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EQUINE DISEASE QUARTERLY

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RESEARCH SPOTLIGHT

Growing Equine 'Mini-Guts' to Investigate Infectious Causes of Intestinal Illness in the Horse

Gastrointestinal disorders represent a range of serious, potentially life-threatening conditions that continue to be a major challenge to horse owners and the equine industry, leading to significant financial costs associated with prevention and treatment as well as the loss of horses of all ages. A major roadblock in studying and understanding disease outcomes associated with infectious causes of colic is a lack of relevant laboratory model systems in which to model intestinal infections. To overcome this obstacle, the Shaffer Laboratory developed microscopic, organ-like systems (termed 'organoids') from a variety of horse tissues to study bacterial and viral equine infectious diseases. Importantly, due to their unlimited self-renewal and tissue expansion, organoids bridge the gap between the laboratory and disease models, providing an attractive alternative to animal experimentation. In addition, organoids have emerged as an invaluable tool for accurately predicting drug metabolism and response, such that they represent an ideal platform for therapeutic discovery and pre-clinical development.

When generated from the intestines, mini-gut organoids (referred to as 'enteroids') reproduce the unique characteristics and microarchitecture of the equine gastrointestinal tract, providing a robust laboratory model to mimic the horse intestine. While enteroids grown in a three-dimensional (3D) configuration are beneficial for long-term culture and functional characterization, host-pathogen interactions are more challenging to model. For example, enteroids grow in a wide range of shapes and sizes, leading to inconsistent approximation of equine cell numbers and inaccurate or unpredictable bacteria-to-cell ratios. Moreover, pathogens invade tissues using receptors localized to the luminal host cell surface. Unfortunately, ensuring that bacteria or viruses gain access to the enteroid interior where luminal cell receptors are located is technically challenging, time-consuming and difficult to standardize. To address this challenge, our lab developed methods to manipulate enteroids to allow bacteria or viruses access to critical cell receptors. Our first approach involves imbedding parts of enteroids onto a semipermeable membrane submerged into specialized media that encourages normal cell division and architecture. In this system, enteroids self-assemble such that the luminal cell surfaces (those that would normally be on the inside of the intestine) are exposed and accessible from the top of the culture dish. This technique provides a more convenient, consistent and precise way to control equine cell numbers and allows us to easily infect enteroid-derived tissues.

Our second method involves attaching enteroid tissue onto microfluidic chip devices capable of directional fluid flow and mechanical deformation that applies physical stretch across the chip. Critically, these normal, physiologic forces generate a tissue microenvironment that more accurately mimics the directional flow of digesta traveling through the gut via wavelike peristalsis. These chips developed in our lab consist of two parallel channels separated by a semipermeable membrane that enables "cross-talk" between the interfaced gut tissue and an artificial vasculature created by an equine endothelial cell barrier.

Therefore, these gut-on-a-chip models recreate a multi-tissue system similar to blood vessel lined organs in a living horse. Notably, this system affords the opportunity to study the frontline defense to infectious agents; introduction of immune cells through the endothelial channel allows us to monitor pathogen-induced cellular migration from the artificial vasculature into the gut tissue. In this configuration, immune cells exit the vasculature channel to neutralize invading pathogens in the gut tissue channel, thereby replicating host defenses elicited during infection in the horse.

Using advanced laboratory techniques, we validated and demonstrated the functionality of our enteroids. For example, we determined that, similar to tissues within the horse gastrointestinal intestinal tract, equine enteroids exhibit barrier functions, relevant brush border enzyme activities and mucus production. Further, we compared cellular differentiation patterns within enteroid systems using immunohistochemistry and advanced RNA sequencing analyses. Across our various techniques, we determined that our polarized enteroid model exhibits increased cellular differentiation compared to 3D enteroids. However, the addition of biomechanical forces (stretch and flow) in the gut-on-a-chip system demonstrated the highest level of cell type variation, reinforcing our hypothesis that microfluidic devices generate the most life-like laboratory environment in which to study equine infectious disease outcomes.

Our aim is to develop versatile enteroid models for both bacterial and viral infections. Since Salmonella is one of the most common causes of bacterial enteritis in the horse, we first used our enteroid platform to evaluate the effects of bacterial infection on host inflammatory responses. In initial studies, we analyzed how *S. Typhimurium* uses bacterial nanomachines to inject virulence proteins into intestinal cells to manipulate host immunity and enable colonization. Building on our success, we are using our unique models to investigate additional aspects of microbial pathogenesis and to accelerate the development of novel vaccines to combat infectious disease threats to the equine industry. For example, equine rotavirus B has emerged as a significant health and economic concern and continues to cause foal diarrhea outbreaks in Kentucky and other states including New York and Pennsylvania.

We are the first laboratory to demonstrate productive equine rotavirus replication in our intestinal enteroid cultures – a critical experimental step that will enable future vaccine development.

Overall, our organoid technologies can be used to understand how pathogens interact with specific equine tissues, to explore new ways to prevent infection and to discover effective drugs to combat infectious disease. Furthermore, developing additional equine organoid models will provide the experimental foundation for numerous studies focused on tissue injury and regeneration, inflammation and infection control, pre-clinical analysis of new therapeutics, toxicology and drug metabolism studies and the identification of genetic factors that determine disease outcome in the horse. Our organoid-to-microfluidic chip pipeline is rapidly pioneering the way towards successful equine precision medicine that will allow us to develop effective medical intervention strategies in the lab. Ultimately, our platform will reduce animal experimentation and is accelerating progress in disease modeling, vaccine design and development, understanding infectious disease outcomes and investigating regenerative medicine in the context of the dynamic physiology of the horse.

Lynn M. Leedhanachoke, MS, DVM
Department of Veterinary Science
108 Gluck Equine Research Center
Lexington, KY 40546
lmle224@uky.edu

Carrie L. Shaffer, PhD
Department of Veterinary Science
108 Gluck Equine Research Center
Lexington, KY 40546
carrie.shaffer@uky.edu

Phone: +1(859) 218-1168

Please visit our Gluck Website:

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EDITORS
Lynne Cassone
Lutz Goehring
Allen Page

STAFF
Holly Wiemers
Hendri Smeenk

First Quarter 2024

International report on equine infectious diseases.

This report collates information provided by diagnostic laboratories in Lexington, Kentucky, University of Kentucky Veterinary Diagnostic Laboratory (UKVDL) and Equine Diagnostic Solutions, Inc. (EDS). We want to thank IDEXX laboratories providing data for Germany.

We have further included information from the International Thoroughbred Breeders Federation, the International Collating Centre (ICC) in Newmarket/Cambridge, United Kingdom, and information from the American Association of Equine Practitioners' Equine Disease Communication Center (EDCC). This report summarizes heightened activity of several relevant contagious or environment-linked diseases among equids. We encourage everyone to report laboratory-confirmed (toxico)infectious disease of *Equidae* to the ICC in Cambridge, UK, or EDCC, USA. With few exceptions, reports are from Europe and North America.

Information from all our sources shows *Strep. equi spp. equi* (Strangles) as the most frequently and consistently diagnosed equine pathogen for the first quarter of 2024. Reports from various sources reflect 'newly diagnosed' infection.

There have been sporadic reports of equine influenza virus, both from Europe and North America.

Two equine Rabies cases were reported from the Eastern United States, and single cases of EIA throughout North America. In Europe, EIA was diagnosed in Belgium, Germany and Hungary. A case of Glanders *(Burkholderia mallei)* was reported from Iran.

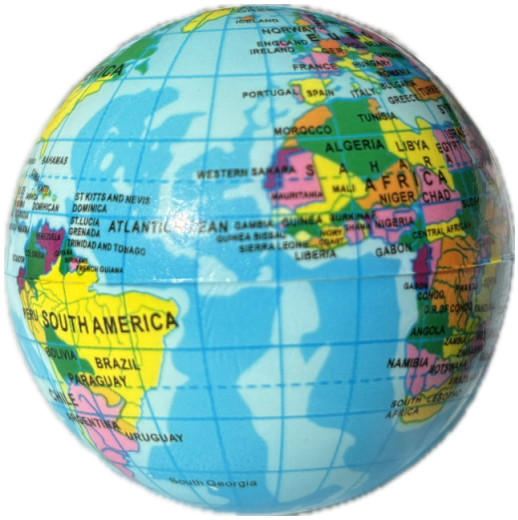
Many mares are in or entering their third trimester of pregnancies. Not surprisingly, the numbers for EHV-1 (and EHV-4) abortions are up for the first quarter. All reports of EHV abortion originate from Europe, while there are no reports from North America. However, this is likely because of underreporting as UKVDL reports five fetuses aborted due to EHV-1 for the last quarter. Their data shows abortions in various breeds.

For the first quarter of 2024 there has been a drastic increase in EHV-1 neurologic disease (EHM) cases/outbreaks, both in North America, more than 20, and (primarily) Northern Europe (about 15). Our German sources, on the other hand, are reporting very low EHM activity for the first quarter. Affected breeds were Thoroughbred, Standardbred, Warmblood, Draft horse, Quarter Horse and "Pony." While EHM outbreaks are often associated with "traveling horses," there were a number of reports without this association, suggesting spontaneous and differently triggered reactivation from latent carriers.

Lutz Goehring
Edward O. Olajide

Gluck Equine Research Center – Department of Veterinary Science - University of Kentucky

l.goehring@uky.edu



Maxwell H. Gluck
Equine Research Center
Lexington, Kentucky USA,
40546-0099
Telephone (859) 257-4757
Fax (859) 257-8542
gluck.ca.uky.edu

Vesicular Stomatitis Returns with a New Bag of Tricks

If there's one thing we're learning about climate change's effects on vector-borne diseases, it's that the diseases will do something different and we can't predict quite what that is going to be. When vesicular stomatitis reappeared in the United States in 2023 after a three-year hiatus, the fact that a new incursion occurred was not a surprise; rather, everything else about the outbreak was out of character, from the geographic locations affected, to the species of animals that developed lesions, to the natural disasters that altered the trajectory of disease occurrence.

Vesicular stomatitis virus (VSV), spread by black flies, sand flies and biting midges, causes blister-like lesions on the muzzle, tongue and coronary bands of affected livestock. The virus circulates year-round in southern Mexico and only occasionally moves into the U.S. when climate factors support expansion of the infected vectors northward. Equids are most commonly infected, but cattle cases also occur. While other livestock species are susceptible, such as llamas, alpacas, pigs, sheep and goats, only a handful of cases in those species are diagnosed. The painful lesions heal on their own in a couple of weeks and most affected animals only need supportive care, but the trade ramifications and movement restrictions for livestock during an outbreak are significant. International movement of susceptible livestock may be halted from affected states, and even states without cases may have to test animals prior to export. Shows, events and county fairs may be canceled, and non-affected states restrict movement of livestock from affected states. The outbreak usually lasts for months through the vector season (summer to fall), and often continues into winter as vectors die off or go dormant. Meanwhile, horse owners struggle to meet requirements to move to their shows and events and, as cases spread, they may suddenly find themselves in a newly-affected area and cannot return home. Simultaneously, they're frantically applying fly spray to horses and implementing aggressive fly mitigation strategies on farms to prevent their animals from contracting the disease. Vesicular stomatitis virus is zoonotic, so humans can get infected from handling livestock with lesions, (yet another flu-like illness to worry about!)

The 2023 VSV outbreak that produced 319 infected premises (311 had infected equids), ran from May 17, 2023, to Jan. 18, 2024, and primarily impacted California, a state which had never previously recorded a natural incursion of the disease from Mexico. For decades, Southern California had been protected from incursions by extreme drought conditions, but the Winter of 2022 saw heavy snow and Spring of 2023 brought buckets of rain. Lush vegetation abounded; the flies and midges thrived and expanded their range over the border carrying the virus. The only previously recorded VSV cases in California were caused by human error, not a natural incursion, and occurred in the 1982-83 outbreak when infected dairy cattle were moved from Colorado. As the case counts increased quickly in horses in Southern California in Summer 2023, another new development occurred when a wildlife park in the region discovered multiple VSV-lesioned rhinoceros in their herd. Rhinos are a species that had never been identified as susceptible, and, ultimately, all 26 of the park's rhinos became affected and struggled with lesion healing. Some of the rhinos sloughed their foot pads and became badly lame, in addition to not wanting to eat or drink with sloughing tongue mucosa. Rhinoceros share ancient genetic lineage with horses, so perhaps we should have guessed their susceptibility.

Climate change wasn't done surprising us as Hurricane Hillary formed in August and took shockingly rare aim at Southern California. The resulting flooding seemed to flush the vector eggs and larvae out to the Pacific, which stopped the outbreak in Southern California. Cases continued, however, to move northward from the Central Valley and eventually as far as Sacramento County before the cold snaps in early January 2024 finally ended the outbreak. VSV incursion years are often followed by overwintering of the virus, and expansion year outbreaks, so it remains to be seen if this will occur in such a novel outbreak region like California. As a previously drought-riddled region becomes verdant (a new lake has formed in Death Valley!), climate change continues to keep us guessing about what new tricks an old vector-borne disease can perform. A multi-disciplinary research team including USDA, state, laboratory and university collaborators has begun studying the impacts of climate parameters on VSV occurrence hoping to better predict what the virus has in store for us in the future.

Angela Pelzel-McCluskey, DVM, MS
Angela.M.Pelzel-McCluskey@usda.gov
USDA-APHIS-Veterinary Services
Fort Collins, Colorado

The 2022 Kentucky Equine Survey: Measuring a Decade of Change

In any business or industry, good records help decision makers make good decisions. However, in general, the equine industry is one where market data is scarce. Industry-specific studies are not inexpensive, but they are worth the investment. A decade ago, the University of Kentucky and the Kentucky Horse Council joined forces to conduct the 2012 Kentucky Equine Survey, a comprehensive, statewide survey of horses, ponies, donkeys and mules. Fast-forward 10 years: UK and KHC teamed up again to produce the 2022 Kentucky Equine Survey (KyES), allowing us to learn how the industry was impacted by events of the past decade, including emerging from the most significant contraction since the Great Depression and World War II, economic uncertainty following the COVID-19 pandemic and the highest inflation levels seen since 1980. The information generated by these studies is intended to be a resource for a broad set of decision makers, including policymakers, elected officials, entrepreneurs and business owners, researchers, educators, and veterinarians.

Statewide and national equine-specific studies are essential because, due to the diverse nature of equine enterprises, traditional agricultural surveys miss significant parts of the industry. The United States Department of Agriculture (USDA) Census of Agriculture, conducted every five years, measures equine production on "farms," defined as any establishment that has at least $1,000 in cash receipts on the sale of agricultural products annually or at least five equids. This definition largely excludes places that do not engage in the production of horses, ponies, mules or donkeys (breeding and/or selling). As a result, we believe that Census of Agriculture estimates undercount equids in Kentucky by more than 50%.

The purpose of the 2022 KyES was to determine inventory estimates of Kentucky's equine operations and equids, and to measure economic activity on equine operations. Data collection was conducted by the Kentucky Field Office of the National Agricultural Statistics Service, an agency of the USDA. The unit of observation was an "equine operation" (as opposed to a "farm"), which was defined as an address at which at least one equid (horse, pony, mule or donkey) resided. This definition encompasses places including commercial breeding and training facilities, competition facilities, lesson barns and boarding facilities, nonprofit operations and personal residences.

Surveys were distributed by mail to 15,000 equine operations in Kentucky. Of those, 8,337 were returned with usable data (56% response rate). Results from the survey indicate that there were approximately 31,000 equine operations in Kentucky. Farms or ranches comprise the largest type of equine operation (18,000), followed by personal residences (10,000); boarding, training or riding facilities (1,000); and breeding operations (600). Another 1,400 operations indicated other primary functions.

There were an estimated 209,500 horses, ponies, mules and donkeys in the state on July 1, 2022, which is down more than 13% from 2012. Thoroughbreds (48,500) were the most prevalent breed, followed by Quarter Horses (35,000), Walking Horses (28,500), Donkeys and Mules (13,500) and Saddlebreds (12,000). Most equids in Kentucky are used for trail and pleasure riding (62,500), followed by breeding (33,500 broodmares; 22,000 "growing" horses; and 4,000 breeding stallions) and 32,500 equids are idle, retired or otherwise not working. More than 45% of Kentucky's equids are between 5 and 15 years old, with nearly one-quarter (24.8%) over 15 years old; about 20% 1 to 4 years old; and about 10% foals and weanlings. The value of all equids in Kentucky was $6.5 billion. The total value of all assets, including equine and equine-related assets (such as land and buildings, vehicles and equipment and so on), was estimated at $27.7 billion.

In 2021, income from sales of equids was nearly $1.1 billion, while income from equine-related services was $990 million ($450 million from breeding services and $540 million from non-breeding services). Accordingly, total equine-related income from sales and services was almost $2.1 billion 2021. In the same time frame, total equine-related expenditures amounted to $1.6 billion ($535 million in capital expenditures and $1.03 billion in operating expenditures, excluding labor). Of these operating expenses, 82% were spent in Kentucky.

Kentucky's equine operations employed 12,500 workers (6,300 full-time and 6,200 part-time employees), with total payroll expenses of $322 million and non-wage benefits of $30 million. Labor expenses were highest for breeding-related activities (35%), followed by racing (13%), recreation (11%) and competition (9%).

Equine owners were asked to identify which equine health issues are most important. The top three equine health concerns were caring for senior equine (27.1%), musculoskeletal issues (22.4%) and digestive problems (17.4%).

Comparing the results from the last two surveys suggests some strengths: an increase in the value of equids sold; equine operation income increased more than expenses; and a smaller supply of equids, coupled with a decline in non-paid transfers, suggests that the risk of horses becoming unwanted has lessened.

The decline in the number of equids and equine operations in Kentucky is being experienced by all facets of animal agriculture across the nation, providing research opportunities to explore the driving factors of these declines within the entire agricultural industry.

Understanding the number and location of equids is critical to ensuring the health of these animals and the future sustainability of the industry. This information drives provision of veterinary and other business services, research programs and educational outreach. For Kentucky, the 2022 KyES reinforces the economic significance of equine agriculture, which remains vibrant and strong as a signature industry in Kentucky, generating investment, tourism, recreation and positive symbolism for the state.

The full report and supplementary materials (detailed methodology, county fact sheets and additional detailed state- and county-level analyses) are available online at https://equine.ca.uky.edu/kyequinesurvey.

C. Jill Stowe
Professor
Department of Agricultural Economics
jill.stowe@uky.edu

Insulin Dysregulation (ID) and the Link to Laminitis: Why Diagnostic Testing is Important

Over the past two decades, research has improved our understanding of the most common equine endocrine diseases: pituitary pars intermedia dysfunction (PPID, formerly referred to as Equine Cushing's disease), and equine metabolic syndrome (EMS). One of the most critical new findings is that insulin dysregulation (ID) is the key driver of horses developing endocrinopathic laminitis, or now called hyperinsulinemia-associated laminitis (HAL), formerly referred to as founder. As we learn new information about these disorders, we continue to change the guidelines and recommendations for diagnostic testing. If a horse is suspected to have one of these endocrine disorders, timely and accurate diagnostic testing is key for developing a management and dietary plan to lower the risk of these horses developing laminitis. Diagnostic testing and outcomes are not a "one-size-fits-all" model, as there are different metabolic types. Thus, evaluating the complete picture, including both clinical signs of disease and diagnostic testing, is critical.

Brief Review of PPID, EMS and the involvement of ID

ID is the key feature and top concern for equids with EMS, and ID can coexist in equids with PPID. PPID is a progressive and debilitating disease affecting older horses of all breeds with a reported prevalence of more than 20% in horses older than 15 years and more than 25% in horses older than 20 years. PPID is characterized by abnormal growth and function of the pituitary gland in the pars intermedia region, causing abnormal secretion of various hormones.

This then contributes to the clinical signs seen with PPID, which can be varied and include hypertrichosis/hirsutism (overabundant hair growth and failure to shed hair), weight loss, abnormal fat redistribution, muscle wastage/atrophy, lethargy and depression, polyuria/polydipsia (increased drinking and urination) and increased susceptibility to infections such as sinusitis and hoof abscesses being most common.

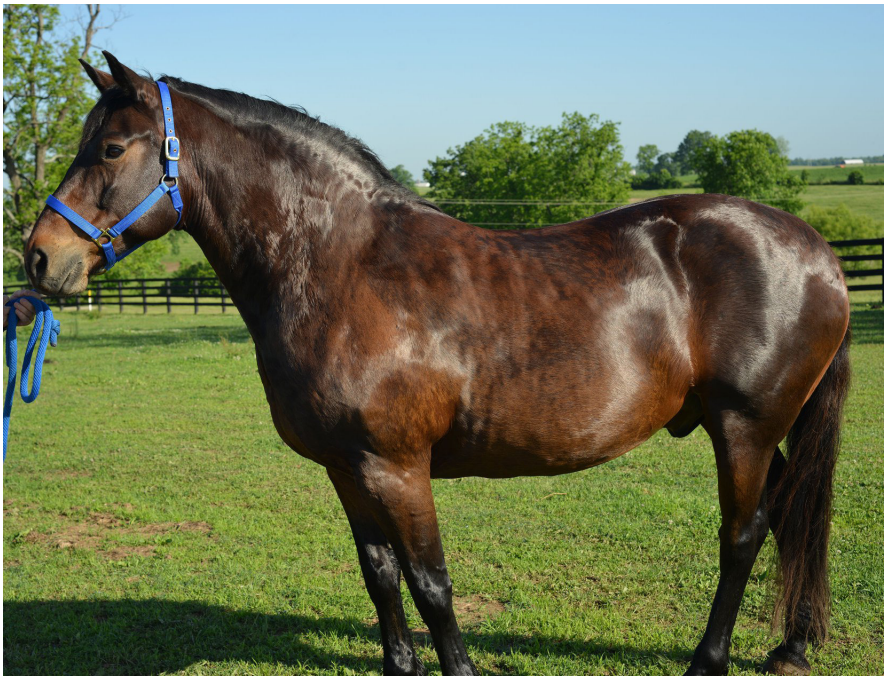
Whilst most PPID cases can occur without EMS/ID being present, PPID can coexist in a subset of horses with both EMS/ID. Horses with PPID and ID will be at a higher risk for laminitis, but at this time the specific link between PPID and ID has not been fully clarified.

EMS is extremely common, with a recent study of ponies and cobs in the United Kingdom reporting a prevalence of 23%. The breeds most at risk of developing EMS include the pony breeds, Spanish breeds, gaited breeds, Morgans, miniature horses and warmbloods. These breeds have a high-genetic risk of developing EMS with only mild environmental influences, although other horses with lower genetic risk can still develop EMS if pushed by environmental factors including diet and lack of exercise. Ultimately, EMS is not a single disease but a collection of risk factors for endocrinopathic laminitis - the leading cause of laminitis. At the core of this group of risk factors is ID and generalized or regional adiposity (cresty neck and subcutaneous deposits of adipose tissue behind the shoulders and at the tail head). That being said, we now know that not all obese animals are ID. Indeed, some animals may have a lean phenotype and are considered the non-obese manifestation of EMS, while these are often horses with PPID and EMS.

ID is characterized by an abnormal, postprandial (after feeding) metabolic response, especially with diets containing increased levels of carbohydrates. It is this abnormal insulin response that increases the risk of horses developing HAL, a painful hoof condition which often results in the need for euthanasia. While increased insulin is concerning when sustained hyperinsulinemia over ~100 uIU/mL is observed, precise cut-offs are unknown at this time. Regardless, the key to preventing laminitis is the appropriate diagnosis of ID and subsequent management changes to maintain healthy postprandial responses to diets by lowering circulating insulin responses.

How to diagnose ID?

One should incorporate diagnostic testing for ID if an equid has history or clinical signs of EMS, or if one suspects an equid to be PPID and ID. ID diagnostic testing should be considered during a wellness or pre-purchase exam, when considering corticosteroid use, or as a tool to help guide decisions with nutritional management and monitoring of an equid that has been diagnosed with ID.



ID is defined as any combination of the following: basal (resting) hyperinsulinemia, postprandial hyperinsulinemia (response to the oral sugar test or consumed feeds) or tissue insulin resistance (IR). The most accepted and practical in-field diagnostic tests for ID are measuring resting (basal) insulin and/or performing dynamic testing using the oral sugar test (OST). Measuring resting or basal insulin requires collecting a single blood sample from an equid in the fed state (hay or pasture, but not grain, which is explained further below); either plasma or serum insulin concentrations are then used to detect resting hyperinsulinemia (HI). This approach is really used for convenience sampling or monitoring, as it has a low sensitivity/high specificity, meaning it can detect more severely affected equids but does a poor job identifying mildly affected equids. Unfortunately, resting insulin levels can be normal in some ID animals, which emphasizes the importance of dynamic testing using the OST. Resting insulin is also useful when assessing postprandial responses to a horse's current diet regimen or monitoring responses to management changes.

The two currently recommended dynamic tests for diagnosing ID are the OST and the insulin tolerance test (ITT). The OST is preferred because the insulin response reflects the natural sequence of events that mimic the response to a meal, including digestion, absorption, hormone responses, secretion of insulin from the pancreas and risk of HAL, versus the ITT which measures hepatic and/or tissue insulin sensitivity. To conduct the OST, it is currently recommended that the equid undergo a three to six-hour fast, followed by administering either a low dose (0.15 mL/kg) or high dose (0.45 mL/kg) of lite corn syrup. Recent research characterizing the OST test has indicated a few important considerations when testing, including the fed vs fasted state of the animal prior to the OST; low vs high dose of corn syrup; season; and stress. For more information about these studies, contact Dr. Amanda Adams (Amanda.adams@uky.edu). Research has shown that winter and spring can exacerbate ID, thus one may avoid this time of year for testing or at least keep this in mind when testing during these times. Currently there are no seasonal reference ranges for diagnosing ID.

In cases where ID is thought to coexist with PPID, diagnostic testing for PPID is important. Today, the most recommended and frequently used diagnostic tests for PPID are 1) measuring baseline plasma ACTH concentrations and/or 2) measuring ACTH concentrations following the thyrotropin-releasing hormone (TRH) stimulation test, which is believed to be the most discriminating method. Determining which test is most appropriate for an individual case should be guided by clinical signs or stage of disease. If early stage is suspected, the TRH stimulation test is preferred. If moderate or advanced PPID is suspected, assessing baseline ACTH concentration may be sufficient. Retesting is recommended if results are not consistent with clinical signs. If results fall within equivocal range, the TRH stim is recommended. Given that season has an impact on ACTH levels, if possible, it's best to avoid fall testing.

For all diagnostic testing purposes (PPID and/or ID), it is critical to send samples to a reputable lab that has established reference ranges for each of the tests. Since not all labs and assays are the same, the same facility for sample testing should be used, especially if comparing results. Additionally, consider reviewing the Equine Endocrinology Group's published guidelines for up-to-date diagnostic testing protocols, and recommendations for reference ranges and interpretation of testing results - https://equineendocrinologygroup.org/.

Additional information regarding the Adams' Lab research going on at the University of Kentucky's Aged Horse Research Center can be found at www.seniorhorsehealth.com.

Dr. Amanda Adams
Associate Professor
Gluck Equine Research Center
University of Kentucky
Email: Amanda.adams@uky.edu

Phone: +1(859) 218-1168