AN ABSTRACT OF THE THESIS OF

Melissa M. Esser for the degree of Master of Science in Veterinary Science presented on September 12, 2013.

Title: Serum Biochemistry and Immunoglobulin Dynamics in Multi-day Endurance Racing Horses

Abstract approved:

Erica C. McKenzie

Endurance riding reflects a relatively new sport among equine athletic disciplines and is growing rapidly. Of all the equine athletic disciplines, endurance riding is the most closely interrelated with veterinary assessment and control to ensure that horses are not exercised beyond their athletic capacity. As endurance riding competition has evolved, the average speeds at which horses typically complete their events have increased. Faster speeds offer greater challenges for veterinarians judging and treating horses in these competitions since horses are at greater risk of potentially life-threatening metabolic compromise or orthopedic injury. Since endurance racing is comprised of prolonged submaximal exercise, often in warm environmental temperatures, participating horses experience a substantial thermal load that must be dissipated largely through evaporative cooling. Competing horses may lose as much as 12 liters of sweat per hour during endurance racing which, without replacement, can result in severe electrolyte

derangements and metabolic compromise. Though multiple studies have evaluated the effect of a single day of racing activity on biochemical variables in endurance horses, the impact of multi-day endurance events has not been assessed.

In addition, the strenuous training and racing schedules endurance horses typically experience may place them at greater risk of infectious disease. Previous studies of other athletic species have identified changes in immune function and infectious disease prevalence with training and competition, including alterations in serum and mucosal immunoglobulin concentrations. Though some studies have evaluated specific aspects of immune function in endurance horses, there have been no studies of immunoglobulin dynamics in response to training and athletic competition in this population.

The purpose of this study was to assess the effects of multiple day endurance activity on serum biochemical values. Additionally, serum immunoglobulin fractions were examined in this group of horses before and during the multi-day endurance event, and were compared to values measured in untrained age and breed matched horses.

Changes in serum biochemistry variables noted in competing horses in the study were mild and reflected the loss of water and electrolytes in sweat, including mild but significant increases in serum urea nitrogen, creatinine, and phosphorus concentrations, which persisted across multiple days. In addition, serum creatine kinase increased significantly after exercise in the horses racing 25 miles and while CK and AST persistently remained elevated over multiple days in the horses racing 50 miles. Bilirubin

concentration and sorbitol dehydrogenase activity also increased, although these elevations were mild. Few horses displayed changes in these variables that exceeded reference intervals. In regard to serum immunoglobulin fractions, serum concentration of immunoglobulin Gb isotype was slightly but significantly higher in endurance horses prior to racing than compared to untrained control horses. The cause for this difference remains undetermined, and there was no significant difference in serum concentrations of the other measured immunoglobulin isotypes (IgA, IgM, IgG(T), IgGa) between trained or untrained horses, or in response to the number of days raced.

The results of these studies help to define the changes in multiple routinely measured serum biochemistry variables during prolonged multi-day endurance exercise and also provide foundation data regarding serum immunoglobulin isotype fractions in endurance horses. In the current study there was no evidence that horses undergoing multiple days of endurance competition have more substantial metabolic derangements than horses racing once, or that endurance horses develop significantly lower serum immunoglobulin concentrations in response to training or racing.

Serum Biochemistry and Immunoglobulin Dynamics in Multi-day Endurance Racing Horses

by
Melissa M. Esser

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented September 12, 2013
Commencement June 2014

Master of Science thesis of Melissa M. Esser presented on September 12, 2013.

APPROVED:

Major Professor, representing Veterinary Science

Dean of the College of Veterinary Medicine

Dean of the Graduate School

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Melissa M. Esser, Author

ACKNOWLEDGEMENTS

The author expresses sincere appreciation to my advisor, Dr. Erica McKenzie, for her guidance with this project and assistance in manuscript preparation.

The technical assistance of Bernadette Stang with the ELISA assays was critical to the success of this thesis, and she consequently deserves my heartfelt gratitude.

The author would also like to thank Dr. Danielle Drennen for assistance with field sample collection and processing.

Financial support was provided by the Oregon State University College of Veterinary Medicine Department of Clinical Sciences.

CONTRIBUTION OF AUTHORS

Dr. Erica McKenzie assisted with project design, field sample collection logistics, and manuscript preparation.

Dr. Mark Payton, Oklahoma State University, assisted with statistical analysis.

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Chapter 1: Introduction

Endurance riding represents a relatively new sport among equine athletic disciplines and is growing rapidly. The American Endurance Ride Conference (AERC) reported 22,956 horses participating in competition in 2007, representing a substantial increase in numbers from1996 when there were 16,590 horses participating. Endurance competition is a relatively well known sport in North America due to the prestigious 100 mile Tevis Cup race that commences in Nevada and ends in California, and which has been held annually since 1955. Other prestigious equine endurance events exist outside of North America and have promoted endurance racing as a global sport. These events include the Tom Quilty Gold Cup in Australia (held since 1966), and the President's Cup in the United Arab Emirates which was established in 2002. Federation Equestre Internationale (FEI) first recognized endurance racing as a competitive equine discipline in 1982, and the Rules for Endurance Events describe the event as 'a test of the competitor's ability to safely manage the stamina and fitness of the horse over an endurance course in a competition against the track, the distance, the climate, the terrain and the clock' (FEI website, 2013).

Of all the equine athletic disciplines, endurance riding is the most closely interrelated with veterinary assessment and control to ensure horses are not exercised beyond their athletic capacity. Typical distances of endurance races range from 80 to 160 km (50 to 100 miles). According to AERC Rules and Regulations, a horse and rider pair have a maximum of 12 hours to complete 80 km, and up to 24 hours to complete 160 km, to avoid disqualification. During racing, horse and rider pairs will

also pass through ‘vet gates’ where a mandatory stop is required of the horse and rider pair to allow veterinary assessment of the horse in order to determine its fitness to continue the event. According to FEI rules each “phase” or loop of an endurance event before a horse encounters a ‘vet gate’ is not to exceed 40 km (25 miles) in distance. For course distances of 80-119 km (50 to 74 miles), there must be at least two vet gates plus a final veterinary examination at completion of the race; for distances of 120-139 km (75 to 87 miles), there must be at least three vet gates plus a final veterinary examination; and for distances of 140 -160 km (87.5 to 100 miles) there must be at least five vet gates plus the final examination. These distances are typically raced over one to two days. As endurance riding competition has evolved, the average speeds at which horses typically complete their events have increased. Although variability of terrain and weather conditions in different parts of the world affect average finish times in individual events, horses competing on courses that are considered relatively flat and fast have achieved average speeds exceeding 25 km/hr over total distances of 120 to160 km (Nagy, et al. 2012). Faster speeds offer greater challenges for veterinarians judging and treating horses in these competitions since horses may be at increased risk of metabolic disorders or orthopedic injury. Events with mountainous terrain and significant cumulative feet of gain also pose challenges for horses and veterinarians, where technically difficult and dangerous trails, extreme elevation changes (such as the 2550 feet of gain encountered over 4.5 miles of distance in the Western States 100 mile ride), and extreme weather changes (with swings of as much as 80^{o}F reported at the Western States 100 mile ride) offer unique tests of the horse’s capacity to maintain metabolic homeostasis and musculoskeletal

soundness.

1.1 Horses

Horses that participate in endurance racing typically comprise a variety of breeds including ponies, Thoroughbreds, sport horse breeds, gaited breeds, and even draft horse crosses. However, most horses competing at an amateur level, and the vast majority competing at an elite level, are Arabian or Arabian-cross. Consequently, the typical endurance racing horse has an average height of 14 to 15 hands (56-60 cm) at the withers, and weighs between 350 to 450 kg. In national-level rides (i.e. AERC sanctioned rides), competition requirements prior to entry in a ride do not exist beyond the need for horses to meet the minimum age requirement (typically five years) and to pass an initial veterinary examination at the race site. The owner/rider maintains responsibility for preparing the horse accordingly for the distance and the speed at which the rider intends to travel the course. This common situation presents the risk of novice riders commencing races for which their horse is not appropriately conditioned, which is further exacerbated by excitement and lack of experience which may result in racing the course at inappropriately high speeds. Therefore horses competing in lower level events may be at risk of greater physical and metabolic stress compared to horses that are selected for competition in larger more prestigious events (Nagy, et al. 2012; AERC website-Rules and Regulations, 2013). Minimum requirements have been established in order to compete within an FEI sanctioned event. The horse and rider

(although not necessarily together) must successfully complete two rides of distances between 40 to 79 km and two rides of between 80 to 90 km at speeds of 16 km/h or less, before successfully completing at least one ride of 80 to 90 km at an unregulated speed. All of these prerequisites must be accomplished within 24 months prior to competing in an FEI sanctioned event.

1.2 Veterinary Control

During endurance racing events, horses are typically examined by a veterinarian multiple times throughout the competition. All horses are examined before commencing the event, at periodic intervals during the event as previously described, and at completion of the event. Horses are also typically examined at any time on the course that they display possible clinical signs of disease or difficulty. During this examination veterinarians are expected to evaluate competing horses for musculoskeletal disease as well as signs of systemic compromise (dehydration, multi-organ dysfunction, hypovolemic shock) or metabolic disorders which are deduced via assessment of heart rate and respiratory rate, cardiac recovery index (Ridgeway, 1994), color and moisture of mucous membranes, capillary refill time, skin tent time, and the presence and intensity of gastrointestinal borborygmi (AERC Guidelines for Veterinary Control Judging Handbook, 2011). Horses can be eliminated from endurance events at any time during the race for a variety of reasons including signs of systemic compromise, musculoskeletal disease or distress. Horses are allowed to

continue after examination if they have been deemed ‘fit to continue’ by the examining veterinarian and are not showing lameness or stiffness reflecting musculoskeletal disease, or clinical signs consistent with systemic illness and/or metabolic imbalances (exhaustion, rhabdomyolysis, colic, synchronous diaphragmatic flutter). Veterinarians therefore play a critical role in maintaining the safety and public perception of endurance riding events, and the collection and provision of appropriate scientific data relevant to the health of the endurance horse represents a critical means of providing information which veterinarians may utilize to make potentially life-threatening decisions.

1.3 Metabolic Disorders

Endurance horses can develop several disorders secondary to dehydration, electrolyte and acid-base derangements, heat accumulation, and substrate depletion, which can result in life-threatening illness. Exhaustion (also called ‘the exhausted horse syndrome’) is due to the compound effects of dehydration, electrolyte imbalances, heat accumulation and substrate depletion (Foreman, 1998). Horses may present for signs such as depression, stumbling or weakness, lameness, hyporexia or anorexia, dehydration, unwillingness to drink, and a facial expression described as ‘grimaced’ or ‘blank’. Mucous membrane characteristics often reflect peripheral congestion, and tachycardia is a common finding, with the heart rate of affected horses rarely dropping below the required 64 beats/minute during a ‘cardiac recovery

index' examination. A 'cardiac recovery index' examination is a brief examination which consists of taking a heart rate, trotting the horse approximately 250 yards, and then taking a second measurement of heart rate one minute after the first. Horses typically will not take the entire minute to travel the designated distance, and during the remainder of the time until the second measurement of heart rate, they may rest. It is ideal for the second measurement of heart rate to be the same or lower than the first, and for the initial heart rate to not exceed 60 beats per minute. Gastrointestinal sounds may be decreased or absent in exhausted horses, and signs of abdominal pain may occur. Other issues including hyperthermia, synchronous diaphragmatic flutter (SDF), and myositis are complicating factors which may concurrently exist. Clinicopathologic abnormalities that commonly arise in affected horses include hemoconcentration (as evidenced by increased packed cell volume [PCV] and serum total protein concentration), decreased blood glucose, elevated muscle enzymes (creatine kinase [CK] and aspartate transaminase [AST]), and increased serum bicarbonate concentration (alkalemia). Additional electrolyte abnormalities that are frequently present include hyponatremia, hypochloremia, hypokalemia, hypocalcemia and hypomagnesemia (Foreman, 1998; Foss and Wickler, 2004).

Sustained exercise in horses leads to the accumulation of substantial heat (Jones and Carlson, 1995). Consequently, the failure to counteract heat accumulation with appropriate thermoregulatory mechanisms can lead to hyperthermia and decreased athletic performance, and can be potentially fatal (Foss and Wickler, 2004). Horses are

at the greatest risk for hyperthermia when the gradient for convective, conductive and radiant heat loss is small. This would be typical for a sunny, hot and humid day, when the only heat loss mechanism that can be appropriately effective is evaporation, a process which is substantially impeded by high humidity. In addition, due to dehydration reducing cardiac output, some horses will lose the ability to sweat effectively, exacerbating hyperthermia. Hyperthermic horses may be tachycardic and may have a shallow, rapid respiratory rate. Rectal temperature often exceeds 104^{o}F (40^{o}C). Borborygmi may be decreased to absent and there may be signs of concurrent dehydration (Foss and Wickler, 2004).

Endurance horses with metabolic imbalance often present with signs of abdominal pain (colic). The etiology is unknown, but is thought to be related to dehydration and acid-base and electrolyte abnormalities contributing to gastrointestinal dysfunction, and promoting ileus. Affected horses can display signs consistent with mild to severe abdominal pain. Decreased to absent borborygmi are typical, and horses are often anorexic and will not drink. Mucous membranes are often dry and may be congested. Laboratory findings are non-specific but may include evidence of metabolic alkalosis, hypokalemia, hypocalcemia, hypochloremia and hemoconcentration (Foss and Wickler, 2004). In a study by Fielding et al., (2009) the primary reason for metabolic elimination was colic, which occurred in 40% of horses eliminated for metabolic disorders during a 100 mile race.

Exertional rhabdomyolysis (muscle necrosis occurring with exercise) is

characterized among the common metabolic reasons for endurance horses to be eliminated from an event. A recent study investigating factors related to elimination of elite endurance horses at a high level event found that 23% of horses eliminated for metabolic problems had evidence of myopathy (Fielding, et al. 2009). In this study, horses that presented for myopathy had pale mucous membranes, decreased or absent borborygmi, and prolonged capillary refill time. Horses may exhibit marked clinical signs of muscular disease including swelling or firmness of major muscle groups (such as the gluteal muscles, lumbar muscles and triceps muscles) or may display signs that are subtle and non-specific such as tachycardia and reluctance to trot. Clinical pathology is helpful in the diagnosis of myopathy which can be made via demonstration of substantially increased serum muscle enzyme activities (Foss and Wickler, 2004). The pathophysiology of exertional rhabdomyolysis in endurance horses has not yet been elucidated, but research is underway (McKenzie and Valberg, unpublished). Horses which have a history of repeated episodes of exertional rhabdomyolysis have been successfully managed by feeding a higher fat, lower starch diet, and providing a regular daily training schedule (McKenzie, et al. 2003).

Synchronous diaphragmatic flutter (SDF, or ‘thumps’) is another relatively common cause of metabolic imbalances in endurance horses, which was reported in one study in 10% of elite endurance horses removed from a 100 mile ride for metabolic problems (Fielding, et al. 2009). Clinical signs of SDF may develop at any time during racing, and the condition is often the sequela to fluid, acid-base, and electrolyte disturbances, particularly hypocalcemia (Foss and Wickler, 2004). The

striking clinical feature of SDF is a rhythmic contraction of the diaphragm and flank that coincides with the heart rate. In affected horses, the heart rate may be elevated, the mucous membranes may be pale, membrane capillary refill time may be prolonged, and gastrointestinal borborygmi will be diminished to absent (Fielding, et al. 2009).

1.4 Equine Serum Immunoglobulins

The immune system is composed of a highly complex organization of cellular and soluble components, which are diffusely located throughout nearly all other organ systems, to provide immunity and protection against microbial infection. The major soluble components responsible for humoral immunity are the immunoglobulins, which are composed of glycoprotein molecules that have a unique ability to combine specifically with antigen. Antibodies are produced by plasma cells, which are end-stage B lymphocytes, in response to foreign substances within the body. A complex interaction between macrophages (antigen presenting cells), T cells and B cells results in the initiating signal for antibody production from a plasma cell. These interactions are under tight control of major histocompatability complex (MHC) II and involve interleukin (IL)-1 and IL-2. Lymphokines involved in B cell differentiation and population expansion also include IL-3, IL-4, IL-5, IL-6, IL-7 and interferon-gamma (Nieman and Nehlsen-Cannarella, 1991). However, this signaling pathway can vary greatly and depends on the initiating antigen. B cell activation may take place without the assistance of T cells; however full development of B cells only takes place in the

presence of T cells and their interleukin products (Tizard, 2009; Janeway, 2001). The primary role of the antibody is to combine with antigens to form free immune complexes which are then quickly removed from circulation, or to combine with tissue, triggering various effector functions such as complement activation and phagocytosis (Janeway, 2001; Nieman and Nehlsen-Cannarella, 1991).
Extensive studies of antibodies and their structure and role in humoral immunity followed their initial discovery by Emil v. Behring and Shibasaburo Kitasato in 1890 (von Behring and Kitasato, 1890). The regular use of equine serum in human patients with infectious diseases and the subsequent complication of serum sickness that arose after repeated injections of hyperimmune equine serum into humans led to fundamental studies of the structure and function of equine immunoglobulins in the 1960's and 1970's (Hill and Cebra, 1965; Weir, et al. 1966; Helms and Allen, 1970; Vaerman, et al. 1971). Equine immunology reappeared as a research interest in the 1990's when characterization of immunoglobulin genes, generation of isotype specific monoclonal antibodies, and the role of antibodies in equine disease and experimental models was explored (Wagner, 2006; Ford, et al. 1994; Home, et al. 1992; Sitnikova and Su, 1998).

These investigations gave rise to the knowledge that equine immunoglobulins are based on a monomer consisting of two long (heavy) and two short (light) polypeptide chains. This basic monomer can be divided into three functional units. Two units, which are called the Fab fragments, have antigen binding activity, while the third unit, called the Fc fragment (fragment crystalline), is associated with binding to

molecules (i.e. complement) and to receptors on cells including macrophages, neutrophils and lymphocytes, to trigger further host defenses. The Fc region, which is located at the terminal end of the immunoglobulin, binds to receptors found on the cell types mentioned above, which subsequently facilitates or stimulates functions such as phagocytosis and antibody dependent cellular cytotoxicity.

The original nomenclature of equine immunoglobulins, especially immunoglobulin G (IgG), was based on their antigenic differences and their serological and electrophoretic properties. Using this methodology, the subclasses IgGa, IgGb, IgGc, IgG(T), and IgG(B) were defined. Currently, most of the early immunoglobulin subclasses have been correlated to their corresponding immunoglobulin heavy chain constant (IGHC) genes. Immunoglobulin M, IgD, IgE and IgA retained their original nomenclature, and have corresponding genes indentified as IGHM, IGHD, IGHE, and IGHA respectively. Seven equine genes encoding gamma heavy chains have been identified (IGHG 1-7), which account for more IgG isotypes than previously assumed to exist (Wagner, et al. 2004). The horse has the greatest number of IGHG genes of any mammalian species examined to date and all seven IgG subclasses appear to be expressed *in vivo* (Wagner, et al. 2004). The previously named IgGa corresponds to IgG1, IgG(T) corresponds to IgG3 and IgG5, IgGb corresponds to IgG4 and IgG7, and IgGc corresponds to IgG6 (Wagner, et al. 2002, 2004). For the purposes of this paper, however, the previous nomenclature (utilizing letters rather than numbers) will be used to remain consistent with the nomenclature used by the manufacturer of the reference and coating antibodies used

for the analyses described in chapter three of this thesis.

Investigation of the effector function capabilities of the IgG subclasses is not only beneficial for the design and production of effective equine vaccines, but also to provide a greater understanding of the equine immune system and its response to infectious disease. According to the work of Lewis, et al. (2008), maximal vaccination protection is achieved through Fc gammaR- and complement mediated elimination mechanisms, which stimulate antibodies of the IgG1, IgG3, IgG4, and IgG7 subclasses. Vaccines that stimulate only IgG2, IgG5 or IgG6 antibodies are predicted to offer less effective protection. Additionally, since IgG plays an important role in serum and mucosal compartments in the horse (Sheoran, et al. 2000), these considerations are applicable to both systemic and mucosal vaccinations.

Previous investigations into the role of individual subclasses in immunity have reported that IgGa and IgGb (i.e. isotypes IgG1, IgG4, IgG7) contribute to protection against equine influenza virus (Nelson, et al. 1998; Breathnach, et al. 2006; Soboll, et al. 2003), *Streptococcus equi* (Sheoran, et al. 1997) and *Rhodococcus equi* (Lopez, et al. 2002). It has also been suggested that IgGb (IgG4 and IgG7) plays the most important role in equine protective antibody-mediated immune responses to intracellular pathogens (Nelson, et al. 1998; Lopez, et al. 2002; Goodman, et al. 2006), where conversely IgGc (IgG6) is suggested to be least important (Sheoran, et al. 2000) The study by Lewis, et al. (2008) helps to confirm these suggestions, demonstrating that both IgG4 and IgG7 are able to stimulate a potent respiratory burst and activate

complement via the classical pathway. Moreover, opsonization by specific IgG is necessary for efficient phagocytosis of *Rhodococcus equi*, *Escherichia coli*, and *Actinobacillus equuli* by neutrophils and alveolar macrophages (Hietala and Ardans, 1987; Grohndahl, et al. 2001; Cauchard, et al. 2004) and IgG mediated neutralization of equine arteritis virus (Balasuriya and MacLachlan, 2004) and acute phase equine herpes virus-1 (Snyder, et al. 1981) is complement dependent. The effector functional capabilities of IgG1, IgG3, IgG4 and IgG7, therefore, are well equipped and play a critical role in protective immunity.

The study of immunoglobulin isotype fractions during exercise may help to define strengths and weaknesses of immune function in endurance racing horses that may be at greater risk of infectious respiratory disease. Additionally, establishing normal values for this subset of the equine population may provide a baseline from which to investigate disease risk in exercising horses, and where vaccines designed to specifically promote certain immunoglobulin isotypes may be beneficial.

1.5 Serum Biochemistry and Exercise

Blood is an essential constituent in the support of the dramatically increased metabolic demands encountered during endurance exercise, and is responsible for the transportation of oxygen, water, electrolytes, nutrients, and hormones to working muscles and tissues, and the removal of carbon dioxide and other waste products (Kingston, 2004). The serum or plasma portion of the blood is typically evaluated for

protein fractions (total protein, albumin, globulin), indicators of renal function (serum urea nitrogen, creatinine), enzymes related to the tissue integrity of muscular and hepatobiliary systems (CK, AST, gamma glutamyl transferase (GGT), sorbitol dehydrogenase (SDH), lactate dehydrogenase (LDH), and bilirubin), electrolytes (sodium, potassium, chloride, bicarbonate), macro minerals (magnesium, calcium and phosphorous), and triacylglycerol (Kingston, 2004; McKenzie, unpublished). Due to convenience of sampling and analysis, serum biochemistry is often an initial diagnostic test that may reveal expected aberrations in athletic horses that are not present in resting or untrained horses.

As a result of exercise, fluid, electrolytes and a small amount of protein can move transiently between compartments in order to maintain homeostasis and as a protective mechanism for cells (McKeever, 2008). These shifts in plasma fluid volume have different mechanisms and degrees depending on the type of exercise performed, and the exercise duration and intensity (McKeever, 2008). As a consequence of fluid shifts, an increase in total protein and albumin concentration may occur in exercising endurance horses (hemoconcentration), with the severity dependent on volume of sweat loss and duration of the event (McKeever, 1998; Convertino, 1987; McKeever, et al. 1993; Harrison, 1985). Initial fluid shifts promoting mild increases in serum protein concentrations are normal and appear to be due to a redistribution of fluid from capacitance vessels to increase venous return and increase cardiac output (McKeever, 1998; Rowell, 1983, 1993; Schott and Hinchcliffe, 1993; McKeever, 1997). Larger

increases in these values are often a sign of dehydration and can be an indicator of systemic compromise (Barton, et al. 2003; Munoz,et al. 2010; Trigo, et al. 2010; Barnes, et al. 2010).

Aberrations of renal function and renal blood flow are also a direct result of decreased plasma volume, and similarly to circulating protein fractions, the degree of alteration is dependent on the duration and intensity of exercise. Acute exercise results in decreased renal perfusion and glomerular filtration rate in order to spare cardiac output, and affects mechanisms associated with tubular reabsorption of water and electrolytes (McKeever, 1998; Zambraski, 1990). Prolonged exercise leading to dehydration can exacerbate these phenomena, resulting in increases in serum urea nitrogen, creatinine, and phosphorus concentrations.

Creatine kinase (CK) is a muscle specific cytosolic enzyme with a short half-life of approximately two hours and which peaks approximately four-to-six hours after a muscular insult (Cardinet, et al. 1967). This enzyme assists the production of an immediate source of energy for muscle cell contraction via the phosphorylation of adenosine diphosphate (ADP) from creatine phosphate (Cardinet, 1997). The enzyme AST is also used as an indicator of muscular damage in horses, and has a prolonged half-life of 7 to 8 days (Cardinet, 1967). However this enzyme is not muscle specific and may be found in many tissues including muscle and liver (Harris, et al. 1990), and therefore must be interpreted in combination with other liver enzymes to accurately determine the contributing organ (Hall and Bender, 2011). Elevations of LDH can also

indicate muscular injury, however this enzyme does not provide tissue specific information unless individual isoenzyme fractions are analyzed (Hall and Bender, 2011). Endurance exercise is known to increase serum CK activity several thousand units per liter in apparently healthy exercising endurance horses (Schott, et al. 2006; Munoz, et al. 2010).

The liver is a diverse organ with a variety of functions. It has a critical role in the metabolism and synthesis of proteins, carbohydrates, and fats, and produces most plasma proteins, including albumin, fibrinogen, clotting factors and many globulins (excluding immunoglobulins). The liver also plays a key role in energy metabolism, via storage of fat and glycogen, with release or metabolism of these substances to provide energy during exercise (Bain, 2011; Hinchcliff, 2008). Though energy supply pathways within the liver are dramatically upregulated during exercise, exercise has minimal impact on serum activity of most liver-derived enzymes. Increases in serum GGT activity were reported in horses after a 160 km endurance race (Rose, et al. 1983). In humans elevations in hepatocellular enzymes have also been reported after ultra-long distance running (Nagel, et al. 1990) and also strenuous exercise (Foigt, et al. 1976).

Electrolyte and mineral balance in the body can also be affected by exercise. The most significant derangements of serum electrolyte and mineral concentrations tend to occur during high intensity or prolonged submaximal exercise events, though differences may be evident between these two forms of exercise due to the nature of

the exercise and resulting fluid/electrolyte shifts or losses. High intensity exercise in horses produces mild to moderate hyperkalemia, marked hypernatremia, and decreased arterial bicarbonate concentrations (Bayly, et al. 2006). These changes are a result of plasma volume contraction as well as metabolic and respiratory acidosis associated with an intense exercise effort, and typically resolve rapidly once exercise ceases (Bayly, et al. 2006). Electrolyte and mineral imbalances resulting from prolonged submaximal endurance exercise are mainly the result of sweat loss. The equine sweat gland is not responsive to aldosterone like the human sweat gland is, and therefore cannot conserve sodium (McCutcheon, et al. 1998). Consequently, the equine sweat gland allows a hypertonic solution containing sodium, potassium, calcium, and high concentrations of chloride to move from the interstitial space to the surface of the skin. This has multiple important benefits. The production of hypertonic sweat facilitates the movement of a greater amount of water outward to support heat loss. Second, the extra salt in equine sweat, in addition to the unique protein 'latherin' that horses secrete, alters the evaporation point to enhance evaporative cooling. This is likely a functionally important aspect, as horses have a greater volume to surface area compared to humans. Unfortunately, the consequence of these adaptations is the potential for large fluid and electrolyte losses (McKeever, 2008) with subsequent systemic effects. Prolonged voluminous sweating in horses promotes hypochloremic metabolic alkalosis, which has been identified in endurance horses with metabolic compromise during racing in several studies (Munoz, et al. 2010; Fielding, et al. 2009;

Barnes, et al. 2010)

Endurance horses experience a multitude of stressors during competition, including inherent physiologic changes in organ function, fluid and electrolyte balance, and immune function. Collectively, many studies have suggested that submaximal endurance exercise may produce alterations in these variables that may place an equine endurance athlete at risk of metabolic imbalance or infectious disease. Therefore establishing immunological and serum biochemical values in horses racing multiple consecutive days, and comparing values between horses that compete successfully and unsuccessfully, may provide information from which to base decisions regarding vaccination for disease, management, and treatment.

Chapter 2: Serum Biochemistry Dynamics in Multiday Endurance Racing Horses

Melissa M. Esser, DVM; Erica C. McKenzie, BSc, BVMS, PhD, DACVIM; Mark Payton, PhD

From the Department of Clinical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331 (Esser, McKenzie).
From the Department of Statistics, Oklahoma State University, Stillwater, OK 74078 (Payton).

2.1 Introduction

Endurance racing requires that horses perform prolonged submaximal exercise, typically over distances ranging from 50 to 100 miles (80-160 km). Prolonged submaximal exercise in horses can increase the metabolic rate by 10- to 20-fold (Rose, et al. 1977). Since 75 to 80% of energy produced by catabolism of stored substrates is converted to heat during physical work (Brody, et al. 1945), competing horses encounter massive thermal loads that must be dissipated mainly through evaporative processes (Carlson, 1985; Kerr and Snow, 1982), promoting fluid losses that can approach 12 liters per hour in volume. Hence competing endurance horses are at substantial risk of potentially life-threatening metabolic illnesses secondary to dehydration, electrolyte and acid-base derangements, heat accumulation and substrate depletion (Foreman, 1998; Fielding, et al. 2011; Fielding, et al. 2009; Fielding and Dechant, 2012). Horses that are exercised at a level of exertion that exceeds their conditioning for prolonged periods of time may achieve physiologic exhaustion (Whiting, 2009; Fielding, et al. 2009; Fielding, et al. 2011), epitomized by a nebulous condition colloquially referred to as 'the exhausted horse syndrome'. Clinical signs and laboratory findings in affected horses typically reflect hyperthermia, fluid and electrolyte losses, acid base disturbances, muscle damage and gastrointestinal dysfunction. Overexertion, suboptimal body composition, low grade lameness, rhabdomyolysis and other potentially occult problems are all factors which might contribute to occurrence of the exhausted horse syndrome (Whiting, 2009). Affected

horses typically display derangements in serum biochemical variables and acid-base status, including hyponatremia, hypochloremia, metabolic alkalosis and hemoconcentration (Whiting, 2009; Trigo, et al. 2010; Viu, et al. 2010; Fielding, et al. 2009). Even elite equine competitors are at risk of severe metabolic disorders, with the prestigious Western States 100 mile race reporting a prevalence of elimination due to severe metabolic disorders of 7.6% in competing horses over a two year span (Fielding, et al. 2009). In another study of a much larger number of horses (Fielding, et al. 2011) the incidence of metabolic disorders was reported to be 4.2%.

Multiple studies have evaluated biochemical abnormalities in horses during single day endurance racing events (Trigo, et al. 2010; Rose, et al. 1983; Deldar, et al. 1982; Barnes, et al. 2010; Barton, et al. 2003; Munoz, et al. 2010) and during training (Rose, et al.1982) to identify relationships between serum biochemical abnormalities and race elimination (Fielding, et al. 2009; Trigo, et al. 2010; Schott, et al. 2006). The results of these studies have repeatedly shown predictable changes in serum electrolyte concentrations in competing endurance horses, including decreases in serum sodium, potassium, chloride and calcium. In a study investigating electrolytes in horses racing 160 km, approximately 23% of the horses removed from competition for a metabolic reason also had significantly decreased sodium and chloride at the mid-ride examination (Barnes, et al. 2010). Another study reporting electrolyte values of 25 horses racing 48 km, 33 horses racing 83 km, and 25 horses racing 159 km, found that compared with preride values, all horses had serum potassium, chloride, bicarbonate,

and calcium concentrations significantly decreased with distance in all groups at certain time points (Barton, et al. 2003). Additionally, Viu, et al. (2010) investigated acid-base imbalances in 30 endurance horses racing 120 km, 17 of which completed the race, three were removed for metabolic abnormalities and 10 horses removed for lameness. This study also reported a mild but significant decrease in blood pH detected with increasing distance, while pCO_2, HCO_3-, tCO_2, and calculated base excess showed an initial increase with a tendency to decrease from the race mid-point to the end. In addition, the effect of distance on electrolyte concentrations, characterized by a modest and sustained decrease in sodium, chloride and ionized calcium with a slight decrease in potassium concentration that recovered by the end of the race. However, the ability to predict elimination for metabolic conditions using serum biochemical variables has not been established, perhaps with the exception of greater likelihood of elimination of horses with higher packed cell volume and total protein measurements (Barnes, et al. 2010; Trigo, et al. 2010; Fielding, et al. 2009). Despite the fact that multi-day endurance racing events exist, only a single study (Munoz, et al. 2010) thus far seems to have investigated changes in biochemical variables over multiple days of racing. Munoz, et al. described the pattern of changes in renin (REN), angiotensin (ANG), aldosterone (ALD) and arginine vasopressin (AVP) concentrations and reported that horses eliminated due to metabolic problems were more dehydrated and had a greater activation of the REN-ANG-ALD-AVP axis. The purpose of the current study was to determine if progression of specific serum biochemical abnormalities

would occur in amateur level endurance horses competing in a multi-day endurance event, and whether changes in serum biochemical variables could be used to predict elimination from an event.

2.2 Materials and Methods

Animals

One hundred and thirty horses were entered in a multi-day endurance race in the Northwestern United States in July, 2011. The course elevation was approximately 4236 ft and consisted of soft footing over mild elevation changes. The temperature range was 31^{o} F to 84^{o} F with relative humidity of 76%, and average wind speed of 5 to 10 miles per hour. The race consisted of three consecutive days of racing distances of either 25 or 50 miles (40 or 80 km, respectively) each day, followed by one rest day, and then three more consecutive days of racing the same distances previously listed. Owners could elect on what day and on how many days they would race, though they did not change distance categories between days of racing. Each day prior to competing, every horse underwent a thorough physical examination by an experienced veterinarian and was only allowed to proceed in the event if they were found to be free from clinical signs of significant disease and lameness (defined as greater than Grade 2 lameness according to the American Association of Equine Practitioners lameness scale) (Anon, 1999).

Sample collection

For the purposes of the current study, serum samples for biochemical analyses were obtained from 54 horses including 44 horses completing the 50 mile distance category and 10 horses completing the 25 mile distance category. Samples were obtained from horses the day prior to commencement of racing and again four-to-six hours after the completion of each day of racing coincident with the expected peak of serum creatine kinase (CK) activity induced by exercise (Hall and Bender, 2011). Blood samples were obtained from each horse on each occasion by jugular venipuncture into 10 mL serum separator Vacutainer® (BD Vacutainer Tubes, Franklin Lakes, NJ) tubes.

Procedures in this study were approved by the Institutional Animal Care and Use Committee of Oregon State University. Participating owners signed an informed consent form prior to being entered into the study.

Samples were allowed to clot and were subsequently centrifuged at 1600 x g for ten minutes. Sample supernatant was removed, placed into labeled 4 mL cryovial (Nalgene® cryovial tubes, Rochester, NY) tubes and stored in a liquid nitrogen dry shipper until the time of analysis. Samples were analyzed at the Oregon State University Veterinary Diagnostic Laboratory within 72 hours of collection using a commercially available blood and serum chemistry analyzer (Hitachi 911, Roche-Boehringer Manheim, Indianapolis, IN). Variables analyzed included serum

concentrations of urea nitrogen (BUN), creatinine, glucose, total protein, albumin, total bilirubin and total carbon dioxide (tCO_2). Serum sodium, potassium, chloride, calcium, phosphorus, and magnesium concentrations were also determined in addition to the activities of creatine kinase (CK), gamma glutamyl transferase (GGT), aspartate transaminase (AST), and sorbitol dehydrogenase (SDH). Serum total globulin concentrations were subsequently calculated for all horses by subtracting each individual horse's measured serum albumin concentration from their measured serum total protein concentration.

Statistical Analysis

Statistical analysis was performed utilizing analysis of variance procedures PROC MIXED of SAS Version 9.3 (SAS Institute, Cary, NC). Fixed effects in the model were distance, time, and their interaction. To account for repeated measures within animals, a first-order autoregressive variance-covariance structure was used.. The simple effects of distance given time and time given distance were tested with significance declared at $p \leq 0.05$. Serum CK activity was log transformed prior to analysis due to non-normal distribution. Race days were excluded from statistical analysis if less than five horses raced on a given day. Hence data was only available for 25 mile category horses before racing and after one day of racing, and for 50 mile horses before racing and after one, two and three days of racing. Where appropriate, values are provided as Least Square Means to adjust for missing data points.

2.3 Results

Of the 54 horses sampled in the current study, all 54 completed at least one day of racing including 44 horses in the 50 mile category and 10 horses in the 25 mile category. Twenty horses completed two days of racing including 18 horses in the 50 mile category and two horses in the 25 mile category. Ten horses completed three days of racing, nine of which were in the 50 mile category and one in the 25 mile category. Three horses, all in the 50 mile category, completed four days of racing, and two of these horses went on to complete a fifth and sixth day of racing. No horses within the study were eliminated from racing at any time, and the decision not to continue additional days of racing was voluntary on the part of the owner.

The initial sample population included a variety of breeds; Arabian (29), Arabian cross (16), Mustang (3), Tennessee Walking Horse (2), Missouri Fox Trotter (2), Pinto (1), and Appaloosa (1) and comprised 37 geldings, 16 mares, and one stallion ranging in age from 5-22 years (median 11 years). Values from three horses were excluded from statistical analyses because they were so abnormal it was concluded they could only represent a handling or laboratory processing error.

Prior to exercise, no significant differences were appreciated in serum biochemical variables between horses racing the 25 and 50 mile distances, with the exception of a minor difference in serum SDH activity ($p = 0.0013$), though mean values in both groups were within the reference interval. Following the first day of

exercise, urea nitrogen ($p = 0.002$) and Mg ($p = 0.04$) concentrations were significantly increased in horses traveling 50 miles compared to 25 miles. Total CO_2 ($p = 0.004$) was also significantly different between the two groups, and was lower in the 50 mile category, however mean values of all variables remained within reference intervals.

Within horses racing the 25 mile distance category, serum globulin concentration ($p = 0.038$) and SDH activity ($p = 0.0008$) decreased significantly from before to after exercise, and serum CK activity increased significantly after exercise ($p = 0.019$) (Table 2.1).

Within horses racing the 50 mile distance category, BUN, creatinine, and phosphorus concentrations increased significantly ($p < 0.0001$) from before to after exercise on the first day and remained persistently elevated during consecutive days of racing (Figure 2.1). Blood glucose concentration was also significantly elevated ($p = 0.0032$) after the first day of racing, though significant differences did not occur between day 1 and days 2 and 3 of racing (Table 2.2). Total protein, albumin and globulin concentrations were also significantly higher ($p = 0.0021$, $p = 0.0025$, $p = 0.0004$, respectively) before racing and after the first day of racing compared to days two and three of racing, with the exception of albumin on day 2 (Figure 2.2). Serum bilirubin concentration was also significantly increased ($p < 0.0001$) each day of racing compared to before racing with a further increase documented on day 3. Total CO_2 decreased significantly ($p = 0.0006$) after the first day of racing though values on day 2 and 3 of racing were not different to before racing (Table 2.2). No significant

differences were identified in the activity of GGT from before to after racing in horses in the 50 mile category. Serum SDH activity was significantly elevated ($p = 0.0008$) after the first day of racing compared to before racing, however, the elevation in SDH was mild and values remained within the reference interval (Table 2.2). Log transformed serum CK activity was significantly higher ($p < 0.0001$) after days 1(6.32 ± 0.17), 2 (6.43 ± 0.3), and 3 (6.05 ± 0.29) of racing compared to before racing (5.21 ± 0.1). Log transformed serum AST activity was significantly higher ($p < 0.0001$) after the first day of racing (5.86 ± 0.08), and increased further after racing days 2 (6.21 ± 0.2) and 3 (5.95 ± 0.14). No significant differences were identified in serum concentrations of sodium, potassium, chloride, calcium or magnesium from before to after exercise in horses racing in the 50 mile category (Table 2.2).

2.4 Discussion

The results of the current study demonstrated multiple mild changes in a variety of serum biochemistry variables in endurance horses racing 25 miles for one day, and 50 miles for three days. Observed changes were consistent with those described in previous reports and largely reflected decreases in circulating plasma volume with exercise. Changes in specific serum biochemical variables were more pronounced in horses that traveled 50 miles in distance compared to 25 miles, but did not progress significantly in horses racing 50 miles in distance over multiple days.

Serum biochemistry changes that were most consistent with decreased

circulating volume and subsequent deceases in glomerular filtration included mild elevations in serum concentrations of urea nitrogen, creatinine, and phosphorus from before to after racing. These changes were more prominent in horses traveling 50 versus 25 miles, but were no different when horses raced 50 miles over one, two or three days consecutively, suggesting that healthy endurance horses maintain homeostasis in the face of repeated exercise efforts. Although it was fortunate that no horses sampled during this study failed to complete their event or suffered significant metabolic compromise during racing, it also negated any comparison between successful finishers and horses that failed to finish as a result of disruption of these homeostatic mechanisms. As discussed in the introduction, previous studies investigating biochemical indicators of metabolic elimination in large numbers of horses experiencing single endurance exercise bouts have also had limited success in identifying predictors of metabolic elimination, suggesting that investigation of alternative and practical markers to identify vulnerable horses during endurance competition is warranted.

Horses competing in the 50 mile distance category also displayed significantly increased serum glucose concentrations after the first day of racing, which exceeded the reference interval. This change was attributed to a combination of excitement and anxiety on the first day in horses that had been rested for their competition effort, in addition to reflecting hormonal alterations stimulated by exercise that promote glycogenolysis in liver and muscle tissue, increasing circulating blood glucose concentrations (Poso, et al. 2008). It is possible that higher

serum glucose concentrations on day one of racing compared to subsequent days might reflect differences in glycogen and glucose metabolism and utilization on the first day compared to subsequent days of repeated prolonged submaximal exercise (Poso, et al. 2008). In exercising sled dogs, muscle glycogen utilization on the first day of prolonged exercise is substantial, but reliance on muscle glycogen stores quickly diminishes with repeated exercise efforts on consecutive days in favor of alternative substrates (McKenzie, et al. 2005). The pattern of substrate utilization in endurance horses with consecutive days of exercise effort is currently unknown but worthy of investigation, which would require concurrent collection of blood and muscle tissue to fully elucidate.

Serum bilirubin concentration also increased in horses racing 50 miles on consecutive days. Decreased feed intake is the most common cause of increased serum bilirubin concentration (predominantly unconjugated bilirubin) in horses (Bain, 2011), and it is likely that increased time spent in athletic activity may have contributed to reduced feed intake in horses in the current study, though feed intake was not assessed. Also, though serum bilirubin concentrations increased in racing horses, values remained within the reference interval and were therefore unlikely consistent with substantial cholestasis. Long distance exercise has however, previously been demonstrated to impact liver activity in several species, reflected by alterations in serum bilirubin as well as hepatic enzyme activities. Long distance racing sled dogs display elevations in serum ALP and ALT after exercise, and long distance human runners have demonstrated substantial increases in serum GGT, ALP

and GLDH activities, reflecting both hepatocellular and cholestatic consequences of long distance exercise (McKenzie, et al. 2007; Fallon, et al. 1999; Nagel, et al. 1990). Horses competing over 50 miles in the current study had no significant alterations in serum GGT activity, but displayed a mild increase in serum SDH activity after the first and second day of racing, though values remained within reference interval. However, this enzyme is particularly labile in nature, even in frozen samples, so despite careful handling processes in the field and laboratory, and analysis within 72 hours, it is probable that the analyzed values did not reflect SDH activity at the time of collection, and values might also have been higher if blood had been collected immediately after racing ceased. Sample collection was limited in the current study to four hours after racing ceased; however multiple sampling points following exercise efforts in endurance horses would likely enhance the ability to detect changes in specific biochemical variables. Nonetheless, the increase in serum SDH activity that occurred in horses after racing 50 miles may reflect mild hepatocellular damage. In one study of human ultra-distance runners, transient but large elevations of the hepatocellular enzyme GLDH were reported in more than 50% of competitors, supporting the concept that prolonged endurance exercise causes some degree of hepatocellular damage (Nagel, et al. 1990). In addition, a study by Foigt, et al. (1976) reported increases in hepatocellular enzymes such as alanine aminotransferase (ALT), SDH, and isocitrate dehydrogenase (ICDH) during strenuous physical exercise in humans. Collectively these findings indicate that prolonged exercise commonly induces changes in hepatocellular and cholestatic enzyme activities in athletes, with

minimal impact on hepatic function.

Changes in serum protein fractions including total protein, albumin and globulin have also been reported to occur with prolonged and repeated endurance exercise in a variety of species. In the current study, horses racing 50 miles over consecutive days displayed a mild decrease in serum total protein, serum albumin and serum globulin fractions by the second or third days of racing, though mean values remained within respective reference intervals. Horses racing 25 miles on a single occasion displayed a mild decrease in serum total globulin concentrations only. Racing endurance horses sampled immediately after exercise typically display negligible change or increases in serum protein fractions, which can become pronounced in compromised horses, reflecting decreased plasma volume. Increases in serum protein concentration with exercise however are typically transient in horses, and resolve within hours as internal fluid shifts occur (McKeever, 2008; Fielding, et al. 2009; Schott, et al. 2006), and the timing of sampling in this study likely negated observation of the initial phase of this typical phenomenon. Of interest however is the decline in serum protein fractions with repeated exercise in horses in the current study. The impact of repeated endurance exercise on serum protein fractions has not been studied in endurance horses to the author's knowledge, but in sled dogs undergoing consecutive bouts of endurance exercise, a similar phenomenon is observed with gradual decline in serum total protein, albumin and globulin concentrations over time (McKenzie, et al. 2007). Proposed contributions to the decline in these protein

fractions has included immunosuppressive effects of prolonged exercise, expansion of plasma volume in preparation for repeated exercise efforts, catabolism of proteins for energy supply, and gastrointestinal loss. Studies in humans (Gleeson, et al. 1995; Mashiko, et al. 2004; Saygin, et al. 2006; Umeda, et al. 2004) and dogs (McKenzie, et al. 2009) have demonstrated evidence for immune compromise with decreased serum and mucosal immunoglobulin fractions. In addition, decreased lymphocyte function in endurance horses has been demonstrated after stimulation with mitogen suggesting that endurance horses are at risk of immunosuppression (Cywinska, et al. 2010). Inter-compartmental fluid shifts occur after commencement of exercise, and become more pronounced with endurance exercise as the result of fluid losses into sweat. In addition, small plasma protein shifts also occur in conjunction with the fluid and electrolyte redistribution. In endurance horses exercising multiple days in a row, compensatory increases in plasma volume may occur in preparation for further exercise, contributing to depression of circulating plasma protein concentrations (McKeever, 2008).

In horses racing 25 miles on one day and 50 miles on repeated days, negligible changes occurred in serum electrolyte and mineral concentrations. Changes were limited to a slight decrease in serum total CO_2 concentration on the first day of racing in 50 mile horses, suggesting a transient mild decline of serum bicarbonate concentration in this group, which has been previously reported in endurance horses in single day events (Barton, et al. 2003). Profound decreases in total CO_2 reflecting

severe metabolic compromise can occur in endurance horses but were not observed in any individuals in the current study. (Foreman, 1998; Whiting, 2004). Previous studies (Schott, et al. 2006; Barnes, et al. 2010; Barton, et al. 2003) have demonstrated hyponatremia, hypochloremia and metabolic alkalosis in endurance horses competing races of 100 miles in length, reflecting the unique tendency of the horse to lose massive amounts of hypertonic sweat rich in sodium and chloride. The minor alterations observed in the current study likely reflect the shorter race distance, and possibly more tentative or conservative racing strategies given the lower stakes of amateur competition or the knowledge that effort would need to be conserved for repeated days of exercise, ultimately reducing volume and electrolyte loss required for evaporative cooling. Endurance riders also very frequently supplement horses before, during and after racing with a variety of electrolyte products which may help to reduce changes in serum concentrations of these variables over time. Electrolyte supplementation by owners occurred in this study, but given the frequency with which it occurred, and the variety of products employed, it was deemed impossible to evaluate the effects of such supplementation in the current study. Nonetheless, horses racing 50 miles in distance on repeated days demonstrated negligible changes in serum electrolyte and mineral concentrations, suggesting that homeostatic mechanisms, potentially in addition to owner ministrations, were effective at maintaining serum electrolyte and mineral concentrations in a race distance of 50 miles, even when performed on multiple consecutive days.

Serum AST and CK activities were measured in the current study as indicators of muscle damage, and heavily influenced the timing of sample collection since

rhabdomyolysis is one of the common key indicators of metabolic compromise in horses performing endurance racing activity. Endurance exercise commonly elicits mild to moderate increases in serum CK activity even in healthy endurance horses, though values usually do not increase beyond several thousand units per liter (Schott, et al. 2006; Munoz, et al. 2010). In the current study, serum CK values of horses after competing both 25 and 50 miles in distance were significantly higher than before racing. However, values after racing 25 miles in distance (mean $\pm$ SE; 429 $\pm$ 74 U/L) were typically within the reference interval (145-633 U/L) apart from slightly increased values in two horses (which remained < 1000 U/L). In contrast, mean serum CK and AST in horses competing in the 50 mile distance category was considerably higher. Serum CK values after racing on days 1, 2 and 3 were 2224 $\pm$ 1474 U/L, 1322 $\pm$ 670 U/L, and 700 $\pm$ 306 U/L, respectively. Mean serum AST values after racing on days 1, 2, and 3 were 448 $\pm$ 93 U/L, 720 $\pm$ 247 U/L, and 422 $\pm$ 67, respectively. Sixteen of 44 horses in this group displayed elevations in serum CK that exceeded the reference interval, with 14 horses exceeding 1000 U/L, reflecting greater muscle injury accrued through longer racing distance. In addition, serum AST activity increased significantly and progressively as the number of days raced accumulated; patterns which have also been reported in humans (Fallon, et al. 1999) and racing sled dogs (McKenzie, et al. 2007). Mean serum CK in racing horses tended to be greatest on the first day of racing, and declined with consecutive exercise bouts. A similar phenomenon has been demonstrated in long distance running humans and sled dogs suggesting adaptation of muscle tissue and function to repeated exercise tasks (McKenzie, et al. 2005, 2008; Ross, et al. 1983).

Of concern is the fact that two horses in the 50 mile group had serum CK values after the first day of racing that were abnormally high for competing endurance horses and suggestive of exertional rhabdomyolysis (measuring 6,619 U/L and 60,831 U/L respectively). However, both horses continued the event, competing an additional two days and one day of racing respectively after passing physical examination procedures by race veterinarians, and subsequently displayed a decline in serum CK values indicating rhabdomyolysis was subsiding. Continuing competition in the face of occult unrecognized exertional rhabdomyolysis is a common concern in endurance riding events since exacerbating existing muscle damage could contribute to metabolic compromise and severe renal damage from the combination of dehydration and myoglobinuria. Neither of these horses displayed increases in serum creatinine concentration nor were noted by their owners to have clinical signs of muscle damage, but horses were not examined beyond the end of the race. It was not possible to determine if muscle injury in these horses (one Arabian and one Arabian cross) was a sporadic phenomenon or caused by an underlying muscular disorder that might promote repeated episodes of muscle necrosis with exercise as commonly seen in other equine breeds. However exertional rhabdomyolysis is known to be a common condition in racing endurance horses, occurring with a purported prevalence of 4% (McKenzie, et al. unpublished) matching the prevalence documented in the current study. Investigation of this phenomenon in the Arabian breed is currently underway.

In conclusion, the results of the current study reflect mild changes in a variety

of serum biochemical variables that are largely consistent with results of similar studies evaluating high level endurance horses competing for a single day. Aberrations that were noted were consistent with the effects of prolonged submaximal endurance exercise, and were persistent, but not progressive, when multiple consecutive days of exercise were performed suggesting that healthy horses stabilize serum biochemical variables with repeated days of competition. This study was limited to some degree by sample numbers and the relatively short race distances that reduced the risk of developing metabolic compromise, however assessment of typically analyzed biochemical variables in competing endurance horses appears to be a weak method of detecting horses vulnerable to metabolic compromise, with the exception of creatine kinase activity. Future efforts should focus on identifying superior diagnostic methods of evaluating endurance horses for metabolic compromise and muscular damage.

	Pre	Post	P value	Reference
SDH U/L	6.9 ± 0.1	3.9 ± 0.8	0.0008	2.4-7.2
CK U/L	5.18 ± 0.1	5.91 ± 0.19	0.019	
Globulin g/dL	3.2 ± 0.1	3.0 ± 0.1	0.038	2.5-4.0
T. protein g/dL	6.7 ± 0.1	6.4 ± 0.1	0.052	5.9-7.6

Table 2.1: Serum biochemistry values for endurance horses traveling 25 miles. CK values are log transformed.

	Pre-race	Day 1	Day 2	Day 3	P value	Ref Range
BUN mg/dL	16 ± 0.4^{a}	22 ± 0.7^{b}	23 ± 1.1^{b}	21 ± 1.2^{b}	<0.0001	8-23
Creat mg/dL	0.9 ± 0.02^{a}	1.1 ± 0.02^{b}	1.0 ± 0.05^{b}	1.0 ± 0.04^{b}	<0.0001	0.9-1.7
Glucose mg/dL	103 ± 3^{a}	118 ± 5^{b}	103 ± 5^{ab}	91 ± 4^{a}	0.0032	77-109
TP g/dL	6.6 ± 0.1^{a}	6.6 ± 0.1^{a}	6.3 ± 0.1^{b}	6.4 ± 0.2^{b}	0.0021	5.9-7.6
Albumin g/dL	3.5 ± 0.1^{ab}	3.5 ± 0.1^{a}	$3.4 \pm 0.1b^{c}$	3.3 ± 0.1^{c}	0.0025	2.9-3.8
Globulin g/dL	3.1 ± 0.1^{a}	3.1 ± 0.1^{a}	3.0 ± 0.1^{b}	3.1 ± 0.2^{b}	0.0004	3.0-3.8
Bilirubin mg/dL	0.9 ± 0.1^{a}	1.2 ± 0.1^{b}	1.3 ± 0.2^{c}	1.3 ± 0.2^{c}	<0.0001	0.8-2.6
CK U/L	5.21 ± 0.1^{a}	6.32 ± 0.17^{b}	6.43 ± 0.3^{b}	6.05 ± 0.3^{b}	<0.0001	
GGT U/L	15 ± 0.7	15 ± 0.7	12 ± 1.0	13 ± 1.1	0.9468	7-25
AST U/L	5.66 ± 0.06^{a}	5.86 ± 0.08^{b}	6.21 ± 0.2^{c}	5.95 ± 0.14^{c}	<0.0001	
Sodium mEq/L	136 ± 0.5	136 ± 0.4	137 ± 0.5	136 ± 0.7	0.5171	133-142
Potassium mEq/L	3.9 ± 0.1	3.6 ± 0.1	3.9 ± 0.2	4.1 ± 0.1	0.0529	2.5-4.7
Chloride mEq/L	101 ± 0.5	100 ± 0.6	101 ± 1.1	101 ± 1.1	0.1695	94-105
Calcium mg/dL	11.5 ± 0.1	11.6 ± 0.1	11.4 ± 0.2	11.2 ± 0.2	0.2501	11.5-13.3
Phos mg/dL	2.5 ± 0.1^{a}	3.5 ± 0.1^{b}	3.8 ± 0.3^{b}	3.5 ± 0.2^{b}	<0.0001	1.9-4.1
Mag mg/dL	1.9 ± 0.03	1.9 ± 0.03	1.9 ± 0.05	1.9 ± 0.07	0.6866	1.7-2.9
tCO2 mEq/L	25.3 ± 0.4^{a}	23.8 ± 0.4^{b}	24.7 ± 0.8^{a}	25.2 ± 0.5^{a}	0.0006	21.4-30.2
SDH U/L	3.6 ± 0.2^{a}	4.7 ± 0.3^{b}	3.6 ± 0.3^{ab}	3.4 ± 0.4^{a}	0.0008	1.54-4.72

Table 2.2: Serum biochemistry values reported for horses competing in the 50 mile category. Data displayed as mean $\pm$ SEM. Different superscripts (a, b, or c) indicate statistically significant differences in values across a row. Pre-race: n = 51; Day 1: n = 51; Day 2: n = 17; Day 3: n = 8. CK and AST values are log transformed.

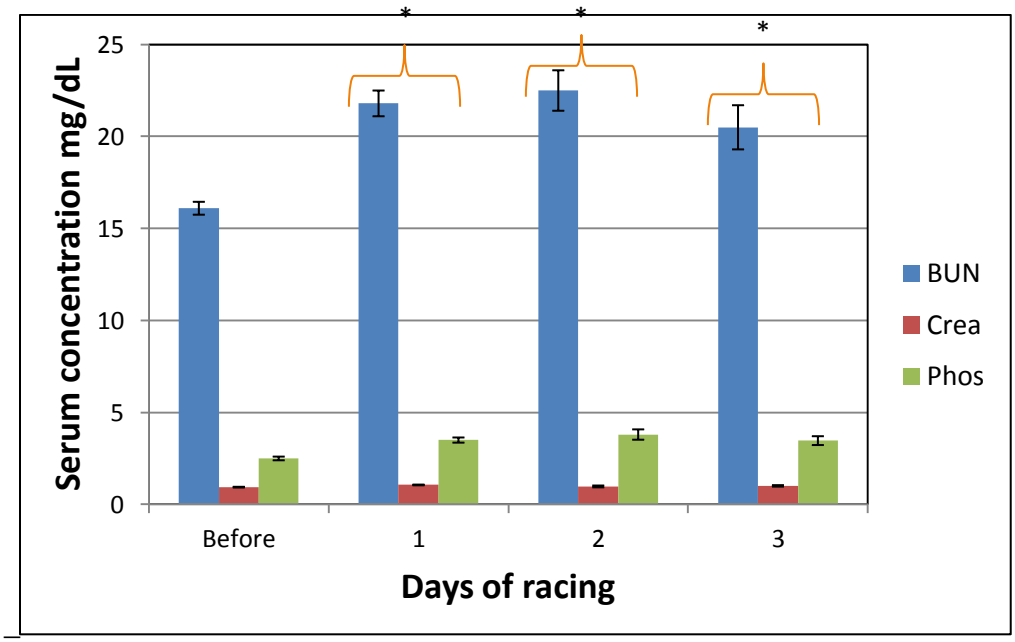

Figure 2.1: Serum concentrations of urea nitrogen (BUN), creatinine, and phosphorus in endurance horses before and after racing 50 miles in distance on three consecutive days.
* Indicates significant increase above the pre-race samples

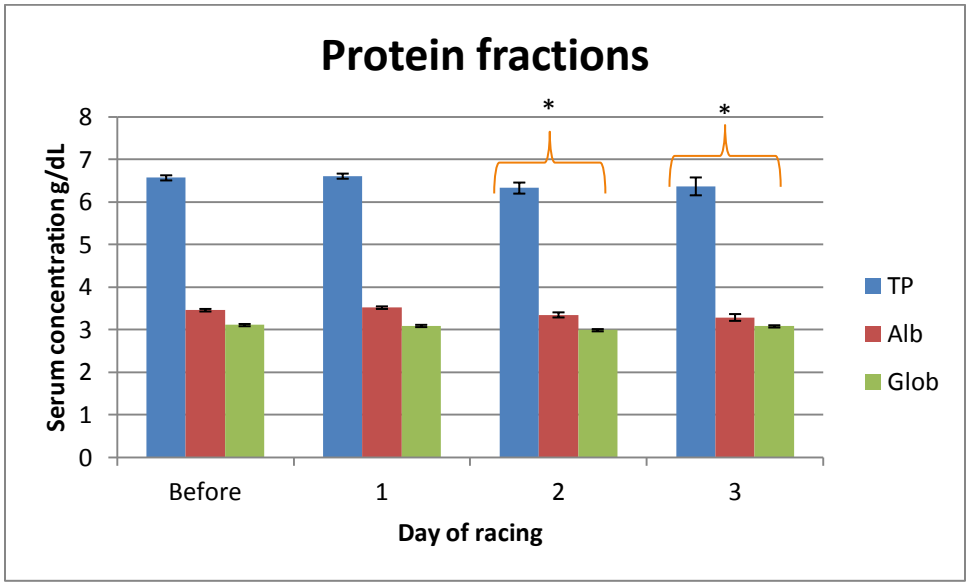

Figure 2.2: Serum concentrations of total protein (TP), albumin (Alb), and globulin (Glob) in endurance horses before and after racing 50 miles in distance on three consecutive days. * Indicates significant decrease from before racing and day 1 values.

Chapter 3: Serum Immunoglobulin Dynamics in Multi-day Endurance Racing Horses

Melissa M. Esser, DVM; Erica C. McKenzie, BSc, BVMS, PhD, DACVIM; Mark Payton, PhD

From the Department of Clinical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331 (Esser, McKenzie).
From the Department of Statistics, Oklahoma State University, Stillwater, OK 74078 (Payton).

3.1 Introduction

Exercise stress has long been recognized to have potentially detrimental effects on immune function, which may increase the vulnerability of athletes to infectious disease. The type of exercise performed, in regard to whether it represents acute high intensity exercise or prolonged submaximal exercise appears to have different effects on the immune system which have been studied in a variety of species including humans, mice, horses, and dogs (Verde, et al. 1992; Nieman, et al. 1989; Mackinnon, et al. 1989; Liu, et al. 1987; Nehlsen-Cannarella, et al. 1991a; Papa, et al. 1989; Eskola, et al. 1978; Nehlsen-Cannarella, et al. 1991b; Cywinska, et al. 2010; Wong, et al. 1992; Buschmann and Baumann, 1991). A study by Liu, et al. (1986) revealed an enhancing effect of exercise on immune function in mice, where moderate exercise in mice resulted in production of antibody titers to *Salmonella typhi* immunization that were more than two times that of sedentary controls. A similar phenomenon was observed in trained human marathon runners who demonstrated increased antibody production after vaccination with tetanus (Eskola, et al. 1978) compared to non-trained subjects. Furthermore, in a study by Verde, et al. (1992), increases in IgG and IgM synthesis were reported in humans after heavy exercise. Conversely, prolonged, repeated submaximal or high intensity exercise has been demonstrated to have a variety of adverse effects on immune function in humans and animals. Lymphocyte stimulation is impaired in humans and mice exposed to prolonged exercise challenge. A study by Hedfors, et al. (1983) reported decreased production of serum IgG, IgM and IgA after lymphocyte stimulation in athletes

undergoing a standardized bicycle ergometer test. Similarly, a study of chronically exercised mice revealed reduced lymphocyte proliferative response in comparison with sedentary mice, which was exacerbated when the chronically exercised mice were challenged with acute exhaustion exercise (Hoffman-Goetz, et al. 1986). Endurance exercise in horses has been shown to have significant and prolonged adverse effects on neutrophil function (Lejune, et al. 2010; Robson, et al. 2003; Donovan, et al. 2007; Jensen-Waern, et al. 1999; Cywinska, et al. 2010). However, perhaps one of the most extensively investigated phenomena in regard to immune function in human athletes has been the impact of exercise on serum and mucosal immunoglobulin fractions (Gleeson, et al. 1995; Mashiko, et al. 2004; Saygin, et al. 2006; Umeda, et al. 2004). Similarly, in a study by McKenzie, et al. (2009) a striking decrease in serum immunoglobulin G concentration was observed in 30% of trained and racing Alaskan sled dogs, which resolved after dogs were rested from exercise for four months. In addition, resting salivary IgA concentrations in elite cross country skiers were shown to be significantly lower than controls (Tomasi, et al. 1982), though trained competitive cyclists were found to have salivary IgA concentrations no different to those of control subjects (MacKinnon, et al. 1989).

While some specific aspects of immune function have been investigated in endurance horses, (Lejune, et al. 2010; Robson, et al. 2003; Donovan, et al. 2007; Jensen-Waern, et al. 1999; Cywinska, et al. 2010; Art, et al. 2006; Franck, et al. 2010), these studies have been limited to assessments of neutrophil and lymphocyte proliferation in

response to mitogen. To the authors' knowledge, no studies exist investigating the effects of training and racing on serum immunoglobulin fractions in racing endurance horses. Endurance horses are commonly transported long distances to competitions, comingle with unfamiliar horses of variable vaccination and disease status, and are exposed to significant physiologic and psychological stressors, all of which may compromise their resistance to infectious disease. Therefore the objective of the current study was to compare the concentration of specific serum immunoglobulin fractions, including IgA, IgM, IgG(T), IgGa, and IgGb in trained endurance horses competing a multi-day endurance race to concentrations measured in non-conditioned healthy horses of similar ages and breeds.

3.2 Materials and Methods

Animals

One hundred and thirty horses were entered in a multi-day endurance race in the Northwestern United States in July of 2011, which consisted of three consecutive days of racing distances of either 25 or 50 miles per day, followed by one rest day, and then three more consecutive days of racing the same distances previously listed. The course elevation was approximately 4236 ft and consisted of soft footing over mild elevation changes. The temperature range was 31^{o} F to 84^{o} F with relative humidity of 76%, and average wind speed of 5-10 miles/hour. Owners could elect on what day and on how many days they would race, though they did not change distance categories during the

event. Each day prior to competing every horse underwent a thorough physical examination by an experienced veterinarian and was only allowed to proceed in the event if they were found to be free from clinical signs of significant disease and lameness (defined as greater than Grade 2 lameness according to the American Association of Equine Practitioners lameness scale) (Anon, 1999).

Sample Collection

For the purposes of the current study, serum samples for immunoglobulin analyses were obtained from 54 horses including 44 horses competing in the 50 mile distance category and 10 horses competing in the 25 mile distance category. A group of fifteen age and breed matched horses used periodically for light pleasure riding were selected to act as a control group. These horses had not been trained for endurance competition or other athletic disciplines within the past three years, and comprised 6 geldings, 1 stallion, and 8 mares, with an age range of 4 to 28 years (median age 10 years). Breeds represented within this group included Arabian (9), Arabian-cross (4), Quarter Horse (1), and Mustang (1). Comparison with this control group of horses was considered necessary for appropriate evaluation of serum immunoglobulin fractions given the potentially slow time course of change in these variables compared to the more rapid dynamics of traditionally studied biochemical variables (which typically allow each individual to act as their own control via comparison to pre-exercise values). Samples were obtained from horses the day prior to commencing any racing, and again four-to-six hours after the completion of each day of racing, coincident with collection of samples

for serum biochemical analyses. Blood samples were obtained from control horses at rest on a single occasion. All blood samples were obtained by jugular venipuncture into 10 mL serum separator Vacutainer® (BD Vacutainer® Tubes, Franklin Lakes, NJ) tubes.

Procedures in this study were approved by the Institutional Animal Care and Use Committee of Oregon State University. Participating owners signed an informed consent form prior to being entered into the study.

Blood samples were allowed to clot after collection and were subsequently centrifuged at 1600 x g for 10 minutes. Sample supernatant was removed, placed into labeled 4 mL cryovial (Nalgene® cryovial tubes, Rochester, NY) tubes and stored in a liquid nitrogen dry shipper for less than 72 hrs, at which point samples were stored at -80 ^{o}F until analysis. Serum concentrations of IgGa, IgGb, IgG(T), IgA, and IgM were determined using a previously validated commercially available horse specific enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Bethyl Laboratories Inc, Montgomery, TX.). Briefly, 96-well microtiter plates were coated with a 1:100 dilution of the appropriate goat anti-horse immunoglobulin isotype antibody. The plates were washed vigorously five times, and subsequently treated with a blocking solution (Tris buffered saline, 1% BSA) and refrigerated overnight at 4^{o} C. The plates were washed five times vigorously before serum samples were added. Serum samples were diluted in sample dilution buffer (Tris buffered saline, 1% BSA, 0.05% Tween 20) at 1:100,000 to 1:500,000 for IgGa, 1:500,000 to 1:1,000,000 for IgGb, 1:100,000for IgG(T), 1:10,000 for IgA, and 1:10,000 for IgM. One hundred microliters

of diluted sample was added in duplicate to each well and incubated for one hour at room temperature. An 8-point standard curve, including a blank negative, was included in duplicate in each plate and for each immunoglobulin isotype. After incubation, plates were washed vigorously five times and horseradish peroxidase (HRP)-conjugated goat anti-horse immunoglobulin isotype diluted in dilution buffer (described above) (1:10,000 for IgGa, 1:60,000 to 1:100,000 for IgGb, 1:50,000 for IgG(T), 1:100,000 for IgA, and 1:35,000 for IgM) was added to each well. Plates were incubated for one hour at room temperature and then washed vigorously five times. After washing, HRP substrate was added to each well and plates were incubated in the dark until the highest standard was deep blue and measured approximately the expected absorbance reported in the manufacturer's instructions, which took 5 to 10 minutes. The reaction was quenched with 1 M H_2SO_4 and plates were read at 450 nM (yellow) with an ELISA plate reader (Thermo Scientific Multiskan™ GO Spectrophotometer, Waltham, MA). The concentration of the sample was determined based on the standard curve values (using only values that fell within the linear portion of the standard curve) and multiplying the concentration of the corresponding OD reading by the sample dilution. The cubic polynomial equation produced the best fit for all immunoglobulin reference isotypes, except IgGa with which the quadratic polynomial equation was used (Thermo Scientific™ SkanIt Software, Waltham, MA). The standard curve was accepted if the coefficient of determination (R^2) was > 0.994. All samples were analyzed in duplicate and individual sample pairs were retested when coefficients of variation (CV) exceeded

10%. The inter-plate CV was performed by selecting one control horse sample (C11; which was excluded from control group analysis due to lack of qualification for age match) and dividing the sample up in to multiple aliquots to be stored frozen with the other samples until used in each new plate. This sample was analyzed in duplicate, as the other samples were, and the value was declined if the duplicate CV exceeded 10%.

Statistical Analysis

Analysis was performed utilizing analysis of variance procedures in PROC MIXED of SAS Version 9.3 (SAS Institute, Cary, NC). Fixed effects in the model were distance, time, and their interaction. To account for repeated measures within animals, a first-order autoregressive variance-covariance structure was used.. Planned contrasts of time given distance, as well as control versus assessment group at time 1, were assessed. Significance was declared at $p \leq 0.05$. Race days were excluded from statistical analysis if less than five horses raced on a given day. Hence data was only available for 25 mile category horses before racing and after one day of racing, and for 50 mile horses before racing and after one, two and three days of racing.

3.3 Results

Of the 54 horses sampled in the current study, all 54 completed at least one day of racing including 44 horses in the 50 mile category and 10 horses in the 25 mile category. Twenty horses completed two days of racing including 18 horses in the 50 mile category

and 2 horses in the 25 mile category. Ten horses completed three days of racing, nine of which were in the 50 mile group and one in the 25 mile group. Three horses completed four days of racing 50 miles, and two of these horses went on to complete a total of six days of racing 50 miles. No horses were eliminated from racing during the study, and horses that did not progress through subsequent days of racing in the event were removed voluntarily by the owners.

The initial sample population included Arabians (29), Arabian crosses (16), Mustangs (3), Tennessee Walking Horses (2), Missouri Fox Trotters (2), and one Pinto (1) and one Appaloosa (1). Horses comprised 37 geldings, 16 mares, and one stallion ranging in age from 5-22 years (median 11 years).

Distance category (25 or 50 miles) and number of days raced was found to have no significant impact on serum concentrations of any immunoglobulin subtype (Table 3.1). Serum concentration of IgA in the 50 mile group was 1.97 ± 0.14 mg/dL in horses prior to racing, and was not significantly different to control horses (1.48 ± 0.19) or after one, two or three days of racing (1.98 ± 0.13, 1.80 ± 0.15 and 1.76 ± 0.26 mg/dL, respectively). Serum concentration of IgM in the 50 mile group was 1.02 ± 0.05 mg/dL in horses prior to racing, and not significantly different to control horses (1.08 ± 0.12) or after one, two or three days of racing (1.03 ± 0.06, 0.91 ± 0.08 and 0.89 ± 0.11 mg/dL, respectively). Similarly, serum concentration of IgG(T) (6.10 ± 0.41 mg/dL before racing) in the 50 mile group was not significantly different prior to racing from control horses (6.29 ± 0.58) or after racing one, two or three days (6.23 ± 0.45, 6.62 ± 0.63, and 6.65 ± 1.34 mg/dL, respectively). Serum concentration of IgGa in the 50 mile group was

2.41 ± 0.10 mg/dL in horses prior to racing, and was not significantly different to control horses (2.45 ± 0.22) or after any day of racing (2.58 ± 0.14, 2.39 ± 0.20, and 2.61 ± 0.22 mg/dL, on days one, two and three of racing, respectively) (Figure 3.1). Serum IgGb concentration was found to be lower in control horses (5.71 ± 0.54 mg/dL) compared to horses racing 25 (7.00 ± 0.57 mg/dL) and 50 miles of distance (7.65 ± 0.41 mg/dL) (Figure 3.2). The inter-assay CV was 21.7% for IgG(T), 10.7% for IgGb, 26% for IgGa, 33.1% for IgA, and 24.4% for IgM (Table 3.2), which for all variables except IgGb slightly exceeded published recommendations of < 20 derived from a human IgG ELISA kit (http://www.vaccine.uab.edu/ELISAProtocol(007sp).pdf, 2013).

3.4 Discussion

In this study, endurance training and racing exercise appeared to have minimal impact on serum immunoglobulin fractions in horses, with the exception of a slightly higher serum IgGb concentration in endurance horses at rest compared to resting untrained control horses. Immunoglobulin G subclass b (which is composed of IgG4 and IgG7 according to the most recent nomenclature) is the most prevalent immunoglobulin isotype in equine serum, followed by IgG(T) and IgGa (IgG1), both of which were also measured in the current study but which were not different between trained and untrained horses (Wagner, 2006; Lewis, et al. 2008; Sheoran, et al. 2000).

Few studies have measured immunoglobulin concentrations in athletes with comparison to a separate rested and untrained control group, making direct comparisons with the current study challenging. In a study by Nehlsen-Cannarella, et al. (1991a), 36

human subjects participating in 45 minutes of brisk walking at 60% heart rate reserve five days a week for 15 weeks had serum immunoglobulin concentrations (IgG, IgM, IgA) comparable to those measured in an untrained resting control group. However, subjects within the exercising group in this study did demonstrate an increase in serum immunoglobulin concentrations after six weeks of training compared to values measured prior to training. These data agree with another study by the same investigator Nehlsen-Cannarella, et al. (1991b) in which a mild increase in serum IgG concentration (7.2%) was measured immediately following conclusion of moderate submaximal exercise (walking for 45 minutes at 60% VO_{2max}). Similar trends were seen with serum IgA and IgM, but did not reach statistical significance. Similar findings have been reported in other studies measuring serum immunoglobulin concentrations immediately after exercise (Stephenson, et al. 1985; Poortmans, et al. 1970; Neiman, et al. 1989), and it has been hypothesized that this phenomenon is attributable to plasma volume changes (Stephenson, et al. 1985) and/or shifting of immunoglobulins from rapidly exchangeable extravascular plasma protein pools. Since endurance horses may be conditioned in the days prior to an endurance race and often encounter prolonged trailer rides to the race site, it is possible that undetermined plasma volume changes may have contributed to their greater serum IgGb concentrations compared to control horses in the current study.

Furthermore, endurance horses are frequently exposed to comingling with unfamiliar horses in the course of their travel and events. As a result, endurance horses are presumably more likely to be vaccinated and to be exposed to other immune stimulating phenomena, and heightened antibody responses may also explain a greater

concentration of a prominent protective serum immunoglobulin fraction in endurance horses compared to the control horses in this study. Trained human runners have been demonstrated to have improved capacity for antibody production in response to tetanus vaccination compared to control subjects (Eskola, et al. 1978).

Mean serum IgGb concentrations in the current study were substantially lower than reported in previous studies (Sheoran, et al. 2000; Halliwell, 1989; Riggs, 1987) (Table 3.1). The methodology used in this study (previously validated polyclonal antibodies produced by affinity purification) was different than that used in prior studies which utilized monoclonal antibodies in the ELISA assays (Sheoran, et al. 2000). Monoclonal antibodies have highly specific binding to a single epitope and therefore are naturally more selective with tighter binding properties. In contrast, polyclonal antibodies may bind to many epitopes, potentially with less affinity, which could lead to falsely elevated background fluorescence. While a greater background fluorescence was noted (OD's of 0.1-0.2) in the blank microtiter wells in the current study, the remainder of the standard curve OD's increased as expected and fit the standard curve equation with high correlation (IgGb R^2 >0.994) indicating that the data were valid. Nevertheless, given the methodology employed in the current study, it is possible that during washing procedures, loosely bound antibody was incidentally removed, ultimately resulting in lower recorded concentrations of IgGb.

A potential weakness in our data is reflected in the values obtained for the inter-plate coefficient of variation (CV). The laboratory was a temperature controlled room, all

samples were run during a single month when there was little variation in ambient temperature and humidity, and all reagents were handled and stored in a similar manner according to the manufacturer's instructions for every experiment. Despite this, high inter-plate CV values were obtained for IgG(T), IgGa, IgM, and IgA. However, inter-plate CV was also calculated using a single sample in duplicate which may have been suboptimal. In spite of this, the inter-plate CV for IgGb was low (10.6%) and therefore unlikely to have been a significant contributing factor to the lower serum IgGb concentrations reported in the current study compared to previous studies.

No significant difference was identified between control horses and racing horses at any time point for serum IgA, IgM, IgGa, IgG(T), and mean concentrations of these variables reported in this study were in accordance with previously published values in horses (Wagner, 2006; Sheoran, et al. 2000; Lewis, et al. 2008). These findings are also in agreement with studies of human athletes where Mackinnon, et al. (1989) reported no significant difference in serum IgA, IgG, and IgM concentrations from before to after maximal endurance exercise. In a study in 1989, Nieman, et al. investigated changes in serum IgG, IgM, and IgA concentrations after graded maximal exercise in athletes and non-athletes and found no significant difference between groups prior to, during or after exercise. Similarly, McKenzie, et al. (2009) reported no significant difference in serum IgA and IgM concentrations in trained, racing or rested long distance sled dogs, despite demonstration of substantially lower serum IgG concentrations in trained and raced sled dogs versus resting sled dogs in the same study.

Respiratory disease is a significant cause of morbidity in athletic horses (Raphel and Beech, 1982; Burrell, et al. 1996; Jensen-Waern, et al. 1998; Nesse, et al. 2002;

Robson, et al. 2003) and humans, prompting studies to investigate mucosal concentrations of secretory immunoglobulins, including IgA, IgG, and IgM, in athletes at various stages of training and competition (Tomasi, et al. 1982; MacKinnon, et al. 1989, 1990, 1991, 1993; Huston, et al. 1986, 1987). The results of these studies have been somewhat equivocal, with some reporting lower mucosal IgA concentrations as a possible contributor to the higher prevalence of upper respiratory disease in athletes who have undergone severe prolonged exercise or who are 'overtrained' (Peters and Bateman, 1983). Unfortunately secretory immunoglobulin concentrations were not measured in the current study but represent an area of interest that could be pursued in future investigations of immune function in endurance horses.

In conclusion, the current study demonstrated increased serum immunoglobulin (IgGb) concentration in trained endurance horses compared with untrained controls. Upper respiratory tract disease susceptibility is multi-factorial, and involves the innate immune system and secretory immunoglobulins, in addition to serum immunoglobulins that were evaluated in the present study. While studies exist regarding effector function of different immunoglobulin fractions in horses and their role in infectious disease, specific cut-off values for immunoglobulin fraction concentrations and disease susceptibility in endurance horses have not been established and require further research.

	IgA	IgM	IgG(T)	IgGa	IgGb
Pre (mg/mL)	1.70 ± 0.20	1.02 ± 0.12	6.07 ± 0.26	2.40 ± 0.28	7.0 ± 0.57
Post (mg/mL)	1.63 ± 0.18	0.87 ± 0.10	5.29 ± 0.54	2.19 ± 0.21	7.09 ± 0.82
Control (mg/mL)	1.48 ± 0.19	1.08 + 0.12	6.29 ± 0.58	2.45 ± 0.22	5.71 ± 0.54*
Reference (mg/mL)	0.4 ± 0.3[a]	1.1 ± 0.4[bc]	4.0 ± 2.5[a]	3.4 ± 0.6[a]	19.2 ± 5.2[a]

Table 3.1: Serum immunoglobulin concentrations in endurance horses before (pre) and after (post) racing 25 miles in distance. Previously reported reference ranges for serum immunoglobulin concentrations in resting horses are also included (reference).
* Indicates statistical significance.
Data obtained from [a] = Sheoran, et al. 2000; [b] = McGuire, et al. 1973; [c] = Perkins, et al. 2003. All concentrations are reported in mg/mL.

	IgG(T)	IgGb	IgGa	IgA	IgM
Plate 1 (mg/mL)	5.40	6.95	3.32	2.96	1.85
Plate 2 (mg/mL)	3.86	5.3	2.76	2.80	1.55
Plate 3 (mg/mL)	4.38	5.75	2.86	5.65	1.27
Plate 4 (mg/mL)	4.53	6.60	3.73	3.87	1.01
Plate 5 (mg/mL)	6.60	6.15	5.03	2.90	1.16
Mean (mg/mL)	4.95	6.15	3.54	3.64	1.37
Std (mg/mL)	1.074048	0.656696	0.918915	1.205044	0.334096
%CV	21.7	10.7	26.0	33.1	24.4

Table 3.2: Control inter-assay sample values from each of the five microtiter plates for each immunoglobulin isotype with inter-plate coefficient of variation (%) calculated. Unless otherwise stated, all values displayed are in mg/mL

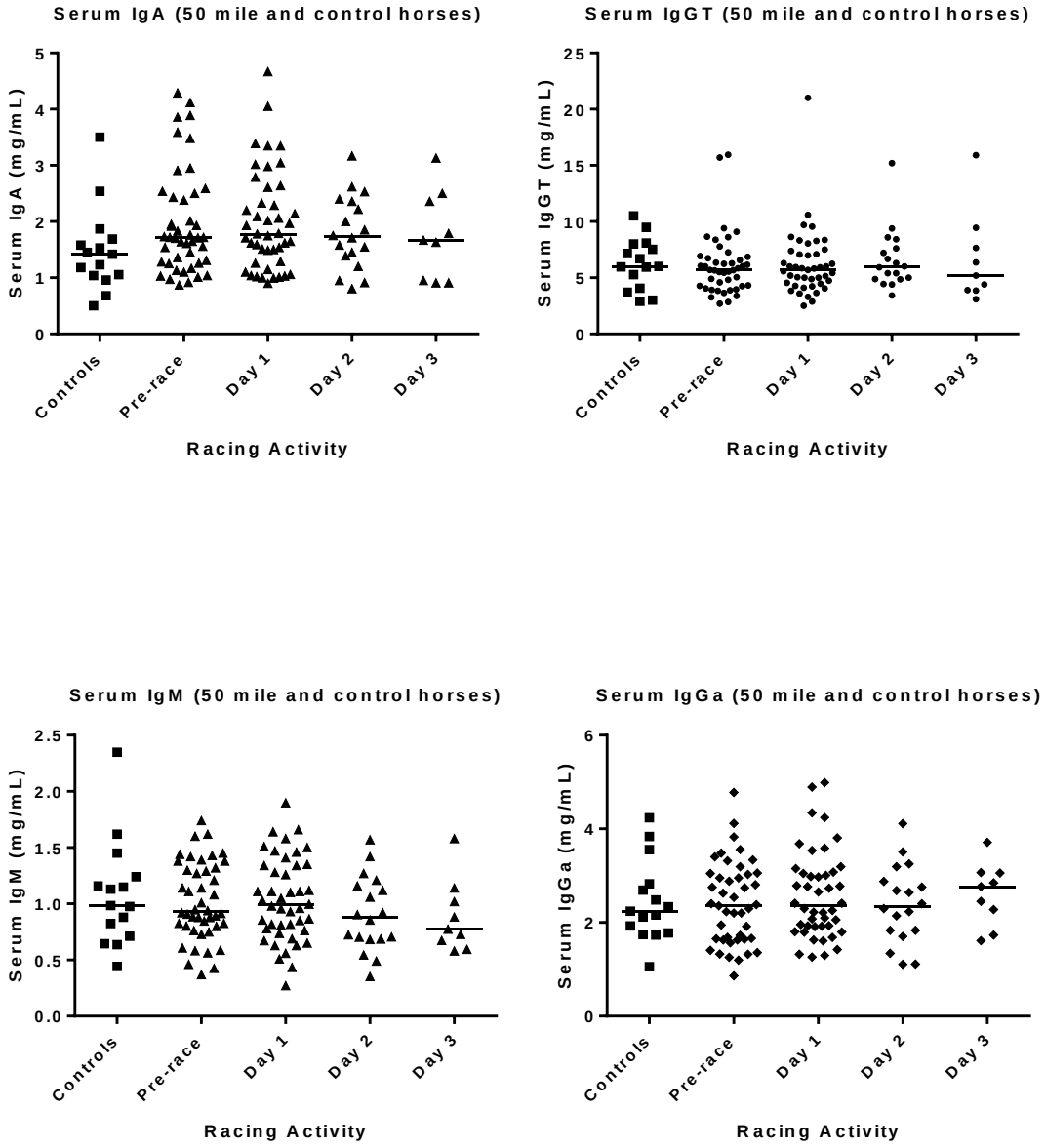

Figure 3.1: Serum concentrations of IgA, IgG(T), IgM, and IgGa (mg/mL) in untrained control horses at rest (n= 15), and in trained endurance horses before (n= 44) and after racing 50 miles in distance for three consecutive days (day 1: n = 44; day 2: n = 18; day 3: n = 9).

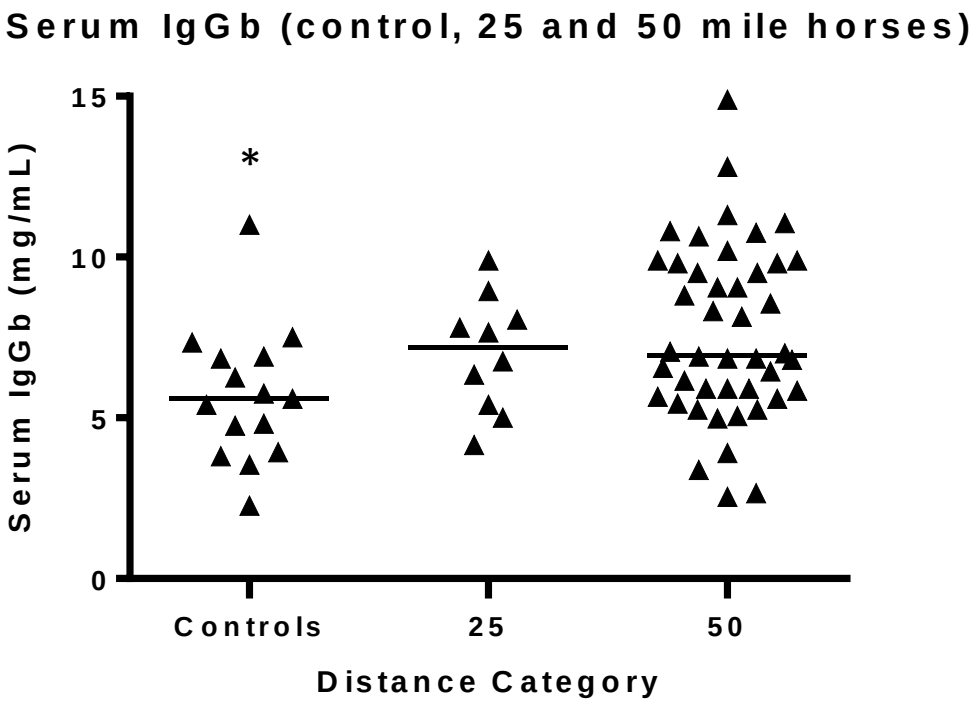

Figure 3.2: Serum IgGb concentration (mg/mL) of untrained control horses and trained endurance horses prior to competing 25 and 50 miles in race distance. Horizontal bar represents the median value for each group. 50 mile: $n = 44$; 25 mile: $n = 10$; control: $n = 15$. * Significant difference ($P < 0.05$) to other groups.

Chapter 4: Conclusion

In these studies the impact of multi-day endurance racing exercise on specific serum biochemistry variables and immunoglobulin isotype concentrations was investigated to elucidate factors that may place horses at greater risk of metabolic and infectious disease. Despite an abundance of research regarding serum biochemistry changes in endurance horses participating in single day endurance events, the effects of multiple days of submaximal exercise on these variables has not been studied. Likewise, the primary focus of the majority of literature evaluating the effects of exercise on the equine immune system has focused on neutrophil function and lymphocyte responses, with no evaluation of serum immunoglobulin dynamics. The initial intent of this project was to take advantage of a multi-day endurance racing event which had a unique construction, whereby horses could race 25 or 50 miles in distance on consecutive days for up to six days. This would potentially allow the same group of horses to be studied on repeated days while traversing similar terrain in similar weather conditions. The objective was to identify, in a clinical setting, abnormalities of specific serum biochemical variables in conditioned endurance horses after repeated days of exercise and to determine if anomalies in these variables progressed with repeated exercise bouts. These variables included measures of liver and muscle damage and renal function, in addition to protein fractions, and electrolytes and minerals. In addition, using this same group of horses and a control group of untrained resting horses, serum concentrations of five circulating immunoglobulin isotypes could be quantified as a partial measure of immune status in trained endurance horses undergoing a multi-day submaximal exercise effort.

Mild but significant increases were identified in several serum biochemical values that primarily reflected decreased circulating plasma volume, likely a result of fluid shifts arising in response to exercise and sweat loss. Additionally, significant and persistent elevation in serum CK activity, reflecting muscle damage, occurred in horses racing 25 and 50 miles in distance, with the most pronounced elevations occurring in the 50 mile group on day 1 of racing. Although the magnitude of increase in this enzyme was considered appropriate for the exercise effort in most horses, two horses in the 50 mile group had values that were considered abnormal and consistent with subclinical rhabdomyolysis. Exertional rhabdomyolysis is a common phenomenon in endurance horses, and a common cause of race elimination. To date, a primary underlying muscular disorder in Arabian and Arabian cross breeds has not been definitively identified and implicated as the cause of exertional rhabdomyolysis in this group, however, current research efforts are focused in this direction, and identifying affected horses is a useful step in constructing a population of diseased horses for study. Biochemical variables relevant to liver dysfunction that were evaluated in this study included bilirubin, SDH, GGT and AST. While statistically significant elevations in bilirubin and SDH were identified, any clinical significance of these changes was considered unlikely since alterations were very slight and values did not exceed reference intervals. The resting liver is responsible for multiple important functions (e.g. protein production, detoxification, metabolism, bile recycling, energy production and storage), some of which require upregulation during endurance exercise, including fat oxidation and glycogenolysis. While the slight increases in bilirubin demonstrated in this study are

likely to be secondary to decreased feed intake during racing, liver function was not evaluated per se.

Mild decreases in protein fractions were identified in the study reported here, though changes were slight and unlikely to be clinically significant. One proposed theory, taking into consideration the mild degree of change, is a relative decrease in serum protein concentration secondary to increases in plasma volume. Blood samples were obtained up to six hours after exercise bouts finished allowing sufficient time for the horses to rest, eat and drink, which may have effectively replenished their circulating plasma volume. In addition, most endurance horse owners regularly administer electrolytes, which may have further promoted fluid consumption and plasma volume expansion. Another proposed theory is that of an acute phase inflammatory response in associated with exercise trauma. Albumin is a negative acute phase protein, and may have decreased in response to the inflammatory stressors that arise with a multi-day athletic endeavor.

The results of studies investigating electrolyte concentrations and acid-base status in endurance racing horses have been variable. While some studies fail to reveal any significant abnormalities in serum electrolyte concentrations, many have demonstrated decreases in serum sodium, potassium, chloride and calcium concentrations with concurrent increases in serum bicarbonate concentration. However, equally as often bicarbonate has been reported to remain within normal limits, or to be decreased. This latter event has been proposed to be secondary to lactic acid accumulation counteracting the expected elevation in serum bicarbonate concentration that should occur as a

consequence of significant losses of chloride in sweat. Cumulatively, the results of the current and other studies indicate that the use of biochemical variables to predict race elimination is not justifiably sensitive or accurate.

In regard to the serum immunoglobulin data, no significant difference was found between untrained and trained horses in any of the studied immunoglobulin isotypes with the exception of IgGb, which was slightly but significantly greater in trained endurance horses. Immunoglobulin Gb is one of the primary immunoglobulins targeted to be produced in response to equine systemic or mucosal vaccines. Horses that are more frequently transported and competed typically have a more frequent and diligent vaccination schedule than sedentary horses. It is therefore possible this phenomenon might explain the slightly higher circulating IgGb concentration in endurance horses compared to the resting control group. It is difficult to compare these results with previous studies. There have been few previously published reports on resting serum immunoglobulin isotype concentrations, specifically the IgGb isotype, in horses. Different methodologies may induce differences in absolute concentrations, which is likely a contributing factor to the results reported in this study in which mean IgGb concentrations were substantially lower than those previously described. Conversely, while IgA was not significantly different between trained or untrained horses, or with racing, values reported in the current study were slightly higher than reported in previous studies of horses. Immunoglobulin A is found in high concentrations at mucosal barriers and in colostrum and may provide first line defense against invading respiratory pathogens. Since some studies have shown compromise to IgA in athletes, associated

with a greater incidence of respiratory infections, future studies investigating mucosal IgA concentrations in athletic horses would be of interest given the significance of respiratory disease in this population. In addition, studies of vaccine responses in athletic equine populations may also provoke interesting results.

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