Exercise and Immunodeficiency Affect Immunoglobulins in Endurance Horses

Kari Elizabeth Krick

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David S. Kronfeld, Co-Chairman

Rhonda M. Hoffman, Co-Chairman

Rebecca K. Splan

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Abstract

Two studies were conducted on endurance horses predominantly of Arabian breeding participating in an 80 km ride dedicated to research in April 2001 (Trial 1) and April 2002 (Trial 2). Objectives were to determine effects of endurance exercise, antioxidant supplementation, and a feed rich in fiber and fat vs. a high fat sweet feed on immunoglobulin A and G concentrations as well as identify selective IgA deficiency in endurance horses of Arabian breeding. There were no effects of distance in Trial 1 on IgA ($P = 0.73$) or IgG ($P = 0.18$) concentrations. In Trial 2, IgA concentrations increased ($P = 0.05$) and IgG concentrations increased ($P = 0.006$) after the start of the race. There were no effects of antioxidant supplementation on IgA ($P = 0.16$), IgG ($P = 0.16$), and IgM ($P = 0.70$) concentrations. There were no diet effects on IgA ($P = 0.80$), IgG ($P = 0.59$), and IgM ($P = 0.54$) concentrations. There were horses in both trials that were deficient in IgA only. Concentrations of IgG and IgM were within normal ranges, and there were no differences in training, performance and transportation variables, IgG concentrations, antioxidant supplementation, and feed supplementation compared to the horses with normal IgA concentrations. The concentration of IgM was higher in IgA deficient horses in Trial 1 ($P = 0.035$) and Trial 2 ($P = 0.017$). Horses with deficient IgA tended to be associated with health problems commonly found in humans and dogs affected with selective IgA deficiency.

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Introduction

Moderate intensity exercise tends to enhance the immune system while high intensity exercise tends to suppress it (Hines et al., 1996). Training also has immunosuppressive effects and can lead to the overtraining syndrome (Sharp and Koutedakis, 1992), which causes a decline in performance. Endurance racing in horses is considered to be exercise of moderate intensity over a long distance, and the conditioning required involves hours and kilometers of training and could lead to the overtraining syndrome. Thus the effects on the immune system of endurance training and endurance events need to be investigated.

Selective IgA deficiency is the most common immunodeficiency in humans and certain breeds of dogs, but has not been documented in horses. Most affected individuals lead normal, asymptomatic lives. However, some individuals suffer from recurring respiratory, skin, and gastrointestinal problems, allergies and chronic diarrhea (Horowitz and Hong, 1975; Moroff et al., 1986). If this immunodeficiency does exist in horses, a breed that is already afflicted with an immunodeficiency (severe combined immunodeficiency), the Arabian and horses with Arabian breeding, would be a likely suspect. Immune deficient horses would not have the same protection that a normal horse would have and therefore should be under a more aggressive health program so that they are still able to perform at optimum levels. This information would be important in practical health management.

The two main objectives of this study were to examine effects of endurance exercise on serum immunoglobulin concentrations and investigate the possibility of selective IgA deficiency

in endurance-trained horses of Arabian breeding. Secondary objectives were to observe the effects of dietary energy sources (fiber and fat versus sugar and starch), antioxidant supplementation and, from histories, transportation, training and previous performance.

Review of Literature

General Overview

The immune system serves to protect the body against foreign cells andsubstances. There are two types of immune responses: nonspecific and specific. Nonspecific responses are the first line of defense and block the entry and spread of pathogens. Skin, mucosal membranes, tears and saliva are nonspecific barriers to entry. Inflammation is also a nonspecific response that functions to protect the body against pathogens.

Specific responses include humoral-mediated immunity and cell-mediated immunity. Humoral immunity is regulated by B cells and protects against invading viruses and bacteria. Cell-mediated immunity is regulated by T cells and defends against viruses, bacteria, parasites, fungi, protozoans and cancerous body cells.

The immune system exhibits two unique characteristics – specificity and memory (Sharp and Koutedakis, 1992). A subset of the immune cells recognizes and responds to the numerous external stimulation an individual might encounter in a lifetime (specificity), and the immune system is capable of mounting a much more vigorous, effective and quicker response in subsequent times a recognized stimulus is received (memory). The immune system regulates itself by means of helper, suppressor and cytotoxic T and B lymphocytes.

Lymphocytes originate from a multipotential stem cell, and either develop from the thymus (T cells) or from bone marrow (B cells). T cells account for 75% and B cells account for

10% of the mononuclear leukocytes (Sharp and Koutedakis, 1992). Mature T and B lymphocytes express surface receptors, T-cell receptor and immunoglobulin, respectively, that allow them to be activated only by a specific foreign molecule or antigen.

Helper T cells are macrophages that become activated when they encounter the antigens displayed on the macrophage surface. Most B cell responses require the assistance of T helper cells. Helper T cells produce cytokines (Interleukin 4, 5) that stimulate the antigen-activated B cells to proliferate and differentiate (Scott, 1993). Activated B cells proliferate, differentiate into plasma cells, and produce large quantities of immunoglobulins capable of binding to an antigen and initiating its removal from the body. T helper cells are recognized by a phenotypic surface marker (CD4) and may constitute a subset of CD4+ T cells called Th2 cells. Helper T cells are not capable of secreting antibodies by themselves.

Suppressor T cells inhibit the immune response of B and T cells, serving as an off switch for the immune system. Although somewhat controversial, some evidence suggests that a population of T lymphocytes is capable of downregulating or suppressing B and T cell functions (Murphy, 1993). Suppressor T cells maintain homeostasis and prevent the immune system from damaging normal host tissue or causing overt hypersensitivity or autoimmune disease.

Cytotoxic, or killer, T cells destroy body cells infected with a virus or bacteria by recognizing viral antigens. Killer T cells attach to the infected cell's plasma membrane and secrete proteins that punch holes in the plasma membrane. The infected cell's cytoplasm leaks

out, the cell dies, and is removed by phagocytes. Memory T cells remain in the body awaiting the reintroduction of the antigen.

Two enzyme groups are responsible for differentiation and normal function of B lymphocytes and T lymphocytes (Perryman, 2000). The first group express recombinase-activating genes (RAG) in lymphoid tissues. The other enzymes are DNA-dependent protein kinases (DNA-PK) found in virtually all cells. B and T lymphocyte precursors cut, rearrange, and anneal the genes that encode antigen-specific receptors expressed on lymphocyte surfaces. Failure to complete these essential gene rearrangement events results in elimination of lymphocyte precursor cells and absence of mature, functional B and T lymphocytes (Perryman, 2000). The RAG and DNA-PK gene products work sequentially to accomplish this vital task.

Phagocytic cells, both polymorphonuclear neutrophil leukocytes (PMNs) and macrophages, play essential roles in the immune response. Tissue-associated macrophages and blood-borne monocyte macrophages can serve as antigen presenting cells. Macrophages present antigen physically on their surface and bind directly to T cells. They also produce cytokines that assist in the activation of T cells (Scott, 1993). Macrophages also produce arachidonic acid metabolites which can downregulate T cell functions. Macrophages, in turn, can be activated by T cell cytokines to become more effective in controlling intracellular pathogens. Macrophages and PMNs can bind and internalize antigen coated with antibody and/or complement proteins and destroy microbes.

An additional cell type, the natural killer (NK) cell, is thought to play an important accessory role in the immune response. Natural killer cells are large, granular lymphocytes that can mediate antibody-dependent cellular cytotoxicity (ADCC) as well as lyse other target cells. Natural killer cells produce cytokines which upregulate the responses of macrophages.

Immunoglobulins (Ig)

Function. The broad functions of immunoglobulins are to recognize, bind, and inactivate antigens. Immunoglobulins recognize many pathogens including bacteria, viruses, and parasites. Once recognition occurs, immunoglobulins stimulate phagocytes and other cytotoxic cells to destroy foreign invaders(Mackinnon, 1996). There are numerous ways in which immunoglobulins inactivate antigens. Immunoglobulins can bind directly to the antigen or to complement, a large series of proteins involved in immune and inflammatory responses. Immunoglobulins may coat the antigen and facilitate its uptake and digestion by phagocytic cells. Immunoglobulin-coated cells bearing foreign antigen may be killed by other lymphocytes or phagocytes by ADCC. Immunoglobulins activate the complement cascade to produce inflammatory molecules that attract phagocytes from the blood. Immunoglobulins can also inactivate antigens by binding to the toxins the antigen might produce, rendering them harmless. Interfering with the movement and binding of an antigen to a host cell as well as inhibiting the uptake of essential nutrients to an antigen are also ways immunoglobulins can inactivate antigens and protect the host's body (Mackinnon, 1996).

Structure. Immunoglobulins are Y-shaped glycoproteins composed of two identical long (or heavy) polypeptide chains and two identical short (or light) polypeptide chains linked

together by disulfide bonds. There are two forms of the light chain (κ and λ) but a specific B cell expresses only one form (Reed and Bayly, 1998). Although the light chains are similar among the Ig classes, the heavy polypeptide chains, which serve as their antigen-binding sites, are unique to each class of immunoglobulin (Mackinnon, 1996). There are several isotypes of heavy chains that have been identified (μ, δ, γ, α, ε) that correspond a class: IgD, IgM, IgG, IgA, and IgE (Reed and Bayly, 1998). Each immunoglobulin structure is composed of variable domains and constant domains. The light chains can be divided into a conserved carboxy-terminal domain and an amino-terminal variable domain. The heavy chains are similar except they exhibit more constant domains than the light chains, usually three (Reed and Bayly, 1998). Immunoglobulin E differs from the other classes because it has one more domain than the others.

The amino-terminal variable domains compose the fragment antigen binding (Fab) segments that can contort into a three-dimensional structure that binds complementary to the antigen. The distance between the first and second constant domain varies, as it acts as a hinge to allow for independent movement of the two antigen-binding sites (Reed and Bayly, 1998).

The carboxy-terminal end of the Y-shaped molecule consists of the fragment crystallization (Fc) portion. The Fc portion does not bind antigen. The Fc portion can bind complement and to Fc receptors on the surface of other immune cells. Fc receptor binding to the antigen-antibody complex stimulates the uptake of the complex by macrophages (Mackinnon, 1996).

B cells are capable of switching the heavy chain isotype during an immune response, thus switching the class of immunoglobulin the B cell secretes (Reed and Bayly, 1998). This is accomplished through the substitution of one heavy chain constant region with another. The chromosome sequentially arranges the genes encoding the five different constant regions of the heavy chain. At first, the genes encoding for IgD and IgM are used to form the heavy chain, permitting these immunoglobulins to be the first to appear during an immune response. When isotype switching occurs, a new constant region is selected and old ones are spliced out or looped out. Thus, a different class of immunoglobulin is now secreted from the B cell.

There are five classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM. Little is known about IgD and IgE. Immunoglobulin D is the antigen receptor of naïve B lymphocytes, and is rarely present in the circulation (Reed and Bayly, 1998). Immunoglobulin E is found in very small amounts in the plasma, and is the primary immunoglobulin responsible for allergic reactions (Reed and Bayly, 1998). Most literature focusing on changes in plasma immunoglobulin concentrations due to effects such as exercise, diet, antioxidants, etc. is concerned with measuring IgA, IgG and IgM.

Immunoglobulin A (IgA)

Although the predominant Ig in mucosal secretions, IgA comprises only 15-20% of total serum Igs (Mackinnon, 1996). Secretory IgA is a dimer composed of two IgA molecules joined by a J chain (Reed and Bayly, 1998). Serum IgA is predominantly monomeric. The presence of IgA in mucosal secretions provides the host with the first line of defense. Newly synthesized IgA is transported across the epithelium and discharged into the secretory lumen by way of the

presence of specific receptors on epithelial cells. Immunoglobulin A has been shown to inhibit attachment and replication of certain pathogens, and to be capable of neutralizing viruses and toxins (Mackinnon et al., 1993). T cells regulate production of IgA. T cell-derived transforming growth factor β has been identified as the primary cytokine that promotes B cells to switch from IgM-bearing cells to IgA-bearing cells (Beagley et al., 1989). Interleukin 5 has been identified as the major cytokine that drives the differentiation of IgA-bearing cells to IgA-antibody secreting cells (Beagley et al., 1989). Studies on the ability of IgA antibodies to regulate the antibody response are scarce (Heyman, 2000).

The reference range of serum IgA concentration in horses, as analyzed by radial immunodiffusion, is 67 to 239 mg/dl (MacLeay et al., 1997). A horse with an IgA concentration of 40 mg/dl or less was considered clinically IgA deficient.

Immunoglobulin G (IgG)

A major Ig in serum, IgG comprises 70-75% of total serum Ig and is the predominant Ig in the secondary immune response (Mackinnon, 1996). After IgM production increases first in response to a challenge, IgG production increases and becomes the main immunoglobulin. This isotype switch is driven by interferon γ, which blocks the switch to IgE from IgM and augments the switch from IgM to IgG (Reed and Bayly, 1998). Immunoglobulin G is the main immunoglobulin found in the lower respiratory airways and alveolar spaces, and a deficiency may cause recurrent and chronic infections. Immunoglobulin functions mainly in opsonization and neutralization, as well as fixing complement and participating in ADCC (Reed and Bayly, 1998). The reference range of serum IgG concentration in horses is 1000 to 2000 mg/dl

(MacLeay et al., 1997). There are four IgG subclasses differing in their heavy chains found in the horse: IgGa, IgGb, IgGc, and IgG(B) (Reed and Bayly, 1998).

Immunoglobulin M (IgM)

Comprising about 10% of total serum Ig, and present in small amounts in mucosal secretions, IgM is the earliest Ig to respond to the first exposure to a given antigen (Mackinnon, 1996). It has been suggested that the binding to antigen and activating of complement by IgM starts the chain of events that lead to antibody responses (Heyman, 2000). A pentamer, IgM is also involved in aggulination, neutralization and opsonization (Reed and Bayly, 1998). Surface IgM is found on naïve, activated and memory B cells. Immunoglobulin M acts as a compensatory Ig, as it usually increases with a deficiency in IgG or IgA (Mackinnon, 1996). The reference range for serum IgM concentration in horses is 100 to 300 mg/dl (MacLeay et al., 1997).

Exercise and immunity with emphasis on the horse

Exercise intensity. Research on the effects of moderate intensity exercise such as endurance racing on immune function has yielded conflicting results (Table 1). Immunoglobulin concentrations in serum (Nehlsen-Cannarella et al., 1991) and saliva (Reid et al., 2001) increased or remained the same (McDowell et al., 1993; Mackinnon and Hooper, 1994) following a bout of moderate exercise in humans. Highly trained runners engaging in moderate intensity exercise showed no significant alterations in IgG, IgA and IgM (Nieman et al., 1989a). Horses had enhanced phagocytic and oxidative burst capacity of blood neutrophils following moderate

exercise. Since the immune system is a complex system, a change in one variable may not necessarily affect the overall immune status of an individual.

Conflicting results also have been found in the effect of intense exercise on immune function (Table 2). Salivary IgA decreased (Mackinnon et al., 1993), remained unchanged (Walsh et al., 1999) or increased (Blannin et al., 1998) following intense exercise in people. A program of intense exercise consisting of 5 min at 50% VO_{2max}, 2 min walking, and then a run to fatigue at 115% VO_{2max} in horses lowered ($P = 0.005$) oxidative burst activity of peripheral blood neutrophils. Another study in horses reported serum IgG, IgA, and IgM unchanged after a bout of intense exercise (Wong et al., 1992). In humans, the immunosuppression found after a 3 h marathon returned to normal levels 21 h into recovery (Nieman et al., 1989b)

Possible reasons for conflicting results in exercise studies focusing on immunoglobulins may include the definition of exercise intensity, and problems in the analysis of serum and salivary immunoglobulins. Some studies have defined the intensity of exercise based on $VO_{2max,}$ others use heart rate, and still others use plasma lactate concentrations (Hines et al., 1996). What is high intensity exercise in one study may be considered high-moderate, or even moderate exercise in other studies. Exercise effects on plasma volumes must be taken into account, as the change in plasma volume will bias immunoglobulin concentration. Salivary immunoglobulin concentrations are affected by saliva flow rate and dryness of the mouth (Walsh et al., 1999). Salivary IgA was found to be affected by relaxation, light, moderate and intense incremental exercise in humans (Reid et al., 2001). However, once the salivary secretion rate was taken into account, the effects of exercise on salivary IgA no longer existed. Another potential problem

with immune/exercise studies is that training effects are confounded with single bouts of exercise.

Training. Continuous exercise over long periods of time (i.e. training) may suppress the immune system, without allowing time to rebound. Compared to their non-athletic counterparts, athletes appear to suffer from a higher incidence of illness, especially upper respiratory tract infections (URTI). Serum IgA, IgG, and IgM were lower ($P < 0.03$) in elite swimmers who trained for more than 25 h/wk compared to control, who trained for less than 4 h/wk over a seven month period (Gleeson et al., 1995). Salivary IgA also decreased over the study period in both pre-exercise ($P = 0.06$) and post-exercise ($P = 0.04$) samples. After individual training sessions, salivary IgA concentration was lower in the trained swimmers (-10%) but not in sedentary controls (+11%) (Pyne and Gleeson, 1998). However, resting concentrations of IgG, IgA and IgM appeared normal in endurance trained human athletes (Mazzeo, 1994; Hines et al., 1996) and well-trained competitive bicyclists (Mackinnon et al., 1987) when compared to untrained age-matched controls. Prodigious amounts of training can lead to the overtraining syndrome, which causes a loss of performance and prolonged fatigue (Sharp and Koutedakis, 1992).

Age. Immunosenescence, the age-related decrease in immune function, has been found in humans, dogs, rodents and horses (McFarlane et al., 2001). The decline in immune function may be due to the loss of thymic and T-cell function, caused by middle-aged thymus involution (Pedersen et al., 1999). However, serum immuoglobulin concentrations tend to increase with age. In humans, a link between salivary IgA concentration and age was found (Kugler et al., 1992). Salivary IgA concentration in children less than seven years old was lower ($P < 0.05$)

than people older than seven years of age, including adults. Children's ages and salivary IgA concentration were positively correlated ($r = 0.49$, $P < 0.05$). Saliva flow rate and salivary IgA secretion rate was lower in elderly people (60-80 yr) than young people (20-30 yr) (Miletic et al., 1996). There were no differences between IgM concentrations in old (> 20 yrs) and young (5 to 12 yrs) horses, but IgG ($P = 0.07$) and IgA ($P = 0.07$) tended to be higher in old horses (McFarlane et al., 2001).

Exercise affects the immune response of young and old horses differently (Horohov et al., 1999). Antibody titers were lower in older horses (25.3 ± 1.1 yr) than younger (7.5 ± 0.7 yr) horses pre-exercise. However, older horses had lower lymphoproliferative responses to mitogens after exercise than their younger counterparts, indicating they were better able to withstand exercise-induced changes in the immune system. So older horses appear to have an immune system that is unable to illicit a response from either a challenge or exercise.

Diet

Lower blood glucose concentrations have been linked to hypothalamic-pituitary-adrenal activation, increased release of adrenocorticotrophic hormone and cortisol, decrease in insulin and a variable effect on blood adrenaline levels (Nieman and Pedersen, 1998). Therefore ingestion of carbohydrates should maintain plasma glucose levels, attenuate increases in stress hormones and diminish exercise-induced changes in immune status (Pedersen et al., 1999). Carbohydrate loading in humans has been shown to increase plasma glucose levels (Nieman et al., 1997b), allow fewer perturbations in blood immune cell counts (Mitchell et al., 1998), lower

granulocyte and monocyte phagocytosis and oxidative burst activity (Nieman et al., 1998), and diminish pro- and anti-inflammatory cytokine release (Nehlsen-Canarella et al., 1997).

While the immune system is enhanced by a low-fat diet (Barone et al., 1989; Kelly et al., 1989), obesity appears to be associated with an impaired immune function (Stallone, 1994). Ten untrained men were given either a carbohydrate-rich diet or fat-rich diet and endurance trained three to four times a week for seven weeks (Pedersen et al., 2000). The carbohydrate-rich diet consisted of 65 % carbohydrate, 15 % protein and 20 % fat. The fat-rich diet consisted of 21 % carbohydrate, 17 % protein and 62 % fat. The subjects on the carbohydrate-rich diet had higher ($P = 0.007$) unstimulated natural killer cell activity than the subjects on the fat-rich diet. Training on a carbohydrate-rich diet enhanced natural immunity compared to a fat-rich diet (Pedersen et al., 2000). While an excessive intake of fatty acids has been shown to suppress the immune function, it has also been shown that polyunsaturated fats are essential for T cell function, and a deficiency of essential fatty acids impairs immune function (Shepard and Shek, 1995). Thus it appears that dietary fat is necessary for immune function, but not in excess.

Antioxidants

Vitamin E. Vitamin E is an essential nutrient for humans and animals. Vitamin E is a term encompassing eight fat-soluble nutrients, called tocopherols. The most abundant and biologically active is α-tocopherol. Mainly found in mitochondria fractions and in the endoplasmic reticulum, α-tocopherol is present in limited amounts in cytosol and peroxisomes (Bjorneboe et al., 1990). Alpha-tocopherol is also found in large quantities in membranes of immune cells because of the high polyunsaturated fatty acid content, which puts the cell at high

risk for oxidative damage (Meydani and Beharka, 1996). Free radical damage may eventually disrupt the immune cell's normal function. However, the presence of α-tocopherol in the membrane will help protect the cell from oxidative damage due to vitamin E's antioxidant capabilities. Vitamin E reacts with peroxyl radicals produced from polyunsaturated fatty acids in the membrane phospholipids or lipoproteins to yield a stable lipid hydroperoxide. Through this reaction, vitamin E effectively reduces harmful lipid-free radicals and protects tissues from free radical attack. Through this antioxidant capability, vitamin E appears to enhance immune function.

Vitamin E deficiency disrupts normal immune function. Both cell-mediated and humoral immunity are diminished by vitamin E deficiency (Meydani and Beharka, 1996). Mice deficient in vitamin E had decreased humoral immune function, relative to mice with adequate vitamin E (Bendich, 1990). Once supplemented with vitamin E, previously deficient mice had restored humoral immune function. Vitamin E deficiency also causes impaired T cell function and moderate impairment of B cell function (Bendich, 1988). Depressed lymphocyte function, hypothesized to be due to an increase in oxygen intermediates, was noted in rats (Eskew et al., 1985). The combined effect of vitamin E deficiency and ozone exposure was tested to determine the effects of increased oxidative stress (Eskew et al., 1986). Rats that underwent either vitamin E deficiency or ozone exposure were found to have no significant decreases in cell-mediated immunity (Eskew et al., 1986). However, the combined effect of vitamin E deficiency and ozone exposure compromised cell-mediated immunity. Therefore, in situations in which lipid peroxidation or oxidative stresses are increased, such as exercise, the vitamin E requirement might be higher for optimal immune function.

Supplementation of vitamin E higher than recommended levels has been shown to increase immune function and resistance to disease (Meydani and Beharka, 1996). Deleterious effects of high vitamin E doses have not been shown (Meydani et al., 1994; 1997). Mares that were supplemented with 160 IU vitamin E/kg intake daily were shown to have increased serum IgG ($P = 0.064$) and tended to have increased serum IgA ($P = 0.13$) compared to mares supplemented with 80 IU vitamin E/kg intake daily (Hoffman et al., 1999). Recommended levels of vitamin E supplementation for horses are 50 IU/kg intake for maintence and 80 IU/kg intake for pregnancy, lactation, growth and exercise (NRC, 1989). Vitamin E supplemented to calves at a dosage of 125 to 500 IU/d increased T cell and B cell mitogenesis (Reddy et al., 1987). Vitamin E supplementation was negatively correlated with clinical mastitis in dairy cows (Weiss et al., 1990).

Vitamin C. Vitamin C, or L-ascorbic acid, is a water-soluble antioxidant vitamin that protects biomembranes against lipid peroxide damage by eliminating peroxyl radicals in the aqueous phase before they can initiate peroxidation (Frei et al., 1989). High concentrations of vitamin C are found in blood leukocytes (Moser, 1987). A high vitamin C concentration is necessary for neutrophil function (Anderson and Lukey, 1987), and ascorbate transporters present in neutrophils actively pump ascorbate from plasma against high concentration gradients (Washko et al., 1989). Suppressive effects of corticoids on cattle neutrophil function have been alleviated with vitamin C supplementation (Roth and Kaeberle, 1985).

It seems that vitamin C would be another antioxidant important in maintaining optimal immune function, but research has shown conflicting results. Vitamin C deficiency reduced (Fraser et al., 1978) or had no effect (Bendich et al., 1984) on lymphocyte mitogenesis in guinea pigs. Supplementation increased (Blair and Cummins, 1984) or had no effect (Cummins and Brunner, 1989) on serum IgG in dairy calves. A decreased incidence of scours was documented in calves supplemented with vitamin C (Cummins and Brunner, 1989).

Vitamin C acts synergistically with α-tocopherol by reducing the tocopheroxyl radical, thereby regenerating membrane-bound vitamin E and restoring the radical scavenging activity of vitamin E (Niki, 1987; Chan, 1993). Supplemental vitamin E and vitamin C also synergistically inhibit the release of arachidonic acid (ElAttar and Lin, 1992). This inhibition of arachidonic acid results in stimulation of immune cell response and suppression of tumor growth in humans and animals (Kubena and McMurray, 1996). Therefore it appears that although vitamin C alone may not conclusively have an effect on immune function, the combination of vitamin E and vitamin C is effective.

Immunodeficiencies in animals

Severe combined immunodeficiency. Severe combined immunodeficiency (SCID) is found in humans, rats, dogs and horses of Arabian breeding (Perryman, 2000). Absence of serum IgM, declining IgG concentration, and lymphopenia (<1000 lymphocytes/μl) are used to diagnose SCID (Perryman, 2000). Both T and B lymphocytes are affected, hence the name combined immunodeficiency. In horses, affected foals survive until about five months of age at which time the maternal antibodies are catabolized and foal antibodies cannot be produced.

Affected horses lack the enzyme DNA-dependent protein kinase (DNA-PK) that results in a five base pair deletion within the catalytic subunit of DNA-PK (DNA-PK_{CS}) (Shin et al., 1997). This mutation leads to a frame shift, resulting in the truncation of 967 amino acids from DNA-PK_{CS} gene product (Perryman, 2000). The truncation results in elimination of lymphocyte precursor cells and absence of mature, functional B and T lymphocytes (Perryman, 2000).

Severe combined immunodeficiency has been identified as autosomal recessive (Perryman, 2000). Heterozygous horses are clinically normal, survive into adulthood and have immunoglobulin concentrations within normal ranges. Since SCID has an incidence of approximately 2% (Perryman and Magnuson, 1982) and inevitably leads to death in homozygous foals, a test has been developed to identify normal or mutated DNA-PK_{CS} in adult horses of Arabian descent (Shin et al., 1997). This test has identified SCID carriers and helped to reduce the incidence of SCID in the Arabian breed.

Agammaglobulinemia. Although not the first immunodeficiency found in horses, agammaglobulinemia was the first to be discovered in humans (Perryman and Magnuson, 1982). Absence of serum IgM, IgA and IgG(T) and markedly reduced concentrations of IgG as low as 16 mg/dl (McGuire et al., 1976) characterize the immunodeficiency found in Thoroughbreds, Quarter Horses and Standardbreds (Perryman, 2000). Other breeds may be susceptible to the immunodeficiency but have yet to be documented. The immunodeficiency is caused by failed production of B-lymphocytes (Banks et al., 1976). Because affected horses can live up to 19 months of age, conclusions have been drawn that the T lymphocytes must be functional (Perryman, 2000) or else the combined immunodeficiency would cause the animals to die before

four months of age (McGuire et al., 1976). Agammaglobulinemia has been proven to be a sex-linked (Y chromosome) immunodeficiency in humans (Perryman and McGuire, 1982) but the low incidence in horses leads to an assumption that only males may be affected (Perryman, 2000). Not enough research has been conducted in the horse to determine the genetic basis of agammaglobulinemia (Perryman, 2000).

Selective IgM deficiency. Quarter Horses and Arabians are afflicted with an immunodeficiency called selective IgM deficiency. This deficiency is characterized by concentrations of IgM greater than two standard deviations below normal levels, but normal levels of the other immunoglobulins (Perryman and McGuire, 1982). The affected horses can be of either gender and usually range in age from two to eight months when diagnosed, but can live to adulthood (Perryman, 2000). Horses more than two years of age commonly acquire neoplasms, which make the IgM deficiency secondary to the neoplasm (Perryman, 2000). The genetic basis, as well as molecular mechanism of selective IgM deficiency is still unknown.

Selective IgA deficiency. The most common immunodeficiency found in man is selective IgA deficiency (Felsburg et al., 1976). Several breeds of dog also are afflicted with the immunodeficiency including German Shepherds (Day and Penhale, 1988), shar-peis (Moroff et al., 1986), beagles (Felsburg et al., 1985) and English cocker spaniels (Day, 1996). Selective IgA deficiency is characterized by reduced or absent IgA, with normal or elevated IgG and IgM concentrations (Felsburg et al., 1976). No such immunodeficiency has been documented in the horse.

Immunoglobulin A deficient humans and dogs are predisposed to respiratory, gastrointestinal, neurologic, skin, and autoimmune problems (Horowitz and Hong, 1975; Moroff et al., 1986). Unlike some other immunodeficiencies, selective IgA deficiency allows most affected individuals to live clinically normal lives into adulthood. An epidemiological study conducted on 830 healthy adult dogs for IgA deficiency found 1% with undetectable IgA concentrations, and 8% had deficient levels of IgA (Felsburg et al., 1976).

There are many theories as to why individuals can be afflicted with an immunodeficiency and yet be asymptomatic. One such theory is that the reduction in IgA concentration is compensated by an increase in the other immunoglobulins such as IgG and IgM (Horowitz and Hong, 1975; Moroff et al., 1986). Another theory is that the contribution of IgA subclasses to either the serum or mucosal concentrations fluctuates (Moroff et al., 1986). It can be hypothesized that the mucosal concentration of IgA in asymptomatic IgA deficient individuals would be greater than in symptomatic individuals.

Since animals with IgA deficiencies can be clinically normal, it has been suggested that the deficiency is not caused by a diseased state (Day, 1996). Deficient dogs have been shown to remain deficient throughout their lives (Felsburg et al., 1976; Day, 1996). This would lead to the conclusion that selective IgA deficiency is due in part to the inability to produce sufficient IgA. Plasma cells need T cells to convert them from IgM bearing cells to IgA bearing cells (Whitbread et al., 1984). Therefore, if T cells were not able to transform plasma cells, a deficiency in IgA would be met with a corresponding increase in IgM.

The incidence of IgA deficiency varies, with reports as high as 76.9% in a canine breeding colony and as low as 0.003% in the entire human population (Moroff et al., 1986). There is a familial inheritance of selective IgA deficiency found in humans (Felsburg et al., 1985). A strong heritability was found in beagles (Felsburg et al., 1985) and English cocker spaniels (Day, 1996). In the latter, the deficiency was passed down through three generations. The exact genetic basis is still unknown though, as several differing patterns of inheritance have been shown, including both autosomal recessive and autosomal dominant (Horowitz and Hong, 1975), and sporadic patterns (Moroff et al., 1986).

Table 1. Effects of moderate exercise on the immune system

Subject	**Immune variable**	**Response**	**Reference**
Horse	Neutrophils	Enhanced activity	Radial et al., 2000
Marathon runner and Jogger	IgA secretion rate	Unchanged	Mackinnon and Hooper, 1994
College men	Salivary IgA conc.	Unchanged	McDowell et al., 1993
Walkers	Total leukocytes Total lymphocytes IgG conc. IgA, IgM conc.	Increased Increased Increased Unchanged	Nehlsen-Cannarella et al., 1991
Humans	Salivary IgA	Increased	Reid et al., 2001
Marathon runner	IgA, IgG, IgM conc.	Unchanged	Nieman et al., 1989

Table 2. Effects of high intensity exercise on the immune system

Subject	**Immune variable**	**Response**	**Reference**
Horse	Neutrophils	Decreased activity	Radial et al., 2000
Horse	IgA, IgG, IgM conc.	Unchanged	Wong et al., 1992
Elite kayakers	Salivary IgA secretion rate and conc. IgG, IgM secretion rate and conc.	Decreased Unchanged	Mackinnon et al., 1993
Male humans	Salivary IgA secretion rate and conc.	Increased	Blannin et al., 1998
Well-trained male athletes	Salivary IgA conc.	Unchanged	Walsh et al., 1999

Objectives

The objectives for the present study are as follows: 1) To assess moderate exercise effects on serum immunoglobulins A and G in the endurance horse. 2) To determine if a supplement high in vitamin E or vitamins E and C would increase immunoglobulin concentrations in endurance horses. 3) To determine if a diet rich in fiber and fat or a high fat sweet feed would influence serum immunoglobulin concentrations. 4) To investigate the possibility of an immunodeficiency in the endurance-trained Arabian by quantifying serum IgA levels and obtaining data on possible symptoms of selective IgA deficiency from the riders based on their horses' health.

Materials and Methods

Trial 1

Surveys. Endurance riders were first contacted via telephone to determine their interest in an endurance race dedicated solely to research, the Middleburg Research Ride. Based on interest, 65 surveys were mailed requesting detailed nutritional and performance histories as well as rider opinions of regarding nutritional supplements, antioxidants and electrolyte replacement (Appendix). More specifically, questions addressed the following: horse information (age, sex, breed, height, existing medical problems), performance and training history (number, distance, and completion rate of past races, training times and distance exercised during the month, week and day before a race), transportation time and distance to the Middleburg Agricultural Research and Extension Center (MARE Center), and feed and supplement information (feed/supplement immediately before, during and after race, amount and brand of feed/supplements fed while at home) (Williams, personal communication).

Of the 65 surveys mailed, 55 were returned. Changes in rider interest and/or unforeseen circumstances (e.g., injury) in the weeks before The Ride reduced the horse entries and surveys of interest to 46. The survey results indicated that approximately half (n = 21) of the horses consumed grain at less than 12 %, and the other 25 horses more than 17 % of their total dietary intake. These groups were statistically different ($P < 0.0001$). Since grain intake has been associated with a series of disorders (Kronfeld, 2001a) and may exacerbate other performance stresses, oxidative stress and acid-base balance, horses with different grain intakes were paired prior to forming experimental groups. The survey results also aided the evaluation of transportation and training effects on plasma immunoglobulins.

Horses. Forty-six endurance horses, 10.6 ± 0.6 years of age, of mainly Arabian or Arabian mixed breeding, and an average body weight of 421 ± 5.9 kg, were paired by dietary history and divided into two groups. Main effects were a 2x2 factorial design with effects electrolytes (commercial vs test) and antioxidant (vitamin E only vs both vitamins E and C).

Horses were first divided based on their rider's willingness to use Virginia Tech formulated electrolytes. Riders chose between using a commercial electrolyte supplement to which their horse was already adapted, or our formulated electrolyte mixtures (Kronfeld, 2001b), including a potassium-free mixture that was to be given during exercise and a resting/potassium-replacement formula to be given pre- and post-exercise. Riders choosing to continue with their commercial electrolytes were instructed to follow their normal administration procedures. Riders that chose to use the test electrolytes were given measured doses and guidelines for administration, but the specific times and method of dosing were left to the riders' best judgement.

Within the electrolyte groups, horses were randomly divided into two vitamin supplementation groups. Horses received 5,000 IU/d vitamin E only, in the form of dl-alpha tocopherol acetate, or both vitamin E (5,000 IU/d) and vitamin C (7 g/d). The vitamins were supplied in measured, pre-packaged, daily doses (SmartPak Equine, Pembroke, Massachusetts), with instructions to top dress on daily feed for 3-4 weeks prior to research ride.

Exercise Protocol. The Middleburg Research Ride, held on April 1, 2001, was an 80-km race that followed American Endurance Ride Conference (AERC) rules and covered terrain

ranging from 121 to 442 m elevation in northern Virginia. The institution's Animal Care and Use Committee approved the protocol. Veterinary checkpoints were set up the day before the race and at km 0 (pre-race), 21, 37, 56, 72, and 80 (finish). Pulse, respiration, and rectal temperature were taken as well as standard veterinary procedures to evaluate fitness to continue. Body weight of the horse without tack, and the rider with tack, was also taken the day before, and at km 37 and 80.

Blood Sample Collection. Venous blood samples were taken the day before the ride, and 56 and 80 km. Heart rate and rectal temperature were recorded each time blood was taken. Samples were pulled into heparinized 10 ml Vacutainer™ (Becton Dickinson and Company, Franklin Lakes, NJ) tubes. The samples were centrifuged at 3000 g for 10 min. Plasma was removed and frozen at -80° C pending analyses.

Serum Immunoglobulin Quantification. Plasma immunoglobulins A, G, and M were assayed by single radial immunodiffusion kits (VMRD, Pullman, WA). Three µl of reference standard is pipetted into a well in agarose plates. Three µl of sample is then pipetted into the remaining wells. Antiserum specific for the immunoglobulin to be measured is incorporated into the agarose gel. The sample antigen diffuses into the gel containing the antibody, and a ring of precipitation forms that is proportional in size to the concentration of the antigen. The precipitation ring can be directly measured in mm 18 to 24 hr after pipetting. A linear relationship exists between the diameter of the ring and the concentration of the antigen when plotted on semi-log graph paper. Duplicate samples were run for all classes of immunoglobulins.

Albumin. A sister study collected blood samples at all veterinary checkpoints, and analyzed the samples for albumin (Hess, personal communication). The data were used in this study to adjust IgA and IgG concentrations for water shifts during exercise. Calculation of adjusted immunoglobulin (Ig_{56ADJ}) concentrations for 56 km used albumin data from 0 (alb_0), and 56 (alb_{56}) and immunoglobulin concentrations at 56 km (Ig_{56}):

$$Ig_{56ADJ} = (1/(alb_{56}/alb_0)) * Ig_{56}$$

Calculation of adjusted immunoglobulin (Ig_{80ADJ}) concentrations for 80 km used albumin data from 0 (alb_0), and 80 (alb_{80}) and immunoglobulin concentrations at 80 km (Ig80):

$$Ig_{80ADJ} = (1/(alb_{80}/alb_0)) * Ig_{80}$$

Statistical Analysis. Data were summarized as least squares means and standard errors and plotted over distance. Data were tested for normal distribution by the Shapiro-Wilk statistic. Analysis of variance was used to evaluate effects of treatment (electrolyte and vitamin supplementation), distance, treatment x treatment, and treatment x distance interaction effects. Interactions that were non-significant ($P > 0.10$) were dropped from the model. There were no treatment x treatment interaction effects found between electrolytes or vitamins. When treatment x distance interaction effects were found ($P = 0.10$) a Student's t-test was applied to examine treatment effects at each distance.

Associations of blood variables and survey data with immunoglobulin concentrations were tested as Pearson's product-moment correlations.

Empirical regression for IgA, IgG and IgM was determined by stepwise selection ($P < 0.15$). Physiological regression was determined by multiple regression using only variables of likely physiological influence and previously determined to be correlated with the specific immunoglobulin at the $P < 0.10$ level. The relative influence of the selected variables on immunoglobulin concentration was determined by the standardized β coefficients (*t* statistics). All statistical procedures were assisted by the SAS system (SAS Inst. Inc., Cary NC).

Trial 2

Surveys. Riders completed surveys detailing performance and nutritional histories, similar to those used in Trial 1 (Appendix). Riders answered an additional five questions pertaining to respiratory, skin, and gastrointestinal problems as well as allergies and chronic diarrhea. These disorders are common symptoms found in humans and dogs with selective IgA deficiency, so were of interest in this trial (Horowitz and Hong, 1975; Moroff et al., 1986).

Horses. Forty endurance horses, 12.1 ± 0.7 years of age, of mainly Arabian or Arabian mixed breeding, and an average pre-race body weight of 438 ± 7.1 kg, were randomly divided into four groups. Main effects were a 2x2 factorial design with effects of diet (multi-fiber and fat vs sweet feed), and electrolytes (commercial vs test).

Horses were first paired based on their riders' willingness to use Virginia Tech formulated electrolytes. Riders chose between using a commercial electrolyte supplement to which their horse was already adapted, or our formulated electrolyte mixtures (Kronfeld, 2001b), including a potassium-free mixture that was to be given during exercise and a resting/potassium-

replacement formula to be given post-exercise. Riders choosing to continue with their commercial electrolytes were instructed to follow their normal administration procedures. Riders that chose to use the test electrolytes were given measured doses and guidelines for administration, but the specific times and method of dosing were left to the riders' best judgement.

Within the electrolyte group, riders willing to feed their horses feed formulated by Virginia Tech were randomly divided into two groups and given a feed either rich in fiber and fat, or a high fat sweet feed, our copy of a typical best-selling endurance horse feed on the market (Table 3). Dairy One proximate analysis is in Table 4. Riders were given feeding instructions and began feeding the VT feeds three months prior to the race.

Exercise Protocol. The Middleburg Research Ride 2002, held on April 14, 2002, was an 80-km race that followed American Endurance Ride Conference (AERC) rules. The institution's Animal Care and Use Committee approved the protocol. Veterinary checkpoints were set up the day before the race and at km 27, 50, 72 and 80 (finish). Pulse, respiration, rectal temperature and body weight were taken as well as standard veterinary procedures to evaluate fitness to continue.

Blood Sample Collection. Venous blood samples were taken the day before the ride, and 27 and 80 km. Samples were collected into heparinized 10 ml Vacutainer™ (Becton Dickinson and Company, Franklin Lakes, NJ) tubes. The samples were centrifuged at 3000 g for 10 min. Plasma was removed and frozen at -80° C pending analyses.

Serum Immunoglobulin Quantification. Plasma concentrations of immunoglobulins A, G, and M were assayed by single radial immunodiffusion kits (VMRD, Pullman, WA). Three µl of reference standard is pipetted into a well in agarose plates. Three µl of sample is then pipetted into the remaining wells. Antiserum specific for the immunoglobulin to be measured is incorporated into the agarose gel. The sample antigen diffuses into the gel containing the antibody, and a ring of precipitation forms that is proportional in size to the concentration of the antigen. The precipitation ring can be directly measured in mm 18 to 24 hr after pipetting. A linear relationship exists between the diameter of the ring and the concentration of the antigen when plotted on semi-log graph paper. Duplicate samples were run for all classes of immunoglobulins.

Albumin. A sister study collected blood samples at all veterinary checkpoints and analyzed them for albumin (Hess, personal communication). The data were used in this study to adjust IgA and IgG concentrations for water shifts during exercise. Calculation of adjusted immunoglobulin (Ig_{527DJ}) concentrations for 27 km used albumin data from 0 (alb_0), and 27 (alb_{27}) and immunoglobulin concentrations at 27 km (Ig_{27}):

$$Ig_{27ADJ} = (1/(alb_{27}/alb_0)) * Ig_{27}$$

Calculation of adjusted immunoglobulin (Ig_{527DJ}) concentrations for 80 km used albumin data from 0 (alb_0), and 80 (alb_{80}) and immunoglobulin concentrations at 80 km (Ig80):

$$Ig_{80ADJ} = (1/(alb_{80}/alb_0)) * Ig_{80}$$

Statistical Analysis. Data were summarized as least squares means and standard errors and plotted over distance. Data were tested for normal distribution by the Shapiro-Wilk statistic. Analysis of variance was used to evaluate the effects of treatment (electrolyte and feed supplementation), distance, treatment x treatment, and treatment x distance interaction effects. Interactions that were non-significant ($P > 0.10$) were dropped from the model. There were no treatment x treatment interaction effects found between electrolytes or feed. When treatment x distance interaction effects were found ($P = 0.10$) a Student's t-test was applied to examine treatment effects at each distance.

Associations of blood variables and survey data with immunoglobulin concentrations were tested as Pearson's product-moment correlations.

Empirical regression for IgA, IgG and IgM was determined by stepwise selection ($P < 0.15$). Physiological regression was determined by multiple regression using only variables of likely physiological influence and previously determined to be correlated with the specific immunoglobulin at the $P < 0.10$ level. The relative influence of the selected variables on immunoglobulin concentration was determined by the standardized β coefficients (*t* statistics). All statistical procedures listed above were assisted by the SAS system (SAS Inst. Inc., Cary NC).

The Two by Two Tables procedure was used to compute tests of association for two by two contingency tables. The procedure was used to test for association between IgA deficiency and results of the survey relating to health problems. Fisher exact test, two-tailed, was used to determine association. This procedure was assisted by Statistix 7 (Analytical Software, Tallahassee, FL).

Table 3. Ingredient composition of fiber and fat feed and high fat sweet feed given to endurance horses (Trial 2)

	Ingredients, %	
	Fiber and fat feed (n = 2)	**High fat sweet feed (n = 2)**
Processed cereal by-product, 10%	40	0
VT corn oil	6	4
VT mineral premix	0.5	0.5
VT vitamin premix	0.5	0.5
Alfalfa hay, early bloom	8	0
Corn, dent yellow, cracked	4	29
Oat grain	0	35
Soybean meal, 48%	0	5
Molasses, cane	5	10
Timothy, full bloom	12	8.5
Soybean hulls	12	4
Beet pulp	12	0
DiCal-Phos	0	2
Limestone	0	1.5
TOTALS	**100**	**100**

Table 4. Nutrient composition (mean ± SE) on a dry matter basis of fiber and fat feed and high fat sweet feed in Trial 2

Component	Fiber and fat feed (n = 2)	High fat sweet feed (n = 2)
Crude protein, %	13.8 ± 0.13	11.7 ± 0.13
Crude fat, %	17.7 ± 1.51	7.65 ± 1.51
Acid detergent fiber, %	23.1 ± 0.32	14.6 ± 0.32
Neutral detergent fiber, %	36.4 ± 1.44	26.0 ± 1.44
Non-structural carbohydrate, %	17.5 ± 0.60	43.6 ± 0.60
Starch, %	5.35 ± 0.78	31.9 ± 0.78
Sugar, %	12.2 ± 0.30	11.7 ± 0.30
Ash, %	9.71 ± 0.41	8.7 ± 0.41
Calcium, %	1.81 ± 0.09	1.96 ± 0.09
Phosphorus, %	0.89 ± 0.07	0.79 ± 0.07
Magnesium, %	0.55 ± 0.02	0.19 ± 0.02
Potassium, %	1.17 ± 0.04	1.06 ± 0.04
Sodium, %	0.18 ± 0.04	0.36 ± 0.04
Iron, mg/kg	224 ± 6.08	317 ± 6.08
Zinc, mg/kg	179 ± 12.2	171 ± 12.2
Copper, mg/kg	57.0 ± 7.85	64.5 ± 7.85
Manganese, mg/kg	208 ± 19.7	84.0 ± 19.7

Analysis performed by Dairy One, Ithaca NY

Results

Trial 1

Forty-four horses started the research ride and 34 horses finished. Horses did not finish due to metabolic problems, lameness and rider option. Only data from horses that finished were included in the analysis of correlations and distance effects. Data collected at 0 km were used to compare horses with deficient IgA concentrations to horses with normal IgA concentrations regardless of whether or not they completed the ride. Pre-race and post race means were 421 ± 5.9 and 404 ± 6.3 kg, respectively.

Serum IgG. Serum IgG concentrations at 0, 56 and 80 km were (lsmean ± SE) 2298 ± 126, 2614 ± 124, and 2533 ± 124 mg/dl, respectively. There were no effects of distance (P = 0.18) on serum IgG concentration. There were no effects of distance (P = 0.65) on IgG concentration adjusted for water shifts with albumin data. Adjusted IgG concentrations at 0, 56 and 80 km were (lsmean ± SE) 2301 ± 111, 2367 ± 123, and 2204 ± 130 mg/dl, respectively. There were no effects of antioxidant supplementation (P = 0.16) on IgG concentration. Immunoglobulin G concentration was correlated with age (r = 0.27, P = 0.074), transport time to the race (r = 0.28, P = 0.083), transport distance to the race (r = 0.30, P = 0.057), and number of previous races (r = 0.33, P = 0.041). The best-fitting empirical multiple regression had an adjusted R-square of 0.32, with IgM concentration having the greatest impact on serum IgG, followed by transportation distance (Table 5). The physiologically selected multiple regression had an adjusted R-square of 0.07, with number of previous races having the strongest impact on IgG, followed by transportation distance, transportation time and age. (Table 6).

Serum IgM. Immunoglobulin M concentrations were only measured at 0 km, and the mean concentration was 76.6 ± 34.7 mg/dl. There were no effects of antioxidant supplementation ($P = 0.70$) on IgM concentration. Serum IgM concentration was not correlated with any of the variables studied at the $P < 0.10$ level. At the $P < 0.15$ level IgM was correlated with rank of finish at the end of this race (r = 0.25, $P = 0.11$). The best-fitting empirical multiple regression had an adjusted R-square of 0.56, with training activity the day before the race and IgG concentration having the greatest impact on serum IgM, followed by IgA concentration and rank of finish in this race (Table 7). A physiologically selected multiple regression was not possible as IgM was not correlated with a variable to physiologically explain IgM concentration at the beginning of the race.

Serum IgA. Serum IgA concentrations at 0, 56, and 80 km were (lsmean ± SE) 181 ± 20.3, 170 ± 20.0, and 158 ± 20.0 mg/dl, respectively. There were no effects of distance ($P = 0.73$) on IgA concentration. There were no effects of distance ($P = 0.52$) on serum IgA concentration adjusted for water shifts using albumin data. Adjusted IgA concentrations at 0, 56, and 80 km were (lsmean ± SE) 183 ± 18.9, 162 ± 21.1, and 151 ± 22.5 mg/dl, respectively. There were no effects of antioxidant supplementation ($P = 0.16$) on IgA concentration. IgA concentration was correlated with number of previous races (r = 0.31, $P = 0.058$), training distance the week before the race (r = 0.35, $P = 0.044$) and training time the day before the race (r = 0.33, $P = 0.047$). The best-fitting empirical multiple regression had an adjusted R-square of 0.75, with feed and IgM concentration having the greatest impact on serum IgA, followed by training distance the week before a race, and transportation time to the race (Table 8). The physiologically selected multiple regression had an adjusted R-square of 0.16, with number of

previous races having the strongest impact on IgA, followed by training distance the week before the race and training time the day before the race (Table 9).

IgA Deficient Horses. Sixteen horses (36 %) had IgA concentrations < 40 mg/dl. Of these horses, nine had undetectable concentrations (< 19 mg/dl). The data were placed into two groups based upon IgA concentration: IgA deficient (defA) and IgA normal (normA), 14.1 ± 26.7 and 307 ± 20.1 mg/dl, respectively ($P < 0.0001$).

Data relative to defA and normA are summarized as means and standard errors in Table 10. Immunoglobulin M concentration was higher ($P = 0.035$) in the defA group (91.2 ± 8.31 mg/dl) than the normA group (68.3 ± 6.25). There were no differences ($P > 0.11$) in age, weight, IgG concentration, transport time, transport distance, and training variables.

Trial 2

Forty horses started the research ride and 23 horses finished. Horses did not finiah due to metabolic problems, lameness and rider option. Only data from the horses that finished were included in the analysis of correlations and distance effects. Data collected at 0 km were used to compare horses with deficient IgA concentrations to horses with normal IgA concentrations regardless of whether or not they completed the ride. Pre and post race body weights were 438 ± 7.1 and 419 ± 7.1 kg, respectively.

Serum IgG. Serum IgG concentrations at 0, 27 and 80 km were (lsmean ± SE) 1975 ± 97.2, 2364 ± 94.3 and 2357 ± 94.3 mg/dl, respectively. Serum IgG concentration was lower at 0

than 27 km (P = 0.005) and lower at 0 than 80 km (P = 0.006). Serum IgG concentration was not different between 27 and 80 km (P =0.96). There were no effects of distance (P = 0.15) on serum IgG adjusted for water shifts with albumin data. Adjusted IgG concentrations at 0, 27 and 80 km were (lsmean ± SE) 1962 ± 85.2, 2053 ± 82.6, and 2204 ± 89.7 mg/dl, respectively. There were no effects of feed (P = 0.59) on IgG concentration. IgG was correlated with age (r = 0.43, P = 0.006) and number of previous races (r = 0.39, P = 0.022). The best-fitting empirical multiple regression had an adjusted R-square of 0.68, with skin problems having the greatest impact on serum IgG, followed by training distance the week before the race (Table 11). The physiologically selected multiple regression had an adjusted R-square of 0.15, with age having the strongest impact on IgG concentration, followed by number of past races. (Table 12).

Serum IgM. Immunoglobulin M concentrations were only measured at 0 km, and the mean concentration was 75.9 ± 12.1 mg/dl. There were no effects of feed on IgM concentration (P = 0.54). Serum IgM was correlated with age (r = 0.40, P = 0.011), transport time to this race (r = 0.42, P = 0.01), transport distance to race (r = 0.34, P = 0.053) and IgA concentration (r = -0.33, P = 0.051). The best-fitting empirical multiple regression had an adjusted R-square of 0.92, with training time the day before the race, and gastrointestinal problems having the greatest impact on serum IgM, followed by IgA concentration, age, training distance the week before the race and chronic diarrhea problems (Table 13). The physiologically selected multiple regression had an adjusted R-square of 0.46, with transport time and distance having the strongest impact on IgM concentration, followed by age and IgA concentration (Table 14).

Serum IgA. Serum IgA concentrations at 0, 27 and 80 km were (lsmean ± SE) 113 ± 22.0, 186 ± 21.3 and 135 ± 21.3 mg/dl, respectively. IgA concentration was increased after the

start of the race, but returned to baseline at the finish ($P = 0.05$). There were no effects of distance ($P = 0.16$) on IgA concentration adjusted for water shifts with albumin data. Adjusted IgA concentrations at 0, 27, and 80 km were 115 ± 18.9, 162 ± 18.3, and 122 ± 19.9 mg/dl, respectively. There were no effects of feed on IgA concentration ($P = 0.80$). IgA was correlated with training distance the week before the race (r = 0.36, $P = 0.053$), transport time to the race (r = -0.28, $P = 0.11$), and IgM concentration (r = -0.33, $P = 0.05$). The best-fitting empirical multiple regression had an adjusted R-square of 0.24, with rank at the finish of Trial 2's ride having the only impact on serum IgA (Table 15). The physiologically selected multiple regression had an adjusted R-square of 0.18, with training distance the week before the ride having the strongest impact on IgA concentration, followed by transportation time to the race and IgM concentration (Table 16).

IgA Deficient Horses. Twelve horses (30 %) had IgA concentrations < 40 mg/dl. Of these horses, eight had undetectable concentrations (< 19 mg/dl). The data were placed into two groups based upon IgA concentration: IgA deficient (defA) and IgA normal (normA), with 9.69 ± 27.9 and 252 ± 18.8 mg/dl, respectively. All of the IgA concentrations in the defA group were not different from 0 ($P > 0.35$).

Data relative to defA and normA horses are summarized as means and standard errors in Table 17. Immunoglobulin M concentration was higher ($P = 0.017$) in the defA group (96.4 ± 10.2 mg/dl) than the normA group (64.5 + 7.01). The defA horses were older ($P = 0.03$) compared to the normA horses, 14.2 ± 1.22 and 10.8 ± 0.82 yr, respectively. There were no

differences ($P > 0.11$) in weight, IgG concentration, transport time, transport distance, and training variables.

Health questionnaire. Data from the health questionnaire was collected on 10 defA and 22 normA horses. Two of the 10 defA horses had respiratory problems compared to 0 of 22 normA horses. Four defA horses had skin problems, and two normA horses had skin problems. Three defA horses had allergies, and one normA horse had allergies. Gastrointestinal problems were reported in three defA horses and four normA horses. One horse from each group experienced chronic diarrhea. Fisher exact test indicated an association trend between selective IgA deficiency and respiratory problems ($P = 0.091$), skin problems ($P = 0.06$), and allergies ($P = 0.079$), but not gastrointestinal problems ($P = 0.65$). Associations with chronic diarrhea could not be calculated due to the low numbers used in the calculation.

Six horses from each group had one or more health problems listed on the survey. Five horses from each group experienced two or more health problems. Two defA horses and zero normA horses suffered from three of the health conditions on the survey. Fisher exact test indicated an association between selective IgA deficiency and two or more health problems ($P = 0.019$) and an association trend between selective IgA deficiency and one or more health problems ($P = 0.12$) and three health problems ($P = 0.091$). There were no horses in either group that experienced four or five of the health problems on the survey.

Returning horses. Fourteen horses participated in both trials and their immunoglobulin data were compared between trials. Concentrations of IgG and IgM were lower ($P = 0.003$, $P = 0.042$, respectively) in Trial 2 compared to Trial 1 (Table 18). Concentration of IgA did not

differ between trials ($P = 0.166$). Horses that were deficient in Trial 1 remained deficient in Trial 2 (Table 19).

Table 5. Empirical multiple regression on IgG (Trial 1) (n = 44)

	Mean	**SE**	**t**	**Adjusted R^2**	**P**
Intercept	2735	356	7.68		
IgM	-8.43	4.05	-2.08	0.16	0.098
Transport distance	1.17	0.64	1.83	0.32	0.087

Table 6. Physiological multiple regression on IgG (Trial 1), using variables previously associated ($P < 0.15$) with IgG (n = 44)

	Mean	**SE**	**t**	**P**
Intercept	2008	250	8.02	< 0.0001
Age	7.67	29.5	0.26	0.80
Transportation time	-57.2	111	-0.52	0.61
Transportation distance	1.67	2.27	0.74	0.47
Past races	6.93	8.16	0.85	0.40

Adjusted $R^2 = 0.07$

Table 7. Empirical multiple regression on IgM (Trial 1) (n = 44)

	Mean	SE	t	Adjusted R^2	P
Intercept	86.1	25.6	3.36		
IgG concentration	-0.02	0.01	-2.00	0.15	0.083
IgA concentration	-0.09	0.03	-3.00	0.26	0.12
Activity day before	13.8	5.00	2.76	0.46	0.025
Rank of finish	0.90	0.45	2.00	0.56	0.063

Table 8. Empirical multiple regression on IgA (Trial 1) (n = 44)

	Mean	**SE**	**t**	**Adjusted R^2**	**P**
Intercept	-95.8	135	-0.71		
Feed	194	75.5	2.57	0.30	0.020
IgM	18.3	7.20	2.54	0.46	0.048
Distance week before	17.6	5.40	3.26	0.59	0.056
Transportation time	-159	89.1	-1.78	0.68	0.077
Daily Time	-2.59	0.895	-2.89	0.75	0.099

Table 9. Physiological multiple regression on IgA (Trial 1), using variables previously associated ($P < 0.15$) with IgA (n = 44)

	Mean	**SE**	**t**	**P**
Intercept	-3.41	80.8	-0.04	0.97
Past races	2.46	2.24	1.10	0.28
Distance week before	7.66	3.87	1.98	0.058
Time day before	86.3	69.6	1.24	0.23

Adjusted $R^2 = 0.16$

Table 10. Least squared means ± SE of different variables in IgA deficient horses and normal IgA horses (Trial 1)

Variable	**IgA deficient horses (n = 16)**	**IgA normal horses (n = 28)**	**P**
Age (yr)	10.7 ± 1.00	10.5 ± 0.76	0.83
Weight (kg)	417 ± 8.9	424 ± 6.7	0.52
Transport time (hr)	3.41 ± 1.05	3.68 ± 0.86	0.84
Transport distance (km)	165 ± 51.4	168 ± 42.3	0.96
Training distance month before race (km/wk)	29.0 ± 2.91	29.9 ± 2.25	0.81
Training distance week before race (km/wk)	12.2 ± 2.41	11.6 ± 1.86	0.84
Training time day before race (hr)	0.65 ± 0.12	0.91 ± 0.10	0.11
Number of past races	14.1 ± 3.61	14.9 ± 3.07	0.87
IgA concentration (mg/dl)	14.1 ± 26.7	307 ± 20.1	< 0.0001
IgG concentration (mg/dl)	2316 ± 111	2154 ± 83.3	0.25
IgM concentration (mg/dl)	91.2 ± 8.31	68.3 ± 6.25	0.035
Completion rate of previous races	0.80 ± 0.04	0.77 ± 0.04	0.58
Rank at finish	12.4 ± 2.94	13.6 ± 2.22	0.75

Table 11. Empirical multiple regression on IgG (Trial 2) (n = 40)

	Mean	**SE**	**t**	**Adjusted R^2**	**P**
Intercept	1162	206	5.64		
Skin problems	553	127	4.35	0.48	0.0009
Distance week before	19.3	7.58	2.55	0.68	0.029

Table 12. Physiological multiple regression on IgG (Trial 2), using variables previously associated ($P < 0.15$) with IgG (n = 40)

	Mean	**SE**	**t**	**P**
Intercept	1495	206	7.25	< 0.0001
Age	27.7	18.7	1.48	0.15
Past races	8.22	6.67	1.23	0.23

Adjusted $R^2 = 0.15$

Table 13. Empirical multiple regression on IgM (Trial 2) (n = 40)

	Mean	SE	t	Adjusted R^2	P
Intercept	10.7	33.1	0.32		
Time day before	56.8	15.1	3.76	0.27	0.068
Gastrointestinal problems	29.0	12.5	2.32	0.49	0.062
IgA	4.68	1.22	3.84	0.75	0.11
Distance week before	2.67	0.85	3.14	0.84	0.090
Chronic diarrhea problems	-57.1	24.0	2.38	0.92	0.055

Table 14. Physiological multiple regression on IgM (Trial 2), using variables previously associated ($P < 0.15$) with IgM (n = 40)

	Mean	**SE**	**t**	**P**
Intercept	33.3	17.2	1.93	0.065
Age	2.78	1.34	2.07	0.049
Transport time	57.3	19.0	3.02	0.006
Transport distance	-0.93	0.33	-2.85	0.009
IgA concentration	-0.08	0.04	-2.07	0.050

Adjusted $R^2 = 0.46$

Table 15. Empirical multiple regression on IgA (Trial 2) (n = 40)

	Mean	**SE**	**t**	**Adjusted R^2**	**P**
Intercept	36.9	65.1	0.57		
Rank at finish	9.70	4.83	2.01	0.24	0.066

Table 16. Physiological multiple regression on IgA (Trial 2), using variables previously associated ($P < 0.15$) with IgA (n = 40)

	Mean	**SE**	**t**	**P**
Intercept	130	61.1	2.13	0.014
Distance week before	7.92	3.45	2.29	0.031
Transportation time	-0.85	10.5	-0.08	0.94
IgM	-0.92	0.60	-1.53	0.14

Adjusted $R^2 = 0.18$

Table 17. Least squared means ± SE of different variables in IgA deficient horses and normal IgA horses (Trial 2)

Variable	**IgA deficient horses (n = 12)**	**IgA normal horses (n = 28)**	**P**
Age (yr)	14.2 ± 1.22	10.8 ± 0.82	0.030
Weight (kg)	450 ± 13.6	439 ± 9.13	0.50
Transport time (hr)	2.50 ± 0.57	1.37 ± 0.37	0.11
Transport distance (km)	134 ± 34.7	67 ± 24.4	0.13
Training distance month before race (km/wk)	26.6 ± 3.45	27.1 ± 2.64	0.92
Training distance week before race (km/wk)	8.30 ± 2.05	12.6 ± 1.62	0.11
Training time day before race (hr)	0.49 ± 0.14	0.46 ± 0.10	0.97
Number of past races	12.7 ± 4.14	9.69 ± 2.64	0.54
IgA concentration (mg/dl)	9.69 ± 27.9	252 ± 18.8	< 0.0001
IgG concentration (mg/dl)	2011 ± 136	1888 ± 91.6	0.46
IgM concentration (mg/dl)	96.4 ± 10.2	64.5 ± 7.01	0.017
Completion rate of previous races	0.72 ± 0.100	0.77 ± 0.063	0.69
Rank at finish	10.4 ± 2.49	13.2 ± 1.81	0.37

Table 18. Concentrations of IgA, IgG, and IgM (mean ± SE) in horses (n = 14) that participated in both Trials 1 and 2

Immunoglobulin	**Concentration (mg/dl)**	**P**
IgA		
Trial 1	178 ± 11.4	0.17
Trial 2	154 ± 11.4	
IgG		
Trial 1	2104 ± 38.8	0.0003
Trial 2	1841 ± 38.8	
IgM		
Trial 1	85.7 + 3.28	0.042
Trial 2	75.2 + 3.28	

Table 19. Concentrations of IgA between Trial 1 and Trial 2 in horses that participated in both Trials 1 and 2

Horse	Trial 1 concentration (mg/dl)	Trial 2 concentration (mg/dl)
1	253.55	198.43
2	260.68	181.96
3	< 19	< 19
4	< 19	< 19
5	256.04	199.75
6	207.37	130.40
7	175.92	116.43
8	339.69	469.27
9	28.97	29.70
10	< 19	< 19
11	267.29	153.02
12	< 19	< 19
13	271.79	222.23
14	409.32	438.52

Discussion

The results of this study indicated that there were no effects of exercise, antioxidant supplementation, or diet supplementation with a fiber and fat feed or a high fat sweet feed on serum immunoglobulin concentration. A selective IgA deficiency was found, which is a first for the horse.

Exercise. There were no effects of distance on IgA and IgG in Trial 1. This is consistent with other findings (Nieman et al., 1989). However, IgG increased with distance in Trial 2 and IgA increased after the start, but returned to baseline at the finish of the race. IgG has been reported to increase with moderate exercise while IgA concentrations remain unchanged at the finish, with no changes in plasma volumes (Nehlsen-Cannarella et al., 1991). Once albumin was used to adjust IgA and IgG concentrations for water shifts the exercise-induced increase in immunoglobulin concentrations were nullified. Albumin concentrations increased with exercise, signifying water loss. The immunoglobulins thus became more concentrated. Therefore the increases in immunoglobulin concentrations were biased by water loss and not release of immunoglobulins into the bloodstream. It is difficult to conclusively say that IgG and IgA concentrations increased with distance in Trial 2 when water shifts are taken into account.

Antioxidants. Dietary vitamin E increased serum IgG and IgA in horses (Hoffman et al., 1999), but there have been no such reports relative to vitamin C supplementation. Since vitamin E deficiency has been suggested to contribute to equine exertional rhabdomyolysis (Siciliano et al., 1997) and equine motor neuron disease (Mayhew, 1994), this study supplemented vitamin E

as a positive control, with added vitamin C as the dietary treatment. Our findings indicate that the combination of vitamin E and vitamin C did not influence immunoglobulin concentrations, compared to supplementation of vitamin E only.

Diet. There were no differences in IgA, IgG and IgM concentrations between the diet rich in fiber and fat and high fat sweet feed diet. An enhanced immune system, as measured by unstimulated natural killer cell activity, was found in endurance-trained men consuming a high-carbohydrate diet compared to a high-fat diet (Pedersen et al., 2000). However, the men's high-fat diet consisted of 62 % fat, much higher than the fat percentage in the Virginia Tech equine formulated high fiber and fat feed (17.7 ± 1.51 %) or high fat sweet feed (7.65 ± 1.51 %). Perhaps the high fat diets in this study did not contain enough fat to cause immunosuppression. Pre-diet samples were not taken so the true effect of the diets is unknown.

Selective IgA deficiency. The horses in Trials 1 and 2 could be separated into two groups, defA and normA, based on IgA concentrations and several criteria. All of the IgA concentrations in the defA horses were not statistically different from zero. The defA IgA concentrations were less than 40 mg/dl; previously reported as deficient in the horse (MacLeay et al., 1997). None of the IgA concentrations in the normA group met any of the criteria for deficiency. Frequency numbers for IgA concentrations are displayed for Trial 1 (Figure 2), Trial 2 (Figure 3) and both Trials 1 and 2 (Figure 4).

There were no training differences between defA and normA horses. There were also no transportation differences, either in time or distance. Previous performance history, such as

number and completion rate of previous races also did not differ. Training has been found to affect IgA as well as IgG and IgM (Gleeson et al, 1995), but in this study training could not explain the low IgA concentrations. Moreover, neither could the overtraining syndrome as a loss of performance is a common symptom (Sharp and Koutedakis, 1992), and there were no correlations between rank of finish and IgA concentration. Rank of finish from Trials 1 and 2 for the top 20 finishers are displayed in Figure 1.

DefA horses were older than normA horses in Trial 2, but there were no differences in age in Trial 1. However, immuoglobulin concentrations tend to increase with age (McFarlane et al., 1996). In humans, a link between salivary IgA concentration and age was found (Kugler et al., 1992). Salivary IgA concentration in children less than seven years old was lower ($P < 0.05$) than people older than seven years of age, including adults. Children's ages and salivary IgA concentration were positively correlated ($r = 0.49$, $P < 0.05$). There were no differences between IgM concentrations in old (> 20 yrs) and young (5 to 12 yrs) horses, but IgG ($P = 0.07$) and IgA ($P = 0.07$) tended to be higher in old horses (McFarlane et al., 2001). Therefore, age is not an explanation for the low IgA concentrations in the defA horses because the lower IgA concentration was found in older horses, which goes against previous literature in both horses and humans. Also, age was an effect on IgA only in Trial 2, so it is a poor explanation of the difference, as it would have been different in Trial 1 as well.

The only difference between the IgA groups was IgM concentration. Immunoglobulin M concentration was higher in the defA horses than the normA horses, possibly to compensate for the low IgA concentrations. Another explanation could be a lack of T cell function, or a lack of

T cells themselves. If plasma cells are to be converted to IgA secreting from IgM secreting cells, T cells are needed (Whitbread et al., 1984). If T cells are not available, then a deficiency in IgA results, with a corresponding rise in IgM concentration.

The data collected here appear to represent an immunodeficiency not previously found in horses. It is not agammaglobulinemia (McGuire et al., 1976) as the horses have survived into adulthood and have normal levels of IgG and IgM. Selective IgM deficiency (Perryman and McGuire, 1982) has been ruled out because the levels of IgM are not below normal in the defA horses. All the defA horses are of Arabian breeding, although the most common genetic immunodeficiency afflicting this breed, severe combined immunodeficiency (SCID), is not applicable in this study. Homozygous foals with SCID have both IgM and IgG deficiencies and the foals die by five months of age (Perryman, 2000). Heterozygous SCID horses have normal serum immunoglobulins (McGuire et al., 1974). In this study, data most closely resemble data found in selective IgA deficiency. Serum concentrations of IgG and IgM were within normal ranges, as seen in English cocker spaniels (Day, 1996), beagles (Felsburg et al., 1985) and humans (Horowitz and Hong, 1973).

DefA horses were associated with the most common symptoms found in dogs (Moroff et al., 1986) and humans (Horowitz and Hong, 1975) with selective IgA deficiency – respiratory, and skin problems, and allergies as well as expression of multiple symptoms. It may be argued that the low IgA concentrations were due to the health problems of the horses, and not to an immunodeficiency. However, some of the horses with deficient IgA had no history of the health problems in question. Also, the defA horses continued to have deficient IgA concentrations a

year after they were first measured. Maintaining deficient levels of IgA is consistent with findings in beagles (Felsburg et al., 1985) and English cocker spaniels (Day, 1996) with selective IgA deficiency.

The incidence of horses deficient in IgA (36 % Trial 1, 30 % Trail 2) was higher than the range of 0.0003 % to 0.004 % incidence reported in the human population (Felsburg et al., 1985) but lower than the 76.9 % found in two breeding colonies of shar-pei dogs (Moroff et al., 1986). A possible explanation for the high incidence found in the endurance horses could be shared genetic lines, similar to those found in beagles (Felsburg et al., 1985) and shar-peis (Moroff et al., 1986). There may be a possibility of similar genetic lineage since the horses were predominantly from the same geographic region (Northern Virginia and Maryland) and were bred for endurance racing. These results warrant investigation into the genetic lineage of defA horses.

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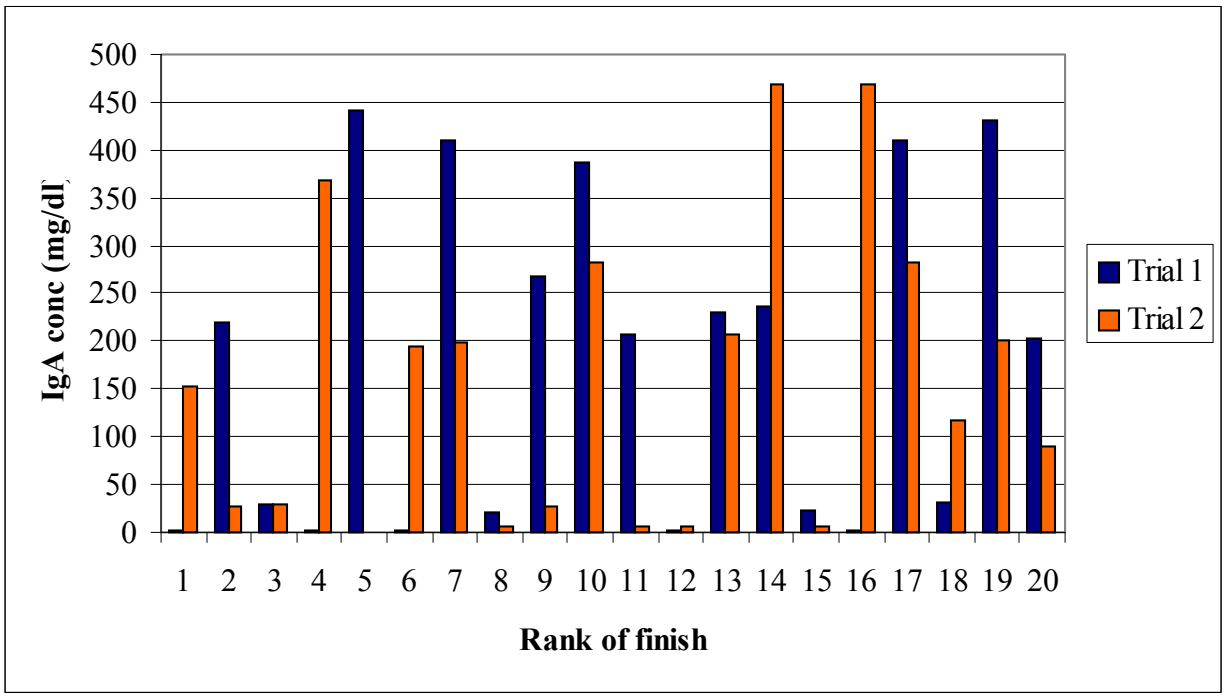

Figure 1. IgA concentration (mg/dl) at each rank of finish (Trials 1 and 2) for the top twenty horses

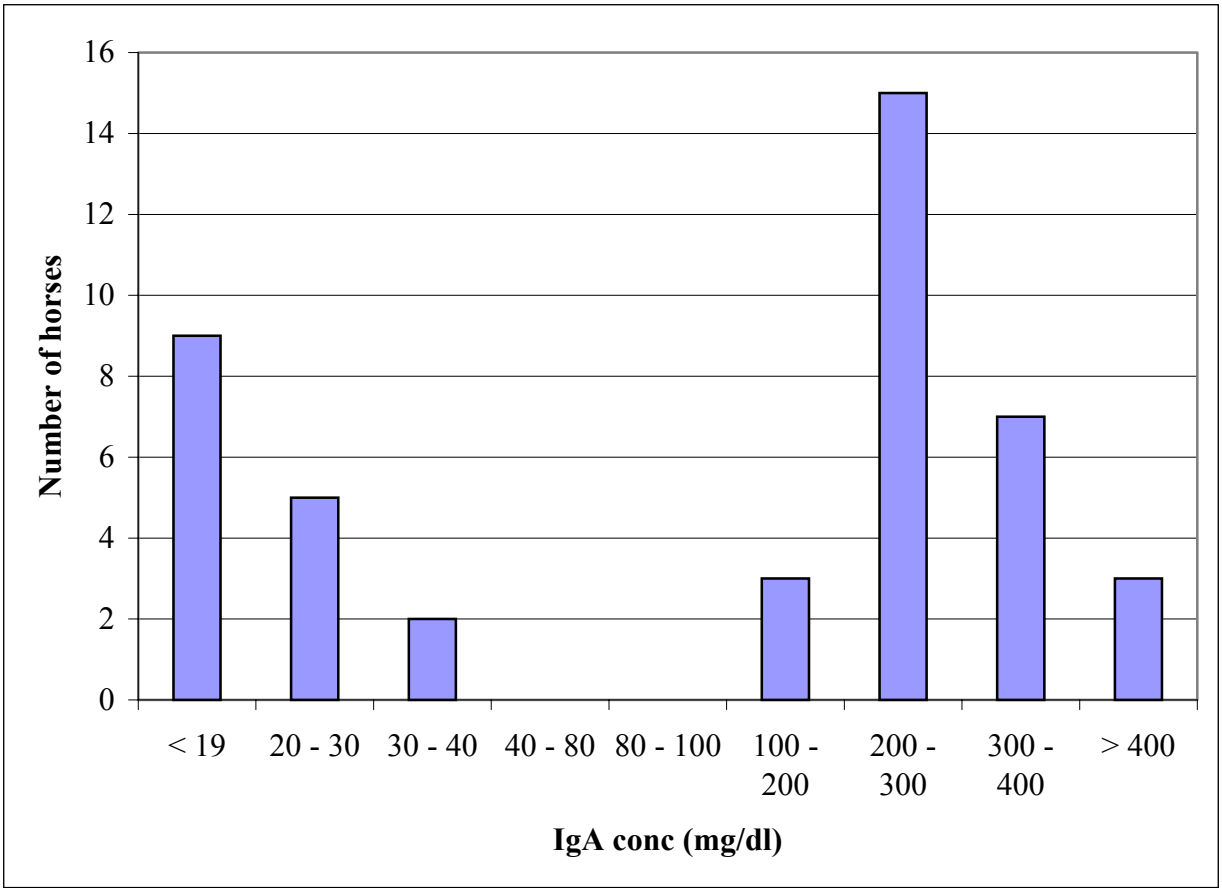

Figure 2. Frequency of IgA concentrations (mg/dl) in endurance horses (Trial 1)

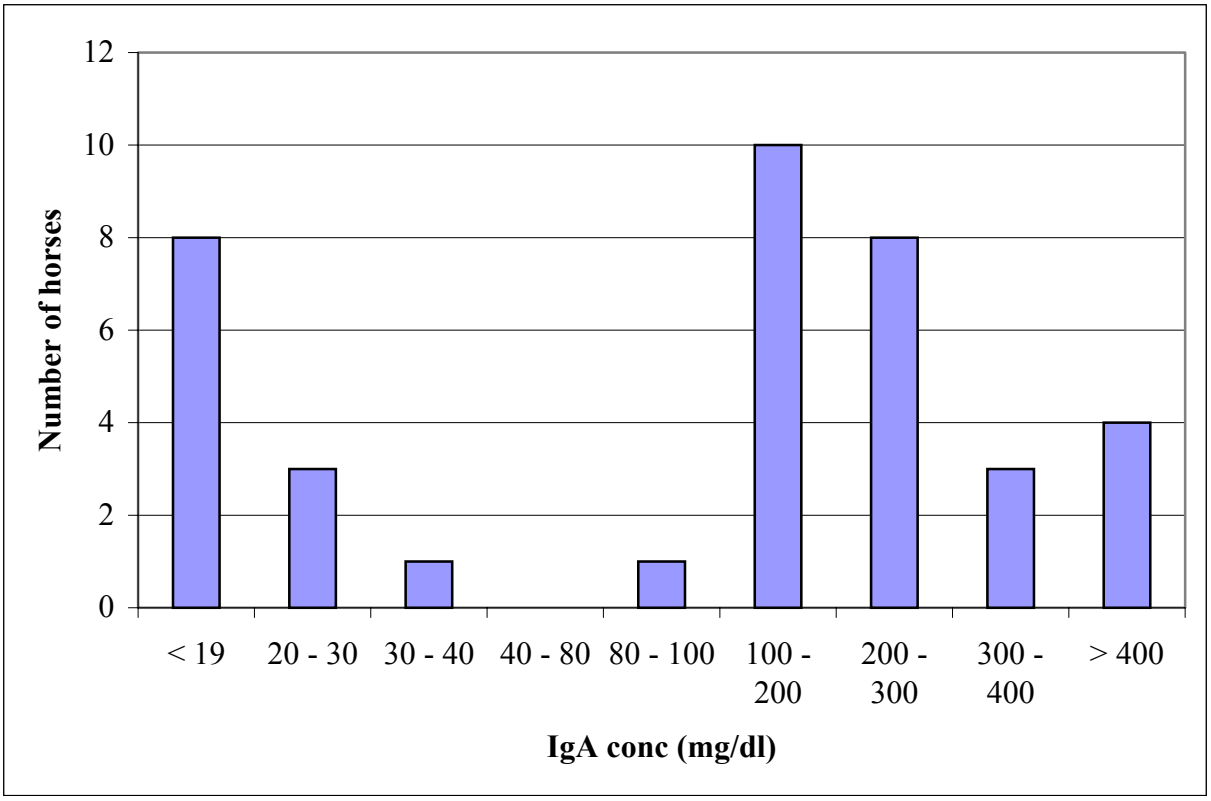

Figure 3. Frequency of IgA concentrations (mg/dl) in endurance horses (Trial 2)

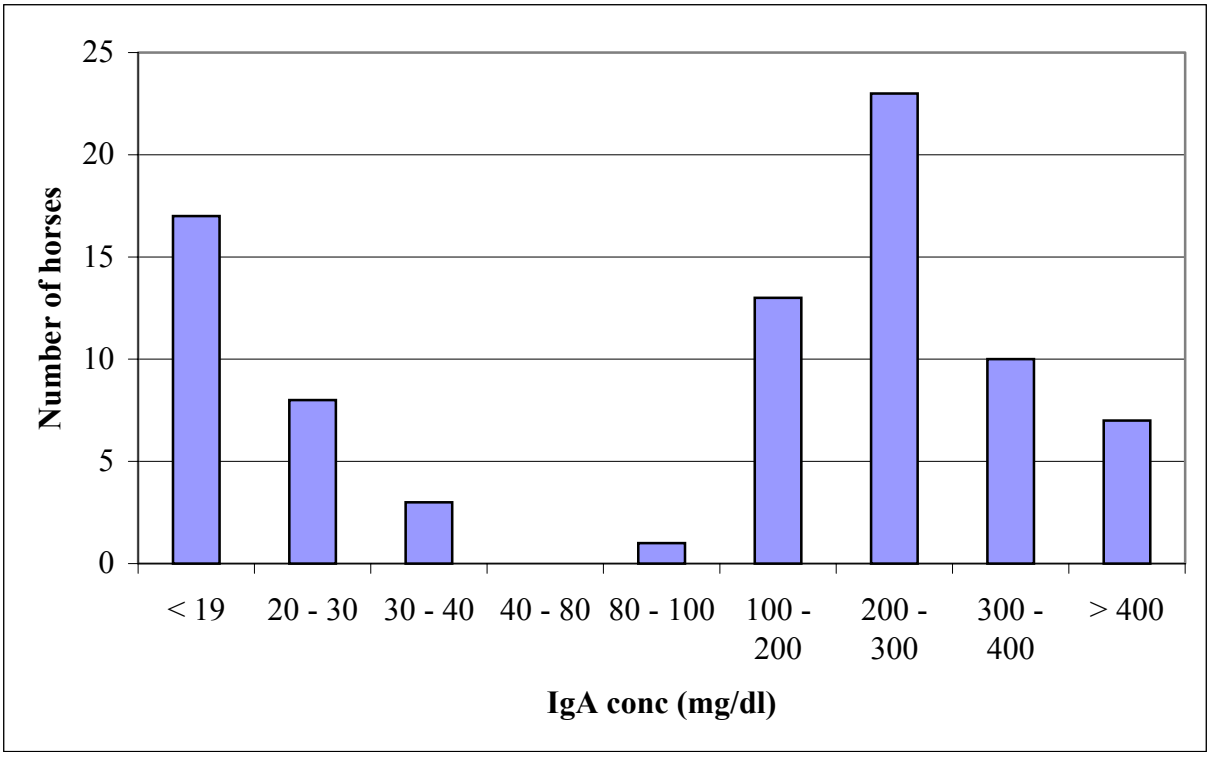

Figure 4. Frequency of IgA concentrations (mg/dl) in endurance horses (Trials 1 and 2)

Implications

Serum IgA and IgG concentrations were not affected by endurance exercise at a distance of 80 km, nor by training, or transport. There appears to be selective IgA deficiency in horses of Arabian breeding that has not been previously reported in any breed of horse. Horses with selective IgA deficiency were more likely to have respiratory problems, skin problems, and allergies. The discovery of selective IgA deficiency in the horse warrants further research into the area.

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Appendix

Survey questions for Trials 1 and 2 included basic animal information, as well as past performance, training and transport data as listed.

Horse information

- Breed:
- Age:
- Performance problems:
- Medical needs:

Performance/race information

- Past performance
  - Number of races:
  - Distances of past races:
  - Completion rate (# starts/ # finishes):

Training

- Months before a race
  - Days/week:
  - Hours/week:
  - Distance/week:
- Week before a race
  - Days/week:
  - Hours/week:
  - Distance/week:

Activity day before race

Type of activity:

Hours or distance:

Transport to research ride: hours/miles to MARE Center:

Survey questions pertaining to selective IgA deficiency symptoms asked in Trial 2

1. Does your horse suffer from respiratory problems? COPD? Are they recurrent?
2. Does your horse ever have skin problems? Is/was it excessive or recurring?
3. Does your horse have any known allergies?
4. Does your horse have a history of colic or other gastrointestinal problems?
5. Does your horse have a history of soft feces or diarrhea?

Vita

Kari Elizabeth Krick

Kari Elizabeth Krick, daughter of Mr. and Mrs. Peter Krick, was born on March 27, 1978 in West Palm Beach, Florida. She graduated from Wellington High School in 1996. Kari attended the University of Florida on a Florida Bright Futures Scholarship, graduated with honors, and received a Bachelor of Science degree in Animal Science, equine option in May 2000. She then accepted a John Lee Pratt Fellowship in Animal Nutrition to pursue graduate studies at Virginia Polytechnic Institute and State University in the field of Equine Nutrition. Upon completion of her Master of Science degree, Kari will pursue her doctorate degree at Michigan State University in Equine Nutrition and Exercise Physiology with Dr. Brian Nielsen.