4.8	[3.8, 6.2]
Driver/hauler (n=1249)			
Drives themself	992	79.4	[77.1, 81.6]
Another member of household	93	7.4	[6.1, 9.0]
Trainer/barn manager	64	5.1	[4.0, 6.5]
Friend	44	3.5	[2.6, 4.7]
Commercial driver	40	3.2	[2.4, 4.3]
Other barn/farm staff	11	0.9	[0.5, 1.6]
Other	5	0.4	[0.1, 1.0]

n = Total number of responses for an individual question

Table 2.2 Demographic of horses transported by road on journeys of 3 hours of less from a survey on equine road transportation in the U.S.

Demographics	Number of Responses	Percentage (%)	95% Confidence Interval
Age (n=920)			
Foal/weanling (<1)	2	0.2	[0.01, 0.8]
Young (1-4)	57	6.2	[4.8, 8.0]
Adult (5-15)	543	59.0	[55.8, 62.2]
Mature (16-20)	207	22.5	[19.9, 25.3]
Senior (21-30)	106	11.5	[9.65, 13.8]
Very Aged (30+)	5	0.5	[0.2, 1.3]
Breed (n=1167)			
Quarter Horse	247	21.1	[18.9, 23.6]
Thoroughbred	195	16.7	[14.7, 19.0]
Warmblood	115	9.9	[8.3, 11.7]
Crossbred/mixed	109	9.3	[7.8, 11.1]
Paint	82	7.0	[5.7, 8.7]
Other	419	36.0	[33.2, 38.7]
Sex (n=1160)			
Gelding	676	58.3	[55.4, 61.1]
Mare	455	39.2	[36.5, 42.1]
Stallion	15	1.3	[0.7, 2.1]
Filly	14	1.2	[0.7, 2.0]
Colt	0	0	N/A
Location (n=1142)			
Southeast	475	41.6	[38.8, 44.5]
Midwest	241	21.2	[18.8, 23.6]
Northeast	170	14.9	[12.9, 17.1]
Southwest	93	8.1	[6.7, 9.9]
Rocky Mountain	82	7.2	[5.8, 8.8]
Pacific	81	7.1	[5.7, 8.7]

n = Total number of responses for an individual question

Table 2.3 Management of a horse within the hour before, during, and in the hour after transportation by road on journeys of 3 hours or less from a survey on equine road transportation in the U.S.

Management	Number of Responses	Percentage (%)	95% Confidence Interval
Before			
Access to (n=1169)			
Feed/concentrate	352	30.1	[27.6, 32.8]
Hay/pasture	1061	90.8	[89.0, 92.3]
Water	1087	93.0	[91.4, 94.4]
Recorded TPR (n=1336)*	92	6.9	[6.5, 9.5]
Administration of (n=1127)			
Ulcer prevention/ treatment	198	17.6	[15.5, 19.9]
Electrolytes	93	8.3	[6.8, 10.0]
Pre/probiotics	44	3.9	[2.9, 5.2]
"Calming" supplement	30	2.7	[1.9, 3.8]
During			
Access to (n=1167)			
Feed/concentrate	25	2.1	[1.4, 3.2]
Hay/forage	955	81.8	[79.5, 83.9]
Water	149	12.8	[11.0, 14.8]
Stopped to rest	132	11.3	[9.6, 13.3]
Tack/equipment (n=1168)			
Halter	1111	95.1	[93.7, 96.2]
Leg/hoof protection	309	26.5	[24.0, 29.1]
Blanket/cooler	193	16.5	[14.5, 18.8]
After			
Access to (n=1171)			
Feed/concentrate	171	14.6	[12.7, 16.8]
Hay/pasture	981	83.9	[81.7, 85.9]
Water	1080	92.4	[90.7, 93.8]
Recorded TPR (n=1360)*	134	9.9	[8.4, 11.6]
Increased frequency of (n=1163)			
Defecation	154	13.2	[11.4, 15.3]
Vocalization	127	10.9	[9.3, 12.9]
Sweating	114	9.8	[8.2, 11.7]

n = Total number of responses for an individual question
*Multiple responses were able to be selected therefore the total number will exceed the number of survey respondents
TPR= Temperature, pulse, and/or respiration

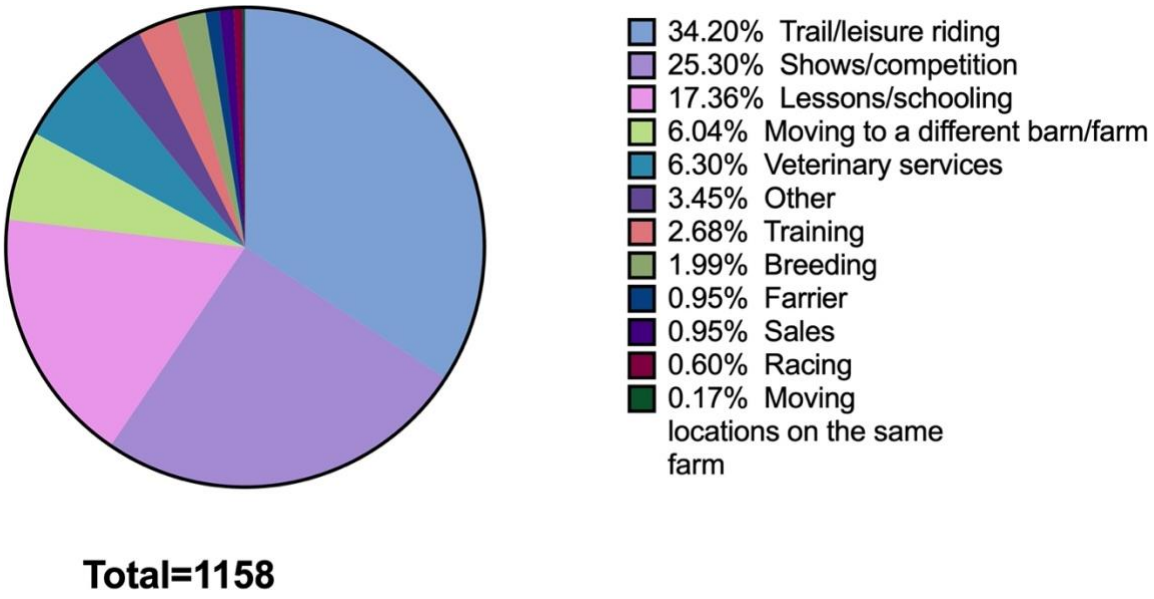

Figure 2.1 The most common reasons for transporting a horse by road in 2021 reported by respondents to a survey on equine transportation in the U.S.

The percentage of trips taken for trail/leisure riding (n=396; 95% CI [31.5, 37.0]), shows/competitions (n=293; 95% CI [22.9, 27.9.]), lessons/schooling (n=201; 95% CI [15.3, 19.6]), moving to different barn/farm (n=72; 95% CI [4.8, 7.6]), veterinary services (n=73; 95% CI [5.9, 7.9]), other (n=40; 95% CI [2.5, 4.7]), training (n=31; 95% CI [1.9, 3.8]), breeding (n=23; 95% CI [0.5, 1.7]), farrier (n=11; 95% CI [0.5, 1.7]), sales (n=11; 95% CI [0.5, 1.7]), racing (n=7; 95% CI [0.3, 1.2]), moving locations on the same farm (n=2; 95% CI [0.03, 0.6]).

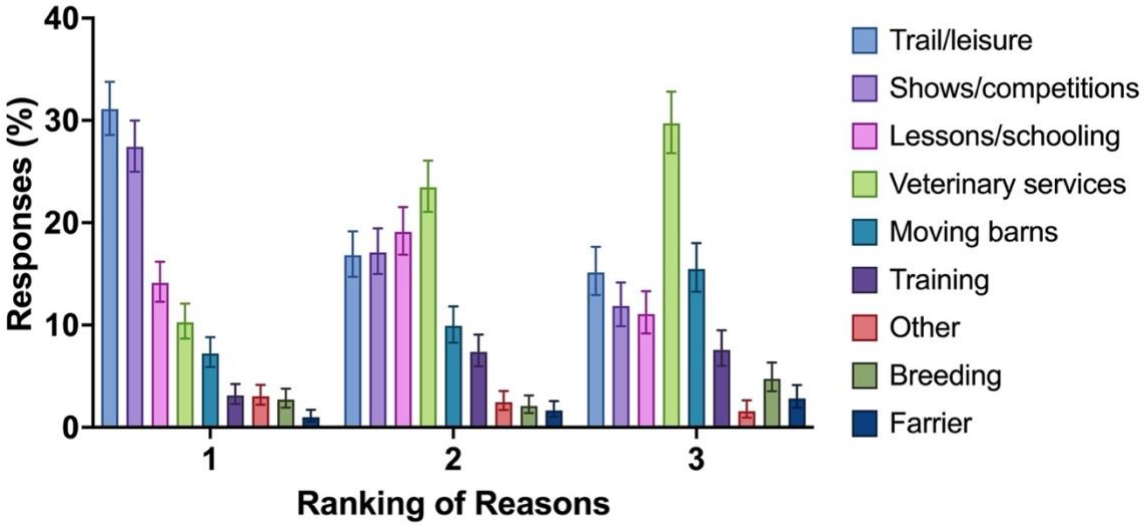

Figure 2.2 The ranking of the top 3 reasons for transporting a horse by road reported by respondents to a survey on equine transportation in the U.S.

The data are presented as percentages with 95% confidence intervals.

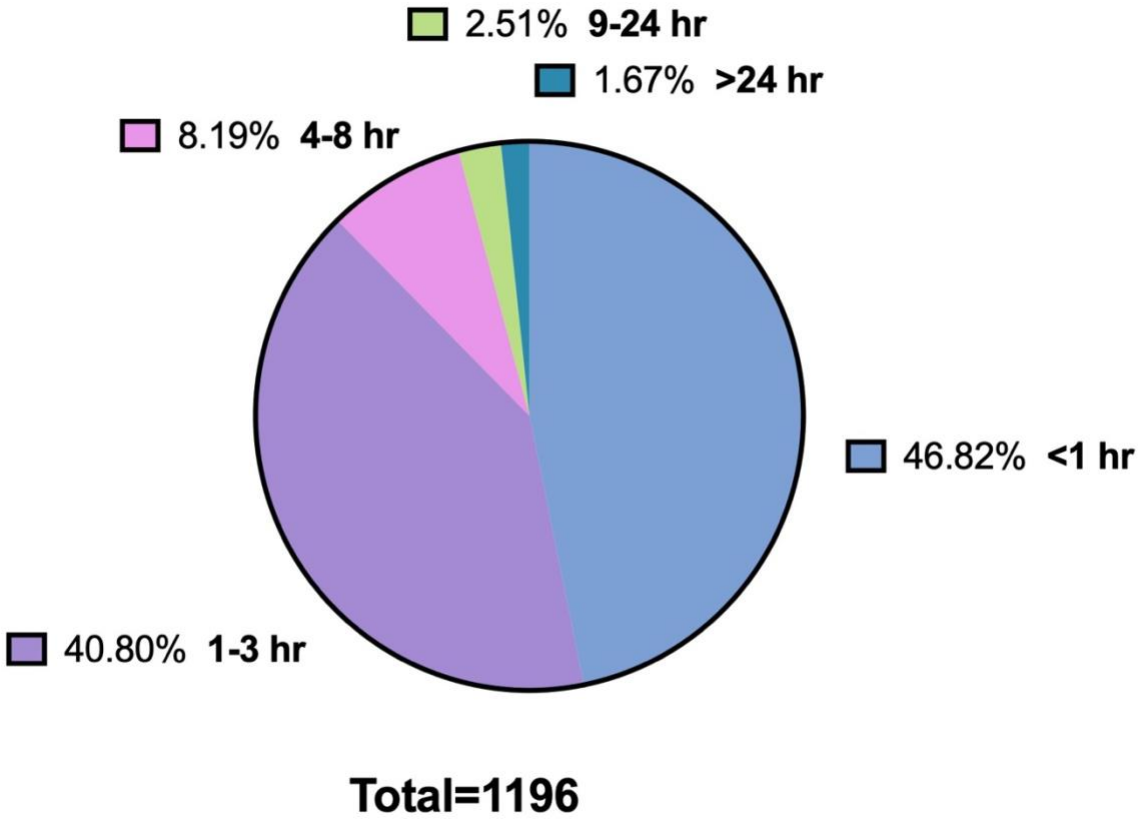

Figure 2.3 The duration of the most common trip taken by road reported by respondents to a survey on equine transportation in the U.S.

The percentages of trips that were <1 hr (n=360, 95% CI [44.0, 49.7]), 1-3 hr (n=488, 95% CI [38.1, 43.6]), 4-8 hr (n=98, 95% CI [6.8, 9.9]), 9-24 hr (n=30, 95% CI [1.8, 3.6]), or >24 hr (n=20, 95% CI [1.1, 2.6]).

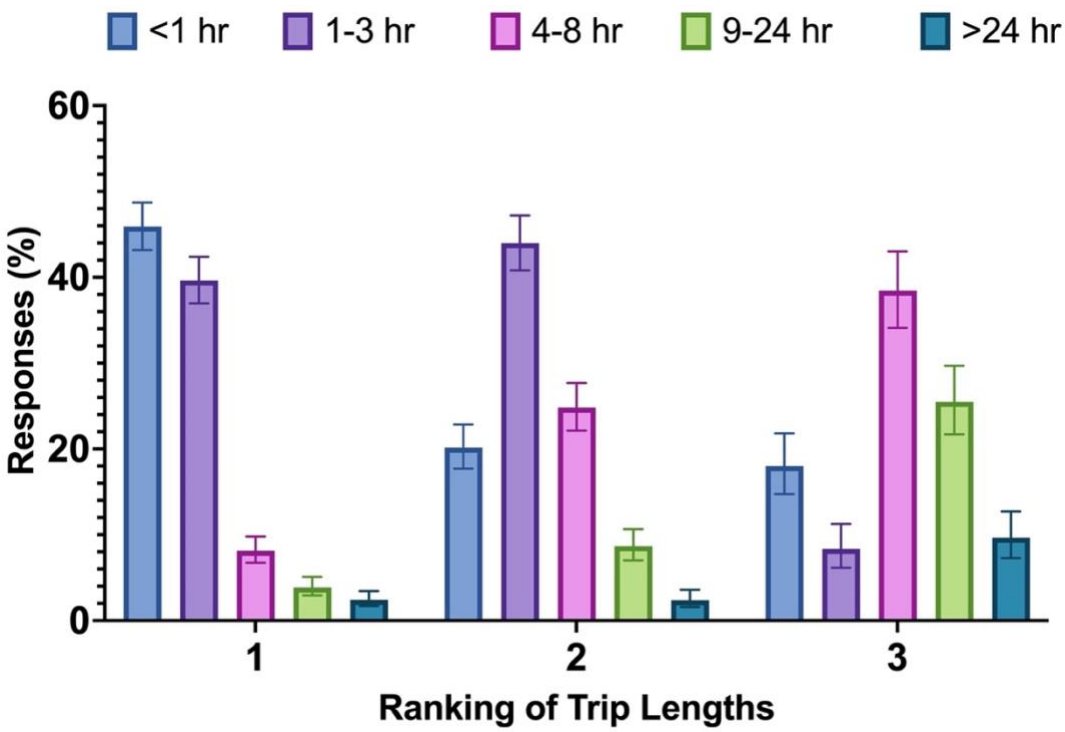

Figure 2.4 The ranking of the top 3 trip lengths taken when transporting a horse by road reported by respondents to a survey on equine transportation in the U.S.

The data are presented as percentages with 95% confidence intervals.

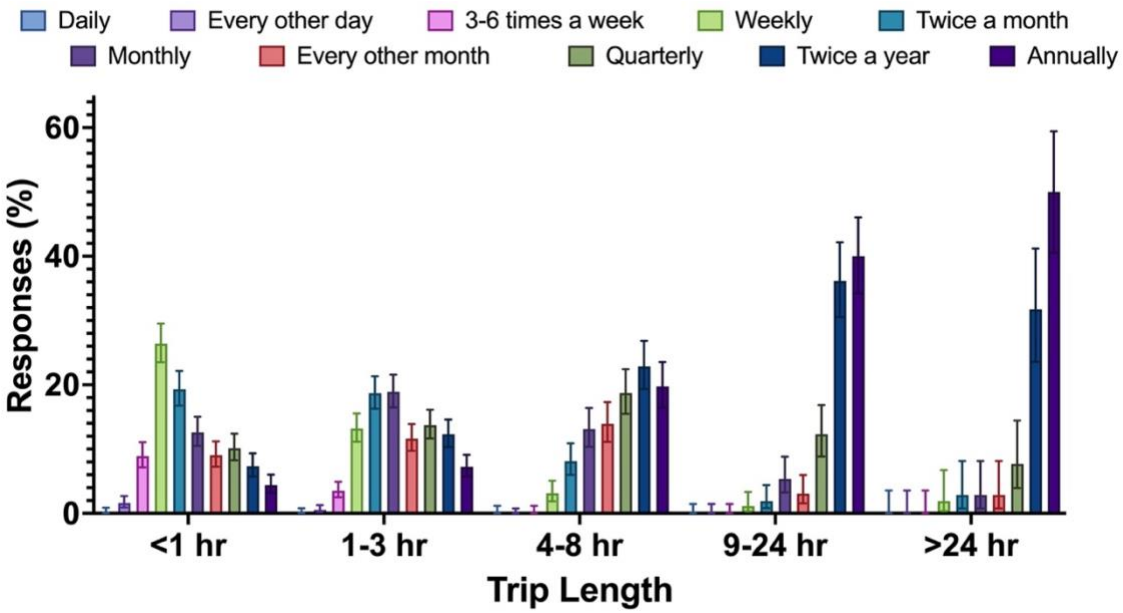

Figure 2.5 The frequency of transporting different distances by road reported by respondents to a survey on equine transportation in the U.S.

The data are presented as percentages with 95% confidence intervals.

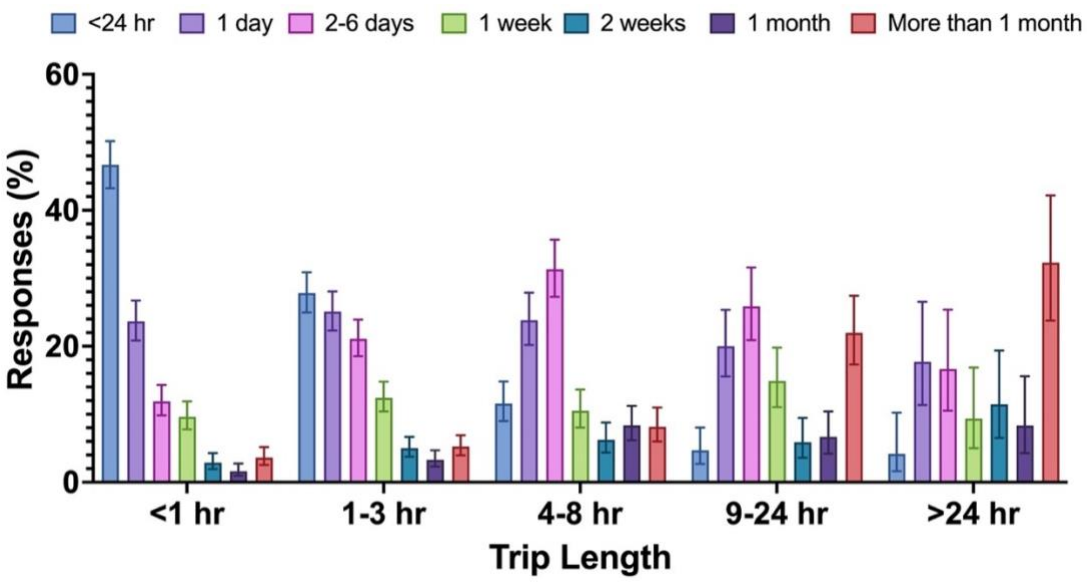

Figure 2.6 The length of time between trips of the same length reported by respondents to a survey on equine road transportation in the U.S.

The data are presented as percentages with 95% confidence intervals.

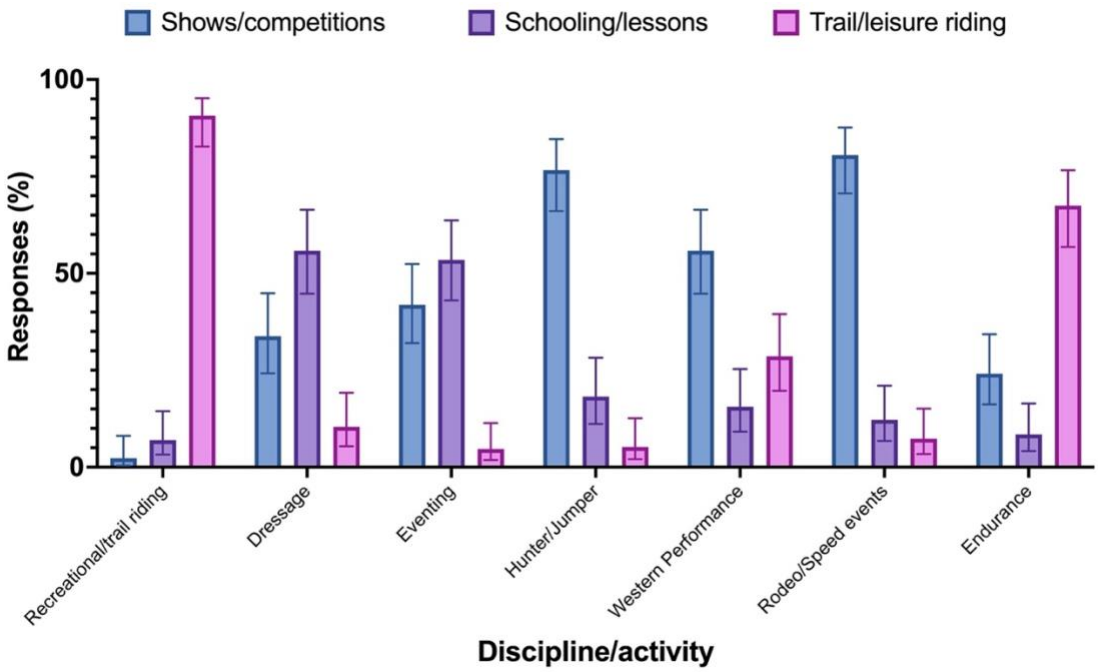

Figure 2.7 Reasons for transporting a horse by road based on discipline/primary activity reported by respondents to a survey on equine road transportation in the U.S.

The data are presented as percentages with 95% confidence intervals.

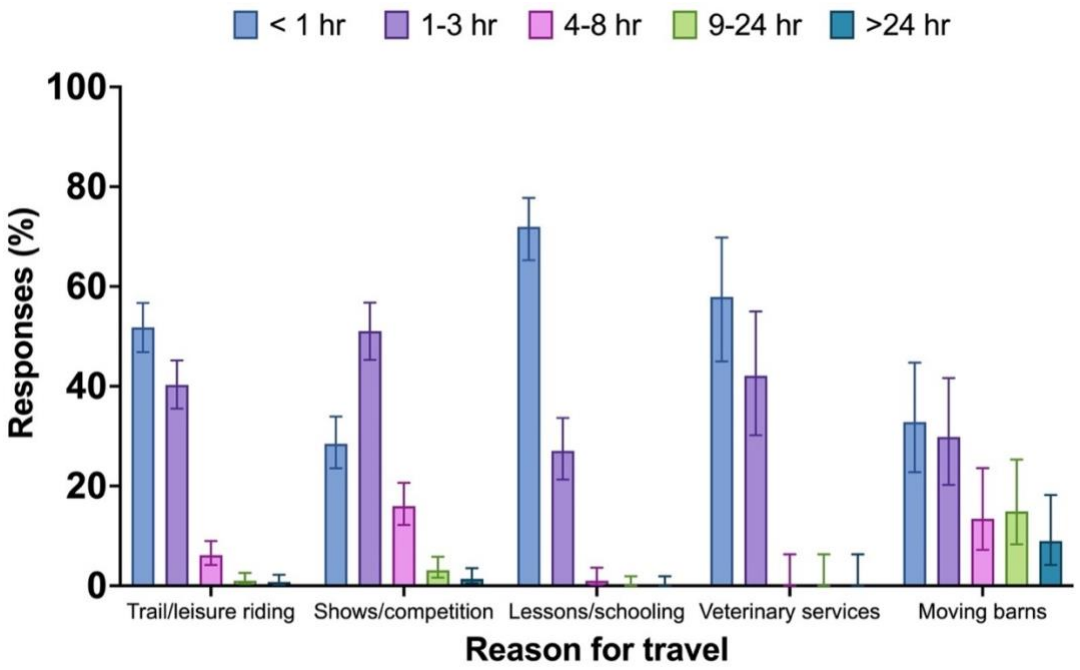

Figure 2.8 Reasons for transporting a horse by road based on trip duration reported by respondents to a survey on equine transportation in the U.S.

The data are presented as percentages with 95% confidence intervals.

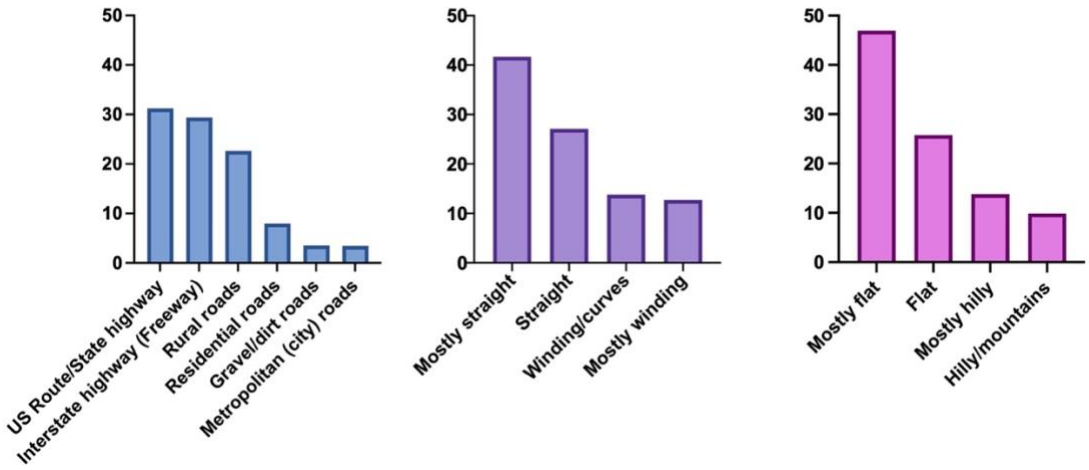

Figure 2.9 Percentage of time spent transporting horses on different road types reported by respondents to a survey on equine transportation in the U.S.

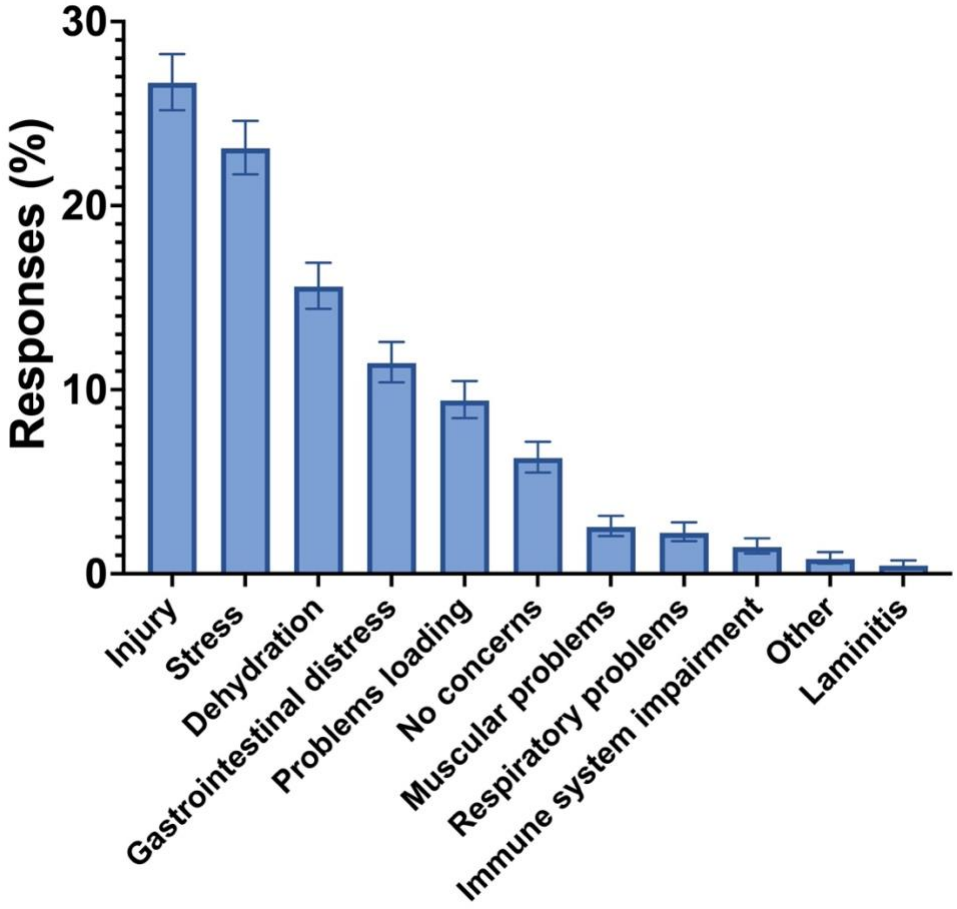

Figure 2.10 Ranking of horse health concerns when transporting horses by road reported by respondents to a survey on equine transportation in the U.S.

Data are presented as percentages with 95% confidence intervals.

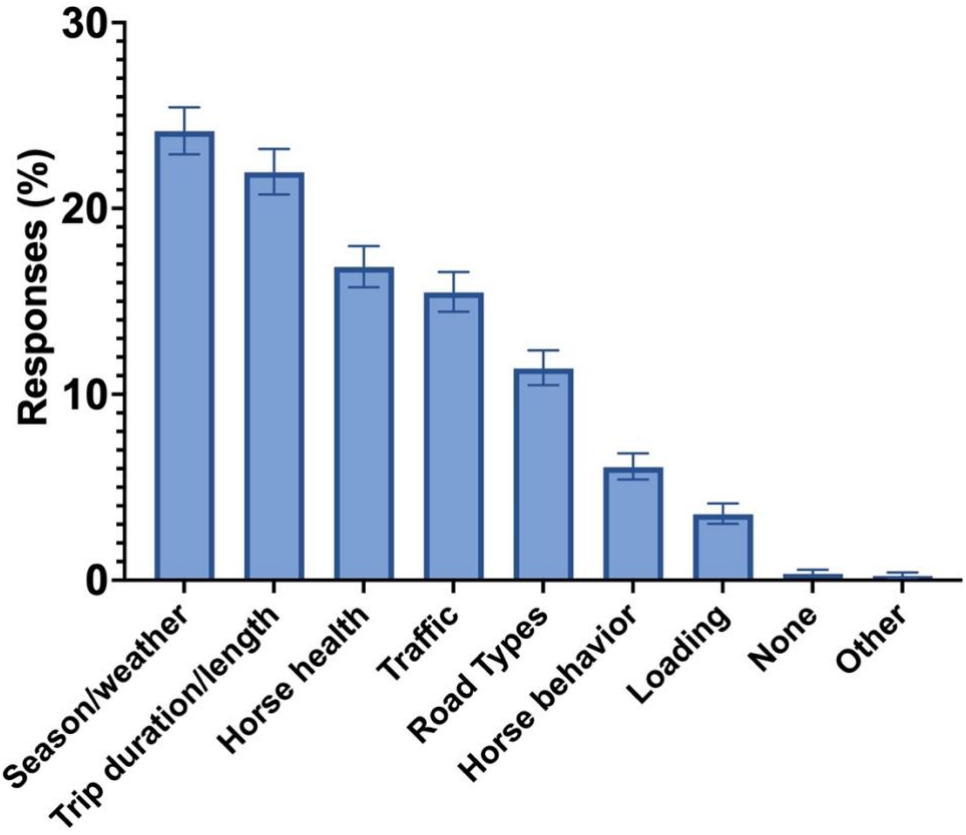

Figure 2.11 Ranking of the main factors considered when planning to transport horses by road reported by respondents to a survey on equine transportation in the U.S.

Data are presented as percentages with 95% confidence intervals.

CHAPTER 3 AGE-RELATED DIFFERENCES IN SHORT-TERM TRANSPORTATION STRESS RESPONSES OF HORSES.

3.1 Abstract

Transportation of horses on short journeys can lead to an increase in stress. There are known age-associated changes in immune and metabolic responses in horses; however, no research exists evaluating how age may influence these responses to transportation stress. Eleven mares within two age groups, aged (n=5, 22 ± 1 year) or young (n=6, 2 ± 1 year), were transported 1 hour and 20 minutes. Peripheral blood and saliva were collected before and after transportation at baseline (2 to 3 weeks prior to transportation), 24 h pre-transport, 1 h before loading, 15 min, 30 min, 1-3 h, 24 h and 8 d post-transport. Heart rates, rectal temperatures, under the tail temperatures, serum cortisol, plasma ACTH, serum insulin, salivary cortisol and IL-6 were measured. Whole blood gene expression of the cytokines IL-1b, IL-2, IL-6, IL-10, IFNγ, and TNFα were determined through qPCR, and peripheral blood mononuclear cells were isolated, stimulated, and stained to determine IFNγ and TNFα production. Serum cortisol ($P<0.0001$), salivary cortisol ($P<0.0001$) and heart rate ($P=0.0002$) increased in response to transportation with no age differences. Rectal ($P=0.03$) and under the tail temperatures ($P=0.02$) were increased in young versus aged horses. ACTH was higher in aged horses ($P=0.007$) and post-transportation ($P=0.0001$). Aged horses showed a greater increase in insulin compared with young horses ($P<0.0001$). While age does not seem to impact cortisol responses to short-term transportation in horses, it did influence the post transportation insulin response to stress in aged horses.

Keywords: Horse; Age; Transportation; Stress; Immune Response

Full reference: Jacquay, E.T., Harris, P.A. and Adams, A.A. (2023). Age-Related Differences in Short-Term Transportation Stress Responses of Horses. J Equine Vet Sci 128. https://doi.org/10.1016/j.jevs.2023.104879

3.2 Introduction

Although horses are frequently transported by road for a wide variety of reasons, there can be an increased stress response and potential impact on the immune system [1,7,16]. Long-distance transportation can decrease lymphocyte proliferation, increase neutrophil counts, and increase interferon gamma (IFNγ) production; all of which are indicative of decreased immune function [1,2]. These alterations in immune function may in turn increase the horse's susceptibility to infection. In particular, long-distance journeys (> 24 h) have been associated with an increased risk of developing transportation related diseases [9,15,50,55]

While most research has previously focused on long-term transport, horses in the United States are more frequently transported by road short a duration of 3 hours or less [118]. Even on short journeys the hypothalamic-pituitary-adrenal (HPA) axis is activated in response to transportation stress as measured through increases in circulating adrenocorticotropic hormone (ACTH) and cortisol concentrations [9,119]. While blood has mainly been used to evaluate changes in HPA axis hormones in response to transportation, there has been an increase in the use of non-invasive techniques to measure stress during transportation, specifically salivary cortisol, and heart rate [6,7]. These non-invasive techniques to monitor horse health during transportation have been promising and should continue to be incorporated.

Furthermore, aged horses (>15 years) are in an increased inflammatory state known as “inflamm-aging”, characterized by having increased inflammatory cytokine production from lymphocytes and increased gene expression of inflammatory cytokines in whole blood [77]. Aged horses also experienced an altered production of inflammatory cytokines following short-term transportation that could potentially put them at a higher risk for developing transport-related illnesses [79]. However, no research currently exists evaluating how the response of senior horses to transport-related stress compares to that of younger horses. The objectives of this study were to (1) understand the differences in stress and immune function between aged and young horses and (2) to utilize non-invasive techniques to monitor stress and immune responses to short-term transportation. It was hypothesized that aged horses would have reduced immune responses compared to young horses observed through changes in inflammatory cytokine gene expression and lymphocyte production of cytokines post-transportation, but that stress responses would not differ between age groups. Furthermore, we hypothesized that non-invasive monitoring techniques could detect changes in stress following short-term transportation in aged and young horses.

3.3 Materials and Methods

This study was approved by the University of Kentucky’s Institutional Animal Care and Use Committee (protocol #2021-3854).

3.3.1 Animals

Twelve idle, non-pregnant mares of mixed light breed from the University of Kentucky’s research herd were initially enrolled in the study. Horses were classified by known age into two groups: aged (n=6, mean age ± SD = 22 ± 1 yr) and young (n=6, mean

age ± SD = 2 ± 1 yr). One month prior to the start of the study body condition (BCS) and cresty neck score (CNS) were determined by two trained personnel and were recorded [120,121]. Bodyweight was measured in kilograms with a calibrated, portable, digital scale (True Test Inc, Mineral Wells, TX). There were no differences in median BCS or CNS between aged (BCS median = 5.1, range= 4.3-5.8; CNS median = 2, range = 1-3) and young (BCS median=6, range=5-6.5; CNS median = 1.8, range = 1-2) horses. Additionally, there were no differences in weight between aged (mean weight ± SD = 562.1 ± 44.1kg) and young (mean weight ± SD = 523.7 ± 75.1kg) horses.

Approximately one-week prior horses being transported, horses were screened for endocrine disorders by measuring basal plasma ACTH and serum insulin. Horses with basal plasma ACTH <30 pg/mL and no signs of hypertrichosis were considered negative for pituitary pars intermedia dysfunction (PPID) [107]. Horses were considered negative for insulin dysregulation if their basal serum insulin was <50 μU/mL and they had no other signs or history of equine metabolic syndrome such as BCS >7, regional adiposity, and/or history of laminitis [84]. Only horses considered negative for PPID and ID were included in the study. Endocrine results were not received until after horses had been transported and one aged mare's bloodwork was above the diagnostic cutoffs for PPID (172.0 pg/mL) and ID (57.4 μU/mL) and was excluded from further analysis resulting a total of 11 horses: aged (n=5) and young (n=6).

This study was conducted in the fall of 2021 from October to November and all horses were owned by and housed at the University of Kentucky's Department of Veterinary Science North Farm. When not being transported horses were group housed on pasture or semi-dry lots with ad libitum access to water and supplemented with hay and

complementary feed to meet NRC requirements of horses at maintenance [122]. None of the horses on the study received any medications from the time they were enrolled until the study was completed.

3.3.2 Study Design

3.3.2.1 Transportation

All animals used in this study had previous experience being transported with no adverse responses and had not been transported in at least 25 days, with their most common reason for transportation being moving pastures/locations on the 2400-acre research farm. Horses were transported in a livestock trailer at the same time of day over three separate days within a two-week period. On each transportation day, two horses from the aged group and two horses from the young group were on the trailer in each trip. Horses were paired with another horse of their age then randomly assigned by age group to the front or back section of the trailer for each trip. The same round-trip route was taken each journey, with an approximate travel time of 1 hour and 20 minutes over a distance of 88.5 km on mainly highway type roads. All horses were tied to the left side of the trailer at eye level and a middle divider separated the front and back half of the trailer. Horses returned to their normal pasture after transportation.

There was no inclement weather on the days of transport and the ambient temperature was an average of 7°C 24 h pre-transport, ranged from -1°C to 19°C on the transport days, and averaged 2°C 24 h post-transport, and 3°C 8 days post-transport at the time of sample collections.

3.3.2.2 Blood and saliva collection

All sample collection occurred prior to horses receiving complementary feed. Blood samples were collected through jugular venipuncture at nine timepoints: Baseline (0900 h, 2-3 weeks prior to transportation) and 24 h before transportation (0900 h; 24 h pre); on the day of transportation: 1 h before loading (0800 h) and 15 min (1030 h), 30 min (1045 h), 1 h (1115 h), 2 h (1215 h) and 3 h (1315 h) after unloading from the trailer; following transportation : 24 h after (0900 h, 24 h post) and 8 days after transportation (0900 h, 8 d post). Blood was evaluated, as described below, for plasma ACTH, serum cortisol and insulin, whole blood gene expression, and peripheral blood mononuclear cells (PBMCs) for intracellular staining of cytokines.

Saliva was collected immediately after blood collections at the same timepoints. Saliva samples were taken using cotton swabs (Salivette, Sastedt, Numbrecht-Rommelsdorf, Germany) placed under the horses' tongue for 60 seconds using a hemostat [123]. After fully saturated, the cotton roll was returned to the polypropylene tube and stored at 4°C until centrifugation at 1000 x g for 2 minutes. Samples were then stored at -20°C until further analysis for cortisol and IL-6.

3.3.2.3 Heart rate and temperature

Heart rate was determined using Polar H10 Equine Heart Rate Monitor (Polar Electro Oy, Kempele, Finland) [124]. Polar Equine heart rate belts were attached around the heart girth of the horse according to manufacturer instructions. The Polar H10 hear rate sensor was then attached and paired to the Polar Beat application on a Bluetooth capable device, with each horse having its own sensor and device. Heart rate data was recorded at the same timepoints immediately before blood and saliva collections.

Rectal temperature was measured with a digital thermometer in degrees Celsius (Neogen, Lansing. MI) at baseline, 24 h pre-transport, the 1 h prior to being transported, 24 h post-transport and 8 d post transportation, prior to blood and saliva collections.

Under the tail temperature monitoring was achieved using VetTrue TailTab (Epona Biotec Ltd., Hong Kong, China) [125]. TailTabs were attached under the tail by cleaning the skin with a 70% isopropyl alcohol wipe (Shenzen 3Leaves Technology Co., LTD., Shenzhen, China) and attaching the adhesive side to the base of the tail. TailTabs were paired via Bluetooth with the VetTrue App v1.2 (Epona Biotech Ltd.). Temperature was recorded at 5-minute intervals over 24 hours, starting one hour prior to transport. Data was then exported to Microsoft excel prior to statistical analysis.

3.3.3 Sample collection & processing

3.3.3.1 Serum Cortisol, Plasma ACTH, and Serum Insulin

Blood collected into serum and EDTA vacutainer tubes was centrifuged at 800 g x 10 minutes, aliquoted, and frozen at -20°C for one week until being shipped on dry ice to Cornell University's Animal Health Diagnostic Center for analysis of cortisol and ACTH using a chemiluminescent immunoassay system (IMMULITE® 1000; Siemens, Munich, Germany), previously validated for use in the horse [126,127], and insulin via a commercially available human insulin radioimmunoassay (EMD, Millipore Corp, Billerica, MA) previously validated for use with equine serum , with a sensitivity of 2.72 µU/mL and mean intra- and inter-assay coefficient of variation (CV) of 7.4% and 6.3% respectively.

3.3.3.2 Salivary cortisol and IL-6

Frozen saliva samples were shipped on dry ice to Salimetrics LLC (State College, PA) for analysis of salivary cortisol in duplicate using an enzyme immune-assay previously validated for use in the horse [128], with a sensitivity of 0.007 ug/dL and mean intra- and inter-assay coefficient of variation (CV) of 4.6% and 6.0% respectively. A subset of salivary samples, pre-transport (baseline or 24 h pre), 15 min post, and 3h post-transport, were additionally tested in duplicate for Interleukin-6 (IL-6) using an enzyme immune-assay with a detection limit of 0.07 pg/mL and mean intra- and inter-assay coefficient of variation (CV) of 4.8% and 10.6% respectively.

3.3.3.3 Cytokine gene expression in whole blood

Whole blood was collected directly into Tempus™ Blood RNA tubes (Applied Biosystems Inc., Foster City, CA) at all nine time points and frozen at –20°C until processing. RNA was isolated and qPCR was performed as previously described [129]. Relative quantities (RQ) were calculated using the $2^{-\Delta\Delta Ct}$ method, where baseline sample ΔCt values from each individual horse was used as the calibrator for each cytokine and β-glucuronidase (β-Gus) was used as the housekeeping gene for all samples [130]. Samples were assayed in duplicate using commercially available primers and probes (Thermo Fischer Scientific Inc., Waltham, MA): IL-1β (Ec04260298_s1), IL-2 (Ec03468864_m1), IL-6 (Ec03468678_m1), IL-10 (Ec03468647_m1), IFNγ (Ec03468606_m1), and TNFα (Ec03467871_m1).

3.3.3.4 PBMC isolation, staining, and flow cytometry

At all nine time points, PBMCs were isolated from heparinized blood using a Ficoll density gradient and frozen at –80°C as previously described [77,131]. PBMCs were counted using a VICELL™ Counter-XR (Beckman Coulter, Miami, FL) and plated at a

concentration of 4 x 10^6 cells/mL in complete media in duplicate on 24-well cell culture plate, all wells received Brefeldin A (10 µg/mL; Sigma-Aldrich) and one well per sample was stimulated with PMA/ionomycin (25 ng/mL; 1uM, Sigma-Aldrich). PBMCs were incubated at 37°C, 5% CO_2 for 4 hours, then transferred to a 96-well plate and fixed in 2% paraformaldehyde overnight. PBMCs were stained for IFNγ and TNFα (monoclonal mouse anti-equine TNFα antibody (100 µL of 1:10 dilution in saponin buffer; antibody [HL801] provided by Dr. Robert McKay, University of Florida) and FITC-conjugated secondary antibody (F(ab')2 goat anti-mouse IgG (H +L)). Cytokine production was determined using flow cytometry (Attune™ NxT Flow Cytometer; Thermo Fisher Scientific) as previously described [132]. A gate was placed around the lymphocyte population, and the percentage of gated cells were analyzed and recorded. The change in the percentage of positively stained gated cells (Δ %) was determined by subtracting the number of gated lymphocytes that were unstimulated from those stimulated with PMA and ionomycin.

3.3.4 Statistical Analysis

All statistical analysis was completed using GraphPad Prism 9.4.0 (GraphPad, San Diego, CA). Age differences in mean weight were determined using unpaired t-Test and median BCS and CNS with Mann-Whitney test. Stress (heart rate, rectal temperature, TailTab temperature, serum cortisol, serum insulin, and salivary cortisol) and immune parameters (salivary IL-6, whole blood gene expression of cytokines: IL-1b, IL-2, IL-6, IL-10, IFNγ, TNFα, and % gated IFNγ+/TNFα+ lymphocytes) were assessed for changes over time (pre- and post-transportation) and age (aged vs young). These were performed using repeated measures ANOVA or a mixed-effect model with the horse as a random

effect and the time of sample collection and age as fixed effects. Geisser-Greenhouse's correlation was used to adjust for lack of sphericity in repeated measures. Q-Q plots were used to determine normal distribution of residuals and data was log transformed if normal assumptions were not met. Residual plots were used to confirm correctness of fit for a linear model. RQ values were natural log (Ln) transformed to meet assumptions of normality. Tukey's or Bonferroni's post hoc adjustments were made to account for multiple comparisons. Simple linear regression and Pearson correlation coefficients were calculated between serum and salivary cortisol concentrations. Results were considered significant at $P < 0.05$.

3.4 Results

All horses were loaded on to the trailer, transported, and unloaded with no adverse effects or incidents.

3.4.1 Rectal temperature, under the tail temperature, and heart rate

Although there were temperature differences on the days horses were transported, there was no effect of the day of transportation on rectal temperatures. There were no differences in rectal temperatures in response to transportation for horses in the young age group and no effect of day transported; however, aged horses had decreased temperatures at 8d post-transportation when compared with baseline samples ($P=0.016$, Figure 3.1).

Young horses had increased under the tail temperatures compared with aged horses ($P=0.015$, Figure 3.2). Under the tail temperature monitoring also showed effects of transportation ($P=0.0023$) and a transportation x age group interaction ($P<0.0001$); however, there were no statistical differences for pairwise comparisons after conducting post hoc analysis, likely due to the high number of factor levels.

There were no age group differences for heart rate, but there was a transportation effect (P=0.0002) and transportation x age group interaction (P=0.005, Figure 3.3). Heart rate was increased in aged horses compared to young horses at 3 h post transportation (P=0.02). Aged horses had increased heart rate compared to baseline (2-3 weeks pre-transport) at 15 min (P=0.05) and 3 h post-transport (P=0.02). Young horses had increased heart rates from baseline at 15 min (P=0.04) and 30 min (P=0.03) post transport, but heart rates were decreased from baseline at 8 d post-transportation (P=0.015).

3.4.2 Salivary IL-6

The majority of young horse salivary IL-6 samples were below the limit of detection (0.07 pg/mL); therefore, a value of 0.035 pg/mL was used for statistical analysis. Salivary IL-6 was increased in aged versus young horses (P=0.003) with no effect of transportation (Figure 3.6).

3.4.3 Serum Insulin and Plasma ACTH

Insulin increased in response to transportation (P<0.0001) with aged horses having increased insulin concentrations compared to young horses from 1 h to 3 h post-transportation (P<0.0001, Figure 3.7A) and an age x transportation interaction (P<0.0001). Aged horses had increased insulin from baseline (25.4 ± 11.0 µU/mL) at 2 hours post-transportation (53.65 ± 3.4 µU/mL, P=0.03) and young horses had increased insulin from baseline (9.87 ± 2.9 µU/mL) at 1 h post-transportation (15.9 ± 4.2 µU/mL). Serum insulin was above the basal diagnostic reference range for insulin dysregulation [133] in all aged horses (57.6 ± 4.2 µU/mL, n=5) at 3 h post-transportation.

There was a significant effect of age (P=0.007) and transportation (P=0.0001) on ACTH (Figure 3.7B), but no age x transportation interaction. Young horses had increased

ACTH at 15 min post transportation when compared with 24 h (P=0.02) and 1 h pre-transport (P=0.03) and decreased ACTH from 15 min at 2 h (P=0.02) and 3 h post-transportation (P=0.04). ACTH was above the reference range for PPID diagnosis [134] in 3 out of the 5 aged horses at 15 min post-transportation (92.9 pg/mL, 61.7 pg/mL, and 110.0 pg/mL).

3.4.4 Gene expression in whole blood

There was no effect of age on gene expression of the cytokines, IL-2, IL-6, IL-1b, IL-10, IFNγ, and TNFα (Figure 3.8A-F). There was also no effect of transportation on IL-6, IL-1b, IL-10, IFNγ, and TNFα (Figure 3.8B-F). There was an effect of transportation for IL-2 gene expression with aged horses having increased expression from baseline at 30 min post-transport (P=0.002, Figure 3.8A) and young horses had increased expression from baseline at 1 h pre-transport (P=0.01) and 30 min post-transport (P=0.008). There was a transportation x age group interaction (P=0.007, Figure 3.8B) for IL-6 gene expression.

3.4.5 PBMCs- Flow cytometry IFNγ and TNFα

Δ % of IFNγ+ and TNFα + lymphocytes were both elevated in aged horses compared with young horses (P<0.0001 and P=0.03 respectively, Figure 3.9A-B). There was no effect of transportation on the Δ % of IFNγ+ and TNFα + lymphocytes.

3.5 Discussion

There are both similarities and differences in how aged and young horses respond physiologically to short-term transportation stress. Increases in temperature and pyrexia have been associated with pleuropneumonia when horses are transported long distance [59]. Conversely in this short-distance transport study, aged horses had a decreased rectal temperature 8 d post-transportation compared to their baseline. This could be attributed to

the decrease in ambient temperatures from 15 °C to 3°C over this time period and the fact that older horses have more challenges with thermoregulation [135]. Aged horses also had a lower rectal temperature than the young horses throughout the study confirming previous research in our lab showing that older horses have decreased rectal temperatures compared with young horses [136]. The decrease in under the tail temperatures in aged horses also further supports the rectal temperature findings. These results suggest that these age-related differences in temperature could be attributed to the aged horses diminished ability to thermoregulate in colder weather, potentially due to changes in metabolism associated with aging, similar with what has been observed in elderly people [137] Additionally, while there were no statistical differences between BCS between aged and young horses in this study, the lower BCS and increased muscle atrophy associated with aging could also be contributing to the reduced thermoregulation ability in older horses [138].

Activation of the sympathetic nervous system in response to transportation stress can be observed by the increase in heart rate 15 minutes post-transportation in aged and young horses that agrees with previous transport studies using aged (>15 years) or young horses (2-7 years) that were transported 1 to 1.5 hours [9,79]. Studies that were able to monitor heart rate during transport have shown that loading on the trailer was the time relative to transport with the highest increase in heart rate from baseline [7,139]. Whilst heart rate was not recorded during transport in this study due to technical difficulties, the increase from baseline at 15 min post-transport could be attributed to the anticipation of unloading and adapting to returning to their resident pasture.

Stimulation of the HPA axis to short-term transportation stress was measured by increased plasma ACTH concentrations in both aged and young horses following

transportation, similar to previous studies evaluating short distance transportation of stallions aged 4 to 20 [12]. The aged horses had overall higher ACTH concentrations compared with young horses, which has been previously reported in horses aged horses (18-24 years) versus adults (5-13 years) at rest [104]. The exact reason for higher ACTH in aged horses is unknown but could possibly be linked to the increased inflammatory state and neurodegeneration associated with aging that could progress to development of PPID [140]. Additionally, this research was conducted in the fall when circulating ACTH is elevated, especially in older horses [104]. It should also be noted that this study was performed using mares whose reproductive phases were unknown and ACTH can be increased during the peri-ovulatory period of the estrous cycle, which could account for some of the variability in ACTH concentrations [141,142]. Importantly, 3 out of 5 aged horses had elevated plasma ACTH above the fall PPID diagnostic cutoff range of > 90 pg/mL (October) or > 50pg/mL (November) post transportation [107]. Therefore, caution should be used if transporting an aged horse to a veterinary clinic for PPID testing as the increased stress response post-transportation could cause misleading results. Additionally, it is not recommended that ACTH results alone should be used to diagnose PPID due to the influence that factors such as age and stress have on ACTH concentrations [104,119]

Although the aged horses had higher ACTH concentrations, which theoretically could stimulate more cortisol production from the adrenal glands, there were no age differences in the serum or salivary cortisol concentrations post-transportation. Previous research evaluating how different parameters, including season and age, influence cortisol has not found an influence of age on circulating cortisol at rest in equine serum or saliva when samples were taken over a period of 6 months to 1 year [143,144]. The lack of

increase in cortisol in aged horses could be attributed to the cortisol that we are measuring being bound to transporter proteins versus more bioavailable free cortisol or the ACTH not being enough of an increase in the aged horses to stimulate the adrenal glands to produce more cortisol. This could also potentially be explained by the cortisol recovery capacity of the aged horses due to the presumed increased experience with being transported.

Serum and salivary cortisol increase in response to transportation stress, with the highest concentrations being immediately after unloading in short-term journeys [7,12]. Similarly to our results, salivary cortisol did not differ from baseline concentrations 1-hour post-transportation on a journey of 50 km [7], suggesting that in the first hour post-transport the horse is still physiologically recovering from the stress of travel. Previous studies have found a strong positive correlation with serum and salivary cortisol RIA in the horse in response to ACTH challenge [145], but to our knowledge this is the first report showing a strong correlation of equine serum cortisol using chemiluminescent enzyme immunoassay and salivary cortisol ELISA in response to short term transportation. Due to the ease of saliva collection and strong correlation with serum using various assays, this data further supports the potential elimination of blood collections when monitoring cortisol in response to transportation.

Although there were increases in stress associated parameters in response to short-term transportation, they all returned to baseline by 24 hours post-transportation in all horses, as seen previously in a study transporting aged horses short-term [79]. There is an acute stress response to short term transportation in horses; however, the overall outcomes appear to be transient and special attention to the horses well-being should focus on the first few hours post-transportation.

Horses tend to become more ID with age and show increased insulin responses to oral ingestion of starch and sugar, as well as increased tissue insulin resistance [104,146]. This is the first report of an increased insulin response following transportation in metabolically normal (not ID or PPID) aged horses. Whilst cortisol is generally considered an insulin antagonist, it is possible that after short-term transportation there could be altered glucose metabolism in aged horses contributing to an increase in blood glucose, that has been previously reported in response to short distance transport, leading to a change in insulin dynamics in aged horses similar to the response seen after a glucose challenge [9]. Importantly these increased insulin concentrations post-transportation could potentially give false positive indications of insulin dysregulation in aged horses if blood is taken after horses are transported to a veterinary clinic. Although insulin returned to baseline levels by 24 h post-transportation, it remains to be determined how long it takes for insulin to normalize post transportation and therefore when endocrine testing should be conducted in aged horses. Moreover, while all the horses used in this study were considered metabolically normal, it is unknown what the impact of short-term stress is on insulin in horses with insulin dysregulation and further research is needed in this area.

Dysregulation of inflammatory cytokines is associated with both ageing and transportation stress in aged horses. Although some changes in gene expression of cytokines in whole blood were observed following transportation, the impact of short-term transportation on inflammatory cytokines remains unclear. Previous research found increases in IL-2 and IL-10 and decreases in gene expression of IL1b, TNFα, and IFNγ in aged horses 15 min post-transportation [79] and the differences in results could be attributed to increased variability between horses in the current study possibly due to

differences in data and statistical analysis. The increase in IL-6 in aged horses 30 minutes post-transportation is consistent with previous work in our lab and in a study transporting horses 50 km measuring plasma IL-6 concentrations using ELISA [73,79]. Expression of IL-6 has also been reported to be increased in healthy aged horses (>16 years) compared with healthy adult horses (6-14 years) [140]. The increase in IL-6 could be linked to activation of the acute phase response in response to transportation stress that could also be supported by measured increases in acute phase proteins following transportation [74,75]. To our knowledge this is the first report that salivary IL-6 is also increased in aged horses versus young horses. Though no oral abnormalities were observed in the horses used in the study, it should be mentioned that the salivary IL-6 measured could be reflective of local versus systemic inflammation. IL-6 and other salivary analytes should continue to be explored to determine if more parameters can be measured in saliva as a way to non-invasively monitor horse health.

Long distance transportation can decrease the immune capacity of horses through altered lymphocyte populations and decreased lymphocyte proliferation [2,147]. Conversely, over short distances we did not see any changes in lymphocyte production of IFNγ and TNFα in either the aged or young horses following transportation, in agreement with previous research in short-term transport of aged horses [79]. Assumingly the differences in immune response between long and short distance transport can be attributed to the additional length of stressors associated with increased distance of travel. However, we did see an increase in Δ % of IFNγ+ and TNFα + lymphocytes in the aged horses consistent with "inflamm-aging" that could put aged horses at an increased risk of developing health problems [77]. Young horses did not show any changes in whole blood

gene expression of cytokines or cytokine production from lymphocytes, suggesting that their inflammatory state was not impacted by the acute stress they experienced following short-term transportation; however, these measurements are just components of overall immune function. Due to the dysregulation of cytokine production in aged horses, special attention should be taken to monitor horse health when transporting aged horses due to potential to exacerbate the effects of inflamm-aging. This study observed changes in aged horses (21 to 22 years); however, further research is needed to determine the exact ages at which horses experience these age-related changes in endocrine and immune responses.

Some limitations to this study include that only mares were used, and it is unknown if there would be any sex differences in stress and immune function post-transportation and as mentioned previously, the reproductive phases were unknown which could have impacted ACTH concentrations. Additionally, although the horses used all had experience being transported, they were not routinely transported which could potentially result in an elevated stress response compared with horses who are more accustomed to being transported on a regular basis. Furthermore, it should be taken into consideration that it is an inherent assumption that older horses might have more transportation experience than young horses as a result of their increased age, which could potentially confound some age differences observed. Horses were also brought into paddocks in the morning before sampling occurred which could have influenced their pre-transportation ACTH and/or cortisol concentrations.

3.6 Conclusion

Short-term transportation causes acute stress in horses regardless of age; however, aged horses have different endocrine and immune responses post-transportation. In

particular, the increase in serum insulin and plasma ACTH in aged horses post-transportation should be considered especially when transporting horses that could have metabolic disorders or when transporting to a veterinary clinic for endocrine disorder diagnostic testing. Future research should be conducted using ID and PPID horses to better understand the impact of transportation stress on horses with altered endocrine function and how it might influence diagnostic testing. Whilst there is no apparent impact of transportation on the immune function in young horses based on the parameters measured, all horses should be monitored for any deviations from normal health parameters when being transported. More careful attention should be given to older horses given the changes seen in both immune and metabolic responses to short-term transportation. Non-invasive techniques should continue to be utilized to monitor horse health while transporting.

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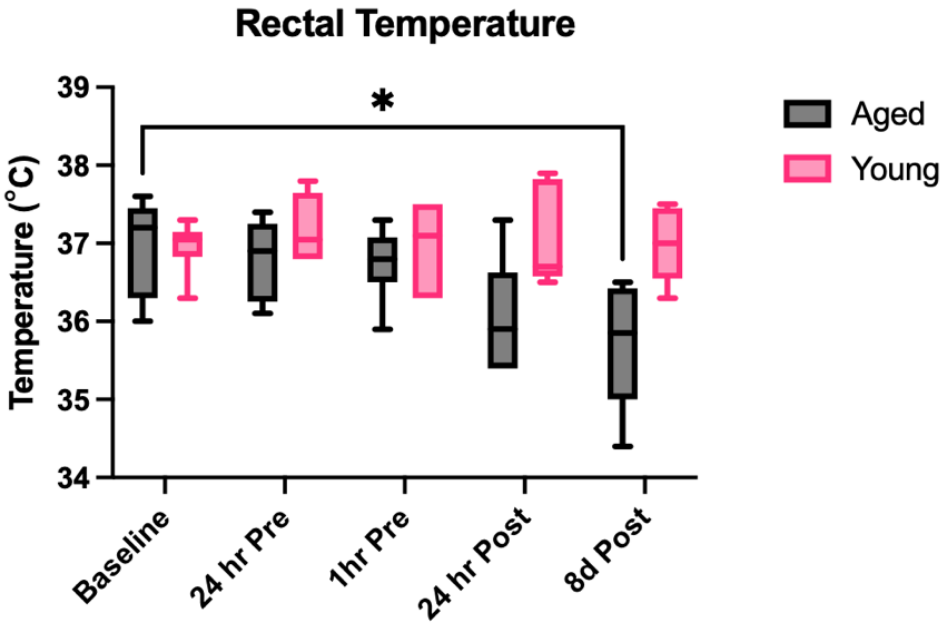

Figure 3.1 Rectal temperature of horses before and after short-term road transportation.

The data are presented as the median and interquartile range (IQR) with min and max. Multiple comparisons were calculated using Bonferroni post-hoc analysis. Significant effects of age (P=0.03), transportation (P=0.03), and age x transportation (P=0.02) were observed. Differences within the aged horse group between baseline and 8 d post-transportation denoted as *, P<0.05.

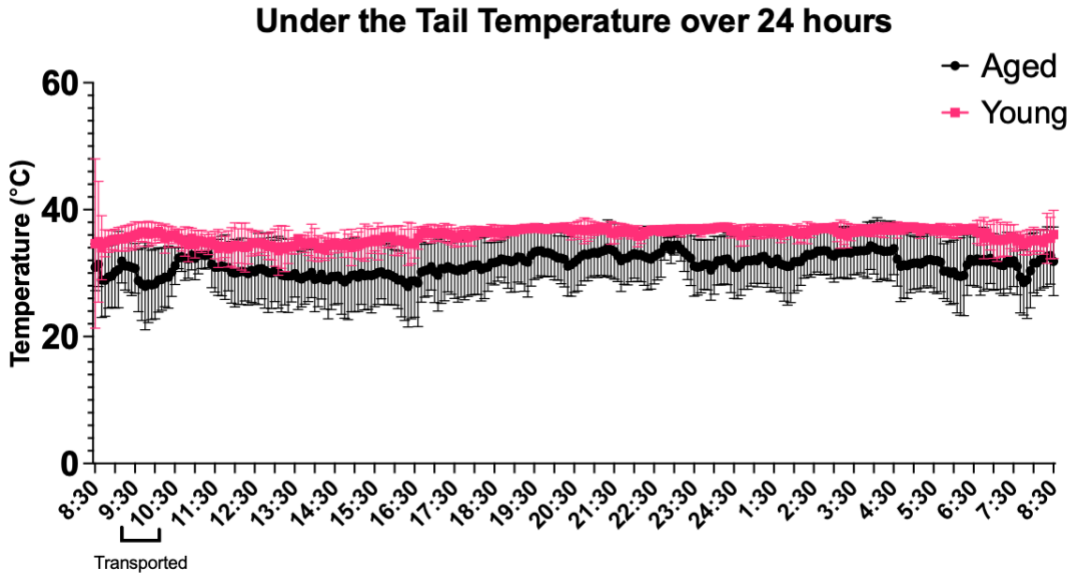

Figure 3.2 Under the tail temperatures of horses 1 h pre - to 24 h post short-term road transportation.

The data are presented as mean ± confidence interval. Significant effects of age (P=0.02), transportation (P=0.002), and age x transportation (P<0.0001) were observed. Mean ± SEM temperature for aged horses was 36.1 ± 0.1°C for young horses and 29.1 ± 0.3°C for aged horses.

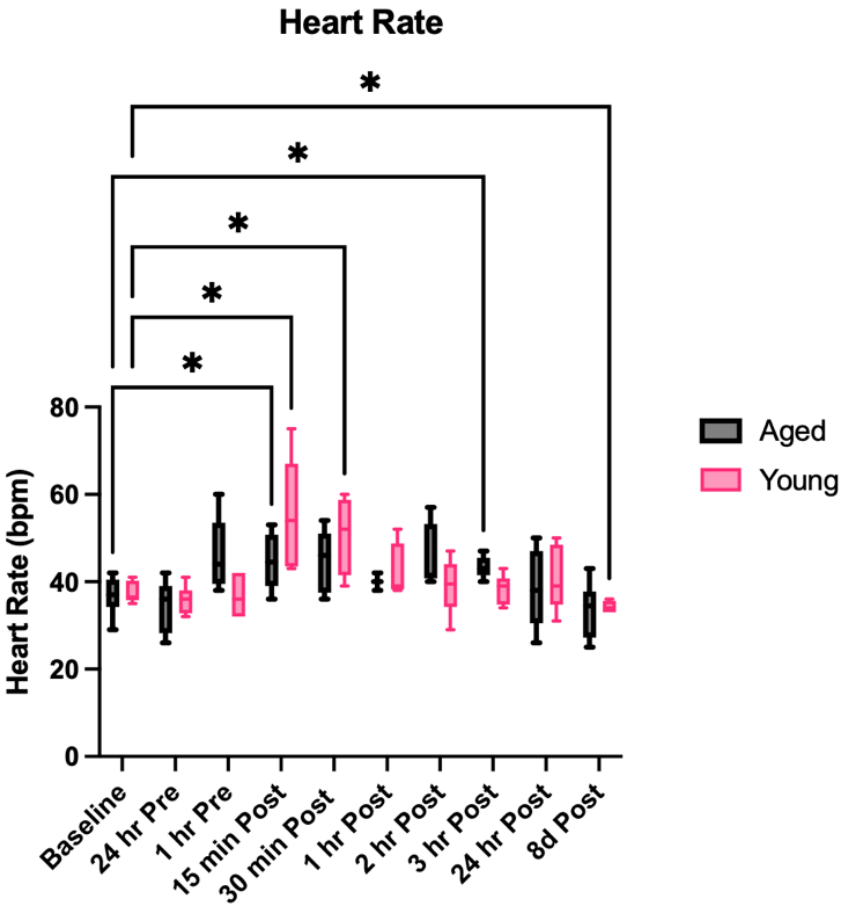

Figure 3.3 Heart rate of horses before and after short-term road transportation.
The data are presented as the median and interquartile range (IQR) with min and max. Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was no significant effect of age, but significant effects of transportation (P=0.0002), and age x transportation interaction (P=0.005). Differences within age groups in relation to baseline are denoted as *, P<0.05.

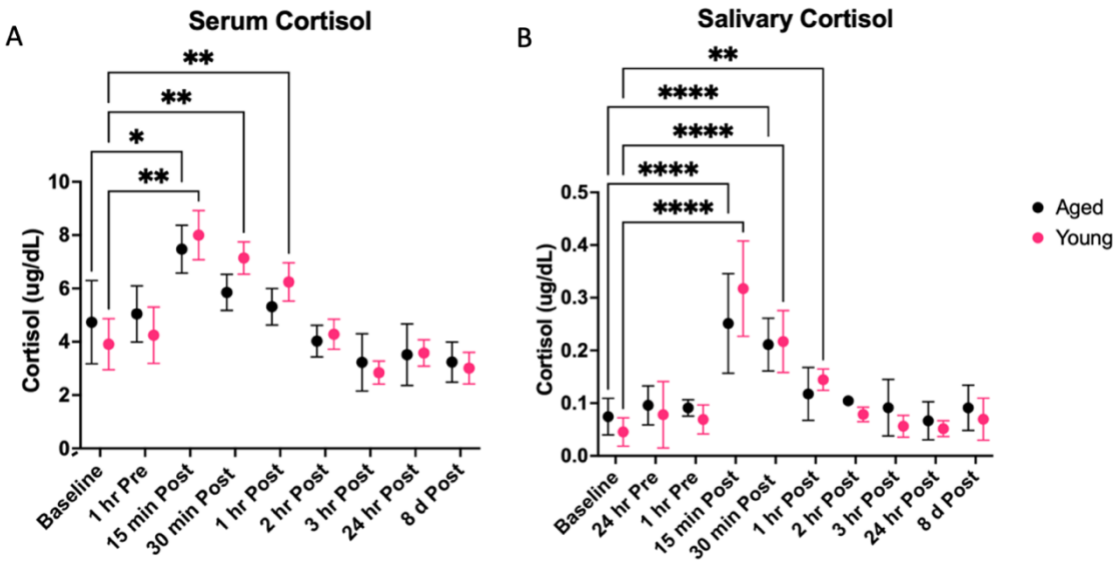

Figure 3.4 Serum (A) and salivary (B) cortisol concentrations of horses before and after short-term road transportation.

The data are presented as the mean ± confidence interval. Multiple comparisons were calculated using Bonferroni post-hoc analysis. There were no significant effects of age, but significant effects of transportation (A: P<0.0001; B: P<0.0001), and age x transportation interaction (A: P=0.0004). Differences within age groups in relation to baseline are denoted as ****, P<0.0001; **, P<0.01; *, P<0.05.

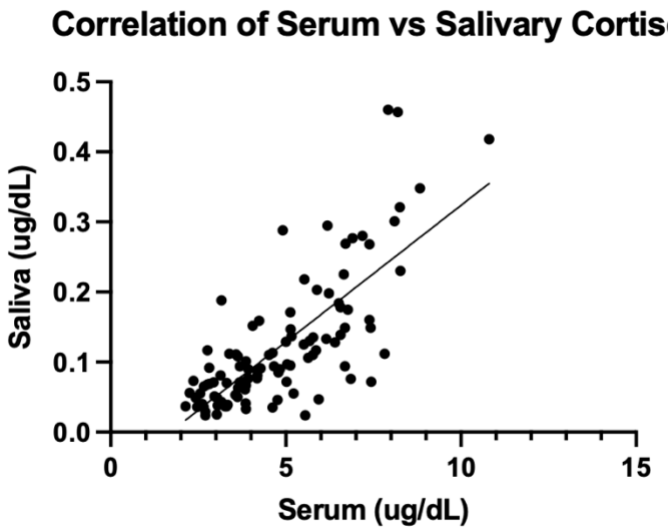

Figure 3.5 Simple linear regression and correlation of serum and salivary cortisol of horses before and after short-term road transportation.

Pearson correlation coefficient r = 0.76, P< 0.001; linear regression R^2 = 0.57, P < 0.001.

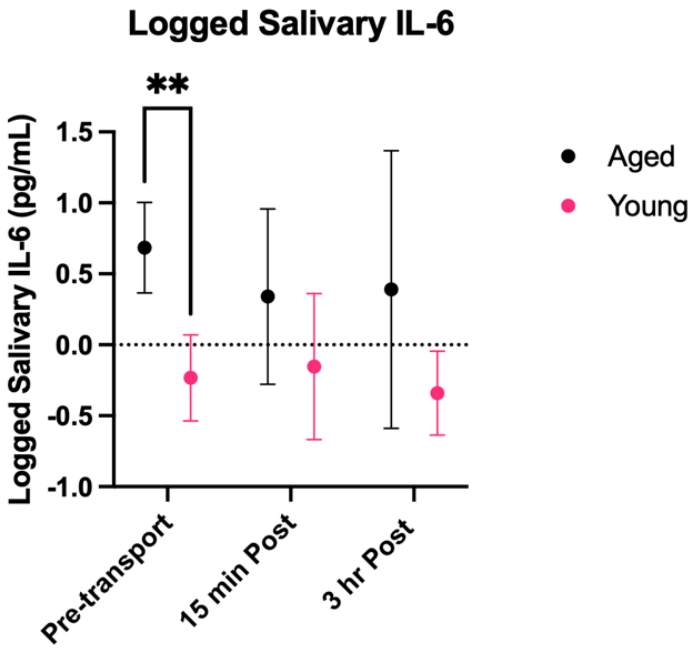

Figure 3.6 Logged salivary IL-6 concentrations of horses before and after short-term road transportation.

The data are presented as the mean ± confidence interval. Multiple comparisons were calculated using Bonferroni post-hoc analysis. Significant effect of age (P=0.003), but not transportation and age x transportation interaction. Differences between age groups are denoted as **, P<0.01.

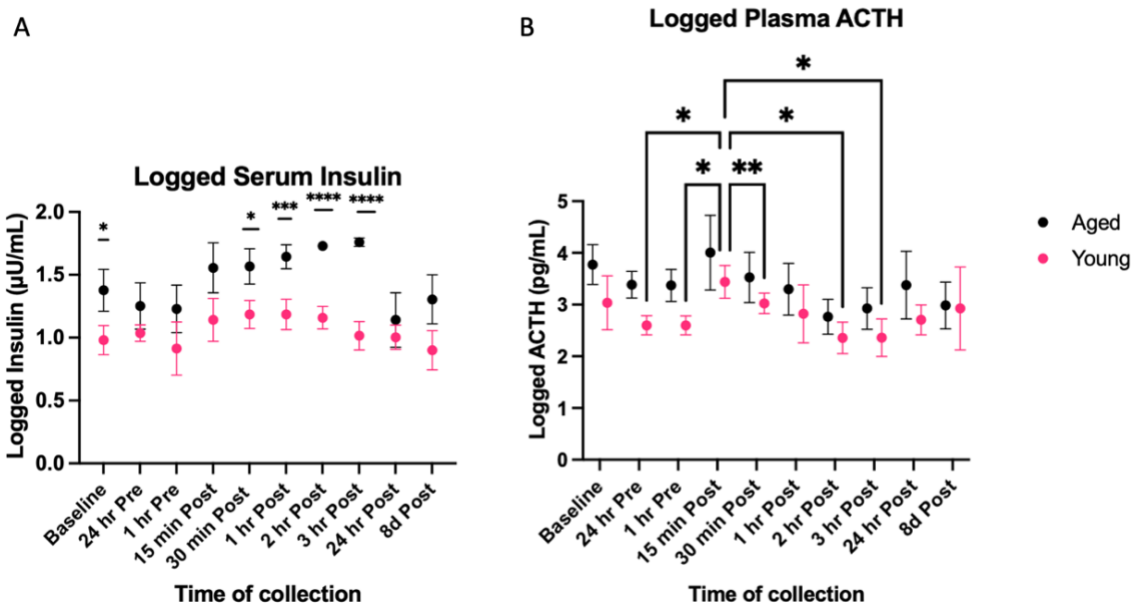

Figure 3.7 Logged serum insulin (A) and logged plasma ACTH (B) concentrations of horses before and after short-term road transportation.
The data are presented as the mean ± CI, and significant differences were calculated using repeated measures ANOVA (A) or mixed-effects model (B) and multiple comparisons were calculated using Bonferroni (A) or Tukey post-hoc analysis. Significant effects of age (A: P<0.0001; B: P=0.007), transportation (A: P<0.0001; B: P=0.0001), and age x transportation (A: P<0.0001) were observed. Differences between age groups (A) and for young horses pre- and post-transport (B) are denoted as ****, P<0.0001; ***, P<0.001; **, P<0.01; *, P<0.05.

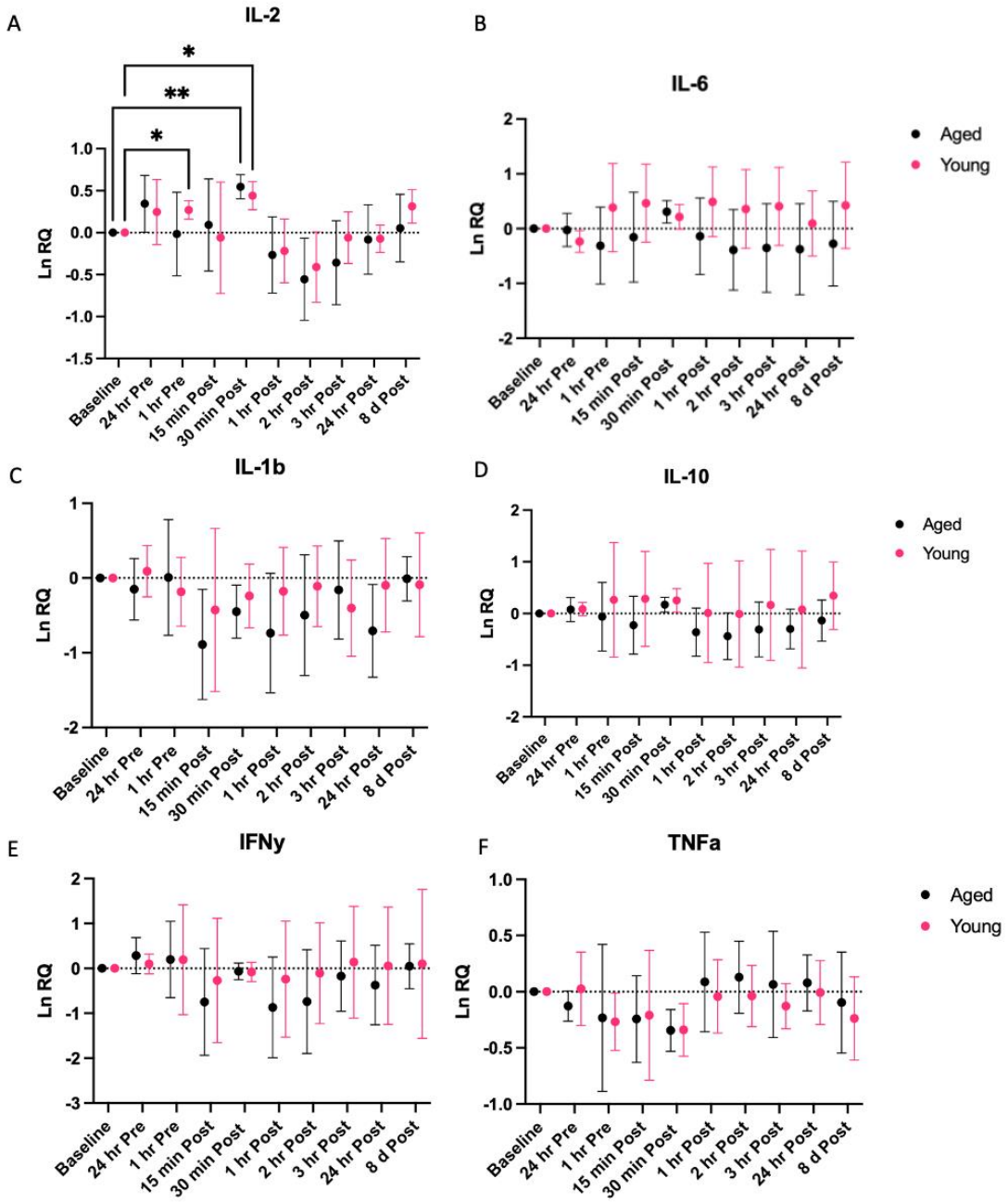

Figure 3.8 Whole blood gene expression of IL-2 (A), IL-6 (B), IL-1b (C), IL-10 (D), IFNγ (E), and TNFα (F) of horses before and after short-term road transportation.

The data are presented as the mean ± CI, and significant differences were calculated using mixed-effects model and multiple comparisons were calculated using Bonferroni post-hoc analysis. There were no significant effects of age, but significant effects of transportation (A: P=0.0003) and age x transportation interactions (B, P=0.007). Differences within age groups in relation to baseline are denoted as **, P<0.01; *, P<0.05. Ln: natural log transported, RQ: relative quantities.

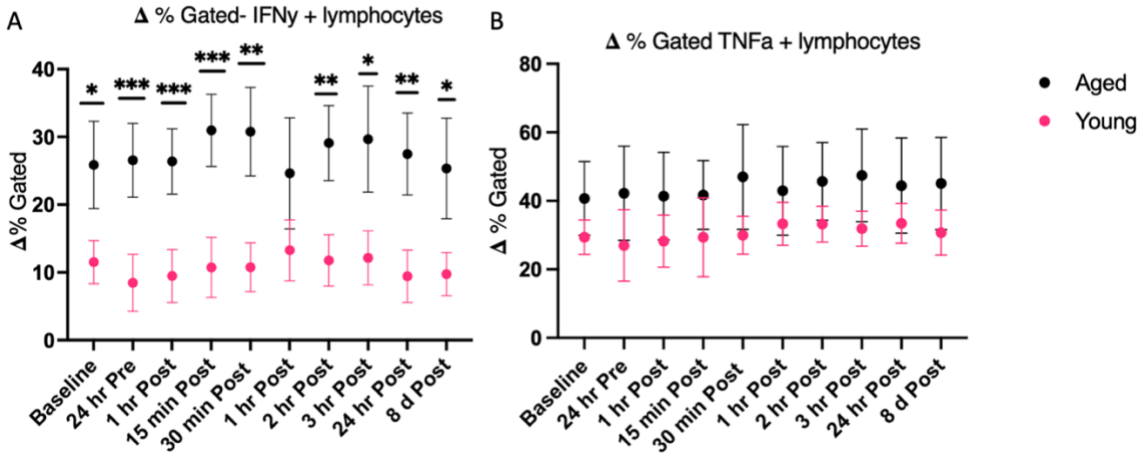

Figure 3.9 Δ % of IFNγ+ and TNFα + gated lymphocytes of horses before and after short-term road transportation.
The data are presented as the mean ± CI, and significant differences were calculated using repeated measures ANOVA (A) or mixed-effects model (B) and multiple comparisons were calculated using Bonferroni post-hoc analysis (A). Significant effect of age (A: P<0.0001; B: P=0.03) were observed, but no significant effects for transportation and age x transportation interactions. Differences between age groups (A) denoted as ***, P<0.001; **, P<0.01; *, P<0.05.

CHAPTER 4 THE IMPACT OF SHORT-TERM TRANSPORTATION STRESS ON INSULIN AND ORAL SUGAR RESPONSES IN INSULIN DYSREGULATED AND NON-INSULIN DYSREGULATED HORSES.

4.1 Summary

Background: It is unknown whether short-term transportation affects endocrine responses similarly in horses with and without insulin dysregulation (ID).

Objectives: To characterize the effect of short-term transportation on stress parameters and insulin responses to an oral sugar test (OST) in horses with and without ID.

Study Design: Longitudinal cohort study.

Methods: Fourteen adult non-pregnant, non-PPID mares of mixed light breeds were grouped as either ID (n=7) or non-ID (n=7) based on endocrine testing. Over 2 weeks, horses were transported once, in groups of 3 to 4 in a horse trailer on a round-trip journey of ~1.5h. Blood and saliva were collected 24 hours and 1h pre-transportation, directly after unloading and 15min ,1h, 3h plus 24h post-transportation. An OST was performed 24h pre-transportation and 3h post-transportation with a pre- (T0) and post-OST sample collected 60 min later (T60). Heart rates and rectal temperatures were also collected throughout the study. Serum insulin, serum cortisol, and plasma glucose were measured using validated assays. Repeated measures ANOVA were used to determine differences after transportation and between ID and non-ID horses. Non-normal data were log-transformed and multiple comparisons were adjusted using Bonferroni post-hoc tests.

Results: Mean serum insulin concentration was higher in ID horses versus non-ID horses (mean = 109.9 µU/mL vs 30.2 µU/mL , $P<0.001$; 95% CI for mean difference = [55.6-107.7 µU/mL). Mean serum insulin concentration increased following OST at T60 in ID horses pre- (154.6 µU/mL, $P=0.04$; 95% CI = [86.3-223.0 µU/mL]) and post-transportation

(284.6 µU/mL , P=0.03; 95% CI = [114.3-454.8 µU/mL]). NID horses had a mean OST T60 insulin post-transportation of 56.6 µU/mL (95% CI=[29.1-84.1 µU/mL]; above recognized threshold [45 µU/mL] for ID diagnosis).

Main limitations: Small number of horses, only mares used, and OST not performed immediately post-transportation.

Conclusions: Performing an OST 3 hours following short-term transportation may result in inaccurate ID status.

Keywords: horse, transportation, stress, insulin dysregulation, ID diagnosis

Full reference: Jacquay, E.T., Harris, P.A. and Adams, A.A. Short-term transportation stress on insulin and oral sugar responses in insulin dysregulated versus non-insulin dysregulated horses. *Equine Veterinary Journal, 2024, In revision.*

4.2 Introduction

Insulin dysregulation (ID) is characterized by hyperinsulinemia, and/or tissue insulin resistance [80,82]. ID horses are at an increased risk of developing laminitis, making it important to correctly diagnose and manage these animals [82]. Currently, ID diagnosis is made based on basal circulating insulin concentration or using dynamic testing, such as the postprandial insulin response to an oral sugar test (OST) [84,87]. Horses may be transported to a veterinary clinic for such diagnostic testing and yet it remains unknown how transportation stress may impact endocrine responses in ID horses.

Short-term transportation (<3 hours) stress activates the hypothalamic-pituitary-adrenal (HPA) axis in horses resulting in elevated heart rates and increased circulating cortisol [9]. Resting heart rates have been shown to be increased in EMS horses compared to non-EMS horses [148]; however, it is unclear if transportation stress would exacerbate

this effect. Cortisol stimulates gluconeogenesis, subsequently raising blood glucose concentrations in horses following transport [53]. Insulin is responsible for maintaining homeostasis through uptake of glucose into tissues [81]. One study showed that serum insulin increased post-transportation of 1-3 hours, in both horses classified as having equine metabolic syndrome (EMS) and in healthy horses; with the EMS horses having higher pre- and post-transport insulin concentrations [92]. However, this study did not carry out any dynamic testing to determine ID status and only took one blood sample before and after the transport at an unknown time relative to transportation. In another study, metabolically normal aged horses (>20 years) had an increase serum insulin after 1.5 hours of transportation, while younger animals (<4 years old) did not [115]. This suggests that even short-term transportation can influence basal insulin concentrations in older horses. However, it is currently unknown how transportation stress might affect dynamic diagnostic testing results for suspected ID horses or healthy horses. In a survey on equine road transportation in the United States, transporting a horse to a veterinary clinic was ranked as the second most common reason for travel, with transportation typically lasting <1 hour (57.9%) or 1-3 hours (42.1%) [149]. Therefore, understanding the stress and endocrine responses to transport in horses with ID could help veterinarians determine the best timing of ID diagnostic tests if horses are transported to a veterinary clinic.

While serum insulin is a useful diagnostic tool for ID, salivary insulin could be a non-invasive way to monitor ID horses. Insulin has been shown to be detectable in equine saliva [36] and this study aimed to explore if concentrations could be correlated with OST results and accurately detect differences between ID and non-ID horses.

The objectives of this study were therefore to understand how short-term transportation stress affects serum and salivary insulin responses in horses with and without insulin dysregulation. It was hypothesized that ID horses would have an altered basal insulin and post-OST insulin responses following short-term transportation when compared to non-ID horses.

4.3 Materials and Methods

This study was approved by the University of Kentucky's Institutional Animal Care and Use Committee (protocol #2021-3854).

4.3.1 Horses

Fourteen non-pregnant, idle mares of mixed age and light horse breed from the University of Kentucky's Department of Veterinary Science research herd were enrolled in the study. Approximately one month prior to horses being transported (April 2022), body condition score (BCS, 1-9) and cresty neck score (CNS, 0-5) were recorded by 2 trained personnel and averaged [120,121]. Additionally, bodyweight (BW) was measured in kilograms with a calibrated, portable, digital weigh bridge (model 700, True Test Inc).

At the same time, horses underwent dynamic testing for insulin dysregulation and pituitary pars intermedia dysfunction (PPID), over two separate days. All horses were considered non-PPID based on basal ACTH (chemiluminescence immunoassay (IMMULITE® 2000) <50 pg/mL and 10-minute post-thyrotropin releasing hormone (TRH) stimulation test ACTH <200 pg/mL [150], with no clinical signs of hypertrichosis and no previous clinical history [151].

Horses were grouped by insulin status as determined by an oral sugar test (OST). OST was performed on horses housed in semi-dry lot pastures, prior to receiving

complementary feed, in the morning by collecting a blood sample (T0) via jugular venipuncture using a vacutainer needle followed by oral administration of 0.15 ml/kg of Karo Light Corn Syrup (AHC Food Companies Inc., Chicago, IL, USA) and collection of a second blood sample via jugular venipuncture 60 minutes later (T60). Horses with T60 post-OST insulin concentrations (radioimmunoassay, EMD Millipore Corp., Burlington, MA, USA) >45 µU/mL were considered insulin dysregulated (ID, n=7) and horses with T60 insulin ≤45 µU/mL were classified as non-insulin dysregulated (non-ID, n=7)[84]. Demographics, morphometrics, and endocrine screening results of horses used in the study are described in Table 4.1.

Based on sample size of similar groups in cohort studies determining short-term transportation stress responses in horses (n=6) [79,115], a sample size of n=7 for each group was considered sufficiently powered to determine endocrine differences between ID and non-ID. This was confirmed through sample size calculation using differences in T60 post-OST insulin concentrations for ID and non-ID horses (effect size = 2.66) with $\alpha = 0.05$ and 80% power [85].

4.3.2 Study Design

This study was conducted in May of 2022 at the University of Kentucky's Department of Veterinary Science's North Farm Unit. Prior to and after transportation, horses were group housed on semi-dry lots with ad libitum access to water and supplemented with hay (non-structural carbohydrates, NSC < 12% Dry Matter, DM) and received 0.3% BW of a commercial complementary feed (McCauley's Alam ®: Digestible Energy 89% DM, NSC 12% DM) in the mornings to meet NRC Requirements of horses at maintenance [122]. Horses did not receive complementary feed prior to the 24 h pre-transportation OST, in the

morning on the day of transportation, or prior to 24 h post-transportation sampling. Additionally, horses did not have access to hay during transportation.

All 14 horses had previous experience being transported but had not been transported within 14 days prior to the start of the study. Horses were transported in a livestock trailer over 4 different days within a two-week period. Three to four horses from the same herd were transported per trip with a combination of both ID and non-ID horses on each day randomly assigned to their position on the trailer. The same driver drove a round-trip route of with a mean duration of 75.5 minutes (SD ± 6.4 min) over a distance of 88.5 km of mostly highway departing from and arriving back to the same location. All horses were tied to the left side of the trailer at eye level and a middle divider separated the front and back half of the trailer. Horses were group housed in a paddock following transportation for sample collections and then returned to their normal pastures.

4.3.2.1 Blood and Saliva Collection

Blood was collected via jugular venipuncture on fasted horses 24 hours before the day of transportation (starting at 0800 h). On the day of transportation samples were collected: 1 h before loading (0800 h), directly after horses were unloaded from the trailer (1020 h), plus 15 min (1035 h), 1 h (1120 h), and 3 h (1320 h) after unloading from the trailer (Figure 1). All samples were collected within 10 minutes of the designated sample time points. Additionally, an OST was performed at the 24 h pre transportation and the 3 h post transportation time points with the T60 collected an hour later (at ~ 0900 h and ~1420 h post-respectively). The final blood collection was 24 h after transportation (0800 h) the following day. Blood was collected into serum and EDTA vacutainer tubes and either transported to Rood and Riddle Equine Hospital (Lexington, KY) for hematology

(complete blood count, CBC) and biochemistry analysis or centrifuged at 800 g x 10 minutes, aliquoted, and frozen at -20°C prior to being analyzed for plasma glucose, serum insulin and serum cortisol using methods described below.

Saliva samples were collected at the same time points immediately after the blood had been collected using cotton swabs (SalivaBio Oral Swab, Salimetrics) held with a hemostat and placed under the horses' tongue for 60 seconds [115]. After being fully saturated with saliva, the cotton swab was returned to its labelled polypropylene tube and then stored at 4°C until centrifugation at 2000 x g for 5 minutes. Samples were then stored at -20°C until further analysis for cortisol and insulin.

4.3.2.2 Heart rate and rectal temperature

Rectal temperature was measured with a digital thermometer in degrees Celsius (Neogen) 24 h pre-transport, 1 h prior to being transported, 4 hours post-transport and 24 h post-transport. Ambient temperatures were also recorded at the time horses were loaded onto the trailer.

Heart rate was determined using Polar H10 Equine Heart Rate Monitor (Polar Electro Oy). Polar Equine heart rate belts were attached around the heart girth of each horse according to manufacturer instructions. The Polar H10 hear rate sensor was then attached and paired to the Polar Beat application on a Bluetooth capable device, with each horse having its own sensor and device. Heart rate data was recorded using the Polar H10 heart rate sensor for one minute at 24 h pre- and post-transport and on the day of transport starting 1 hour prior to transport continuously until 4 hours post-transport. The heart rate belt being removed following the final blood and saliva collection. Data was recorded at 1-minute

intervals and exported from Polar Diary to Microsoft Excel and then averaged over 10-minute intervals and when horses were loaded and unloaded from the trailer.

4.3.3 Blood and Salivary Assays

4.3.3.1 CBC and chemistry analysis

Peripheral blood samples taken at 1 h pre-, 1 h post- and 24 h post-transportation were analyzed at the Rood and Riddle Equine Hospital Laboratory (Lexington, KY) for CBC (Beckman Coulter ACT Diff) and serum biochemistry (Beckman Coulter AU480) as a baseline health assessment and to determine any changes associated with transportation.

4.3.3.2 Serum cortisol, serum insulin, and plasma glucose

Frozen serum and plasma samples were shipped on dry ice to Cornell University's Animal Health Diagnostic Center for analysis of serum cortisol using a chemiluminescent immunoassay system (IMMULITE® 2000; Siemens), previously validated for use in the horse [152], plasma glucose using an automated chemistry analyzer (Roche Cobra c501), and insulin via a commercially available human insulin radioimmunoassay (EMD Millipore Corp), validated for use with equine serum [153].

4.3.3.3 Salivary cortisol and insulin

Salivary cortisol analysis was performed in duplicate using an enzyme-linked immunoassay (ELISA; Salimetrics, State College PA) previously validated for use in the horse [128]. With mean inter-assay coefficient of variation (CV) of 6.4% and intra-assay CV of 9.4%.

For salivary insulin analysis, frozen saliva samples were shipped on dry ice to Salimetrics LLC (State College, PA) and ran in duplicate using a human enzyme

immunoassay (EIA) with mean inter-assay CV of 4.7%, intra-assay CV of 4.2%, and sensitivity of 7.5 pg/mL.

4.3.4 Data analysis

Statistical analysis was performed using GraphPad Prism (version 9.5.1). Unpaired t-test were used to determine pre-transport differences in mean age, weight, basal insulin and post-OST insulin between ID and non-ID horses while Mann-Whitney U tests were used to determine differences in median BCS and CNS with a Fisher's exact for distribution of breeds. Repeated measures ANOVA, with horse as random effect and time of sample collection and insulin status as fixed effects, was used to evaluate the effect of transportation (pre- versus post-transportation) and differences between ID and non-ID horses (ID status) for rectal temperature, heart rate, CBC/Chemistry analytes, serum cortisol, salivary cortisol, plasma glucose, serum insulin, and salivary insulin. Data that did not meet normal assumptions, through Q-Q plots and Shapiro Wilk's test for normality, were log transformed and confirmed to meet normality through linear Q-Q plots. Multiple comparisons were performed using Bonferroni post-hoc analysis. Simple linear regression and Pearson correlation coefficients were calculated between serum x salivary cortisol and serum x salivary insulin concentrations. Statistical significance was considered at $P<0.05$.

4.4 Results

There were no adverse responses to transportation and all horses loaded and unloaded from the trailer uneventfully.

4.4.1 Serum and salivary insulin

The ID horses overall had higher basal serum insulin concentrations compared to non-ID horses ($P<0.0001$) with an effect of transportation ($P=0.02$, Figure 4.2). Specifically,

ID horses had higher serum insulin than non-ID horses at all time points except unloading and 3 h post transportation when there was greater individual animal variability. There was also an effect of transportation (P=0.01) and insulin status (P<0.0001) on the serum insulin response to the OST both pre- and post-transportation (Figure 4.3). In ID horses, as expected the insulin had increased at the T60 following the OST both pre- (mean = 74.5 µU/mL vs. 154.6 µU/mL, P=0.04, 95% CI for mean difference = [175.8,16.5 µU/mL]) and post-transportation (mean = 137.1 µU/mL vs. 284.6 µU/mL, P=0.03, 95% CI for mean difference = -285.4,-9.7 µU/mL], Figure 4.3). Non-ID horses had a mean (95% CI) OST T60 serum insulin of 56.6 µU/mL (29.1-84.1 µU/mL) post-transportation with 5/7 non-ID horses' insulins above the cutoff for ID diagnosis of 45 µU/mL 60 min following OST [84].

Overall salivary insulin was also higher in ID versus non-ID horses (P=0.001) with no effect of transportation (Figure 4.4). There was also an effect of transportation (P=0.02) and insulin status (P=0.03) on salivary insulin response to the OST (Figure 5). In ID horses, salivary insulin increased from OST T0 pre-transport to the T60 pre-transport (mean = 1089.7 pg/mL vs. 7007.5 pg/mL, P=0.03; 95% CI of differences = [-2.563,-0.1869 pg/mL]) as well as T60 post-transportation (mean = 1089.7 pg/mL vs. 4672.8 pg/mL, P=0.04; 95% CI of differences = [-2.840,-0.1442 pg/mL]).

There was no correlation between serum and salivary insulin when comparing all samples collected (R=0.14, data not shown) or when comparing serum and salivary insulin in response to OST (R=0.47, data not shown).

4.4.2 Serum and salivary cortisol

Serum and salivary cortisol both increased in response to transportation (P<0.0001), with no differences between ID and non-ID horses (Figure 4.6). Serum cortisol concentrations were highest at unloading (mean, 95% CI = ID: 10.9 ug/dL, 8.8-13.06 ug/dL; non-ID: 10.7 ug/dL, 4.4-8.0 ug/dL) and were increased from 24 h pre concentrations at unloading (mean = 5.6 ug/dL vs. 10.8 ug/dL, P<0.0001, 95% CI for mean difference = [-6.8,-3.7 ug/dL]), 15m post (mean = 5.6 ug/dL vs. 9.2 ug/dL, P<0.0001, 95% CI for mean difference = [-5.0,-2.2 ug/dL]), and 1 h post transportation (mean = 5.6 ug/dL vs. 10.8 ug/dL, P=0.01, 95% CI for mean difference = [-2.6,-0.3 ug/dL]; Figure 4.6A). Serum cortisol decreased from 24 h pre concentrations at the OST T60 the day before transport (mean = 5.6 ug/dL vs. 3.7 ug/dL, P=0.0001, 95% CI for mean difference = [1.0, 2.9 ug/dL]), as well as 3 h post-transportation (mean = 5.6 ug/dL vs. 2.3 ug/dL, P=0.0001, 95% CI for mean difference = 1.7,3.9 ug/dL]) and 4 h post/T60 OST the day of transport (mean = 5.6 ug/dL vs. 2.0 ug/dL, P=0.0001, 95% CI for mean difference = [2.5,4,7 ug/dL], Figure 4.6A).

Salivary cortisol was increased from 24 h pre concentrations at unloading (P<0.0001), 15m post (P<0.0001), and 1 h post (P=0.001) transportation (Figure 4.6B), with the highest concentrations being at unloading in ID horses (mean, 95% CI = 0.49 ug/dL, 0.25-0.72 ug/dL), and at 15 min post-transportation in non-ID horses (mean, 95% CI = 0.45 ug/dL, 0.25-0.52 ug/dL), although the concentrations were very similar in both groups. A positive correlation was found between serum and salivary cortisol concentrations (r = 0.72, Supplementary File 4.1).

4.4.3 Plasma glucose

Plasma glucose concentrations were increased post-transportation (P=0.0003; Figure 4.7), with this effect observed in both the ID and Non-ID horses. Plasma glucose was increased from 24 h pre concentrations at 1 h post-transportation (mean = 102.0 mg/dL vs. 123.0 mg/dL, P=0.02, 95% CI for mean difference = [-37.48, -4.524 mg/dL]).

4.4.4 Rectal temperature and heart rate

Neither transportation nor ID status any association with rectal temperatures of horses before or after short-term transportation (Supplementary File 4.2). Ambient temperatures ranged from 16.6°C- 31.1°C.

There was an effect of transportation on heart rate (P<0.0001); however, there were no differences in heart rate between ID and non-ID horses (Figure 4.8). Mean heart rates were lowest prior to loading (mean, 95% CI = ID: 41.5 bpm, 35.8-47.2 bpm; non-ID: 39.2 bpm, 36.7-41.3 bpm), and highest at loading (mean, 95% CI = ID: 97.2 bpm, 70.9-123.4 bpm, non-ID: 93.0 bpm, 66.7-119.3 bpm), remained elevated from pre-transportation and increased again at unloading (mean, 95% CI = ID: 78.2 bpm, 63.9-92.5 bpm, non-ID: 79.3 bpm, 61.5-97.1 bpm).

4.4.5 CBC/Chemistry

Overall, this study found minor changes in CBC and serum biochemistry results. There was no influence of transportation or ID status on most serum chemistry parameters, and all were within the normal equine reference range (data not shown). ID status did not impact any of the CBC parameters measured ;however, there were slight increases in white blood cell (WBC) count, Neutrophil to Lymphocyte (N:L) ratio, Serum Glutamic

Oxaloacetic Transaminase (SGOT) / Aspartate Aminotransferase (AST), Creatine Kinase (CK) and Sorbitol Dehydrogenase (SDH) 1 h post-transportation (Supplementary File 4.3).

4.5 Discussion

Short-term transportation produced a similar stress response, as measured by cortisol concentrations and heart rate, but resulted in different insulin responses in horses with and without insulin dysregulation.

Overall, the hypothesis was supported with insulin concentrations being higher in ID versus non-ID horses pre-transport, post-OST, and at the majority of time points post-transportation, but not at unloading and with poor agreement between serum and saliva concentrations. It is understood that corticosteroids antagonize the effects of insulin [90,154], and therefore with the increase in cortisol it may have been expected that insulin concentrations would have decreased. However, this might not be the case in horses, especially those with metabolic disorders such as insulin dysregulation, when they experience acute stress. Serum insulin has previously been reported to increase post-transportation in both EMS and non-EMS aged (>20 years) horses [92,115]. While the current study observed that ID horses had increased serum insulin concentrations versus non-ID horses, there was no obvious increase in basal insulin post-transportation. However, in another study, horses classified as EMS had increased serum insulin concentrations following transport compared with pre-transport [92]. While we did not detect differences in insulin pre- and post-transportation in ID horses in the current study, this could be due to the timing of blood collection following transportation, differences in selection criteria for ID and EMS horses between the studies, or because horses in the current study did not have access to forage during transportation. In addition to

transportation, stress can be associated with confinement and hospitalization, influencing insulin responses as displayed through decreased insulin tissue sensitivity in horses [91,155]. This indicates that insulin response to stress in horses can be influenced by various stressful events or changes in environment. In the current study horses returned to their resident pastures following transportation, but it is possible that insulin responses to stress could be heightened if transporting to a novel environment, such as a veterinary clinic. Therefore, further research should focus on evaluating how stress influences insulin responses in ID horses, and implementing monitoring strategies during events that may elevate insulin levels.

Importantly, transportation altered the insulin responses to dynamic testing for insulin dysregulation post-transportation, with changes in serum insulin concentrations in both ID and non-ID horses. Most notably, 5/7 non-ID horses had post-transportation OST T60 serum insulin above the recommended cutoff range [84]. Also concerning, some ID horses had OST T60 insulins greater than 2-fold higher than their pre-transportation OST T60. While insulin concentrations correlate with the severity of laminitis as determined by radiograph findings [156], it remains unclear whether specific insulin levels or durations trigger hyperinsulinemia-associated laminitis (HAL). Therefore, special care should be given when transporting horses with ID, especially those with a history of HAL.

It is currently unclear from this study if salivary insulin could be used as a non-invasive diagnostic tool for detection of insulin dysregulation in horses. Differences between ID and non-ID horses were able to be detected in saliva, but results were not consistent with serum concentrations. Differences in salivary insulin can be detected in humans following a low versus high carbohydrate meal [32] and strong correlations between serum and salivary

insulin have been found in humans, with a 30-minute delay between peak serum insulin and salivary insulin following an oral glucose tolerance test [33,157]**.** Since only a T60 post-OST sample was taken in this study, it is possible the peak salivary insulin concentration was missed. Recently, salivary insulin was also able to be detected using an automated assay in the UK with low inter- and intra-assay variability; however, like our study, they did not find a correlation with serum insulin collected at the same time points [36]. The discrepancies found could be due to differences in timing of sampling between serum and saliva, differences in assays, antibodies, and/or magnitude being measured. Additionally, collection methods could have contributed to differences in salivary insulin concentrations. The use of salivary insulin in diagnostic testing for ID in horses would be extremely beneficial to veterinarians and horse owners by being less invasive due to not needing a blood collection; however, further research is needed to determine the best sampling protocols, timing, and assays to measure equine salivary insulin.

Stress associated with short-term transportation is corroborated by the increase in serum and salivary cortisol seen post-transport. Serum cortisol was highest immediately after unloading with a gradual decline; however, salivary cortisol remained elevated at 15 min post-transportation in both ID and non-ID horses. Additionally, serum cortisol decreased at pre-transport T60 OST, 3- and 4-hours following transportation compared with 24 h pre-transport, potentially due to being brought into paddocks away from their normal pasture 24 h pre-transportation and a decreased stress response after returning to their familiar environment with herd mates following transportation. It is possible that although correlated with serum cortisol, salivary cortisol has both a delayed response and is less sensitive to minor changes in stress versus serum due to smaller concentrations being

measured [158]. Both serum and salivary cortisol returned to or decreased from pre-transport concentrations by 3 hours post-transport, consistent with previous studies transporting horses' short distances [7,115]. The lack of differences in serum or salivary cortisol between ID and non-ID horses could be explained by the fact that total cortisol was measured, which is not different between ID and non-ID horses, rather than free cortisol that has been reported to be increased in horses with endocrinopathies such as ID and PPID [97,143]. It should be noted that horses used in this study were selected based on having been previously transported without issue and traveled with their herd mates. While this was done to reduce any additional added stressors in the study, these responses could be different for horses who have not been transported before, traveling alone, or with unfamiliar horses [6,159].

Blood glucose increases in response to transportation stress in horses [9]. Increased cortisol drives gluconeogenesis thus increasing blood glucose under stressful conditions to increase energy availability to the horse. In addition to the current study, hyperglycemia has been observed following both long and short-distance transportation [2,9,89] and following the combination of post-transportation and exercise when compared to exercise alone [89]. However, the lack of differences in glucose between ID and non-ID horses observed suggest that the increase in glucose is not the main driver of increased insulin post-transportation.

Short-distance transportation, as illustrated in this study, does not seem to impact rectal temperatures when ambient temperatures were not extreme. While this study took place in late spring (May), it has also been shown that rectal temperatures of aged and young horses were not changed 24 h post-transportation in the fall (October/November)[115]. However,

it is unknown how short-term transportation would impact rectal temperature in extreme heat or cold and it has been well documented that increasing trip length can increase incidence of shipping fever post-transportation [59,160].

Stimulation of the HPA axis can be monitored through changes in stress response before, during, and after transportation. However, unlike previous studies in EMS horses we did not see elevated heart rates in ID horses [148]. Heart rate for both ID and non-ID horses was highest at the time of loading in agreement with previous research, where the highest heart rates have been found at loading regardless of trip length [7,40,139]. However, it is possible that increases in heart rate reflect horse movement getting on and off the trailer or maintaining balance versus stress response alone [5]. While heart rate variability (HRV) is a more accurate measure of stress [124], unfortunately for technical reasons we were not able to consistently measure HRV in this study. Although it is unknown if there would be differences between ID and non-ID horses.

While changes in CBC and serum chemistry parameters have been documented in horses following long-distance (8+ hours) transport, [8,147] there were no differences in immune parameters between ID and non-ID horses in response to short-term transportation stress in this study. The minor changes in immune cell counts for WBC and N:L ratio, observed in this study post-transportation, are possibly caused by the increase in cortisol and catecholamines in response to transportation stress [161].

Some limitations in this study include that only mares were used, as they were the population of horses available that fit the criteria for inclusion in the study. Although it is not believed that there would be sex differences in insulin responses measured [162], there could be some hormonal changes attributed to the stage at which a mare is in her estrous

cycle [163]. Additionally, the OSTs were performed a few hours after transportation and in the early afternoon which could have potentially augmented some of the insulin responses observed, as our pre-transportation OST were performed in the morning the day prior to avoid any possible interference with the effects of transportation. However, this timing was chosen to both measure basal insulin responses post-transportation and allow for immediate stress to be resolved, as cortisol can remain elevated for up to an hour after short-term transportation [79,115]. Although these horses were turned out on semi-dry lot paddocks presumed to be low in NSC, the pasture was not directly tested at time of sample collections, and therefore some changes in diet post-transportation could have influenced OST results. Further studies should evaluate various time points post-transportation for performing an OST to determine the best timing of diagnostic testing if transporting to a veterinary clinic. Also, while OST insulin results have been shown to be fairly repeatable when performed within 7 days [164], it is less clear what the repeatability is when OSTs are performed within 24 hours, or at different times of day.

4.6 Conclusion

Short-term transportation could be stressful to horses regardless of their ID status. ID horses have increased insulin responses to an OST both pre- and post-transportation compared with non-ID horses. In some individuals the insulin response to the OST post transportation may be even more augmented, indicating that ID horses with a history of hyperinsulinemia-associated laminitis should be more closely monitored post-transportation. Performing dynamic endocrine testing, such as an OST, post-transportation could potentially result in misleading ID status categorization in non-ID horses. Further work is needed to clarify exactly when is the best time to perform endocrine testing for ID

diagnosis following transportation for ID diagnosis. Salivary insulin might be a future tool for ID diagnosis in horses, but it does not appear to correlate with serum insulin as measured in this study.

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Table 4.1 Horse demographics, morphometrics, and endocrine screening of insulin dysregulated (ID) and non-insulin dysregulated (Non-ID) horses prior to transportation.

Parameter	ID	Non-ID	P-value
Breeds	1 TB, 5 QH, 1 QHX	1 TB, 3 QH, 2 QHX, 1 TWH	0.6
Age (years)	14.6 ± 3.8	13.3 ± 5.6	0.6
BW (kg)	562.8 ± 38.6	547.9 ± 57.3	0.6
BCS	7 (5-8)	6 (4-7)	0.08
CNS	2 (1-3)	2 (1-3)	0.8
Insulin OST T0 (µU/mL)	32.9 ± 10.3	14.1 ± 8.3	**0.003**
Insulin OST T60 (µU/mL)	106.7 ± 12.5	28.5 ± 7.8	**<0.0001**
ACTH TRH T0 (pg/mL)	17.3 ± 5.5	20.25 ± 12.4	0.6
ACTH TRH T10 (pg/mL)	67.8 ± 28.4	41.3 ± 19.5	0.06

Values for age, body weight (BW), oral sugar test (OST) T0 and T60 insulin, and thyrotropin releasing hormone (TRH) stimulation test T0 and T10 adrenocorticotropin hormone (ACTH) are presented as means ± standard deviations; body condition score (BCS) and cresty neck score (CNS) are presented as median (range); breeds are listed by distribution. P-values represent statistical differences between ID and non-ID horses within a row.

Abbreviations: TB, Thoroughbred; QH, Quarter Horse; QHX, Quarter Horse cross; TWH, Tennessee Walking Horse.

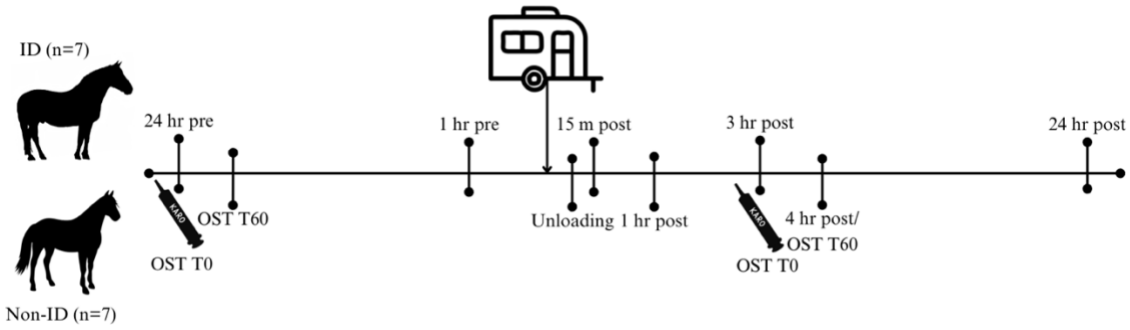

Figure 4.1 Timeline of blood/saliva collections and oral sugar tests (OST) in relation to transportation (pre- and post-) of insulin dysregulated (ID) and non-insulin dysregulated (Non-ID) horses.

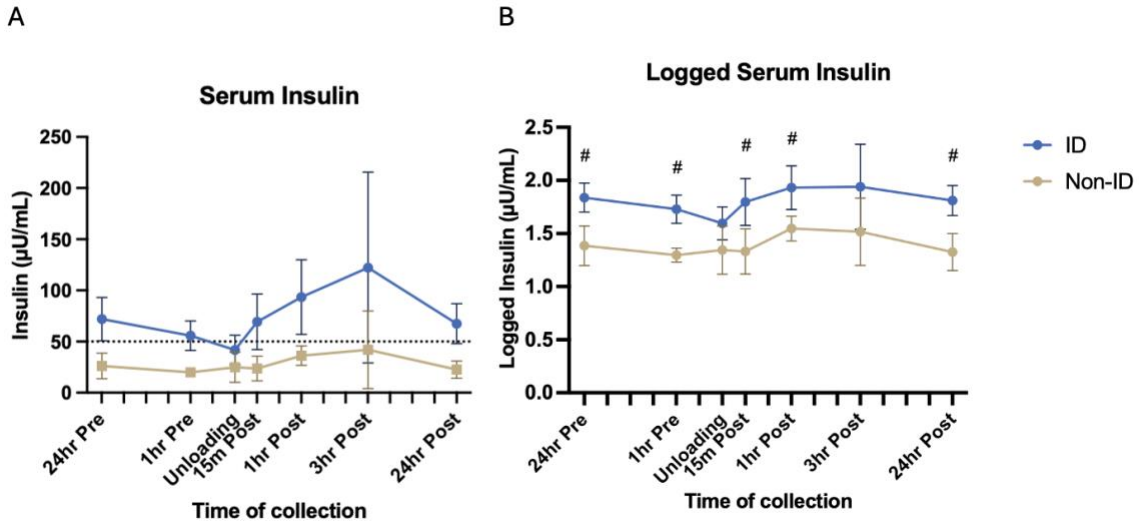

Figure 4.2 Serum insulin (A) and logged serum insulin (B) of insulin dysregulated (ID) and non-insulin dysregulated (Non-ID) horses before and after short-term transportation.

The data are presented as the mean with 95% confidence interval. Dashed line represents diagnostic cutoff for basal insulin (50 µU/mL). Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was an effect of transportation (P=0.02) and insulin status (P<0.0001), but no transportation x time interaction for serum insulin. Differences in serum insulin between ID and non-ID horses at a specific time are denoted as #, P<0.05.

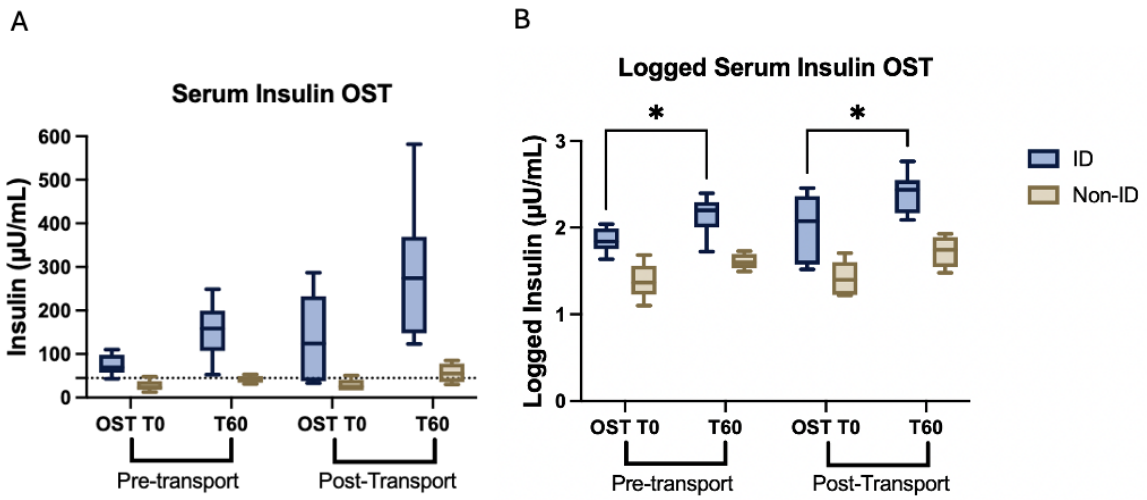

Figure 4.3 Serum insulin (A) and logged serum insulin (B) of insulin dysregulated (ID) and non-insulin dysregulated (non-ID) horses in response to oral sugar test (OST) performed pre- and post-transportation.

The data are presented as the median and IQR with min and max. Dashed line represents diagnostic cutoff for OST T60 insulin (45 µU/mL). Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was an effect of transportation ($P=0.01$) and insulin status (A: $P<0.0001$), but no transportation x insulin status interaction. Differences between T0 and T60 in ID horses are denoted as *, $P<0.05$.

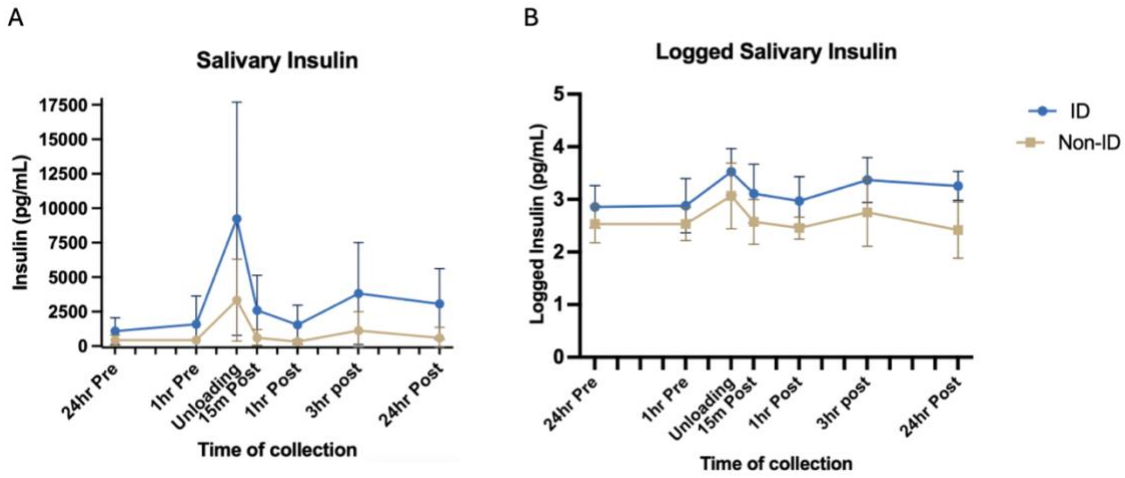

Figure 4.4 Salivary insulin (A) and logged salivary insulin (B) of insulin dysregulated (ID) and non-insulin dysregulated (Non-ID) horses before and after short-term transportation.

The data are presented as the mean with 95% confidence interval. Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was an effect of insulin status (P=0.001), but no transportation effect or transportation x insulin status interaction.

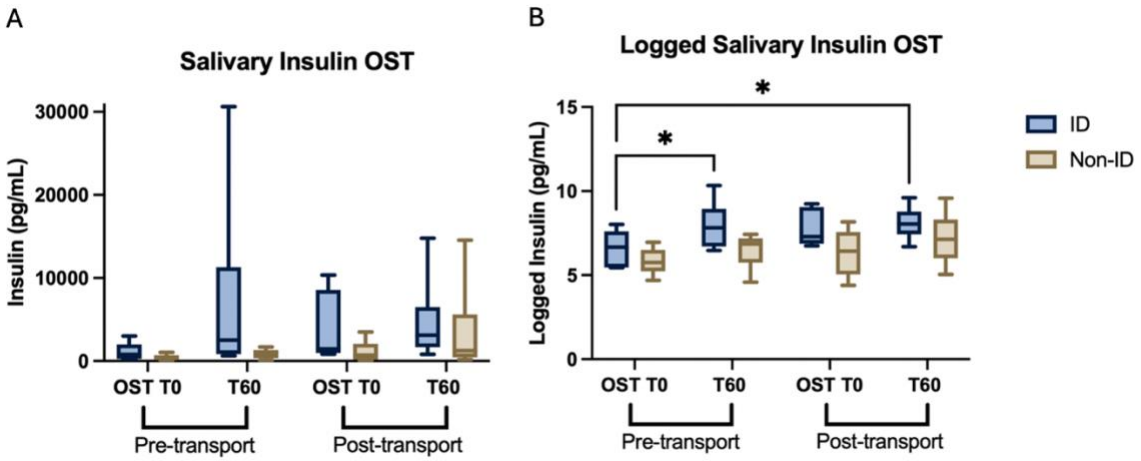

Figure 4.5 Salivary insulin (A) and logged salivary insulin (B) of insulin dysregulated (ID) and non-insulin dysregulated (non-ID) horses in response to oral sugar test (OST) performed pre- and post-transportation.

The data are presented as the median and IQR with min and max. Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was an effect of transportation ($P=0.02$) and insulin status ($P=0.03$), but no transportation x insulin status interaction. Differences between T0 and T60 in ID horses are denoted as *, $P<0.05$.

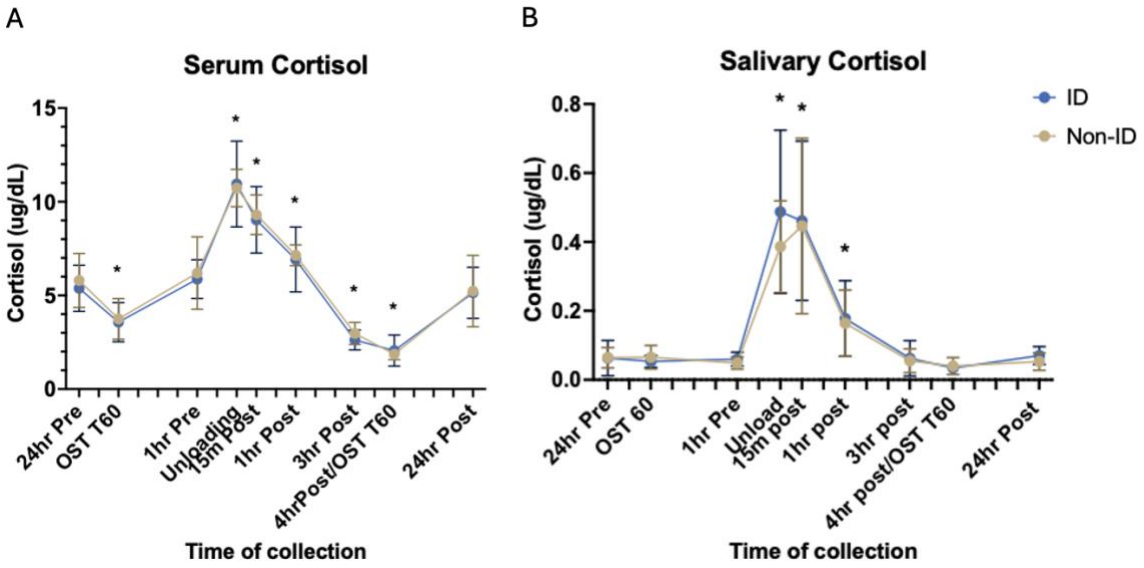

Figure 4.6 Serum cortisol (A) and salivary cortisol (B) of insulin dysregulated (ID) and non-insulin dysregulated (Non-ID) horses before and after short-term transportation.

The data are presented as the mean with 95% confidence interval. Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was an effect of transportation ($P<0.0001$), but no effect of insulin status or transportation x insulin status interaction. Transportation effect differences in relation to 24 hr pre are denoted as *, $P<0.05$.

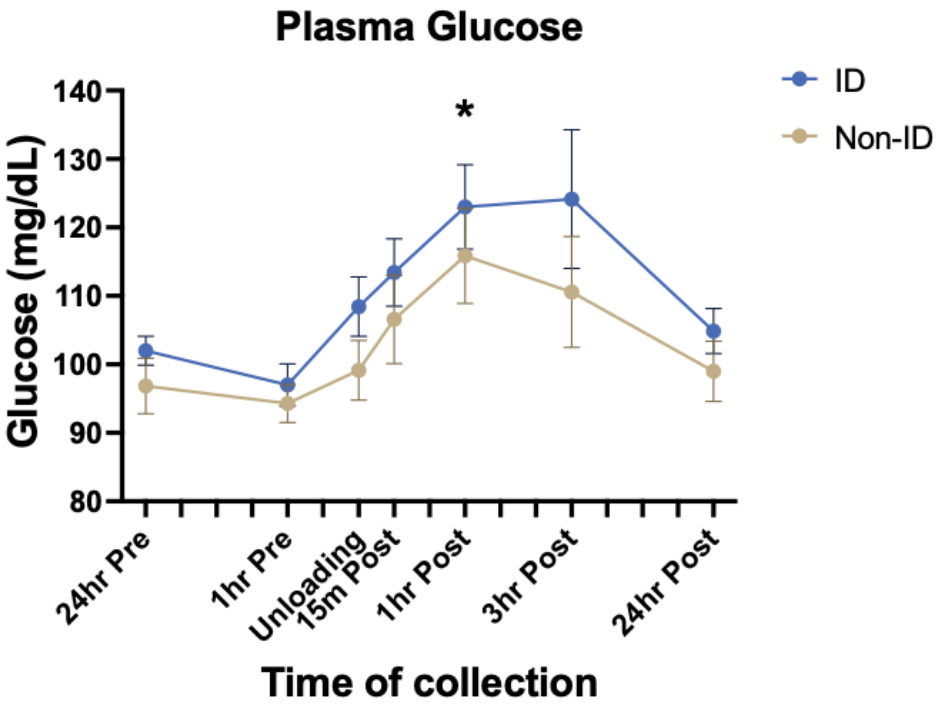

Figure 4.7 Plasma glucose of insulin dysregulated (ID) and non-insulin dysregulated (Non-ID) horses before and after short-term transportation

The data are presented as the mean with 95% confidence interval. Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was an effect of transportation ($P=0.0003$), but no effect of insulin status or transportation x insulin status interaction. Transportation effect differences in relation to 24 hr pre are denoted as *, $P<0.05$.

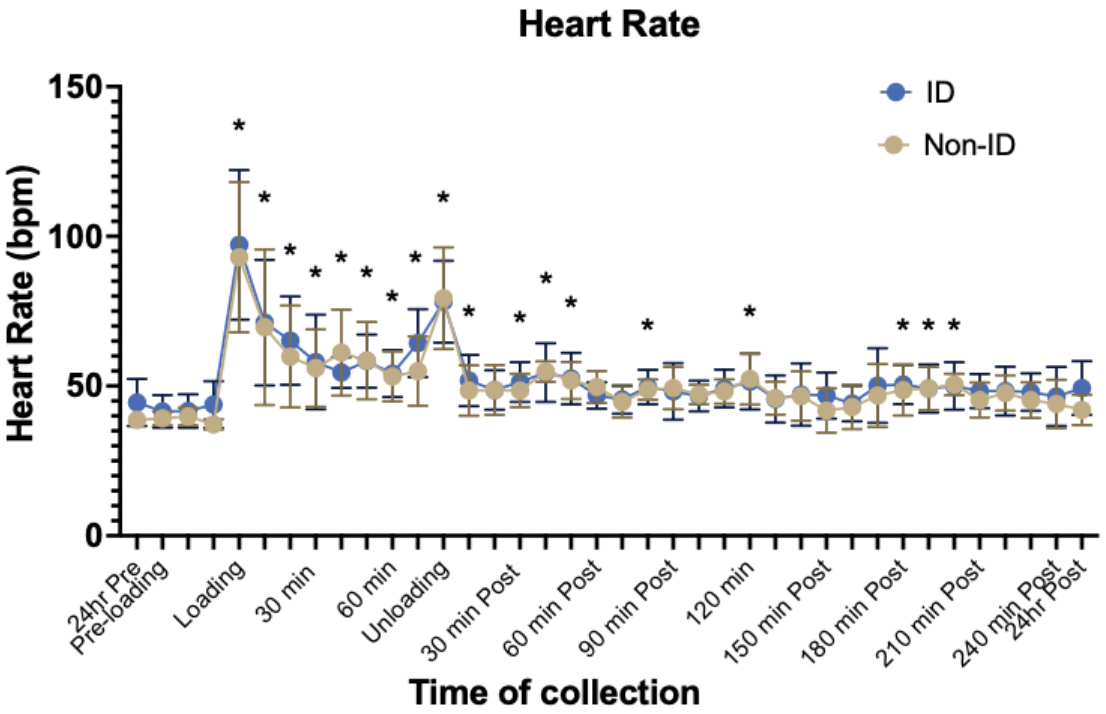

Figure 4.8 Heart rates before, during, and after short-term transportation of insulin dysregulated (ID) and non-insulin dysregulated (Non-ID) horses.

The data are presented as the mean with 95% confidence interval. Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was an effect of transportation ($P<0.0001$), but no effect of insulin status or transportation x insulin status interaction. Main effect differences over time in relation to 24 hr pre are denoted as *, $P<0.05$.

CHAPTER 5 AN EVALUATION OF STRESS RESPONSES TO SHORT-DURATION TRANSPORTATION IN HORSES WITH AND WITHOUT PARS PITUITARY INTERMEDIA DYSFUNCTION (PPID).

5.1 Summary

Background: It is unknown how transportation stress may impact horses with PPID.

Objectives: To determine differences in endocrine responses to short duration transportation stress in horses with and without PPID.

Study Design: Longitudinal cohort study.

Methods: Fifteen age-matched, adult, mixed breed horses were transported ~1.5 hours round trip by road. Horses were grouped by PPID status using ACTH 10 minutes (T10) following thyrotropin releasing hormone (TRH) stimulation test and clinical signs of disease. The initial study consisted of 6 non-PPID (T10 TRH ≤200 pg/mL) and 6 PPID (T10 TRH 201-1200 pg/mL) horses, with a supplementary group of 3 additional PPID horses (PPID:A; T10 TRH >1200 pg/mL). For both studies, blood and saliva samples were collected before and after transportation (1 h pre-transportation, after unloading, 15 min, 30 min, 1 h, 2 h, and 24 h post-transportation). Additionally, heart rates, rectal temperatures and respiratory rates were monitored. Plasma ACTH, serum insulin, serum cortisol, and salivary cortisol concentrations were measured using validated assays. Data were analyzed using repeated measures ANOVA, transforming any non-normal data, with Bonferroni adjustments for multiple comparisons.

Results: In the initial study, PPID horses had higher mean ACTH concentrations compared with non-PPID horses at unloading (mean = 93.5 pg/mL vs 40.1 pg/mL, P<0.01; 95% CI for mean difference = [-267.3,160.5 pg/mL]) through 30 minutes post-transportation (mean = 504. pg/mL vs 23.2 pg/mL, P=0.03; 95% CI for mean difference = [-241.1,186.7

pg/mL]). However, 2/6 non-PPID horses had plasma ACTH above the diagnostic cutoff for PPID (> 40 pg/mL) at unloading. Serum and salivary cortisol were increased following transportation in PPID and non-PPID horses of the initial study, with a return to pre-transportation levels by 2 hours. Conversely, in the supplementary group (PPID:A) ACTH remained elevated, with no increase in cortisol in response to transportation. There were no differences in serum insulin concentrations based on PPID status or following transportation in either study.

Main limitations: Small number of horses, supplemental study conducted in fall.

Conclusions: Short-duration transportation may alter diagnostic testing for PPID in non-PPID horses.

Keywords: horse, transportation, stress, PPID

5.2 Introduction

Pituitary pars intermedia dysfunction (PPID) is the most common endocrinopathy in aged horses [60], with a large study finding it present in more than 20% of horses over the age of 15 years [93]. Horses with PPID have altered hormone responses due to hyperplasia of the pituitary pars intermedia that causes an excessive production of proopiomelanocortin (POMC) derived hormones [96,165]. This can be measured in the blood due to the increase in basal adrenocorticotropic hormone (ACTH) as well as increased ACTH in response to dynamic diagnostic testing, such as a thyrotropin releasing hormone (TRH) stimulation test [95,96]. There is a spectrum of clinical signs for horses with PPID, including abnormal hair coat changes, specifically hypertrichosis or delayed shedding, and muscle atrophy, loss of topline muscling, along with chronic infections, infertility and laminitis in some cases [96]. The diagnosis of PPID relies on a combination of clinical signs and ACTH concentrations,

as various factors can contribute to increased ACTH concentrations such as season, diet, sedation, and stress that can interfere with PPID diagnostic results [103,105,106,108].

Short-term transportation has been associated with an increase in stress-related hormones, such as ACTH and cortisol, in aged (>15 years), non-PPID horses [79,115]. It is currently not recommended to collect blood to determine ACTH concentrations for the use of PPID diagnosis within 30 minutes after trailering as the stress response to transportation of 45 minutes has been shown to cause false positive results in some non-PPID horses due to elevated ACTH post-transportation [107,112]. However, it is unclear how the acute stress from short-duration transportation would impact stress hormones and/or diagnostic testing of horses with PPID. Therefore, the objective of this study was to evaluate the stress response of horses with and without PPID to short-duration road transportation. It was hypothesized that PPID horses would have altered stress responses following short-duration transportation compared with non-PPID horses due to the dysregulation in pituitary hormones.

5.3 Materials and Methods

This study was approved by the University of Kentucky's Institutional Animal Care and Use Committee (protocol #2021-3854 and #2023-4340).

5.3.1 Horses

Initially, twelve idle non-pregnant mares of mixed light breeds from the University of Kentucky's Department of Veterinary Science research herd were enrolled in the study. Approximately one month prior to horses being transported (April 2023), body condition score (BCS, 1-9), cresty neck score (CNS, 0-5), hair score (HS,1-3) and muscle atrophy score (MAS) were recorded by 2 trained personnel and averaged [120,121,166,167].

Additionally, bodyweight was measured in kilograms with a calibrated, portable, digital weigh bridge (model 700, True Test Inc).

During this period, horses underwent dynamic testing for insulin dysregulation and PPID. Horses were tested in their resident pastures in the morning prior to receiving complementary feed. An oral sugar test (OST) was performed on grain fasted horses in the morning by collecting a blood sample (T0) via jugular venipuncture using a vacutainer needle followed by oral administration of 0.15 ml/kg of Karo Light Corn Syrup (AHC Food Companies Inc., Chicago, IL, USA) and collection of a second blood sample via jugular venipuncture 60 minutes later (T60). All horses enrolled in this study had basal insulin <50 μU/mL and/or T60 post-OST insulin concentrations <60 μU/mL (Tosoh 360 AIA) with no other clinical signs of equine metabolic syndrome (EMS) at the time of the study [80].

In the same week, but on a separate day from OST, horses were screened for PPID using a thyrotropin-releasing hormone (TRH) test performed on a separate day from the OST. During the TRH simulation test, peripheral blood was collected prior (T0) to intravenous administration of 1 mg TRH (Sigma-Aldrich), followed by blood collection 10 minutes post-injection (T10).

Horses were grouped by PPID status as non-PPID (T10 ACTH ≤200 pg/mL, no signs of hypertrichosis; n=6) and 6 PPID (T10 ACTH 201-1200 pg/mL, HS ≥1/3; n=6) based on recommended diagnostic cutoff values (post-TRH T10 ACTH; IMMULITE® 2000) and presence of hypertrichosis [107].

Following the initial study, it was discovered that the horse with the highest plasma ACTH concentrations in the PPID group also had the lowest serum cortisol values (Figure 5.1). To explore whether this pattern was unique to this horse or related to the elevated

circulating ACTH, a supplementary pilot study was conducted with 3 additional PPID horses (PPID:A) that were available and suitable for transportation (October 2023). These horses underwent the same body measurements and endocrine screening and had markedly elevated T10 post-TRH ACTH >1200 pg/mL, along with other clinical signs including hypertrichosis and muscle atrophy. However, it should be noted this supplementary study was conducted in the fall, when ACTH concentrations are known to be elevated [103,168,169].

Ages, body morphometrics, and endocrine screening of all horses prior to transportation are described in Table 5.1.

Hematology (CBC; Beckman Coulter ACT Diff) and serum biochemistry (Beckman Coulter AU480) was performed at Rood and Riddle Equine Hospital (Lexington, KY) for all animals prior to the start of the study to confirm all horses were otherwise clinically normal outside of PPID/ID diagnosis prior to being transported.

A priori sample size was calculated with a significance level (α) of 0.05, Type II error rate (β) or 0.2, and desired power of 90%. Estimated basal ACTH differences between PPID (mean =197.3 ± 78.9 pg/mL) and non-PPID horses (mean =54.6 pg/mL) were used [97], and the sample size was determined to be 6 horses per group [170]. It should be noted that there is a lack power in the supplementary group of PPID horses (PPID:A, n=3).

5.3.2 Study Design

The initial study was conducted in May of 2023, while the supplementary study took place in October 2023 at the University of Kentucky's Department of Veterinary Science's North Farm and Woodford Farm. Prior to and after transportation, horses were group housed on semi-dry lots with *ad libitum* access to water and supplemented with hay

(NSC < 12% DM) and 0.6% BW complementary commercial feed (NSC 12% DM) twice a day to meet NRC Requirements of horses at maintenance [122]. Horses had access to forage but did not receive feed prior to sample collection on the day of transport or 24 hours post-transportation and hay was not provided on the trailer during transportation. All horses had previous experience being transported but had not been transported within 7 days prior to the start of the study. Horses were transported in a livestock trailer and tied to the left side of the trailer at eye level and a middle divider separated the front and back half of the trailer, similar to previously reported [115]. In the initial study, 3 horses were transported per trip, with a combination of PPID and non-PPID horses from the same pasture. This was repeated over 4 different days within a two-week period. Whereas the supplementary group had 3 PPID horses transported on one day. The same driver completed a round-trip route averaging 1 hour and 13.2 minutes (SD ± 7 minutes), covering 88.5 km mostly on highways, starting and ending at the same location. After unloading from the trailer, horses were group housed in a paddock attached to their pasture for sample collections and were then returned to their resident pastures.

5.3.2.1 Rectal temperature, respiratory rate, and heart rate

Rectal temperature was measured with a digital thermometer in degrees Celsius (Neogen) 1 h prior to being transported and 24 h post-transportation. Directly after, respiratory rates were calculated by measuring breaths, rise and fall of abdomen, for 20 seconds and multiplying by 3 to obtain breaths per minute, and then recorded. Ambient temperatures were also recorded at the time horses were loaded onto the trailer.

Heart rate was monitored using Polar H10 Equine Heart Rate Monitor (Polar Electro Oy) as previously described [115]. Heart rate data collection was collected using the Polar

H10 heart rate sensor and began on the day of transport starting 1 hour prior to transport and then being continuously monitored until 2 hours post-transport, when the monitor was removed following the final sample collection. Heart rate was also recorded at 24 hours post-transportation for 10 minutes. Data was recorded at 1-minute intervals and exported from Polar Diary to Microsoft Excel and then averaged over 10-minute intervals, including when horses were loaded and unloaded from the trailer.

5.3.2.2 Blood and Saliva Collection

For both studies, on the day of transportation samples were collected: 1 h before loading (0800 h), directly after horses were unloaded from the trailer (1020 h), plus 15 min (1035 h), 30 min (1050 h), 1 h (1120 h), and 2 h (1220 h) after unloading from the trailer and 24 h (0800 h) post-transportation the day after. All samples were collected within 10 minutes of the designated sample time points. Blood was centrifuged at 800 g x 10 minutes, aliquoted, and frozen at -20°C prior to being analyzed for plasma ACTH, serum insulin, and serum cortisol using methods described below.

Saliva samples were collected at the same time points immediately after the blood had been collected using cotton swabs (SalivaBio Oral Swab, Salimetrics) held with a hemostat and placed under the horses' tongue for 60 seconds [115]. After being fully saturated with saliva, the cotton swab was returned to its labelled polypropylene tube and then stored at 4°C for a maximum of 6 hours until centrifugation at 2000 x g for 5 minutes. Samples were then stored at -20°C until further analysis for cortisol and insulin.

5.3.3 Blood and Salivary Assays

5.3.3.1 Serum cortisol, serum insulin, and plasma ACTH

Frozen serum and plasma samples were shipped on dry ice to Cornell University's Animal Health Diagnostic Center for analysis of serum cortisol, previously validated for use in the horse [126] and plasma ACTH, both using chemiluminescent immunoassay (CIA) system (IMMULITE® 2000; Siemens),

Plasma ACTH concentrations were validated for use on equine plasma samples with a commercially available human ACTH CIA (IMMULITE® 2000; Siemens, Seattle, WA, USA) at the University of Cornell's Animal Health Diagnostic Center Endocrinology Laboratory. Serial dilutions of 4 equine samples with assay buffer were parallel to the standard curve, and samples that were spiked with four different quantities of ACTH (Sigma, St. Louis, MO, USA) had observed concentrations that averaged 90% of expected. The manufacturer did not report the cross-reactivity of the CIA antibody for equine ACTH. The sensitivity of the assay, as reported by the manufacturer is 5 pg/mL. The mean intra- and inter-assay coefficients of variation were 3.8% and 5.0%, respectively.

Serum insulin was measured using an automated immunofluorescent assay (IRI Insulin, Tosoh AIA 360, Tosoh Bioscience) previously validated for use in the horse [171].

5.3.3.2 Salivary cortisol

Salivary cortisol analysis was performed in duplicate using an enzyme-linked immunoassay (ELISA; Salimetrics, State College PA) previously validated for use in the horse [128]. With a limit of detection <0.007 ug/dL, mean inter-assay coefficient of variation (CV) of 9.0%, and intra-assay CV of 14.3%.

5.3.4 Data analysis

Statistical analysis was performed using GraphPad Prism (version 10.2.2). One-way ANOVA was used to determine pre-transport differences in mean age, weight, basal insulin, post-OST T60 insulin, basal ACTH, and post-TRH ACTH; while Kruskal-Wallis tests were used to determine differences in median BCS, CNS, HS, and MAS between non-PPID, PPID and PPID:A groups. Repeated measures ANOVA, with horse as random effect and time of sample collection and PPID status as fixed effects, was used to evaluate the effect of transportation (pre- versus post-transportation) and differences between moderate PPID and non-PPID horses (PPID status) for rectal temperature, respiratory rate, heart rate, plasma ACTH, serum cortisol, salivary cortisol, and serum insulin. Data that did not meet normal assumptions, through Q-Q plots and/or Shapiro Wilk's test, were log transformed and confirmed to meet normality through linear Q-Q plots. Multiple comparisons were performed using Bonferroni (ANOVA) or Dunn's (Kruskal-Wallis) post-hoc analysis. Statistical significance was considered at $P<0.05$.

5.4 Results

Overall, the transportation of the horses was uneventful with minor incidents including 2 horses having to be reloaded onto the trailer and one horse from the PPID group experiencing herd separation anxiety before transportation, despite being transported with pasture mates, that resulted in slightly elevated pre-transportation ACTH and cortisol concentrations.

5.4.1 Plasma ACTH

ACTH concentrations were influenced by transportation ($P<0.001$) and PPID status ($P<0.0001$) with a transportation x PPID status interaction ($P=0.04$; Figure 5.2 and 5.3). In

the initial study, PPID horses had increased ACTH concentrations compared with non-PPID horses at unloading (mean = 93.5 pg/mL vs. 40.1 pg/mL, P=0.006, 95% CI for mean difference = [-267.3,160.5 pg/mL]), 15 min post (mean = 62.8 pg/mL vs. 28.0 pg/mL, P=0.007, 95% CI for mean difference = [-248,179.0 pg/mL]) and 30 minutes post-transportation (mean = 50.4 pg/mL vs. 23.2 pg/mL, P=0.03, 95% CI for mean difference = [-241.1, 186.7 pg/mL]). While ACTH peak mean and 95% CI were at highest unloading (PPID = 93.5 pg/mL [55.6,131.4 pg/mL], non-PPID = 40.1 pg/mL [28.8,51.4 pg/mL]), two non-PPID horses (n=6) had ACTH concentrations above the diagnostic cutoff for PPID (>40 pg/mL) at the time of unloading [107].

Additionally, ACTH concentrations for the supplementary group (PPID:A) horses were higher than PPID and non-PPID horses in the initial study at all time points (Figure 5.3). ACTH peak mean and 95% CI at 15 min post-transportation in the supplementary group (PPID:A = 893.3 pg/mL [135.4, 1651.3 pg/mL]); however, ACTH remained elevated with no differences between pre- and post-transportation.

5.4.2 Serum and salivary cortisol

Serum cortisol was impacted by transportation (P<0.001) and PPID status (P=0.003) with a transportation x PPID interaction (P<0.001; Figure 5.4). In the initial study, mean serum cortisol was increased compared with 1 h pre-transportation (non-PPID = 6.1 ug/dL, 95% CI = [4.3,7.9 ug/dL], PPID = 6.4 ug/dL, 95% CI = [3.6,9.2 ug/dL]) at unloading, (non-PPID = 11.6 ug/dL, P<0.001, 95% CI = [7.6,15.5 ug/dL], PPID = 10.2 ug/dL, P<0.001, 95% CI = [7.6,12.8 ug/dL]), 15 min (non-PPID = 9.6 ug/dL, P<0.001, 95% CI = [6.5,12.7 ug/dL], PPID = 8.7 ug/dL, P=0.03, 95% CI = [6.5,11.0 ug/dL]), and 30 minutes

post (non-PPID = 8.7 ug/dL, P=0.01, 95% CI = [6.2,11.4 ug/dL]]). For the supplementary group (PPID:A), there was no increase in cortisol in response to transportation.

Similarly, salivary cortisol was also affected by transportation (P<0.001) and PPID status (P=0.003) with an interaction between transportation and PPID status (P<0.001, Figure 5.5). In the initial study, mean salivary cortisol was increased compared with 1 h pre-transportation in non-PPID horses (0.07 ug/dL, 95% CI = [0.03,0.1 ug/dL]), at unloading (0.3 ug/dL, P=0.01, 95% CI = [0.2,0.5 ug/dL]), 15 min post (0.4 ug/dL, P=0.001, 95% CI = [0.3,0.4 ug/dL]), 30 minutes post (ug/dL, P=0.03, 95% CI = [0.1,0.4 ug/dL]), and one hour post-transportation (ug/dL, P<0.05, 95% CI = [0.1,0.2 ug/dL]). While there were no statistical differences for PPID horses in the initial study, salivary cortisol followed a similar pattern as serum cortisol with a numerical increase in response to transportation; however, the supplementary group of horses (PPID:A) had no change in salivary cortisol following transportation.

5.4.3 Serum insulin

There were no differences in insulin concentrations based on PPID status or following transportation (Figure 5.6). While all horses initially had basal and T60 post-OST insulin levels below the diagnostic cut off for ID when screened prior to the day of transportation, one horse in the supplementary group (PPID:A) had elevated insulin concentrations above basal diagnostic cutoffs at all time points. Additionally, one horse in the PPID group of the initial study had insulin above the suggested diagnostic cut off for ID [84] at 1 and 2 h post-transportation.

5.4.4 Rectal temperature, respiratory rate, and heart rate

There were no differences in rectal temperatures or respiratory rates 1 hour before loading on to the trailer compared with 24 hours post-transportation for non-PPID or PPID horses in either study (Figure 5.7). Ambient temperatures during transportation ranged from 14-18°C for the initial study and was 7°C for supplementary group.

Heart rates increase in response to transportation ($P<0.0001$) but were not influenced by PPID status (Figure 5.8). For the initial study, peak mean and 95% CI heart rates were at loading (PPID = 129.8 bpm [91.1,167.8 bpm], non-PPID = 115.3 bpm [99.2,131.5 bpm]) and unloading (PPID = 85.7 bpm [51.5,120.2 bpm], non-PPID = 96.5 bpm [68.6,124.3 bpm]). While, in the supplementary study mean and 95% CI heart rates were highest at loading (PPID:A = 127.0 bpm [53.5, 200.5 bpm]).

5.5 Discussion

Short distance transportation stress impacted ACTH and cortisol concentrations, with differences between PPID and non-PPID horses.

PPID horses had increased basal ACTH concentrations from non-PPID horses following transportation. The lack of differences observed in pre-transportation basal ACTH in this study between PPID and non-PPID horses in the initial study could be explained by the influence of environmental factors on ACTH and/or the TRH stimulation test's greater sensitivity in detecting early cases of PPID. To our knowledge this is the first study transporting PPID horses by road over a short distance; however, various studies have evaluated the ACTH response to short distance transportation in non-PPID horses [11,112,115]. The consensus is that there is a period following transportation, approximately 30 minutes after unloading, where some non-PPID horses could have

ACTH concentrations elevated above the diagnostic cutoff for PPID [79,112,115]. However, these studies also had horses of varying ages and were performed at different times of the year. The current study found 2/6 non-PPID horses with post-transportation ACTH above the diagnostic cutoff at unloading. While not all horses have elevated ACTH following transportation, it should be noted that ACTH could be elevated in certain individuals. Additionally, in this study horses were returned to their resident farm after transportation and stress responses could be further exacerbated by being transported to a novel location, such as a veterinary clinic, for diagnostic testing.

Not evaluated in this study was whether transportation stress would alter diagnostic results when using dynamic testing, such as a TRH stimulation test. Previous research has shown that basal ACTH can be increased in non-PPID horses following transportation [112,113,115]. However, a study that performed TRH stimulation tests at various times following short distance transportation determined that ACTH concentrations were not elevated above the diagnostic cutoff in non-PPID horses, and these horses were correctly screened non-PPID [111]. Given that ACTH is already elevated in PPID horses [109,172], it is not expected that there would be a change in the diagnostic results since these levels should already be above the cutoff; however, additional investigation would be needed to confirm.

While the PPID:A horses in the supplementary group had elevated ACTH concentrations compared with PPID horses in the initial study, they did not exhibit a further increase and instead maintained an elevated ACTH concentration post-transportation. The differences in PPID:A horses could be due to adrenal exhaustion as they already have chronically high ACTH concentrations and therefore did not show a

further response to the stress of transportation. There is high seasonal variability with ACTH concentrations of horses and changing diagnostic thresholds depending on the time of year with the highest being in fall [103,173]. While the supplementary study was conducted in the fall, these horses had also previously been diagnosed as PPID in the spring based on basal and T10 TRH ACTH. However, the ACTH values of the PPID:A horses in the supplementary group might not be directly comparable to the PPID horses in the initial study, conducted in spring, due to the seasonal increase in ACTH. Despite this, the three horses in the supplementary group (PPID:A) showed the same lack of response as the one horse in the initial Spring PPID group that had the highest ACTH values. Additionally, circulating ACTH tends to increase with age [104,169], emphasizing the importance of using age-matched control when evaluating basal ACTH concentrations between PPID and non-PPID horses [101].

Cortisol was increased immediately following transportation in non-PPID and PPID horses in the initial study and returned to baseline within two hours post-transportation. Similarly, aged horses without clinical signs of PPID showed an increase in cortisol concentrations following short distance transportation [115]. However, horses in the supplementary group (PPID:A) had no increase in cortisol in response to transportation. Some explanation for this phenomenon could be due to an exhaustion of the adrenal glands or non-responsiveness due to the increase in circulating ACTH in this group of PPID horses, that had elevated basal ACTH, compared with the PPID horses in the initial study. A lack of change in cortisol can also be seen in PPID horses following dexamethasone suppression test, where ACTH is uninhibited by the negative feedback of dexamethasone due to ACTH production by the pars intermedia [172]. A concern regarding this lack of

cortisol response to the acute stress from transportation in the PPID:A horses is that it could be associated with altered activation of the immune system. While typically viewed as a negative, acute stress can be protective by activating the innate immune response and inflammatory mediators to defend against potential threats [67]. Although not evaluated in this study, transportation stress has been shown to modulate immune response in non-PPID horses post-transportation [76,79,115]. Since PPID horses are known to be immunosuppressed [138], it is possible that transportation stress could potentially have compounding effects further compromising immune function in these horses [140,174,175]. While further research is needed to determine how transportation stress directly impacts immune function in PPID horses, this study suggests that special attention and care is advisable when transporting horses with PPID to minimize any negative effects of transportation stress.

The increase in circulating ACTH has been shown to correspond with severity of disease, with ACTH concentrations correlating with postmortem pituitary grade, the gold standard for diagnosis of horses with PPID [176]. The differences in ACTH and cortisol responses to transportation stress between the PPID:A horses in the supplementary study and PPID horses in the initial study could be attributed to PPID:A horses having more severe or being in later stages of PPID. Future studies should continue evaluating endocrine responses in PPID horses to classify differences based on possible severity of disease.

Insulin was not impacted by PPID status or transportation in this study. These results contrast with previous research in our lab that showed an increase in insulin concentrations in aged horses (>20 years) following transportation of the same distance [115]. These

differences could potentially be attributed to the horses in the aforementioned study not undergoing dynamic testing, such as an OST, to confirm they were non-ID prior to transportation or due to different insulin assays used between the studies.

The lack of differences found between rectal temperature and respiratory rates before loading and 24 hours post-transportation were expected and in agreement with previous research transporting horses the same distance at similar times of the year [79,115]. Whilst heart rates increased at loading and unloading, they returned to normal resting levels within 24 hours post-transportation, supporting that there is an acute stress response to short distance transportation, as previously reported [115]. The results from this study also further support the suggestion that loading and unloading might be the time in transportation where a horse is most active [1,5].

The main limitation in this study is the small number of horses used, particularly in the supplementary group. However, the differences in results between the PPID:A horses in the supplementary group and non-PPID and PPID horses in the initial study suggest these changes could be due to increasing severity of the disease. It is possible that due to low numbers in the supplementary group of horses (PPID:A) there may not have been sufficient power to detect additional differences, due to the increased risk of Type II errors. As mentioned previously, ACTH concentrations in the supplementary group of PPID horses were elevated partially due to being taken in fall where ACTH is typically higher [103]; however, their PPID diagnosis is consistent with previous basal (T0) and T10 post-TRH ACTH taken on the same horses in spring that were above the diagnostic cutoff. In addition, all showed classic clinical signs of PPID [107]. It should also be noted that while both clinical signs and ACTH, basal and post-TRH test, were used to categorize horses,

the only definitive diagnosis of PPID is through pituitary gland evaluation postmortem and there is a gradual progression of disease associated with aging making it difficult to differentiate between individuals with and without PPID [101]. Finally, mostly mares and only one gelding were used in this study and there could have been some differences in ACTH based on the stage of a mares estrous cycle [141], but estrus monitoring was not within this study's scope.

5.6 Conclusion

The responses of stress hormones to short duration transportation in this study varied between non-PPID and PPID horses. PPID horses had increased ACTH concentrations following transportation compared with non-PPID horses. Additionally, some non-PPID horses may have post-transportation ACTH concentrations above the diagnostic cutoff values. PPID horses with markedly elevated basal and T10 TRH ACTH had sustained elevated ACTH and did not have a typical increase in cortisol in response to transportation. This could be concerning due to the protective nature of cortisol to maintain homeostasis during a stressful event, and special care should be taken to monitor PPID horses when transporting them.

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Table 5.1 Ages, body morphometrics, and endocrine screening results of pituitary pars intermedia (PPID) and non-PPID horses prior to short duration transportation.

Parameter	**Non-PPID** (n=6)	**PPID** (n=6)	**PPID:A*** (n=3)	**P-value**
Age (years)	22 ± 4.0[a]	25.4 ± 2.3[a]	27.0 ± 2.0[a]	0.09
BW (kg)	573.4± 38.5[a]	539.5 ± 49.4[a]	556.8 ± 77.4[a]	0.6
BCS	5 (4-6)[a]	5 (5-6)[a]	5(5-6)[a]	0.3
CNS	1 (1-2)[a]	2 (1-2)[a]	1 (1-2)[a]	0.5
HS	0[a]	1.5 (1-2)[b]	2 (1-2)[b]	**0.02**
MAS				
Neck	1 (1-2)[a]	1 (1-2)[a]	2 (1-2)[a]	>0.9
Back	1 (1-2)[a]	1 (1-2)[a]	2 (2-3)[a]	0.09
Hind	1 (1-2)[a]	1.5 (1-2)[a]	2 (1-2)[a]	0.2
Total	3 (3-6)[a]	3.5 (3-5)[a]	4 (4-7)[a]	0.3
Insulin OST T0 (μU/mL)	20.8 ± 13.6[a]	20.7 ± 14.8[a]	19.6 ± 18.3[a]	>0.9
Insulin OST T60 (μU/mL)	26.7 ± 10.2[a]	38.7 ± 11.5[a]	38.7 ± 21.5[a]	0.3
ACTH TRH T0 (pg/mL)	23.8 ± 15.95[a]	65.2 ± 86.3[a]	948.0 ± 419.3[b]	**<0.001**
ACTH TRH T10 (pg/mL)	135.4 ± 73.4[a]	618.0 ± 288.9[b]	2029.0 ± 739.3[c]	**<0.001**

Values for age, body weight (BW), oral sugar test (OST) T0/T60 insulin, and post-thyrotropin releasing hormone (TRH) stimulation test T0 /T10 ACTH are presented as means ± standard deviations; body condition score (BCS), cresty neck score (CNS), hair score (HS), and muscle atrophy score (MAS), are presented as median (range).
P-values represent differences within a row, with differences between groups ($P < .05$) represented by different "a,b,c" superscripts.
*ACTH taken in fall (October).

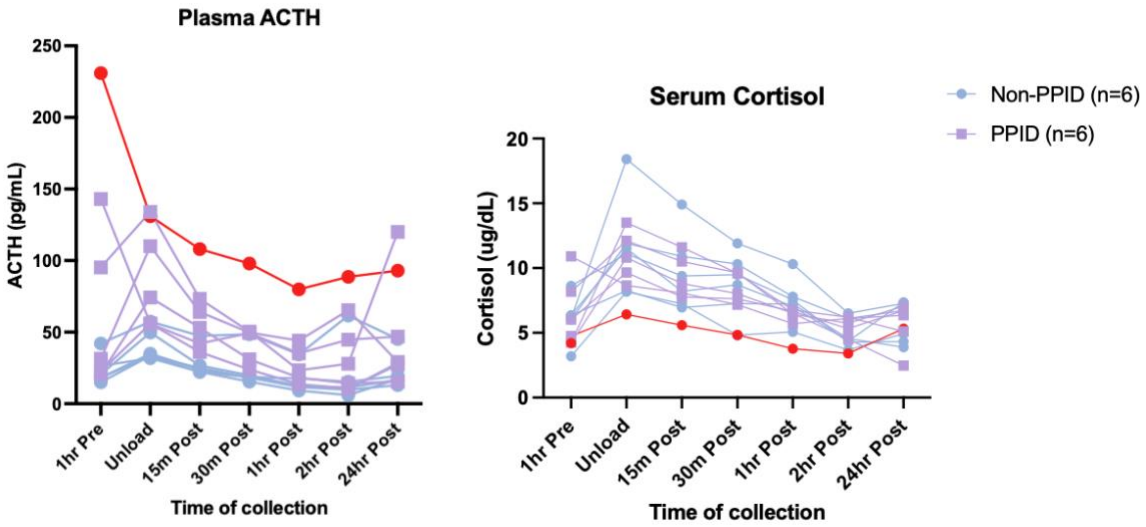

Figure 5.1 Individual plasma adrenocorticotropin hormone (ACTH) and serum cortisol concentrations of horses transported a short duration.

The horse in the pituitary pars intermedia (PPID) group of the initial study with the highest ACTH and lowest cortisol concentrations is highlighted in red.

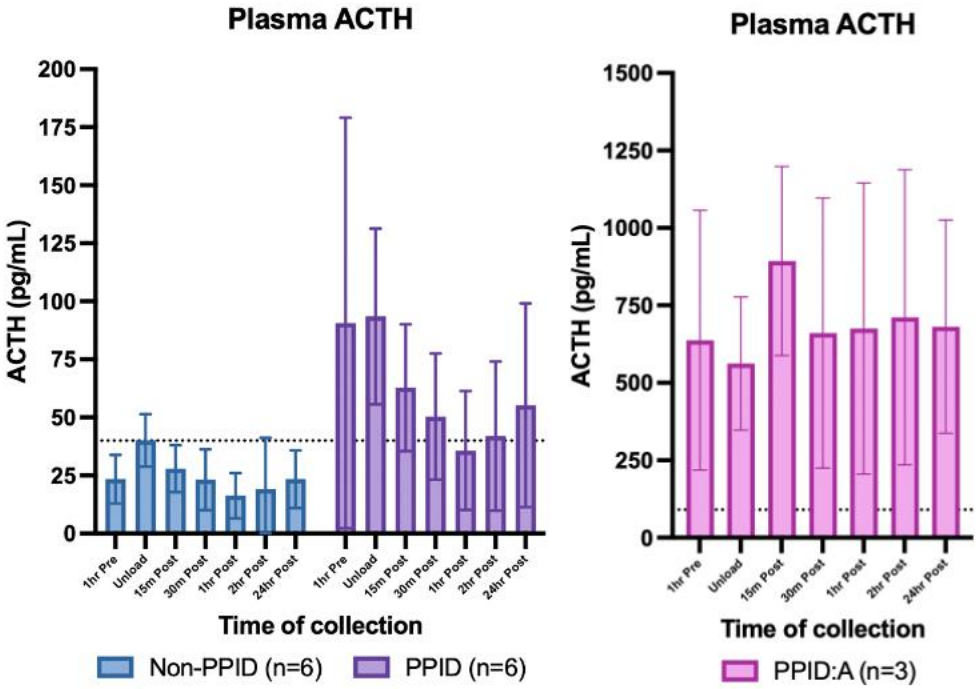

Figure 5.2 Plasma ACTH of horses with and without pars pituitary intermedia dysfunction (PPID) before and after short-term transportation.

The data are presented as the mean and 95% CI. Dashed lines represent diagnostic cutoff values of basal ACTH for PPID based on season of study (40 pg/mL and 90 pg/mL, respectively).

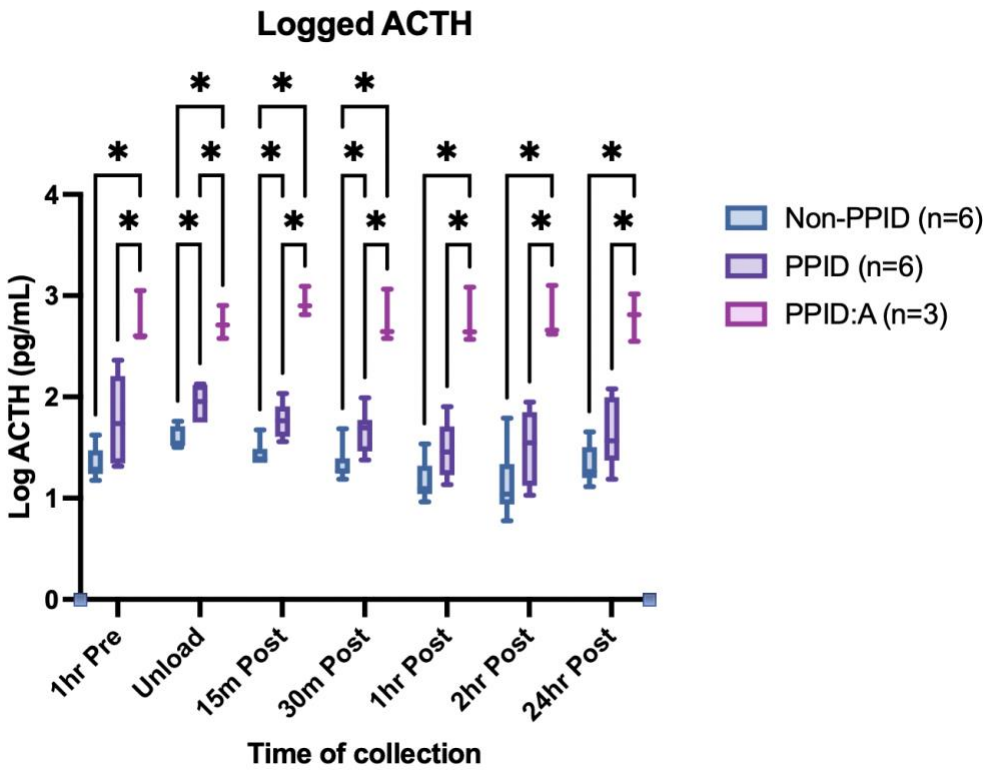

Figure 5.3 Plasma ACTH (logged) of horses with and without pars pituitary intermedia dysfunction (PPID) before and after short-term transportation.

The data are presented as the mean and IQR. Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was an effect of transportation ($P<0.001$), PPID status ($P<0.0001$) and transportation x PPID status interaction ($P=0.04$). Differences between groups (PPID status) at specific times are denoted as *, $P<0.05$.

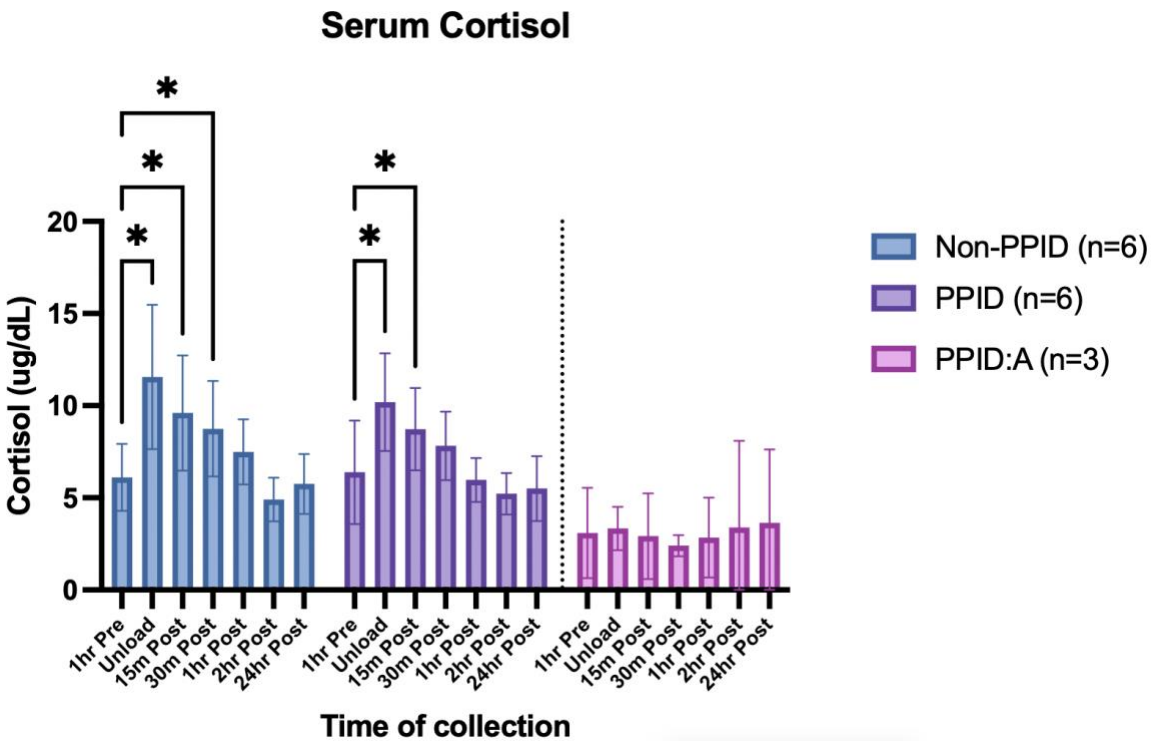

Figure 5.4 Serum cortisol of horses with and without pars pituitary intermedia dysfunction (PPID) before and after short-term transportation.

The data are presented as the mean with 95% confidence interval. Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was an effect of transportation ($P<0.001$), PPID status ($P=0.003$) and transportation x PPID status interaction ($P<0.001$). Transportation effect differences in relation to 1 hr pre within a group are denoted as *, $P<0.05$.

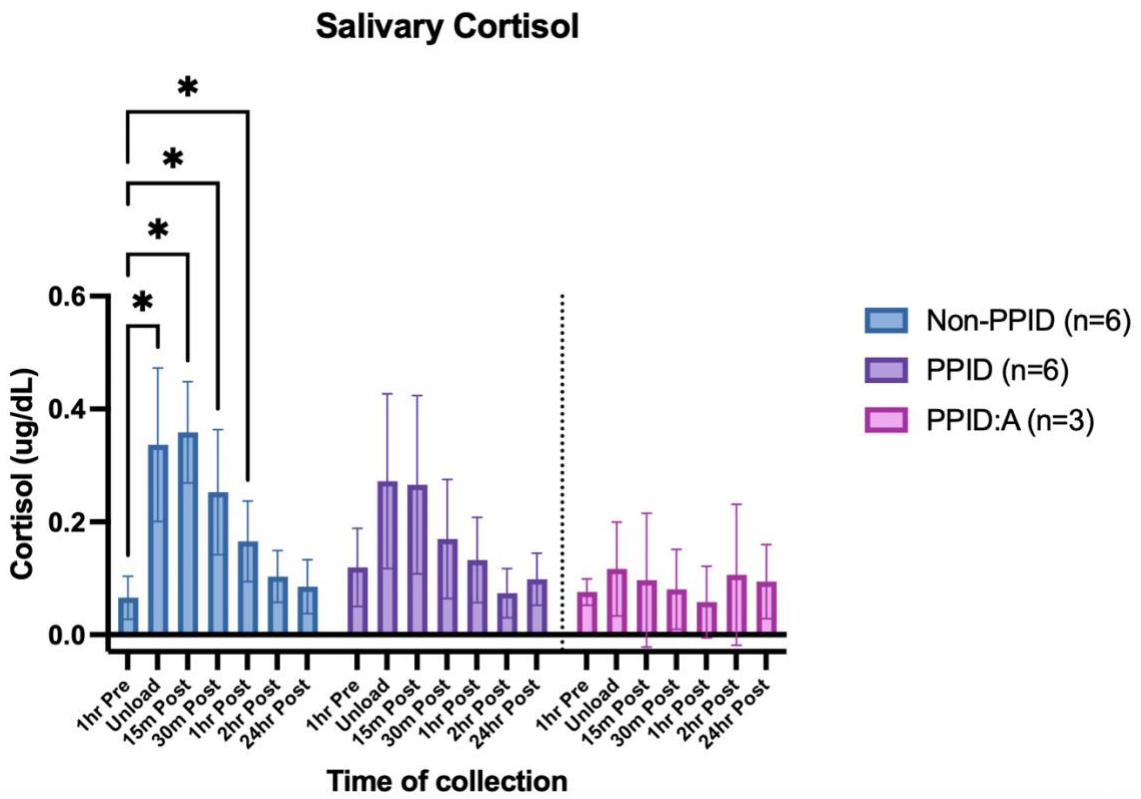

Figure 5.5 Salivary cortisol of horses with and without pars pituitary intermedia dysfunction (PPID) before and after short-term transportation.

The data are presented as the mean with 95% confidence interval. Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was an effect of transportation ($P<0.0001$), PPID status ($P=0.003$) and transportation x PPID status interaction ($P<0.001$). Transportation effect differences in relation to 1 hr pre within group are denoted as *, $P<0.05$.

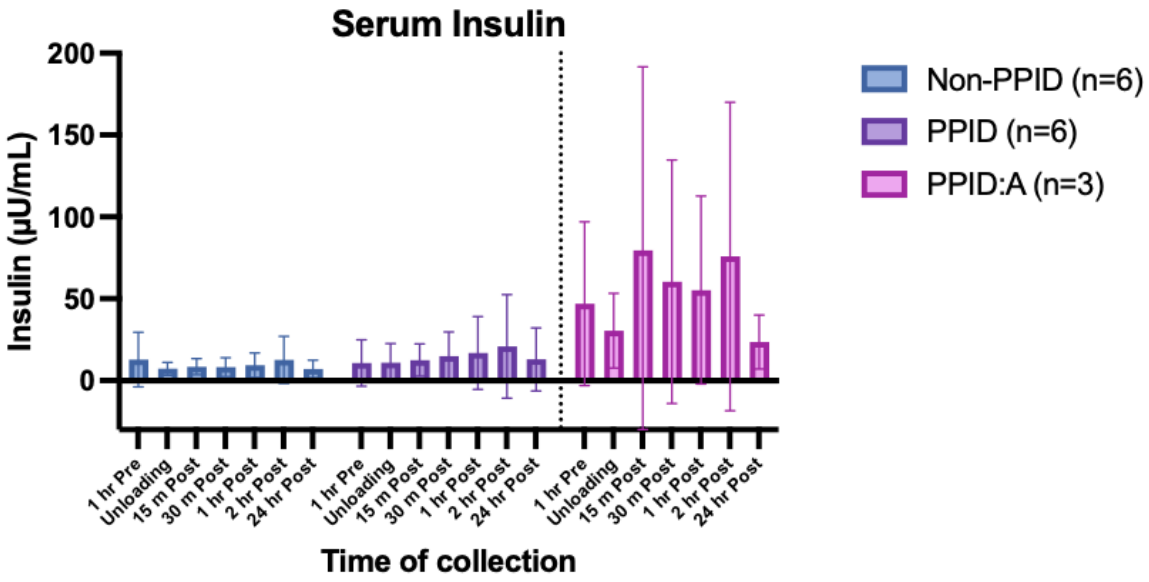

Figure 5.6 Serum insulin of horses with and without pars pituitary intermedia dysfunction (PPID) before and after short-term transportation.

The data are presented as the mean with 95% confidence interval. There was no effect of transportation or PPID status.

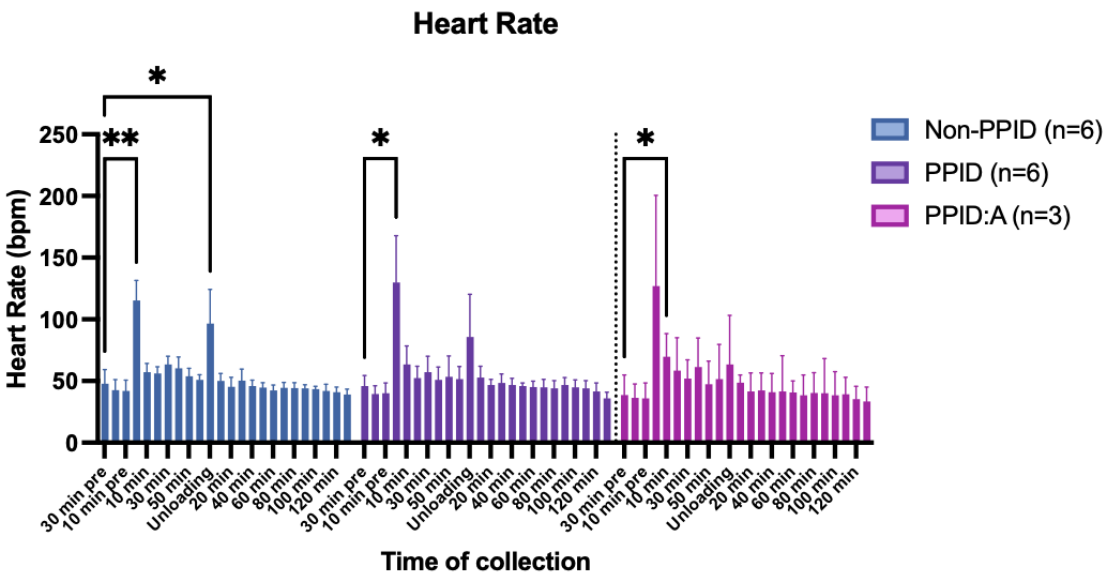

Figure 5.7 Heart rates before, during, and after short-term transportation of horses with and without pituitary pars intermedia dysfunction (PPID).

The data are presented as the mean with 95% confidence interval. Multiple comparisons were calculated using Bonferroni post-hoc analysis. There was an effect of transportation ($P<0.0001$), but no effect of PPID status or transportation x PPID status interaction. Transportation effect differences in relation to 30 min pre within a group are denoted as *, $P<0.05$.

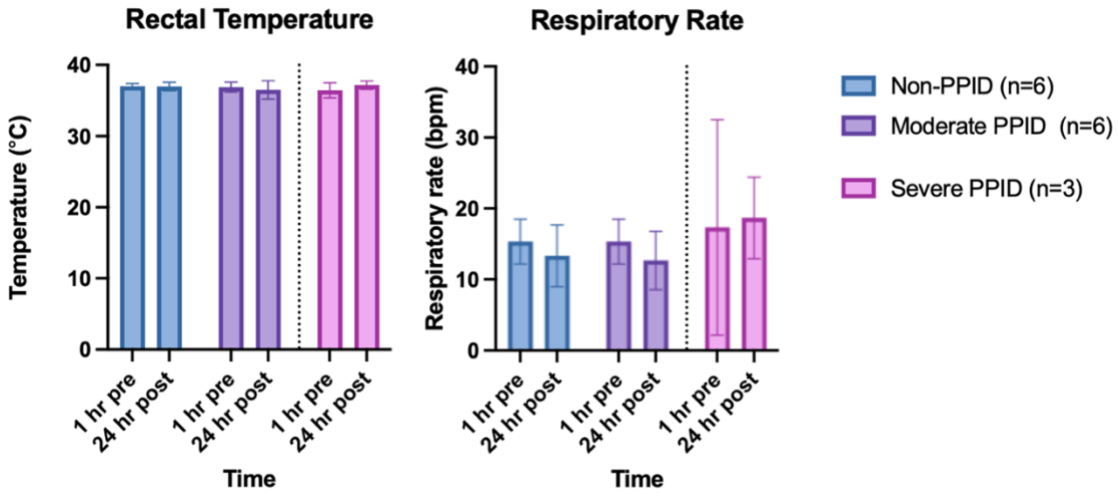

Figure 5.8 Rectal temperatures and respiratory rates of horses with and without pituitary pars intermedia dysfunction (PPID) 1 hour before and 24 hours after short-term transportation.

The data are presented as mean and 95% confidence interval. There was no effect of transportation or PPID status.

CHAPTER 6 CONCLUSIONS AND FUTURE DIRECTIONS

While horses are commonly and frequently transported short distances by road, it was still unclear how stress impacts endocrine and immune function. Few studies had previously evaluated horses' responses to short-distance transportation, with a focus on cortisol, heart rate, and stress-related behaviors [6–9]. Our lab previous conducted the only study specifically using senior horses to understand how age relates to physiological responses to being transported a short duration [79]. Furthermore, only one study had examined how transportation influences the insulin responses of horses with and without EMS [92]. The results reported in this dissertation helped bridge these gaps in knowledge while also raising additional questions on how to further improve management when transporting horses over short durations.

This dissertation explored how and why horses are transported, with an emphasis on short-duration transportation. A survey of equine road transportation practices and 3 animal studies were conducted to evaluate how different groups of horses, based on age and presence or absence of ID/PPID, responded to transportation by measuring selected stress-associated hormones, cytokines, TPR, glucose, and/or insulin. This research highlights the influence that short-duration transportation can have on the stress response and its downstream effects in horses and how it can alter hormones used to monitor and diagnose endocrine diseases.

To better understand the most common journeys horses travel, a nationwide survey was conducted. This survey revealed that horses are more commonly transported a short distance of 3 hours or less, further emphasizing the importance to better understand how horses respond physiologically to these shorter trips. While increasing length of travel is

associated with an increased risk of developing transportation related health problems [16], management practices might vary based on the length of trip. Due to keeping the length of the survey reasonable as to not lose interest in participation, questions about management of a specific horse were only asked about short trips (<3 hours). Additional research comparing management practices across different trip length would be beneficial, as longer trips may require more critical management to minimizing these problems. Also, the survey did not fully capture commercial drivers and professional riders, the latter of which transported their horses on longer trips compared with amateurs according to the survey results. These demographics likely have more extensive management strategies for transporting horses over longer durations and an additional survey targeting these groups specifically would provide more beneficial information.

The results on the management of horses during transportation, particularly in the trailer, served as a basis for how the horses in the subsequent studies presented in this dissertation were trailered. For instance, horses were tied at or above eye level to match how horses were reported to be transported based on the survey results. One notable difference was that the survey showed most horses had access to hay during transport; however, our studies did not provide forage to avoid potential interference with hormones being measures, particularly insulin. This is an area that warrants further exploration, as access to forage could affect post-transportation insulin concentrations, especially given the varying levels of NSC in forages, which are thought to be the main driver in post-prandial insulin response [85]. This could also possibly explain some of the differences in insulin concentrations post-transportation in the studies presented in this dissertation compared with previous research [92].

Most survey respondents did not monitor vital signs such as temperature, pulse, and respiration before or after transporting their horse short distances. Although not surprising, these are easy and practical ways to monitor aspects of stress response and overall horse health, especially considering that stress to the horse was found to be one of the main concerns when transporting. Previous studies have used various monitoring techniques during transportation, including ocular, rectal, and under the tail thermometers for temperature [59,125,177], and heart rate monitors for pulse and HRV [6,40]. While results from the studies in this dissertation did not show any concerning increases in temperature post-transportation, these projects were performed in temperate weather conditions and where horses returned to their resident farm. Monitoring changes in TPR could be more beneficial to identifying disturbances when transporting longer distances, in extreme heat, and/or to new locations. In a study transporting horses over long durations (24+ hours) they found that temperature monitoring at multiple intervals post-arrival was necessary to identify horses with transportation-related pyrexia and subclinical pneumonia [125]. Additionally, while not evaluated in this dissertation, monitoring behavioral changes following transportation could also aid in early detection problems or used to more closely monitor horses that do not adapt well to transportation. While there were no immediate health concerns for any horses transported on studies in this dissertation, a basic physical exam should be performed on all horses to ensure horse well-being prior to transport [178,179] and to establish a baseline for comparison post-transportation.

The survey revealed that senior horses (16 years or older) made up 34.6% of those transported on short trips, highlighting the need to properly monitor and manage this population of horses. The increasing number of senior horses has also prompted interest in

their proper care and management [60,62]. It was surprising to see the percentage of horses being transported similar to the proportion in the general horse population, considering that many are expected to be retired and out of work [61,116]. Because this dissertation only evaluated some areas of stress and immune response to transportation, senior horses still might need altered management post-transportation. The horses in the studies conducted in this dissertation returned to their normal pastures and most parameters measured returned to those of pre-transportation within a few hours; however, it is unknown if senior horses might need a longer recovery time post-transportation if being transported for other reasons, such as exercise or competition. The responses of senior horses post-transportation in different settings warrants further research.

Since senior horses are commonly transported, the stress and immune responses of aged horses were compared to young horses transported short distances. Eleven horses, 5 aged (22 ± 1) and 6 young (2 ± 1) were transported a round trip of 1.5 hours with temperature, heart rate, blood and saliva collected before and after. This study showed that cortisol increased post-transportation but with no age differences. In addition to stress, cortisol can increase based on time of day due to circadian rhythm and with physical activity such as exercise [180]; however, there does not seem to be an obvious relationship between age and cortisol in horses [143].

Salivary cortisol showed a strong positive relationship with serum cortisol across all studies in this dissertation, further supporting its use as a non-invasive measure of stress. While saliva collections are less invasive than blood collections, they may not necessarily be easier to perform in all horses, and each horse should be acclimated to saliva sampling techniques. Salivary cortisol can be useful in cases when collecting a blood sample is not

possible, can reduce the number and/or need for blood collections, and be considered more favorable in terms of animal welfare [180].

Aged horses generally had increased markers of inflammation when compared to young horses, but these were not always influenced by transportation. There were some similarities and differences to the previous study using aged horses [79]. Another recent study found that horses transported 4 hours experienced an acute inflammatory status in response to transportation stress [76]. These studies all evaluated components of immune response and found some changes associated with age and/or in response to transportation; however, none of these comprehensively evaluated the immune system or the impact of these changes on disease occurrence or susceptibility. This could be further evaluated by challenging horses to vaccination post-transportation and comparing antibody titers to non-transported horses. While not conclusive, these results still emphasize that caution should be taken when transporting aged horses due to inflamm-aging.

Aged horses (non-ID) had increased insulin after transportation, causing concern for the impact of transportation stress on both metabolic health and diagnostic results for endocrine disorders. Changes in insulin dynamics have also been reported in horses confined and hospitalized [91]. While the relationship between transportation stress as insulin was evaluated in the remaining studies, our understanding of how stress affects insulin in horses is still limited.

The relationship between transportation stress and metabolic function was further investigated using ID (n=7) and non-ID (n=7), that were transported with the same parameters as the previous study. To investigate the impact of transportation stress on dynamic testing, an OST was performed 24 hours before and 3 hours post-transportation.

While OST results have been shown to be repeatable at 72 hours [164] further research should evaluate OSTs performed on consecutive days as well as how the time-of-day influences OST results. Similar to the previous study, there was an increase in cortisol following transportation; however, this was not impacted by insulin dysregulation status. Total cortisol does not seem to increase in horses with EMS or hyperinsulinemia; however, free cortisol fraction (FCF) is higher in horses with hyperinsulinemia [143]. It is unclear what the implications of these observed differences is and whether it is contributing to insulin responses to stress in ID horses. Additional studies should evaluate stress-related differences in FCF to see if this can more accurately explain the differences in insulin between ID and non-ID horses post-transportation. Glucose was also increased following transportation, most likely in response to the increased cortisol. However, the lack of differences between ID and non-ID horses makes the mechanism for why insulin increases in ID horses following transportation less straightforward than simply a response to increased blood glucose.

Insulin was elevated in ID horses before transportation, with an increase following both pre- and post-transportation OST. The increase in post-transportation OST insulin in ID horses was concerning considering hyperinsulinemia is highly associated with laminitis. While it is unknown if there is a threshold level and/or duration of insulin that causes HAL [84], horses with a history of laminitis should be closely monitored for any lameness or other signs of distress when being transported due to the potential for an increased insulin response. Notably, insulin was above diagnostic cutoff in non-ID horses' when an OST was performed after transportation that could cause misleading diagnostic results for ID. However, non-EMS horses have showed to have increased basal insulin following

transportation in contrast with the results of this dissertation [92]. While our results suggest basal insulin might be more accurate in diagnosis of ID compared to OST post-transportation, this discrepancy makes it difficult to make a recommendation towards using basal insulin for ID diagnosis post-transportation, warranting further research.

Since diagnostic testing for ID was not performed immediately after transportation it is unclear if there is a specific time point post-transportation where dynamic testing would not be impacted. Additional studies should perform OSTs at various time points post-transportation. Furthermore, while this study used a control group of non-ID horses who were also transported, follow up studies taking should also take blood samples at the same time of day prior to transportation to better confirm that changes observed are reflective of a transportation effect and not time of day.

While there were some differences observed between ID and non-ID horses, the use of salivary insulin as a diagnostic tool for needs further investigation as a non-invasive way to monitor insulin in EMS/ID horses. In human saliva, there can be a 30-minute delayed response in the increase in insulin following a challenge compared with blood [32]. Preliminary data (not included in this dissertation) show that even when taken at various timepoints beyond 60 minutes post-OST there is not a clear difference in salivary insulin between ID and non-ID horses and further changes to sample collection and analysis are continuing to be evaluated. In addition to the ease of saliva collection for some horse owners, lateral flow point-of-care insulin tests have been recently made available [181]. Evaluating salivary with these stall side insulin tests is another area that should be explored due to their ease and convivence. Easier testing methods for veterinarians, and possibly horse owners, adds tools to improve the ability to monitor and manage these horses.

The endocrine responses of PPID horses to short distance transportation were explored to determine if the measured hormones in the HPA axis are further impacted by transportation-associated stress. Fifteen horses, 6 non-PPID and 6 PPID, with a supplementary group of 3 additional PPID horses (PPID:A) were transported with the same parameters of the previous two studies. The PPID:A horses had elevated ACTH prior to transportation and lacked a cortisol response to transportation, raising concerns about their ability to mount a proper stress response that could be protective in function. However, the exact mechanism behind the lack of cortisol response in PPID horses and the downstream impact on immune function needs additional research and could possibly be attributed to adrenal exhaustion from the constant ACTH. Further research should also evaluate parameters of the immune response in PPID horses under stressful conditions, such as being transported, as stress could exacerbate their already immunosuppressive state. While the exact impact on immune response is unknown, special care should be taken to monitor these horses during transportation making sure that they have access to water, and biosecurity measures are followed when traveling to new places [178].

ACTH was above the diagnostic cutoff after transportation in 5/11 non-PPID horses in the studies described in this dissertation, further emphasizing that diagnostic testing using basal ACTH should not be conducted immediately after transportation. While not all horses show increased ACTH following transportation, some horses can have elevated ACTH up to 30 minutes following transportation [111–113]. The current recommendations are to not take basal ACTH for use in PPID diagnosis when horses are experiencing stress and/or pain and within 30 minutes of transportation [107]. In non-PPID horses, TRH post-transportation did not cause false positive results, even when performed at varying times

[111]. It could be assumed PPID horses would have elevated ACTH regardless of transportation, supporting that TRH may be a better option if a horse needs to be transported to a veterinary clinic for PPID screening.

Management of horses on studies in this dissertation during transportation was kept constant to control for compounding factors; however, there are many aspects of management that could be explored further to potentially minimize stress. This includes how horses are tied in the trailer [182], spacing available [44], and positioning in which horses are facing (forwards vs backwards) [3,8,183]. Alterations in these areas have showed positive results towards improving horse welfare in long journeys but have not been evaluated in horses transported a short duration. Additionally other countries provide outlined guidelines for minimum requirements that are not as specifically defined in the U.S. For example, Australia and the UK have guidelines [184,185] that are worth exploring to compare with how horses are managed during transportation in the U.S.

Overall, the results from this dissertation highlight the prevalence of horses being transported short durations and provides further insight into areas of the endocrine and immune responses of horses to short-distance transportation. Additionally, there are changes in various aspects of the stress and immune response to transportation depending on the age of the horse and their endocrine status, specifically horses with ID and/or PPID. These alterations can cause misleading diagnostic tests in horses without these endocrine disorders if taken directly after transportation and there are increases in hormones that could be potentially concerning to horse health. Continued research is needed evaluating non-invasive monitoring techniques and the timing of diagnostic testing for endocrine disorders due to the influence stress can have. Based on the results of this dissertation and

previous research, for short-duration transportation of horses it is recommended to assess overall horse health prior to and following transportation. This includes monitoring vital signs and paying special attention to senior horses and those with endocrine disorders to identify and address any potential health concerns that may arise as a result of transportation. Collectively these studies highlight the importance for further research into short duration transportation to improve best practices for management and monitoring of horses to minimize stress and maximize horse health.

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EDUCATION

University of Kentucky
PhD in Veterinary Science — Expected August 2024

Kansas State University
Master of Science in Animal Sciences — 2017

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PROFESSIONAL POSITIONS

Adjunct Instructor, Midway University	Aug 2023 – Present
Graduate Research Assistant, University of Kentucky	Aug 2020 – Present
Laboratory Technologist, Equine Diagnostic Solutions	Oct 2019 – Aug 2020
Reproduction Center Groom/Research Coordinator, Spy Coast Farm	Jun 2018 – Oct 2019
Assistant/Young Horse Dressage Trainer, Kynynmont Connemaras	Aug 2017 – June 2018
Graduate Teaching Assistant, Kansas State University	Aug 2014– Aug 2017

AWARDS & HONORS

2023	University of Kentucky Outstanding Teaching Assistant Award - Finalist
2022	University of Kentucky GradResearch Live - 3 Minute Thesis - 3rd Place
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PROFESSIONAL PUBLICATIONS

Jacquay, E.T., Harris, P.A., Adams, A.A. Age-Related Differences in Short-Term Transportation Stress Responses of Horses. J Equine Vet Sci. 2023 Jul 1;128. doi: 10.1016/j.jevs.2023.104879.

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