

**Vitamin E Status of Thoroughbred Horses and the Antioxidant
Status of Endurance Horses**

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University in partial fulfillment of the requirements for the degree of**

**Doctor of Philosophy
in
Animal and Poultry Science
(Equine Nutrition)**

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(ABSTRACT)

Two times are critical for the horse — the first few days of its life and the last few moments of a race. Vitamin E is critical in regard to immune competence in the first and antioxidant status in the latter. Studies conducted at the Middleburg Agricultural Research and Extension (MARE) Center include the development of horse feeds that replace sugar and starch with fat and fiber. The previous fat source of the pasture supplement under development was corn oil, which contains much vitamin E, was replaced with a cereal by product, which contains relatively little. Vitamin E has been studied in horses to a limited degree but not in grazing Thoroughbreds, thus the MARE Center gave me the opportunity to study vitamin E in Thoroughbred mares and foals. Middleburg is located in the Blue Ridge Mountains on Northern Virginia close to the site of one of the toughest endurance races in the world. This allowed me the opportunity to study vitamin E and antioxidant status in the horse during endurance racing.

Initial studies of vitamin E supplementation to mares during the last trimester of gestation were disconcerting with no changes in serum concentrations of α -tocopherol (vitamin E). Studies conducted during the post-partum period revealed evidence of responses to vitamin E supplementation, as increased α -tocopherol concentrations were observed in mares' milk and in foal serum. Foals are born with virtually no circulatory antibodies and the supplementation of a synthetic form of vitamin E to mares demonstrated an increased passive transfer of immunoglobulins to foals. Natural vitamin E has shown a greater bioavailability than synthetic forms, in other species and was tested here at the MARE Center on mares. A greater passive transfer of immunoglobulins was observed with natural vitamin E supplementation compared with the synthetic forms, with immunoglobulin M concentrations in foal serum remaining higher

for a longer period after birth compared to foals of non-supplemented mares. The transfer of α -tocopherol via the milk was also increased in concentration and duration in mares supplemented with natural vitamin E.

Bioavailability of five oral forms of vitamin E (3 natural and 2 synthetic) were tested and one natural form was also administered intravenously so that clearance of vitamin E could be used to calculate the efficiency of absorption of the oral forms. Efficiency of absorption for oral treatments was not determined because of the slow turnover time of the intravenously administered vitamin E, which confounded all subsequent baseline serum α -tocopherol concentrations. Of the salvageable data, serum α -tocopherol concentrations were higher in grouped treatments at 9 and 12 h post dosing. Lipid fractions revealed possible insufficient absorption of the oral doses of vitamin E and possibly tissue saturation following intravenous doses of vitamin E. Serum concentrations of α -tocopherol were generally higher following natural forms of oral vitamin E administration.

As vitamin E is the most important antioxidant in cells, it is often supplemented to endurance horses competing in 80 and 160 km races. Vitamin E protects lipid cell membranes from peroxidation by free radicals, which are increased during strenuous exercise resulting in oxidative stress. The antioxidant status of horses is severely tested during endurance racing and so a study was conducted to monitor changes in circulating antioxidants during three endurance races. Interesting novel findings in the horse were the maintenance of serum α -tocopherol and the depletion of erythrocyte glutathione and plasma ascorbate during two 80 km and one 160 km races. Associations were found between increased muscle cell enzyme leakage and decreased antioxidant status during endurance exercise and although associations do not prove a causation of oxidative stress, they do provide motivation to search for a cause and it is tempting to propose that oxidative stress damaged muscle cell membranes in endurance horses. Further, these findings propose a connection between muscle cell damage and a new form of exertional rhabdomyolysis (ER) that has been observed in endurance horses, where oxidative fibers are damaged compared to the typical glycolytic fiber damage associated with known forms of ER.

An increased understanding of vitamin E utilization in the horse will improve the health and welfare of all horses, but especially newborn foals and the athletic endurance horses.

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CHAPTER 1:

INTRODUCTION

Two times are critical for the horse — the first few days of its life and the last few moments of a race. Vitamin E is critical in regard to immune competence in the first and antioxidant status in the latter. The change from corn oil, which contains much vitamin E, to a cereal by product, which contains relatively little, in the pasture supplement under development at the Middleburg Agricultural Research and Extension Center gave me the opportunity to study vitamin E in the mare and foal. Middleburg is located in the Blue Ridge Mountains on Northern Virginia close to the site of one of the toughest endurance races in the world. This allowed me the opportunity to study vitamin E and antioxidant status in the horse during endurance racing.

Foals are born with essentially no circulating antibodies and acquire immunity from ingestion of colostrum of their dam. Enhancing passive transfer of immunoglobulins to foals would decrease the risk of septicemia and bacterial infection in neonatal foals. One study in horses has shown that concentrations of immunoglobulins in mares' milk were increased when mares were supplemented with synthetic vitamin E. Research on other species has revealed greater increases in the absorption of naturally occurring vitamin E over the synthetic forms. Consequently, if naturally occurring vitamin E were more bioavailable than synthetic vitamin E, perhaps supplementation with the naturally occurring vitamin E would further increase passive transfer.

Vitamin E is an important antioxidant located in cell membranes and supplementation of vitamin E to endurance horses is common practice during competition. Endurance horses compete in 80 and 160 km races over difficult terrain and in adverse ambient conditions. Oxidative stress associated with exercise is not fully understood in horses and has not been measured previously in endurance horses. Monitoring changes in blood concentrations of antioxidants and possible indicators of oxidative damage to muscle cells will offer the endurance competitors vital information on the status of their mounts during a race so that adjustments could be made to supplementation techniques before and perhaps during an endurance event.

Vitamin E has been studied little in the horse. Thus preliminary studies on dose rates that might affect vitamin E status and an experiment on bioavailabilities of commercially available vitamin E sources were undertaken.

CHAPTER 2:

REVIEW OF LITERATURE

History of Vitamin E

Vitamin E was discovered in 1922 by Herbert M. Evans at the University of California, while conducting research in nutrition and fertility (Evans and Bishop, 1922). Rat pregnancies would not go full term and fetal resorption occurred when rats were fed a vitamin E deficient diet. Fetal resorption was prevented when rats were fed fresh lettuce or wheat germ. Consequently, vitamin E was first described as a lipid extract from plants that was essential for maintaining fertility. In 1936, an alcohol was isolated, and the chemical formula for α -tocopherol was proposed. The term ' α -tocopherol' came from *Tocos*, the Greek word for 'birth', *Ferein*, the Greek verb for 'bringing', the ending *ol* represents an 'alcohol', and alpha is the first letter of the Greek alphabet and is used for the first isolated tocopherol (Papas, 1999a). In 1938, Hoffman-La Roche laboratories in Basel, Switzerland synthesized α -tocopherol. One year later, a method was developed in Holland to measure vitamin E in foods, body tissues and body fluids, replacing the laborious and lengthy rat fetal resorption bioassay (Papas, 1999a). It was in 1940 when Distillation Products Industries (DPI) produced and marketed a natural source vitamin E extracted from a vegetable oil by-product. In later years, soybean oil distillate was the raw material used because it contained higher concentrations of tocopherols. Soybean oil is rich in gamma-tocopherol, and with the high demand for α -tocopherol, a chemical methylation process for converting other tocopherols to α -tocopherol was developed by DPI scientists.

Vitamin E is comprised of eight compounds, four tocopherols and four tocotrienols. The four tocopherols were isolated and characterized during the late 1930s (Evans et al., 1936), and the four tocotrienols were isolated many years later (Pennock et al., 1964). Researchers from DPI assembled and distributed to other scientists and physicians all the scientific reports on vitamin E, and this network of researchers eventually became the foundation of the Vitamin E Research and Information Service (VERIS), which is still functioning today. Natural source vitamin E was produced by DPI until 1996, and since then Archer Daniels Midland started producing natural vitamin E. Hoffman-La Roche remain the largest producer of synthetic

vitamin E today. Annual total vitamin E production exceeds 15,000 tons, of which 3000 tons of natural source vitamin E is mainly used in humans. A large proportion of synthetic vitamin E is used to fortify animal feeds, and the remaining vitamin E is used in nutritional supplements, food fortification and in cosmetics (Papas, 1999a).

Chemical Forms of Vitamin E

Vitamin E is the generic descriptor for all eight compounds of vitamin E, the four tocopherol and four tocotrienol derivatives that qualitatively exhibit the biological activity of α -tocopherol (International Union of Pure and Applied Chemistry and International Union of Biochemistry and Molecular Biology –IUPAC-IUB, 1973).

The four tocopherols have a chroman ring with a saturated phytyl chain, and are named α , β , γ , and δ , differing only in the number and position of the methyl groups on the chroman ring. Tocotrienols are also named α , β , γ , and δ , and have a chroman ring, but an unsaturated phytyl chain with double bonds in the 3', 7' and 11' positions.

In nature, the only tocopherol synthesized is the RRR stereoisomer, with asymmetric carbons at the 2', 4' and 8' positions. Chemically synthesized tocopherols are equimolar racemic mixtures (all-rac) of all eight stereoisomers of α -tocopherol, which result from the random positioning of the methyl groups on the 2', 4' and 8' asymmetric carbons.

Biopotency of Natural and Synthetic Forms of Vitamin E

The biopotencies of vitamin E are measured in international units (IU) and are based on the rat fetal resorption test, the classical assay for vitamin E activity (Desai, 1980). The natural RRR- α -tocopherol stereoisomer is extracted commercially from vegetable oil seeds, such as soybeans, and is marketed as *d*- α -tocopherol, *d*- α -tocopheryl acetate and *d*- α -tocopheryl succinate. The synthetic *all-rac*- α -tocopherol is commercially termed *dl*- α -tocopherol and *dl*- α -tocopheryl acetate (Ames, 1979). The specific activity of 1.0 mg of synthetic all-rac *dl*- α -tocopheryl acetate is equivalent to 1.00 IU and represents the international standard for animal nutrition (National Formulary of the American Pharmaceutical Association, 1960; United States

Pharmacopoeia, 1995). Based on this standard the biological activity of *d*- α -tocopherol is 1.49 IU/mg, *d*- α -tocopheryl acetate is 1.36 IU/mg, *d*- α -tocopheryl succinate is 1.21 IU/mg, *dl*- α -tocopherol is 1.10 IU/mg, and *dl*- α -tocopheryl succinate is .89 IU/mg (Ames, 1979). Esters of α -tocopherol, particularly acetate, provide stability during food storage and are the common forms used in human and animal supplementation (Papas et al., 1991). The biopotency of RRR α -tocopherol and its esters over the corresponding *all-rac* form is 36 % higher. These biopotencies were developed from tests in rats, and were applied to humans without species adjustments. However, research using deuterated compounds in humans has indicated that these currently used biopotencies significantly underestimated (by 64 %) the biopotency differences of RRR tocopherols and their esters over corresponding *all-rac* tocopherols and their esters (Acuff et al., 1994).

Absorption and Transport of Vitamin E

Vitamin E is a fat-soluble vitamin and is absorbed in the same way as other lipids. If vitamin E is ingested in an esterified form, the esters are hydrolyzed by pancreatic lipases and tocopherols are absorbed in their free form (Figure 2.6). Bile secreted from the liver emulsifies the tocopherols to form micelles. Tocopheryl polyethylene glycol 1000 succinate (TPGS), a synthetic water soluble form of vitamin E, forms its own micelles and can be absorbed without the aid of lipases or bile salts (Traber et al., 1986; Sokol et al., 1987). In the small intestine, the micelles containing the tocopherols are absorbed through the intestinal wall forming chylomicrons, and these are secreted into the lymph. In the lymph, lipoprotein lipases rapidly catabolize the chylomicrons and some tocopherols are transferred from chylomicrons remnants to lipoproteins or tissues. Most chylomicron remnants become bound by apolipoprotein E and travel to the liver, which has specific apolipoprotein E receptors. From the liver, tocopherols from the chylomicron remnants are secreted into very-low-density lipoproteins (VLDL) and circulate in the plasma. In the plasma, VLDL are hydrolyzed by lipases to low-density lipoproteins (LDL), which contain the highest proportion of plasma tocopherols, and LDL readily exchange tocopherols with high-density lipoproteins (HDL). Tocopherols in HDL may be transferred back to chylomicron remnants in the plasma and return to the liver (Papas, 1999b).

Tissue uptake of tocopherols is not completely understood. As VLDL are hydrolyzed,

tocopherols may enter tissues, or tocopherols incorporated in LDL may be transferred to tissues via LDL receptors located on tissue surfaces, or by direct transfer of tocopherol across tissue cell membranes down a concentration gradient (Papas, 1999b). Tocopherols incorporated in LDL appear to be recognized by specific receptors on tissue cell surfaces. Cultured fibroblasts from humans with hereditary LDL receptor deficiency had a 40 % lower uptake of α -tocopherol compared to normal fibroblasts (Traber and Kayden, 1984).

Tissues of rats had tocopherol concentrations that differed according to the rate of LDL receptor clearance (Spady et al., 1985). In muscle, pancreas and nervous tissue of rats that showed low LDL receptor activity, relatively low concentrations of tocopherols were observed. In contrast, adrenal tissue showed high LDL receptor activity and correspondingly higher tocopherol concentrations. These findings were not demonstrated in heart or adipose tissues, suggesting other routes of tocopherol uptake must be present. Indeed, LDL receptor mediated tocopherol uptake in cultured human fibroblasts revealed only 50 to 75 % of the total tocopherol uptake (Traber and Kayden, 1984).

Tocotrienols have a similar absorption pattern to tocopherols, but during their transport in the plasma in chylomicrons, the tocotrienols are deposited in adipose tissue with triglycerides (Hayes et al., 1993).

α -Tocopherol Binding Protein

The concentration of α -tocopherol in human plasma and tissues is 2 to 3 times higher than γ -tocopherol, even though typical diets have a higher γ -tocopherol content (Bieri and Evarts, 1973). Studies that supplemented humans with deuterated RRR- and *all-rac*-tocopherols of vitamin E found higher concentrations of RRR- α -tocopheryl acetate in blood compared to *all-rac*- α -tocopheryl acetate (Acuff et al., 1994; Burton et al., 1998). This biodiscrimination mainly occurs in the liver (Kayden and Traber, 1993), and even though α -tocopherol and γ -tocopherol are absorbed equally, α -tocopherol is preferentially secreted into VLDL in the liver. In addition, RRR- α -tocopherol and a synthetic stereoisomer of α -tocopherol are equally absorbed, but higher blood and tissue concentrations of RRR- α -tocopherol were observed. An α -

tocopherol binding protein has been identified in the rat (Dutta Roy et al., 1993; Sato et al., 1993) and human (Gordon et al., 1996) and is proposed to preferentially incorporate RRR- α -tocopherol into VLDL in the liver (Kayden and Traber, 1993). Patients with familial isolated vitamin E deficiency have a defective tocopherol transfer protein or no tocopherol transfer protein, and have impaired biodiscrimination of tocopherol stereoisomers (Traber et al., 1993).

Bioavailability of Natural and Synthetic Forms of Vitamin E

The biopotency of natural RRR- and synthetic *all-rac*- α -tocopherol in humans is based on the rat fetal resorption assay hence biodiscrimination across species is assumed equal. In humans, the relative bioavailability of RRR- and *all-rac*- α -tocopheryl acetate was calculated from the area under the blood concentration curve for each tocopherol form following oral supplementation of deuterium labeled tocopheryl acetate esters (Acuff et al., 1994). The accepted biopotency ratio of RRR- to *all-rac*- α -tocopheryl acetate was 1.36:1 for the rat, but the human studies using deuterium labeled tocopheryl acetate revealed a biopotency ratio of 2:1.

In tapir, iguanas, sheep, cattle, humans, and in horses, RRR- α -tocopheryl acetate had a greater activity than the expected IU of *all-rac*- α -tocopheryl acetate (Hidioglou et al., 1988, 1989; Burton and Traber, 1990; Papas et al., 1990). In horses, the relative effectiveness of *d*- α -tocopheryl acetate was more than 300% in comparison to *dl*- α -tocopheryl acetate and the expected 36% increase in effectiveness of natural over synthetic acetate forms was only found in rats (Papas, 1991).

Sheep supplemented with *d*- α -tocopherol had higher serum and tissue α -tocopherol concentrations than those fed *d*- α -tocopheryl acetate, *dl*- α -tocopherol or *dl*- α -tocopheryl acetate (Hidioglou et al., 1988).

Captive elephants and black rhinoceroses supplemented daily with *dl*- α -tocopheryl acetate had low circulating α -tocopherol levels (0.3 μ g/ml). When both species were supplemented with increased *dl*- α -tocopheryl acetate, or with *d*- α -tocopheryl acetate or *d*- α -tocopherol, the circulating α -tocopherol increased by less than 0.2 μ g/ml indicating that these

species were unable to absorb these forms efficiently. However, *d*- α -tocopheryl polyethylene glycol 1000 succinate (TPGS) increased circulating α -tocopherol levels (0.3 to 1.9 $\mu\text{g/ml}$) even though much lower TPGS doses were used (Papas et al., 1991).

Calves were supplemented daily for 35 d with one of four α -tocopherol supplements, *d*- α -tocopheryl acetate, *dl*- α -tocopheryl acetate, TPGS, or an experimental blend containing *d*- α -tocopheryl acetate and TPGS, and a control group received no supplement (Roquet et al., 1992). Blood samples were collected for 35 d. Plasma and red blood cell concentrations of α -tocopherol of control calves remained unchanged. Plasma and red blood cell α -tocopherol concentrations increased ($P < 0.05$) in all supplemented calves. Highest α -tocopherol concentrations were observed in calves supplemented with *d*- α -tocopheryl acetate or the blend of *d*- α -tocopheryl acetate and TPGS.

Horses were supplemented for 35 d with 2000 IU *dl*- α -tocopheryl acetate or with similar amounts of *d*- α -tocopheryl acetate, TPGS, or an experimental blend which consisted of *d*- α -tocopheryl acetate oil formulated in a solid form (Papas et al., 1990). After 5 d, plasma α -tocopherol concentration increases from base-line concentrations were 0.3 $\mu\text{g/ml}$ (*dl*- α -tocopheryl acetate), 0.4 $\mu\text{g/ml}$ (*d*- α -tocopheryl acetate), 0.8 $\mu\text{g/ml}$ (*d*- α -tocopheryl acetate, solid form), and 1.0 $\mu\text{g/ml}$ (TPGS). The plasma α -tocopherol increase resulting from TPGS was much greater than the expected 36% increase of a *d*-source over a *dl*-source. After 35 d all *d*-forms increased α -tocopherol concentrations by at least 1.4 $\mu\text{g/ml}$ above base-line values, whereas the *dl*-form increase from base-line value remained at 0.3 $\mu\text{g/ml}$.

An oral vitamin E tolerance test (OVETT) used in humans was modified for use in horses (Craig et al., 1991). Three forms of vitamin E oral supplementation were tested, two dosages were compared, and three methods of administration were tested. The vitamin E forms were *dl*- α -tocopheryl acetate, *dl*- α -tocopheryl acetate plus polyethylene glycol and *dl*- α -tocopherol. The dosages were 45 IU/kg BW and 90 IU/kg BW. The three methods of administration were a paste delivered to back of tongue, added to 1 kg of grain and fed orally, and added to 2 L of water and

delivered by stomach tube.

Absorption was measured as the area under the time concentration curve following a dosage of 90 IU/kg BW of *dl*- α -tocopherol administered in 1 kg of grain. The area under the curve was 32.2 $\mu\text{g}\cdot\text{h}/\text{ml}$ and peak serum vitamin E concentration increase from baseline was 4.0 $\mu\text{g}/\text{ml}$. In comparison, the maximal absorption observed following the 45 IU/kg BW of for the same form and method of administration was 4.3 $\mu\text{g}\cdot\text{h}/\text{ml}$ and peak serum vitamin E concentration increase from baseline was 0.4 $\mu\text{g}/\text{ml}$. The authors suggested that although horses lack a gall bladder, bile secretions are increased at meal times, and because vitamin E absorption is dependent on bile secretions, the addition of the vitamin E dose to the grain would allow higher peak absorption of vitamin E.

Vitamin E Metabolism

The regulation of vitamin E concentrations in the blood and tissues of the body are not well understood. The α -tocopherol binding protein is involved in the regulation of individual tocopherols by preferentially secreting RRR- α -tocopherol over *all-rac*- α -tocopherols into the circulation, as observed in blood and tissue concentrations. Infusions of lipid emulsions containing high γ -tocopherol concentrations increased blood concentrations of γ -tocopherol 10-fold during the infusion. Concentrations of γ -tocopherol returned to baseline, which is lower than α -tocopherol concentrations, within 24 h (Traber, 1996).

The liver is not the main storage organ for vitamin E, unlike vitamin A, where ~95 % of the body reserves are stored. The low vitamin E storage in the liver may explain the low toxicity of vitamin E (Kappus and Diplock, 1992). Information on tissue concentrations of vitamin E in humans is limited, but shows that increased tissue concentrations occur following vitamin E supplementation although large amounts tocopherols did not accumulate in tissues (Burton et al., 1998). Highest concentrations of tocopherols were found in subcellular fractions of tissues within membranous organelles such as microsomes and mitochondria, which are involved in highly oxidative-reductive actions (Taylor et al., 1976).

The metabolic fate of vitamin E is not well understood. In humans it has been hypothesized, but not confirmed, that tocopherols other than α -tocopherol are excreted with the bile in digesta (Papadopoulos, 1999b). Also in humans, concentrations of tocopherols were highest in adipose tissue and γ -tocopherol concentrations were 30% of total tocopherols with higher concentrations in adipose, muscle and skin tissues than expected from concentrations in the blood (Burton et al., 1998). In the blood cells, liver, spleen, heart, kidney and muscles of rats, γ -tocopherols were catabolized more rapidly than α -tocopherols (Tran and Chan, 1992).

Placental transfer of α -tocopherol is limited in ruminants (Hidiroglou et al., 1969) and pigs (Mahan, 1991) resulting in neonates that are susceptible to vitamin E deficiency. Ruminant fetal serum concentrations of α -tocopherol decreased with increased fetal age (Van Saun et al., 1989). Plasma concentrations of α -tocopherol were higher in calves following colostrum consumption. Pre-colostral plasma α -tocopherol concentrations were 0.2 $\mu\text{g/ml}$ and increased to 3.3 $\mu\text{g/ml}$ after one wk (Nockels, 1991).

Most whole organ tissue concentrations of α -tocopherol were directly proportional to dietary intake (Bieri, 1972). Exceptions are tissues with high fat storage cells, such as adipose tissue and liver, which have high α -tocopherol concentrations that increase with age even with constant dietary vitamin E intake. Adipose tissue is the storage tissue for up to 90 % of the body concentrations of vitamin E, with liver and skeletal muscle accounting for much of the balance (Combs, 1991a).

Rates of tissue tocopherol depletion vary in vitamin E deficient rats. The turnover time of α -tocopherol in rat lung and liver was 7 to 10 d, but in the spinal cord was 76 d (Ingold et al., 1987). Guinea pig adipose tissue tocopherol concentrations were mobilized slowly over four months of vitamin E deficient diets. Plasma tocopherol concentrations decreased to $< 0.5 \mu\text{g/ml}$ and clinical signs of deficiency were observed (Machlin et al., 1979).

Vitamin E deficiency has been suggested to contribute to the development of equine motor neuron disease (EMND), a neuromuscular disorder that affects motor neurons in the spinal cord and brainstem of horses (Divers et al., 1992, 1994; Mohammed et al., 1993). In a case-control study to investigate the association between plasma vitamin E levels and the risk of EMND, plasma vitamin E levels and information relative to dietary and management practices were collected from 53 horses diagnosed with EMND and 69 controls (De La Rua-Domenech et al., 1997). An association (odds ratio 0.17, $P \leq .10$) between plasma vitamin E levels and EMND was found, with the likelihood of the disease increasing as vitamin E levels decreased. Vitamin E deficiency is also thought to predispose horses to develop equine degenerative myeloencephalopathy (EDM), a degenerative disease of the spinal cord and brain stem, typically affecting horses under 12 mo of age (Mayhew et al., 1978). Positive clinical responses have been found after vitamin E supplementation.

Vitamin E as an Antioxidant

Vitamin E is the major lipid-soluble antioxidant in cell membranes. It is a radical scavenging antioxidant that attenuates oxidative damage by inhibiting the chain initiation and propagation of lipid peroxidation in cell membranes. Vitamin E plays an essential role as an intercellular and intracellular antioxidant. Oxidative stress has been defined as a disturbance in the balance of pro-oxidant and antioxidant systems in intact cells (Sies, 1985). Reactive oxygen species (ROS) include reactive non-radical oxygen species and radicals of oxygen, carbon, nitrogen or sulfur (Holmberg, 1984; Jenkins, 1988). These ROS are produced during aerobic metabolism, and approximately 3-5% of oxygen consumed is reduced to ROS in cells, with the remaining oxygen converted to water and carbon dioxide in the electron transport chain (Chance et al., 1979). Oxygen consumption is a direct function of workload in skeletal muscle. Aerobic organisms have evolved antioxidant systems to render the continuous production of ROS harmless under normal, resting conditions (Davies et al., 1982). Cell injury or death may occur if the production of ROS is substantially increased, either by reduced antioxidant mechanisms, such as in vitamin E deficiency, or during exercise.

Free radical mediated chain oxidation proceeds by a chain reaction consisting of three steps, chain initiation, chain propagation and chain termination (Noguchi and Niki, 1999). The chain initiation involves the formation of a free radical and its attack on a lipid. The free radical may arise from light, heat or through physiological reactions during aerobic reactions. The free radical attack on the lipid will result in lipid radicals. Lipid radicals react with oxygen molecules becoming peroxy radicals, and these peroxy radicals then attack more lipid radicals, which react with oxygen and propagate the chain reaction. Furthermore, although a chain initiating radical may cause the oxidation of many molecules, the chain propagating radical is a lipid peroxy radical, regardless of the initiating radical species. The initiating radical may result from a hydroxyl radical or a nitrogen dioxide radical. In addition, PUFA with more than two double bonds are selectively oxidized (Porter et al., 1995). Chain termination occurs when a lipid radical or a lipid peroxy radical is scavenged by an antioxidant such as vitamins E and C, or when two lipid peroxy radicals react to produce non-radical ketones and alcohols.

Aerobic animals have numerous interrelated antioxidant systems. These may prevent oxidant damage in several ways (Jenkins, 1993; Sen, 1995): a) scavenging of ROS and reactive derivatives; b) decreasing the conversion of less reactive ROS to more reactive ROS; c) facilitating repair of damage caused by ROS; and d) providing an environment favorable for activity of other antioxidants. In conjunction with the vitamin E, vitamin C functions to reduce superoxide and hydroxyl radicals present in cell membranes and quench free radicals and ROS, preventing lipid peroxidation (Niki et al., 1995). A synergy between vitamin E and C results in the regeneration of tocopheroxyl radicals produced by ROS reactions (Niki et al., 1982).

Lipid peroxidation occurs in tissues with a high concentration of polyunsaturated fatty acids (PUFA), such as cell and organelle membranes, lipoproteins, adipose tissue and brain. Membrane concentration of α -tocopherol is approximately one α -tocopherol molecule to 1000 lipid molecules. The phytyl tail of the tocopherol molecule allows the positioning of the molecule within the membrane bilayer so that the active chroman ring lies close to the surface of the membrane (Kagan et al., 1990).

Chylomicrons carrying various tocopherols in the blood may play a role as antioxidants to lipids, adipose tissue and the liver (Papas, 1999b). However, through biodiscrimination of tocopherols in the liver, the preferential secretion of α -tocopherol into VLDL and subsequently LDL, reveals the major importance of α -tocopherol.

Relationship between Selenium and Vitamin E

Selenium is a mineral element that exists as inorganic salts and as organic selenomethionine in yeast. Metabolic interrelationships in cellular antioxidant protection have been demonstrated between vitamin E and Se (Combs, 1991b). Selenium functions in the cytosol and mitochondrial matrix of cells via the enzyme glutathione peroxidase (GPX), a selenoenzyme. Reduced glutathione (GSH) is its mandatory substrate (Deneke and Fanburg, 1989; Lu et al., 1990). Glutathione peroxidase catalyzes the reaction of GSH and hydrogen peroxide (H_2O_2) to water and oxidized glutathione (glutathione disulfide, GSSG). Glutathione reductase (GR) regenerates GSH from GSSG and NADPH. The intracellular ratio of GSH to GSSG (>10:1) is usually maintained by GR, but if a tissue is exposed to large quantities of hydroperoxides or other free radicals, GSSG will accumulate and lead to oxidative stress (Ji and Fu, 1992; Ji et al., 1992).

Reduced GSH, a tri-peptide with an active thiol of cysteine, is synthesized intracellularly and functions as an antioxidant primarily as a component of the enzymes GPX and GR (Ji et al., 1995). A function of GPX is to reduce hydrogen peroxide and protect cells from oxidant damage. Glutathione also functions to reduce ascorbyl radicals and similar reactions have been proposed for the reduction of tocopheryl radicals (Papas 1999b). Consequently, reduced GSH allows the regeneration of ascorbate and vitamin E (Niki et al., 1995). The biological mode of action for Se is therefore, as a component of the major antioxidant enzyme GPX, where it functions as part of the cellular defense system against damage induced by free radicals.

Selenium is important as the second line of defense, after vitamin E, against peroxidation of phospholipids, and to a certain level, these nutrients are mutually replaceable (McDowell, 1989). Supplemental vitamin E and Se act together in maintaining the integrity of cells by

protecting against lipid peroxidation especially during times of increased oxidant stress, such as strenuous prolonged exercise, infections, exhaustion and disease states.

Free radicals formed when electrons are added to molecular oxygen interact with membrane lipids. Superoxide anion and peroxide radicals react with PUFAs of membrane phospholipids and when ferric iron is present, these radicals react with hydroperoxides to form highly reactive hydroxyl radicals and singlet oxygen. Singlet oxygen can initiate lipid peroxidation and cause structural and functional damage to cell membranes. Singlet oxygen is converted by superoxide dismutase to hydrogen peroxide. Hydrogen peroxide is either converted by GPX to water or by catalase to water and oxygen. However, GPX is located in the cytosol and mitochondrial matrix, yet catalase is limited to the peroxisomes, so GPX serves to remove hydrogen peroxide more readily (Combs and Combs, 1984). Consequently, GPX eliminates initiator free radicals and attenuates lipid peroxidation. Interestingly, GPX is not able to reduce lipid peroxides when lipid peroxides are within phospholipid membranes. First, the peroxidized fatty acids must be separated from the glycerol component, allowing GPX to act.

Vitamin E and Se have nutritional complementary roles in the antioxidant protection of cells (Combs, 1991b). The location of the lipophilic vitamin E in the membranes of cells and organelles acts in relation to selenoenzyme GPX located in the aqueous portion of the cell. This positioning allows these antioxidants to act in concert to reduce the initiators of lipid peroxidation and protect cells.

The interrelationship of Se and vitamin E may be associated with lymphoid cell membrane fluidity and influence immune response mechanisms (Sheffy and Schultz, 1979). Both nutrients are able to protect leukocytes and macrophages during phagocytosis (Badwey and Karnovsky, 1980; Baboir, 1984; Hogan et al., 1990,1992).

Immunity and Vitamin E in Foals and Other Species

An epidemiological study of 2,468 foals determined that approximately 27 % of all foals born alive contracted an infection or disease and approximately 5 % of these foals died (Cohen, 1994). Septicemia was the principal cause of death (30.2 %) in foals less than 7 d of age.

Foals are born with virtually no immunoglobulins, only minimal immunoglobulin M (IgM) is provided in utero (McGuire and Crawford, 1973). Immunoglobulin G (IgG) and IgM are absorbed by the neonatal foal from colostrum and provide transient protection against bacterial infections. This passive transfer is considered adequate when a minimum concentration of 400 to 800 mg/dl of IgG is present in foal serum (McGuire et al., 1977). Failure of passive transfer of adequate concentrations of immunoglobulin may occur when colostrum has low concentrations of immunoglobulin because the mare prematurely lactated, by failure of the foal to ingest enough colostrum because of weakness, or from death of the mare, or failure of the foal to absorb enough colostrum within the first ~12 h. Both colostrum production and the ability of the foal to absorb immunoglobulins decline from birth to 12 h. Failure of passive transfer can result in neonatal septicemias and bacterial infections (Jeffcot, 1974; McGuire et al., 1977).

Failure of passive transfer of immunoglobulin was found in nine of 87 Thoroughbred foals, and seven (78 %) of these foals acquired infections (McGuire et al., 1977). Twelve foals had partial failure of passive transfer, and three of these foals had infections. Analyses of pre-suckle colostrum revealed low colostral IgG content. Of eleven other foals that died from infection within 2 wk of age, six had failure of colostral IgG transfer (< 200 mg IgG/100 ml serum), and 4 foals had partial failure (200 to 400 mg IgG/100 ml serum).

Immediately after parturition, colostral specific gravity decreases as colostral crude protein concentrations decrease and for the first 3 h of lactation (Ullrey et al., 1966), 40 % of total colostral crude protein is IgG (Rouse and Ingram, 1970). Colostral specific gravity was determined using a hydrometer calibrated so that distilled water at 24 °C had a specific gravity of 1.000. Pre-suckle colostrum samples collected from 100 mares revealed correlations ($r = 0.90$) between colostral specific gravities and IgG concentrations. Foal serum IgG concentrations were also correlated ($r = 0.82$) with specific gravities of ingested colostrum (LeBlanc et al., 1986). A colostral sample specific gravity of 1.06 corresponded to adequate passive transfer of IgG. Eight of 48 (17 %) foals had serum IgG concentrations < 400 mg/dl. Corresponding dams had colostral specific gravities of < 1.06 and colostral IgG concentrations of < 3000 mg/dl. Foals had serum IgG concentrations of > 520 mg/dl 24 h post parturition when colostral specific

gravities of their dams was ≥ 1.06 and colostral IgG concentrations were 3000 to 6000 mg/dl.

Vitamin E and Se are essential nutrients that function to maintain antioxidant defense in tissues and cells. Deficiencies of either nutrient in cows have resulted in increased incidence and severity of mastitis (Hogan et al., 1993). Neutrophils are a primary defense mechanism to bacterial infections in the bovine mammary gland. Deficiencies of α -tocopherol or Se have resulted in reduced neutrophil activity (Smith et al., 1984). Vitamin E supplementation of early lactation cows resulted in increased bactericidal activity of blood neutrophils, and increased neutrophil α -tocopherol concentrations during the periparturient period (Hogan et al., 1993).

Pregnant dairy cows were supplemented with vitamin E and Se during the last 3 wk of gestation to determine the effects of these nutrients on plasma immunoglobulin concentrations and passive immunity in calves, and colostrum and milk production of cows (Lacetera et al., 1996). In the first 36 h post parturition, supplemented cows produced 22 % more colostrum than non-supplemented cows, but percentages of colostral immunoglobulins were not different. Selenium status, measured by erythrocyte GPX activities, was higher in calves from supplemented cows. Plasma immunoglobulin concentrations from birth to 56 d of age did not differ between calves of supplemented and non-supplemented cows.

Calves were fed either normal colostrum and milk or skimmed colostrum and skimmed milk supplemented with coconut oil for 7 d postpartum to determine the role of fat soluble vitamins in colostrum and milk in immune function development (Rajaraman et al., 1997). Calves fed the coconut oil replacement (a fat source lacking detectable fat soluble vitamins), did not incur serum increases in fat soluble vitamins, including α -tocopherol, unlike the calves fed normal colostrum and milk. In both groups of calves, IgG concentrations were similar and not affected by treatments. Populations of collected leukocytes were functionally and compositionally similar between the two groups and not affected by treatments. Changes in fat soluble vitamin content of serum therefore did not affect passive immunity or immune function in calves.

Pregnant sows were injected with vitamin E and Se to determine concentrations of immunoglobulin A, IgG and IgM in colostrum and serum of sows and serum of piglets (Hayek et al., 1989). Prepartum serum concentrations of immunoglobulins in treated and untreated sows were not different. Colostral IgM concentrations were higher in sows injected with vitamin E and Se, but only significantly ($P < 0.05$) from Se. Serum of piglets at birth, from vitamin E and Se treated sows, was higher in IgM concentration but not IgA or IgG concentrations. Serum concentrations of IgM on d 14 postpartum were higher in piglets from sows treated with vitamin E and Se or Se alone, and IgG concentrations were higher in piglets from sows treated with E and Se, Se alone and vitamin E alone. Supplementation of vitamin E and Se to sows prepartum enhanced passive transfer of immunoglobulins to their piglets.

Pregnant and lactating sows were supplemented with vitamin E to determine the effect of vitamin E on serum vitamin E concentration and immune response of suckling piglets (Babinszky et al., 1990). Sows were fed a control diet (no added vitamin E) or a high vitamin E supplemented diet daily during pregnancy. Piglets were weaned at 4 wk of age, and at 5 wk of age piglets were immunized and antibody production was measured. Sows fed the high vitamin E supplemented diet had piglets with increased serum vitamin E concentration, total number of leukocytes, T- and B-cell lymphocytes, and IgG concentrations compared to piglets of non-supplemented sows.

A study was conducted on gilts to determine the effects of dietary fat on milk concentrations of α -tocopherol and the effect of intramuscular doses of α -tocopherol in piglets on their plasma α -tocopherol concentrations and serum IgG concentrations (Hidiroglou et al., 1995b). Gilts were fed a non-fat or a 5 % fat diet from 57 d of gestation, and each group was supplemented daily with Se and vitamin E. At farrowing, three piglets from each gilt were supplemented with intramuscular *d*- α -tocopherol at birth, on d 7 and d 14. Colostrum and milk samples from gilts, and blood samples from piglets were collected weekly. One piglet from each group of vitamin E supplemented and non-supplemented piglets was killed for tissue analyses of α -tocopherol concentration.

Concentrations of α -tocopherol in the colostrum were significantly higher than in the milk and there was no effect of dietary fat. Milk α -tocopherol concentrations decreased as lactation continued in all groups. Plasma α -tocopherol concentrations of piglets following colostrum ingestion and α -tocopherol injection were higher than in piglets without the injection and before colostrum intake. Plasma α -tocopherol concentrations of piglets injected with α -tocopherol at birth, d 7 and d 14 were significantly higher than non-injected piglets. Serum IgG concentrations in piglets were not different between α -tocopherol injected piglets and non-injected piglets, or between piglets from sows fed a fat supplemented or non-fat diet. The α -tocopherol concentrations of all tissues (spleen, liver, kidney, heart, lung, hip muscle) were significantly higher from piglets injected with α -tocopherol than the non-injected piglets, but the effect of dietary fat was not significant (Hidiroglou et al., 1995b). Therefore, the injection of α -tocopherol to piglets post-partum had the greatest influence on piglet tissue concentrations of α -tocopherol.

Calves were supplemented with vitamin C and vitamin E in combination to determine the effects of these supplements on immune responses (Hidiroglou et al., 1995a). Calves were supplemented daily from birth to 6 wk of age. Plasma concentrations of α -tocopherol were significantly higher for supplemented calves than non-supplemented calves, but concentrations of IgG and IgM were not different, although IgM concentrations tended to be higher in supplemented calves compared to non-supplemented calves.

Few studies have been made to evaluate the amount of vitamin E required for optimum immune function in horses. Norwegian horses were supplemented daily with 600 IU of vitamin E, or 5 mg of Se, or both nutrients (Baalsrud and Øvernes, 1986). An improved humoral immune response to tetanus toxoid or equine influenza virus was observed in horses supplemented with vitamin E alone, or Se plus vitamin E, but no improvement with Se alone.

Mares (n = 12) were supplemented with vitamin E added to the daily feed for 8 wk before parturition to determine the effects of vitamin E supplementation on mare serum, colostrum and milk immunoglobulin concentrations, and on foal serum immunoglobulin concentration

(Hoffman et al., 1999). Colostrum samples were collected at pre-suckle, 6, 12 and 24 h after foaling. Serum samples from mares and foals were collected within 1 h of foaling, at 6, 12 and 24 h after foaling and weekly for 4 wk. Supplemented mare serum IgG concentrations were greater ($P = 0.06$) and IgA tended to be greater ($P = 0.13$) 2 wk before foaling than non-supplemented mares. Supplemented mares had higher IgG ($P = 0.005$), IgM ($P = 0.12$) and IgA ($P = 0.013$) concentrations in pre-suckled colostrum than non-supplemented mares. Pre-suckle foal serum concentrations of IgG, IgM and immunoglobulin A (IgA) were not different between foals from supplemented or non-supplemented mares. Following colostrum ingestion, foals from supplemented mares had higher serum concentrations of IgG ($P = 0.085$) and IgA ($P = 0.16$) than foals from non-supplemented mares. Higher concentrations of immunoglobulins in foal serum may reflect improved passive transfer in foals from mares supplemented with vitamin E. Concentrations of α -tocopherol were not measured in mares' milk, or mare and foal serum.

Vitamin E in Colostrum and Milk of Mares and Other Species

Concentrations of α -tocopherol were determined in colostrum and milk of cows that were supplemented with vitamin E or not supplemented during the last 2 wk of gestation (Parrish et al., 1947). Cows supplemented with 0.5 to 1.0 g/d vitamin E had colostrum α -tocopherol concentrations of 1.4-fold higher than non-supplemented cows. Cows supplemented with 10 g/d vitamin E had colostrum α -tocopherol concentrations that were 4.5-fold higher than non-supplemented cows. Concentrations of α -tocopherol in colostrum of cows supplemented with 0.5 to 1.0 g/d and 10 g/d vitamin E were 9- and 12-fold higher than milk (d 8 postpartum) concentrations, respectively. Concentrations of α -tocopherol in colostrum of non-supplemented cows were 7-fold higher than milk (d 8 postpartum) concentrations.

Sows supplemented with vitamin E and Se during gestation and lactation had higher plasma concentrations of both nutrients compared to non-supplemented sows (Loudenslager et al., 1986). Colostrum concentrations of α -tocopherol and Se were higher than milk concentrations in all sows. Mean concentrations of α -tocopherol of supplemented sows were significantly higher ($P = 0.001$) than non-supplemented sows in colostrum (8.7 $\mu\text{g/ml}$ vs 4.6 $\mu\text{g/ml}$), and in milk at 2 d (2.9 $\mu\text{g/ml}$ vs 1.8 $\mu\text{g/ml}$). Milk concentrations of α -tocopherol in both

supplemented and non-supplemented groups rapidly decreased ($P < 0.01$) by d 2 of lactation.

Calves were fed normal colostrum and milk, or skimmed colostrum and milk plus coconut oil from birth to 1 wk postpartum (Rajaraman et al., 1997). Concentrations of α -tocopherol in normal colostrum (4.6 $\mu\text{g/ml}$) were 13-fold higher than in skimmed colostrum (0.3 $\mu\text{g/ml}$). Concentrations of α -tocopherol in normal milk (1.8 $\mu\text{g/ml}$) were 8-fold higher than in skimmed milk (0.2 $\mu\text{g/ml}$). No differences were observed in serum IgG concentrations of calves after consuming either normal or skimmed colostrum and milk for 1 wk.

Colostrum concentrations of α -tocopherol from sows fed a non-fat diet were (mean \pm SE) $10.30 \pm 1.2 \mu\text{g/ml}$ and were significantly higher ($P < 0.05$) than milk α -tocopherol concentrations at 1 wk ($3.3 \pm 0.4 \mu\text{g/ml}$). Colostrum concentrations of α -tocopherol from sows fed a 5% fat diet were $11.2 \pm 1.2 \mu\text{g/ml}$ and were significantly higher ($P < 0.05$) than milk α -tocopherol concentrations at 1 wk ($2.3 \pm 0.4 \mu\text{g/ml}$). Milk concentrations of α -tocopherol decreased over the first 4 wk of lactation in all sows and were $1.5 \pm 0.2 \mu\text{g/ml}$ in sows fed the non-fat diet and $1.9 \pm 0.2 \mu\text{g/ml}$ in sows fed the fat diet (Hidiroglou et al., 1995b).

Ewes were supplemented with vitamin E for 28 d prepartum to determine placental and mammary gland transfer of α -tocopherol to lambs (Njeru et al., 1994). Four groups of pregnant ewes were either non-supplemented or supplemented with *dl*- α -tocopheryl acetate at 15, 30 or 60 $\text{IU} \cdot \text{ewe}^{-1} \cdot \text{d}^{-1}$. Colostrum samples were collected immediately postpartum and milk samples were collected 1, 3, 14 and 28 d postpartum. Serum α -tocopherol concentrations of ewes increased during the 28 d prepartum supplementation period, but placental transfer was not observed because serum α -tocopherol concentration of neonatal lambs were not different between groups ($P > 0.05$). On d 1 postpartum, α -tocopherol concentrations in colostrum of non-supplemented ewes ($3.30 \pm 0.13 \mu\text{g/ml}$) were significantly lower than colostrum concentrations of α -tocopherol of ewes supplemented with 60 $\text{IU} \cdot \text{ewe}^{-1} \cdot \text{d}^{-1}$ ($9.55 \pm 0.13 \mu\text{g/ml}$). Concentrations of α -tocopherol declined in milk by d 28 and milk of ewes receiving the 60 IU per ewe per d of vitamin E supplement was 85% higher than control ewes and 34% higher than

milk of ewes receiving 30 IU per ewe per d. Mean concentrations of α -tocopherol in milk at the four time intervals (d 1, 3, 14 and 28) were 1.65, 2.95, 3.87 and 4.70 $\mu\text{g/ml}$ for non-supplemented, 15, 30 and 60 IU per ewe per d vitamin E supplements. If all non-supplemented and supplemented groups were averaged by day, mean α -tocopherol concentrations of milk were $6.94 \pm 0.13 \mu\text{g/ml}$ on d 1, $3.26 \pm 0.15 \mu\text{g/ml}$ on d 3, $1.72 \pm 0.14 \mu\text{g/ml}$ on d 14 and $1.22 \pm 0.16 \mu\text{g/ml}$ on d 28. Serum α -tocopherol concentrations of lamb increased over 28 d postpartum indicating efficient mammary gland transfer.

Vitamin E concentrations in milk and milk-fractions of cows and humans during different lactation stages and seasons were compared (Deschuytere et al., 1987). In cows, vitamin E concentrations decreased significantly ($P < 0.05$) with time postpartum. Concentrations of α -tocopherol were higher ($P < 0.05$) in cow's colostrum in the spring, mean \pm sd ($5.2 \pm 0.7 \mu\text{g/ml}$) and summer ($3.6 \pm 1.0 \mu\text{g/ml}$), than in the winter ($2.9 \pm 0.3 \mu\text{g/ml}$). Cow's milk (9 d postpartum) concentrations of α -tocopherol in spring ($1.5 \pm 1.0 \mu\text{g/ml}$) and summer ($1.8 \pm 0.8 \mu\text{g/ml}$) were similar to winter concentrations ($0.9 \pm 0.2 \mu\text{g/ml}$). A significant difference ($P < 0.005$) was found between cow's milk and human milk in vitamin E content. Human α -tocopherol concentrations were significantly higher ($P < 0.05$) in colostrum ($15.4 \pm 4.6 \mu\text{g/ml}$) than in milk (5 to 8 d postpartum) concentrations ($6.2 \pm 2.4 \mu\text{g/ml}$).

Colostrum and milk concentrations of α -tocopherol were measured in mares for 6 wk postpartum (Schweigert and Gottwald, 1999). Horses were fed a ration to meet NRC requirements (Anon, 1989) and which included 30 mg vitamin E/kg ration. Colostrum samples were collected immediately postpartum and milk samples were collected at 1, 3 and 6 wk postpartum. Highest concentrations of α -tocopherol were found in colostrum collected immediately postpartum. Concentrations of α -tocopherol in colostrum ($1.66 \pm 0.34 \mu\text{g/ml}$) were 5.7-fold higher ($P < 0.05$) than milk collected 3 wk postpartum ($0.33 \pm 0.05 \mu\text{g/ml}$), and milk collected 6 wk postpartum ($0.39 \pm 0.06 \mu\text{g/ml}$). Concentrations of α -tocopherol in milk collected 2 d ($0.83 \pm 0.16 \mu\text{g/ml}$), 4 d ($0.67 \pm 0.14 \mu\text{g/ml}$), 7d ($0.47 \pm 0.06 \mu\text{g/ml}$), 3 wk ($0.34 \pm 0.06 \mu\text{g/ml}$) and 6 wk ($0.39 \pm 0.07 \mu\text{g/ml}$) postpartum were not different ($P > 0.05$).

In contrast to studies on cows (Schweigert, 1990) where increased colostrum concentrations of α -tocopherol correspond to a decline in plasma concentrations during the parturition period, horse colostrum concentrations of α -tocopherol were not affected by and had no effect on plasma concentrations (Schweigert and Gottwald, 1999).

Vitamin E in Forages and Concentrates

Plants synthesize the tocopherols and tocotrienols of vitamin E. Vitamin E is a lipophilic vitamin and is stored in the lipid portion of plants. Vitamin E dietary requirements are increased by polyunsaturated fatty acids (PUFA), about 0.4 to 0.6 IU of vitamin E per gram of PUFA (Food and Nutrition Board, 1989). The richest sources of vitamin E are vegetable oils, whole cereal grains (particularly the germ), legumes and most green plants. Different proportions of the various tocopherols and tocotrienols are synthesized in different plants and so different species and grains are varied in their vitamin E content (Lynch, 1991).

Green forages containing the natural form of vitamin E, tend to have 5 to 10 times more vitamin E content than cereal grains. This is because the majority of the vitamin E is stored in the fresh leaves of grasses, where 20 to 50 times higher vitamin E content is observed compared to the stem concentrations. Consequently, as grasses mature, the vitamin E activity decreases by 70-90% from early growth to maturity. Legume vitamin E content decreases from 34 to 65% from the leafy to the post-bloom stage (Bauernfiend, 1980).

Different plant species have differing vitamin E activities, therefore, the inherent structural differences of these vitamin E molecules will have varying responses to any curing and processing of forages and grains. Natural tocopherols, in the free alcohol form are more susceptible to oxidant stress because the active site on both tocopherols and tocotrienols is the 6-hydroxyl group on their chroman ring (Papap, 1999b). Free forms are not protected by esterification and are rapidly oxidized. Tocopherols and tocotrienols are rapidly oxidized when exposed to oxidizing agents such as atmospheric oxygen, light, heat and trace mineral salts. Decomposition of natural tocopherols in forages is increased by heat, ultraviolet light exposure, prolonged storage time, high moisture content and mechanical stress. If high microbial

populations are present in forages, especially forages with a high-moisture content, large losses of vitamin E activity occur (Bauernfeind, 1980). Natural tocopherols are sometimes used as the natural antioxidant of the feed, protecting the feed from oxidant stress, not necessarily providing a vitamin E source to the animal consuming the feed. Synthetic forms, or any esterified form of vitamin E, typically used in feed concentrates, will not protect the fats of the feed from peroxidation but will provide a vitamin E source for the animal consuming the feed once it has been ingested and absorbed in the small intestine (Papas, 1991).

Artificial drying methods (105 °C) used on forages can result in losses of 25 to 45 % of tocopherol activity, so during long-term storage of dried forage (hay) in winter months the tocopherol content will diminish. Artificially drying corn may deplete tocopherol levels by 40%. Vitamin E activity of alfalfa hay stored at 33°C for 12 wk was decreased by 54 to 73 % and commercial dehydration of alfalfa decreased vitamin E activity by 5 to 33 % (Bauernfeind, 1980). Curing forage also incurs the time for oxidation of tocopherols to occur, with the cut hay sitting on the field for a few days, and because of the variability in cutting times between farms and states. Consequently, there is a huge variability in the vitamin E content of hays compared to grains, which are harvested more systematically.

Premixing is a processing mechanism that enhances the distribution of nutrients. Pelletizing and extrusion processing methods enhance the digestibility of carbohydrates. However, these methods can damage vitamin E stability through oxidation processes. Vitamin stability must be evaluated at each stage of processing because losses occur at each stage in varying degrees. Temperature, humidity, conditioning time, redox reactions vary during different parts of the processing treatments. Trace minerals exert large redox reactions, and friction tends to erode the protective coating of the vitamin. Pelletizing exerts a high level of friction, pressure and heat. The friction and pressure incurred during pelletizing may expose vitamin E to chemical destruction, and the heat and humidity will enhance these chemical reactions. The damaging effects of extrusion are the very high heat, the humidity and redox reactions (Coelho, 1991).

The stability of naturally occurring tocopherols is poor with substantial losses incurred during processing and storage of feed ingredients and during the manufacturing and storage of finished products. Oxidation of tocopherols in feed ingredients occur through heat, oxygen, moisture, oxidizing fats and trace minerals, and also during processing techniques such as grinding, mixing with fat, pelleting and extrusion. The tocopherol content of high moisture corn was analyzed after 8 mo of storage following various processing treatments (Young et al., 1977). High moisture corn was artificially dried, naturally dried, preserved with propionic acid or ensiled. The highest tocopherol content was observed in the artificially dried corn (91 % retained), compared to ensiled corn, which lost 80 % of the tocopherol content over the 8 mo storage. The instability of the high moisture grains therefore require higher supplemental vitamin E than similar rations containing dried grains.

In order to evaluate the vitamin E activity of supplements, the initial α -tocopherol content of the feedstuff and any further processing that was conducted, such as drying, heat exposure, pelleting or grinding must be considered. In premixes, the stability of the supplemental vitamin E acetate is determined by the particle size and the exposure of the vitamin E acetate, and the pH and mineral content of the premix (Coelho, 1991). The various forms of vitamin E in supplements have characteristics that protect the vitamin E from oxidation. Esterified forms of vitamin E are generally used in premixes and supplement feeds to preserve the content of the vitamin E in the supplement. Esterified forms can withstand the high steam and high temperature used in preparation of the premix. Supplements that have a high pH with high magnesium oxide quantities may destabilize the vitamin E. Natural tocopherols are not recommended for premixes because of the high oxidation caused by trace minerals. Esterified forms were found to be fairly stable in both ambient and heat stressed environments, compared to natural tocopherols in a premix, which after 4 wk of storage, lost 55 % at ambient temperatures, but 87 % under heat stressed conditions (Schneider, 1988). In order to compensate for the high losses incurred during the processing of supplements, vitamin E application is applied after pelleting and extrusion, usually by spraying. This method also has complications, for example, the difficulty of maintaining a fat-soluble vitamin in solution, protection of the vitamin in solution, and uneven coating, so that distribution through the premix has a high

coefficient of variation (20-50 %). Finally, because the vitamins are coated on the outer part of the pellets, they are exposed to more stresses and storage life will decrease.

Vitamin E Status in Horses

Erythrocyte stability in equine blood was measured by a layering hemolysis (LH) test to determine the requirement of vitamin E for maintaining stability (Stowe, 1968). Foals were tocopherol depleted by feeding a pelleted semi-purified ration devoid of vitamin E. Blood was collected and the LH test was used to estimate α -tocopherol requirements for erythrocyte stability. After foals were depleted for 200 d, serum tocopherol concentrations were $< 1.15 \mu\text{g/ml}$ and the LH test revealed weakened erythrocyte stability. The amount of α -tocopherol required to maintain erythrocyte stability was $27 \mu\text{g/kg BW}$ of parenteral or $233 \mu\text{g/kg BW}$ orally per day. Erythrocytes deficient in tocopherol were protected from LH within 4 to 5 h following parenteral administration of tocopherol.

Vitamin E requirements of Standardbred horses were evaluated by tissue depletion and repletion (Roneus et al., 1986). After a 2.5 mo depletion period horses were supplemented daily for 112 d with one of four levels of vitamin E (200, 600, 1800 and 5400 mg total diet), or were placed in a non-supplemented control group. A second depletion period followed the supplementation period. A daily oral supplement of 600 to 1800 mg of *dl*- α -tocopheryl acetate in the total diet was required to meet the nutrient requirements of Standardbreds at maintenance. Tissue concentrations of α -tocopherol increased relative to level of supplementation, but tissue γ -tocopherol concentrations decreased. Diets contained a mixture of vitamin E isomers, but tissue concentrations were mainly α -tocopherol with only traces of γ -tocopherol. The authors suggested that this difference might be due to the more rapid turnover of γ -tocopherol or to a dilution effect of increased dietary α -tocopherols. Daily supplementation of vitamin E was recommended to maintain a constant level of vitamin E in serum, liver and skeletal muscle, although this assertion was not supported by data for non-daily dosing.

Plasma concentrations and adipose tissue concentrations of α -tocopherol were determined in various breeds of horses (Steiss et al., 1994). Blood samples and adipose tissue

biopsies were collected during a 3 wk period in late summer. Horses were housed together for a minimum of 6 mo and fed pasture, hay and alfalfa pellets. Plasma α -tocopherol concentrations (mean \pm sd) were 2.8 ± 0.9 $\mu\text{g/ml}$ (range, 1.0 to 5.3 $\mu\text{g/ml}$). Adipose tissue concentrations were 22 ± 15 ng/mg wet wt (range 5 to 62, median 16.3). Plasma and adipose tissue α -tocopherol concentrations were linearly related (plasma = $1.66 + 0.05 \times$ wet wt value, $r = 0.82$, $P < 0.0001$). Breed differences were observed in α -tocopherol concentrations of plasma ($P < 0.001$) and adipose tissue ($P = 0.011$), with Thoroughbred horses having lower concentrations of α -tocopherol compared to Quarter Horses, Arabian, Percheron and some crossbreds. Low lipid levels have been observed in horses and remained unchanged during a vitamin E depletion and repletion study (Roneus et al., 1986). Serum lipid levels of chickens, cows and mink were 1.5 to 3 times higher than horses and pigs (Hakkarainen et al., 1984), and serum vitamin E concentrations of horses and pigs were correspondingly lower (Roneus et al., 1986).

Seasonal differences in serum α -tocopherol concentrations were observed in Finnish mares and foals grazing pasture in summer months and fed hay and oats during winter months (Mäenpää et al., 1988a). Higher concentrations ($P < 0.001$) were found in August and September (summer) and lowest in April and May (winter) in both mares and foals. Tocopherol concentrations of mares ranged from approximately 1.5 to 2.8 $\mu\text{g/ml}$ (read from charts) and foals ranged from approximately 1.1 to 2.1 $\mu\text{g/ml}$ (read from charts), with lower values representing winter months and higher values representing summer months. Seasonal differences remained after mares were supplemented with ~ 275 mg vitamin E/d during winter months and no significant increases in serum α -tocopherol concentrations were found (Mäenpää et al., 1988b). However, foals supplemented with 200 mg vitamin E/d over the same period had a 66.7 % increase ($P < 0.001$) in serum α -tocopherol concentrations in April and May. Foals fed the non-supplemented diet had serum tocopherol concentrations that ranged from 1.1 to 2.2 $\mu\text{g/ml}$ (read from charts) and foals fed the vitamin E supplemented diet (200 mg/d) had serum tocopherol concentrations that ranged from 1.4 to 2.4 $\mu\text{g/ml}$ (read from charts). It is not clear why foals responded to vitamin E supplementation and mares did show any changes in serum concentrations. Perhaps the requirements of the foals were being met in the spring of their yearling year as they were stabled and receiving little or no exercise. The mares, however, were

in the last trimester of their following pregnancy at this time and would have a higher requirement for vitamin E.

Serum α -tocopherol concentrations of racing Standardbred horses ($n = 142$) were determined from January to July (Mäenpää and Lappeteläinen, 1987). Serum concentrations of α -tocopherol in the winter months were (mean \pm se) $1.94 \pm 0.14 \mu\text{g/ml}$ and increased ($P < 0.001$) by 36.1 % to $2.64 \pm 0.14 \mu\text{g/ml}$ in summer months. These values in the horses are approximately a third of serum α -tocopherol concentrations in man ($\sim 8.0 \mu\text{g/ml}$). Differences were also observed in serum α -tocopherol concentrations in horses from different stables during both winter and summer seasons. In winter the mean range was 0.95 to 3.06 $\mu\text{g/ml}$ (3.2-fold, $P < 0.001$) and in summer the mean range was 1.86 to 3.46 $\mu\text{g/ml}$ (1.9-fold, $P < 0.01$). Vitamin supplements were fed to many of the racehorses, but information on the amounts and form of vitamins fed was not known. Differences in serum concentrations of α -tocopherol of the racehorses may partially reflect differences in vitamin supplements fed especially as the horses were not racing in the winter and so were most likely fed less vitamin supplements. Also the lack of pasture during the winter months would lower the tocopherol intake because the hay fed in place of pasture was cut and stored in July and tocopherols would be readily oxidized.

Serum α -tocopherol concentrations of Norwegian horses during a 24 wk period were (mean \pm sd) $1.85 \pm 0.25 \mu\text{g/ml}$ when fed no supplemental vitamin E, $1.80 \pm 0.21 \mu\text{g/ml}$ when fed 600 mg/d α -tocopheryl acetate, $1.70 \pm 0.40 \mu\text{g/ml}$ when fed 5 mg/d sodium selenite, and $2.00 \pm 0.34 \mu\text{g/ml}$ when fed 600 mg/d of α -tocopheryl acetate and 5 mg/d sodium selenite (Baalsrud and Øvernes, 1986).

Vitamin E deficiency has been proposed as a cause of equine degenerative myeloencephalopathy (EDM), a degenerative disease of the spinal cord (Mayhew et al., 1987). Consequently, serum vitamin E concentrations of horses with and without EDM were determined in 89 horses, 40 of which were subsequently confirmed to have EDM by histological examination of the spinal cord, and 49 control horses without EDM (Dill et al., 1989). In horses aged < 12 mo, with EDM, serum vitamin E concentrations (range 1.9 to 4.8 $\mu\text{g/ml}$, median 3.1

µg/ml) were similar ($P = 0.58$) to the same aged horses without EDM (range 1.4 to 6.1 µg/ml, median 2.5 µg/ml). Similar findings were observed in horses aged 12 to 18 mo, where serum vitamin E concentrations of EDM horses (range 2.7 to 8.1 µg/ml, median 3.8 µg/ml) were similar ($P = 0.59$) to horses without EDM (range 2.6 to 10.5 µg/ml, median 3.5 µg/ml). Horses older than 18 mo and diagnosed with EDM had serum vitamin E concentrations (range 1.5 to 8.2 µg/ml, median 400 µg/ml) that were similar ($P = 0.73$) to horses without EDM (range 2.0 to 6.5 µg/ml, median 395 µg/ml). These findings did not support the reported role of vitamin E deficiency as a cause of EDM.

Vitamin E status of horses in Alberta and Saskatchewan, Canada was determined to identify environmental and biological factors that may have influenced plasma vitamin E concentrations (Blakley and Bell, 1994). Blood samples were collected from ~400 horses in 24 locations during a 2 y period and plasma α -tocopherol concentrations were determined. Over the 2 y experimental period normal baseline plasma α -tocopherol concentrations were 7.65 µmol/L (3.29 µg/ml). Younger horses (6 to 12 mo) had lower ($P < 0.0001$) plasma α -tocopherol concentrations (mean \pm se) 5.62 ± 0.52 µmol/L (2.42 µg/ml) than older horses (> 72 mo) 8.79 ± 0.28 µmol/L (3.78 µg/ml). However, foals (< 6 mo) had higher ($P < 0.0001$) plasma α -tocopherol concentrations 8.60 ± 1.19 µmol/L (3.70 µg/ml). Seasonal effects on plasma α -tocopherol concentrations were also observed with May to August concentrations, 9.68 ± 0.44 µmol/L (4.17 µg/ml) appearing higher ($P < 0.0001$) than September to December 6.98 ± 0.46 µmol/L (3.00 µg/ml) and January to April 7.00 ± 0.17 µmol/L (3.01 µg/ml). Seasonal variability was mainly attributed to the diets. In western Canada the period between May and August is the only time of year horses have access to pasture, which has substantially higher levels of vitamin E than dried forages and many processed feeds (Schryver and Hintz, 1987; Blakley and Bell, 1994). The older horses had higher plasma concentrations of α -tocopherol. Lower levels in young horses may reflect their high nutritional demands for vitamins during periods of rapid growth. Foals less than 6 mo had higher concentrations and may reflect the 4 to 6 mo age period, July to August, where foals would be grazing vitamin E rich pastures (Blakley and Bell, 1994).

Plasma α -tocopherol concentrations of Przewalski horses, which are semi-free-ranging horses in Ukraine (Dierenfeld et al., 1997), were lower ($P < 0.05$) in foals (mean \pm sd; 4.74 ± 1.40 $\mu\text{g/ml}$) than adult horses (6.58 ± 1.14 $\mu\text{g/ml}$). These findings did not correspond to a previous study (Dierenfeld and Raphael, 1992) where foals had higher plasma α -tocopherol concentrations (2.25 ± 0.70 $\mu\text{g/ml}$) than older horses (1.80 ± 0.64 $\mu\text{g/ml}$). The differences may be due to diet because in the previous study the foals were 3 mo older and would have been grazing pasture which was assumed to be richer in vitamin E at that time.

Serum α -tocopherol concentrations, cholesterol and total serum lipid concentrations were measured in young and adult horses over a 72 h period (Craig et al., 1989). Blood samples were collected at 3 h intervals for 72 h from two groups of horses, young (≤ 1 y) and adult (2 to 18 y). Serum α -tocopherol concentrations were variable within horse. Within young horses a mean 13 % coefficient of variation (CV) and in adult horses a 10 % CV was observed. Total serum lipid concentrations were also variable with mean CV of 15 % (range 7 to 22 %) for both groups. Cholesterol concentrations were less variable with a mean CV of 5 % and range of 2 to 7 %. Mean serum α -tocopherol concentrations over the 72 h period for young horses ($n = 6$) were 2.58 $\mu\text{g/ml}$, mean cholesterol concentration for young horses was 77.2 mg/dl, and mean total serum lipid concentration was 312.6 mg/dl. Mean serum α -tocopherol concentration over the 72 h period for adult horses ($n = 6$) was 3.59 $\mu\text{g/ml}$, cholesterol concentration was 88.2 mg/dl and total serum lipid concentration was 283.7 mg/dl. Within animal variance was attributed to variation within the animal and not to errors in laboratory measurement. Serum α -tocopherol concentrations of young horses were lower ($P < 0.05$) than adult horses. In human studies, ratios of serum α -tocopherol and cholesterol concentration have been used to determine vitamin E status, and serum α -tocopherol and serum lipid concentrations were correlated (Horwitt et al., 1972). In horses serum α -tocopherol concentrations were not correlated with cholesterol or serum lipid concentrations (Craig et al., 1989).

Vitamin E deficiency symptoms have been observed in many species fed diets high in PUFA (Grant, 1961; Dam, 1962; Blaxter, 1962). Accordingly, the effects of added dietary

soybean oil on vitamin E status of 2 y old non-exercising horses was investigated (Siciliano and Wood, 1993). Horses were either fed a control diet or a diet supplemented with 6.4 % soybean oil. Blood samples were collected on d 0, 30, 60 and 90 and analyzed for serum α -tocopherol, serum cholesterol and serum triglyceride concentrations. Mean (\pm se) serum α -tocopherol concentrations were greater ($P < 0.001$) at 90 d in the soybean oil supplemented horses ($6.10 \pm 0.25 \mu\text{g/ml}$) than control horses ($4.70 \pm 0.25 \mu\text{g/ml}$). Mean serum α -tocopherol concentrations on d 0 (before supplementation) were $4.00 \pm 0.25 \mu\text{g/ml}$ in soybean oil supplemented group and ($3.80 \pm 0.25 \mu\text{g/ml}$) in controls, but serum α -tocopherol concentrations increased ($P < 0.001$) in control horses during the experimental period also. The authors suggested that the study diet might have been providing more vitamin E than the pre-study diet. Serum cholesterol concentrations were higher ($P < 0.001$) in the soybean oil supplemented horses at 30, 60 and 90 d and serum triglyceride concentrations were not affected by diet. Serum total lipids (measured as the sum of serum cholesterol and serum triglyceride concentrations) were correlated ($r = 0.62$, $P < 0.001$) with serum α -tocopherol concentrations in soybean oil supplemented horses, but not in the control horses.

Plasma α -tocopherol concentrations of pregnant Thoroughbred and Warmblood mares were determined during 6 wk prepartum and 6 wk postpartum (Schweigert and Gottwald, 1999). Mean (\pm se) plasma α -tocopherol concentrations in mares 6 wk prepartum were $1.4 \pm 0.3 \mu\text{g/ml}$, and $1.4 \pm 0.1 \mu\text{g/ml}$ at 21 d prepartum, $1.9 \pm 0.2 \mu\text{g/ml}$ at 7 d prepartum, $1.7 \pm 0.1 \mu\text{g/ml}$ at parturition, $1.4 \pm 0.09 \mu\text{g/ml}$ at 7 d postpartum, $1.2 \pm 0.08 \mu\text{g/ml}$ at 21 d postpartum, and $1.2 \pm 0.1 \mu\text{g/ml}$ at 6 wk postpartum. Plasma α -tocopherol concentrations remained unchanged ($P > 0.05$) during the 12 wk periparturient period.

Oxidative Stress and Antioxidant Status during Endurance Exercise

Oxidative stress is an important factor in cancer, aging and cell membrane damage, and increases during strenuous exercise. Oxidative stress is a damaging imbalance in the oxidative-antioxidative system of cells. Metabolic advantages of increased oxygen supply during exercise may paradoxically be implicated in oxidative injury to muscle cells by free radicals (oxygen species with unpaired electron) and other reactive oxygen species (ROS) produced by oxidative

reactions (Sjodin et al., 1990; Sen, 1995).

Vitamin E is the major lipid-soluble antioxidant in cell membranes. It is a radical scavenging antioxidant that attenuates oxidative damage by inhibiting the chain initiation and propagation of lipid peroxidation in cell membranes. Vitamin C is a water-soluble antioxidant and reducing agent for chemical reactions that occur either intracellularly or extracellularly. Vitamin C reduces superoxide and hydroxyl radicals and acts in conjunction with the vitamin E present in cell membranes to quench free radicals and ROS, preventing lipid peroxidation (Niki et al., 1995). A synergy between vitamin E and vitamin C results in the regeneration of tocopheryl radicals produced by ROS reactions (Niki et al., 1982).

Glutathione, a tri-peptide with an active thiol of cysteine, is synthesized intracellularly and functions as an antioxidant primarily as a component of the enzymes glutathione peroxidase (GPX) and glutathione reductase. Glutathione serves as the substrate for GPX and is rapidly oxidized to glutathione disulfide (GSSG) during oxidative stress. Glutathione also functions to reduce ascorbyl radicals (Meister, 1994; Winkler et al., 1994), and similar reactions have been proposed for the reduction of tocopheryl radicals (Papas, 1999b). Consequently, erythrocyte GSH allows the regeneration of vitamin C and E.

Human plasma tocopherol concentrations increased during intense exercise (Pincemail et al., 1988), but exercise-induced changes in plasma volume were not accounted for. Humans running a half-marathon had unchanged plasma tocopherol, increased plasma ascorbate, and decreased GSH concentrations in samples taken immediately after running the race and 120 h post-race (Duthie et al., 1990). Human plasma ascorbate concentrations increased immediately after a 21 km race and decreased at 24 h post-race to 20 % below pre-exercise values and remained at this level for 48 h (Gleeson et al., 1987). Prolonged submaximal exercise resulted in increased GSSG and decreased erythrocyte GSH concentrations in human athletes (Gohil et al., 1988).

Sled dogs supplemented with vitamin E and vitamin C, or a placebo, had decreased plasma tocopherol and increased plasma ascorbate concentrations during 3 d of endurance

exercise (Piercy et al., 2000), but plasma volume changes were not reported.

Standardbred horses had decreased erythrocyte GSH concentrations and unchanged GPX activities immediately after intense exercise (Gallagher and Stowe, 1980). Studies investigating relationships between physical exercises, oxidative stress and antioxidant capacity have been conducted without antioxidant supplementation on Maremmana (Italian breed) racehorses (Chiaradia et al., 1998) and Thoroughbred racehorses (Ishida et al., 1999). After three months of training, Maremmana racehorses completed a series of exercise tests of increasing intensity. Increased concentrations of malondialdehyde (MDA, an end product of lipid peroxidation and assayed by separating the thiobarbituric acid adduct by HPLC and fluorescence detection), an indicator of lipid peroxidation, and plasma glutathione were observed after short duration exercise, and no changes in creatine kinase (CK) activities were observed (Chiaradia et al., 1998). Exercise-induced mobilization of GSH from the liver into the blood for muscle uptake may account for increased plasma GSH concentrations immediately following exercise (Leeuwenburgh and Ji, 1995). However, the exercise tests performed (Chiaradia et al., 1998) were of short duration relative to endurance exercise, where GSH demands of increased GPX activity would presumably result in decreased plasma GSH concentrations. Blood samples collected from Thoroughbred racehorses before and after a race revealed increased lipid peroxide concentrations and superoxide dismutase (SOD) activities (Ishida et al., 1999).

Maremmana racehorses were supplemented with vitamin E and selenium and completed a series of exercise tests before and after 70 d of training. Horses receiving dietary supplements and training had increased antioxidant defenses, demonstrated by increased resistance to erythrocyte peroxidative stress and increased GPX activity in lymphocytes, measured *in vitro*, and decreased plasma MDA concentrations (Avellini et al., 1999).

Erythrocyte GPX activity decreased and plasma lipid peroxide concentrations increased in Thoroughbred racehorses during sprint exercise (Ono et al., 1990). When horses were supplemented intravenously with selenium and vitamin E, lipid peroxide concentrations decreased and GPX activity decreases were minimized.

Thoroughbred racehorses supplemented with intravenous vitamin C before a race had reduced lipid peroxidation, measured by thiobarbiturate reactive substances (TBARS) after racing (TBARS measure the level of lipid peroxidation in serum using spectrofluorimetric methods). Plasma ascorbate concentrations were unchanged after the race and plasma CK activity increases were similar in supplemented and non-supplemented horses (White et al., 2001).

The antioxidative status of five endurance horses was assessed during a 160 km race (Frankiewicz-Jozko and Szarska, 2000). Systemic measurements revealed increased lipid peroxidation measured by TBARS, increased plasma CK activity, decreased total antioxidant status, and increased activity of enzymatic antioxidants GPX, SOD and glutathione reductase. Nonenzymatic antioxidant status, hydration status, or changes in body weight were not reported.

The antioxidant status of 40 horses competing in a 140 km endurance race was studied (Marlin et al., In press). Blood samples were collected before, immediately after the race and after 16 h recovery. Plasma antioxidants were maintained during the race (vitamin C and E), but vitamin C decreased during recovery. Total erythrocyte GSH concentrations decreased during the race, and CK and AST activities increased during the race and recovery.

Dissimilar responses in exercise-induced changes in variables measuring oxidative and antioxidant status may reflect differences in species, exercise mode, intensity and duration, ambient conditions and laboratory analyses, or whether values were adjusted for exercise-induced changes in plasma volume.

GENERAL OBJECTIVES

The following five general objectives correspond to the five sections of research conducted at the Middleburg Agricultural Research and Extension Center in 1999 and 2000. Each section will include specific objectives for the particular research studies conducted:

1. To determine a suitable dose and frequency of dosing of vitamin E supplement to alter vitamin E status of horses.
2. Measure the effectiveness of various natural and synthetic forms of vitamin E supplementation on vitamin E status of horses.
3. Evaluate the vitamin E and Se status of groups of horses over one year and to compare the different groups of horses through the seasons.
4. To test the supplementation of mares during late gestation with synthetic and natural forms of vitamin E on mare milk vitamin E and immunoglobulin status and foal serum vitamin E and immunoglobulin status.
5. To determine the antioxidant status and muscle cell leakage of horses during two 80 km and a 160 km endurance race, and to evaluate the relationship of muscle cell leakage to changes in antioxidant status.

TIME-LINE OF STUDIES

- **Experiment 1.1:**conducted in January 1999 over a 6 d period using 6 pregnant mares.
- **Experiment 1.2:**conducted in March 1999 over a 7 d period using 6 geldings.
- **Experiment 2:**conducted in October and November 1999 over a 6 wk period using 6 geldings.
- **Experiment 3.1:**conducted from January to October 1999 using 24 mares during gestation and lactation, and 22 foals from birth until 12 mo of age (April/May 1999 to April 2000), and 6 geldings from January to October 1999.
- **Experiment 3.2:**conducted in January, April, July and October 1999 using 12 groups of horses.
- **Experiment 4.1:**conducted from January to October 1999 using 24 gestating or lactating mares and 22 foals.
- **Experiment 4.2:**conducted from January to June/July 2000 using 24 gestating or lactating mares and 23 foals.
- **Experiment 5.1:**conducted in April and June 2000 using 12 endurance horses in April and 18 endurance horses in June.
- **Experiment 5.2:**conducted in June 2000 using 35 endurance horses.

CHAPTER 3

PRELIMINARY TESTING OF VITAMIN E DOSAGE

ABSTRACT: Two preliminary studies were conducted to first determine if fluctuations in circulating α -tocopherol concentrations would result from a dosing frequency of twice a week and secondly to test if collecting blood samples 2 d after a dose was sufficient to measure consistent serum α -tocopherol concentrations. Vitamin E was administered orally to the horses in a syringe paste mixed with molasses. The dosing frequency test showed that serum α -tocopherol concentrations did not fluctuate and consistent serum α -tocopherol concentrations were maintained. No increases in serum α -tocopherol concentrations were observed in horses that were administered vitamin E. However, only two doses were given. Mare serum α -tocopherol concentration appeared near the lower end of normal concentrations reported in the literature and so these doses may have been rapidly taken up by the tissues.

A dose response test on geldings with 2 incremental doses of vitamin E revealed small but significant increases in serum α -tocopherol at 9 h post dosing. These doses were high but have been reported in the literature as necessary to measure the difference between increased concentrations from supplementation and endogenous circulating concentrations. Perhaps the paste method of dosing was not suitable for vitamin E administration and that administering the vitamin E in the feed would elicit a better response. A feed stimulates an increase in bile secretion and bile is required for vitamin E absorption. Horses lack a gall bladder and secrete low amounts of bile continuously from the liver with increases only at meal times. Therefore a paste dose of vitamin E between meal times may not induce an increase in bile secretion and inhibit the absorption of vitamin E. This experiment also suggested that serum α -tocopherol may not be a sensitive index of vitamin E status in the mature horse.

INTRODUCTION

Groups of Thoroughbred broodmares available at the Middleburg Agricultural Research and Extension Center (MARE Center) are maintained on pastures and live out all year round.

Mares are divided into two groups of 12 mares and each group receives a specified feed supplement concurrent with ongoing studies at the MARE Center. Mares are fed their respective feed supplements two times a day, morning and evening, in their groups of twelve and no individual feeding is practiced. A practical system needed to be developed to supplement the groups of mares living out on pastures with vitamin E. A dosing frequency test was conducted on 6 mares to test if large fluctuations in serum α -tocopherol concentrations would result from a single dose compared to daily dosing (Experiment 1.1).

After the first two months of vitamin E supplementation, blood samples of mares revealed no changes in serum α -tocopherol concentrations of vitamin E supplemented mares (Figure 3.1). Accordingly, a dose response test was conducted on eight geldings to estimate a dose that would elicit an increase in serum α -tocopherol concentration (Experiment 1.2)

OBJECTIVES

The objective of Experiment 1.1 was to test if dosing mares by hand two times per week would be sufficient to measure changes in serum α -tocopherol concentrations on a weekly basis. The objective of Experiment 1.2 was to determine which dosage of vitamin E would elicit an increase in serum α -tocopherol concentration.

MATERIALS AND METHODS

Experiment 1.1

Six pregnant Thoroughbred mares were used for the dose frequency testing. Two baseline samples were collected using jugular venipuncture into two 10 ml Vacutainer sterile blood tubes (Vacutainer Systems, Becton Dickinson, NJ). Three mares were dosed mid-afternoon on a Monday and again on the mid-morning of the following Friday with three times the recommended (NRC) amount of vitamin E for pregnant mares. Vitamin E was administered orally by syringe with molasses added to disguise the taste of the vitamin E oil. Each dose was

2.1 ml or 2010 IU of synthetic *dl*- α -tocopheryl acetate (Hoffman La-Roche, NJ). The other three mares were controls, receiving only the molasses vehicle. Blood samples were collected daily from each horse for 6 d. Mares remained on pastures and received their usual feed supplements each morning and afternoon.

Blood tubes were protected from light, heat and moisture during collection (placed under dark towel inside portable black plastic container in field), sample preparation (minimal light in laboratory) and storage (wrapped in black plastic bags inside freezer with no lights). Blood tubes were placed in the dark at room temperature for 2 h to allow blood to clot. Blood was centrifuged at 3,000 x g (Centrifichem System 600, Baker Instruments Corporation, PA) for 10 min. Serum was separated from blood and placed in 12 x 55 mm polypropylene vials (Sarstedt, Newton, NC) and a few crystals of hydroquinone 1,4-Benzenediol 99+% (Sigma Chemical, MO) was added as an antioxidant. Serum vials were wrapped in black plastic and stored at -80°C .

Sample Analyses

Serum α -tocopherol concentrations were determined in duplicate by high-pressure liquid chromatography (HPLC, Waters Model 600) procedures modified from Miller and Yang (1985) and Craig *et al.* (1989). Standard curves using 2.0, 4.0, 6.0 and 10.0 $\mu\text{l/ml}$ were run before and after each batch of samples. An extraction solution of 650 μl hexane was added to 100 μl plasma and 100 μl internal standard tocol in ethanol. Samples were vortexed for 1 min, placed on a shaker plate for 5 min, followed by centrifugation at 1000 g for 30 to 60 sec. The clear top layer containing fat-soluble vitamins was removed by Pasteur pipette into a separate tube and dried down over N_2 . Dried samples were resuspended in 100 μl ethanol, vortexed and placed in autosampler vials, and 40 μl aliquots of sample were injected into the HPLC. A mobile phase of 99% methanol and 1% H_2O at a flow rate of 1 ml/min was used. Aliquots were chromatographed using a Microsorb MV 100 Angstrom pore, reverse phase C_{18} column (4.6 mm x 100 mm) and detected at 292 nm.

Experiment 1.2

Eight geldings were used in the dose response test. Two baseline samples were collected from each horse at the start of the study. Dosage calculations started at 90 IU (IU are international units representing the activity of vitamin E) of vitamin E/kg BW, based on a study that modified the oral vitamin E tolerance test of humans for horses (Craig et al., 1991), and increased dosages were administered to 2 horses each. One oral syringe dose of synthetic *dl*- α -tocopheryl acetate, with molasses added to disguise the taste of the vitamin E oil, was administered to each horse in the following dosages (2 horses per dosage level): 8000 IU, 16000 IU, 32000 IU and 48000 IU. Blood samples were collected 9, 24 and 168 h post-dosing. Horses were maintained on pastures during the study.

Collection of blood samples, sample storage and analyses procedures follow those of Experiment 1.1 exactly.

Statistical Analyses

Data are summarized as means \pm s.e. Analysis of variance was used to determine changes with time and a *post hoc* Fisher's protected LSD test was performed to test for differences between means. Paired t tests were used in Experiment 1.2 to test for differences between treatments (SAS Inst. Inc., Cary, NC).

RESULTS

Experiment 1.1

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) for the three mares administered vitamin E and the three control mares are shown in Table 1.1. No increases were observed and no fluctuations in serum α -tocopherol concentrations were found ($P = 0.996$).

Experiment 1.2

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) for geldings during the dose response test are shown in Table 1.2. No differences were found in serum α -tocopherol concentrations between horses treated with different dosages at 9, 24 or 168 h post-dosing ($P = 0.91$). Serum α -tocopherol concentrations of all geldings increased ($P = 0.04$) from baseline to 9 h after dosing. Concentrations of serum α -tocopherol were slightly higher ($P = 0.005$) at 24 h post dosing. Differences from baseline (pre-dosing) to 9, 24 and 168 h post-dosing are shown in Figure 1.1.

DISCUSSION

The dosing of mares with vitamin E two times a week at 3.5 d apart and collecting blood samples in-between these dosing times demonstrated that no large fluctuations occurred in serum α -tocopherol concentrations and that serum concentrations were consistent during the 6 d. No differences were found between the supplemented and non-supplemented groups and this may indicate that the method of dosing (oral paste) was not appropriate and that dosing closer to feed times would be an improvement. Since horses do not have a gall bladder, bile is secreted at a low level continuously from the liver but increases at mealtimes (Anwer et al., 1975; Gronwall et al., 1975). Vitamin E is a fat-soluble vitamin and requires bile for absorption, therefore dosing during the mealtime would most likely improve absorption. Only two doses were administered and perhaps a longer period of time is required before serum concentrations increase. Another explanation could be that the vitamin E was absorbed rapidly and taken up by the tissues. The first post administration blood samples were collected 18 h after dosing and with that sampling interval, an earlier peak in circulating vitamin E may have been missed.

The results of the dose response test revealed only minimal increases in serum α -tocopherol and no difference between treatments were observed. Fecal samples were not collected during this study, which is unfortunate. Explanations for the minimal response are that vitamin E was rapidly absorbed and taken up by the tissues before the 9 h sample was collected,

or the vitamin E was not absorbed and was excreted in the feces, or the sensitivity of the α -tocopherol analyses methodology is not sufficient, or losses occurred during sample preparation and storage. The study by Craig et al., (1991) revealed an increase of 2.8 $\mu\text{g}/\text{ml}$ in horses at 9 h post dosing, using the same 90 IU/kg BW dosage, which corresponded to the lower dosage in this study. However, in the study by Craig et al., (1991) the vitamin E was added to a kg of feed supplement and this may have been important in the absorption of the vitamin E doses.

CHAPTER 4

BIOAVAILABILITY AND KINETICS OF NATURAL AND SYNTHETIC FORMS OF VITAMIN E IN THOROUGHBRED HORSES

ABSTRACT: Supplementation of vitamin E may be beneficial to horses experiencing oxidative stress, such as during parturition, endurance or racing exercise. Biosynthesis of vitamin E in nature yields only the RRR- α -tocopherol stereoisomer, the most bioactive form in human and animal tissue. Conversely, tocopherols synthesized chemically are equimolar racemic mixtures of 8 stereoisomers of α -tocopherol (*all-rac- α -tocopherol*). The biopotency of RRR α -tocopherol is about 36 % higher than synthetic vitamin E in the rat (Anon, 1960; 1980). Research conducted on humans has shown that the biopotencies of natural RRR- α -tocopherol forms of vitamin E have higher relative effectiveness in comparison to synthetic *all-rac- α -tocopherol* forms (Acuff et al., 1994; Burton et al., 1998).

Accordingly, a study was conducted to determine the effectiveness of various vitamin E forms in increasing serum α -tocopherol concentrations in grazing horses. Six mature, healthy Thoroughbred horses were randomly assigned to a 6 x 6 Latin square design of six treatments and six one-week periods. Horses were maintained on bluegrass/white clover pasture and offered 1 kg of a grain supplement (with vitamin E removed from the premix) once daily. Three natural (*d*-) and 2 synthetic (*dl*-) vitamin E forms were given orally, and one of the natural forms (*d- α -tocopherol*) was also administered intravenously (IV). Natural forms were *d- α -tocopherol*, *d- α -tocopheryl acetate* and *d- α -tocopherol polyethylene glycol succinate (TPGS)*, and synthetic forms were *dl- α -tocopherol* and *dl- α -tocopheryl acetate*. Dosages were calculated for each vitamin E treatment on a molar basis, and oral treatments were administered to each horse in 1 kg of supplement. Baseline blood serum samples were collected 24 h and immediately before dosing, and at 0.5, 1, 3, 6, 9, 12, 24, 48 and 168 h post-dosing. Bioavailabilities were calculated from comparisons of magnitudes of responses measured by areas under the concentration versus time curves (AUC) and kinetic analysis was determined from the IV curve and a simple exponential model.

Mean baseline serum α -tocopherol concentrations were 4.61 ± 0.12 $\mu\text{g/ml}$ for the 5 oral

vitamin E treatments. Serum α -tocopherol concentration increases were different ($P = 0.003$) between treatments and were higher ($P < 0.05$) in the *d*- α -tocopherol form of vitamin E than the other four forms. Kinetic analyses of the five oral products revealed a greater AUC after administration of *d*- α -tocopherol over 24 h but AUC and the efficiency of absorption for oral treatments was not well determined because of the slow turnover time of the intravenously administered α -tocopherol, which confounded all subsequent baseline serum α -tocopherol concentrations. Of the salvageable data (three horses from each treatment that had not yet received the intravenous treatment), serum α -tocopherol concentrations were higher in grouped treatments at 9 and 12 h post dosing. Lipid fractions revealed possible insufficient absorption of the oral doses of vitamin E and possibly tissue saturation following intravenous doses of vitamin E. Serum concentrations of α -tocopherol were generally higher following *d*- α -tocopherol and *d*- α -TPGS forms of oral vitamin E administration. This experiment reinforced the previous suggestion that serum α -tocopherol is not a sensitive index of vitamin E status in mature horses.

INTRODUCTION

Experiment 2

Supplemental dietary vitamin E in most animals is in the esterified form, usually acetate, which offers protection of the tocopherol molecule in feeds and supplements. Tocopherol acetate is inactive as an antioxidant, or biologically, and must be hydrolyzed to the free phenol in the small intestine before absorption.

Naturally occurring α -tocopherol exists only as the RRR form. The structure of the phytyl tail of α -tocopherol molecules includes three chiral centers, which allows a possible eight stereoisomers. Synthetic *all-rac* α -tocopherol is a mixture of approximately equal amounts of all eight stereoisomers (Ames, 1979).

The fetal resorption assay was used to compare the biopotencies of the natural and synthetic forms of α -tocopherol (Weiser and Vecchi, 1982; Weiser et al., 1986) and RRR- α -

tocopherol had a greater biopotency than the *all-rac* α -tocopherol form. In addition, the synthetic stereoisomers of α -tocopherol that make up 7/8 of the *all-rac* α -tocopherol form, had lower activities than the RRR- α -tocopherol form in the resorption bioassay. These differences in the stereochemistry of the phytyl tail explain the biopotency differences because the phytyl tail affects the bioavailabilities of these compounds (Burton and Traber, 1990).

The RRR- α -tocopherol stereoisomer and the SRR- α -tocopherol stereoisomer are different only at the 2 position, which is the junction where the chroman ring and the phytyl tail are joined. Competitive uptake studies in rats using these two stereoisomers in the esterified form and deuterated revealed the importance of the stereochemistry of the ring-tail junction, showing that the 2-position plays a major role in the differences in biopotencies of the various α -tocopherol stereoisomers (Burton and Traber, 1990). The liver contained twice as much SRR- α -tocopherol as RRR- α -tocopherol at the start of the 5 mo study, but after 3 wk the RRR- α -tocopherol concentrations were higher. However, plasma and erythrocytes and all other organ concentrations showed an immediate preference for the RRR- α -tocopherol form. Ratios of the two stereoisomers varied in tissues but ranged from 1.2 in the liver to 3.6 in erythrocytes. The relative bioavailabilities of the RRR- and SRR- α -tocopheryl acetates are dependent on the uptakes by specific tissues and on the varying lengths of time to reach tissue stores. Therefore, the traditionally determined biopotencies of the different forms of tocopherol using the fetal resorption assay may not yield accurate results (Burton and Traber, 1990).

Species differences have also demonstrated different bioavailabilities of RRR- α -tocopherol compared with *all-rac* α -tocopherols. In many species, including the horse, the RRR- α -tocopheryl acetate form had a greater activity than the expected IU of the synthetic *all rac*- α -tocopheryl acetate form (Hidiroglou et al., 1988; 1989; Burton and Traber, 1990; Papas et al., 1990).

In chapter 6, a study (Experiment 4.1) is described that was conducted in our laboratory from January to July 1999, where pregnant mares were supplemented with the *all-rac*- α -tocopheryl acetate form. Weekly blood samples did not reveal plasma α -tocopherol increases

over the experimental period. Following this study, a dose response test conducted on geldings (chapter 3, Experiment 1.2) revealed a poor efficiency of absorption of the *all-rac- α -tocopheryl* acetate form. These findings, in addition to the reports of the various relative bioavailabilities of different forms of vitamin E in other species, prompted the design of an experiment to compare the bioavailability and kinetics of natural and synthetic forms of vitamin E in grazing Thoroughbred horses. This study was conducted in October and November 1999 so that the results could be used to design the supplementation for Experiment 4.2 (chapter 6), which was conducted from January to July 2000.

OBJECTIVES

The objective of this study was to determine the effectiveness of various vitamin E forms in increasing serum α -tocopherol concentrations in grazing Thoroughbreds. Analyses of serum data were used to determine the bioavailability and kinetics of vitamin E in the horse.

MATERIALS AND METHODS

Animals

Six Thoroughbred geldings aged between 6 and 12 y were available for this study at the Middleburg Agricultural Research and Extension Center, Middleburg, VA. Horses were maintained full-time on pasture and were supplemented with orchardgrass-alfalfa roundbale hay (Table 3.2) and a sugar and starch grain supplement (Table 3.3). Grain supplements were fed to provide one-third to one-half of current NRC (1989) recommendations for horses at maintenance. The vitamin and mineral premix (vitamin E free) added to the diets was balanced to complement the pastures and meet or exceed current recommendations. Body weights of horses were recorded monthly and diets adjusted to maintain body condition scores of between 5 and 7 (Henneke et al., 1983).

Experimental Protocol

Horses were randomly assigned to a 6 x 6 Latin square design with 6 treatments and 6 one-week periods.

The 6 treatments consisted of 5 oral treatments and one intravenous treatment:

Treatment 1: *d*- α -tocopherol (Intravenous)

Treatment 2: *d*- α -tocopherol (Oral)

Treatment 3: *d*- α -tocopherol polyethylene glycol 1000 succinate (TPGS, Oral)

Treatment 4: *d*- α -tocopheryl acetate (Oral)

Treatment 5: *dl*- α -tocopherol (Oral)

Treatment 6: *dl*- α -tocopheryl acetate (Oral)

A completely counterbalanced Latin square design assumes no carry over effect between treatments (Table 2.1). Each week horses were weighed prior to the bioavailability test so that dosages can be calculated. During the 24 h period before each test, 2 baseline blood samples were collected using jugular venipuncture into two 10 ml Vacutainer sterile blood tubes (Vacutainer Systems, Becton Dickinson, NJ). At 0700 h on test days, a jugular venous catheter was inserted into each horse. Horses were then placed in stalls and fecal collection bags were fitted. Following a 1 h adjustment period, a third baseline blood sample was collected from each horse via the catheter. Horses were administered one of the vitamin E treatments. Intravenous treatments were administered via an intravenous drip slowly over a 30 min period. At the same time oral treatments mixed into 1 kg of concentrate and fed to each horse. Immediately after vitamin E administration all horses were provided *ad libitum* access to mixed grass hay and water.

Blood samples were collected at 0.5, 1, 3, 6, 9, and 12 h post administration and then catheters were removed. Additional blood samples were collected via jugular venipuncture at 24, 48 h and at 168 h post vitamin E administration. The latter sample also served as the first

baseline sample of the subsequent bioavailability test. Two base-line fecal samples were collected before each test, followed by samples at 6, 12, 24, 48 and 168 h post vitamin E administration with an additional sample collected at 24 h as a mixed sample of the total 24 h fecal collection.

Dosage Calculations

Oral dosages were calculated on an equimolar basis so that the lower activity of the esterified and synthetic forms could be administered at equivalent molar concentration to the RRR-*d*- α -tocopherol free form. Dosages were initially based on the amounts of α -tocopherol administered to horses in the evaluation of the oral vitamin E absorption test (OVETT) in horses (Craig et al., 1991). The recommended dosage for performing the OVETT in horses was 90 IU/kg BW. In order to supplement the different treatment forms in this study in equimolar amounts the biopotency of each tocopherol form was divided into 90 to calculate the IU dosage in mg/kg BW, then the amount in mg/kg BW was divided by the molecular wt of each specific tocopherol form, resulting in a dosage in Mmoles. Subsequently, all tocopherol treatment dosages were supplemented at equivalent molar concentrations.

Intravenous dosages were calculated to be 10 % of the oral dose of the natural *d*- α -tocopherol.

Intravenous Treatment Preparation

Finding an intravenous form of *d*- α -tocopherol that did not include selenium was not possible. A series of tests were conducted in our laboratory to produce an intravenous treatment. In all, eight laboratory tests and two *in vivo* tests on horses were conducted before a suitable product was developed. The main difficulty was in emulsification of the α -tocopherol (oil form). Final ingredients were *d*- α -tocopherol, polysorbate 80 NF (Tween 20; Professional Compounding Centers of America Inc., Houston, Texas), alcohol (95% by Vol., 190% proof), saline (0.9 %) and hydroquinone 1,4-Benzenediol 99+% (Sigma Chemical, MO). Total mixture

was approximately 40 ml. This intravenous product was then administered to each horse diluted in 500 ml of saline (0.9 %) via intravenous drip (over 30 min period).

Sample Analyses

Serum α -tocopherol concentrations were measured in our laboratory using high performance liquid chromatography (HPLC, Waters Model 600). The methods followed closely Miller and Yang (1985) and Craig et al., (1989) and are described in detail in Chapter 3. Concentrate and hay samples were frozen at -80°C before sending to a commercial laboratory (Woodson Tenent Inc., Memphis, TN) to be analyzed for α -tocopherol. Fecal samples were frozen and a subset of samples were analyzed for α -tocopherol concentration (Woodson Tennant Inc., TN).

Statistical Analyses

Data were summarized as means \pm s.e. Mean baseline values refer to the mean of the three baseline samples. Different vitamin E sources were evaluated using analysis of variance (ANOVA) for a Latin square (Table 2.3). A completely counterbalanced Latin square design assumes no carry over effect (Table 2.1) and ensures that each treatment is preceded by every other treatment once. The following model was used in the ANOVA for a Latin Square:

$$y = \mu + \alpha_i + \beta_j + \gamma_k + \epsilon_{ijk}$$

where α_i = effect of i^{th} treatment, β_j = effect of j^{th} horse in i^{th} treatment, γ_k = effect of k^{th} week, and ϵ_{ijk} = random error. Because of the effect of the intravenous treatment on the serum α -tocopherol concentrations the period since intravenous administration was added to the model only after examination of the data:

$$y = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + \epsilon_{ijkl}$$

where δ = period since intravenous treatment.

The bioavailability of each vitamin E source will be measured as a) maximum serum α -tocopherol concentration (peak of serum α -tocopherol concentration curve), b) time of maximum serum α -tocopherol concentration, and c) the area under the serum α -tocopherol concentration time curve (AUC). Bioavailabilities were calculated from comparisons of magnitudes of responses (AUC). The AUC were determined by trapezoidal approximations and kinetics were determined on the intravenous curve by fitting an exponential (SlideWrite 5.0 for Windows, Advanced graphics Software, Encinitas, CA).

RESULTS

Mean baseline serum α -tocopherol concentrations ($\mu\text{g/ml}$) of six horses in wk 1 of the 6 wk study ($2.74 \pm 0.33 \mu\text{g/ml}$) were lower ($P = 0.0006$) than mean baseline serum α -tocopherol concentrations ($\mu\text{g/ml}$) of the subsequent 5 wk (4.61 ± 0.12). Serum α -tocopherol concentrations at 9 h post dosing in 6 horses following the six treatments are shown in Table 2.2. Analysis of variance results (Table 2.4) revealed differences in mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) between treatments ($F = 29.6, P < 0.05$) and between horses ($F = 11.8, P < 0.05$), but not among periods ($F = 0.40, P > 0.05$).

Intravenous treatment increased serum α -tocopherol concentrations from baseline ($3.55 \pm 0.36 \mu\text{g/ml}$) to 1 h post intravenous administration ($13.19 \pm 1.33 \mu\text{g/ml}$) to 9 h post dosing ($12.23 \pm 0.92 \mu\text{g/ml}$) and remained higher at 7 d post dosing ($7.54 \pm 0.74 \mu\text{g/ml}$) than the mean of the five oral treatments at all of the sample collection points ($P < 0.0001$). The ANOVA table for the Latin Square Design of treatments with the period since intravenous injection added to the model is shown in Table 2.5 and revealed no effect ($F = 1.42, P > 0.05$) for period since intravenous treatment. Increases in serum α -tocopherol concentrations $\mu\text{g/ml}$ from baseline were fitted to an exponential (Figure 2.1) and showed that serum concentrations of α -tocopherol had not returned to baseline values at 7 d and a turnover time of 212 h (8.85 d) was predicted. At 7 d post-intravenous treatment an oral treatment was administered and this severely confounded

the results of the oral treatments. Data from three horses receiving each of the five oral treatments, that had not yet received the intravenous treatment, were used to calculate the AUC and efficiency of absorption for 7 d (168 h) of the five oral treatments (Table 2.6) calculating the AUC for the oral treatments by trapezoidal approximations and the AUC for the intravenous treatment by fitting an exponential. The AUC for the intravenous treatment was also calculated using trapezoidal approximations (Table 2.7), which increased the efficiency of absorption slightly. The AUC over 24 h was also calculated for each of the 5 oral treatments (Table 2.8) and reveals a higher AUC for the *d*- α -tocopherol treatment compared to the other 4 oral treatments ($P = 0.04$).

Mean increases in serum α -tocopherol concentrations ($\mu\text{g/ml}$) for the six horses over 6 wk were different ($P = 0.003$) between treatments (Figure 2.2), and only *d*- α -tocopherol was higher ($P < 0.05$) than the other four treatments. No differences were found in serum α -tocopherol concentration increases from baseline to 9 h ($P = 0.21$) and 12 h ($P = 0.77$) post dosing of oral treatments of vitamin E in three horses prior to intravenous treatment (Figure 2.3). When treatments were grouped (Figure 2.4), the serum α -tocopherol concentrations were higher at 9 h ($P = 0.01$) and 12 h ($P = 0.04$) post dosing in three horses prior to intravenous treatment. Mean serum α -tocopherol concentrations for the 6 wk over 7 d for each oral treatment is shown in Figure 2.5.

DISCUSSION

The clearance of serum α -tocopherol concentration from the blood following an intravenous dose of *d*- α -tocopherol allows the efficiency of absorption of oral supplements to be calculated. A commercial intravenous form of α -tocopherol without selenium was not available for use in the horse and the product developed in our laboratory had not been tested on horses prior to this study. The intravenous treatment of one horse per wk confounded the results of this study because the clearance of circulating α -tocopherol did not occur until after the 7 d period, making the baseline samples of the subsequent OVETT abnormally high. Some of the data was salvaged, from three horses per oral treatment that had not yet received the intravenous treatment

and these data will be discussed.

Oral Vitamin E Treatments

Any increases in serum α -tocopherol concentration in horses following oral treatments were minimal, especially considering the amount of vitamin E administered per dose. Any increases in serum α -tocopherol concentrations would have been masked by the abnormally high baseline values following intravenous treatments. However, in the first week of the study, following the first oral dose of vitamin E (except the one horse receiving intravenous vitamin E in wk 1), serum α -tocopherol concentrations of the five horses increased by 0.14 to 1.15 $\mu\text{g/ml}$. Baseline serum α -tocopherol concentrations on wk 1 in the six horses ranged from 1.86 to 3.52 $\mu\text{g/ml}$. Interestingly, vitamin E status prior to supplementation did not affect serum α -tocopherol increases in rats, or the relative absorption and transport of different forms of vitamin E (Burton and Traber, 1990). The mean increase in serum α -tocopherol concentration (after oral treatments) in the 3 horses prior to intravenous treatment was $0.50 \pm 0.12 \mu\text{g/ml}$ at 9 h and $0.49 \pm 0.14 \mu\text{g/ml}$ at 12 h post dosing.

Doses approximated 30,000 IU per horse, which is ~35 times NRC (1989) recommended amounts, and the fate of the oral treatment doses of α -tocopherol are unknown. Lipid fractions were determined in the serum samples of two horses collected at 9 h post dosing. Separating the lipid fractions by their densities allows a glimpse at the location of the circulating α -tocopherol. The two serum samples revealed low α -tocopherol concentrations in chylomicrons and very-low-density-lipoproteins (VLDL), and only slightly above baseline values located in the low-density-lipoproteins (LDL) and low concentrations in the high-density lipoprotein fraction (HDL). Orally ingested α -tocopherol as described earlier, and shown in Figure 2.6, is absorbed in micelles through the small intestine and enters chylomicrons and passes into the lymphatic system. For micelles to form, bile is needed from the liver and perhaps with these large doses, enough bile could not be secreted, especially as horses lack a gall bladder and small amounts of bile are continuously secreted (Anwer et al., 1975; Gronwall et al., 1975). A possible explanation for the low concentrations in chylomicrons at 9 h post dosing could be that α -

tocopherol was immediately taken up by the tissues. In rats, 15 % of the dose was found in chylomicrons, and > 92 % was found in the liver, muscle and adipose tissue after a single dose, indicating rapid uptake by the liver of chylomicrons. Chylomicron clearance was completed within ~6 h (Burton and Traber, 1990). However, with such large doses administered in this study, chylomicron concentrations would be expected to be high for more than 9 h and the low concentrations in LDL and HDL at 9 h post dosing indicate a lack of available α -tocopherol for absorption into the tissues.

Fecal excretion of the α -tocopherol dose is another possible explanation. A few fecal samples were analyzed, three samples (0, 24 and 48 h post dosing) from a horse in wk 1 following oral supplementation of *d*- α -tocopherol and three similar samples from a horse following the intravenous treatment. The horse receiving oral treatment had fecal concentrations of α -tocopherol of 130 IU/kg in the baseline sample, 3080 IU/kg at 24 h and 128 IU/kg at 48 h. The horse receiving intravenous treatment had fecal concentrations of α -tocopherol of 141 IU/kg in the baseline sample, 95 IU/kg at 24 h and 70 IU/kg at 48 h. It is likely that a large amount of the oral doses were excreted in the feces.

Intravenous Vitamin E Treatment

The long turnover time (> 7 d) of the intravenously administered α -tocopherol in serum confounded the efficiency of absorption of the oral forms of vitamin E. In the rat, plasma clearance of intravenous vitamin E was ~12 min (Burton and Traber, 1990). The intravenous dose was approximately 3000 IU for each horse, depending on individual BW (10 % of the oral dose of each horse). Average circulating concentrations of α -tocopherol in horses is 3 to 6 μ g/ml. The 3000 IU in 40 L of plasma, typical of a 500 kg horse, would increase serum α -tocopherol concentrations to ~75 μ g/ml, yet the highest serum concentrations measured in horses after an intravenous dose was ~20 μ g/ml. Explanations could be that the validity of the intravenous product may not have been adequate, in that insufficient emulsification would prevent the α -tocopherol from being recognized by the tissues. Secondly, the lipid fractions were measured in the serum of a horse following intravenous treatment and revealed

chylomicrons and VLDL with very low concentrations of α -tocopherol. This is expected following an intravenous dose because no intestinal absorption of α -tocopherol was necessary and no uptake of α -tocopherol by chylomicrons would occur. However, high concentrations of α -tocopherol were found in LDL and HDL, which would indicate that the intravenous α -tocopherol was recognized by the body and incorporated into these lipoproteins. This may not infer that the tissues recognized the tocopherols. Insufficient LDL receptors for this large dose may have prevented tissue uptake of LDL containing α -tocopherol and allowed circulating serum concentrations to remain higher for more than a week. Perhaps tissues were saturated so that further uptake of α -tocopherol was inhibited, or tissues re-circulated α -tocopherol back into LDL and HDL so that concentrations of circulating α -tocopherol remained high.

Other possible reasons for the slow clearance of intravenously administered α -tocopherol could be that the absorption system was overloaded especially as these horses received no added fat in their diet, and may not have had sufficient activity of lipoprotein lipases, pancreatic lipases or bile secretions to cope with the large α -tocopherol dose. The d- α -TPGS is a water-soluble form of vitamin E and forms its own micelles, but improved absorption of this form was not observed. The reason for the large doses administered in this study were to mimic the doses tested previously in horses in the modified OVETT test (Craig et al., 1991). A lower dose of 45 IU/kg BW (this dose was 90 IU/kg BW) was tested in the modification of the OVETT test but no increases in serum α -tocopherol were found. Increases of 2.8 $\mu\text{g/ml}$ were observed at 9 h post dosing of *dl*- α -tocopherol using the 90 IU/kg dose. Large doses need to be used to attempt to distinguish and changes relative to endogenous levels. However, large doses may not be physiologically relevant and may overload the capabilities of the small intestine to absorb the α -tocopherol. Further, a large dose of α -tocopherol may inhibit the absorption of γ -tocopherol and other tocopherols or tocotrienols, or mask normal absorptive mechanisms, or result in adverse interactions with other nutrients. This experiment also showed that serum α -tocopherol concentrations are not a sensitive index of vitamin E status in mature horses. Studies in humans have tested stable isotope labeling techniques such as deuterated tocopherols that are safely ingested and allow the measurement of absorption, transport, uptake and retention of tocopherols (Burton and Traber, 1990). Perhaps this is the future for further studies in horses.

CHAPTER 5

VITAMIN E AND SELENIUM STATUS OF THOROUGHBRED HORSES THROUGH THE SEASONS

ABSTRACT: Vitamin E and Se status of Thoroughbred horses grazing pastures was assessed by serum α -tocopherol and whole blood Se concentrations during one year. Serum α -tocopherol and blood Se concentrations were collected from mares during the last 20 wk of gestation and no changes were observed during this period. Immediately after parturition, serum α -tocopherol and blood Se concentrations were at their highest and then rapidly decreased in the first 2 wk of lactation. Changes at parturition most likely reflect fluid shifts and a decreased plasma volume, which may have exacerbated the tocopherol and Se increases. However, both serum α -tocopherol and blood Se concentrations decreased in mares during lactation and this suggests a critical period to study in future investigations.

Serum α -tocopherol concentrations in foals were highest during August, which corresponded to the highest pasture vitamin E concentrations, and August is the first month that foals spend time grazing pastures. Selenium concentrations in foals were the lowest at birth, which is typical of this species, and increased over the months to a peak at 9 mo, almost reaching the blood concentrations of their dams.

Pasture concentrations of vitamin E fluctuate through the seasons with the lowest content in January and the highest in August. Pasture concentrations of vitamin E were not reflected in serum tocopherol concentrations of mature horses, but were perhaps related to foal increases in August. Pasture vitamin E content did not correspond to the crude protein or crude fat content of the pastures at any time.

Snapshot vitamin E and Se status was measured by a sample collected from all groups of horses on the same day, once during each season of the year. Differences in both tocopherol and Se concentrations were observed between the groups and no pattern in serum tocopherol concentrations was noted between the groups and seasons. Blood Se concentration appeared to reflect the dietary intake of Se provided in the feed supplements. Geldings in particular had lower blood concentrations in the Fall than foals had at birth. Geldings had not received feed supplements since the Spring. Assessment of circulating vitamin E and Se status through the

seasons provides information for calculating nutrient requirements and formulating rations for horses that obtain the majority of their energy requirements from pasture so that requirements can be adjusted to reflect seasonal changes.

INTRODUCTION

Estimating the nutrient requirements of vitamin E in the horse is difficult because little information is available in the literature on the vitamin E status of horses. Depletion-repletion studies were conducted using Standardbred horses and vitamin E requirements were evaluated from tissue biopsies collected at different stages of repletion and depletion (Roneus et al., 1986). This study was conducted in Sweden on 12 horses that were housed in stalls and restricted from grazing pastures.

Seasonal differences were observed in serum α -tocopherol concentrations in mares and foals grazing pasture in Finland from June until October and fed hay and grain during the winter (Maenpaa et al., 1988a). Highest serum α -tocopherol concentrations were found in August and September and lowest in April and May. However, pastures, hays and grains were not tested for their vitamin E content. Similarly, horses grazing pasture in Western Canada had higher plasma α -tocopherol concentrations during summer months compared to winter months and seasonal differences were assumed to be the result of higher vitamin E availability in pastures compared to stored forages and grains (Blakley and Bell., 1994). Pasture, hays and grains were not measured for vitamin E content.

No studies in North America have reported serum α -tocopherol concentrations of Thoroughbred horses grazing pastures year round. In addition, pasture concentrations of vitamin E through the seasons have not been measured. Accurately recommended nutrient requirements for growing horses are essential but in order to accurately recommend a balanced ration for the growing horse, the nutrient content of the ration consumed by the growing horse needs to be determined, especially pasture, which may meet 75 to 100 % of their digestible energy requirements (NRC, 1989), particularly during summer months.

Determining serum α -tocopherol concentrations of foals from birth to 12 mo of age may provide important information on seasonal effects or age (of horse) related changes that may correspond to variations in pasture concentrations of vitamin E. Furthermore, as vitamin E and selenium are interrelated, the blood concentrations of selenium over the same time period could offer valuable assistance in determining the requirements of these vital nutrients and circulatory status through the seasons. Results from this study will only be applicable to horses grazing pastures and eating hays from pastures of Northern Virginia.

OBJECTIVES

The objectives of this study were to determine the vitamin E and Se status of foals from birth to 12 mo of age, and of pregnant mares during the last trimester of gestation, through parturition, and during 6 mo of lactation, and of geldings at maintenance for a one year period. The vitamin E content of pastures were determined monthly, and hays and feeds every two months to determine seasonal variation or changes in vitamin E content with forage storage (Experiment 3.1). In addition, a snapshot of vitamin E and Se status from different groups of horses during each season of the year, specifically, foals, weanlings, yearlings, long yearlings, geldings, stallions, barren mares, pregnant mares during mid- and late gestation and mid- and late lactation will be determined (Experiment 3.2).

MATERIALS AND METHODS –Experiment 3.1

Animals

Twenty-four Thoroughbred mares due to foal from March to May, 1999 were available for this study at the Middleburg Agricultural Research and Extension Center, Middleburg, VA. Mares were assigned randomly to two dietary groups, and each of these groups was sub-divided into two groups of six mares for vitamin E supplementation or a placebo. Twenty-two Thoroughbred foals born between March and May 1999 were available for this study. Foals

remained with their dams from birth until weaning at 6 mo of age. Weanlings remained in the original grouping of their dams and received the same diet of their dam groups until 12 mo of age. Eight Thoroughbred geldings aged 6 to 12 y were available for this study from January to October 1999. Geldings were maintained on pasture as one group. Body weights of mares and foals were recorded monthly and diets adjusted to maintain body condition scores between 5 and 7 (Henneke et al., 1983).

Diets

Mares were grazing orchardgrass/bluegrass/white-clover mixed pastures until foaling, and then bluegrass/white clover pastures until weaning in late October and early November 1999. Mares were supplemented with hay (harvested from orchardgrass-alfalfa pastures on same farm, Table 3.2) and feeds, a sugar and starch (SS) feed (a copy of Omolene-200, Purina Mills) or a fat-and-fiber (FF) feed with a cereal byproduct as a fat source (Table 3.3). Supplements were isonitrogenous and isoenergetic, and were fed to provide one-third to one-half of nutrient requirements recommended for pregnant mares during the third trimester or during lactation (NRC, 1989), with pasture and hay intake assumed to meet the remainder of their requirements. The vitamin and mineral premix (vitamin E free) added to the diets was balanced to complement the pastures and meet or exceed current recommendations (NRC, 1989). Foals nursed their dams milk, grazed pasture and nibbled at their dams feed until weaning, then received the same diet as their dams, including the vitamin E free premix, to provide one-third to one-half of current recommendations for weanlings and then yearlings until the end of the study. Geldings grazed orchardgrass/bluegrass/white-clover mixed pastures throughout the study and received hay (orchardgrass-alfalfa hay harvested on farm from January to March) plus the SS feed (from November to March) to meet current recommendations for mature horses at maintenance (NRC, 1989).

Vitamin E Supplementation

Six mares from each group of 12 were individually administered vitamin E (*dl*- α -

tocopheryl acetate, 995 IU/g; Hoffman-LaRoche, Nutley, NJ) every 3.5 d during the last trimester of pregnancy and for one month during lactation. Vitamin E oil was mixed with molasses and each horse received three times the NRC (1989) requirement for pregnant/lactating mares (2.1 g/d or 2010 IU/d for last trimester, and 3.0 g/d or 2985 IU/d during lactation). The other six mares from each group received a vehicle (molasses only) and served as the controls. Hay and grain samples were analyzed for endogenous α -tocopherol content to ensure non-supplemented groups of mares were receiving sufficient vitamin E to meet the NRC (1989) minimum requirements. Mares were supplemented with the vitamin or vehicle from 16 to 20 wk before foaling, at 1500 on Mondays, and at 0900 on Fridays. The total amount per supplementation (3.5 d x daily amount) was 7.5 ml or 7035 IU (last trimester) and 10.6 ml or 10,101 IU (lactation) of *dl*- α -tocopheryl acetate. Foals and geldings were not administered supplementary vitamin E.

Sample Collection

Blood Serum: Blood samples for α -tocopherol analyses were collected from mares at 20, 13, 9, 6, 5, 4, 2, and 1 wk pre-partum, immediately post-partum, and at 2, 4, 8, 12, 16, 20 and 24 wk of lactation (January to October 1999). Blood samples for α -tocopherol analyses were collected from geldings monthly for 10 mo from January to October 1999. Foal blood samples for α -tocopherol analyses were collected immediately after birth, and at 2, 4, 8, 12, 16, 20, and 24 wk of age, then monthly for 6 mo (foals were born in April and May 1999 and blood samples were collected from birth until March and April 2000). Samples of venous blood were collected into three 10 ml sterile blood tubes (Vacutainer Systems, Becton Dickinson, NJ). Blood tubes were protected from light, heat and moisture during collection (inside black container), sample preparation (minimal lighting in laboratory) and storage (covered in black plastic in unlit freezer). Blood tubes were placed in the dark at room temperature for 2 h to allow blood to clot. Blood was centrifuged at 3,000 x g (Centrifichem System 600, Baker Instruments Corporation, PA) for 10 min. Serum was separated from blood and placed in 12 x 55 mm polypropylene vials (Sarstedt, Newton, NC) and a few crystals of hydroquinone 1,4-Benzenediol 99+% (Sigma Chemical, MO) was added as an antioxidant. Serum vials were wrapped in black plastic and

stored at -80°C .

Whole Blood: Blood samples for Se analyses were collected from mares at 20, 15, 10, 5 and 1 wk pre-partum, immediately post-partum, and at 2, 4, 8, 12, 16, 20 and 24 wk of lactation (January to October 1999). Blood samples for Se analyses were collected from geldings monthly for 1 y, from January to December 1999. Foal blood samples for Se analyses were collected immediately after birth, and at 24 h, and 2, 4, 8, 12, 16, 20 and 24 wk of age, then monthly for 6 mo (foals were born in April and May 1999 and blood samples were collected from birth until March and April 2000). Blood samples were collected using jugular venipuncture into three 10 ml Vacutainer heparinized blood tubes (Vacutainer Systems, Becton Dickinson, NJ). Blood samples were placed in the refrigerator at 4°C .

Pasture: Pasture samples were collected monthly from each of the pastures maintaining mares, foals, weanlings and geldings from January until December 1999. Each month pasture samples were split into two parts, one for vitamin E analysis and the other for ration evaluation. Pasture samples for vitamin E analysis were placed in quart size plastic bags, labeled and stored at -80°C . Samples for ration evaluation were dried in a forced air oven, and ground through a .5 mm screen Wiley Mill (Model 4, Thomas Scientific, Swedesboro, NJ) and stored in airtight plastic tubes.

Hay: Hay samples were collected monthly during winter months from hay offered to pregnant mares, geldings, mares with foals, and weanlings. Samples for vitamin E analysis were placed in quart size plastic bags and stored at -80°C . Samples for ration evaluation were prepared and stored as described for pasture samples.

Feed Supplement: Samples of supplements offered to pregnant mares and weanlings (SS and FF), and geldings (SS) were collected every 4 wk and composites were made every two months. Samples for vitamin E analysis were placed in quart size plastic bags and stored at -80°C . Samples for ration evaluation were prepared and stored as described for pasture samples.

Sample Analyses

Serum α -tocopherol concentrations were determined in duplicate by HPLC procedures modified from Miller and Yang (1985) and Craig *et al.* (1989). Details of procedures are described in chapter 3.

Selenium concentrations of whole blood were analyzed using a fluorimetric method (Whetter and Ullrey, 1978) after wet digestion (AOAC, 1995) at the Virginia-Maryland College of Veterinary Medicine, Blacksburg, VA.

Frozen pasture, hay and feed supplement samples for vitamin E content were submitted for analyses to a commercial laboratory (Woodson Tenent Laboratories, Inc. Memphis, TN). Ground pasture, hay and feed supplement samples for ration evaluation were submitted for duplicate analyses to a commercial laboratory (Dairy One, Ithaca, NY).

Statistical Analyses

Data are summarized as means \pm s.e. Analysis of variance with repeated measures was used to evaluate effects of treatments, sampling times, diets and their interactions. When the F-statistic indicated differences between groups a *post hoc* Fisher's protected LSD test was performed to test for differences between means. Simple regression analysis was used to determine relationships between pasture concentrations of α -tocopherol and crude protein or crude fat content in pastures (SAS, Inst. Inc., Cary, NC).

RESULTS – Experiment 3.1

Pregnant mare mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) were not different ($P = 0.218$) during the last 20 wk of gestation from January until parturition in April or May (Figure 3.1). Serum α -tocopherol concentrations were not different ($P = 0.794$) between vitamin E supplemented and non-supplemented groups during the last 20 wk of gestation until parturition, or during the last 8 wk of gestation and the first 6 wk of lactation ($P = 0.946$), or during the last 20 wk of gestation and 24 wk of lactation from January to October ($P = 0.890$). No differences

were found between groups fed the SS diet or the FF diet during the last 20 wk of gestation from January to April or May ($P = 0.949$).

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of mares decreased ($P < 0.0001$) during 24 wk of lactation and were $5.19 \pm 0.25 \mu\text{g/ml}$ immediately post parturition and $2.58 \pm 0.13 \mu\text{g/ml}$ after 24 wk of lactation (Figure 3.1). Serum α -tocopherol concentrations during 24 wk of lactation were not different ($P = 0.961$) between vitamin E supplemented and non-supplemented mares, or between groups fed the SS diet or the FF diet during the 24 wk lactation period ($P = 0.773$).

Pregnant mare mean whole blood Se concentrations (ppb) were not different ($P = 0.302$) during the last 20 wk of gestation from January until parturition in April or May (Figure 3.2). Blood Se concentrations of pregnant mares were not different ($P = 0.177$) between vitamin E supplemented and non-supplemented groups during the last 20 wk of gestation until parturition. Blood Se concentrations of pregnant mares were different ($P < 0.0001$) during the last 20 wk of gestation and during 24 wk of lactation (Figure 3.2).

Lactating mare mean whole blood Se concentrations (ppb) decreased ($P < 0.0001$) during 24 wk of lactation and were 216.79 ± 7.48 ppb immediately post parturition and decreased ($P = 0.0001$) to 175.52 ± 6.16 ppb at 8 wk of lactation and increased ($P = 0.011$) to 201.42 ± 6.37 ppb at 20 wk, then decreased ($P < 0.0001$) to 150.42 ± 4.18 ppb at 24 wk of lactation (Figure 3.2). Blood Se concentrations of mares during 24 wk of lactation were not different ($P = 0.334$) between vitamin E supplemented and non-supplemented groups.

Gelding mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) were different ($P < 0.0001$) during the 10 mo period from January to October (Figure 3.3). Highest serum α -tocopherol concentrations of geldings were in March ($4.26 \pm 0.41 \mu\text{g/ml}$) and lowest concentrations were in August ($2.01 \pm 0.19 \mu\text{g/ml}$).

Gelding mean whole blood concentrations of Se (ppb) differed ($P < 0.0001$) during the

12 mo period (Figure 3.4). Highest blood Se concentrations in geldings were in April (148.5 ± 6.06 ppb) and lowest concentrations were in October (75.75 ± 3.2 ppb).

Foal mean serum α -tocopherol concentrations were different ($P < 0.0001$) from 0 to 12 mo of age (Figure 3.5). Highest serum α -tocopherol concentrations were found in August (4.74 ± 0.36 $\mu\text{g/ml}$) and lowest concentrations were observed in April 2000 when foals (yearlings) were 12 mo of age (2.29 ± 0.11 $\mu\text{g/ml}$). No differences were found in serum α -tocopherol concentrations between foals of vitamin E supplemented dams and foals of non-supplemented dams ($P = 0.457$), or between foals fed the SS or FF diets ($P = 0.496$).

Foal mean whole blood Se concentrations (ppb) were different ($P < 0.0001$) from 0 to 12 mo of age (Figure 3.6). Highest blood Se concentrations were found in January when foals were aged 9 mo (169 ± 5 ppb) and lowest concentrations were observed in June when foals were aged 2 mo (83 ± 2 ppb). Blood Se concentrations decreased ($P < 0.0001$) from September (148 ± 6 ppb), when foals were aged 5 mo, to weaning time in October (110 ± 3 ppb), when foals were aged 6 mo, but increased ($P < 0.0001$) from October to the peak blood Se concentrations in January (aged 9 mo). No differences were found in blood Se concentrations between foals of vitamin E supplemented dams and foals of non-supplemented dams ($P = 0.889$).

Pasture mean vitamin E concentrations (IU/kg) were different ($P < 0.0001$) over the 12 mo of 1999 (Figure 3.7). Highest concentrations of vitamin E were found in July (207 ± 36 IU/kg) and lowest concentrations were found in January (31 ± 5 IU/kg). No differences were found in pasture vitamin E concentrations between the different pastures grazed during the year. Simple regression analysis on pasture concentrations of vitamin E and the pasture content of crude protein or crude fat revealed no correlations ($P = 0.60$), and pasture vitamin E concentrations were highest when pasture crude protein and crude fat content were at their lowest concentrations (Figure 3.8). Pasture nutrient composition analyses are shown in Table 3.1. Hay nutrient composition including vitamin E content are shown in Table 3.2 and feed supplement nutrient compositions and vitamin E content are shown in Table 3.3.

DISCUSSION - Experiment 3.1

Mare serum concentrations of α -tocopherol did not change during the last 20 wk of gestation despite half of the mares receiving supplemental vitamin E at three times the recommended dose (NRC, 1989) for pregnant mares twice weekly. No differences were found between the supplemented and non-supplemented groups at anytime during the 20 wk of late gestation and 24 wk of lactation. This may indicate that serum tocopherol concentrations may not accurately reflect vitamin E status in mature horses or perhaps supplemental vitamin was absorbed rapidly and taken up by the tissues and stored. During the lactation period, serum α -tocopherol concentrations in all mares were highest immediately after foaling then rapidly decreased. These higher concentrations immediately after foaling may be the result of fluid shifts associated with parturition. Total plasma protein concentrations were not measured during this study and so plasma volume changes could not be calculated. However, it is clear that a huge physiological event such as parturition would result in considerable fluid shifts during parturition and with the onset of lactation, and these shifts may have diluted serum α -tocopherol measurements at this time. Decreased α -tocopherol concentrations throughout the lactation period may represent a need for vitamin E supplementation during this period. Mares in this study were supplemented twice weekly for only 4 wk of lactation. The lactation period for mares is stressful relative to the early gestation period and the large production of milk at this time (10 to 30 L milk/d) may increase the requirement of vitamin E and other nutrients.

Selenium concentrations of mares were not different between vitamin E supplemented and non-supplemented mares at any time during the last 20 wk of gestation and during 24 wk of lactation. Like serum α -tocopherol concentrations during the last 20 wk of gestation, the Se concentrations did not change over this period. Immediately post-partum, Se concentrations were at their highest and like serum α -tocopherol concentrations, decreased rapidly in the first weeks of lactation. Fluid shifts during parturition may have played a role in the measured increases in Se at this time. The reason Se concentrations decreased for the first 8 wk post-partum and then increased until 20 wk of lactation is unclear, but the subsequent decrease between 20 and 24 wk may be explained by the weaning schedule. During October the foals are

weaned from their dams and dams are moved to the other side of the farm. This period is quite stressful for the majority of the mares, including the end of lactation, and decreases in Se concentrations at this time may reflect this stress.

Geldings grazed pastures year round and so it is unclear why their serum α -tocopherol concentrations were highest in the spring and lowest in the summer months. June, July and August concentrations were low even though pasture concentrations were highest during these months. Reasons for these anomalies may be poor sample collection or incorrect laboratory analyses because the horses were observed grazing the pastures and their general demeanor was monitored daily, with no unusual or stressful activities observed. Selenium concentrations of the gelding were also low compared to all other groups of horses during the months that serum α -tocopherol concentrations were at their lowest. Concentrations of blood Se in October were dangerously low in geldings (normal concentrations for horses are 140 ppb and concentrations below 65 ppb are indicative of Se deficiency). Lowest concentrations of blood Se in mares were the equivalent to the highest concentration in geldings. An explanation for the low concentrations of Se in gelding is the lack of supplementary feed during the summer months. Blood concentrations of Se are considered indicative of Se status in horses and as Se is not stored in the body and pastures of Northern Virginia are deficient in Se, it is likely that the geldings were depleted during the summer months.

Foal serum α -tocopherol concentrations were highest in August, which corresponds to the highest levels found in pastures. At this time the foals are 3 to 4 mo of age and are grazing pastures more than in the previous months, which is when their dams milk is more important. Lowest concentrations were observed in April 2000, when foals became yearlings (12 mo) and just at the end of their first winter without their dam. Pasture concentrations were lowest during January to March and provided approximately the minimum amount of vitamin E recommended (80 IU kg total ration) for growing horses (NRC, 1989). Feed supplements fed to foals had the vitamin E free premix added so only endogenous vitamin E in feeds were available (Table 3.4).

Foal blood Se concentrations were lowest at 2 mo of age and gradually increased until 5

mo. A reason for the decreased Se concentrations observed at 2 mo of age is unknown, but perhaps the milk of the dam at this point does not provide enough Se to meet their requirements and this is the age that the foals are starting to eat their dams grain ration (with supplemental Se), which may be associated with the increase in foal blood Se from 2 to 5 mo of age. The decreased Se concentrations observed at 6 mo may be indicative of stress associated with weaning. Highest Se concentrations were found at 9 mo but these were still slightly lower than the concentrations of their dams.

Pasture vitamin E concentrations fluctuated during the year but were above recommended amount for most groups of horses (except growing foals/weanlings and pregnant mares) during 10 mo of the year. Nutrient composition of the pastures was measured but no correlations were found between vitamin E content and any other nutrients. One explanation could be that if the pasture crude protein and fat concentrations were low, perhaps the pastures were stressed at these times, however drought conditions were apparent only in September. In contrast, vitamin E is stored in the lipid portion of the plant so the lower fat content during the peak vitamin E concentration is confusing and may reflect poor sample collection or analyses problems, especially the lower protein concentrations observed in July may reflect poor sample collection (Table 3.1).

Round bale orchardgrass/alfalfa hay (fed to mares and geldings during winter months) concentrations of vitamin E appear to be incorrect (Table 3.2), as literature values report an average of 10 to 60 IU/kg DM (Lynch, 1991). Two separate samples, which were collected from approximately 15 round bales each, had concentrations of vitamin E over 100 IU/kg. Also these high concentrations were higher than alfalfa-timothy square bales of much greener color, which is indicative of higher vitamin E concentrations (Lynch, 1991). A survey of more than 40 hays from different states reported < 50 IU/kg DM in the majority of the hays and only 15 % of the hays had higher than 80 IU/kg DM (Hall et al., 1991).

MATERIALS AND METHODS - Experiment 3.2

Animals

Twelve groups of horses were available for this study at the Middleburg Agricultural Research and Extension Center in 1999. Horse groups included foals (28), weanlings (28), yearlings (28), long yearlings (12), geldings (8), stallions (3), barren mares (8), pregnant mares mid-gestation (22), pregnant mares late-gestation (26), periparturient mares (17), mid lactation mares (28) and late lactation mares (28).

Diets

All horses were grazing orchardgrass/bluegrass/white-clover mixed pastures or bluegrass/white clover pastures and each group was supplemented with hay (orchardgrass-alfalfa hay harvested on farm, Table 3.2) and feeds (either the SS or FF diet with a vitamin and mineral premix which included vitamin E and Se, Table 3.3) to provide one-third to one-half of nutrient requirements recommended for their groups (NRC, 1989) with pasture and hay assumed to meet the remainder of their requirements (Table 3.1). Only 24 pregnant mares (receiving vitamin E free premix in their SS and FF diets) were supplemented with additional vitamin E during late gestation and early lactation because they were on the study described in Chapter 6.

Sample Collection and Analyses

Each mid season period (Spring = April; Summer = July; Fall = October; Winter = January), one blood sample was collected from each horse for serum α -tocopherol concentration ($\mu\text{g/ml}$) and one for whole blood selenium concentration (ppb) on the same day for a snapshot analyses of vitamin E and Se status. Collection, storage and analyses procedures follow Experiment 3.1 exactly.

Statistical Analyses

Data are summarized as means \pm s.e. Analysis of variance was used to evaluate serum and blood concentrations of α -tocopherol and Se in groups of horses during the different seasons (SAS, Inst. Inc., Cary, NC).

RESULTS - Experiment 3.2

Serum α -tocopherol concentrations ($\mu\text{g/ml}$) in horses from all groups were different ($P < 0.001$) between seasons (Figure 3.9). Highest concentrations of serum α -tocopherol were observed in horses during the Spring ($3.56 \pm 0.11 \mu\text{g/ml}$) and lowest concentrations were observed during the Fall ($2.73 \pm 0.07 \mu\text{g/ml}$). In Spring, serum α -tocopherol concentrations of the different groups of horses (Figure 3.10) were different ($P = 0.007$) and were highest in geldings ($4.06 \pm 0.3 \mu\text{g/ml}$) and lowest in stallions ($2.61 \pm 0.1 \mu\text{g/ml}$). In the Summer, serum α -tocopherol concentrations of the different groups of horses (Figure 3.11) were different ($P < 0.0001$) and were highest in foals ($4.32 \pm 0.25 \mu\text{g/ml}$) and lowest in geldings ($2.15 \pm 0.15 \mu\text{g/ml}$). In the Fall, serum α -tocopherol concentrations of the different groups of horses (Figure 3.12) were different ($P < 0.0001$) and were highest in weanlings ($3.31 \pm 0.16 \mu\text{g/ml}$) and lowest in barren mares ($2.02 \pm 0.12 \mu\text{g/ml}$). In the Winter, serum α -tocopherol concentrations of the different groups of horses (Figure 3.13) were different ($P < 0.0001$) and were highest in geldings ($4.19 \pm 0.39 \mu\text{g/ml}$) and lowest in stallions ($2.50 \pm 0.13 \mu\text{g/ml}$).

Whole blood Se concentrations (ppb) in horses from all groups were different ($P < 0.001$) between seasons (Figure 3.14). Highest concentrations of blood Se were observed in horses during the Spring ($155 \pm 3 \text{ ppb}$) and lowest concentrations were observed during the Fall ($131 \pm 4 \text{ ppb}$). In Spring, blood Se concentrations of the different groups of horses (Figure 3.15) were different ($P < 0.001$) and were highest in mares at 1 mo lactation ($187 \pm 4 \text{ ppb}$) and lowest in geldings ($125 \pm 3 \text{ ppb}$). In the Summer, blood Se concentrations of the different groups of horses (Figure 3.16) were different ($P < 0.0001$) and were highest in periparturient mares ($197 \pm$

12 ppb) and lowest in geldings (105 ± 3 ppb). In the Fall, blood Se concentrations of the different groups of horses (Figure 3.17) were different ($P < 0.0001$) and were highest in a (n = 1 this season) stallion (216 ppb) and lowest in geldings (76 ± 3 ppb). In the Winter, blood Se concentrations of the different groups of horses (Figure 3.18) were different ($P < 0.0001$) and were highest in late gestation mares (186 ± 6 ppb) and lowest in geldings (109 ± 3 ppb).

DISCUSSION - Experiment 3.2

When all groups of horses were combined the highest concentrations of serum α -tocopherol were found in the Spring and lowest concentrations in the Fall. It is not clear why the geldings had such variations in their serum α -tocopherol concentrations, especially as their highest levels were in the winter and lowest in the Fall, which did not correspond to changes in pasture content. Barren mares were not receiving feed supplements in the Fall and this may explain the lower serum α -tocopherol concentrations at this time, although vitamin E stores should have been adequate from grazing the summer pastures. Circulating tocopherol concentrations may not represent adequate stores most likely because membrane bound α -tocopherol represents the stored vitamin E whereas circulating α -tocopherol in lipoproteins reflects the transport of vitamin E between tissues. Weaning stress on both the lactating mares and the foals may be a reason for the lower serum α -tocopherol concentrations in the Fall.

Selenium status through the seasons also varied between groups of horses and was highest in all groups of horses in the Spring and lowest in the Fall. These changes most likely reflect dietary intake of feed supplements. Pregnant mares weanlings and stallions are the only groups that are fed supplements in the Fall, which is the only source of Se for these horses. Pastures and hays are deficient of Se in Northern Virginia. Pregnant and lactating mares that were fed the feed supplements, which contain 0.5 ppm of Se (as Sodium Selenite), were receiving 2 mg/d of Se and weanlings were receiving 1.82 mg/d. Other groups of horses did not receive feed supplements until January. Geldings were started on a feed supplement in mid-October to prepare for the bioavailability study (chapter 4) and blood concentrations increased in November.

Overall, serum α -tocopherol concentrations were different among groups and did not reflect the changes in pasture concentrations through the seasons. This suggests that serum α -tocopherol concentrations are not a reliable measure of vitamin E status in mature horses. Selenium concentrations were also different among groups and seasons and most likely reflect dietary intake of Se in feed supplements.

CHAPTER 6

EFFECTS OF SUPPLEMENTARY VITAMIN E ON SERUM AND MILK VITAMIN E STATUS AND IMMUNOGLOBULIN STATUS IN THROUGHbred MARES AND FOALS

EXPERIMENT 4.1

ABSTRACT: Synthetic vitamin E *dl*- α -tocopheryl acetate was administered to pregnant mares during their last trimester on a twice-weekly basis at three times the NRC (1989) recommended amount. The study was designed to test the effects of vitamin E supplementation on serum and milk α -tocopherol and immunoglobulin status in mares and their foals. Mare serum α -tocopherol status was not affected by vitamin E supplementation and decreased in both groups during 1 mo of lactation. However, vitamin E supplemented mares had higher milk concentrations of α -tocopherol and higher immunoglobulin G and M concentrations post-partum, which influenced foal serum α -tocopherol and immunoglobulin status following colostral ingestion. Consequently, it appears that serum vitamin E concentrations are not a valid assessment of vitamin E status in mares and milk concentrations may better demonstrate the influence of supplementary vitamin E. Perhaps serum α -tocopherol concentrations of foals at this early stage in life do reflect vitamin E status because major storage sites such as adipose tissue would not have had time to develop. Enhancement of passive transfer of immunoglobulins to foals by vitamin E supplemented mares may help protect these neonatal foals from life threatening septicemias during this critical period.

INTRODUCTION

The susceptibility of neonatal foals to septicemia and possible death is considered high, especially if an insufficient passive transfer of vital immunoglobulins has occurred. Vitamin E supplementation enhanced immune responses (increased serum IgA, IgM and IgG) in piglets (Babinsky et al., 1990; Hayek et al., 1989), and increased mammary concentrations of α -

tocopherol in sheep and serum α -tocopherol concentrations of their lambs following colostrum and milk consumption (Njeru et al., 1994).

The diffuse placenta of the horse does not allow the transfer of fat-soluble vitamins and immunoglobulins to the fetus and it is essential that the neonatal foal absorb these nutrients from colostrum. One study has shown that vitamin E supplementation of mares, at two times the NRC (1989) recommended amount for pregnant mares, enhanced concentrations of immunoglobulin G and M in colostrum and foal serum following the ingestion of colostrum by the foals (Hoffman et al., 1999).

OBJECTIVES

This study was designed to test the supplementation of pregnant Thoroughbred mares with a synthetic form of vitamin E to evaluate the influence of vitamin E on the serum and colostrum α -tocopherol and immunoglobulin status of mares, and serum α -tocopherol and immunoglobulin status of foals before and after colostrum ingestion. The hypothesis was that mare serum and colostrum concentrations of α -tocopherol would increase with supplementation and immunoglobulin concentrations in colostrum of supplemented mares would enhance passive transfer to foals.

MATERIALS AND METHODS

Animals

Twenty-four Thoroughbred mares due to foal from March to May, 1999 were available for this study at the Middleburg Agricultural Research and Extension Center, Middleburg, VA. Mares were paired by age, parity and breeding date, and assigned randomly to two groups, one given a sugar-and-starch sweet feed (SS) and the other a fat-and-fiber feed (FF). Each of these groups was sub-divided into two groups of six mares, one supplemented with vitamin E, the other six received a placebo. Body weights of mares were recorded monthly and diets adjusted

to maintain body condition scores between 5 and 7 (Henneke et al., 1983).

Diets

Mares were grazing orchardgrass/bluegrass/white-clover mixed pastures until foaling, and then bluegrass/white clover pastures until weaning in late October and early November 1999. Mares were supplemented with hay (round bale orchardgrass-alfalfa from January to April and square bale alfalfa-timothy from October to December; Table 3.2) and feeds, a SS feed (a copy of Omolene-200, Purina Mills) or FF (a fat-and-fiber feed with a cereal byproduct as a fat source). Nutrient composition of feeds, including endogenous vitamin E content is shown in Table 3.3. Supplements were isonitrogenous and isoenergetic, and were fed to provide one-third to one-half of nutrient requirements recommended for pregnant mares during the third trimester or during lactation (NRC, 1989). The vitamin and mineral premix (vitamin E free) added to the diets was balanced to complement the pastures and meet or exceed current recommendations (NRC, 1989).

Vitamin E Administration

Six mares from each group of 12 were individually administered vitamin E (*dl*- α -tocopheryl acetate, 950 IU/g; Hoffman-LaRoche, Nutley, NJ) every 3.5 d during the last trimester of pregnancy and for one month during lactation. Vitamin E oil was mixed with molasses (same amount for all horses) and each horse received three times the NRC (1989) requirement for pregnant/lactating mares (2.1 ml/d or 2010 IU/d for last trimester, and 3.0 ml/d or 2886 IU/d during lactation). Dosages were calculated from the recommended amount of vitamin E (IU) per total ration and the Mcal content of the dietary intake. The other six mares from each group received a vehicle (molasses only) and served as the controls. Hay and grain samples were analyzed for endogenous α -tocopherol content to ensure non-supplemented groups of mares were receiving sufficient vitamin E to meet the NRC (1989) minimum requirements (Table 3.3 and 3.4). Mares were supplemented with the vitamin or vehicle from 12 to 20 wk before foaling, at 1500 on Mondays, and at 0900 on Fridays. The total amount per

supplementation (3.5 d x daily amount) was 7.5 ml or 7035 IU (last trimester) and 10.6 ml or 10,101 IU (lactation) of *dl*- α -tocopheryl acetate.

Sample Collection

Physical signs of impending parturition were monitored and mammary secretions were tested for Ca^+ concentration (FoilWatch test kits, CHEMetrics, Inc., Calverton, VA). When foaling was imminent mares were monitored continuously.

Initial blood samples were collected from all 24 mares (Dec 1998) that were grazing pastures with no supplemental hay or grain to measure baseline concentrations of serum α -tocopherol. Blood samples were collected after two weeks of dietary accommodation and vitamin E supplementation. Samples of venous blood were collected for α -tocopherol analyses into three 10 ml sterile blood tubes (Vacutainer Systems, Becton Dickinson, NJ). Blood samples were collected monthly and then weekly before parturition, at 1 h post-parturition, at 6, 12, 24, and 48 h post-parturition, and after 2 and 4 wk of lactation. Blood tubes were protected from light, heat and moisture during collection, sample preparation and storage. Blood tubes were placed in the dark at room temperature for 2 h to allow blood to clot. Blood was centrifuged at 3,000 g (Centrifichem System 600, Baker Instruments Corporation, PA) for 10 min. Serum was separated from blood and placed in 12 x 55 mm polypropylene vials (Sarstedt, Newton, NC) and a few crystals of hydroquinone 1,4-Benzenediol 99+% (Sigma Chemical, MO) was added as an antioxidant. Serum vials were wrapped in black plastic and stored at -80°C .

Colostrum and milk samples were collected for α -tocopherol analyses from the mares within 30 min of foaling (pre-suckled), at 6, 12, 24, and 48 h after foaling, and at 2 and 4 wk of lactation. Immunoglobulin analyses was conducted on pre-suckle samples and on samples collected at 24 h post-partum. Colostrum and milk samples were filtered and placed in plastic tubes, and stored at -20°C .

Blood samples were collected from foals using jugular venipuncture into two 10 ml

Vacutainer sterile blood tubes to determine α -tocopherol and immunoglobulin G and M concentration, within 1 h of birth for base-line values, then at the same times as mare colostrum and milk samples were collected. Blood samples were prepared and stored as described for mares. Foal blood samples were also collected for immunoglobulin G and M analyses immediately after birth before colostrum ingestion and at 24 h of age.

Sample Analyses

Serum α -tocopherol concentrations were determined in duplicate by HPLC procedures modified from Miller and Yang (1985) and Craig *et al.* (1989). Details of procedures are described in chapter 1. Colostrum and milk samples were analyzed in duplicate for α -tocopherol concentration in duplicate using HPLC procedures. Samples (0.5 ml) of colostrum or milk were thawed and 1.0 ml of ethanol and 0.3 g of solid potassium hydroxide were added. Samples were placed in a bath at 37 °C for 30 min followed by 60 °C for 60 min and agitated periodically. Samples were removed from bath and 1.0 ml of water and 2.0 ml of hexane was added. Samples were placed on a shaker plate for 20 min, then the hexane layer was removed and samples were dried with nitrogen. Samples were resuspended in 0.5 ml of ethanol, vortexed and placed in autosampler vials, and 40 μ l/ml aliquots were injected into the HPLC. A mobile phase of 99 % methanol and 1 % H₂O at a flow rate of 1 ml/min was used. Aliquots were chromatographed using a Microsorb MV 100 Angstrom pore, reverse phase C₁₈ column (4.6 mm x 100 mm) and detected at 292 nm. Serum, colostrum and milk IgG and IgM concentrations were analyzed in duplicate using radial immunodiffusion kits (VMRD, Pullman, WA).

Statistical Analyses

Data are summarized as means \pm s.e. Changes in response variables (α -tocopherol, IgG and IgM) in serum and colostrum/milk were evaluated with repeated measures analysis of variance for differences between treatments and time with diet and vitamin E treatment in the model. When the F-statistic indicated differences between groups a *post hoc* Fisher's protected LSD test was performed to test for differences between means over time (SAS, Inst. Inc., Cary,

NC).

RESULTS

Mare mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) were not different ($P = 0.218$) during the last 20 wk of gestation from January until parturition in April or May (Figure 3.1). Serum α -tocopherol concentrations were not different ($P = 0.794$) between vitamin E supplemented and non-supplemented groups during the last 20 wk of gestation until parturition. Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of mares from foaling to 1 mo of lactation are shown in Table 4.1. Mean serum α -tocopherol concentrations decreased from foaling to 1 mo of lactation in vitamin E supplemented mares ($P = 0.0016$) and in non-supplemented mare ($P = 0.0025$). Mean serum α -tocopherol concentrations were not different between vitamin E supplemented and non-supplemented mares immediately after foaling ($P = 0.937$), at 6 h ($P = 0.459$), 12 h ($P = 0.842$), 24 h ($P = 0.962$), 48 h ($P = 0.703$), 2 wk ($P = 0.433$) and 4 wk ($P = 0.876$) post-partum.

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of mares fed the SS diet or the FF diet were not different at any sample point post partum (Table 4.3).

Changes in mean milk concentrations of α -tocopherol ($\mu\text{g/ml}$) of mares from foaling to 1 mo of lactation are shown in Figure 4.1. Mean milk α -tocopherol concentrations were different from foaling to 1 mo of lactation in vitamin E supplemented mares ($P = 0.0006$) and in non-supplemented mares ($P = 0.0001$). Mean milk α -tocopherol concentrations were not different between vitamin E supplemented and non-supplemented mares immediately after foaling ($P = 0.617$), but α -tocopherol concentrations increased in supplemented mares at 6 h ($P = 0.032$), and were not different at 12 h ($P = 0.539$), 24 h ($P = 0.446$), 48 h ($P = 0.900$), 2 wk ($P = 0.212$) and 4 wk ($P = 0.991$) post-partum. Mean milk α -tocopherol concentrations of mares fed the SS diet or the FF diet were not different at any sample point post partum (Table 4.3).

Milk concentrations of IgG (mg/dl) of mares during 24 h post-partum are shown in Figure 4.2. Mean milk IgG concentrations decreased during the 24 h post-partum in vitamin E

supplemented mares ($P = 0.0001$) and in non-supplemented mares ($P = 0.0001$). Mean milk IgG concentrations were higher in vitamin E supplemented mares compared to non-supplemented mares immediately after (0 h) parturition ($P = 0.023$), but no differences were found between the two groups at 6 h ($P = 0.755$), 12 h ($P = 0.514$) or 24 h ($P = 0.165$) post-partum. Milk concentrations of IgM (mg/dl) of mares during 24 h post-partum are shown in Figure 4.3. Mean milk IgM concentrations decreased during the 24 h post-partum in vitamin E supplemented mares ($P = 0.0013$) and in non-supplemented mares ($P = 0.001$). Mean milk IgM concentrations were higher in vitamin E supplemented mares compared to non-supplemented mares immediately after (0 h) parturition ($P = 0.048$), but no differences were found between the two groups at 6 h ($P = 0.826$), 12 h ($P = 0.592$) or 24 h ($P = 0.138$) post-partum. Mean milk IgG and IgM concentrations of mares fed the SS diet or the FF diet were not different at any sample point post partum (Table 4.3).

Foal serum α -tocopherol concentrations ($\mu\text{g/ml}$) from birth to 1 mo of age are shown in Figure 4.4. Mean serum α -tocopherol concentrations of foals immediately after birth were not different between foals from vitamin E supplemented dams and non-supplemented dams ($P = 0.1010$), but increased at 6 h in foals from vitamin E supplemented dams ($P = 0.049$) compared to foals from non-supplemented dams. No differences were found in serum α -tocopherol concentrations between the two groups of foals at 12 h ($P = 0.198$), 24 h ($P = 0.356$), 48 h ($P = 0.521$), 2 wk ($P = 0.666$) or 1 mo ($P = 0.647$) from birth. Mean serum α -tocopherol concentrations of foals from mares fed the SS diet or the FF diet were not different at any sample point after birth (Table 4.3).

Foal serum IgG concentrations (mg/dl) immediately after birth and at 24 h of age are shown in Figure 4.5. Immediately after birth there were no concentrations of IgG in serum of foals. At 24 h of age serum IgG concentrations were higher in foals of vitamin E supplemented dams compared to foals from non-supplemented dams ($P = 0.036$). Foal serum IgM concentrations (mg/dl) immediately after birth and at 24 h of age are shown in Figure 4.6. Immediately after birth concentrations of IgM in serum of foals were not different between foals from vitamin E supplemented and non-supplemented groups ($P = 0.811$), but at 24 h of age

serum IgM concentrations were higher in foals of vitamin E supplemented dams compared to foals from non-supplemented dams ($P = 0.015$). Mean serum IgG and IgM concentrations of foals from mares fed the SS diet or the FF diet were not different at any sample point after birth (Table 4.3).

DISCUSSION

Vitamin E supplementation with the synthetic form *dl*- α -tocopheryl acetate to pregnant mares during the last trimester increased α -tocopherol concentrations in mares' milk at 6 h post-partum. This increase in milk α -tocopherol concentration corresponded to the nursing foal serum α -tocopherol increase. Immunoglobulin G and M concentrations of colostrum increased in all mares but were enhanced in vitamin E supplemented mares compared to non-supplemented mares only immediately post-partum, the critical period where neonatal foals must absorb these antibodies. Foals from both the vitamin E supplemented and the non-supplemented groups were born with undetectable serum IgG concentrations and extremely low IgM concentrations. Following colostrum ingestion, foals from vitamin E supplemented dams absorbed higher levels of IgG and IgM than the foals from non-supplemented dams. Although differences in α -tocopherol concentrations of milk were only observed at 6 h post-partum and post-suckle immunoglobulin status of foals was only measured at 24 h of age, it appears that vitamin E absorption by the mare may enhance mammary transfer of immunoglobulins.

The mechanism for increasing immunoglobulin concentration in colostrum with supplemental vitamin E is unknown but it is tempting to suggest that because the transport of immunoglobulins to the milk is most likely by exocytosis, which may be inhibited by ROS, the supplemental vitamin E may facilitate this transport by protecting against ROS. Furthermore, since immunoglobulins are secreted into milk during the period leading up to parturition, vitamin E supplementation may serve to protect the immunoglobulins against damage by ROS.

Mare serum α -tocopherol concentrations were not different between vitamin E supplemented and non-supplemented groups during the last 20 wk of gestation, during

parturition and 1 mo of lactation, but decreased in both groups from immediately post-partum to the end of 1 mo of lactation, despite the continuation of supplementation. Consequently, vitamin E status of pregnant mares was not adequately assessed by serum concentrations and suggests milk α -tocopherol concentrations may serve as a function test to assess vitamin E status. It is conceivable that serum α -tocopherol concentrations of foals at this early stage in life do reflect vitamin E status because major storage sites such as adipose tissue would not have had time to develop. The enhancement of passive transfer of immunoglobulins demonstrated in foals by vitamin E supplemented dams may reduce the susceptibility of the foal to septicemias during this critical neonatal stage.

Diet did not affect mare serum α -tocopherol, milk α -tocopherol, milk IgG and IgM, foal serum α -tocopherol or foal serum IgG or IgM concentration at any time after foaling. In the study by Hoffman et al., (1999) mares fed the FF diet (which contained corn oil as a fat source) had higher milk immunoglobulin concentrations which indicated a response to the high vitamin E content of corn oil (483 IU/kg). The FF diet in this study contained a cereal by product as the fat source, which has a lower vitamin E content (66 IU/kg).

EXPERIMENT 4.2

ABSTRACT: Natural vitamin E *d*- α -tocopherol was administered to pregnant mares during their last trimester on a daily basis at three times the NRC (1989) recommended amount. The study was designed to follow Experiment 4.1 in testing the effects of vitamin E supplementation on serum and milk α -tocopherol and immunoglobulin status in mares and their foals. Even though vitamin E was supplemented daily, mare serum α -tocopherol status was not different between the supplemented and non-supplemented groups. Interestingly, natural vitamin E supplementation to mares increased milk concentrations of α -tocopherol similar to synthetic vitamin E supplementation, but this natural form increased milk concentrations for 24 h post-partum as compared to only 6 h in the synthetic vitamin E supplemented mares. Enhanced α -tocopherol concentrations in milk corresponded to higher immunoglobulin G and M concentrations immediately post-partum and were similar to findings in both experiments, but in natural vitamin E supplemented mares, IgM concentrations of milk were higher than in non-supplemented mares at each time point (except 24 h) until 1 mo post-partum. This may have influenced foal serum IgM concentrations at 12 h of age compared to foals from non-supplemented dams and compared to foals from dams supplemented with synthetic vitamin E which had increased serum IgM concentrations only immediately after birth.

Major biological and physiological changes occurring in the mare during the periparturient period may prevent an accurate measure of vitamin E status through fluid shifts that decrease plasma volume and may infer an incorrect increase in serum α -tocopherol concentrations at this time. Therefore, perhaps milk concentrations may better reflect the assessment of vitamin E status. It is not known if the daily supplementation of vitamin E or the form of vitamin E being supplemented influences the vitamin E and immunoglobulin status of neonatal foals, but any enhancement of passive transfer may confer an advantage especially during these critical first few hours of life.

INTRODUCTION

Supplementary *dl* α -tocopheryl acetate, at 3 times the recommended amount, twice

weekly to pregnant mares during the last trimester influenced milk and foal serum α -tocopherol and immunoglobulin status in Experiment 4.1. Similar findings were previously reported for immunoglobulin status of foals after daily supplementation of *dl* α -tocopheryl acetate at two times the recommended amount to dams (Hoffman et al., 1999). These findings, together with the results of the bioavailability and kinetics study of different forms of vitamin E in chapter 2, influenced the designing of a further experiment to investigate the effects of natural vitamin E supplementation to mares. The preferential biodiscrimination of α -tocopherol over other tocopherols and of the seven stereoisomers of α -tocopherol used to form the synthetic *dl*- α -tocopheryl acetate may influence the effects of dietary vitamin E supplementation on milk and foal serum α -tocopherol and immunoglobulin status. Greater transfer of immunoglobulins from the dam to the neonatal foal via the colostrum would improve foal health and provide better protection against life threatening septicemias.

OBJECTIVES

Pregnant Thoroughbred mares were supplemented with a natural form of vitamin E (*d*- α -tocopherol) on a daily basis during the last trimester to evaluate the influence of natural vitamin E on blood and milk concentrations of α -tocopherol and immunoglobulins of mares and their foals. Data from mares supplemented with the synthetic *dl*- α -tocopheryl acetate in 1999 will be compared to mares supplemented with the natural *d*- α -tocopherol in 2000.

MATERIALS AND METHODS

Animals

Twenty-four Thoroughbred mares due to foal from March to May, 2000 were available for this study at the Middleburg Agricultural Research and Extension Center, Middleburg, VA. Mares were paired by age, parity and breeding date, and assigned randomly to two groups, one given a sugar-and-starch sweet feed (SS) and the other a fat-and-fiber feed (FF). In between Experiment 4.1, which ended when foals were 1 mo of age, mares and foals remained in their

dietary groups and were fed the same diets until weaning in October and November 1999. No supplementary vitamin E was added to the diets of either group during this time. In Experiment 4.2 each of the two diet groups was sub-divided into two groups of six mares, one supplemented with vitamin E, the other six received a placebo. Body weights of mares were recorded monthly and diets adjusted to maintain body condition scores between 5 and 7 (Henneke et al., 1983).

Diets

Mares were grazing orchardgrass/bluegrass/white-clover mixed pastures until foaling, and then bluegrass/white clover pastures until weaning in late October and early November 2000. Mares were supplemented with hay (round bale orchardgrass-alfalfa from January to April, Table 3.2) and feeds, a SS feed (a copy of Omolene-200, Purina Mills) or FF2 (a fat-and-fiber feed with a cereal byproduct as a fat source). Nutrient composition of feeds, including endogenous vitamin E content is shown in Table 3.3. Supplements were isonitrogenous and isoenergetic, and were fed to provide one-third to one-half of current nutrient recommendations for pregnant mares during the third trimester or during lactation (NRC, 1989). The vitamin and mineral premix (vitamin E free) added to the diets was balanced to complement the pastures and meet or exceed (some minerals) current recommendations (NRC, 1989).

Vitamin E Administration

Six mares from each group of 12 were individually administered natural vitamin E (*d*- α -tocopherol, 1,459 IU/g; Archer Daniels Midland, Decatur, Illinois) on a daily basis at the same time as their morning feed. Supplementation of vitamin E was conducted during the last 3 to 4 months of pregnancy and continued for one month during lactation. Vitamin E oil was mixed with molasses and a large handful of their grain and hand fed to each horse once daily. Dosages were calculated to be three times the NRC (1989) requirement for pregnant/lactating mares and were determined in a similar manner to the dosages of Experiment 4.1. The other six mares from each group received a vehicle (molasses mixed with handful of grain) and served as the controls. Hay and grain samples were analyzed for endogenous α -tocopherol content to ensure non-

supplemented groups of mares were receiving sufficient vitamin E to meet the NRC (1989) minimum requirements (Table 3.2 and 3.3).

Sample Collection

All sample collection procedures were identical to the procedures described in Experiment 4.1 except that blood samples were collected only once in February and April, and not collected at 6, 12 and 48 h post-partum. Milk samples were collected at the same time points as in Experiment 4.1 except at 6 and 48 h post-partum. The number of samples collected was reduced to minimize the number of times the foals needed to have a venous needle inserted.

Sample Analyses

Serum and milk α -tocopherol and immunoglobulin concentrations were determined using the same methods previously described in Experiment 4.1 and detailed serum α -tocopherol analyses are described in chapter 3.

Statistical Analyses

Analyses are described previously in Experiment 4.1. One-way analysis of variance was performed on data from Experiment 4.1 to compare to Experiment 4.2 for milk α -tocopherol concentrations and milk immunoglobulin G and M concentrations (SAS, Inst. Inc., Cary, NC).

RESULTS

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of mares in January 2000 before vitamin E supplementation were 2.21 ± 0.19 and 2.19 ± 0.15 $\mu\text{g/ml}$ in Vitamin E supplementation group and non-supplementation groups respectively. In February, after 3 wk of vitamin supplementation, mean serum α -tocopherol concentrations were 2.02 ± 0.17 and 2.06 ± 0.17 $\mu\text{g/ml}$ in vitamin E supplemented and non-supplemented groups, respectively. In April,

serum α -tocopherol concentrations of the vitamin E and non-vitamin E groups had increased ($P < 0.001$) to 3.39 ± 0.32 and 3.41 ± 0.17 $\mu\text{g/ml}$, respectively. Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of mares from foaling to 1 mo of lactation are shown in Table 4.2. Mean serum α -tocopherol concentrations decreased during the first 2 wk of lactation in vitamin E supplemented mares ($P = 0.0005$) and in non-supplemented mare ($P = 0.0011$). Mean serum α -tocopherol concentrations were not different between vitamin E supplemented and non-supplemented mares immediately after foaling ($P = 0.805$), 24 h ($P = 0.425$), 2 wk ($P = 0.294$) and 4 wk ($P = 0.492$) post-partum. Mean serum α -tocopherol concentrations of mares fed the SS diet or the FF diet were not different at any sample point after foaling (Table 4.4).

Changes in mean milk concentrations of α -tocopherol ($\mu\text{g/ml}$) of mares from foaling to 1 mo of lactation are shown in Figure 4.7. Mean milk α -tocopherol concentrations were different from foaling to 1 mo of lactation in vitamin E supplemented mares ($P = 0.0001$) and in non-supplemented mares ($P = 0.001$). Mean milk α -tocopherol concentrations were higher in vitamin E supplemented than non-supplemented mares immediately after foaling ($P = 0.036$), and at 12 h ($P = 0.044$) post-partum, and tended to be higher at 24 h ($P = 0.067$) post-partum. No differences were found between supplemented and non-supplemented groups at 2 wk ($P = 0.096$) or at 4 wk ($P = 0.932$) post-partum. Milk α -tocopherol concentrations immediately after foaling were higher ($P = 0.021$) in natural *d*- α -tocopherol supplemented mares in 2000 compared to mares supplemented with *dl*- α -tocopheryl acetate in 1999 (3.64 ± 0.62 vs 2.15 ± 0.17 $\mu\text{g/ml}$). Similarly, at 12 h post-partum, milk α -tocopherol concentrations were higher ($P = 0.022$) in the natural *d*- α -tocopherol supplemented mares in 2000 compared to mares supplemented with *dl*- α -tocopheryl acetate in 1999 (6.43 ± 0.85 vs 3.91 ± 0.56 $\mu\text{g/ml}$). No differences between natural and synthetic forms of vitamin E were observed at 24 h post-partum ($P = 0.143$), or 2 wk ($P = 0.769$), or at 1 mo ($P = 0.289$) post-partum. Mean milk α -tocopherol concentrations of mares fed the SS diet or the FF diet were not different at any sample point post partum (Table 4.4).

Milk concentrations of IgG (mg/dl) of mares during 24 h post-partum are shown in Figure 4.8. Mean milk IgG concentrations decreased during the 24 h post-partum in vitamin E

supplemented mares ($P = 0.0001$) and in non-supplemented mares ($P = 0.0001$). Mean milk IgG concentrations were higher in vitamin E supplemented mares compared to non-supplemented mares immediately after parturition ($P = 0.0002$), but no differences were found between the two groups at 12 h ($P = 0.249$) or 24 h ($P = 0.424$) post-partum. Milk concentrations of IgM (mg/dl) of mares during 24 h post-partum are shown in Figure 4.9. Mean milk IgM concentrations decreased during the 24 h post-partum in vitamin E supplemented mares ($P = 0.0001$) and in non-supplemented mares ($P = 0.0017$). Mean milk IgM concentrations were higher in vitamin E supplemented mares compared to non-supplemented mares immediately after parturition ($P = 0.049$), and at 12 h ($P = 0.043$), but no differences were found between the two groups at 24 h ($P = 0.156$) post-partum. Milk concentrations of IgM in this study remained detectable at 2 wk and were higher in milk of vitamin E supplemented compared to non-supplemented mares ($P = 0.008$), and tended to be higher at 1 mo post-partum ($P = 0.063$). Milk IgG concentration immediately after foaling was higher ($P = 0.026$) in natural *d*- α -tocopherol supplemented mares in 2000 compared to mares supplemented with *dl*- α -tocopheryl acetate in 1999 (8821 ± 261 vs 7796 ± 329 $\mu\text{g/ml}$). No differences were found between the two forms of supplemental vitamin E on milk concentrations of IgG at 12 h ($P = 0.775$) or at 24 h ($P = 0.382$) post-partum. No differences were found between the two forms of supplemental vitamin E on milk concentrations of IgM at 0 h ($P = 0.228$), at 12 h ($P = 0.561$) or at 24 h ($P = 0.301$) post partum. Mean milk IgG and IgM concentrations of mares fed the SS diet or the FF diet were not different at any sample point after foaling (Table 4.4).

Foal serum α -tocopherol concentrations ($\mu\text{g/ml}$) from birth to 1 mo of age are shown in Figure 4.10. Mean serum α -tocopherol concentration of foals immediately after birth were not different between foals from vitamin E supplemented dams and non-supplemented dams ($P = 0.731$), but increased at 24 h in foals from vitamin E supplemented dams ($P = 0.043$) compared to foals from non-supplemented dams. No differences were found in serum α -tocopherol concentrations between the two groups of foals at 2 wk ($P = 0.862$) or 1 mo ($P = 0.954$) of age. Mean serum α -tocopherol concentrations of foals from mares fed the SS diet or the FF diet were not different at any sample point after birth (Table 4.4).

Foal serum IgG concentrations (mg/dl) immediately after birth and at 24 h of age are shown in Figure 4.5. Immediately after birth concentrations of IgG were undetectable in foal serum. At 24 h of age serum IgG concentrations tended to be higher in foals of vitamin E supplemented dams compared to foals from non-supplemented dams ($P = 0.064$). Foal serum IgM concentrations (mg/dl) immediately after birth and at 24 h of age are shown in Figure 4.6. Immediately after birth concentrations of IgM in serum of foals were not different between foals from vitamin E supplemented and non-supplemented groups ($P = 0.568$), but at 24 h of age serum IgM concentrations were higher in foals of vitamin E supplemented dams compared to foals from non-supplemented dams ($P = 0.039$). Mean serum IgG and IgM concentrations of foals from mares fed the SS diet or the FF diet were not different at any sample point after birth (Table 4.4).

DISCUSSION

Supplementation of pregnant mares with natural *d*- α -tocopherol increased milk α -tocopherol concentrations immediately post-partum, at 12 h and tended to increase concentrations at 24 h post-partum which is not only different from non-supplemented mares, but is also different from mares supplemented with synthetic *dl*- α -tocopheryl acetate the previous year, where increases in milk α -tocopherol concentration were only observed at 6 h post-partum. Furthermore, concentrations of α -tocopherol in the milk were higher in mares supplemented with natural *d*- α -tocopherol compared to those supplemented with synthetic *dl*- α -tocopheryl acetate.

Increased α -tocopherol concentrations in mares' milk corresponded to the enhanced immunoglobulin status of the milk. Immunoglobulin G concentrations were higher in milk only immediately post-partum, which was similar to increased IgG of mares supplemented with synthetic *dl*- α -tocopheryl acetate the previous year. However, IgM concentrations in milk of horses supplemented with natural *d*- α -tocopherol were higher immediately post-partum, as in Experiment 4.1, but were also higher at 12 h post partum, unlike in the mares milk of the synthetic *dl*- α -tocopheryl acetate the previous year. Interestingly, IgM concentrations were detectable at 2 and 4 wk post-partum, unlike in Experiment 4.1 where no IgM levels were

detected at 48 h or afterwards, and concentrations were higher at both time points. However, it must be pointed out the concentrations were low and the number of samples were also low and may therefore not reflect a true difference.

Serum α -tocopherol concentrations were unchanged in mares supplemented daily with three times the recommended amount of vitamin E for pregnant mares. This suggests that the removal of serum α -tocopherol and uptake by the tissues was occurring at a similar rate to the serum uptake. Increased milk concentrations of α -tocopherol post partum in supplemented mares compared to non-supplemented mares demonstrates a response to vitamin E supplementation because more had to be absorbed, thus milk serves as a function test for vitamin E status of Thoroughbred mares.

The reason for the supplemental vitamin E increasing milk concentrations and simultaneously increasing immunoglobulin concentration in the mammary gland during the periparturient period is not understood. Enhanced mobilization of fatty acids to the mammary gland during the periparturient period for colostrum and milk production may concomitantly mobilize vitamin E from adipose stores. If stores of vitamin E are greater in vitamin E supplemented mares then vitamin E may be mobilized more readily. The transport of immunoglobulins to the milk occurs through exocytosis and this process may be inhibited by ROS. Therefore, vitamin E supplementation may facilitate the transport of immunoglobulins to the milk by protecting against ROS damage. Furthermore, since immunoglobulins are secreted into the mammary gland during the period leading up to parturition, it is likely that the presence of vitamin E would protect the immunoglobulins against damage by ROS.

Natural vitamin E supplementation to mares may have influenced the serum α -tocopherol concentrations of their foals because foal serum α -tocopherol concentrations were higher at 24 h of age in foals from supplemented mares compared to non-supplemented mares, unlike in Experiment 4.1 where serum concentrations were only higher at 6 h of age. Unfortunately, serum concentrations of foals were not analyzed for α -tocopherol concentration at 12 h of age and were not collected at 6 h of age. The greater concentrations at 24 h though may infer an

enhanced vitamin E status of these foals. Immunoglobulin status of foals following colostrum ingestion was improved, both in IgG and IgM concentrations at 24 h of age. These findings are similar to the results of Experiment 4.1 at 24 h. Unfortunately, IgG and IgM concentrations in foal serum were not measured after 24 h so influences of the higher milk IgM concentrations at 2 and 4 wk post-partum on foal immunoglobulin status could not be determined.

Mare serum α -tocopherol concentrations were not different between vitamin E supplemented and non-supplemented groups during the last 3 mo of gestation or during parturition and 1 mo of lactation, but decreased in both groups from 24 h to 2 wk post-partum, and then increased by 4 wk post-partum to previous concentrations. These changes occurred despite the continuation of daily supplementation with vitamin E. These findings, together with the results of Experiment 4.1, suggest that vitamin E status of pregnant mares is not accurately assessed by serum tocopherol concentrations and proposes milk α -tocopherol concentrations as an indicator of vitamin E status. However, the periparturient period is a highly dynamic phase both biologically and physiologically and so measuring vitamin E status accurately may not be a possibility at this time.

As suggested earlier, perhaps in foals the vitamin E status is easier to assess, especially at this extremely early stage when under developed adipose tissue, the main storage site of vitamin E, is unlikely to influence the assessment vitamin E status.

The daily supplementation of vitamin E during this study compared to the twice-weekly supplementation of vitamin E in Experiment 4.1 may have influenced the results of this study, although it did not affect the serum α -tocopherol concentrations of mares. Diet did not affect serum α -tocopherol, milk α -tocopherol, milk IgG and IgM, foal serum α -tocopherol or foal serum IgG or IgM concentration at any time after foaling. This was not consistent with the study by Hoffman et al., (1999) where mares were fed an FF diet that contained corn oil as a fat source. Corn oil has a relatively high vitamin E content, which may have influenced the higher milk immunoglobulin concentrations observed in mares fed the FF diet. In this study, the FF diet contained 66 IU vitamin E/kg and the SS diet contained 38 IU vitamin E/kg.

Overall, greater passive transfer of immunoglobulins to foals by vitamin E supplemented dams should serve to reduce the susceptibility of neonatal foals to septicemias and may provide a boost to the initial vitamin E supply for newly developing muscle tissues.

CHAPTER 7

ANTIOXIDANT STATUS AND MUSCLE CELL LEAKAGE OF HORSES DURING ENDURANCE EXERCISE

ABSTRACT: Antioxidant status was evaluated in 30 horses during the No Frills (NF80), or the Old Dominion (OD80), 80 km endurance races held in April and June 2000. Each race had a time limit of 12 h and both races were conducted over similar terrain but dissimilar ambient conditions (Experiment 5.1). The results from horses competing in OD80 were subsequently compared with 17 horses competing in the Old Dominion 160 km (OD160) endurance race. The OD160 race was held in June 2000 and had a time limit of 24 h. In addition, indicators of oxidative stress as well as antioxidant status were evaluated in horses during OD160 (Experiment 5.2). Packed cell volume (PCV), total plasma protein (TPP), plasma α -tocopherol (VIT E), erythrocyte glutathione (GSH) concentrations, and erythrocyte glutathione peroxidase (GPX) activities were measured in all three endurance races. Plasma ascorbic acid (VIT C), plasma aspartate aminotransferase (AST), and plasma creatine kinase (CK) activities were measured at OD80 and OD160. Samples were collected at 0, 80 km, and 60 min of recovery (REC) at NF, at 0, 40, 80 km and REC at OD80, and at 0, 64, 106, 142, 160 km and REC at OD160. In all three races, no changes were found in plasma VIT E concentration. In OD80 and OD160 concentrations of plasma VIT C decreased ($P < 0.05$). In all three races erythrocyte GSH concentrations decreased ($P < 0.05$), and mean erythrocyte GPX activities increased. At NF, erythrocyte GPX activities were greater than OD80 from 0 to REC, most likely because of the colder ambient conditions at NF. In OD80 and OD160, plasma AST and CK activities increased from 0 km ($P < 0.05$). Indices of muscle cell leakage (plasma AST and CK) were correlated with indices of antioxidant status (VIT C, GSH and GPX) in OD80 and OD160. Associations between increased muscle leakage and decreased antioxidant status may in part reflect oxidative stress as oxidative damage to cell membranes. These findings suggest the testing of antioxidant supplements in endurance horses to improve performance and welfare.

INTRODUCTION

Increased oxygen demands during strenuous exercise provide a metabolic advantage for energy production, but paradoxically cause oxidative injury to muscle cells. Oxygen consumption during exercise may increase 10 to 20 times in humans, and 30 times in horses (Butler et al., 1993). Oxygen flux through muscle fibers may increase 100 times from rest to maximal exercise (Milnor, 1980). Reactive oxygen species (ROS) produced by oxidative reactions, and free radicals, which are molecules that contain an unpaired electron, damage important cellular components resulting in the loss of cellular function (Sjodin et al., 1990; Sen, 1995). Free radicals formed during oxygen reduction can trigger a chain reaction of lipid peroxidation so that membrane bound enzyme and receptor function is lost (Halliwell and Gutteridge, 1986).

Under non-stressed physiological conditions, extensive enzymatic and nonenzymatic antioxidant defense systems in muscle tissue allow effective scavenging of free radicals and ROS before cellular components are damaged. Antioxidants are positioned in specific cellular locations to facilitate a comprehensive protection against oxidant stress. Glutathione peroxidase, an important enzymatic antioxidant located in both mitochondria and cytosol, removes hydrogen peroxide and organic hydroperoxides. Important nonenzymatic antioxidants include vitamin E, a lipid soluble and major chain-breaking antioxidant in cell membranes, and vitamin C, located in the aqueous phase of cells, scavenges radicals and recycles vitamin E. Glutathione is an abundant cellular nonprotein thiol, with multiple antioxidant functions including donating hydrogen atoms to hydroxyl radicals, and removing hydrogen and lipid peroxides in conjunction with glutathione peroxidase (Ji, 1995). Glutathione is also postulated to reduce vitamin E tocopheroxyl radicals, and is implicated in the reduction of vitamin C semidehydroascorbate radicals during vitamin E recycling (Packer, 1991). Creatine kinase (CK) and aspartate aminotransferase (AST) are two enzymes found primarily in skeletal muscle. Damaged skeletal muscle cells allow leakage of these enzymes into the blood and increased plasma activities of CK and AST are indicators of skeletal muscle damage in horses (Lindholm, 1987; Noakes, 1987). Lipid hydroperoxides are the result of free radical or other ROS attacks on lipid cell membranes. Enhanced free radical and ROS production during strenuous exercise increases

lipid peroxidation of cell membranes.

Prolonged strenuous exercise increases the production of free radicals and ROS and may overwhelm antioxidant defenses, resulting in oxidative stress. If antioxidant systems become depleted during an exercise bout, the susceptibility of cells and tissues to ROS damage is enhanced. Exercise-induced oxidative damage to cell membranes contributes to muscle damage, fatigue and several pathological conditions (Sjodin et al., 1990; Sen, 1999).

The antioxidant defenses of endurance horses may be severely tested during prolonged and strenuous endurance exercise. The No Frills 80 km and the Old Dominion 80 km endurance races will be compared in Experiment 5.1. Subsequently, the Old Dominion 80 km race from Experiment 5.1 will be compared with the Old Dominion 160 km endurance race in Experiment 5.2.

OBJECTIVES

The objectives of this study were to determine the antioxidant status and level of muscle cell leakage in endurance horses competing in 80 km or 160 km endurance races, and to evaluate the relationship of muscle cell leakage to changes in antioxidant status.

MATERIALS AND METHODS

Experiment 5.1: Antioxidant Status and Muscle Cell Leakage of Horses During Two 80 km Endurance Races

Thirty horses were studied during either the No Frills (NF80, n = 12) or the Old Dominion (OD80, n = 18) 80-km endurance races, in the Blue Ridge Mountains near Front Royal, VA. Horses competing in the NF80 had a mean age of (mean \pm sd) 10 \pm 3 y (range 7 to 18 y) and were Arabian (n = 8), part-bred Arabian (n = 3) or Morgan (n = 1), geldings (n = 10) or mares (n = 2). Horses competing in OD80 had a mean age of (mean \pm sd) 11 \pm 4 y (range 6 to 21 y) and were Arabian (n = 9), part-bred Arabian (n = 5), Quarter Horse (n = 2), Morgan (n = 1)

or Appaloosa (n = 1), geldings (n = 15) or mares (n = 3). The NF80 and OD80 races were held in April and June 2000, respectively, and each race had a time limit of 12 h. Both races were conducted over similar terrain but dissimilar ambient conditions. Mean ambient temperature during NF80 was 5.5°C (4°C during pre-race sample collection), and during OD80 was 28°C (range 18 to 34°C). Information on the dietary intake of antioxidants was not uniformly reliable and represents a limitation of this study. However, most endurance horses are provided with abundant vitamin and mineral supplementation (Ralston 1988). Veterinarians examined horses before and after the races, and during 3 rest stops. The Institutional Animal Care and Use Committee of Virginia Tech approved this protocol.

Race times, minus hold-times at rest stops, and mean speeds were calculated. At NF80, a plastic weight tape, placed behind the elbow, was used to measure heart girth and estimate pre-race BW. At OD80, pre- and post-race BW was measured by portable weigh-scales (Tyrel platform, Alweights Hamilton Scale Corp. VA).

Blood samples were collected at 0, 80 km and after 60 min recovery (REC) at NF80, and at 0, 40, 80 km and REC at OD80. Blood samples were analyzed for packed cell volume (PCV), total plasma protein (TPP), plasma α -tocopherol (VIT E), erythrocyte glutathione (GSH) and glutathione peroxidase (GPX) at both NF80 and OD80, and ascorbic acid (VIT C), aspartate aminotransferase (AST), and creatine kinase (CK) were also analyzed at OD80.

A portable laboratory was set up at the race and blood samples were analyzed for PCV (microhematocrit centrifugation) and TPP (refractometer) on site, and all other samples were prepared and frozen on dry ice for later analyses. Plasma α -tocopherol (VIT E) concentrations were determined using HPLC procedures modified from Miller and Yang (1985) and Craig et al., (1989) and are described in detail in Chapter 3 for Experiment 1.1. Plasma ascorbate concentrations (VIT C) were determined using HPLC procedures of Schiiep et al., (1987) and detailed methods are described in the materials and methods section of Experiment 5.2. Erythrocyte concentrations of GSH and GPX activities were determined in triplicate using BIOXYTECH GSH-420 and GPx-340 colorimetric assays, respectively (Oxis International, Inc., Portland, OR). Plasma AST and CK activities were determined in duplicate using routine

clinical chemistry procedures on a Beckman CX5 chemical analyzer (Beckman Instruments Inc., Brea, CA).

Statistical Analyses

Data are summarized as means \pm SE. Changes with time were evaluated with ANOVA, and significance was inferred at $P < 0.05$. A *post hoc* Fisher's protected LSD test was performed to test for differences between means. Logarithmic transformations were applied to data not normally distributed. Data from NF and OD were not statistically compared. Simple regressions ($y = a + bx$) of indices of muscle leakage (y ; CK and AST) on indices of antioxidant status (x ; VIT C, GSH, GPX) were performed. Statistical tests were performed using SAS (SAS Inst. Inc., Cary, NC).

RESULTS – Experiment 5.1

Mean race times were 7.44 h (range 6.02 to 9.12 h) at NF80 and 9.10 h (range 7.38 to 10.23 h) at OD80 (Appendix Table 5.1). Mean speeds were 10.8 and 8.8 km/h at NF80 and OD80, respectively. Mean BW of horses competing at NF80 was 420 ± 10 kg. Mean pre- and post-race BW of horses competing at OD80 was 444 ± 12 and 421 ± 12 kg, respectively (Appendix Table 5.2).

During NF80, mean PCV at 0 km was 39 ± 1.7 % and increased ($P = 0.0004$) to 52 ± 2.0 % at 80 km (Table 5.1). At OD80, mean PCV at 0 km was 41 ± 1.1 % and no changes ($P = 0.901$) were found at 40 km, 80 km or REC (Table 5.1). At NF80, mean TPP concentration at 0 km was 5.8 ± 0.2 g/dl and tended to increase ($P = 0.06$) to 6.4 ± 0.2 g/dl at 80 km (Table 5.2). At OD80, mean TPP concentration was 5.7 ± 0.1 g/dl and no changes ($P = 0.076$) were found at 40 km, 80 km or REC (Table 5.2).

At NF80, α -tocopherol concentration was 5.8 ± 0.5 μ g/ml at 0 km and no changes were found ($P = 0.913$) at 80 km or at REC (Figure 5.1). At OD80, α -tocopherol concentration was

5.0 ± 0.4 µg/ml at 0 km and no changes were found ($P = 0.955$) at 40, 80 km or REC (Figure 5.1). At OD80, mean VIT C concentration at 0 km was 4.6 ± 0.1 µg/ml and decreased ($P = 0.002$) by 15 % at REC (Figure 5.2).

Erythrocyte GSH concentration at 0 km at NF80 was 223 ± 30 µmol/g and decreased ($P = 0.031$) by 36 % at REC (Figure 5.3). At OD80, erythrocyte concentration was 171 ± 29 µmol/g and decreased ($P = 0.0001$) by 59 % at REC (Figure 5.3). At NF80, GPX activity at 0 km was 33 ± 5 mU/mg and no changes ($P = 0.14$) were found at 80 km (Figure 5.4). At OD80, GPX activity at 0 km was 7.5 ± 0.9 mU/mg and increased ($P = 0.013$) to 21.5 ± 4.2 mU/mg at 80 km (Figure 5.4).

At OD80, AST activity at 0 km was 280 ± 14 IU/L and increased ($P = 0.010$) to 352 ± 17 IU/L at 80 km (Figure 5.5). Plasma CK activity at 0 km was 277 ± 36 IU/L and increased ($P = 0.011$) to 611 ± 70 IU/L at 80 km (Figure 5.6).

Plasma AST activities were correlated with erythrocyte GPX activities and GSH concentrations, but not with plasma VIT C concentrations. Plasma CK activities were correlated with erythrocyte GPX activities, erythrocyte GSH and plasma VIT C concentrations. The regressions are summarized in Table 5.5.

MATERIALS AND METHODS

Experiment 5.2: Antioxidant Status and Muscle Cell Leakage of Horses during an 80 km and 160 km Endurance Race.

Horses were studied during the Old Dominion 80 km (OD80) and 160 km (OD160) endurance races in the Blue Ridge Mountains near Front Royal, VA in June 2000. Both races took place on the same day. The OD80 and OD160 races must be completed in 12 and 24 h, respectively. Mean ambient temperature was 28°C with a range of 18 to 34°C over the 24 h period. Thirty-five horses were studied during OD80 ($n = 18$) and OD160 ($n = 17$). Horses

competing in OD80 had a mean age of (mean \pm sd) 11 ± 4 y, range 6 to 21 y, and were Arabian (n = 9), part-bred Arabian (n = 5), Quarter Horse (n = 2), Morgan (n = 1) or Appaloosa (n = 1) geldings (n = 15) or mares (n = 3). Horses competing in OD160 had a mean age of 10 ± 2.4 y, range 8 to 14 y, and were Arabian (n = 12) or part-bred Arabian (n = 5) geldings (n = 15) or mares (n = 2).

Horses consumed water and wet feeds at various points during both races. Information on the specific dietary intake of antioxidants was not uniformly reliable and represents a limitation of this study. However, most endurance horses are provided with abundant vitamin and mineral supplementation (Ralston 1988). Veterinarians examined horses before and after each race, and at four and eight rest stops during OD80 and OD160, respectively. The Institutional Animal Care and Use Committee of Virginia Tech approved this protocol.

Race times, minus hold-times at rest stops, were recorded and mean speeds were calculated. Pre- and post-race BW was measured by portable weigh-scales (Tyrel platform, Alweights Hamilton Scale Corp. VA). Samples of venous blood were collected into lithium heparin tubes (Vacutainer Becton Dickinson and Co., Rutherford, NJ) at 0, 40, 80 km, and after 60 min of recovery (REC) at OD80, and at 0, 64, 106, 142, 160 km and REC at OD160. Blood samples were analyzed for PCV, TPP, VIT E, VIT C, GSH, GPX, AST and CK at both OD80 and OD160. Blood samples were placed in crushed ice, and centrifuged (Centrifichem System 600, Baker Instruments Corp., PA) at 3,000 g for 5 min, within 60 min of collection. A portable laboratory was set up at the race. Plasma and erythrocyte samples were analyzed on site or prepared and frozen on dry ice for later analyses.

Packed cell volume (PCV) was measured in duplicate by microhematocrit centrifugation and TPP concentration was measured using a refractometer.

Plasma α -tocopherol (VIT E) concentrations were determined in duplicate by HPLC procedures modified from Miller and Yang (1985) and Craig *et al.* (1989). Detailed methods for vitamin E analyses are described in Chapter 3 for Experiment 1.1.

Ascorbic acid (VIT C) concentrations were determined in duplicate by HPLC procedures (Schiiep *et al.* 1987). Plasma, 1.0 ml, was diluted with 4.0 ml 5% metaphosphoric acid in distilled water, centrifuged at 2000 g for 10 min, and supernatant transferred and reacted to form 2,4-dinitrophenylhydrazone of VIT C. Following extraction with 2.0 ml of ethyl acetate containing 2% acetic acid, aliquots were chromatographed on an E. Merck Lichrosorb Si-60 column and detected at 520 nm.

Erythrocyte GSH concentration and GPX activities were determined in triplicate using BIOXYTECH GSH-420 and GPx-340 colorimetric assays (OXIS International, Inc., Portland, OR), respectively. Plasma AST and CK activities were determined in duplicate using routine clinical chemistry procedures on a Beckman CX5 chemical analyzer (Beckman Instruments Inc., Brea, CA).

Statistical Analyses

Data are summarized as mean \pm s.e. Antioxidant variables were adjusted for exercise-induced changes in plasma volume at time points where TPP concentration was significantly increased. Changes with time were evaluated with ANOVA, and a *post hoc* Fisher's protected LSD test was performed to test for differences between means (SAS, Inst. Inc., Cary, NC). Logarithmic transformations were applied to CK data, which were not normally distributed. Only data from horses that completed the races were used. Data from OD80 and OD160 were not statistically compared. Simple regressions ($y = a + bx$) of indices of muscle leakage (y; CK and AST) on indices of antioxidant status (x; VIT E, VIT C, GSH, GPX) were performed using pooled data for each race, because ANOVA revealed that horse had no effect on these variables.

RESULTS – Experiment 5.2

Twelve of 18 horses finished OD80, and 10 of 17 horses finished OD160. Of the non-finishers of OD80, one was a rider option and five were because of lameness. In OD160, four of

the non-finishers were because of lameness, one due to a sore back, one rider option and one because of metabolic problems.

Mean race time was 9.10 h (range 7.38 to 10.23 h) and mean speed was 8.8 km/h in OD80 (Appendix Table 5.3). Mean race time was 19.43 h (range 18.01 to 20.54 h) and mean speed was 8.2 km/h in OD160 (Appendix Table 5.3). Mean pre-race BW was 453 ± 12 kg and post-race BW was 421 ± 12 kg in OD80 (Appendix Table 5.4). Mean pre-race BW was 446 ± 8 kg and post-race BW was 425 ± 10 kg in OD160 (Appendix Table 5.4).

Old Dominion 80 km

Mean PCV and TPP concentrations at OD80 were 41 ± 1 % and 5.7 ± 0.1 g/dl at 0 km respectively, and no changes in PCV ($P = 0.90$) or TPP ($P = 0.08$) were found at 40, 80 km or REC (Table 5.3).

Plasma VIT E concentration at 0 km was 5.0 ± 0.4 µg/ml and no change ($P = 0.95$) was found at 40, 80 km or REC (Figure 5.7). Plasma VIT C concentration at 0 km was 4.6 ± 0.1 µg/ml and decreased ($P = 0.002$) by 15 % at REC (Figure 5.8).

Mean erythrocyte GSH concentration at 0 km was 171 ± 29 µmol/g and decreased ($P = 0.0001$) by 59 % at REC (Figure 5.9). Mean erythrocyte GPX activity at 0 km was 7.5 ± 0.9 mU/mg and increased ($P = 0.01$) by 185 % at 80 km (Figure 5.10).

Mean plasma AST activity at 0 km was 280 ± 14 IU/L and increased ($P = 0.01$) by 26 % at 80 km (Figure 5.11), and mean CK activity at 0 km was 277 ± 36 IU/L and increased ($P = 0.01$) by 121 % at 80 km (Figure 5.12).

Plasma AST activities were correlated with erythrocyte GSH concentrations and GPX activities, but not with plasma VIT E concentrations. Plasma CK activities were correlated with plasma VIT C and erythrocyte GSH concentrations, and erythrocyte GPX activities. The regressions are summarized in Table 5.5.

Old Dominion 160 km

Mean PCV at 0 km of OD160 was 38 ± 1 % and increased ($P = 0.04$) by 13 % at 64 km and 16 % at 106 km (Table 5.3). Mean TPP concentration at 0 km was 6.0 ± 0.1 g/dl and increased ($P < 0.0001$) by 8 % at 64 km and 6 % at 106 km (Table 5.4).

Plasma VIT E concentration at 0 km was 5.6 ± 0.5 $\mu\text{g/ml}$ and no change ($P = 0.95$) was found at 64, 106, 142, 160 km or REC (Figure 5.7). One horse was 3.5 to 5.6 $\mu\text{g/ml}$ and 4.8 to 6.8 standard deviations from the mean, hence removed from the analysis as an outlier. Plasma VIT C concentration at 0 km was 4.4 ± 0.2 $\mu\text{g/ml}$ and decreased ($P < 0.0001$) by 23 % at REC (Figure 5.8).

Mean erythrocyte GSH concentration at 0 km was 135 ± 14 $\mu\text{mol/g}$ and decreased ($P < 0.0001$) by 84 % at REC (Figure 5.9). Mean erythrocyte GPX activity at 0 km were 7 ± 1 mU/mg and increased ($P = 0.003$) by 214 % at 160 km (Figure 5.10).

Mean plasma AST activity at 0 km was 294 ± 13 IU/L and increased ($P = 0.0003$) by 46 % at REC (Figure 5.11), and mean plasma CK activity at 0 km was 237 ± 20 IU/L and increased ($P = 0.02$) by 327 % at 160 km (Figure 5.12). Plasma CK activity was especially increased during exercise in 1 horse during OD80 and 2 horses during OD160 (Figure 5.13).

Plasma AST activities were correlated with plasma VIT C (Figure 5.14), erythrocyte GSH concentrations and GPX activities, but not with plasma VIT E concentrations. Plasma CK activities were correlated with erythrocyte GSH concentrations (Figure 5.15). The regressions are summarized in Table 5.5.

DISCUSSION – Experiment 5.1 and 5.2

Evaluating the antioxidant status of horses competing in the NF80 and the antioxidant status and muscle cell enzyme leakage of endurance horses competing in the OD80 and OD160

provides valuable information on the ability of horses to cope with oxidative stress. The results reveal associations between muscle cell leakage and diminished antioxidative status in endurance horses. An unexpected finding was the maintenance of plasma α -tocopherol concentration during all three endurance races.

Increases in PCV and TPP during NF80 compared to no changes in these variables observed during OD80 (Table 5.1 and 5.2) may result from the time of sample collection during the rest stops. At NF80, blood samples were collected immediately (within 60 sec) as the horses pulled into the rest stops, whereas at OD80 blood samples were collected 30 min after the horses pulled into the rest stop and had been offered water to drink. The procedures followed at OD80 regarding blood sample collection were also applied to OD160. During OD160 hydration status was maintained in general, except the 64 and 106 km sample collection points (Table 5.3). These points were between ~10:00 and 14:00 h and between ~14:00 and 20:30 h, respectively. Ambient conditions during these periods were ~32 to 34 °C and may have contributed to the increases in these hydration variables. Further increases in PCV and TPP were not found at the remaining sample collection points during the night.

Plasma α -tocopherol concentrations were maintained during all three races. Similarly, horses competing in a 140 km endurance race had no changes in α -tocopherol concentrations during the race and during 16 h of recovery (Marlin et al., In press). Human plasma tocopherol concentrations increased during intense exercise (Pincemail et al., 1988), but exercise-induced changes in plasma volume were not accounted for. Sled dogs supplemented with vitamin E and vitamin C, or a placebo, had decreased plasma tocopherol and increased plasma ascorbate concentrations during 3 d of endurance exercise (Piercy et al., 2000). Humans running a half-marathon had unchanged plasma tocopherol, increased plasma ascorbate, and decreased GSH concentrations in samples taken immediately after running the race and 120 h post-race (Duthie et al., 1990). Human plasma ascorbate concentrations increased immediately after a 21 km race and decreased at 24 h post-race to 20 % below pre-exercise values for 48 h (Gleeson et al., 1987). Dissimilar responses in exercise-induced concentrations of plasma vitamin E and C, and GSH concentrations may reflect differences in species, exercise mode, intensity and duration, or

whether values were adjusted for exercise-induced changes in plasma volume.

In these studies (Experiment 5.1 and 5.2), the maintenance of circulating α -tocopherol may be explained by the concomitant mobilization of VIT E with fatty acids from adipose tissue stores (Rokitski et al., 1994a), especially as fat is a major energy source during endurance exercise. Plasma ascorbic acid concentrations decreased during both races, presumably through radical scavenging and through regeneration of α -tocopherol (Niki et al., 1982; 1995), therefore supporting circulatory concentrations of α -tocopherol. In contrast, endurance horses had no changes in plasma ascorbic acid concentrations during a 140 km race but significant decreases during 16 h of recovery (Marlin *et al.* In press). Similarly, erythrocyte GSH concentrations decreased during OD80 and OD160, most likely through the regeneration of VIT C by GSH (Meister, 1994; Winkler et al., 1994). Consequently, concurrent decreases in VIT C and GSH during prolonged strenuous endurance exercise may serve to sustain circulating α -tocopherol concentrations.

Furthermore, this ‘sparing effect’ of VIT E may have attenuated muscle CK leakage during the races, as reported in humans after intense endurance training (Rokitzki et al., 1994b; Itoh et al., 2000), since many horses in OD80 and OD160 had only minimal changes in circulating CK levels during the race. The high plasma CK activities observed in three horses in Experiment 5.2 (Figure 5.13) were not associated with clinically evident exertional rhabdomyolysis, according to the veterinarians’ records, but may reflect underlying susceptibility to this disorder.

Mean erythrocyte GPX activities at the start of NF80 in Experiment 5.1 were 4-fold greater than GPX activities during OD80 indicating that horses were experiencing oxidative stress prior to the race. This was possibly in response to the lower ambient temperatures because all horses were shivering when pre-race blood samples were collected at NF80. During NF, erythrocyte GPX activities remained unchanged, but were higher than erythrocyte GPX activities during OD80 and OD160. Another explanation could also be the difference in the time blood was collected (as described for the hydration status) so that immediately after pulling up the

erythrocyte activities were higher in horses during NF80 compared to 30 min later in horses during OD80 and OD160. The erythrocyte GPX activities after 60 min recovery at NF80 had decreased and were similar to values in the OD80 horses at 80 km. Higher activities of erythrocyte GPX reflect the high demand on the antioxidant system to scavenge free radicals and ROS. Similarly, GPX activities increased in horses during a 160 km endurance race in Poland (Frankiewicz-Jozko and Szarska, 2000). In OD80 and OD160, elevated erythrocyte GPX activities with increasing distance corresponded to concurrent depleting erythrocyte GSH concentrations, most likely because GPX requires a supply of GSH as an electron donor. Decreases in GPX activities at REC in all three races may reflect diminished supplies of the GSH substrate at REC, or decreased glutathione reductase activity, the enzyme required to reduce oxidized glutathione (not measured in these experiments).

Elevated plasma AST and CK activities during OD80 and OD160 demonstrated muscle cell damage and leakage of these enzymes into the circulatory system. Maremmana racehorses revealed no changes in plasma CK activities during short duration intense exercise (Chiaradia et al., 1998). Increased plasma CK activities were observed in Thoroughbred racehorses following a 1.2 km maximum velocity race (White et al., 2001), however, these exercise-induced increases in plasma CK activity remained within the normal range for horses at rest (150 to 400 IU/L). In this study (Experiment 5.2), elevated plasma AST and CK activities were indicative of muscle cell leakage in both OD80 and OD160. Horses that were conditioned for eight weeks had attenuated increases in CK and AST activities during a submaximal exercise test (Siciliano et al., 1995). The large variability in plasma CK activities between horses during OD160 suggests differences in fitness condition (Figure 5.12 and 5.13), although fitness was not measured in this study. Variability in plasma AST and CK activity was greater during OD160 compared to OD80 and reflects the extensive demands on these horses during this test of endurance.

Plasma AST and CK activities in horses competing in a 140 km endurance race (Marlin *et al.* In press) were higher than in the present study and may reflect the greater mean speed of these horses (16.5 ± 1.6 km/h), compared to horses in OD160 (8.2 km/h) and horses in OD80 (8.8 km/h). The relatively slower speed of horses in these two races most likely reflects the

mountainous terrain and high ambient temperatures present during these races, compared to the minimal changes in altitude and cool-warm ambient conditions present during the 140 km race reported by Marlin *et al.* (In press). Endurance horses completing a 160 km race in Poland revealed increases in serum CK activities (Frankiewicz-Jozko and Szarska 2000) that were similar to plasma CK activities observed in the horses during OD160 in this study. A positive correlation ($r = 0.47$, $P < 0.05$) between serum CK activity and the level of lipid peroxidation was observed in horses during the endurance race in Poland (Frankiewicz-Jozko and Szarska 2000). In the OD80 and OD160 races of Experiment 5.2, associations were found between increases in plasma AST and CK activities and changes in plasma and erythrocyte antioxidants (Table 5.5). These associations are consistent with increased muscle cell leakage and decreased antioxidant status and may in part reflect oxidative stress during endurance exercise. Alternatively, increased muscle cell leakage may result from cell necrosis, impaired enzyme clearance, increased enzyme synthesis, or from transient or permanent permeability changes in muscle cell membranes (Halliwell and Gutteridge, 1989).

In conclusion, decreased plasma VIT C and erythrocyte GSH concentrations with increasing distance represents antioxidant depletion, and may reflect radical scavenging and regeneration of vitamin C and E derivatives. These responses may serve to maintain circulating α -tocopherol concentrations in an attempt to sustain antioxidant status. Interestingly, five of the 10 horses that completed the OD160 received 20 g/d of ascorbic acid for 3 d prior to the start, but no differences were found between supplemented and non-supplemented groups in plasma ascorbate concentration, or in other plasma or erythrocyte antioxidant concentrations, or muscle cell enzyme activities, before or during the race.

Associations between increased muscle cell leakage and decreased antioxidant status emphasize the demands on the delicate oxidative-antioxidative balance and encourage the testing of antioxidant supplements, especially ascorbic acid supplementation during the race, to improve the performance and welfare of endurance horses.

VITAMIN E STATUS OF THOROUGHBRED HORSES AND THE ANTIOXIDANT STATUS OF ENDURANCE HORSES

GENERAL DISCUSSION

Concentrations of α -tocopherol in the milk of lactating mares appear more indicative of vitamin E status than serum α -tocopherol concentrations. Vitamin E supplementation of mares increased milk α -tocopherol concentrations and immunoglobulins G and M concentrations. It is not clear how increased vitamin E is associated with immunoglobulin concentration in the mammary gland. Enhanced mobilization of fatty acids to the mammary gland during the periparturient period for colostrum and milk production may concomitantly mobilize vitamin E from adipose stores. If stores of vitamin E are greater in vitamin E supplemented mares then vitamin E may be mobilized more readily. The transport of immunoglobulins to the milk occurs through exocytosis and this process may be inhibited by ROS. Therefore, vitamin E supplementation may facilitate the transport of immunoglobulins to the milk by protecting against ROS damage. Furthermore, since immunoglobulins are secreted into the mammary gland during the period leading up to parturition, it is likely that the presence of vitamin E would protect the immunoglobulins against damage by ROS.

Serum α -tocopherol concentrations were unchanged in mares supplemented daily with three times the recommended amount of vitamin E for pregnant mares. This suggests that the removal of serum α -tocopherol and uptake by the tissues was occurring at a similar rate to the serum uptake. Increased milk concentrations of α -tocopherol post partum in supplemented mares compared to non-supplemented mares demonstrates a response to vitamin E supplementation because more had to be absorbed, thus milk serves as a function test for vitamin E status of Thoroughbred mares.

Vitamin E is the most important antioxidant in lipid cell membranes and its radical scavenging capabilities are tested in conditions of stress such as parturition and during lactation, particularly in older mares. Increased free radical production during stress creates a greater

demand for vitamin E action and the supplementation of vitamin E in the horse and other species has been implicated in the stimulation of antibody synthesis. Perhaps it is the physiological changes associated with parturition and tissue recovery post-partum that trigger a surge of vitamin E mobilization from stores to all tissue cells and that supplemented mares, with possibly greater stores, elicit a better response than mares with adequate stores. Circulating concentrations of α -tocopherol were maintained during strenuous endurance exercise despite large decreases in other antioxidants and so it appears that other unknown factors must be influencing the system to maintain circulating concentrations in times of stress, and these factors may explain the lack of change in mares serum during the supplementation period prior to parturition.

Bioavailability of different forms of vitamin E may need to be tested with lower doses over longer periods and with adipose tissue biopsies to better understand the transport and metabolism of vitamin E in the horse. It also seems that circulating concentrations of α -tocopherol do fluctuate through the seasons and these changes do not necessarily correspond to pasture concentrations. However, it most likely takes a period of weeks or months of decreased intake to elicit a decrease in circulating concentrations.

Blood Se concentrations immediately post-partum and during lactation were similar to serum α -tocopherol concentrations. In other groups of horses, particularly the foals, yearlings and geldings, the Se status did not appear to be related to vitamin E status.

During endurance exercise the depletion of plasma vitamin C and erythrocyte GSH concentrations with increasing distance represented antioxidant depletion through the radical scavenging and regeneration of vitamin E and C derivatives. Circulating concentrations of α -tocopherol were maintained through these actions and possibly through fatty acid mobilization during the race so that antioxidant status could be sustained.

Evidence of associations between increased muscle cell leakage and decreased antioxidant status in endurance horses during a race do not prove causation but motivates a

search for causation. In this case it is tempting to propose that oxidative stress damages the muscle cell membranes of horses during endurance racing.

Although the measurement of α -tocopherol concentration in the blood serum is a convenient and minimally invasive tool, it may not be an indication of membrane bound α -tocopherol concentration, the site of action, but may simply reflect circulating lipoproteins, the transporters of α -tocopherol to the tissues. Consequently, when serum α -tocopherol concentrations are maintained during strenuous endurance exercise, the membrane bound concentrations could in fact be decreasing. Serum concentrations were only measured after 1 h of recovery and therefore a true resting circulatory status after a stressful event was not measured. In mares during lactation, the decreased serum concentrations may reflect a strain on the antioxidant system over a 6 mo period.

IMPLICATIONS

Future studies on Thoroughbred mares and foals are needed which include supplementation of mares during the lactation period and perhaps foals during weaning. Adipose tissue sampling would provide more information on the transport and metabolism of vitamin E and may be a better measure of vitamin E status in horses. Studies on endurance horses should test the supplementation of horses with vitamin C or glutathione during the race to determine if supplementation can sustain the plasma concentrations of these variables during racing and the effect these variables would have on circulatory vitamin E.

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TABLES

Table 1.1

Serum α -tocopherol concentrations ($\mu\text{g/ml}$) of mares during the 6 d of the dose frequency test. Mares 1 to 3 were dosed with 2010 IU of *dl*- α -tocopherol acetate on d1 and d5 and mares 4 to 6 received a placebo of molasses. Experiment 1.1

Serum α -tocopherol concentrations, $\mu\text{g/ml}$						
Horse	d 1	d 2	d 3	d 4	d 5	d 6
1	2.57	2.45	2.51	2.30	2.16	2.25
2	1.83	1.84	1.99	2.03	2.10	1.91
3	1.96	1.77	1.80	1.82	1.79	1.89
4	3.06	2.91	2.89	2.76	2.98	2.57
5	1.56	1.66	1.62	1.78	2.01	1.55
6	2.15	2.04	2.20	2.52	2.29	2.46
Mean \pm s.e.	2.19 \pm 0.22	2.11 \pm 0.20	2.17 \pm 0.19	2.20 \pm 0.16	2.22 \pm 0.17	2.11 \pm 0.16

Table 1.2

Serum α -tocopherol concentrations ($\mu\text{g/ml}$) of geldings during the dose response test. Horses 1 and 2 were dosed with 8000 IU, horses 3 and 4 were dosed with 16000 IU, horses 5 and 6 were dosed with 32000 IU and horses 7 and 8 were dosed with 48000 IU of *dl*- α -tocopheryl acetate immediately after baseline samples were collected. Experiment 1.2

Serum α -tocopherol concentrations, $\mu\text{g/ml}$					
Horse	Baseline	Dose (IU)	9 h	24 h	168 h
1	5.86	8000	6.57	6.11	7.02
2	2.52	8000	3.01	3.12	3.19
3	3.65	16000	3.72	3.72	3.76
4	3.19	16000	3.61	4.02	3.72
5	4.81	32000	5.08	5.47	4.70
6	3.46	32000	3.52	3.66	3.63
7	4.40	48000	4.12	4.56	4.27
8	3.93	48000	4.25	4.22	3.04
Mean \pm s.e.	3.98 \pm 0.36		4.23 \pm 0.39	4.36 \pm 0.35	4.16 \pm 0.44

Table 2.1

A 6 x 6 Latin Square design assignment of six treatments for six horses and six 1-wk periods. T1 is *d*- α -tocopherol (Intravenous), T2 is *d*- α -tocopherol (Oral), T3 is *d*- α -tocopherol polyethylene glycol 1000 succinate (Oral), T4 is *d*- α -tocopheryl acetate (Oral), T5 is *dl*- α -tocopherol (Oral) and T6 is *dl*- α -tocopheryl acetate (Oral).

HORSE	PERIOD					
	1	2	3	4	5	6
1	T1	T2	T3	T4	T5	T6
2	T6	T1	T2	T3	T4	T5
3	T2	T3	T4	T5	T6	T1
4	T5	T6	T1	T2	T3	T4
5	T3	T4	T5	T6	T1	T2
6	T4	T5	T6	T1	T2	T3

Table 2.2

Peak serum α -tocopherol concentration ($\mu\text{g/ml}$) at 9 h post vitamin E administration in the 6 x 6 Latin Square design assignment of treatments. Treatments correspond to Table 2.1 above, T1 is *d*- α -tocopherol (Intravenous), T2 is *d*- α -tocopherol (Oral), T3 is *d*- α -tocopherol polyethylene glycol 1000 succinate (Oral), T4 is *d*- α -tocopheryl acetate (Oral), T5 is *dl*- α -tocopherol (Oral) and T6 is *dl*- α -tocopheryl acetate (Oral).

HORSE	PERIOD (wk)						Total
	1	2	3	4	5	6	
1	15.9	3.7	2.9	2.4	4.6	4.6	34.1
2	9.3	10.9	4.1	2.8	3.7	4.6	35.4
3	7.8	6.8	3.2	3	3.5	14	38.3
4	7.4	4.3	10.3	2.8	4.9	9.7	39.4
5	7.4	3.6	4.7	2.8	11.8	7.7	38.0
6	6.9	3.9	3.7	10.5	6.7	5.5	37.2
Total	54.7	33.2	28.9	24.3	35.2	46.1	222.4

Table 2.3

Analyses of Variance table for a treatment x treatment Latin Square Design

SOURCE	df	SS	MS	F
TREATMENTS	$t - 1$	SST	MST = SST/df	MST/MSE
ROWS	$t - 1$	SSR	MSR = SSR/df	MSR/MSE
COLUMNS	$t - 1$	SSC	MSC = SSC/df	MSC/MSE
ERROR	$t^2 - 3t + 2$	SSE	MSE = SSE/df	
TOTALS	$t^2 - 1$	TSS		

Table 2.4

Analyses of Variance table for six treatments of vitamin E administered to six horses (one horse per treatment) over a 6-wk period.

SOURCE	df	SS	MS	F
TREATMENTS	5	266.5	53.3	29.6
HORSES	5	106.8	21.4	11.8
PERIODS	5	3.3	0.7	0.4
ERROR	20	35.2	1.8	
TOTALS	35	411.8		

Table 2.5

Analyses of Variance table for six treatments of vitamin E administered to six horses (one horse per treatment) over a 6-wk period with period since intravenous (IV) treatment added to the model.

SOURCE	df	SS	MS	F
TREATMENTS	5	266.5	53.3	8.56
HORSES	5	106.8	21.4	3.44
PERIODS	5	3.3	0.7	0.11
PERIOD SINCE IV	6	53	8.8	1.42
ERROR	14	87.2	6.2	
TOTALS	35	516.8		

Table 2.6

Area under the time concentration curve over 168 h (AUC_{168}) of serum α -tocopherol ($\mu\text{g/ml}$) and the efficiency of absorption of five vitamin E treatments administered to three horses prior to the intravenous treatment. Efficiency of absorption was calculated as the AUC of the oral treatments, calculated by trapezoidal approximations, divided by the AUC of the intravenous treatment, calculated by fitting an exponential.

Treatment	AUC_{168} $\text{h}\cdot\mu\text{g/ml}^*$	Efficiency of Absorption %*
<i>d</i> -a-tocopherol	8.72 \pm 0.25	0.33 \pm 0.01
<i>d</i> -a-TPGS	6.40 \pm 3.24	0.23 \pm 0.18
<i>d</i> -a-tocopheryl acetate	1.12 \pm 1.06	0.06 \pm 0.06
<i>dl</i> -a-tocopherol	3.59 \pm 1.32	0.19 \pm 0.07
<i>dl</i> -a-tocopheryl acetate	5.39 \pm 3.64	0.29 \pm 0.19

* Mean \pm s.e. (n = 3)

Table 2.7

Area under the time concentration curve over 168 h (AUC_{168}) of serum α -tocopherol ($\mu\text{g/ml}$) and the efficiency of absorption of five vitamin E treatments administered to three horses prior to the intravenous treatment. Efficiency of absorption was calculated as the AUC of the oral treatments divided by the AUC of the intravenous treatment. Both AUC were calculated by trapezoidal approximations.

Treatment	AUC_{168} h·$\mu\text{g/ml}^*$	Efficiency of Absorption %*
d-a-tocopherol	8.72 ± 0.25	1.07 ± 0.03
d-a-TPGS	6.40 ± 3.24	0.79 ± 0.40
d-a-tocopheryl acetate	1.12 ± 1.06	0.14 ± 0.13
dl-a-tocopherol	3.59 ± 1.32	0.44 ± 0.33
dl-a-tocopheryl acetate	5.39 ± 3.64	0.66 ± 0.45

* Mean \pm s.e. (n = 3)

Table 2.8

Area under the time concentration curve over 24 h (AUC_{24}) of serum α -tocopherol ($\mu\text{g/ml}$) following five oral vitamin E treatments administered to three horses prior to the intravenous treatment.

Treatment	AUC_{24}, h·$\mu\text{g/ml}^*$
d-a-tocopherol	21.60 ± 0.09
d-a-TPGS	9.47 ± 4.81
d-a-tocopheryl acetate	0.76 ± 0.13
dl-a-tocopherol	8.22 ± 3.01
dl-a-tocopheryl acetate	8.49 ± 8.09

* Mean \pm s.e. (n = 3)

Table 2.9

Difference in serum α -tocopherol concentrations ($\mu\text{g/ml}$) from baseline to 9 h and 12 h post dosing of vitamin E in three horses prior to intravenous treatment.

Treatment	Difference from baseline at 9 h post dosing *	Difference from baseline at 12 h post dosing *
d-a-tocopherol	0.73 ± 0.51	0.85 ± 0.52
d-a-TPGS	0.70 ± 0.12	0.50 ± 0.25
d-a-tocopheryl acetate	0.09 ± 0.10	-0.03 ± 0.25
dl-a-tocopherol	0.65 ± 0.21	0.49 ± 0.24
dl-a-tocopheryl acetate	0.32 ± 0.43	0.25 ± 0.36

* Mean \pm s.e (n = 3)

Table 3.1 Nutrient composition of pastures where mares and foals grazed in 1999 and weanlings grazed in 2000.

DM Basis ^b	Nutrient Composition of Pastures in 1999 and 2000 ^a											
	1999											2000
	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	
Crude Protein %	21 ± 0.9	15 ± 0.5	11 ± 0.2	14 ± 0.6	15 ± 0.4	23 ± 1.2	22 ± 1.1	17 ± 2.2	13 ± 0.5	16 ± 0.9	17 ± 1.4	
Crude Fat %	4 ± 0.2	3 ± 0.1	3 ± 0.1	3 ± 0.2	4 ± 0.2	4 ± 0.4	3 ± 0.2	3 ± 0.3	2 ± 0.1	2 ± 0.1	4 ± 0.2	
Acid Detergent Fiber %	28 ± 2	33 ± 0.5	37 ± 0.7	37 ± 0.5	35 ± 0.5	25 ± 0.7	25 ± 1.5	31 ± 2	38 ± 0.7	36 ± 0.8	34 ± 2	
Neutral Detergent Fiber %	52 ± 2	66 ± 0.6	66 ± 0.5	67 ± 1	63 ± 2	51 ± 1.4	50 ± 2	54 ± 2	65 ± 1	52 ± 2	52 ± 0.9	
Calcium %	0.7 ± 0.04	0.6 ± 0.05	0.5 ± 0.02	0.7 ± 0.04	0.7 ± 0.03	0.5 ± 0.04	0.5 ± 0.01	0.6 ± 0.05	0.6 ± 0.02	0.7 ± 0.02	0.5 ± 0.03	
Phosphorus %	0.4 ± 0.02	0.3 ± 0.02	0.3 ± 0.01	0.3 ± 0.04	0.3 ± 0.02	0.4 ± 0.03	0.4 ± 0.01	0.3 ± 0.03	0.2 ± 0.01	0.3 ± 0.01	0.3 ± 0.03	

^aMean ± se. (n = 4); ^bAnalysis performed by Dairy One, Ithaca, NY.

Table 3.2

Nutrient composition (DM basis) of round bale orchardgrass-alfalfa hays fed to mares and square bale alfalfa-timothy hays fed to mares and foals in 1999.

Nutrient Composition (DM basis) of Hays 1999^a		
	Orchardgrass-alfalfa	Alfalfa-timothy
Crude Protein %	14 ± 0.1	14 ± 1.8
Crude Fat %	2 ± 0.1	1.7 ± 0.1
Acid Detergent Fiber %	46 ± 1	41 ± 4
Neutral Detergent Fiber %	55 ± 2	56 ± 7
Calcium %	1.1 ± 0.02	1.1 ± 0.03
Phosphorus %	0.2 ± 0.02	0.2 ± 0.01
Vitamin E, IU/kg ^b	15.4*	22 ± 5**

Mean ± se. All nutrients except vitamin E (n = 4); *(n = 1); **(n = 2)

^aAnalysis performed by Dairy One, Ithaca, NY.

^bAnalysis performed by Woodson Tenent Laboratories, Memphis, TN.

Table 3.3

Nutrient composition (DM basis) of the sugar and starch (SS) and the fat and fiber (FF) feeds fed to mares and foals in 1999 and the fat and fiber feed (FF2) fed to mares in 2000.

Nutrient Composition of Feed Supplements, 1999^a			
	SS	FF (1999)	FF2 (2000)
Crude Protein, %	16 ± 1.6	15 ± 0.2	14 ± 0.1
Crude Fat, %	3 ± 0.2	13 ± 0.7	9 ± 0.3
Acid Detergent Fiber, %	8 ± 1.5	19 ± 0.5	24 ± 0.5
Neutral Detergent Fiber, %	15 ± 2.7	37 ± 1.3	31 ± 0.4
Calcium, %	1 ± 0.2	3 ± 0.03	2 ± 0.03
Phosphorus, %	1 ± 0.1	1 ± 0.05	1 ± 0.1
Zinc, ppm	132 ± 32	188 ± 10	151 ± 24
Copper, ppm	28 ± 7	35 ± 4	26 ± 3
Vitamin E, IU/kg ^b	35 ± 5*	63 ± 6*	63**

Mean ± se. All nutrients except vitamin E (n = 4); * (n = 4); **(n = 1)

^aAnalysis performed by Dairy One, Ithaca, NY.

^bAnalysis performed by Woodson Tenent Laboratories, Memphis, TN.

Table 4.1

Serum α -tocopherol concentrations ($\mu\text{g/ml}$) of post-partum mares that received either a placebo or an oral dose of *dl*- α -tocopheryl acetate every 3.5 d during the last trimester (3 mo) in 1999. Experiment 4.1.

Serum α-Tocopherol of Mares at Parturition in 1999		
Sample^b	Vitamin E Supplemented^a	Non-Vitamin E Supplemented^a
0 h	5.23 \pm 0.39	5.18 \pm 0.54
6 h	4.58 \pm 0.35	4.94 \pm 0.32
12 h	4.59 \pm 0.34	4.68 \pm 0.34
24 h	4.62 \pm 0.42	4.64 \pm 0.29
48 h	4.32 \pm 0.43	4.55 \pm 0.41
2 wk	3.31 \pm 0.18	3.52 \pm 0.36
1 mo	3.29 \pm 0.26	3.34 \pm 0.17

^aMean \pm se (n = 12 in each group); ^bSamples collected post-partum.

Table 4.2

Serum α -tocopherol concentrations ($\mu\text{g/ml}$) of post-partum mares that received either a placebo or a daily oral dose of *d*- α -tocopherol during the last trimester (3 mo) in 2000. Experiment 4.2.

Serum α-Tocopherol of Mares at Parturition in 2000		
Sample^b	Vitamin E Supplemented^a	Non-Vitamin E Supplemented^a
0 h	3.15 \pm 0.32	3.27 \pm 0.36
24 h	3.79 \pm 0.40	3.30 \pm 0.46
2 wk	1.97 \pm 0.20	1.61 \pm 0.26
1 mo	3.32 \pm 0.25	3.55 \pm 0.23

^aMean \pm se (n = 12); ^bSamples collected post-partum.

Table 4.3

Mare serum α -tocopherol concentrations ($\mu\text{g/ml}$), milk α -tocopherol concentrations ($\mu\text{g/ml}$), milk immunoglobulin G and M (IgG and IgM) concentrations (mg/dl), foal serum α -tocopherol concentrations ($\mu\text{g/ml}$) and foal serum IgG and IgM concentrations (mg/dl) when fed the sugar-and-starch (SS) supplement, the fat-and-fiber (FF) supplement in 1999. Experiment 4.1

1999						
Horse Group	Variable	n	Sample	SS Diet^a	FF Diet^a	P-value
Mares	Serum α -tocopherol	19	0 h	5.2 \pm 0.5	5.8 \pm 0.6	0.46
	Serum α -tocopherol	23	6 h	4.5 \pm 0.4	5.1 \pm 0.4	0.58
	Serum α -tocopherol	22	12 h	4.7 \pm 0.4	4.9 \pm 0.4	0.72
	Serum α -tocopherol	23	24 h	4.6 \pm 0.4	5.1 \pm 0.4	0.41
	Serum α -tocopherol	22	48 h	4.3 \pm 0.5	4.9 \pm 0.5	0.36
	Serum α -tocopherol	22	2 wk	3.6 \pm 0.3	3.3 \pm 0.3	0.38
	Serum α -tocopherol	21	1 mo	3.6 \pm 0.3	3.3 \pm 0.3	0.48
Mares	Milk α -tocopherol	19	0 h	1.9 \pm 0.3	2.1 \pm 0.4	0.86
	Milk α -tocopherol	19	6 h	3.8 \pm 0.5	3.5 \pm 0.5	0.72
	Milk α -tocopherol	20	12 h	4.3 \pm 0.6	3.2 \pm 0.5	0.18
	Milk α -tocopherol	18	24 h	4.4 \pm 0.5	3.4 \pm 0.5	0.18
	Milk α -tocopherol	21	48 h	1.9 \pm 0.2	1.8 \pm 0.6	0.59
	Milk α -tocopherol	23	2 wk	1.1 \pm 0.1	0.9 \pm 0.1	0.61
	Milk α -tocopherol	22	1 mo	0.8 \pm 0.1	0.8 \pm 0.1	0.96
Mares	Milk IgG	18	0 h	7351 \pm 568	7623 \pm 568	0.74
	Milk IgG	21	6 h	6297 \pm 753	4621 \pm 721	0.13
	Milk IgG	14	12 h	708 \pm 949	1784 \pm 800	0.41
	Milk IgG	19	24 h	190 \pm 60	231 \pm 51	0.62
Mares	Milk IgM	17	0 h	268 \pm 38	220 \pm 40	0.40
	Milk IgM	21	6 h	167 \pm 55	157 \pm 53	0.69
	Milk IgM	11	12 h	20 \pm 46	63 \pm 30	0.46
	Milk IgM	13	24 h	18 \pm 21	30 \pm 14	0.65
Foal	Serum α -tocopherol	17	0 h	3.9 \pm 0.4	3.9 \pm 0.5	0.94
	Serum α -tocopherol	22	6 h	4.3 \pm 0.4	4.3 \pm 0.4	0.98
	Serum α -tocopherol	21	12 h	4.4 \pm 0.5	4.5 \pm 0.5	0.77
	Serum α -tocopherol	22	24 h	5.7 \pm 0.5	6.1 \pm 0.5	0.60
	Serum α -tocopherol	19	48 h	6.7 \pm 0.8	7.7 \pm 0.7	0.35
	Serum α -tocopherol	22	2 wk	4.5 \pm 0.5	4.4 \pm 0.5	0.79
	Serum α -tocopherol	19	1 mo	2.8 \pm 0.3	3.2 \pm 0.3	0.45
Foal	Serum IgG	16	24 h	1399 \pm 94	1422 \pm 101	0.87
Foal	Serum IgM	17	24 h	81 \pm 7	84 \pm 8	0.72

^aMean \pm se

Table 4.4

Mare serum α -tocopherol concentrations ($\mu\text{g/ml}$), milk α -tocopherol concentrations ($\mu\text{g/ml}$), milk immunoglobulin G and M (IgG and IgM) concentrations (mg/dl), foal serum α -tocopherol concentrations ($\mu\text{g/ml}$) and foal serum IgG and IgM concentrations (mg/dl) when fed the sugar-and-starch (SS) supplement, the fat-and-fiber (FF2) supplement in 2000. Experiment 4.2

2000						
Horse Group	Variable	n	Sample	SS Diet^a	FF2 Diet^a	P-value
Mares	Serum α -tocopherol	21	Feb	2.2 \pm 0.2	1.9 \pm 0.2	0.44
	Serum α -tocopherol	21	April	3.4 \pm 0.3	3.3 \pm 0.3	0.83
	Serum α -tocopherol	18	0 h	2.6 \pm 0.3	3.2 \pm 0.2	0.12
	Serum α -tocopherol	20	24 h	3.1 \pm 0.4	3.9 \pm 0.4	0.19
	Serum α -tocopherol	20	2 wk	1.5 \pm 0.2	2.1 \pm 0.2	0.07
Mares	Milk α -tocopherol	15	0 h	2.5 \pm 0.6	2.4 \pm 0.6	0.89
	Milk α -tocopherol	18	12 h	4.8 \pm 0.9	4.8 \pm 0.9	0.96
	Milk α -tocopherol	19	24 h	3.0 \pm 3.2	3.2 \pm 0.3	0.66
	Milk α -tocopherol	21	2 wk	1.0 \pm 0.1	1.0 \pm 0.1	0.89
	Milk α -tocopherol	21	1 mo	0.9 \pm 0.1	0.9 \pm 0.1	0.75
Mares	Milk IgG	16	0 h	8546 \pm 399	8676 \pm 350	0.81
	Milk IgG	18	12 h	910 \pm 288	1073 \pm 250	0.67
	Milk IgG	19	24 h	207 \pm 165	307 \pm 141	0.65
Mares	Milk IgM	16	0 h	204 \pm 26	256 \pm 20	0.15
	Milk IgM	16	12 h	25 \pm 18	41 \pm 14	0.50
	Milk IgM	11	24 h	7 \pm 12	22 \pm 7	0.32
	Milk IgM	16	2 wk	11 \pm 4	12 \pm 4	0.84
	Milk IgM	9	1 mo	21 \pm 15	18 \pm 16	0.88
Foal	Serum α -tocopherol	19	0 h	2.2 \pm 0.5	2.5 \pm 0.5	0.60
	Serum α -tocopherol	19	24 h	3.1 \pm 0.5	3.6 \pm 0.5	0.49
	Serum α -tocopherol	18	2 wk	1.8 \pm 0.2	2.1 \pm 0.2	0.35
Foal	Serum IgG	22	24 h	1524 \pm 127	1557 \pm 119	0.85
Foal	Serum IgM	12	0 h	11 \pm 5	12 \pm 4	0.79
	Serum IgM	19	24 h	89 \pm 9	75 \pm 10	0.32

^aMean \pm se

Table 5.1 Packed cell volume (PCV) at No Frills 80 km (NF80) and Old Dominion 80 km (OD80) endurance races at 0, 80 km and after 60 min recovery (REC) at NF80 and at 0, 40, 80 km and REC at OD80. Experiment 5.1

	Packed Cell Volume (%)			
	Distance (km)			
	0	40	80	REC
NF80*	39 ± 1.7	-	52 ^a ± 2.0	42 ± 1.9
OD80*	40 ± 1.0	43 ± 0.8	42 ± 0.8	41 ± 0.8

*Mean ± standard error. (NF80, n = 10) (OD80, n = 11).

^aMean PCV are different ($P < 0.05$) from values within the same row.

Table 5.2 Total plasma protein (TPP) concentration at No Frills 80 km (NF80) and Old Dominion 80 km (OD80) endurance races at 0, 80 km and after 60 min recovery (REC) at NF80 and at 0, 40, 80 km and REC at OD80. Experiment 5.1

	Total Plasma Protein (g/dl)			
	Distance (km)			
	0	40	80	REC
NF80*	5.8 ± 0.2	-	6.4 ^a ± 0.2	6.2 ± 0.2
OD80*	5.7 ± 0.1	5.7 ± 0.2	6.1 ± 0.2	6.1 ± 0.2

*Mean ± standard error. (NF80, n = 10) (OD80, n = 11).

^aMean TPP concentrations tend to be different ($P = 0.06$) from values within the same row.

TABLE 5.3 Packed cell volume (PCV) and total plasma protein (TPP) concentration at 0, 40, 80 km and after 60 min recovery (REC) during the 80 km endurance race in Experiment 5.2

	Distance (km)			
	0	40	80	REC
PCV (%)*	41 ± 1.1	41 ± 0.7	42 ± 0.8	41 ± 0.8
TPP (g/dl)*	5.7 ± 0.1	5.7 ± 0.2	6.1 ± 0.2	6.1 ± 0.2

*Mean ± standard error (n = 12)

TABLE 5.4 Packed cell volume (PCV) and total plasma protein (TPP) concentration at 0, 64, 106, 142, 160 km and after 60 min recovery (REC) during the 160 km endurance race in Experiment 5.2

	Distance (km)					
	0	64	106	142	160	REC
PCV (%)*	38 ± 1.4	43 ^a ± 1.4	44 ^a ± 2.5	39 ± 1.5	38 ± 1.3	39 ± 1.6
TPP (g/dl)*	6.0 ± 0.1	6.3 ^a ± 0.1	6.3 ^a ± 0.1	5.8 ± 0.1	5.9 ± 0.1	5.9 ± 0.1

*Mean ± standard error. (n = 10) ^aMean PCV ($P = 0.04$) and TPP ($P < 0.0001$) concentrations are different from values within same row.

TABLE 5.5 Regressions ($y = a + bx$) of indices of muscle leakage (y) on indices of antioxidant status (x) during OD80 and OD160 km endurance races in Experiment 5.2

Race (km)	Muscle (y)	Antioxidant (x)	n	a	b	r	P
OD160	AST	VIT C	28	518.4	-42.89	-0.44	0.0194
	AST	GSH	38	390.8	-0.48	-0.44	0.0056
	AST	GPX	32	310.9	+4.18	0.48	0.0055
	CK*	GSH	42	6.4	-0.005	-0.55	0.0009
OD80	AST	GSH	35	324.4	-0.27	-0.46	0.0060
	AST	GPX	28	271.0	+1.60	0.42	0.0276
	CK*	VIT C	38	7.4	-0.37	-0.40	0.0140
	CK*	GSH	38	6.1	-0.002	-0.36	0.0268
	CK*	GPX	30	5.4	+0.03	0.67	<0.0001

*Data for CK are logarithmically transformed

FIGURES

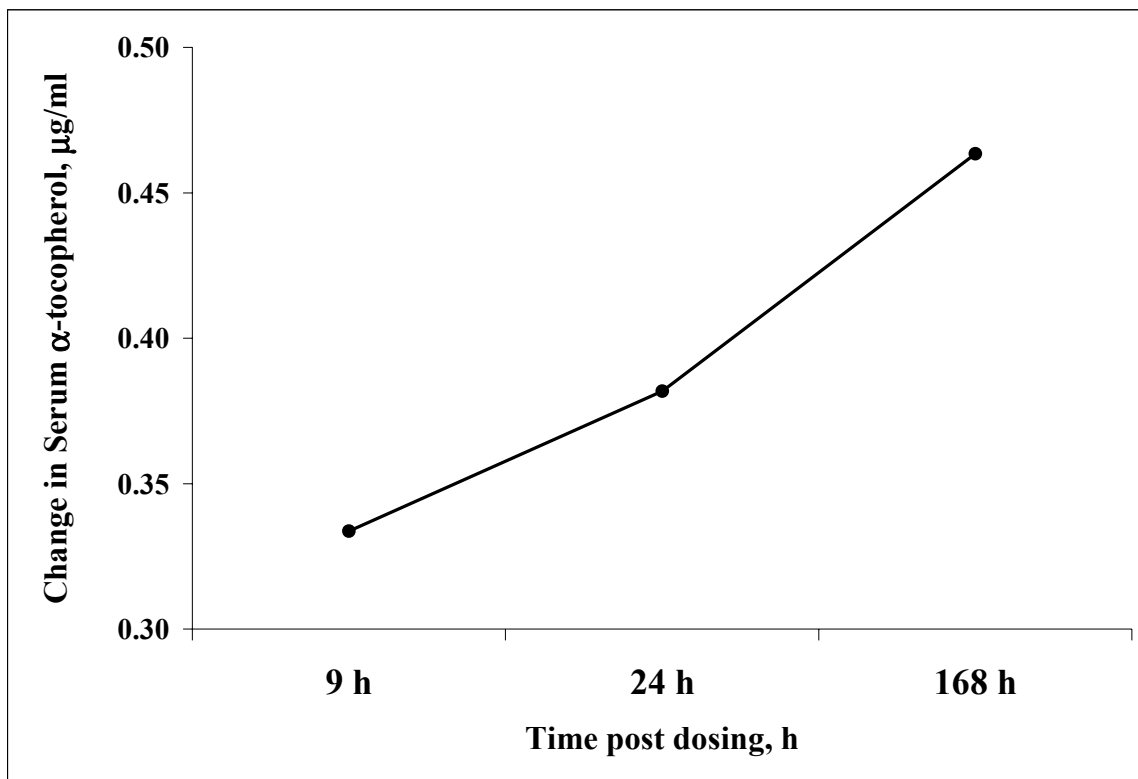


Figure 1.1

Mean change in serum α -tocopherol concentrations ($\mu\text{g/ml}$) of 8 horses after receiving a dose of *dl*- α -tocopheryl acetate. Experiment 1.2

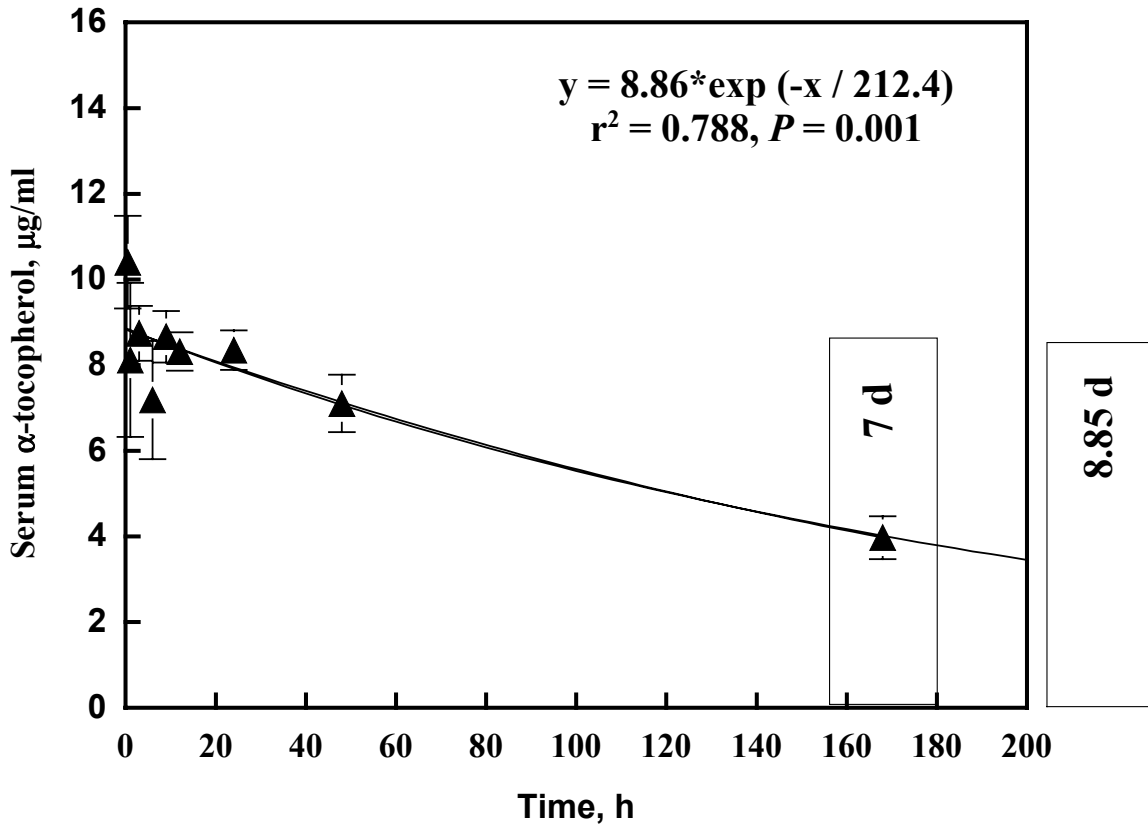


Figure 2.1

Increases (mean \pm s.e.) from baseline in serum α -tocopherol concentrations ($\mu\text{g/ml}$) following intravenous administration of d- α -tocopherol in 6 horses. Bar indicating 7 d represents the time when the following oral treatment oral vitamin E tolerance test was conducted. Bar indicating 8.85 d represents the predicted turnover time when intravenously administered α -tocopherol would reach baseline values.

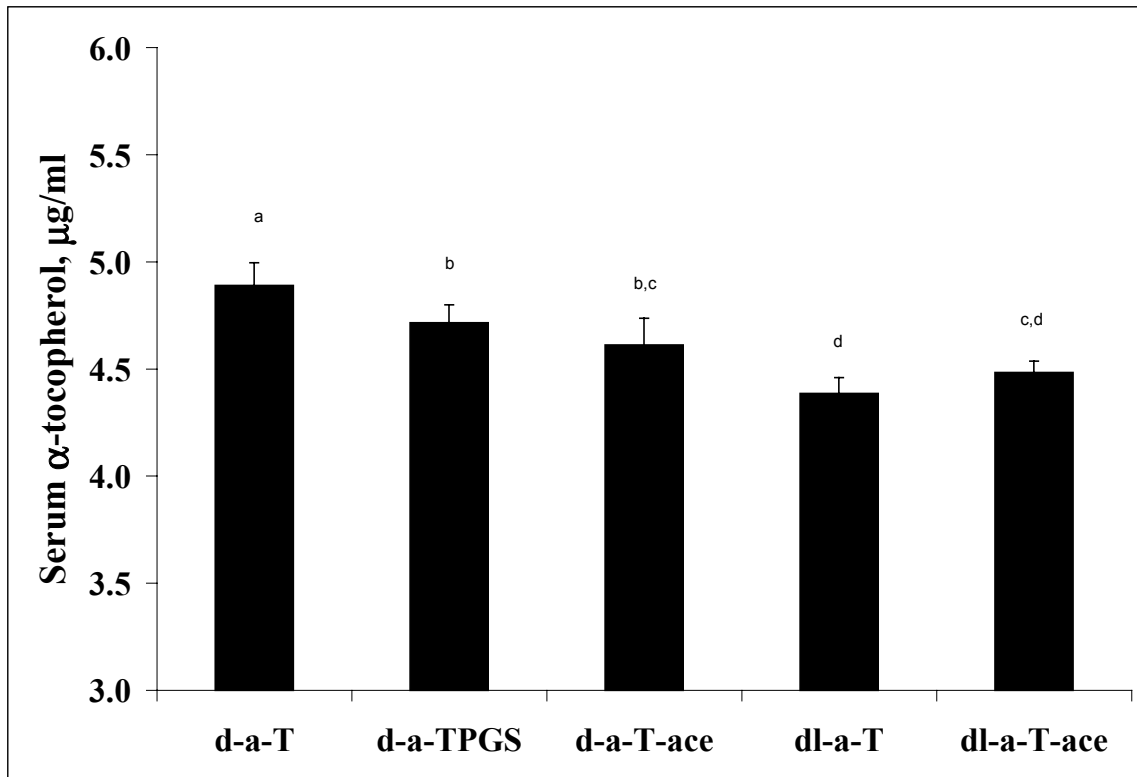


Figure 2.2

Serum α -tocopherol concentrations ($\mu\text{g/ml}$) of six horses over 7 d following five oral treatments of vitamin E. The five treatments are d- α -tocopherol (d- α -T), d- α -tocopherol polyethylene glycol 1000 succinate (d- α -TPGS), d- α -tocopheryl acetate (d-a-T-ace), dl- α -tocopherol (dl-a-T), and dl- α -tocopheryl acetate (dl-a-T-ace). Bars are means, flags are standard errors of the mean. Means with unlike letters are significantly different ($P < 0.05$). Experiment 2.

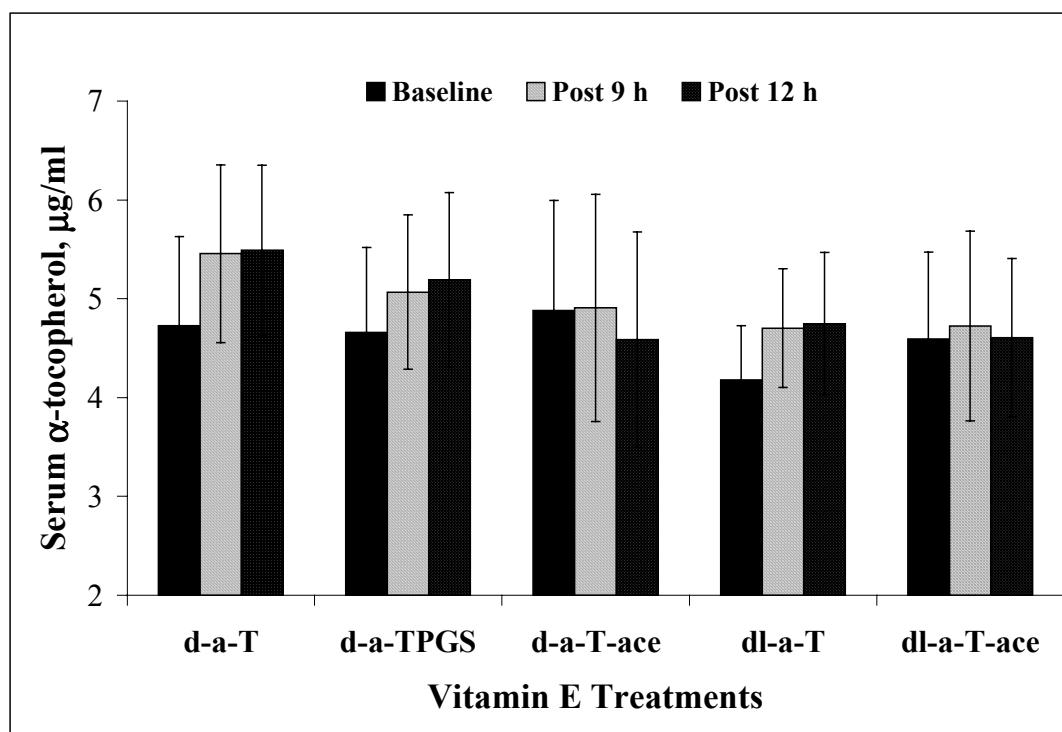


Figure 2.3

Serum α -tocopherol concentrations ($\mu\text{g/ml}$), in three horses prior to intravenous treatment, from baseline (black bars) to 9 h (hatched bars) and 12 h (spotted bars) post dosing with 5 oral treatments of vitamin E. The five treatments are d- α -tocopherol (d- α -T), d- α -tocopherol polyethylene glycol 1000 succinate (d- α -TPGS), d- α -tocopheryl acetate (d-a-T-ace), dl- α -tocopherol (dl-a-T), and dl- α -tocopheryl acetate (dl-a-T-ace). Bars are means, flags are standard errors of the mean. Experiment 2.

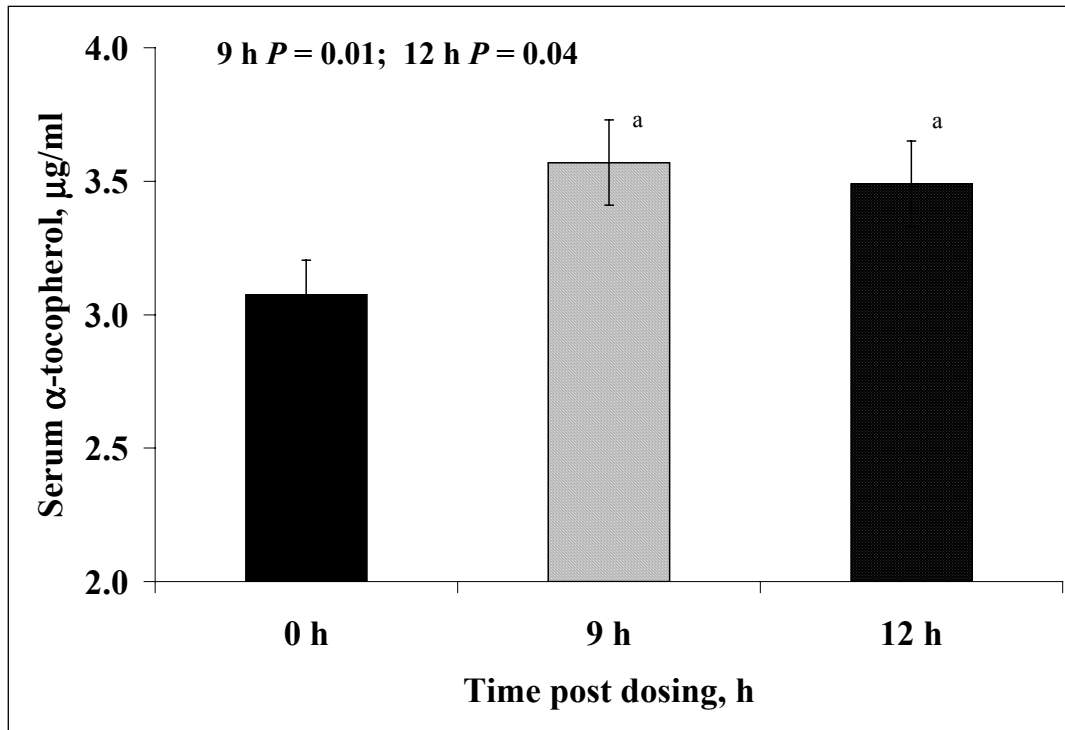


Figure 2.4

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) for three horses prior to intravenous treatment of five oral treatments (grouped) of vitamin E from baseline (black bar) to 9 hr (hatched bar) and 12 h (spotted bar) post dosing. The five treatments are d- α -tocopherol (d- α -T), d- α -tocopherol polyethylene glycol 1000 succinate (d- α -TPGS), d- α -tocopheryl acetate (d-a-T-ace), dl- α -tocopherol (dl-a-T), and dl- α -tocopheryl acetate (dl-a-T-ace). Bars are means, flags are standard errors of the mean. Means with unlike letters are significantly different ($P < 0.05$). Experiment 2.

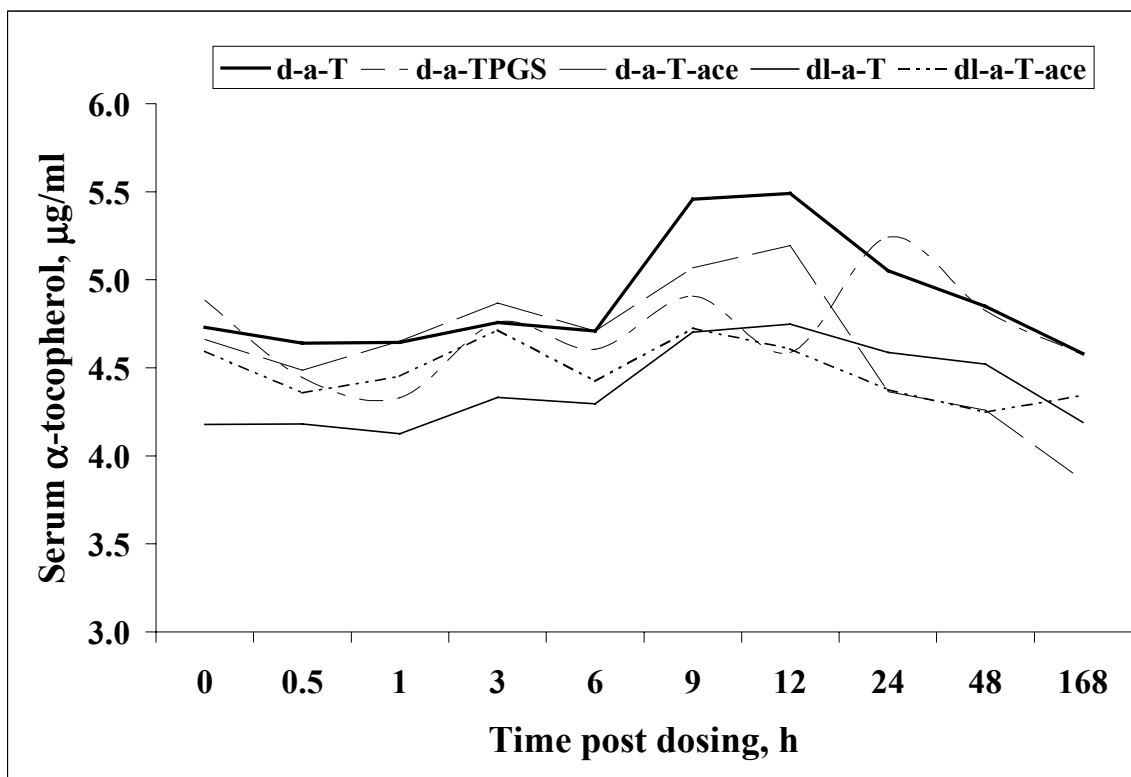


Figure 2.5

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of six horses over 168 h following 5 oral vitamin E treatments (one horse per treatment per wk) over a 6-wk period. Five treatments were d- α -tocopherol (d-a-T), d- α -tocopherol polyethylene glycol 1000 succinate (d-a-TPGS), d- α -tocopheryl acetate (d-a-T-ace), dl- α -tocopherol (dl-a-T), and dl- α -tocopheryl acetate (dl-a-T-ace). Experiment 2

Papas, 1999b

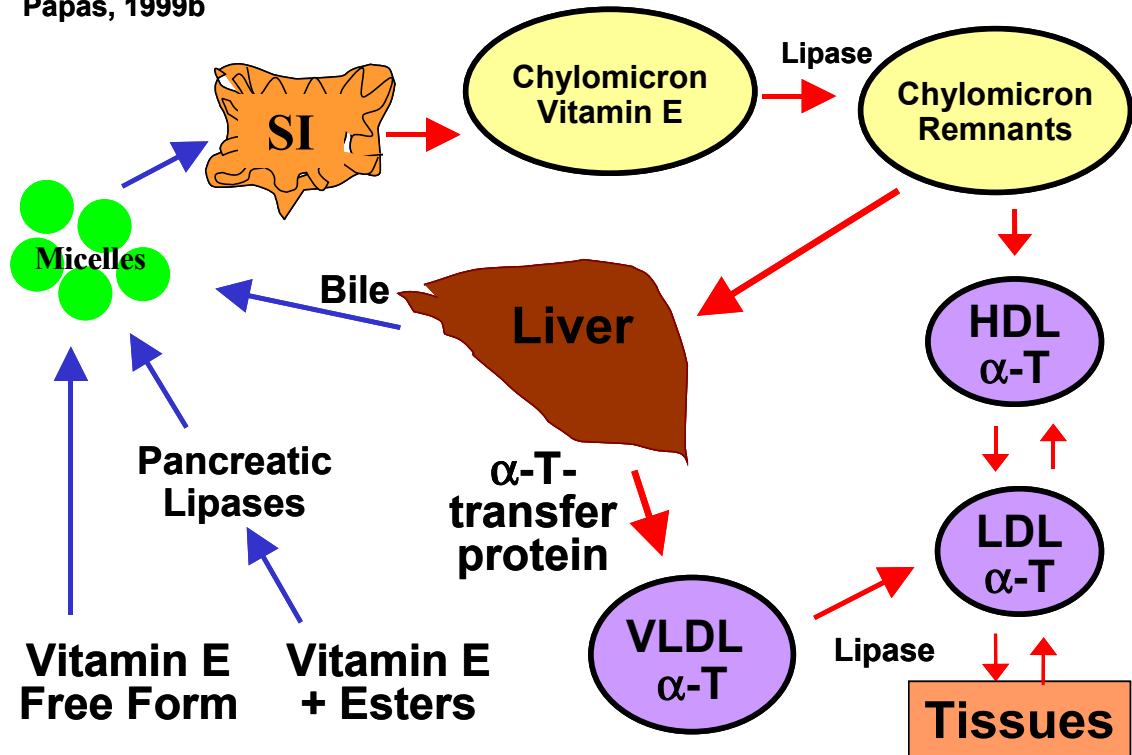


Figure 2.6 Absorption and transport of vitamin E.

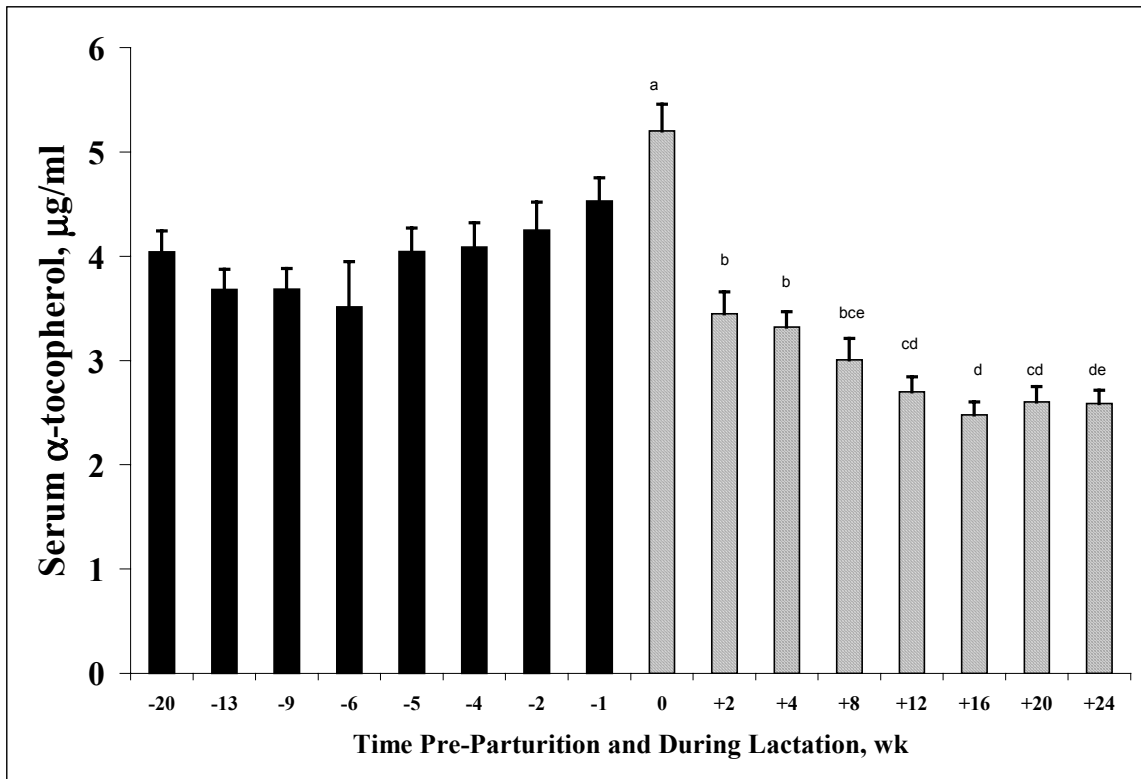


Figure 3.1

Serum α -tocopherol concentrations ($\mu\text{g/ml}$) of mares during the last 20 wk of gestation (black bars) and during 24 wk of lactation (hatched bars). Parturition was at 0 wk. Bars are means and flags are standard errors of the mean. Means with unlike letters are different ($P < 0.0001$) from other means in similar shaded bars. Experiment 3.1

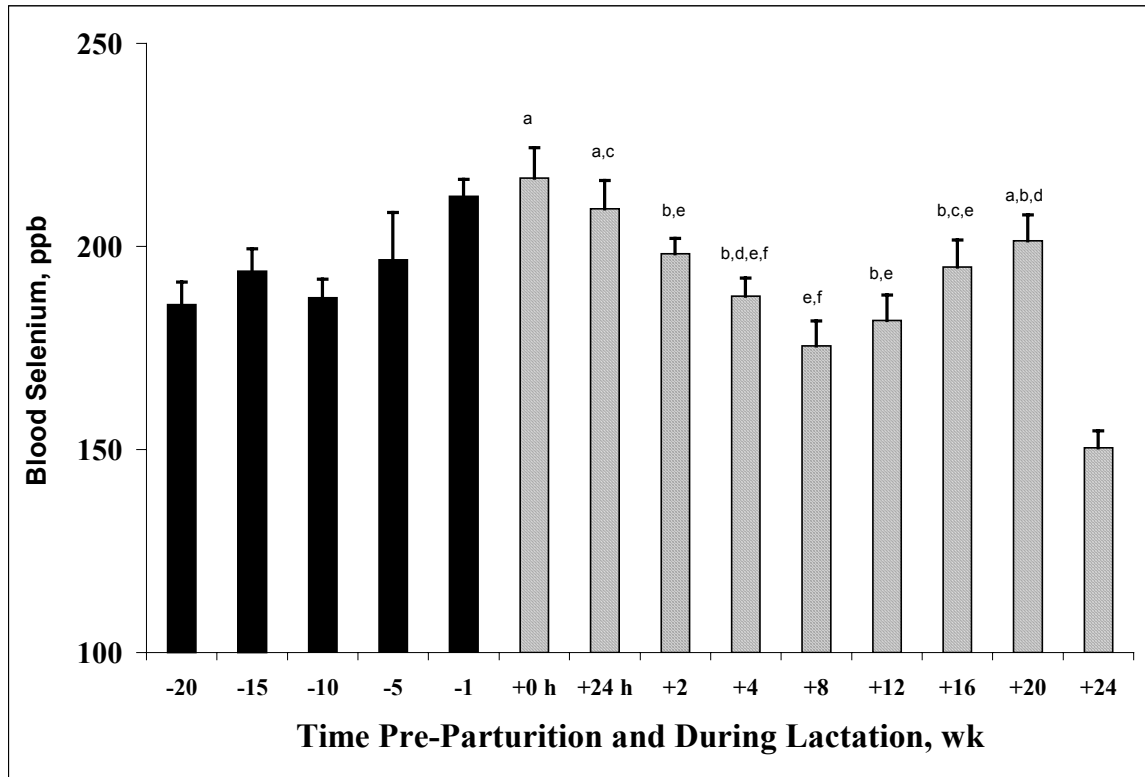


Figure 3.2

Whole blood Se concentrations (ppb) in mares during the last 20 wk of gestation (black bars) and during 24 wk of lactation (hatched bars). Bars are means and flags are standard errors of the mean. Means with unlike letters are different ($P < 0.0001$) from means in similar shaded bars. Experiment 3.1

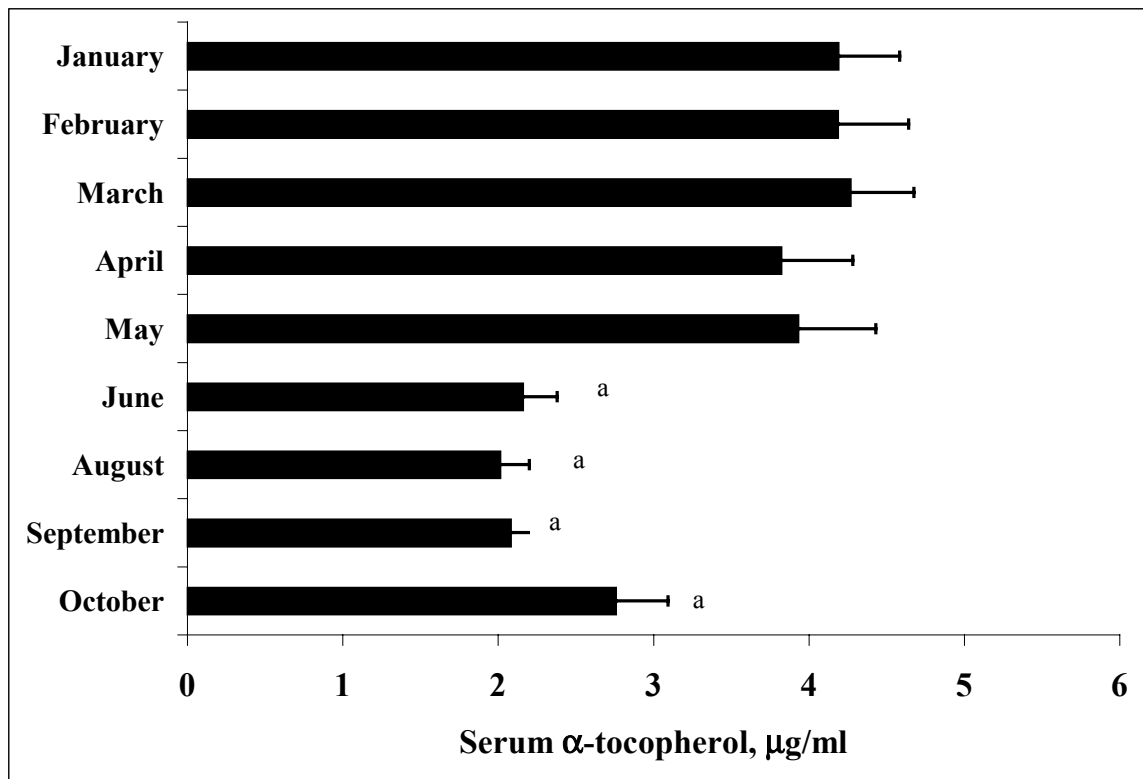


Figure 3.3

Serum α -tocopherol concentrations ($\mu\text{g/ml}$) in geldings from January to October in 1999. Data for July, November and December were unavailable. Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different ($P < 0.05$). Experiment 3.1

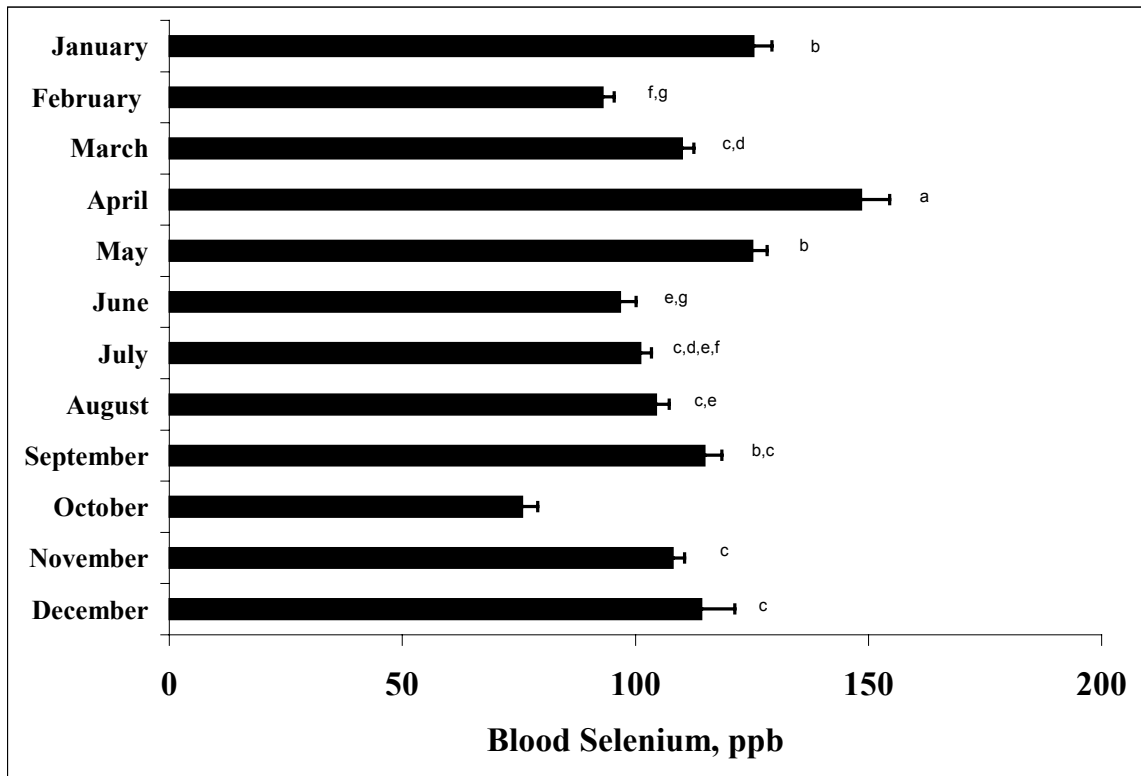


Figure 3.4

Whole blood Se concentrations (ppb) of geldings from January to December, 1999. Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different ($P < 0.05$). Experiment 3.1

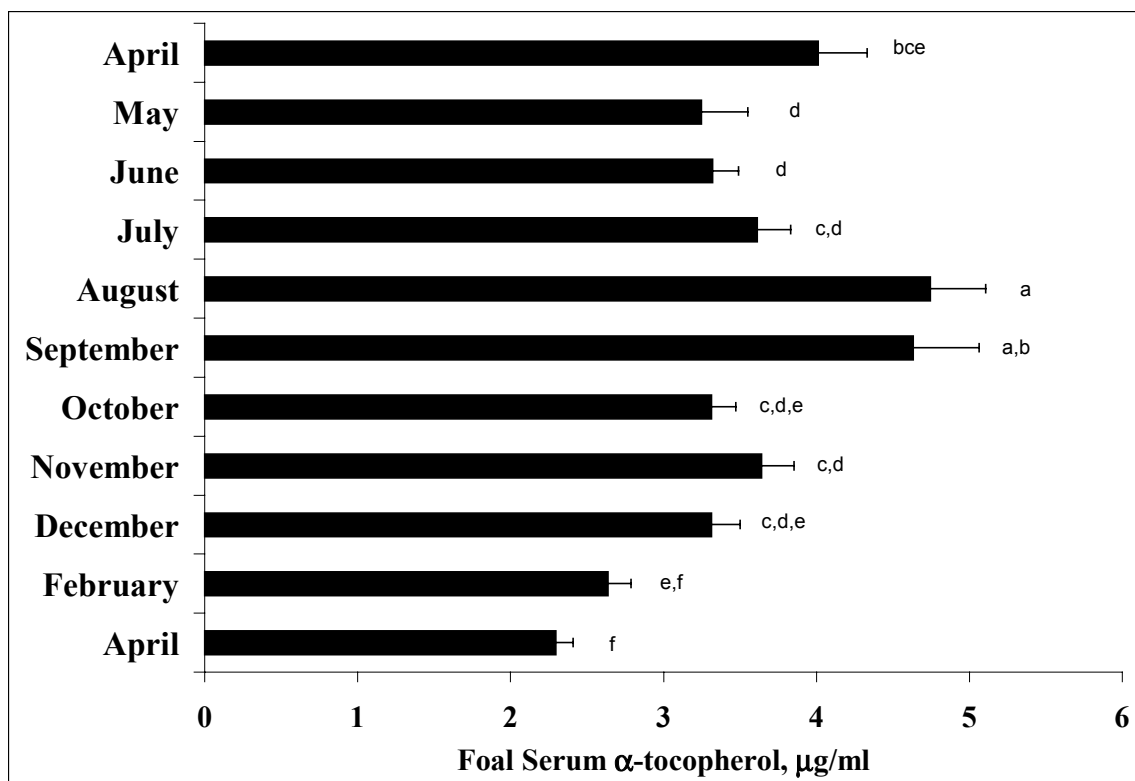


Figure 3.5

Foal serum α -tocopherol concentrations from birth in April/May 1999 to 12 mo of age in April 2000. Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different ($P < 0.05$) Experiment 3.1

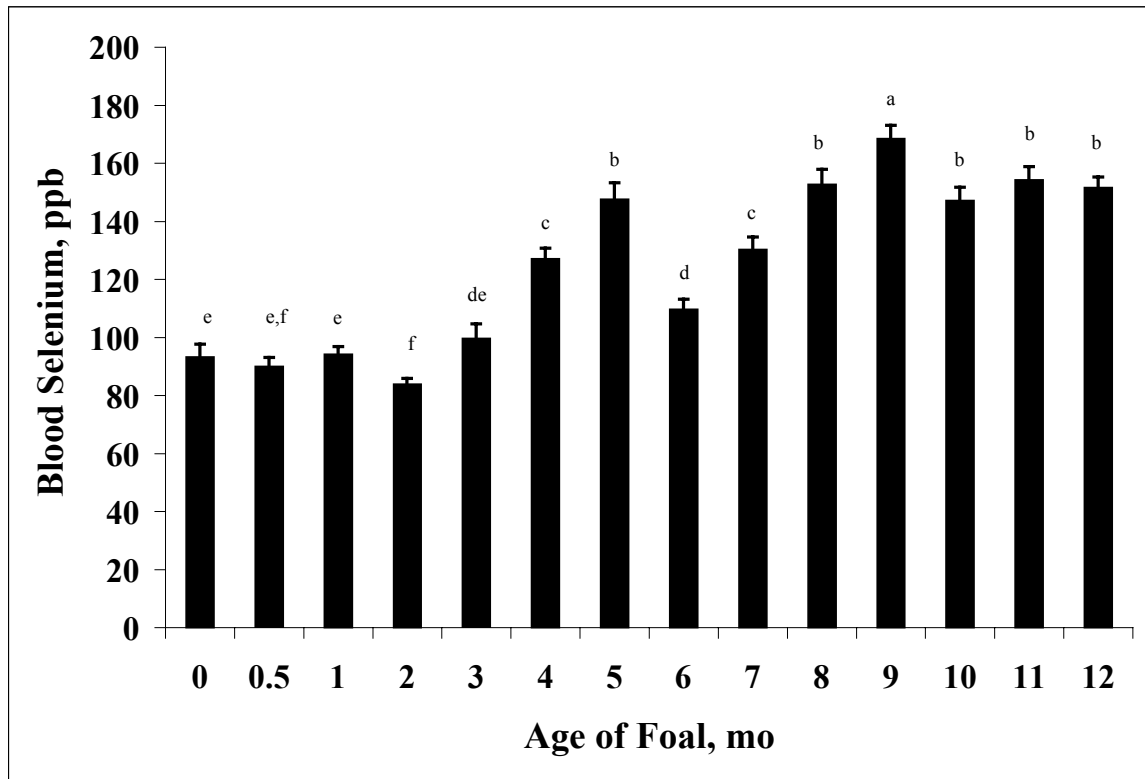


Figure 3.6

Whole blood Se concentrations (ppb) of foals from birth in April/May 1999 to 12 mo of age in April 2000. Bars are means and flags are standard errors of the mean. Means with unlike letters are different ($P < 0.05$). Experiment 3.1

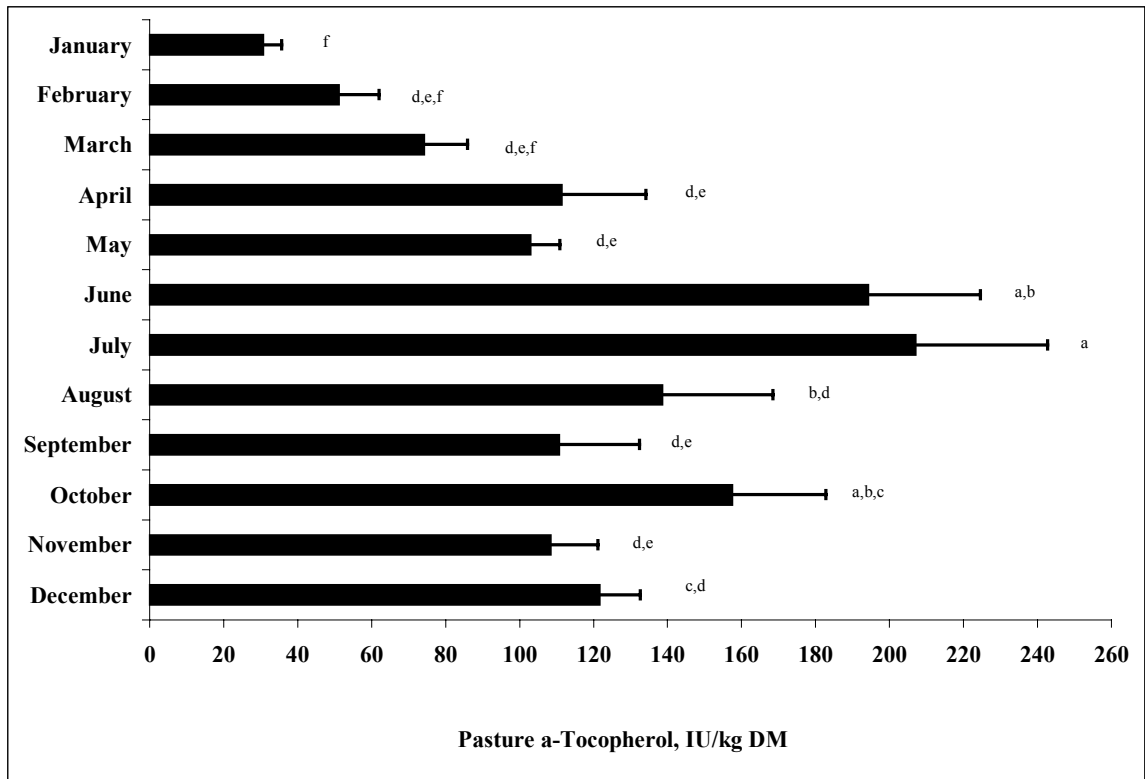


Figure 3.7

Vitamin E content (IU/kg DM) of pastures grazed by horses during 1999. Means with unlike letters are different ($P < 0.05$). Experiment 3.1

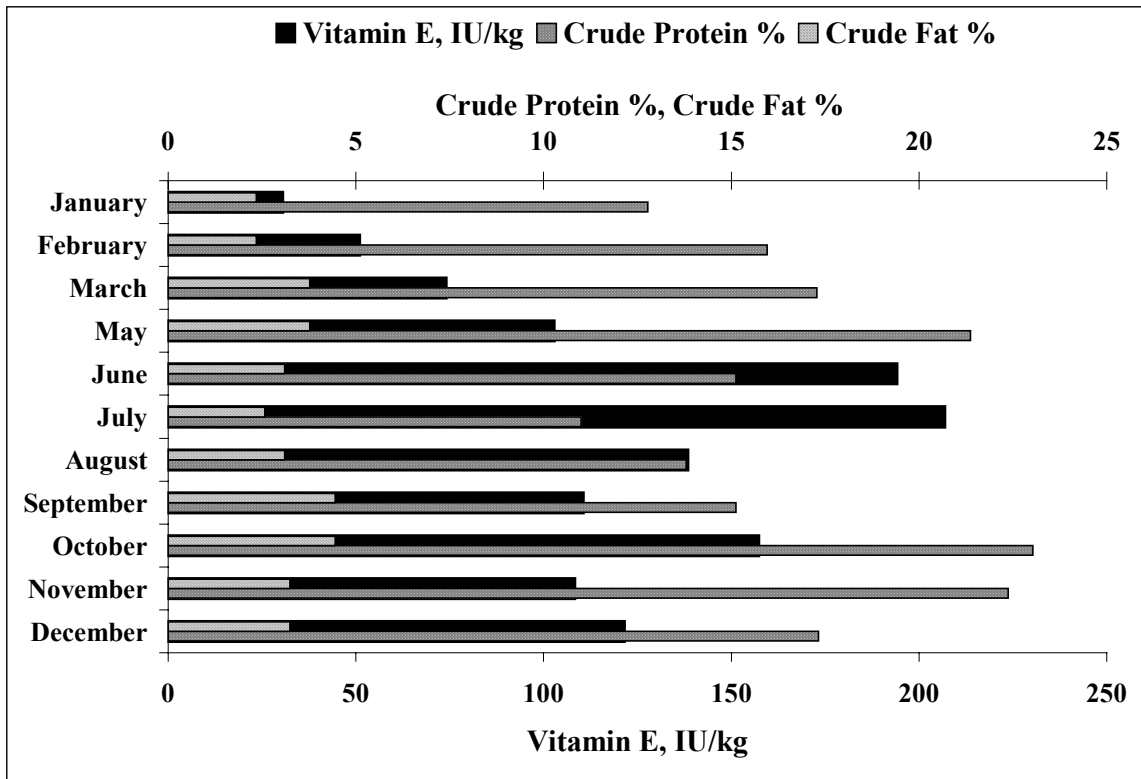


Figure 3.8

Concentrations of vitamin E (IU/kg DM), crude protein (%) and crude fat (%) of pastures grazed by horses during 1999. Experiment 3.1

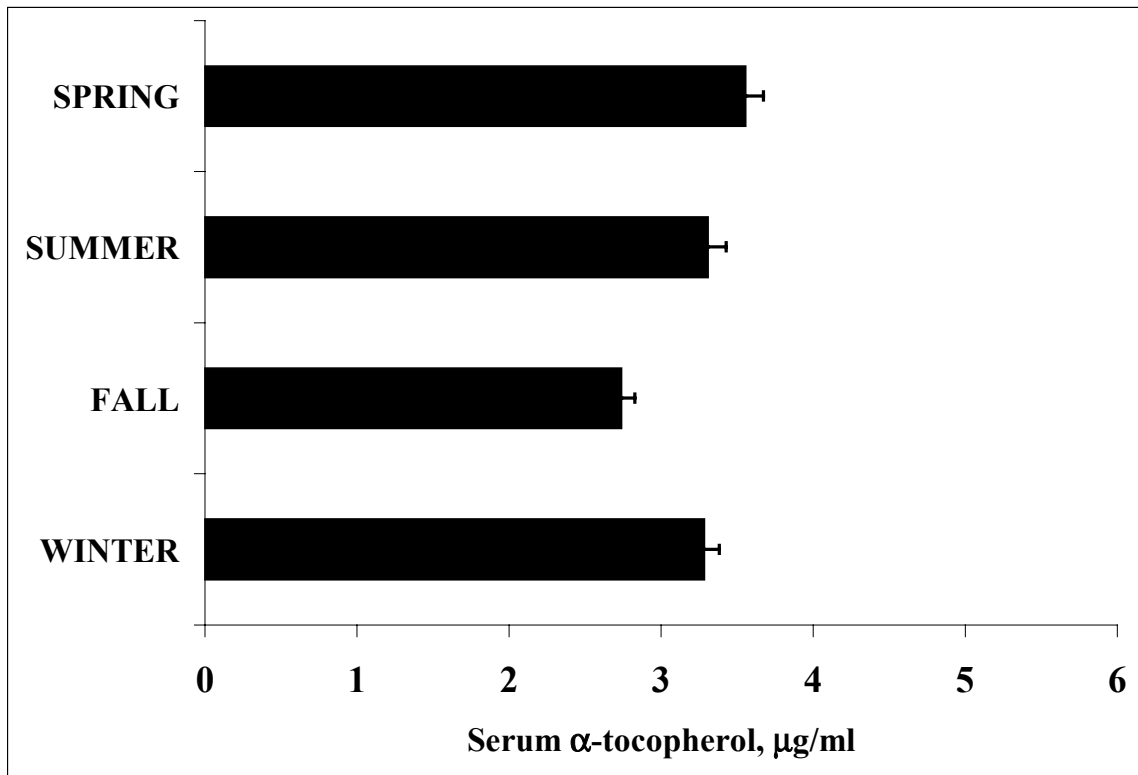


Figure 3.9

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of all groups of horses during each season of 1999 ($P < 0.001$). Experiment 3.2.

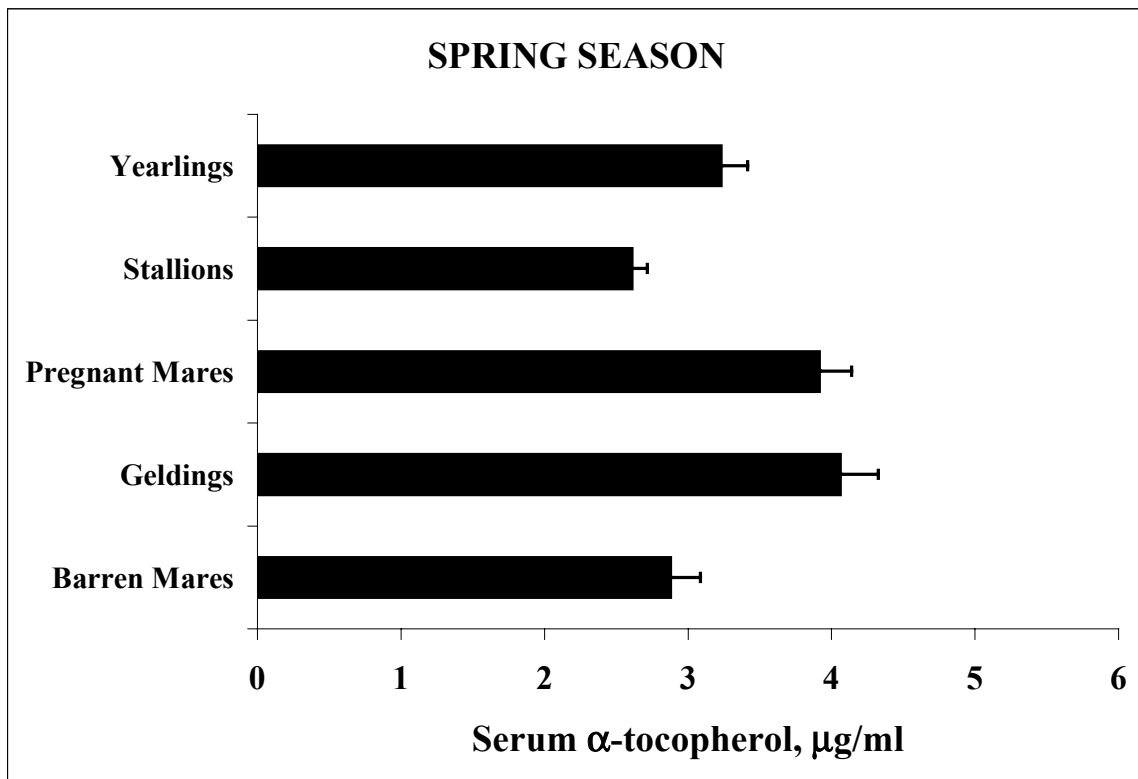


Figure 3.10

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of five groups of horses during the Spring of 1999 ($P = 0.007$). Experiment 3.2

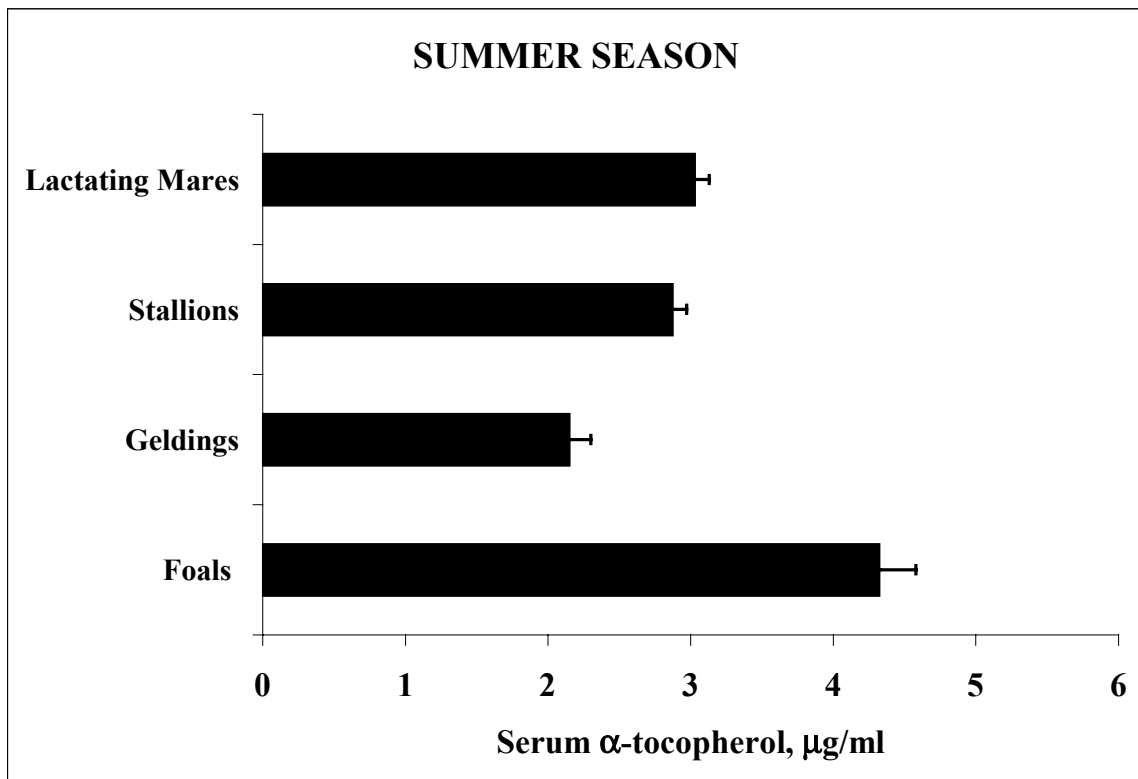


Figure 3.11

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of four groups of horses during the Summer of 1999 ($P < 0.0001$). Experiment 3.2

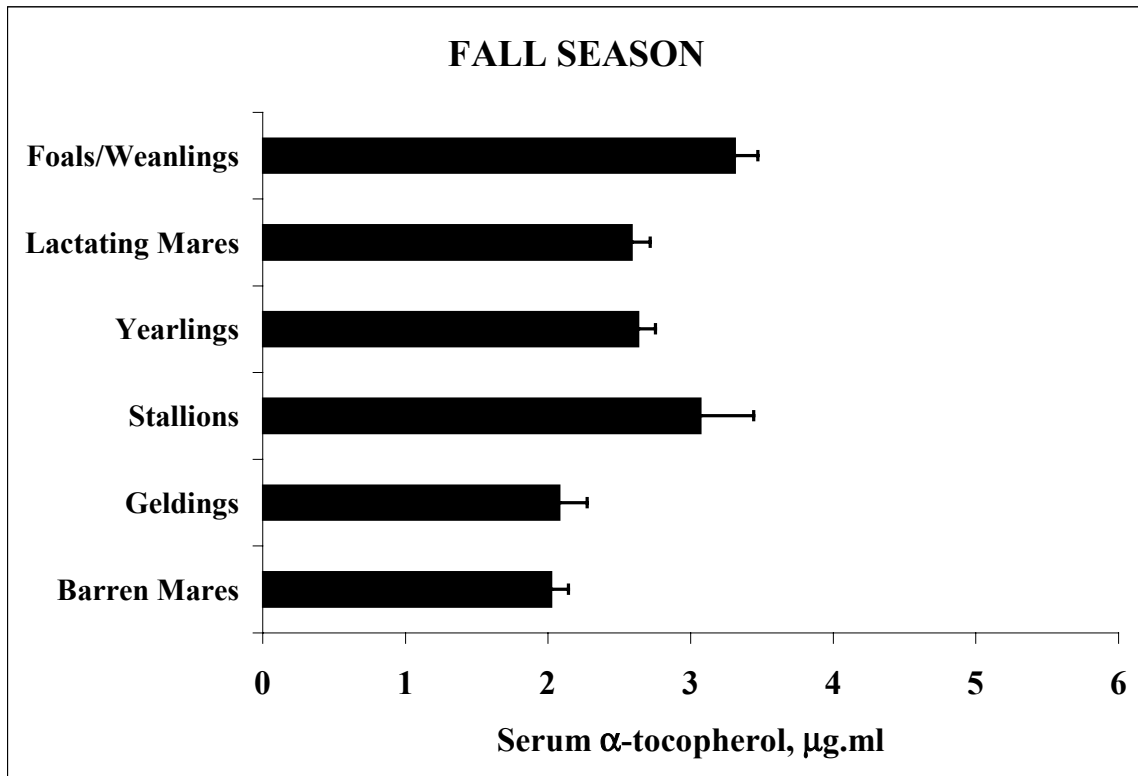


Figure 3.12

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of six groups of horses during the Fall of 1999 ($P = 0.0001$). Experiment 3.2

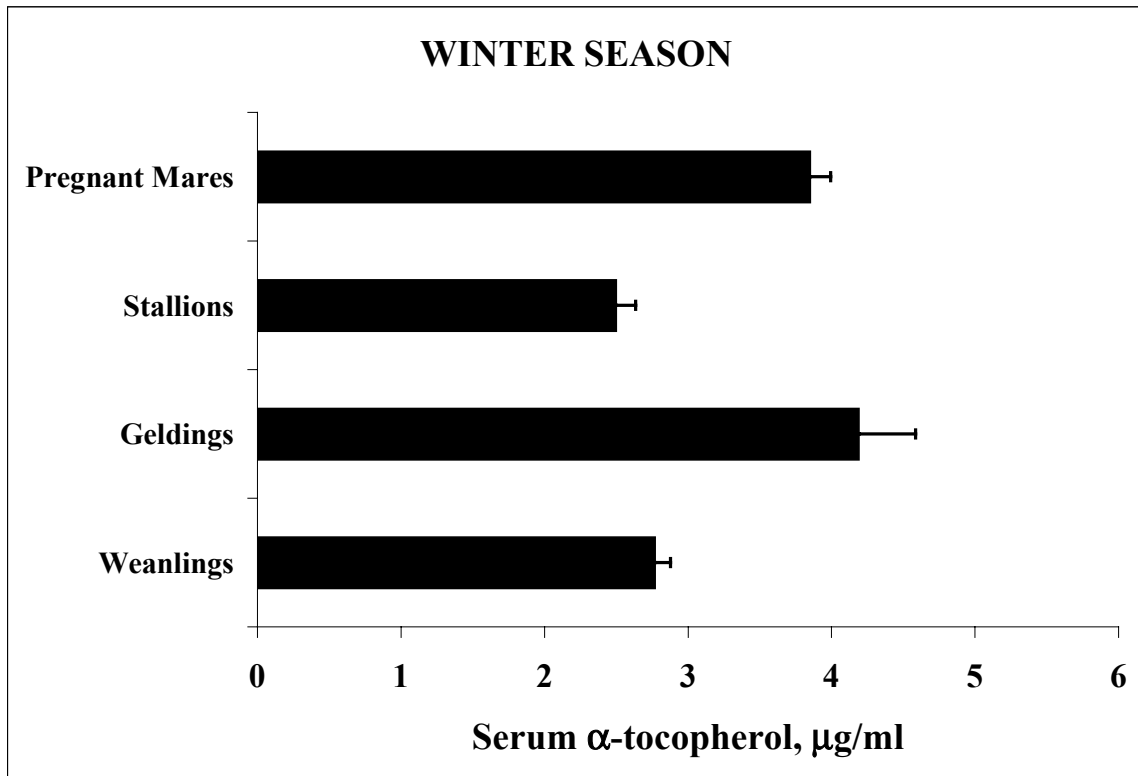


Figure 3.13

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of four groups of horses during the Winter of 1999 ($P < 0.0001$). Experiment 3.2

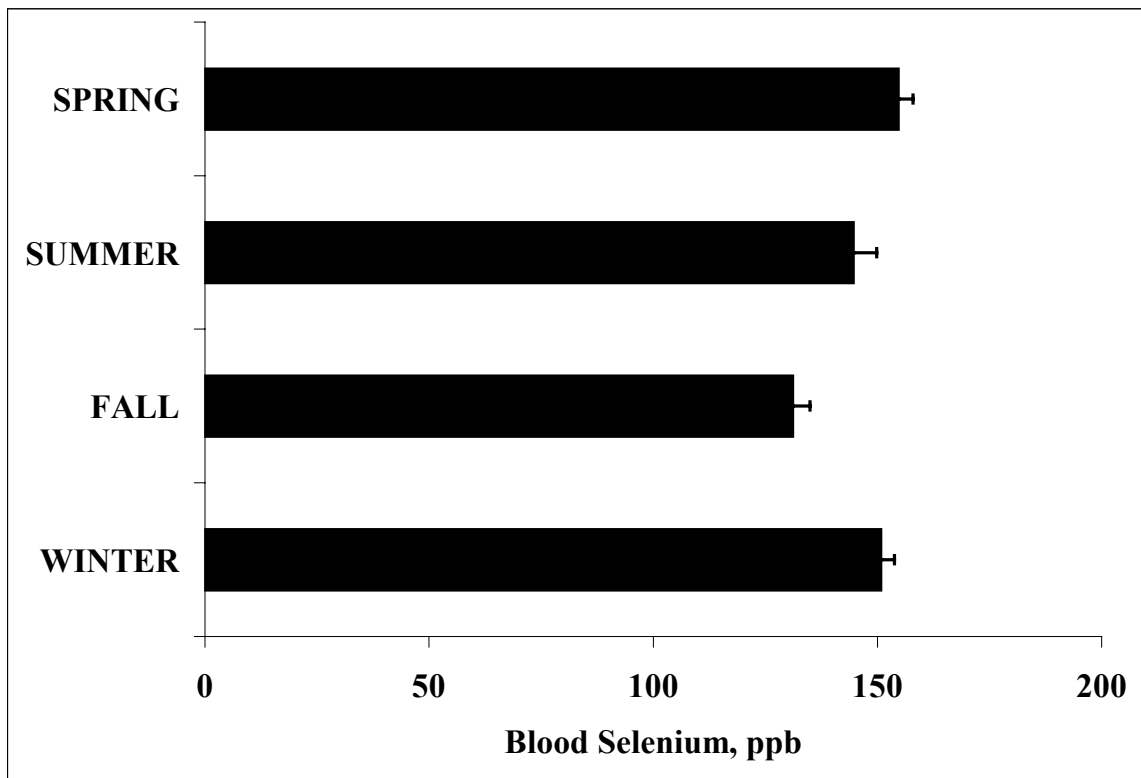


Figure 3.14

Mean whole blood Se concentrations (ppb) for all groups of horses during the seasons of 1999 ($P < 0.001$). Experiment 3.2

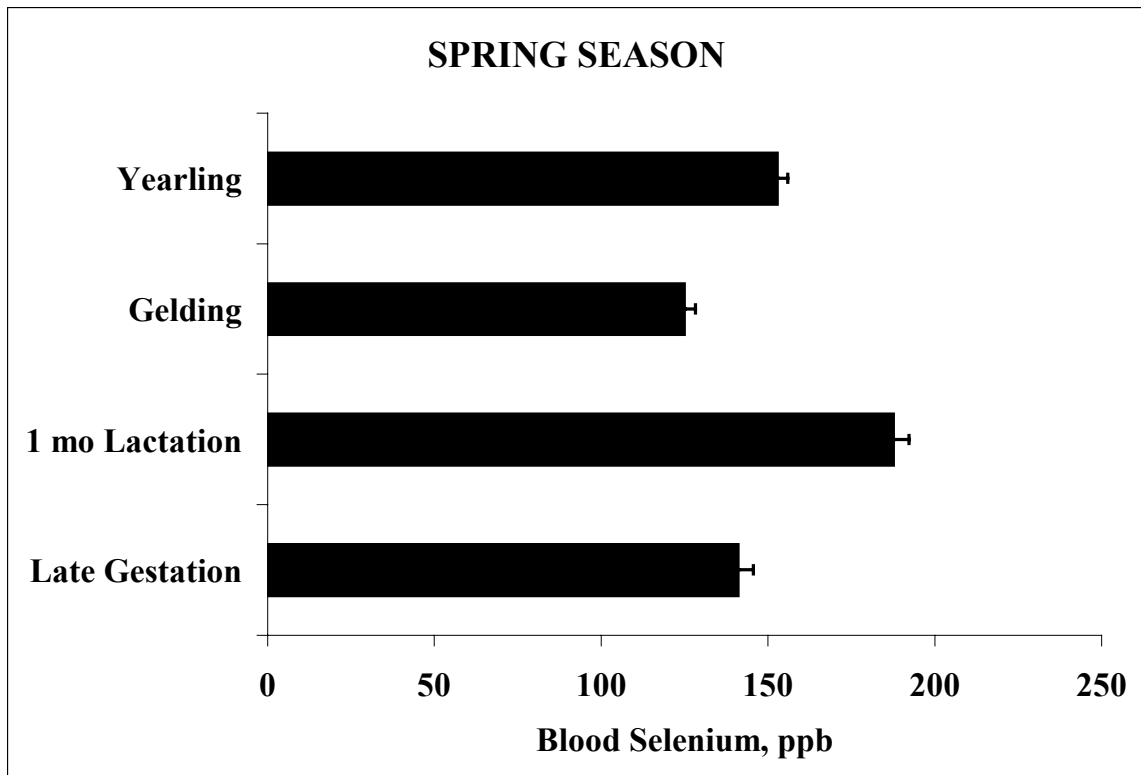


Figure 3.15

Mean whole blood Se concentrations (ppb) for four groups of horses during the Spring season of 1999 ($P < 0.001$). Experiment 3.2

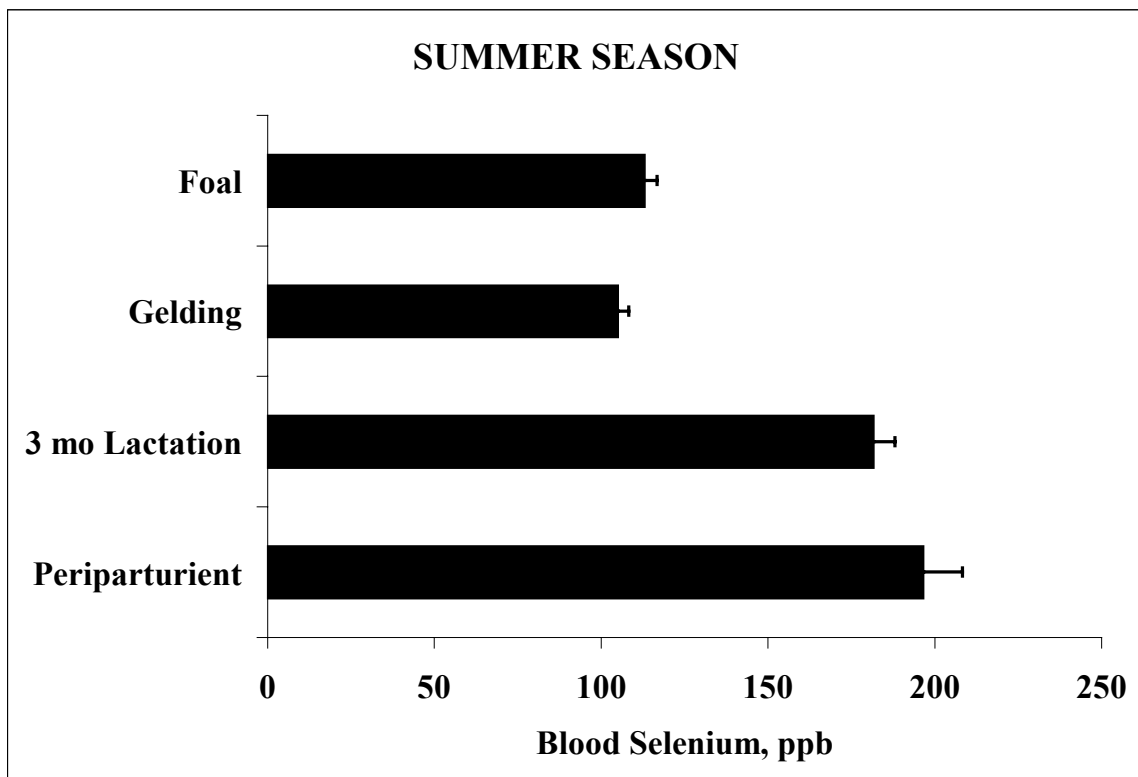


Figure 3.16

Mean whole blood Se concentrations (ppb) for four groups of horses during the Summer season of 1999 ($P < 0.001$). Experiment 3.2

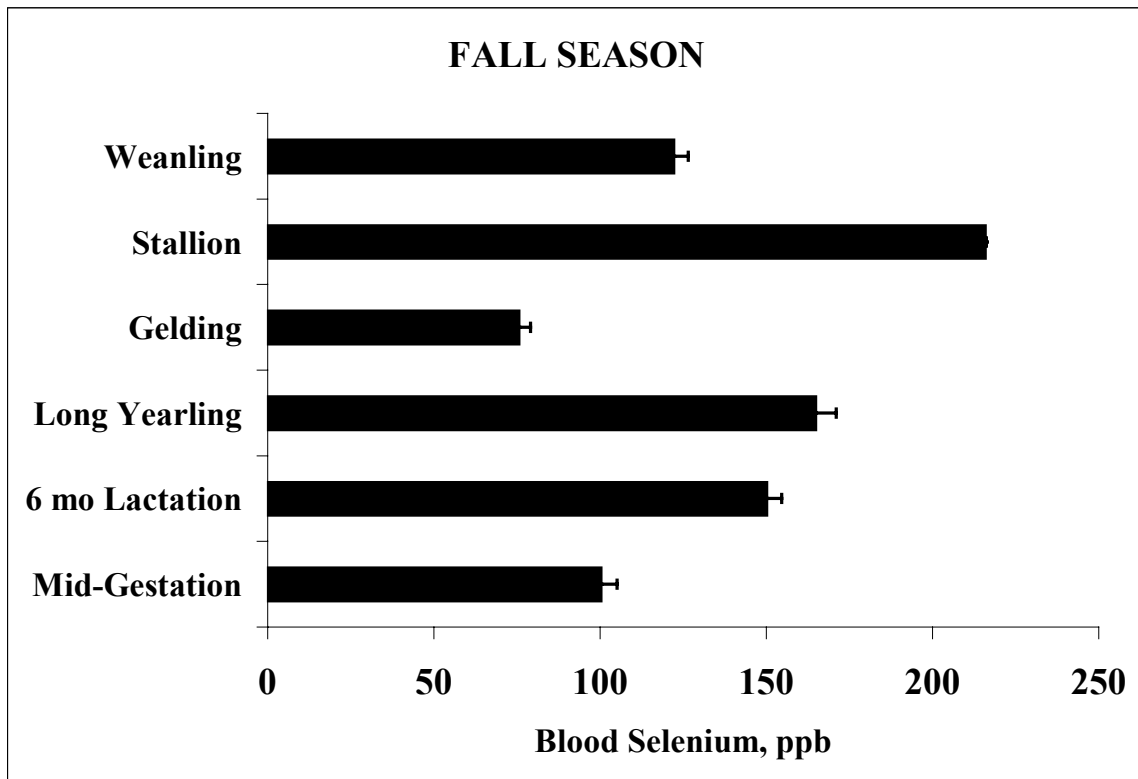


Figure 3.17

Mean whole blood Se concentrations (ppb) for six groups of horses during the Fall season of 1999 ($P < 0.0001$). Experiment 3.2

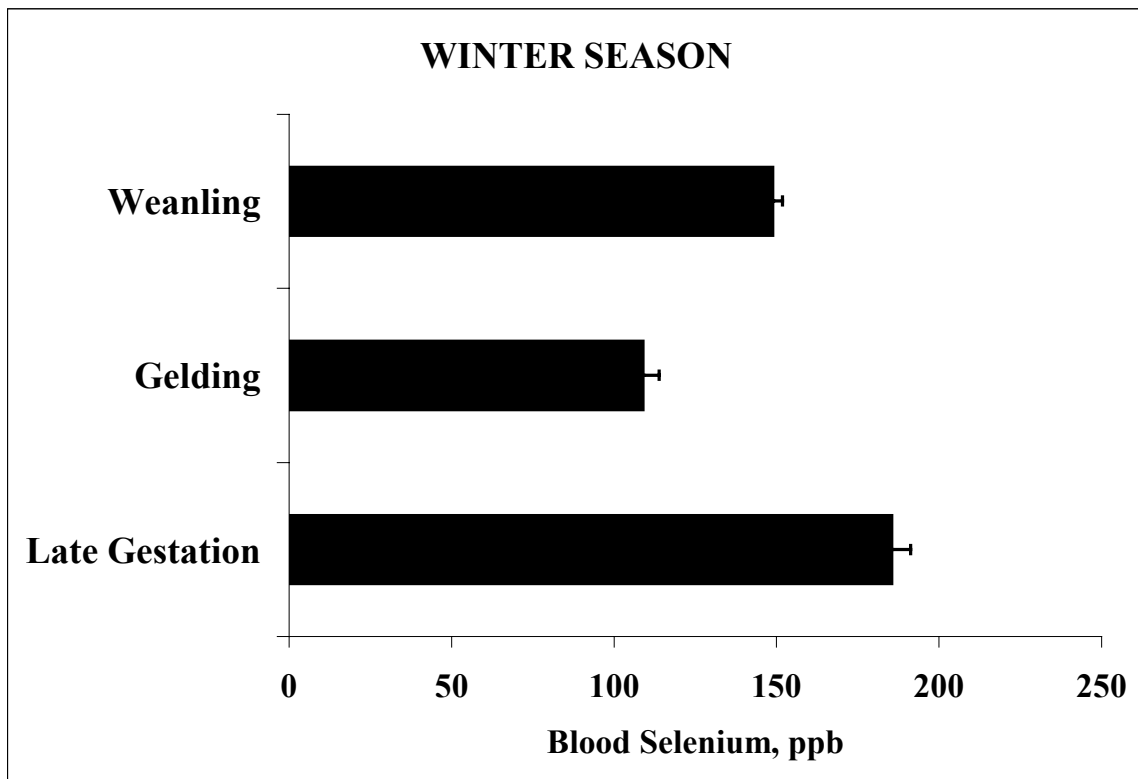


Figure 3.18

Mean whole blood Se concentrations (ppb) for three groups of horses during the Winter season of 1999 ($P < 0.0001$). Experiment 3.2

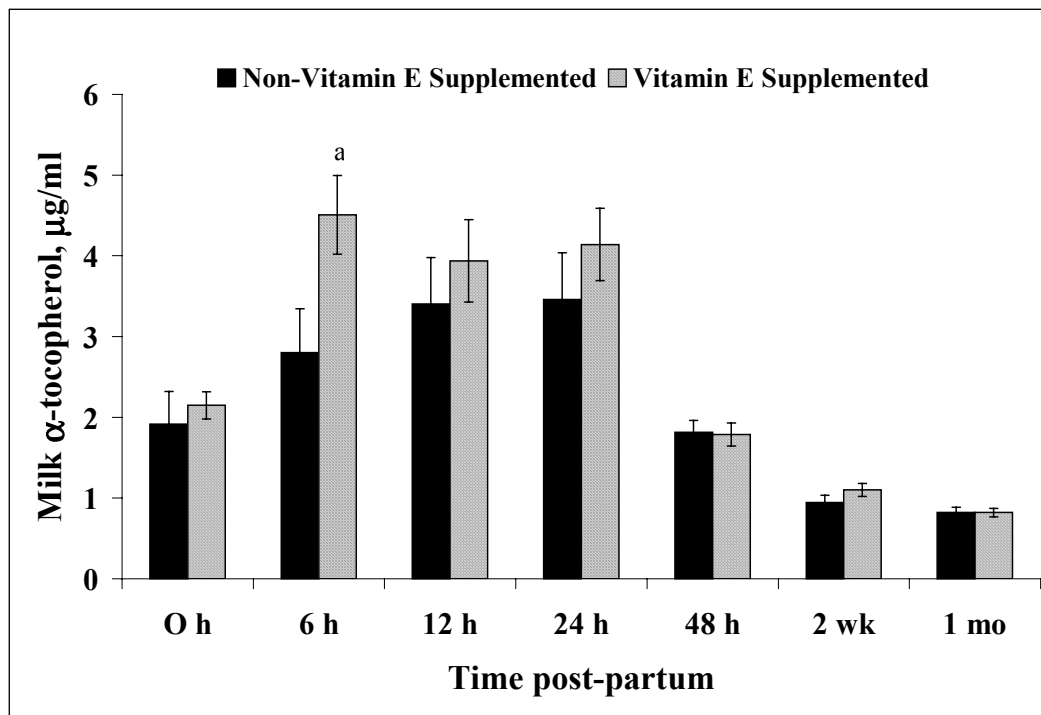


Figure 4.1

Mean milk α -tocopherol concentrations ($\mu\text{g/ml}$) in post-partum mares per group) that received either a placebo (black bars, $n = 12$) or an oral dose of *dl*- α -tocopheryl acetate (hatched bars; $n = 12$) every 3.5 d during the last trimester (3 mo) in 1999. Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different at that sample time ($P < 0.05$). Experiment 4.1.

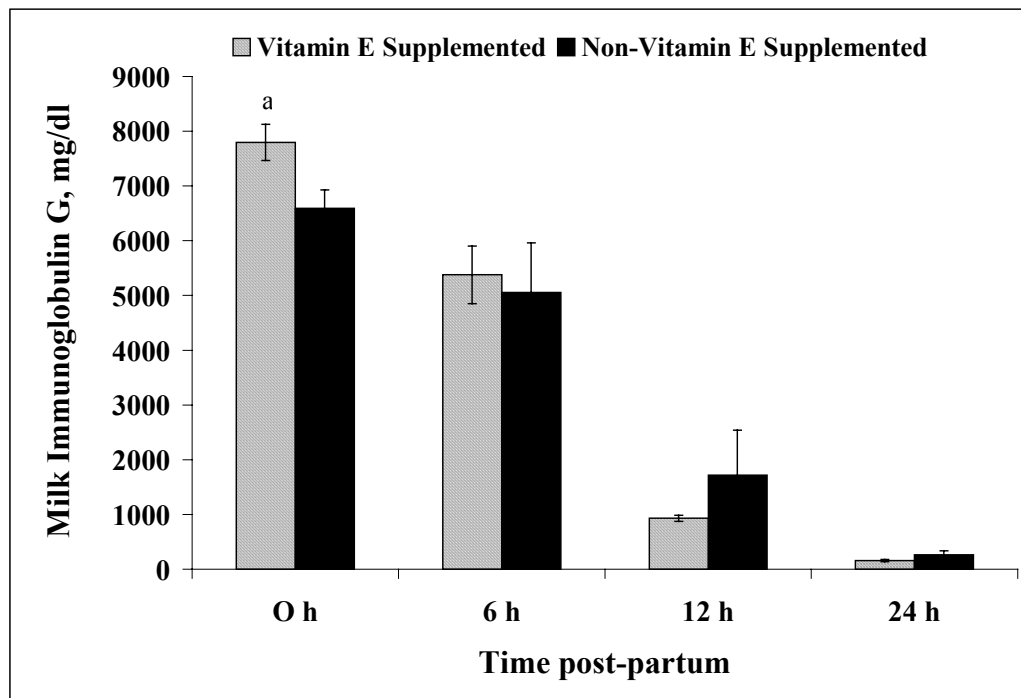


Figure 4.2

Mean immunoglobulin G concentrations (mg/dl) in colostrum and milk of post-partum mares that received either a placebo (black bars, n = 12) or an oral dose of *dl*- α -tocopheryl acetate (hatched bars, n = 12) every 3.5 d during the last trimester (3 mo) in 1999. Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different at that sample time ($P < 0.05$). Experiment 4.1.

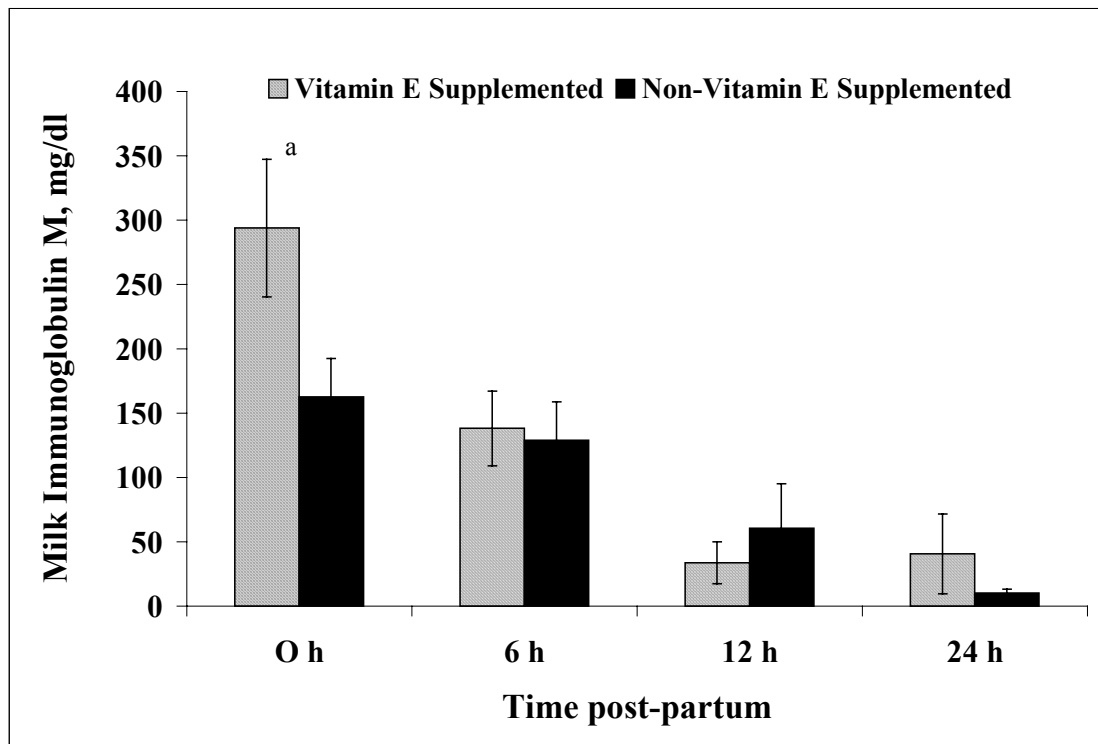


Figure 4.3

Mean immunoglobulin M concentrations (mg/dl) in colostrum and milk of post-partum mares that received either a placebo (black bars, n = 12) or an oral dose of *dl*- α -tocopheryl acetate (hatched bars, n = 12) every 3.5 d during the last trimester (3 mo) in 1999. Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different at that sample time ($P < 0.05$). Experiment 4.1.

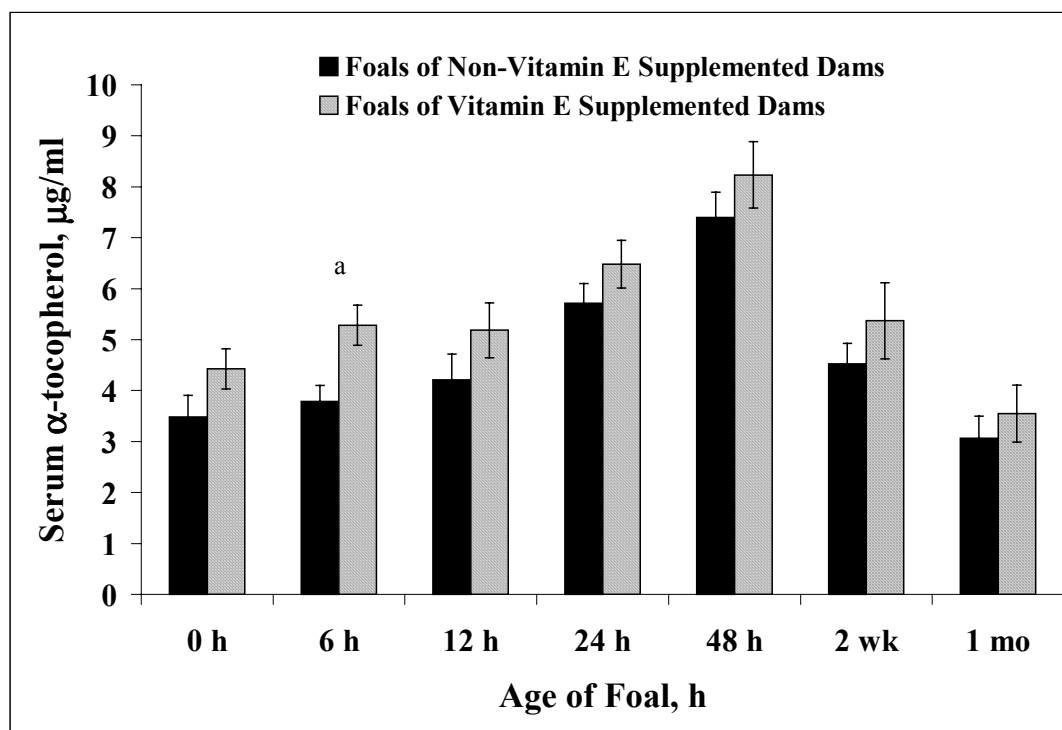


Figure 4.4

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of foals from dams that received either a placebo (black bars, $n = 12$) or an oral dose of *dl*- α -tocopheryl acetate (hatched bars, $n = 12$) every 3.5 d during the last trimester (3 mo) in 1999. Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different at that sample time ($P < 0.05$). Experiment 4.1.

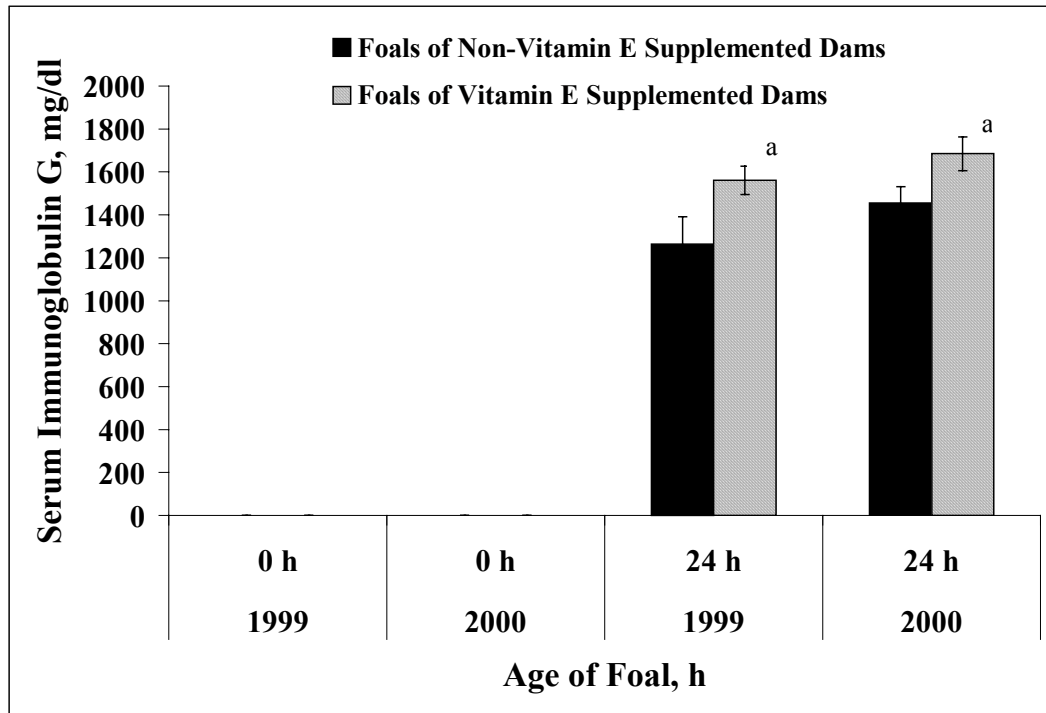


Figure 4.5

Mean serum immunoglobulin G concentration (mg/dl) of foals from dams that received either a placebo (black bars, n = 12) or an oral dose (hatched bars, n = 12) of *dl*- α -tocopheryl acetate every 3.5 d during the last trimester (3 mo) in 1999 (Experiment 4.1), or a daily oral dose of *d*- α -tocopherol during the last trimester in 2000 (Experiment 4.2). Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different at that sample time ($P < 0.05$).

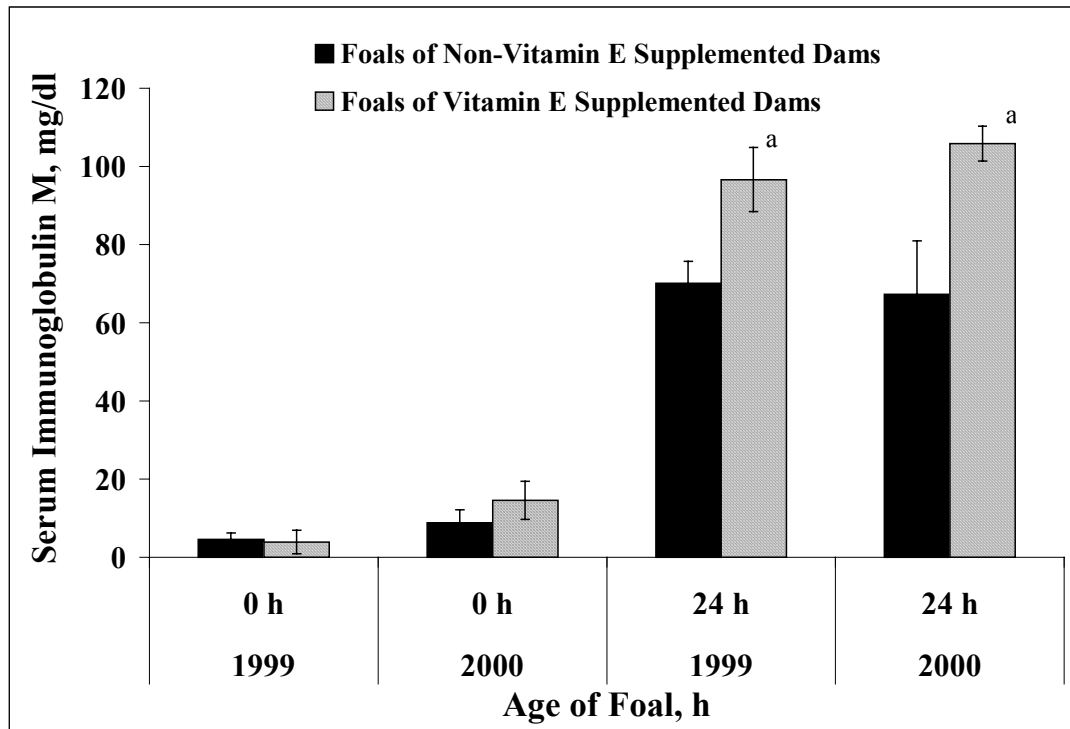


Figure 4.6

Mean serum immunoglobulin M concentration (mg/dl) of foals from dams that received either a placebo (black bars, n = 12) or an oral dose (hatched bars, n = 12) of *dl*- α -tocopheryl acetate every 3.5 d during the last trimester (3 mo) in 1999 (Experiment 4.1), or a daily oral dose of *d*- α -tocopherol during the last trimester in 2000 (Experiment 4.2). Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different at that sample time ($P < 0.05$).

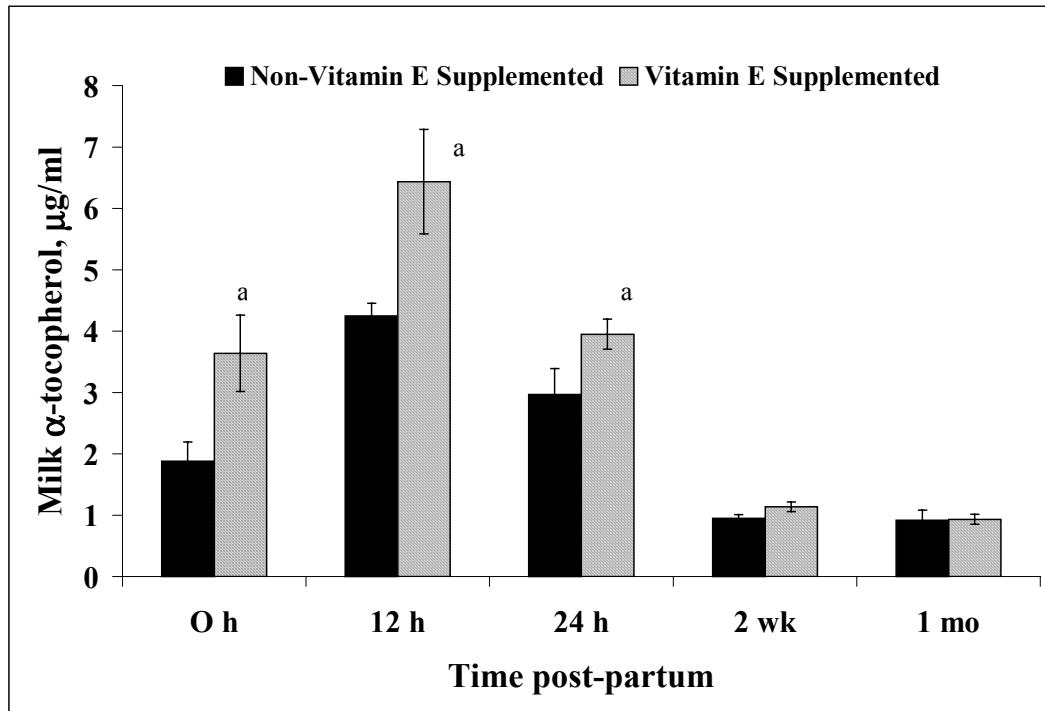


Figure 4.7

Mean milk α -tocopherol concentrations ($\mu\text{g/ml}$) in post-partum mares that received either a placebo (black bars, $n = 12$) or a daily oral dose of d - α -tocopherol (hatched bars, $n = 12$) during the last trimester (3 mo) in 2000. Experiment 4.2. Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different at that sample time ($P < 0.05$).

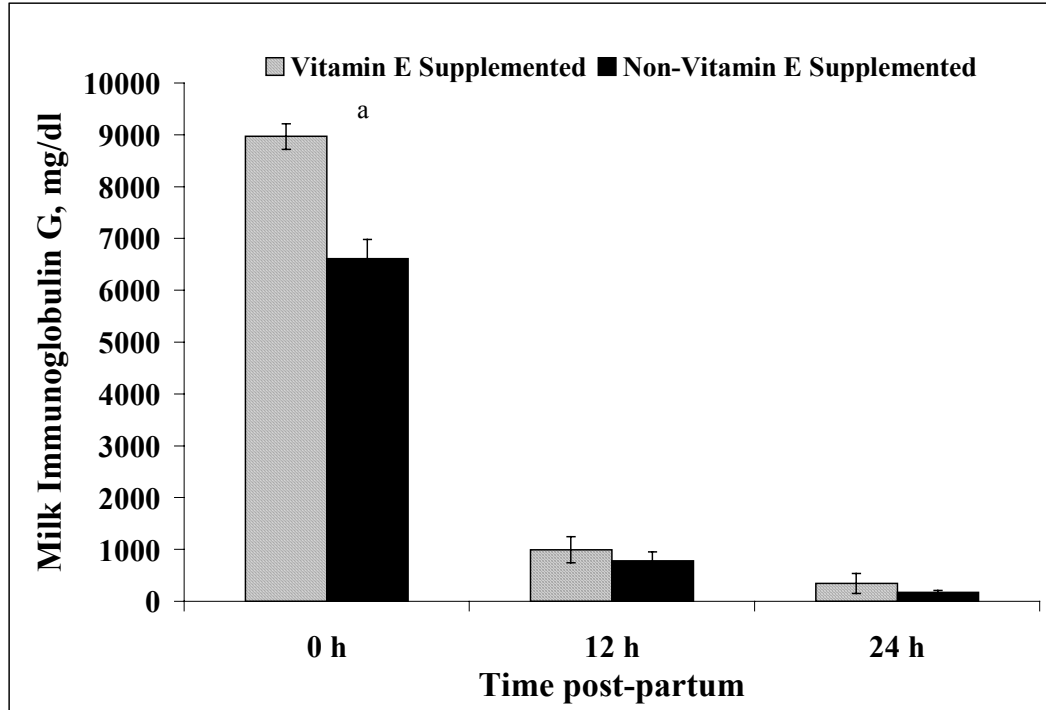


Figure 4.8

Mean immunoglobulin G concentration (mg/dl) in colostrum and milk of post-partum mares that received either a placebo (black bars, n = 12), or a daily oral dose of *d*- α -tocopherol (hatched bars, n = 12) during the last trimester (3 mo) in 2000. Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different at that sample time ($P < 0.05$). Experiment 4.2.

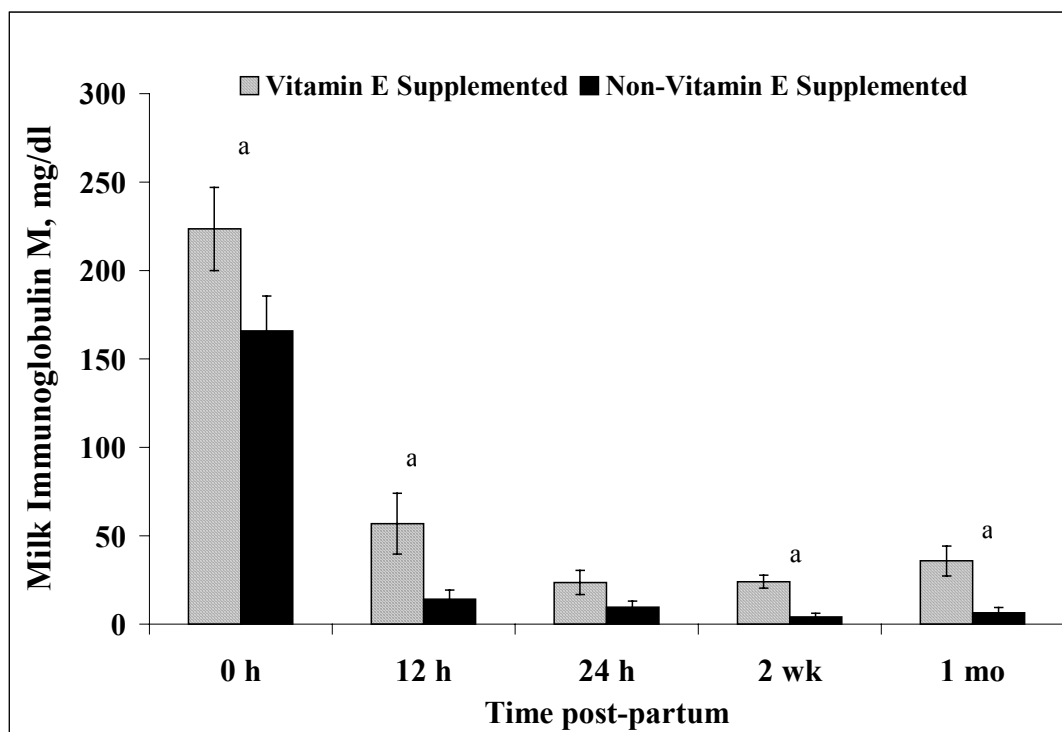


Figure 4.9

Mean immunoglobulin M concentration (mg/dl) in colostrum and milk of post-partum mares that received either a placebo (black bars, n = 12), or a daily oral dose of *d*- α -tocopherol (hatched bars, n = 12) during the last trimester (3 mo) in 2000. Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different at that sample time ($P < 0.05$). Experiment 4.2

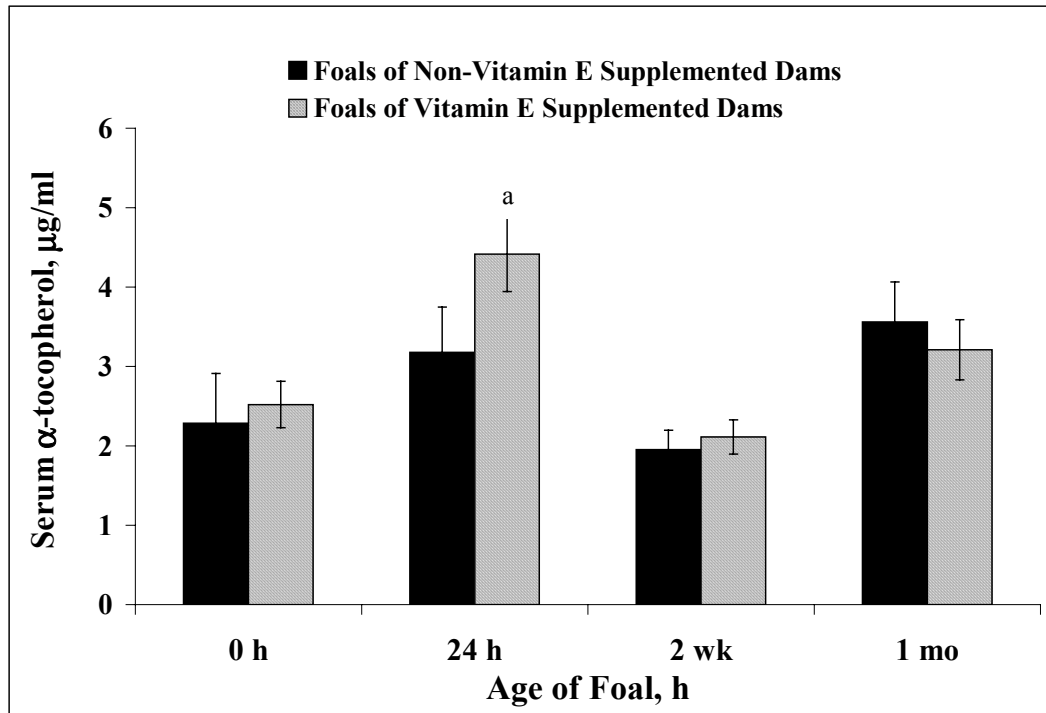


Figure 4.10

Mean serum α -tocopherol concentrations ($\mu\text{g/ml}$) of foals from dams that received either a placebo (black bars, $n = 12$) or a daily oral dose of d - α -tocopherol (hatched bars, $n = 12$) during the last trimester (3 mo) in 2000. Bars are means and flags are standard errors of the mean. Means with unlike letters are significantly different at that sample time ($P < 0.05$). Experiment 4.2.

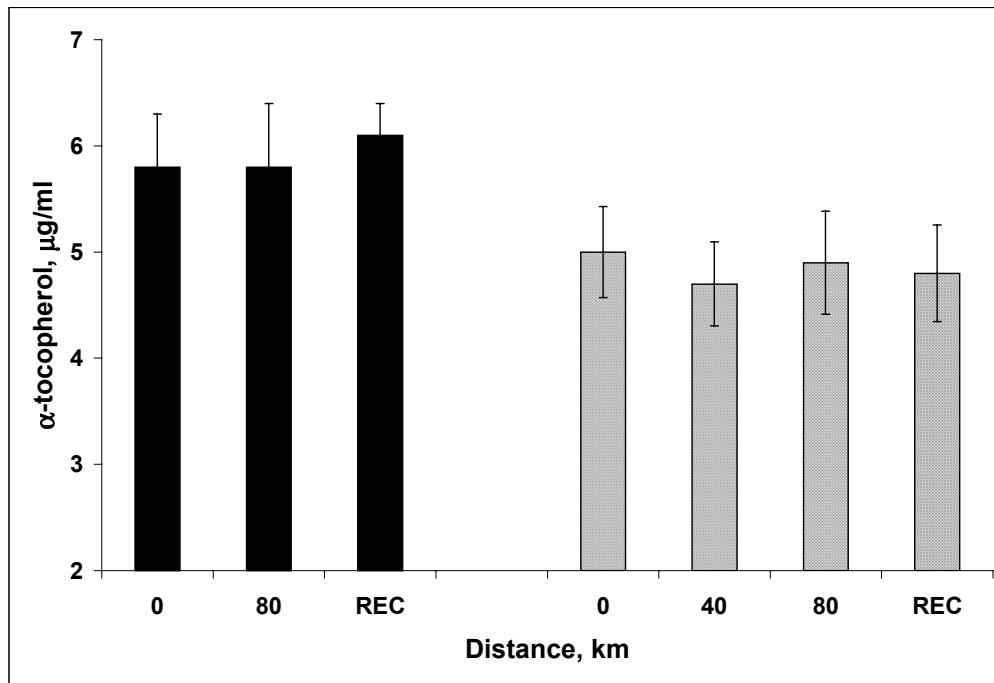


Figure 5.1

Plasma vitamin E concentrations (α -tocopherol $\mu\text{g/ml}$) for horses that completed the 80 km No Frills (NF80, $n = 10$, black bars) and Old Dominion (OD80, $n = 11$, hatched bars) endurance races at 0, 80 km and 60 min recovery (REC) at NF, and at 0, 40, 80 km and REC at OD. Bars are means, flags are standard errors of the mean. Experiment 5.1

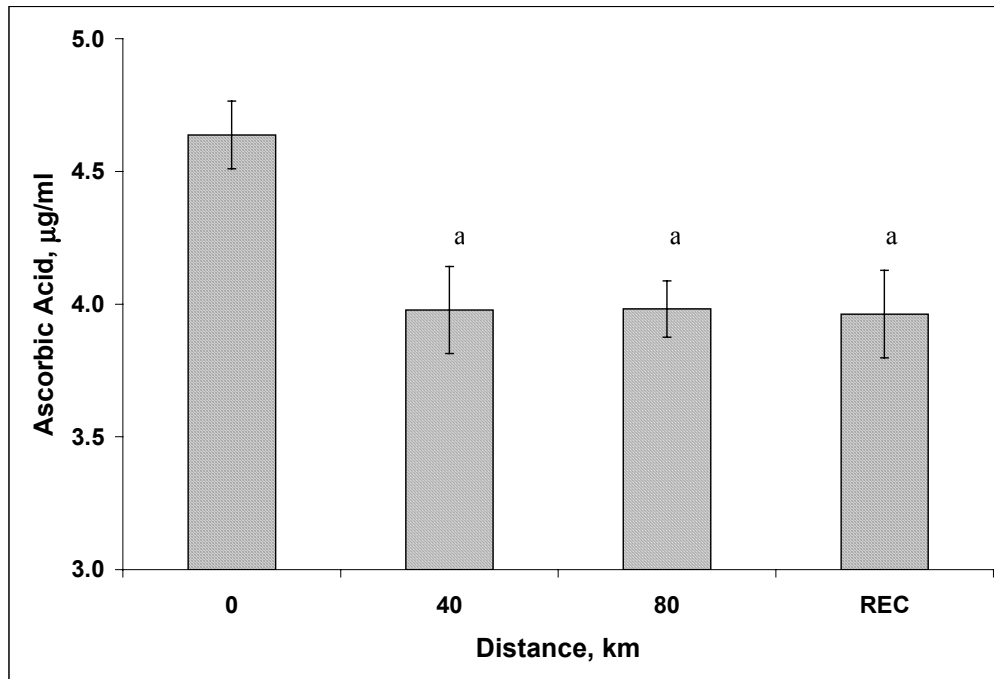


Figure 5.2

Plasma vitamin C concentrations (ascorbic acid µg/ml) for horses that completed the 80 km Old Dominion (OD, n = 11) endurance race at 0, 40, 80 km and 60 min recovery (REC). Bars are means, flags are standard errors of the mean. Means with unlike letters are significantly different ($P < 0.05$) Experiment 5.1

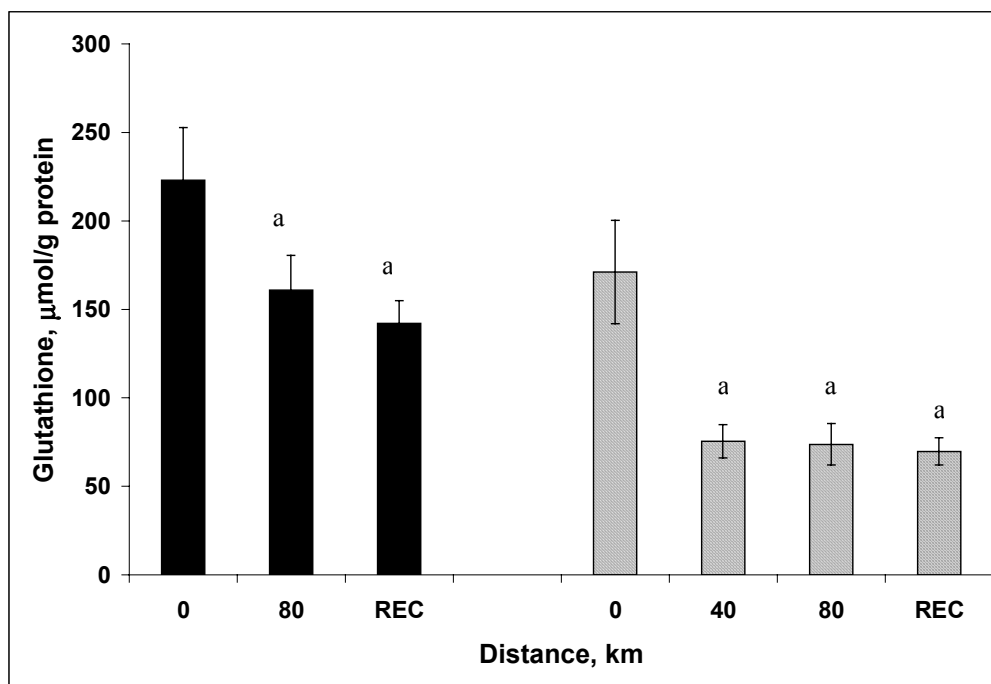


Figure 5.3

Erythrocyte glutathione concentrations ($\mu\text{mol/g}$ protein) for horses that completed the 80 km No Frills (NF, $n = 10$, black bars) and Old Dominion (OD, $n = 11$, hatched bars) endurance races at 0, 80 km and 60 min recovery (REC) at NF, and at 0, 40, 80 km and REC at OD. Bars are means, flags are standard errors of the mean. Means within a race with unlike letters are significantly different ($P < 0.05$). Experiment 5.1

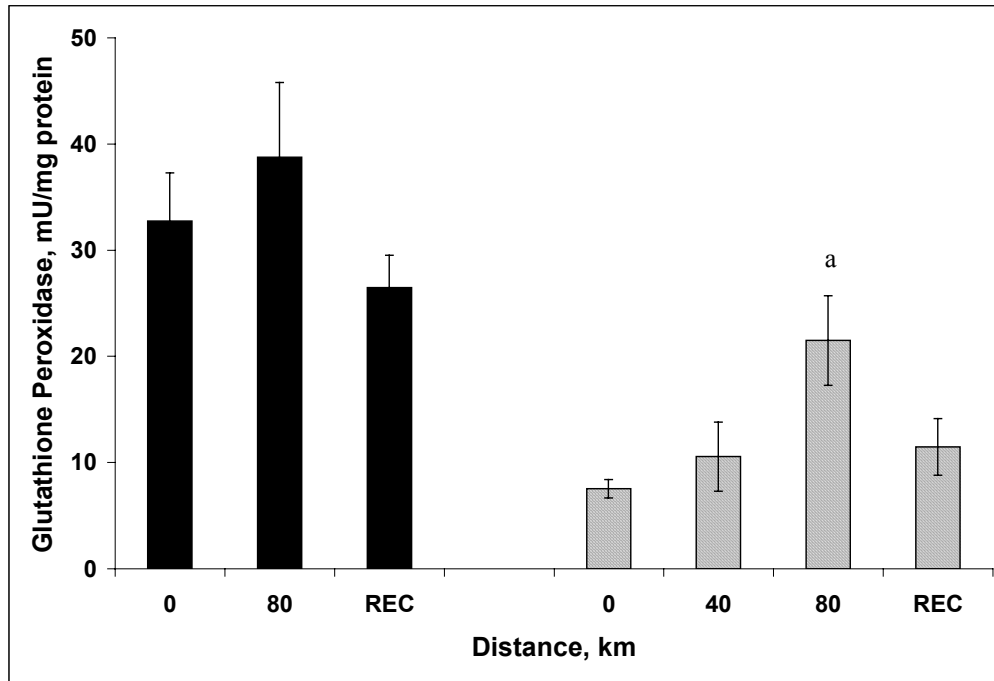


Figure 5.4

Erythrocyte glutathione peroxidase activities (mU/mg protein) for horses that completed the 80 km No Frills (NF, n = 10, black bars) and Old Dominion (OD, n = 11, hatched bars) endurance races at 0, 80 km and 60 min recovery (REC) at NF, and at 0, 40, 80 km and REC at OD. Bars are means, flags are standard errors of the mean. Means within a race with unlike letters are significantly different ($P < 0.05$). Experiment 5.1

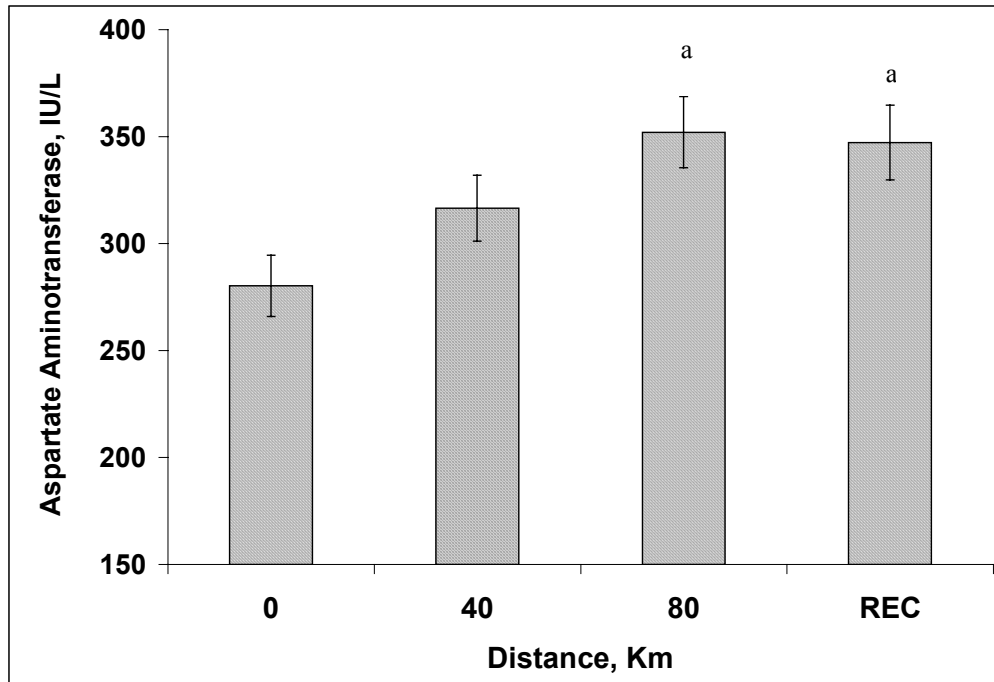


Figure 5.5

Plasma aspartate aminotransferase activities (IU/L) for horses that completed the 80 km Old Dominion endurance race at 0, 40, 80 km and recovery (REC). Bars are means, flags are standard errors of the mean. Means with unlike letters are significantly different ($P < 0.05$).
Experiment 5.1

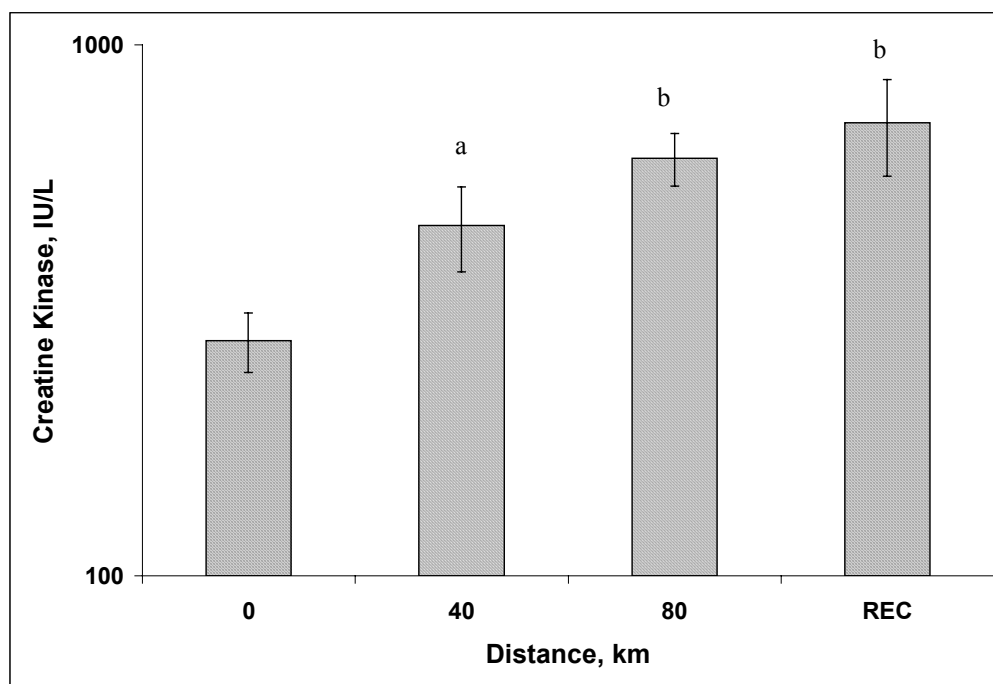


Figure 5.6

Plasma creatine kinase activity (IU/L) for horses that completed the 80 km Old Dominion (OD, n = 11) endurance race at 0, 40, 80 km and 60 min recovery (REC). Bars are means, flags are standard errors of the mean. Means with unlike letters are significantly different ($P < 0.05$). Experiment 5.1

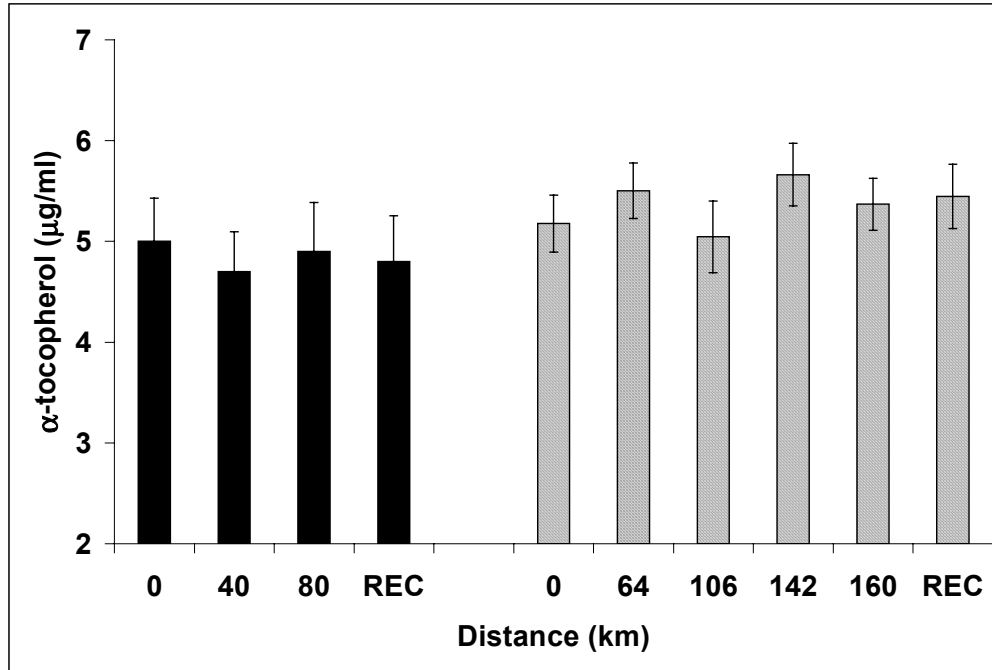


Figure 5.7

Plasma vitamin E concentrations (α -tocopherol $\mu\text{g/ml}$) for horses that completed the 80 km ($n = 12$) endurance race (OD80, black bars) at 0, 40, 80 km and 60 min recovery (REC), and 160 km ($n = 10$) endurance race (OD160, hatched bars) at 0, 64, 106, 142, 160 km and REC. Bars are means, flags are standard errors of the mean in Experiment 5.2

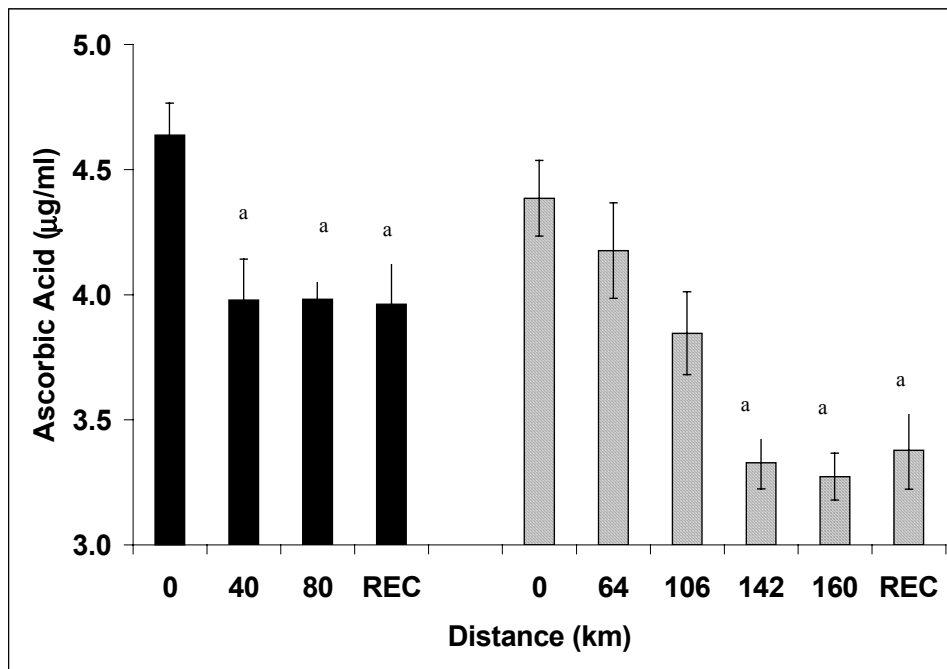


Figure 5.8

Plasma vitamin C concentrations (ascorbic acid µg/ml) for horses that completed the 80 km (n = 12) endurance race (OD80, black bars) at 0, 40, 80 km and 60 min recovery (REC), and 160 km (n = 10) endurance race (OD160, hatched bars) at 0, 64, 106, 142, 160 km and REC. Bars are means, flags are standard errors of the mean. Means within a race with unlike letters are significantly different (P < 0.05). Experiment 5.2

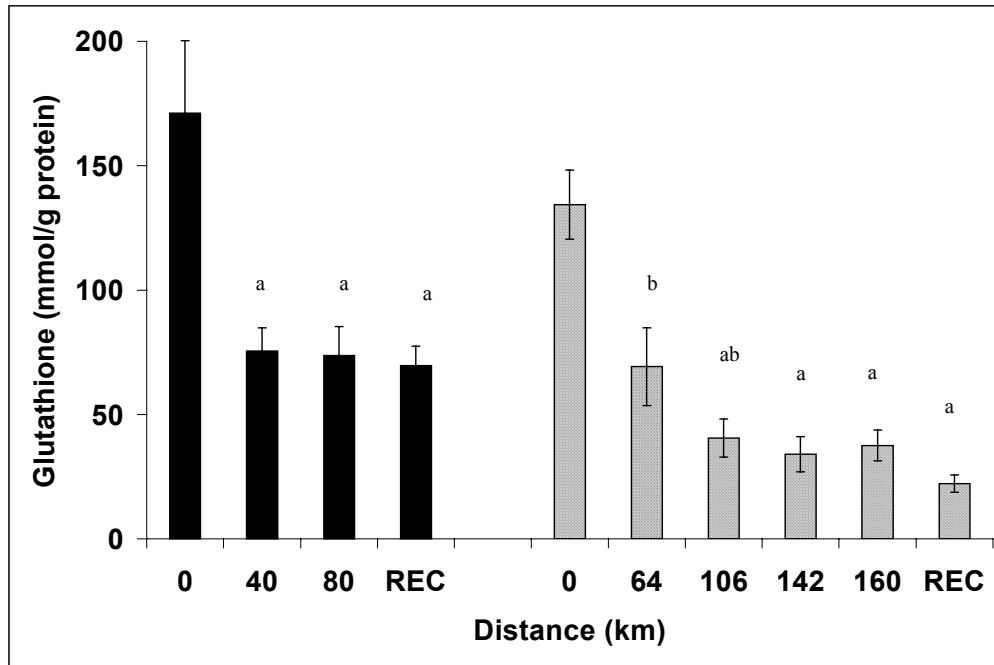


Figure 5.9

Erythrocyte glutathione ($\mu\text{mol/g}$ protein) concentrations for horses that completed the 80 km ($n = 12$) endurance race (OD80, black bars) at 0, 40, 80 km and 60 min recovery (REC), and 160 km ($n = 10$) endurance race (OD160, hatched bars) at 0, 64, 106, 142, 160 km and REC. Bars are means, flags are standard errors of the mean. Means within a race with unlike letters are significantly different ($P < 0.05$). Experiment 5.2

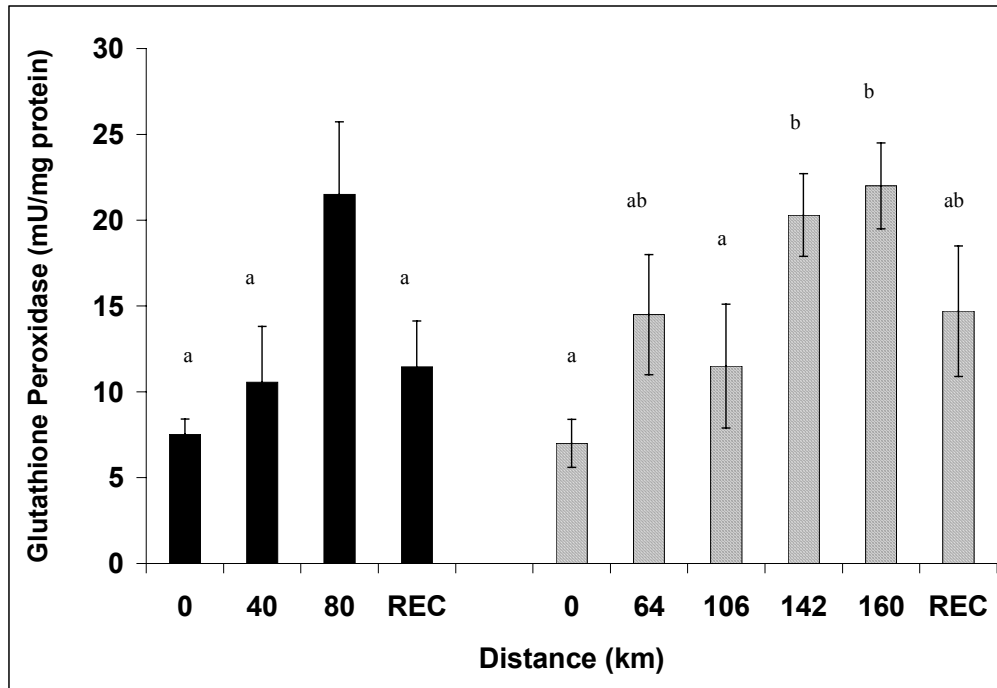


Figure 5.10

Erythrocyte glutathione peroxidase (mU/mg protein) activity for horses that completed the 80 km (n = 12) endurance race (OD80, black bars) at 0, 40, 80 km and 60 min recovery (REC), and 160 km (n = 10) endurance race (OD160, hatched bars) at 0, 64, 106, 142, 160 km and REC. Bars are means, flags are standard errors of the mean. Means within a race with unlike letters are significantly different ($P < 0.05$). Experiment 5.2

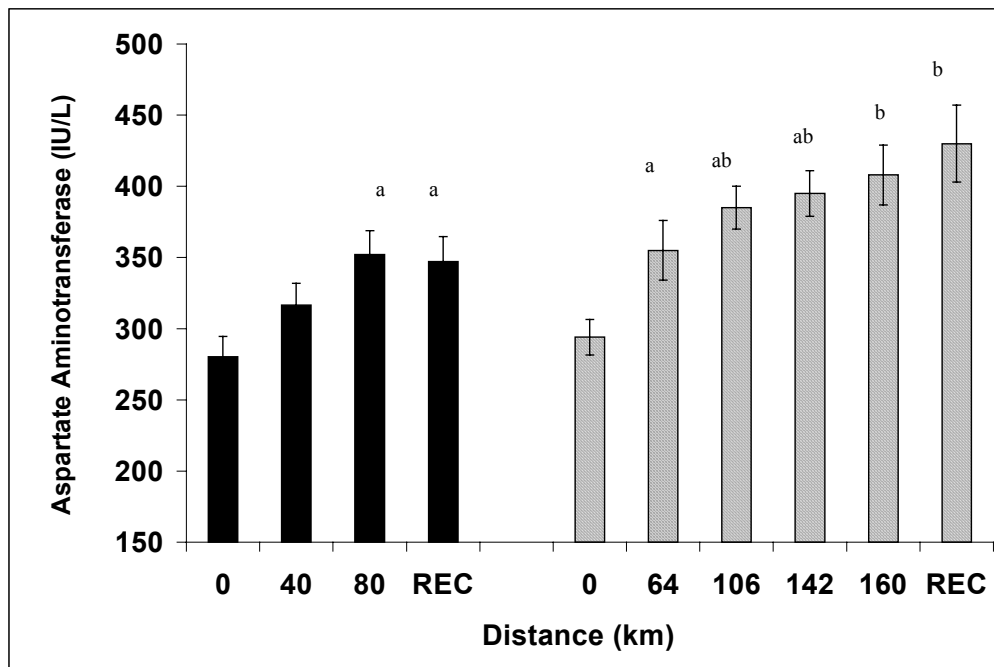


Figure 5.11

Plasma aspartate aminotransferase (IU/L) activity for horses that completed the 80 km (n = 12) endurance race (OD80, black bars) at 0, 40, 80 km and 60 min recovery (REC), and 160 km (n = 10) endurance race (OD160, hatched bars) at 0, 64, 106, 142, 160 km and REC. Bars are means, flags are standard errors of the mean. Means within a race with unlike letters are significantly different (P < 0.05). Experiment 5.2

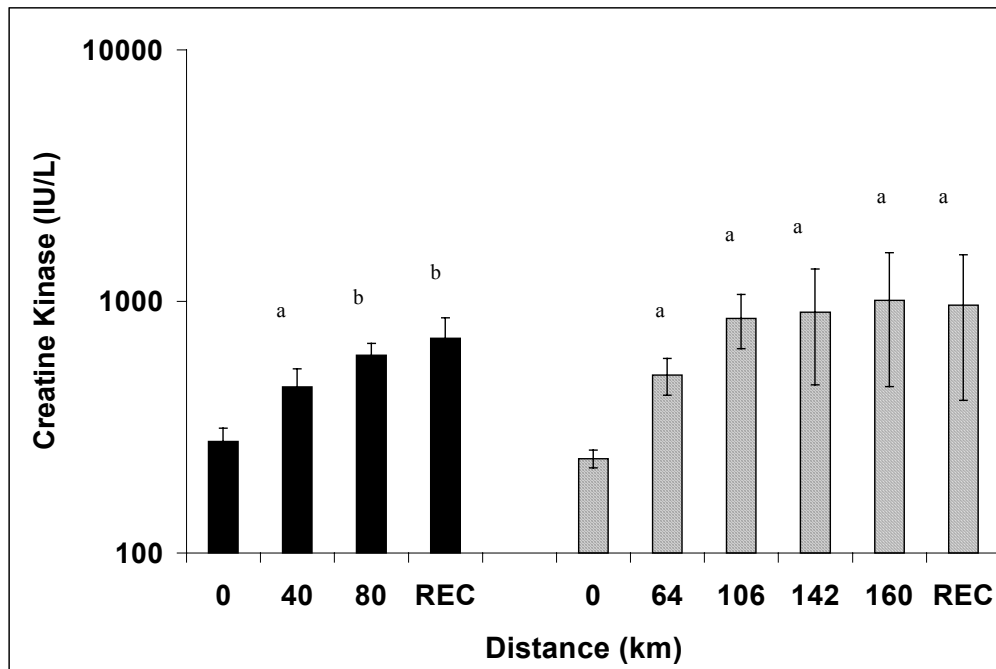


Figure 5.12

Plasma creatine kinase (IU/L) activity for horses that completed the 80 km (n = 12) endurance race (OD80, black bars) at 0, 40, 80 km and 60 min recovery (REC), and 160 km (n = 10) endurance race (OD160, hatched bars) at 0, 64, 106, 142, 160 km and REC. Bars are means, flags are standard errors of the mean. Means within a race with unlike letters are significantly different (P < 0.05). Experiment 5.2

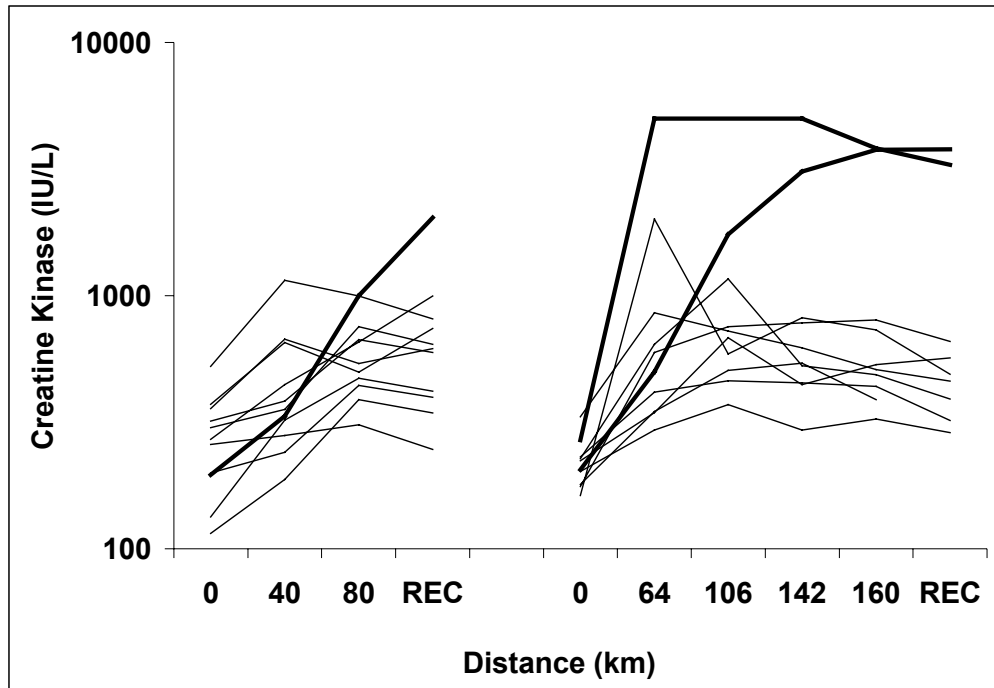


Figure 5.13

Plasma creatine kinase (IU/L) activity for individual horses that completed the 80 km (n = 12) endurance race (OD80, left side chart) at 0, 40, 80 km and 60 min recovery (REC), and 160 km (n = 10) endurance race (OD160, right side chart) at 0, 64, 106, 142, 160 km and REC. Three horses represented by heavier lines were outliers. Experiment 5.2

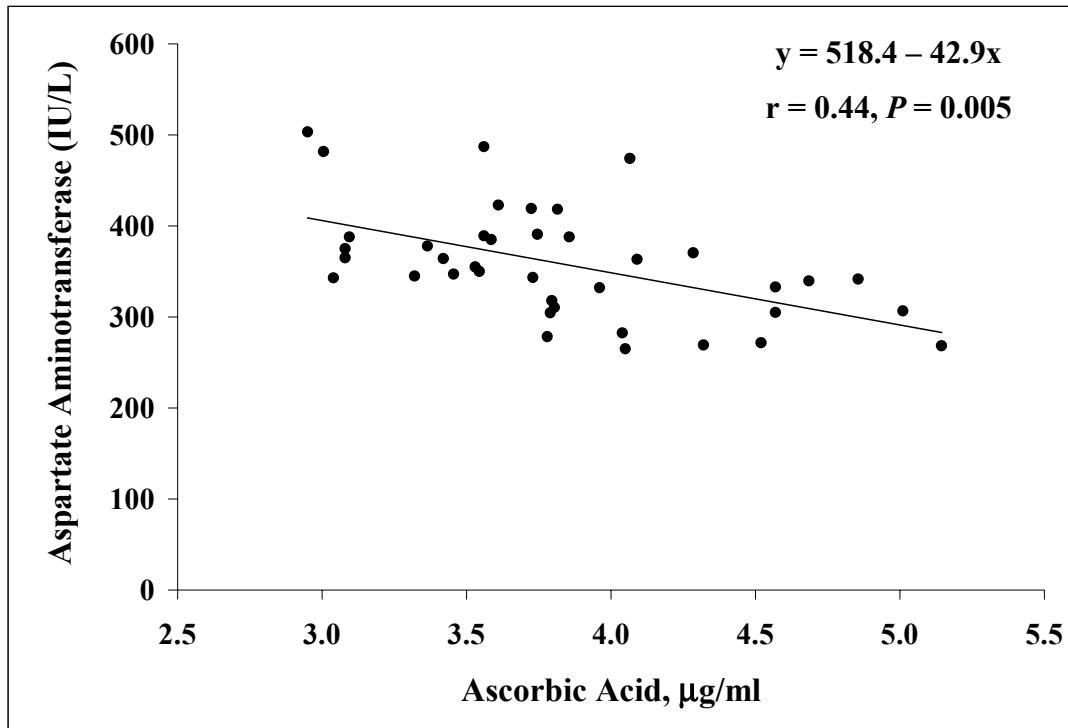


Figure 5.14

Regression ($y = a + bx$) of pooled data for plasma aspartate aminotransferase (IU/L) activities on plasma ascorbic acid ($\mu\text{g/ml}$) concentrations for horses that completed the Old Dominion 160 km endurance race. Experiment 5.2

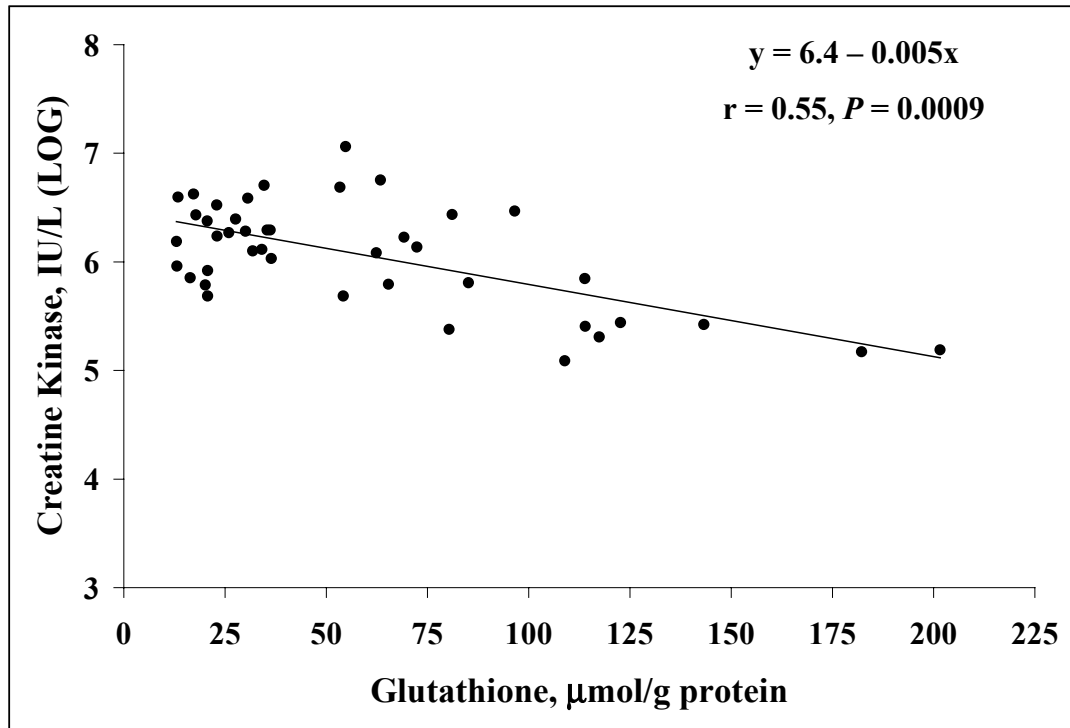


Figure 5.15

Regression ($y = a + bx$) of pooled data for plasma creatine kinase (IU/L) activities on erythrocyte glutathione ($\mu\text{mol/g}$) concentrations for horses that completed the Old Dominion 160 km endurance race. Creatine kinase data were logarithmically transformed. Experiment 5.2

APPENDIX

Appendix Table 5.1

Ride times (completion time minus hold times at veterinary check points) for horses completing the No Frills (NF80) 80 km and the Old Dominion (OD80) endurance races.

Ride Time (h)		
Horse	OD80	NF80
1	7:38:53	8:31:47
2	8:08:43	6:41:43
3	8:20:26	6:02:48
4	8:20:29	7:50:42
5	8:33:24	9:12:41
6	8:37:47	6:41:44
7	9:25:06	8:54:26
8	9:27:07	9:12:39
9	9:47:09	6:35:28
10	10:07:05	
11	10:07:40	
12	10:23:17	
13	10:23:26	
Mean	9:10:49	7:44:53

Appendix Table 5.2

Body weight (BW) of horses competing in the No Frills (NF80) 80 km and the Old Dominion (OD80) endurance races. Post race BW were not measured at NF80.

Horse	OD80		NF80
	Pre-Race BW (kg)	Post-Race BW (kg)	Pre-Race BW (kg)
1	395	376	391
2	376	359	436
3	512	-	436
4	522	510	410
5	473	434	404
6	384	362	390
7	483	439	519
8	510	489	410
9	489	473	379
10	470	448	436
11	410	393	410
12	524	-	410
13	458	-	
14	443	422	
15	436	405	
16	385	378	
17	445	422	
18	435	402	
Mean	453 ± 12	421 ± 12	419 ± 11

Appendix Table 5.3

Ride times (completion time minus hold times at veterinary check points) for horses completing the Old Dominion 80 km (OD80) and the 160 km (OD160) endurance races.

Horse	Ride Time (h)	
	OD80	OD160
1	7:38:53	18:01:41
2	8:08:43	18:01:42
3	8:20:26	18:37:27
4	8:20:29	19:40:50
5	8:33:24	19:40:51
6	8:37:47	19:40:52
7	9:25:06	20:52:18
8	9:27:07	20:52:19
9	9:47:09	20:54:43
10	10:07:05	20:54:44
11	10:07:40	
12	10:23:17	
13	10:23:26	
Mean	9:10:49	19:43:45

Appendix Table 5.4

Body weight (BW) of horses competing in the Old Dominion 80 km (OD80) and the 160 km (OD160) endurance races.

Horse	OD160	
	Pre-BW (kg)	Post-BW (kg)
1	488	462
2	388	-
3	457	435
4	429	-
5	446	422
6	456	438
7	515	-
8	459	432
9	452	-
10	402	393
11	441	-
12	479	461
13	434	-
14	398	369
15	432	413
16	446	-
17	461	-
Mean	446 ± 8	425 ± 10

VITA

Belinda Hargreaves was born on December 11, 1961 in Plymouth, Devon, U. K. She graduated from Uffculme School, Devon, U. K. in 1978. During the following 14 years she was a professional rider and trainer in Europe and America. She attended the University of Bristol, U. K. in 1992, majoring in Biology and Geography (an interdisciplinary program in environmental science), and received her Bachelor of Science Joint-Honors degree in July 1996. The title of her BS (Hons.) thesis was 'Effects of ambient temperature and relative humidity on responses of horses during an incremental submaximal field exercise test'. In 1997 she was awarded a WALTHAM Fellowship to pursue a Masters degree in Equine Nutrition in the Department of Animal and Poultry Sciences at Virginia Polytechnic Institute and State University. She received her Master of Science degree in December 1998. The title of her MS thesis was 'Fecal Kinetics and Digestibility of Hays and Supplements Estimated by Marker Methods in the Horse'. The Virginia Horse Industry Board awarded her a research grant in December 1998 to study vitamin E supplementation of grazing mares. In 1998 she was awarded a John L. Pratt Fellowship to pursue a doctoral degree in Equine Nutrition and Exercise Physiology for the Department of Animal and Poultry Sciences at Virginia Polytechnic Institute and State University. In 1999 she was awarded a second research grant by the Virginia Horse Industry Board to continue to study vitamin E supplementation of grazing mares. In 2000, she was awarded a research grant by the Virginia Horse Industry Board to study oxidative stress and antioxidant status of horses during endurance exercise.

Belinda J. Hargreaves

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