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Dr. D.L. Harmon, Director of Graduate Studies

THE INFLUENCE OF SELENIUM STATUS ON IMMUNE FUNCTION AND ANTIOXIDANT STATUS IN THE HORSE

DISSERTATION

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

By
Mieke Brummer
Lexington, Kentucky

Director: Dr. L.M. Lawrence, Professor of Animal Science
Lexington, Kentucky
2012

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ABSTRACT OF DISSERTATION

THE INFLUENCE OF SELENIUM STATUS ON IMMUNE FUNCTION AND ANTIOXIDANT STATUS IN THE HORSE

Selenium (Se) has received a lot of attention for its antioxidant and immune modulating properties. Yet, comparably few studies have focused on the horse. Therefore the objectives of this research were to evaluate the influences of Se status on immune function and antioxidant defense in horses. Twenty eight horses were allocated to one of 4 dietary Se treatments: low (LS), adequate (AS), high organic (SP) and high inorganic (SS). First, horses assigned to LS, SP and SS were depleted of Se and received a low Se diet (0.07 ppm Se) for 35 wk, while AS received an adequate Se diet (0.14 ppm Se). During week 28 to 35 immune function was evaluated using a vaccine challenge with keyhole limpet hemocyanin (KLH) and equine influenza as antigens. Then, a 29 wk repletion phase followed. The LS and AS received the same diets described above while SP received an organic Se supplemented diet (0.3 ppm; Sel-Plex, Alltech, Nicholasville, KY) and SS an inorganic Se supplemented diet (0.3 ppm; sodium selenite). Immune function was assessed using a vaccine challenge with ovalbumin (OVA) and equine influenza as antigens during week 22 to 29. Samples collected throughout the depletion and repletion phases were used to assess change in Se status, antioxidant status and oxidative stress. Finally, a mild exercise test served to assess exercise induced oxidative stress. The experimental model responded as hypothesized, evaluated by blood Se and glutathione peroxidase (GPx) activity. Upon vaccination with KLH, antibody response was faster in AS than LS. Antigen specific mRNA expression of T-bet was also higher for AS than LS. Following OVA vaccination humoral and cell-mediated vaccination responses were similar across treatments. However, non-specific stimulation of peripheral blood mononuclear cells indicated suppressed mRNA expression of selected cytokines for LS compared to AS, SP and SS. Antioxidant capacity and oxidative stress were unaffected by change in Se status. A difference in GPx response post exercise was also noted between SP and SS. Low Se status impaired some measures of immune function. Supplementation at 0.3 ppm may benefit horses as indicated by higher GPx activity in idle and exercised horses.

Key words: Equine, keyhole limpet hemocyanin, ovalbumin, glutathione peroxidase, oxidative stress

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July 2012
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THE INFLUENCE OF SELENIUM STATUS ON IMMUNE FUNCTION AND ANTIOXIDANT STATUS IN THE HORSE

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FREQUENTLY USED ABBREVIATIONS

Abbreviations relating to selenium and antioxidant measures

AST - Aspartate amino-transferase

CBC – Complete blood count

CK – Creatine kinase

GPx - Glutathione peroxidase

ID - Iodothyronine deiodinases

H_2O_2 – Hydrogen peroxide

MDA – Malondialdehyde

ROS – Reactive oxygen species

Se - Selenium

Se-Cys - Seleno-cysteine

Se-Met - Selenomethionine

T3 - Triiodothyronine

T4 – Thyroxine

TAC – Total antioxidant capacity

TBARS – Thiobarbituric acid reactive substances

TrxR - Thioredoxin reductase

Abbreviations relating to immunology

CMI – Cell-mediated immune response

Con A – Concanavalin A

ELISA - Enzyme-linked immunosorbent assay

GATA3 – Transcription factor, GATA-binding protein 3

HA titer – Hemagglutination titer

IgG – Immunoglobulin G

IFNγ – Interferon

IL- Interleukin

KLH – Keyhole limpet hemocyanin

OVA - Ovalbumin

PBMC – Peripheral blood mononuclear cells

PHA - Phytohemagglutinin

PMA - Phorbol 12-myristate 13-acetate

RBC – Red blood cell

SRBC – Sheep red blood cell

T-bet – Transcription factor, T-box expressed in T-cells

Th – T-helper cell

TNFα – Tumor necrosis factor alpha

CHAPTER 1: Introduction

Selenium (Se) was recognized as an essential trace mineral in 1957 by Schwarz and Foltz who demonstrated that Se prevented necrotic liver degeneration in rats (Schwarz and Foltz, 1957). They also determined that organic Se could be replaced with inorganic Se salts. Finally, the authors hypothesized that Se could play a part in oxidative metabolism in the liver (Schwarz and Foltz, 1957). Since, Se has been shown to not only be involved in the antioxidant mechanism of the body (Hargreaves et al., 2002), but has also been shown to play a role in immune function (Beck et al., 2001). One of the unique characteristics of Se is the fact that it can be incorporated into proteins. Incorporation into proteins is either non-specific as Se-methionine (Se-Met) into organs and skeletal muscles where it contributes to the Se-stores of the body (Schrauzer, 2000), or specific as Se-cysteine (Se-Cys), during the synthesis of the selenoproteins (Papp et al., 2007). Approximately 25 selenoproteins have been identified, although not all of their functions have been fully characterized (Surai, 2006). It is via these selenoproteins that Se is thought to exert its effects on both the antioxidant (Hargreaves et al., 2002) and the immune system (Goldson et al., 2011).

Because soil Se content tends to vary and the Se content of forages and plants depend on the soil Se content, dietary Se intake can be varied. As a consequence many areas, such as central Kentucky, are marginal in Se (Kubota et al., 1967). Without supplementation the Se status of horses kept in these areas will decline over time.

Selenium is believed to contribute to the antioxidant mechanism of the body via the selenoenzyme glutathione peroxidase (GPx), which functions to neutralize hydrogen peroxide and protect against oxidative damage (Battin and Brumaghim, 2009). Glutathione peroxidase activity as well as serum Se concentration has been shown to be altered post exercise, hypothesized to be indicative of the role of Se in combating exercise induced oxidative stress (Akil et al., 2011; White et al., 2011).

Selenium has also been shown to be important for the optimal functioning of the immune system, although the exact mechanism through which Se and the immune system interact remains unknown. In studies with mice, Se status has been shown to affect the mRNA expression of some immune modulating cytokines (Li and Beck, 2007) and cytokine receptor, e.g. interleukin-2 receptor (Roy et al., 1994). Alteration in lymphocyte proliferation has also been linked with Se status (Roy et al., 1994). Studies investigating the effect of Se on the equine immune system are limited (Knight and Tyznik, 1990; Janicki, 2001; Thorson et al., 2010) and the relationship of Se status to immune related cytokines in the horse has not been investigated.

Currently the NRC (2007) recommends a dietary Se intake of 1 mg/d for a 500 kg mature, idle horse. The research conducted by Janicki (2001) indicated that a higher Se intake of 3 mg/d by broodmares may be beneficial for immune function of their foals. Improved immune function through appropriate Se supplementation can be of benefit to the horse industry, especially if greater protection from exercise induced oxidative stress can be attained at the same time.

The primary hypothesis was that Se status would affect immune function and antioxidant status. To test this hypothesis the first objective of this current research was to evaluate the effect of Se status on oxidative stress and antioxidant status as horses were first depleted of Se, then repleted using two different sources of Se. The second objective was to determine if Se status affected the ability of the immune system to respond to both a novel and previously administered vaccine. To investigate these objectives a three phase experiment was conducted over a period of 18 mo.

Phase 1: The aim of phase 1 was to determine the effect of a low Se diet on indicators of Se status, oxidative stress and immune function.

Phase 2: The aim of phase 2 was to determine if Se repletion with either organic or inorganic Se would result in changes in oxidative stress, antioxidant variables and immune function as assessed by means of a vaccine challenge.

Phase 3: The aim of the final phase was to evaluate the effect of Se status on oxidative stress and indicators of muscle damage in response to mild exercise.

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CHAPTER 2: Literature Review

Soil selenium distribution

Herbivores rely on plants to meet their selenium (Se) requirements (Montgomery et al., 2011). However, the Se content of plants tends to reflect the amount and bioavailability of Se in the soil (Ferguson and Karunasinghe, 2011), with the exception of some plants that are capable of accruing Se such as the *Astragalus* species (Whanger, 2002; Montgomery et al., 2011). Geographically, soil Se concentration and bioavailability can be diverse. Alkaline soils tend to have a higher Se bioavailability and therefore better Se absorption by plants, while acidic soils, especially when combined with high rainfall, tend to have poor Se bioavailability (MacDonald et al., 1976). Acidic soils are therefore often associated with Se deficient pastures and crops (MacDonald et al., 1976). Similarly, low soil Se bioavailability has been reported in areas prone to acid rain (Brown and Arthur, 2001). Regions known to be low in Se include areas in the Eastern United States, New Zealand, North East China (Brown and Arthur, 2001), parts of Europe, Egypt (Montgomery et al., 2011) and parts of South Africa (Van Ryssen, 2001).

Dietary Se intake by humans is thought to be insufficient in many areas around the world (Hesketh, 2008). Dietary Se intake is reported to be influenced by variability in soil Se, resulting in variation in the Se status of humans (Brown and Arthur, 2001; Ferguson and Karunasinghe, 2011) as well as grazing herbivores (Van Ryssen, 2001). For instance, it is hypothesized that the Se status of humans in Europe have declined due

to less wheat being imported from the USA (Brown and Arthur, 2001). Several factors are involved and affect the final *in vivo* effect of Se including the amount consumed, the extent of digestion, absorption, how it is metabolized, and eventually how much is excreted (Ferguson and Karunasinghe, 2011).

Selenium supplementation

Studies showing a relationship between dietary Se and the activity of the Se containing protein (selenoprotein) glutathione peroxidase (GPx) introduced the concept of improving functional Se status by means of dietary Se supplementation (Brown and Arthur, 2001). The functional response to Se supplementation is not always observed, which could be attributed to sufficient endogenous Se supply or the inability to activate the protein of interest (Brown and Arthur, 2001). Recently, it has also been determined that humans may have single-nucleotide polymorphisms that can affect the way in which micronutrients such as Se are metabolized by different persons (Méplan et al., 2007; Hesketh, 2008).

Many different dietary forms of Se supplementation exist, ranging from Se-enriched foods via Se-fertilization of crops to the direct consumption of Se supplements (Brown and Arthur, 2001; Montgomery et al., 2011). Se supplementation is normally provided via the direct addition of Se to the concentrate portion of the animal's diet. Alternatively, it can be provided in the form of a selenized salt block (Hintz, 1999). The source of dietary Se supplementation can either be inorganic or organic. Inorganic Se is normally in the form of a mineral salt such as sodium selenite (Na_2SeO_3) or sodium

selenate (Na_2SeO_4). Selenomethionine (Se-Met) is a well-known organic form of Se (Juniper et al., 2008). Generally, Se-Met is the main form of Se found in Se enriched grains as well as selenized yeast (Whanger, 2002). Selenized yeast products are currently used in the manufacture of organic Se supplements. The production of selenized yeast requires the aerobic fermentation by *Saccharomyces cerevisiae* in Se enriched beet or cane sugar (Rayman, 2007). Different companies tend to use their own specific strains of this yeast. The final yeast cream end product of this fermentation process is pasteurized and dried, and at least 90% of the Se should be bound to the yeast or in the complexed organic form (Rayman, 2007).

Selenium metabolism

Vendeland et al. (1992) conducted a study with rats, investigating the intestinal absorption of different forms of Se (Se-Met, selenite and selenate). The researchers found that although all forms of dietary Se were absorbed in the small intestine (SI), some forms of Se were more readily absorbed (and at different rates) in specific sections of the SI compared to others. Overall, Se-Met was reported to be most rapidly absorbed. When comparing the inorganic forms, selenate was found to be more efficiently absorbed in the distal portions of the small intestine while selenite absorption in the duodenum exceeded that of selenate (Vendeland et al., 1992). A potential reason for these differences may lie in the transport of Se across the intestine. Selenate is actively transported by sodium dependent transporters localized in the distal portion of the small intestine (Vendeland et al., 1992), while selenite is passively absorbed via simple

diffusion throughout the small intestine (Surai, 2006). Absorption of Se-Met occurs via sodium-dependent amino acid transporters (Schrauzer, 2000).

Once absorbed the different sources of Se are not metabolized in the exact same way. Deagen et al. (1987) compared the metabolism of seleno-cysteine (Se-Cys), Se-Met and sodium selenite in mature rats. They reported that dietary Se-Cys was degraded by Se-Cys-lyase to alanine and hydrogen selenide in the liver. Once in the selenide form it followed the exact same metabolic pathway as sodium selenite. Se-Met on the other hand was found to increase tissue Se levels beyond that of Se-Cys or sodium selenite. It was hypothesized that the elevated tissue levels were due to the fact that Se-Met could substitute for Met during protein synthesis, allowing for non-selenoprotein incorporation of Se-Met (Deagen et al., 1987). Studies conducted in species such as pigs (Mahan and Peters, 2004), broilers (Payne and Southern, 2005), cattle (Juniper et al., 2008) and catfish (Wang and Lovell, 1997) have all reported higher tissue Se levels when organic sources of Se were used.

The basic Se metabolism pathway is illustrated in Figure 2.1 (Combs, 2001; Papp et al., 2007; Rayman, 2007; Hesketh, 2008). Once absorbed, Se is transported to the liver (Wichtel, 1998). Se-Cys, whether of dietary origin or derived from Se-Met metabolism, is degraded and converted to selenide (H_2Se) in the liver (Schrauzer, 2000). Similarly, selenate and selenite are reduced to selenide. Selenide will either be used for the production of selenoproteins, or will be excreted via breath (dimethyl selenide; $(CH_3)_2Se$) or urine (trimethyl selenonium ion; $(CH_3)_3Se^+$) as depicted. As stated earlier, excess Se-Met that is not metabolized after absorption can be non-specifically incorporated into

organs such as liver and kidneys or skeletal muscle where it contributes to the body's Se storage pool (Schrauzer, 2000).

The synthesis of selenoproteins from selenide involves several steps. Se-Cys is synthesized directly on the tRNA using selenide, ATP and seryl-tRNA (Hesketh, 2008). Seryl-tRNA$^{[SER]SEC}$ is converted to phosphoseryl-tRNA$^{[SER]SEC}$ by the enzyme phosphoseryl tRNA kinase. Phosphorus is then replaced by Se donated by selenide, a process initiated by selenophosphate synthetase (Papp et al., 2007) and selenocysteine synthetase to transform selenophosphate to selenocysteyl-tRNA (Sec-tRNA$^{[SER]SEC}$) (Hesketh, 2008). Se-Cys is normally incorporated at the functional site of the selenoprotein (Brown and Arthur, 2001). The step of Se incorporation occurs during translation by means of a modified UGA codon, directed by a Se-Cys-insertion-sequence (SECIS) (Lei et al., 2007). The fact that Se-Cys incorporation takes place during translation has caused some controversy regarding the significance of measures of mRNA levels of specific selenoproteins (Hesketh, 2008). Based on its role in selenoprotein synthesis, selenophosphate synthetase has been considered a potential regulator of Se metabolism, controlling the incorporation of Se-Cys into selenoproteins and therefore preventing excessive selenoprotein production, as well as potentially protecting against toxicity (Brown and Arthur, 2001).

Selenoproteins

Of the selenoproteins that have been functionally characterized, approximately half are believed to play a role in the antioxidant mechanism (Ferguson and

Karunasinghe, 2011). The most thoroughly characterized selenoproteins include glutathione peroxidase (GPx), thioredoxin reductase (TrxR), selenoprotein-P (Se-P) and iodothyronine deiodinase (ID) (Hesketh, 2008).

Glutathione peroxidase is a selenoenzyme associated with the antioxidant mechanism by means of regulation of hydroperoxide levels inside the cell (Arthur, 1997; Ferguson and Karunasinghe, 2011). As depicted in Figure 2.2, the function of GPx is to reduce hydrogen peroxide to water using glutathione as a donor of reducing equivalents. (Urso and Clarkson, 2003). Glutathione peroxidase has a substrate specificity for glutathione (Surai, 2006). To maintain glutathione levels in the cell the oxidized glutathione disulfide has to be recycled by glutathione reductase, using NADPH as substrate (Urso and Clarkson, 2003). GPx was one of the very first selenoproteins identified and used as an indicator of Se status (Brown and Arthur, 2001) as GPx activity has been shown to respond to dietary Se supplementation (Surai, 2006). In addition to its role in providing protection against hydroperoxide damage, GPx may also be considered as a storage pool of Se because each GPx enzyme contains 4 Se-Cys residues (Brown and Arthur, 2001). Several different forms of GPx have been identified based on its location and function. These forms include GPx-1 (intracellular); GPx-2 (gastrointestinal); GPx-4 (cell membrane associated and acts on phospholipid hydroperoxide as well as functioning in the sperm capsule); and GPx-3 (plasma) (Brown and Arthur, 2001).

Thioredoxin reductase (TrxR) is involved in the redox system of the cell (Brown and Arthur, 2001). It functions within a system that consists of thioredoxin, thioredoxin peroxidase and TrxR, TrxR being the only selenoprotein in this system. The thioredoxin system can also oxidize glutathione, thereby forming a link between the thioredoxin and

glutathione systems (Surai, 2006). The thioredoxin system regulates transcription factors and provides reducing equivalents for DNA synthesis and regeneration of ubiquinol-10 that helps to prevent peroxidation of lipids in the cell (Ferguson and Karunasinghe, 2011). It also plays a role in apoptosis and modulation of protein phosphorylation via activation of protein kinases (Surai, 2006).

Iodothyronine deiodinase (ID) is a selenoenzyme responsible for the conversion of the prohormone thyroxine (T4) to its active form, triiodothyronine (T3) (Brown and Arthur, 2001; Calamari et al., 2009b; Muirhead et al., 2010). Thyroid hormones are essential for health as these hormones not only affect metabolism but also affect growth, development and differentiation (Surai, 2006). Calculating the ratio between T3 and T4 is thought to be a functional indicator of ID and possibly an indicator of Se status (Brown and Arthur, 2001; Calamari et al., 2009b).

The remaining selenoproteins are less well characterized on a functional basis. Selenoprotein-P (Se-P) seems to be responsible for Se transport (Ferguson and Karunasinghe, 2011). It is estimated that approximately 60% of plasma Se is carried by Se-P, as it can carry 10 Se atoms (Brown and Arthur, 2001). Another selenoprotein of interest is selenoprotein-S (Se-S). It functions in the membrane of the endoplasmic reticulum (ER), and is thought to play an important role in protein folding (Hesketh, 2008). Selenoprotein-W is less well characterized in terms of function but is believed to play a role in muscle metabolism (Brown and Arthur, 2001), as it appears to be more abundant in skeletal and cardiac muscle than other organs (Surai, 2006). Recently, it was reported that selenoprotein K is an ER-localized membrane protein, potentially involved in calcium flux in cells (Verma et al., 2011). Other selenoproteins that have been

identified but not yet functionally characterized include selenoproteins -L, -N, and -M, all thought to be associated with redox function (Hesketh, 2008).

Selenium supplementation and the horse

Selenium toxicity and deficiency

A concern regarding Se supplementation is toxicity, whether acute or chronic. Se toxicity is also referred to as alkali disease or blind staggers (Stowe, 1998). The first case on record of Se toxicosis was in 1856 in Madison, Nebraska (Underwood, 1962). Since, the identification of areas known to be high in Se has decreased the number of Se toxicity cases in both horses and cattle (Hintz, 1999). Chronic toxicosis symptoms include cracked hooves, ataxia and hair loss from mane and tail (Hintz, 1999), while acute signs of toxicity typically involve some neurological signs, including hyper-excitability, ataxia, sweating, shortness of breath, fever and in some cases death (Desta et al., 2011). A much publicized case of acute Se toxicity in horses was the 2009 polo pony case in which 20 polo ponies died within 6 h after the parenteral administration of a vitamin/mineral mix (Desta et al., 2011). During an attempt to reproduce a commercial vitamin-mineral supplement (Biodyl®, Merial), a calculation error resulted in the accidental intravenous administration of approximately 2 g of Se as sodium selenite to each horse (Desta et al., 2011). The lethal injectable amount of Se has been reported to be 1.49 mg/kg BW or 0.75 g / 500kg horse (Néspoli et al., 2001).

Cases of chronic dietary Se toxicosis also exist. Horses kept in a feedlot in Nebraska that were provided with excessively high Se hay exhibited signs of toxicosis.

Removing the hay allowed for full recovery without any recorded deaths (Stowe, 1998). Dietary Se supplementation at appropriate levels is thought to be safer than the parenteral administration of Se, as many unfavorable reactions have been reported with the use of injectable Se in other species (Stowe, 1998). Hintz (1999) stated that the actual mechanism of toxicity is not well understood, but thought to relate to oxidative metabolism in cells. According to the NRC (2007) the maximum tolerable level of dietary Se for horses is 5 mg/kg DM, although earlier versions of the NRC estimated that it may be closer to 2 mg/kg DM (NRC, 2007).

Although Se toxicity can occur, Se deficiency can be a problem in geographically low Se areas, as discussed earlier in this review. Low serum Se concentration is frequently used as an indicator of a Se status, but controversy exists as to whether a deficiency can be diagnosed on serum Se concentration alone when no other symptoms are present (NRC, 2007). White muscle disease has been associated with foals of low Se status (Muirhead et al., 2010). White muscle disease is also a problem in other young livestock animals such as calves and lambs, and Se deficiency has been identified as the contributing factor (Underwood, 1962). In addition, Se deficiency in other livestock species has in general been associated with nutritional myopathy, Se-responsive diseases such as muscular weakness in new-borns, un-thriftiness, weight loss, and diarrhoea (Koller and Exon, 1986).

Selenium requirements

The first investigation into the Se requirements of the horse was conducted in 1967 by Stowe et al. at the University of Kentucky. The research involved the analysis of 542 serum samples obtained from experimentally fed (both oral and parenteral Se supplementation) horses on the research farm, as well as samples from Thoroughbred and Standardbred mares, foals, weanlings, and yearlings from horse farms in the area, as well as Thoroughbreds in training in Lexington, KY and New York. Stowe (1967) noted a tendency for Se supplemented foals to have a higher average daily gain in comparison to the Se deficient foals. From this research it was concluded that a horse required an oral Se intake of 2.4 µg/kg BW per day (1.2 mg / 500 kg horse per day) (Stowe, 1967). Interestingly, Stowe (1967) also found foals to have a lower Se status in comparison to their dams and hypothesized on the existence of a Se-dependent microbial organism that hindered the uptake of milk Se or a component in the milk that affected Se absorption. Similarly, a study that evaluated the Se status of mares and foals under normal management conditions reported that foals had lower Se levels compared to their dams, but mares of higher Se status had foals of higher Se status (Lee et al., 1995). The authors also concluded from their study that whole blood GPx activity was a more accurate long term indicator of Se status than whole blood Se.

The research by Stowe (1967) was followed by that of Shellow et al. (1985), also conducted at the University of Kentucky. Shellow et al. (1985) conducted a depletion-repletion study using 4 different levels of sodium selenite over a 12 week period. Prior to the start of the repletion period the horses were kept on a low Se basal diet (0.06 ppm Se). The researchers reported that plasma Se levels reached a maximum response by 35 d of

repletion. There were no differences between feeding 0.16 or 0.26 ppm Se, and the plateau was observed within the same period of time as reported by Stowe et al. (1967). In addition the maximum whole blood response was observed at 6 wk, with no additional benefit when feeding more than 0.11 ppm Se (Shellow et al., 1985). The slower whole blood response was attributed to the slow red blood cell (RBC) turn-over of the horse which can be from 140 to 160 d (Shellow et al., 1985). The lack of plasma GPx response observed led to the conclusion that the basal diet or endogenous stores provided enough Se to maintain GPx activity, and therefore there was no advantage in supplementing mature idle horses with Se levels greater than 0.1 ppm (Shellow et al., 1985). More recently the reference range for serum Se levels in an adult horse has been established at 130 – 160 ng/mL (Stowe and Herdt, 1992). The reference range for whole blood Se is 180-240 ng/mL with whole blood GPx activity being in the range of 40 – 160 EU/g hb (Stowe, 1998).

Current dietary Se recommendations:

Earlier versions of the NRC have referred to mineral requirements based on a specific dietary concentration sufficient to prevent adverse effects on the animal. The 2007 version of the NRC expresses mineral requirements as a daily amount of the particular mineral per kg body weight required by a particular physiological class based on estimated feed intakes. This change was implemented in an attempt to more clearly define mineral requirements in the horse (NRC, 2007). The current Se requirements for a 500 kg horse is 1 mg per day, and includes horses that are idle, in light exercise, breeding

and non-breeding stallions, as well as pregnant mares (NRC, 2007). This estimated requirement increases to 1.13 mg per day and 1.25 mg per day for horses in moderate to heavy exercise respectively, and 1.25 mg per day for a lactating mare (NRC, 2007). The Se requirement for growing animals starts at less than half of its mature Se requirement and increases over time until the age of 2 years when the daily requirement would be similar to that of the mature animal (NRC, 2007). The FDA permits a total dietary Se intake of 0.3 mg/kg DM (Karren et al., 2010), and keeping in line with that some feed manufacturers produce concentrate feed containing approximately 0.6 mg Se/kg DM to allow for a total dietary intake of 0.3 mg Se/kg DM (Stowe, 1998) when used in combination with low Se forage. Similarly Hintz (1999) stated that using concentrates containing 0.1 mg Se/kg DM may not provide sufficient amounts of Se to meet the Se requirement of horses kept in deficient areas.

Effect of Se source on response variables

The initial studies conducted to establish the Se requirements of the horse used sodium selenite as the source of Se (Stowe, 1967; Shellow et al., 1985). More recently the use of organic Se supplements in horse diets have received a lot of interest and several studies have been conducted using organic sources of Se (Pagan et al., 1999; Janicki, 2001; Richardson et al., 2006; Calamari et al., 2009a; Calamari et al., 2009b; Calamari et al., 2010; Karren et al., 2010; Thorson et al., 2010).

Richardson et al. (2006) compared the effects of an organic source of Se (Zn-L-selenomethionine) fed at 5.1 mg Se/d to that of sodium selenite fed at 4.7 mg Se/d and

included a control group receiving 1.3 mg Se/d. They used eighteen 18 mo old American Quarter Horse and American Paint Horses. The study was conducted over a period of 56 d, preceded by a 28 d adaptation period on the control diet only. A response to supplementation was observed within 28 d as measured by plasma Se, indicating the sensitivity of plasma Se concentration to dietary intake (Richardson et al., 2006). A trend existed for higher plasma Se concentration at 28 d in horses receiving organic Se when compared to inorganic, indicative of a faster response to supplementation. However, over the entire 56 d supplementation period the response to supplementation was similar regardless of Se source (Richardson et al., 2006). Plasma GPx activity was not affected by Se supplementation or source. Middle gluteal muscle samples indicated an increase in Se concentration over time, but this increase was regardless of dietary Se source or intake (Richardson et al., 2006). Red blood cell GPx activity increased faster within the first 28 d in the organic supplementation group than the inorganic group. This response was attributed to a single horse, although not a statistical outlier (Richardson et al., 2006). However, at d 56 there was no difference in GPx activity regardless of treatment. Muscle GPx activity decreased in all treatment groups over time, and although not fully explained was thought to indicate that factors other than Se may play a role in muscle GPx activity (Richardson et al., 2006). White et al. (2011) looked at similar variables, in horses fed sodium selenite at either 0.1 or 0.3 mg/kg DM, even though the supplementation period of 34 d was much shorter. These authors reported an increase in plasma Se, but plasma, RBC, muscle GPx and thioredoxin reductase activity remained unaffected by supplementation (White et al., 2011).

Calamari et al. (2007, 2009a, 2009b, 2010) published several reports from a study with lightly exercised mature horses. The study consisted of a 112 d supplementation period followed by a 112 d withdrawal period. The treatment groups included a low Se control group (0.085 mg/kg DM), 3 Se yeast treatment groups receiving 0.2, 0.3 or 0.4 mg/kg DM, and a sodium selenite treatment group receiving 0.3 mg/kg DM for comparison. The horses responded within 28 days to Se supplementation as measured by whole blood and plasma Se concentration. While plasma Se levels plateaued within 75 d to 90 d, the same plateau was not observed across all treatments for whole blood Se concentration (Calamari et al., 2009b). The researchers reported that the Se yeast treatment groups displayed a faster whole blood Se response in comparison to the sodium selenite group. The Se yeast treatment groups also had higher whole blood and plasma Se-Met levels when compared to the other groups (Calamari et al., 2009a). However, Se source did not affect whole blood GPx activity. A correlation of 0.86 ($P < 0.001$) existed between whole blood Se and GPx activity (Calamari et al., 2009b). Although whole blood GPx activity increased in the supplemented groups, it did not reach a plateau over the 112 d experimental period, leading to the conclusion that the supplementation period was too short to accommodate red blood cell turn over (Calamari et al., 2009b). These results were similar to those reported by Richardson et al. (2006). Upon withdrawal of the supplement (Calamari et al., 2007), GPx activity declined over time. The decline was slower than anticipated and after a 112 d withdrawal period GPx activity was still higher in the supplemented groups compared to the control. This slow decline was also attributed to the slow equine red blood cell turnover of 140-150 d (Calamari et al., 2007). When calculating the rate of GPx activity decline it was found to be greater in the horses

receiving the sodium selenite supplement at 0.3 mg/kg DM, than in the horses receiving selenized yeast at 0.2 or 0.3 mg/kg DM. The authors concluded that organic Se is potentially a better source of Se in terms of maintaining GPx activity during periods of deficiency (Calamari et al., 2007).

A study was conducted investigating the effect of plane of nutrition as well as Se-Met supplementation (0.3 mg/kg DM) in the form of Se yeast on broodmares and their foals (Karren et al., 2010). The 4 treatment groups consisted of mares kept on a pasture-only diet (0.19 mg Se/kg DM), a pasture and Se supplement diet (0.49 mg Se/kg DM), a pasture and grain diet (0.35 mg Se/kg DM) or a pasture, grain and Se supplement diet (0.65 mg Se/kg DM). All diets provided more than the recommended amount of Se. Se supplementation was provided for the final 110 d of pregnancy and was ended at parturition. Blood samples were obtained from the broodmares every 14 d until parturition, and then from the foals prior to nursing and every 14 d until they were 56 d old (Karren et al., 2010). Middle gluteal muscle biopsies were taken every 28 d prior to parturition from the broodmares and from foals when they were 12 h, 28 and 56 d old. Although plasma GPx activity remained similar, plasma, colostrum and muscle Se concentrations were greater in mares receiving Se supplementation compared to the un-supplemented groups (Karren et al., 2010). Foals from supplemented mares reportedly had greater plasma and muscle Se concentrations, yet plasma GPx activity was found to be similar when compared to foals from un-supplemented mares.

Pagan et al. (1999) compared the digestibility of an organic selenized yeast (Sel-plex; 0.4 ppm Se) supplement to that of an inorganic source, sodium selenite (0.41 ppm Se) supplement in exercising thoroughbreds. The selenized yeast supplement was found

to be more digestible than sodium selenite (57.3% vs. 51.1%) and had a higher level of retention (Pagan et al., 1999). Urinary Se excretion was not affected by Se source, except for immediately post exercise, when urinary Se excretion was higher in the sodium selenite supplemented horses (Pagan et al., 1999).

Table 2.1 contains a summary of studies that investigated the effect of Se source or dose on peripheral blood variables used as indicators of Se status in horses. Not all of the studies fed the same amounts of Se or measured every indicator presented in the table, and there is a large amount of variation in the response data. The sensitivity of the enzyme GPx to storage time and temperature of assay conditions does make it difficult to compare absolute values of GPx activity across studies. However, the absolute concentration of Se measured in blood or plasma should allow for comparison across studies. The length of supplementation can also potentially affect study outcomes as plasma Se is thought to be more sensitive to change in dietary Se intake, while whole blood Se and GPx activity should give an indication of long term Se status (Shellow et al., 1985).

The effect of selenium on the immune system

The effect of Se on the immune system has received a lot of attention over the years and research interest has focused on human and animal models. Se has been shown to affect both innate and acquired components of the immune system (Arthur et al., 2003).

The immune system

The immune system can be divided into two major components: The non-specific innate immune system and the highly specific adaptive immune system. The innate immune system functions as the first line of defense. It includes anatomic barriers (skin, mucus membranes), physiological barriers (body temperature, low stomach pH, lysozymes in tears, complement system), phagocytic barriers (neutrophils, macrophages) and inflammatory barriers (acute phase proteins, kinins) (Goldsby et al., 2000; Tosi, 2005). The adaptive immune response involves B- and T- lymphocytes and is capable of detecting antigens by means of antigen specific receptors (Iwasaki and Medzhitov, 2010). Although different, the innate and the adaptive immune components work together to eliminate invading pathogens.

The adaptive immune response can be further subdivided into the humoral and the cell-mediated immune (CMI) components. The humoral immune response is associated with antibody production and involves the B-lymphocytes or B-cells. The CMI response involves T-lymphocytes or T-cells and in contrast to B-cells, antigen presentation via antigen presenting cells (e.g. dendritic cells) is required for activation of the T-cells. While both T- and B-lymphocytes are key to the defense against extracellular pathogens, T-lymphocytes are critical for immunity to intracellular pathogens. T-lymphocytes include both T-helper (Th; CD4) and cytotoxic T cells (CTL's; CD8). While Th cells produce cytokines that stimulate and activate B-cells, CTL's, macrophages etc, CTL's have the ability to destroy intracellular pathogen infected cells (Goldsby et al., 2000; Calder, 2007).

Communication between the different components of the immune system is of utmost importance. One of the major forms of communication is through the cytokines (Calder, 2007). Cytokines are proteins that regulate immune cells by binding cytokine receptors present on cell surfaces (Calder, 2007). Cytokines regulate many stages of the immune response, including the activation, proliferation and differentiation of effector cells (Blecha, 1988; Horohov, 2003). Individual cytokines may have multiple biological functions (Horohov, 2003) and can act in an autocrine, paracrine or endocrine manner (Goldsby et al., 2000). In general, the adaptive immune system is thought to function by mounting different types of responses. One type of response is called a T-helper 1 (Th1) response. It is initiated by interleukin (IL) –12, and involves the cytokines IL-2, interferon γ (IFN γ) and tumor necrosis factor α (TNFα). The Th1 response is associated with CMI responses (Horohov, 2003), and plays a central role in defense during intracellular virus and bacterial invasion (Kaiko et al., 2008). A T-helper 2 (Th2) response is initiated by IL-1β and is associated with the cytokines IL-4, IL-5 and IL-13 (Horohov, 2003). Upon recognition of a specific antigen the decision of the immune system to promote a Th1 or a Th2 type of response results in the activation of the appropriate transcription factors. T-bet is a transcription factor associated with the activation of a Th1 response while the transcription factor GATA-3 is associated with a Th2 response (Chinen et al., 2006). In addition cytokines are also classified as pro- or anti-inflammatory (Opal and DePalo, 2000). Cytokines are regarded as pro-inflammatory if they induce a state of inflammation by stimulating a pro-inflammatory cascade when released (Dinarello, 2000). However, inflammation is a regulated process, and the release of pro-inflammatory cytokines is normally followed by the production of anti-

inflammatory cytokines that control the inflammatory response (Pusterla et al., 2006a). It is the net effect of the balance of the pro- and anti-inflammatory cytokines that will determine the type of inflammatory response observed (Dinarello, 2000; Opal and DePalo, 2000). It should be noted that a combination of many factors affects cytokine response (e.g. when the cytokine is released, the tissue that it is released in, and the other cytokines that are present in that same area). Also, pro- and anti-inflammatory function may not be as definitive as when initially classified, as some cytokines (e.g. IL-6) appear to have both pro- and anti-inflammatory functions (Opal and DePalo, 2000). Table 2.2 contains a summary of a selection of cytokines, their target cells and associated functions.

Antigen recognition is a complex process. The B- and T-lymphocytes have the ability to recognize specific sites on antigens called epitopes. In addition to these epitopes, T-lymphocytes also require these epitopes to be associated with the major histocompatibility complex (MHC) found on antigen presenting cells (APC) (Goldsby et al., 2000). Upon recognition, antigens are internalized and processed for presentation on the MHC together with the release of stimulatory cytokines and other surface molecules. Extracellular antigens (such as bacteria) are normally eliminated by means of antibody production, while intracellular antigens (such as viruses) are more efficiently eliminated by the cytotoxic T-cells (Goldsby et al., 2000). Extracellular antigens are processed within the APC's using the exocytic pathway and are presented in association with MHC II found on professional APC (macrophages, dendritic cells). Intracellular antigens are processed in the endocytic pathway and are presented in association with MHC I found on most nucleated cells (Goldsby et al., 2000; Iwasaki and Medzhitov, 2010).

The development of a primary immune response can be summarized as follows: The APC of the innate immune system (dendritic cells and macrophages) are equipped with "pattern recognition receptors" (PPR) that enables these cells to recognize specific molecular patterns called "pathogen associated molecular patterns" (PAMP's). These are invariable regions found on most bacteria, viruses and other pathogens (Iwasaki and Medzhitov, 2010) (Akira et al., 2006). The PAMP's stimulate cells to produce factors such as cytokines (IL-1 and TNFα) and various chemokines. Once stimulated, the dendritic cells or macrophages migrate to the lymphoid tissues where they can present the naïve Th cells with the antigen-MHC II complex (Kaiko et al., 2008). This complex, together with co-stimulatory factors, induces clonal expansion of the naïve Th cells. Over the next 3-7 d following exposure to the antigen, the number of antigen specific Th cells increase within the secondary lymphoid tissues. Those Th cells that recognize the antigen peptide-MHC presented on antigen specific B-cells move into B-cell follicles to further stimulate additional antibody production (Jenkins et al., 2001).

Assessing immune response:

The humoral immune response is frequently assessed by means of a vaccine challenge. The presence of an adjuvant in the vaccine tends to increase the magnitude of this response. In humans, tetanus toxoid or oral polio vaccinations are often used (Fraker, 1994). Evaluating the CMI response is often done using *in vitro* assays that require culturing lymphocytes with mitogens to induce proliferation, differentiation (Fraker, 1994) and cytokine production (Calder, 2007). Lymphocyte proliferation is a popular functional measure of the ability of the lymphocytes to respond to specific antigens and mitogens. Mitogens can stimulate T cells by bypassing their antigen-specific

receptors thereby producing an oligoclonal response. The mitogens concanavalin A (ConA) and phytohemagglutinin (PHA) are typically used to assess T-cell proliferation, while pokeweed mitogen stimulates both T- and B cells (Calder, 2007).

The nutrient composition of the culture media is of utmost importance, particularly when conducting research using cells from animal models that have been depleted of a specific nutrient (Fraker, 1994). To better reflect the *in vivo* environment in such studies, the use of autologous serum is thought to be more appropriate (Fraker, 1994), and has indeed been shown to affect proliferation results in pigs deficient in either Se or vitamin E (Lessard et al., 1991).

Quantitation of cytokines can provide information on disease or inflammatory progression (Sullivan et al., 2000) and provides a functional assessment of the immune system (Calder, 2007). The most common approaches to evaluate cytokine production include detection of secreted cytokine protein by ELISA or radioimmunoassay, detection of intracellular cytokines of specific cells by flow cytometry, and detecting messenger RNA (Sullivan et al., 2000). Although detection of the cytokine protein may provide the most biologically relevant information (Sullivan et al., 2000), the lack of availability of validated equine specific assays makes these methods unavailable for most equine cytokines. Instead, detection of cytokine-specific mRNA is widely used to assess equine immune function (Swiderski et al., 1999; Spencer et al., 2005; Pusterla et al., 2006b; Horohov et al., 2008; McFarlane and Holbrook, 2008; Riihimaki et al., 2008; Adams et al., 2009).

Selenium and the equine immune system

Although there has been some interest in the interaction between the equine immune system and Se status in the horse, only a limited number of studies have been conducted. The results reported below are summarized in Table 2.3.

One of the first studies to investigate the effect of Se on the immune response of the horse was performed by Baalsrud and Overnes (1986). These researchers were interested in the effect of Se, with or without the addition of supplementary vitamin E, on antibody production. Sodium selenite was used as source of Se, fed at 5 mg Se/d, while vitamin E was supplemented as 600 mg α-tocopheryl acetate. Horses were adapted to their diets for 12 wk, followed by a 12 wk vaccination period. Every 2 wk throughout the 24 wk experimental period the horses were dosed intravenously with *Escherichia coli* culture. During the vaccination period horses were vaccinated against tetanus, as well as equine influenza strain A equi 1 and 2, at the start and again at wk 6 of the vaccination period. Antibody titres against *E. coli* O-antigens, *Clostridium tetani* and equine influenza strains were followed on a weekly basis. Horses supplemented with both Se and vitamin E showed the strongest response to tetanus and influenza, followed by the vitamin E supplementation alone. No difference was detected between the Se only and no supplementation groups (Baalsrud and Overnes, 1986). The *E coli* titres were also similar between the treatments. The authors did note that the group receiving the Se supplement alone consisted of only 3 horses, and none of these horses appeared to be good overall immune responders potentially affecting the study outcome.

In contrast, a later study interested in antigen induced humoral response did see an effect of Se supplementation on immune response (Knight and Tyznik, 1990). Prior to the onset of the study ponies were kept on a low Se diet for at least 9 mo. The ponies were then assigned to either a high Se (0.22 mg/kg DM) or low Se (0.02 mg/kg DM) diet. Upon assignment to dietary treatment the ponies received an i.m. injection of sheep red blood cells (SRBC), followed by a second injection 2 wk later. Blood samples were collected weekly and indicated overall higher serum IgG levels and hemagglutination (HA) titers in response to the SRBC in ponies fed the high Se diet (Knight and Tyznik, 1990). The authors stated that they were unable to provide a possible a mechanism of action to explain the effect of Se on antibody production.

In 1993, a study examined the effects of Se and phosphorus (P) depletion on Thoroughbred mares and weanlings (Greiwe-Crandell et al., 1993). The mares and weanlings received a diet either low or adequate in both P and Se. Dietary P and Se intake was manipulated using a combination of pasture (<0.08 mg Se/kg DM), hay (<0.08 mg Se/kg DM) and concentrate (6 mg Se/kg DM) known to be low or adequate in Se and P. An unexpected outbreak of mild pneumonia with nasal discharge affected the weanlings on the study. The authors noted that all of the 11 Se and P deficient weanlings contracted pneumonia early-October and recovered by early-December. Two of these weanlings also had to be treated to allow for full recovery. In contrast a shorter recovery period was noted for the Se and P adequate weanlings. These weanlings were only affected by mid-December, and with the exception of 2, they all recovered by mid-January. They also did not require additional medical treatment (Greiwe-Crandell et al., 1993). The aim of this study was not to investigate immune response to dietary treatment,

however, the authors stated that their observations may suggest a role of adequate Se nutrition in viral defense (Greiwe-Crandell et al., 1993).

A more recent study conducted on broodmares and foals found that foals from mares receiving 3 mg Se/d had greater serum IgG concentrations at 2 wk of age than foals from mares receiving only 1 mg Se/d (Janicki, 2001). In contrast, Se-Met supplementation (0.3 mg/kg DM in addition to basal diet; 0.49 mg Se/kg DM total dietary intake) of broodmares during the last trimester of pregnancy did not affect colostral IgG concentrations or serum IgG concentration of their foals compared to foals from unsupplemented mares (0.19 mg Se/kg DM total dietary Se intake). However, foals were only sampled at 6 hourly intervals from birth until 24 h post birth, and the diet fed to the unsupplemented mares contained almost twice the recommended Se intake (Thorson et al., 2010).

Selenium and immune function in other species

While relatively few studies have examined the effect of Se on the equine immune system, more information is available in other species. Antibody response was evaluated in broiler chicks utilizing 3 different dietary Se levels (0, 0.1 or 0.2 mg/kg; sodium selenite), with or without the addition of vitamin E (Singh et al., 2006). Se alone was found to have no effect on HA titer against Newcastle disease or total immunoglobulin, but a synergistic effect was observed when Se and vitamin E was provided together (Singh et al., 2006). A study conducted on growing Japanese quail, using a range of different Se treatments (0.2 to 1.0 mg Se/kg DM; sodium selenite) found

HA titers against SRBC to increase as dietary Se intake increased (Biswas et al., 2006). In addition it was reported that cell-mediated immunity, as assessed by the change in foot web thickness induced by intradermal injection of PHA, was also increased with an increase in Se supplementation (Biswas et al., 2006).

Reffett et al. (1988b) evaluated the effect of low Se status on the primary and secondary humoral immune response of calves challenged with infectious bovine rhinotracheitis virus (IBRV). For an 84 d period calves received a diet either deficient (0.03 mg/kg) or adequate (0.2 mg/kg, sodium selenite) in Se. Blood samples were obtained every 14 d to monitor Se status via GPx activity, together with liver samples taken at d 84, also for GPx activity determination. These samples validated the deficient Se status of the calves on the low Se diet. The calves were challenged 28 d later via intranasal inoculation with IBRV, followed by second intranasal inoculation 35 d later (Reffett et al., 1988b). Serum antibody titer and serum immunoglobin (IgG and IgM) levels were evaluated for a 50 d period from the time of the first inoculation. A fever response was observed in both groups within the first 3 d after the first inoculation, although no effect was observed on feed intake. It was noted that whole blood and plasma GPx activity increased in the Se adequate calves after inoculation. The increase was attributed to an improved ability to clear the oxygen metabolites that resulted from the immune challenge in the adequate Se calves when compared to the Se deficient calves (Reffett et al., 1988b). Serum IgM was higher in Se adequate calves at all time points, while serum IgG remained unaffected by Se treatment. After an initial increase in serum IBRV antibody titers, the titers decreased in the Se deficient calves but remained elevated in the Se adequate calves. The second inoculation resulted in an increase in the antibody

titers associated with a normal memory response, though at the end of the challenge period Se deficient calves still exhibited lower antibody titer levels in comparison to the Se adequate calves. It would appear as if Se status affected both primary and secondary response, although the effect was more prominent during the secondary (memory) response phase (Reffett et al., 1988b).

Another bovine immune challenge study (Nemec et al., 1990) looked at the effect of Se supplementation with or without the addition of vitamin E on antibody and HA titer response to vaccination with *brucella abortus* strain 19. The Se supplementation was provided in two 30 g boluses containing 3 g elemental Se each administered 84 d prior to the immune challenge. Blood was collected at various time points prior to and post the immune challenge. No effect of treatment was observed for serum IgG, IgM or total antibody levels post vaccination. Agglutination titers also did not show any change over time. In contrast to Reffett et al. (1988b), the humoral response was not improved in heifers supplemented with Se when compared to the un-supplemented control group (Nemec et al., 1990).

Nicholson et al. (1993) measured antibody response in growing beef cattle fed a basal diet of silage, with or without the addition of Se supplements. The study was conducted in 2 phases. During the first phase Se supplementation was provided from 3 different sources: Se-yeast, Se-fertilized silage or sodium selenite. An un-supplemented group served as the control. After 8 wk on the assigned diets the cattle were challenged with SRBC. Following the SRBC challenge each of the 4 treatment groups were subdivided and regrouped to form two dietary treatment groups with an equal number distribution of animals from previous treatment arrangement in each group. One group

received Se-yeast, while the other received the un-supplemented control diet. After 12 wk on this diet the cattle were vaccinated with 1 mg ovalbumin (OVA) followed by both OVA and SRBC vaccinations 5 wk later (wk 17 of the study). Anti-OVA and anti-SRBC titers were determined by HA assay. No effect of treatment was observed on anti-SRBC titers after the first or the second phase. However, after both initial and booster OVA vaccination the decline in anti-OVA titers was more rapid in the cattle on the low Se control diet than those receiving Se supplementation. It was concluded that Se supplementation did support immune function in cattle (Nicholson et al., 1993), confirming the findings of Reffett et al. (1988b).

The immune response in lambs challenged with parainfluenza$_3$ virus (PI3V) was evaluated in lambs fed diets containing either no Se or vitamin E supplements, a combination of the Se and vitamin E supplements, or Se and vitamin E supplements alone (Reffett et al., 1988a). In the supplemented groups Se supplementation (sodium selenite) was provided at 0.2 mg Se/kg DM while vitamin E was supplemented at a rate of 20 mg alpha-tocopheryl-acetate/kg DM. The lambs were kept on their respective diets for 10 wk, followed by a 70 d immune challenge period. On d 0 and 35 of the challenge period lambs received an intratracheal inoculation with PI3V, and the subsequent immune response was followed by measuring immunoglobulin concentrations as well as PI3V titers (Reffett et al., 1988a). It was stated that the expected fever response was milder than what was normally observed when challenging with PI3V. The results indicated that Se supplementation alone resulted in higher IgM levels on d 14, 35 and 49 when compared to other treatments. Serum IgG concentrations were not affected by Se or vitamin E. Serum titer levels increased in all treatments within 14 d. Between d 14 and 35

serum titer continued to increase in the Se only treatment group while it decreased in the other treatment groups. Then, following the second challenge, the expected increase in titers due to memory response was observed in all treatment groups, except the group receiving Se alone. This was thought to be the result of the high titer that was already present in the Se alone treatment group prior to the second vaccination. The authors concluded that Se supplementation was important for adequate immune response (Reffett et al., 1988a). Humoral immune response was also evaluated in male lambs by Kumar et al. (2009). Lambs were fed diets supplemented with sodium selenite or an unspecified organic form of Se at 0.15 mg/kg DM. An un-supplemented group served as a control (Kumar et al., 2009). At d 0 of the feeding period the lambs were vaccinated i.m. using a killed *Pasturella multocida* vaccine with adjuvant. The antibody response was followed over the next 90 d. Similar to Reffett et al. (1988a) the authors reported that antibody levels were higher in the Se supplemented groups when compared to the control group. However, no effect of Se source was detected (Kumar et al., 2009).

Although the majority of farm animal models reported an effect of Se status on the humoral immune response, human and mouse studies do not show a similar trend. Beck et al. (2001) reported no effect of dietary Se treatment on influenza antibody titers in mice receiving a Se adequate (sodium selenite) or deficient diet. The authors interpreted these results as a lack of effect of Se status on B-lymphocyte function (Beck et al., 2001). Similarly, Se treatment did not affect the polio-antibody levels of human subjects supplemented with various levels of Se (0, 50, 100 µg/d) as sodium selenite (Broome et al., 2004). Likewise, no difference was detected in lung influenza titers obtained from mice challenged with influenza virus. This was in spite of a higher

mortality rate (50%) recorded for the mice on an adequate Se diet when compared to the deficient group (Li and Beck, 2007).

The results reported in the literature are summarized in Table 2.3 and Table 2.4. Table 2.3 contains the results from studies reported above relating to the humoral immune response. Table 2.4 summarizes the results from the studies below, reporting variables that relate to the CMI response and innate immunity.

As shown in Table 2.3 Se supplementation appears to affect the humoral response to a greater extent in livestock than rodents and humans. However, when evaluating the CMI response (Table 2.4) it may be noted that Se supplementation appears to have more of an effect on CMI response when human and rodent models are used, compared to livestock. However, a very important consideration is that, in comparison to the farm animal model, supplementation response studies that have been published using rodent and human subjects have often used more molecular approaches.

Cao et al. (1992) found no difference in the ability of lymphocytes from Se deficient or Se adequate (sodium selenite) cattle to proliferate in response to Con A within the first 36 h of incubation. However, when the incubation period was extended to 48 h, Se deficient cells were not able to proliferate to the same extent as the adequate cells (Cao et al., 1992). In contrast, Aziz and Klesius (1985) evaluated lymphocyte proliferation and IL-2 production in lymphocytes obtained from Se-adequate (source of Se not reported) or deficient goats. They found no difference in ability of the lymphocytes to proliferate or produce IL-2 in response to stimulation with Con A (Aziz and Klesius, 1985).

Wuryastuti et al. (1993) conducted a study on pregnant sows, investigating the effect of Se supplementation (0.3 mg/kg DM as sodium selenite) with or without additional vitamin E (60 mg all-rac-α-tocopheryl acetate/kg DM) on lymphocyte proliferation, using 3 different types of mitogens (PHA; ConA and poke weed), and neutrophil function. The sows were supplemented for 90 d prior to parturition through d 4 of lactation. Similar to Aziz and Klesius (1985) no effect of treatment was observed in the ability of the cells to proliferate (Wuryastuti et al., 1993). However, the authors did note that the phagocytic and microbicidal capabilities of neutrophils were lower in the Se-deficient sows on d 90 and at parturition (Wuryastuti et al., 1993). A 25 d study conducted by Lessard et al. (1991) with weanling pigs investigated the effect of a diet deficient in both Se and vitamin E on immune response, compared to a control group receiving 33 IU DL-alpha-tocopheryl acetate and 0.2 mg Se/kg DM (sodium selenite). The pigs were fed their respective diets for 21 d, at which point they received an oral inoculation with *Samonella typhisius* ($1x10^9$ organisms). After an additional 4 d the pigs were euthanized. Blood was collected on d 21 and 25 to isolate PBMC and granulocytes. Lymphocyte proliferation, cytotoxic activity of natural killer cells, antibody dependent cell mediated cytotoxicity and respiratory burst of granulocytes were evaluated. The samples taken on d 21 indicated no effect of treatment on any of the variables investigated. However, on d 25 lymphocyte proliferation was lower in the pigs on the deficient diet. In contrast, peak respiratory burst activity of granulocytes was observed in the pigs on the deficient diet. Interestingly though, these treatment differences were only observed when heat inactivated autologous serum was used instead of traditional fetal bovine serum (Lessard et al., 1991). Malá et al. (2009) investigated the effect of Se

supplementation on a few innate immune variables in goat kids. The study included 3 dietary Se treatments: organic (Se-lactate-protein complex), inorganic (disodium selenate) or no additional supplementation (control). Dietary treatments were assigned 3 mo prior to parturition and continued during the lactation phase of the does. Upon weaning at 69 d of age the kids were placed on the same treatment as their dams. The innate immune response of the kids was evaluated when they were 120 d old. The researchers did not detect any differences in leukocyte numbers, leukocyte differential counts or phagocytic activity (Malá et al., 2009).

Although limited studies have been conducted looking at the effect of Se on the CMI response in farm animals, more in depth studies have been done using the mouse and human model. A study conducted with human subjects over the age of 65 yr found that Se supplementation (100 µg/d as Se-yeast) over 6 mo period improved lymphocyte proliferation with a 138% increase in response to poke weed mitogen (Peretz et al., 1991). However the same response was not observed when other mitogens such as PHA or OKT3 (anti-CD3 monoclonal antibody) was used. The conflicting results were thought to be due to a combination of the age of the research subjects and the specificity of the mitogens. Elderly humans have been reported to have fewer T-lymphocytes when compared to younger humans. In addition OKT3 stimulates T-lymphocytes, while poke weed stimulates both T and B-cell populations, potentially accounting for the different results (Peretz et al., 1991). Roy et al. (1994) investigated the effect of 8 wk of *in vivo* Se supplementation (200 µg/d as sodium selenite) on lymphocyte proliferation (PHA as mitogen) and IL-2 receptor expression in human subjects previously depleted of Se. IL-2 receptor expression was determined by means of a ^{125}I-IL-2 binding assay (Roy et al.,

1994). Although no treatment differences were observed after 48 h of incubation, at 72 h Se supplementation improved proliferation by 45.1% (Roy et al., 1994). The IL-2 receptor expression was also significantly increased when compared to subjects on the placebo treatment (Roy et al., 1994). In a companion paper, Kiremidjian-Schumacher et al. (1994) reported that the Se supplementation also resulted in a 118% increase in cytotoxic lymphocyte mediated tumor cytotoxicity. In addition, natural killer cell activity was increased by 82.3%. Roy et al. (1994) concluded that supplementation of Se, as sodium selenite, either dietary or *in vitro* to cell cultures, increased lymphocyte proliferation and IL-2 receptor expression, indicating the ability of Se to modulate T-cell response. In addition, Kiremidjian-Schumacher et al. (1994) stated that Se supplementation was capable of inducing changes in immune function. Another study evaluated the response of human lymphocytes to *in vitro* supplementation of Se alone or in combination with vitamin E (Lee and Wan, 2002). In contrast to Roy et al. (1994) they found adding Se *in vitro* (0.5µM sodium selenite) followed by stimulation with PHA or LPS did not improve lymphocyte proliferation. However, when no mitogen was added, i.e. the *in vitro* Se addition alone, proliferation was improved in comparison to the control (Lee and Wan, 2002). An important consideration though is that the Se status of the volunteers from which the lymphocytes were obtained was not reported. In addition, Lee and Wan (2002) reported an increase in total T-cells, Th cells and T-suppressor cells as measured by flow cytometry in response to *in vitro* Se supplementation, again in the absence of mitogens. In the presence of PHA the *in vitro* Se supplementation elevated total T-cells and T-helper cells only (Lee and Wan, 2002). Utilizing human subjects of marginal Se status, Broome et al. (2004) investigated the effect of Se supplementation on

the development of the immune response to a live attenuated poliomyelitis vaccine given as a single oral dose. The vaccine was preceded by 6 wk of supplementation with sodium selenite (0, 50 or 100 µg/d). Se supplemented groups showed a greater proliferative response 7 d after administration of the vaccine. Similarly, stimulated whole blood cultures indicated higher levels of IFNγ and IL-10 at d 7 in Se supplemented groups. Se supplemented groups were also found to have lower fecal shedding of virus, interpreted as enhanced viral clearance (Broome et al., 2004).

Petrie et al. (1989) evaluated cytotoxic T cell and natural killer cell cytotoxicity in mice fed a diet containing either 0.1 mg/kg or 4 mg Se/kg as sodium selenite. The mice were supplemented for 4 wk before splenic cells were harvested. Cytotoxic T lymphocytes were also generated from peritoneal exudate cells (PEC) through intraperitoneal infection of tumor cells, followed by recovery of the cell suspension. They reported a 65% increase in splenic natural killer cell activity in comparison to the control mice. The mice on the high Se diet also showed higher levels of anti-tumor cytotoxicity levels as measured by the cytotoxic T cells generated from PEC (Petrie et al., 1989). To investigate the effect of Se status on macrophage function, Vunta et al. (2008) kept mice on a Se deficient (0.01 mg/kg DM) or adequate (0.4 mg/kg DM; Se source not reported) diet for 100 d. After intraperitoneal injection with LPS, cells were isolated from lung tissue. Macrophage infiltration into the lungs was determined by flow cytometry. A 50% higher lung infiltration was found in Se deficient mice when compared to Se adequate mice, implicating a role of Se in the control of macrophage infiltration of tissues. Upon further investigation both TNFα and cyclooxygenase-2 (COX-2) expression by macrophages were found to be lower in the Se adequate mice (Vunta et al., 2008). It was

concluded that adequate Se status may be of importance in the regulation of pro-inflammatory gene expression in macrophages, which in turn may affect organ tissue infiltration by macrophages (Vunta et al., 2008).

Using mice, Beck et al. (2001) evaluated the effect of Se status on the intensity of an influenza infection after exposure to influenza virus strain A/Bangkok/1/79; H3N2. Mice were kept on a diet that was either deficient or adequate in Se. The source of Se in the adequate diet was sodium selenite. At various time points post infection (intra-nasal) selected mice were euthanized for the evaluation of lung histopathology, bronchoalveolar lavage cells (BAL), and cytokine and chemokine mRNA levels in mediastinal lymph nodes. Lung pathology indicated Se deficient mice had more inflammation at d 4, 6, 10 and 21 d post infection. Lung pathology started to decline in the adequate mice after d 6, while it persisted in the deficient mice until d 21. Evaluation of BAL cells revealed higher numbers of CD8 and macrophages in the Se deficient mice on d 5 post infection, which may relate to those results reported by Lee and Wan (2002) in terms of change in T cell numbers. Finally, IFNγ and IL-2 mRNA levels were overall lower in the Se deficient mice. IL-4 and IL-5 were lower in the deficient mice on d 4, but were comparable to adequate mice by d 14. IL-10 and IL-13 were elevated in the deficient mice by d 6 when compared to adequate mice (Beck et al., 2001). From d 14 onwards IL-4, -5, -10 and -13 were higher in the deficient mice (Beck et al., 2001). The lung pathology results were thought to be linked with the measured changes in mRNA levels of the cytokines in the lungs (Beck et al., 2001). Based on the cytokine responses, the researchers concluded that the immune responses in the lungs of Se-deficient mice were

skewed towards a Th2 response, the opposite of what would be expected after a viral challenge (Beck et al., 2001).

In a follow up study, Sheridan et al. (2007) investigated the effect of selenoprotein levels on immune response in mice after an intra-nasal challenge with the same influenza virus (A/Bangkok/1/79; H3N2). This research group took a different approach from the traditional manipulation of dietary Se intake by using transgenic mice with a mutant (Sec) $tRNA^{[Ser]Sec}$ that resulted in lower levels of selenoprotein production. For control purposes "wild type" mice were used. The mice were infected with the influenza virus, and then euthanized at different intervals to study the development of the immune response over time. Across treatments, an overall decrease in lung mRNA cytokine expression (IFNα, IFNβ, TNFα, IL-6) was observed but chemokine (MCP-1 and MIP-1α) mRNA expression was elevated in the mutant mice at d 2 post infection, and the mRNA expression of cytokine IFNγ was elevated in the mutant mice on d 7 post infection (Sheridan et al., 2007). No difference was observed in lung pathology when comparing the mutant and wild type mice, however the wild type mice exhibited faster viral clearance. Upon closer inspection it was found that although the mutant mice did have lower selenoprotein levels, GPx activity was still 82% of that of the wild type, and higher than that of dietary induced Se deficient mice used in their laboratory. Therefore the authors concluded that there appeared to be a threshold GPx value above which lung pathology is prevented (Sheridan et al., 2007).

Li and Beck (2007) then conducted a similar study to Beck et al. (2001), this time using different influenza strain (A/PuertoRico/8/34). Mice were provided with either a deficient (0.002mg/kg DM) or adequate Se diet (0.2 mg/kg DM, sodium selenite) for 4

wk. As before the mice were infected with the virus (intra-nasal) and a selection of mice were then euthanized at various time points post infection for the evaluation of lung histopathology, lung virus titers, chemokine protein levels and cytokine mRNA levels in lung tissue using RT-PCR (Li and Beck, 2007). In contrast to their previous work (Beck et al., 2001), but similar to Sheridan et al. (2007), the authors did not detect a difference in lung pathology between dietary treatments. Further, at d 7 half of the Se adequate mice had died, while all Se-deficient mice were still alive. Se-deficient mice were found to have lower chemokine (RANTES and MIP-1α) levels in lung tissue as well as higher levels of IL-2 expression followed by elevated IL-4 levels. Apart from a trend for elevated IFNγ levels in the deficient mice, no other cytokines were affected (Li and Beck, 2007). These results were in contrast to those reported earlier by Beck et al. (2001). It was concluded that not only the Se status, but also the virulence of the strain of virus used in the infectious study should be considered when interpreting results (Li and Beck, 2007).

As stated earlier in this review, the results from the studies discussed above are summarized in Table 2.3 and 2.4. Again it is obvious that different responses are observed between rodent and human studies and livestock studies. Again, this difference may simply be due to the different approaches that have been used when studying these different models.

Selenium and immune function: Mechanism of action

The exact mechanism of action through which Se affects the immune response has not yet been defined. The antioxidant role of Se has received a lot of consideration as the potential mechanism of action. More recently other potential mechanisms have been considered. Baalsrud and Overnes (1986) suggested that Se affects the immune system through its ability to reduce peroxides and protect vital organelles and cell processes involved in mounting an immune response. Lessard et al. (1991) thought that the inclusion of Se at appropriate levels may help to control cellular oxidative reactions which may in turn affect the ability of immune cells to function properly.

Cao et al. (1992) reported that a key to the mechanism of action may lie in the role of Se in modulation of the arachidonic acid metabolism. They reported that low Se status was associated with a reduction in the metabolites of the 5-lipoxygenase pathway, as measured in *in vitro* cell cultures (bovine), which in turn was linked to lower lymphocyte proliferation. The suppressed proliferative response was reportedly reversed by the addition of specific lipoxygenase pathway products. This potential mechanism of action is supported by molecular based research done by Vunta et al. (2008) using mice as their research model which showed that Se adequate cells favorably convert arachidonic acid to 15d-PGJ_2 (a cyclopentenone prostaglandin) which has an adverse effect on the pro- inflammatory signal transduction pathways as it inhibits the activation of nuclear factor – kappa-β (NF-κβ) dependent gene expression (Vunta et al., 2008). NF-κβ is a transcription factor that initiates the transcription of numerous cytokines, specifically the pro-inflammatory cytokines (Tosi, 2005).

Many other researchers have since contributed to the body of research suggesting that the mechanism of action lies in the antioxidant properties of Se, preventing the over-activation of NF-κβ by high levels of ROS (Beck et al., 2001; Zeng and Combs Jr, 2008). Beck et al. (2001) proposed two potential mechanisms of action based on their work with Se deficient mice and influenza, that may work in unison. They proposed that Se deficiency resulted in higher levels of ROS leading to more severe oxidative stress in comparison to Se adequate mice. The elevated oxidative stress levels in Se deficient mice stimulate the activation of NF-κβ to a greater extent than in Se adequate mice, resulting in greater levels of inflammation observed in the Se deficient mice. Secondly they proposed that host Se status affected the virus itself in that Se deficiency allowed changes in the viral genome resulting in mutations into more virulent strains, affecting the extent of inflammation observed (Beck et al., 2001). The involvement of the transcription factor NF-κβ is a concept that has been considered by others (Duntas, 2009). The immune system depends on NF-κβ to bind to DNA and activate the expression of the genes that encode the proteins that are involved in the immune response (Maggini et al., 2007), including the production of pro-inflammatory proteins such as TNFα and IL-6 (Duntas, 2009). The activation and movement of NF-κβ into the nucleus is via the phosphorylation of I-kappa-beta-alpha (Iκβα). It is proposed that Se status regulates GPx activity, which in turn regulates intracellular ROS levels. Low ROS levels (due to adequate GPx) preserve Iκβα, resulting in low NF-κβ activity, controlling inflammatory protein levels, and therefore state of inflammation (Duntas, 2009).

The *in vitro* work conducted by Lee and Wan (2002) led them to conclude that the mechanism of action lies in the improved free radical scavenging capabilities of Se

adequate cells. Their studies showed inhibition of lymphocyte proliferation by H_2O_2 in a dose dependent manner, implicating the importance of adequate Se status to neutralize H_2O_2 and allow for optimal proliferation (Lee and Wan, 2002). The research conducted by Saito et al. (2003), utilizing a human cell line, complements the results from Lee and Wan (2002).

Saito et al (2003) showed that the suppressed proliferation response of Se deficient cells could be overturned in a dose dependent manner by the *in vitro* addition of Se. It was further observed that the selenoprotein TrxR was more highly preserved in the cultured cells under Se deficiency conditions than GPx. Cell death occurred after the first cell division, and the proliferated cells were even lower in TrxR and GPx. However, the addition of vitamin E to Se deficient cells also reduced cell death, even though the selenoprotein levels were not increased. It was concluded that the accumulation of lipid hydroperoxides resulted in cell death and that the ability of the selenoproteins to neutralize lipid hydroperoxides was the means through which they supported cell proliferation (Saito et al., 2003). Maggini et al. (2007) stated that the mechanism of action may lie in the maintenance of the redox balance in the cell by the selenoproteins. The selenoenzyme TrxR is involved in the regulation of the redox status of the cells. In turn the redox status affects enzymes, transcription factors as well as NF-κβ (Maggini et al., 2007). However, the balance between selenoproteins and non-selenoproteins contributing to the maintenance of redox status makes it difficult to pinpoint the mechanism of action and therefore selenoprotein knockout mouse models may be essential to fully comprehend the mechanism of action (Conrad and Schweizer, 2010). Hoffmann (2007) also stated that the mechanism of action will not be fully understood

until the specific functions of the different selenoproteins in the immune cell, as well as the response of selenoproteins to an immune challenge have been determined. In addition, selenoproteins could potentially have functions other than the commonly accepted elimination of ROS in immune cells (Hoffmann, 2007; Hoffmann and Berry, 2008).

Regardless of their precise function, the importance of selenoproteins in immune cell function was demonstrated in a mouse knockout model study in which mouse T-lymphocytes were lacking selenoproteins (Shrimali et al., 2008). Proliferation was affected in the selenoprotein knock out T-lymphocytes, however chemically blocking endogenous ROS production *in vitro* by the addition of N-acetyl cysteine resulted in normal proliferative response. It was concluded that selenoproteins potentially play a role in T-cell receptor activation, arguing an antioxidant mechanistic role for Se (Shrimali et al., 2008). Ueno et al. (2008) evaluated the mitogenic response of T-cells derived from spleens of mice kept on a Se deficient or adequate diet for 8 wk. Se was then added *in vitro* to the cells. At the time that the spleens were harvested, tissue Se status was also determined for various other organs. Interestingly it was reported that the decrease of Se concentration in the spleen and thymus was not as great as that of the liver, kidney, heart or whole blood. Glutathione peroxidase activity and TrxR measured in the splenic cells indicated that TrxR activity was conserved to a greater extent than GPx activity. These results indicated a potential resistance to Se deficiency in the lymphoid organs (Ueno et al., 2008). The mitogenic response to ConA, calculated and expressed as cell growth, indicated suppressed cell growth in deficient cells without Se added *in vitro* (Ueno et al., 2008). Cells that received additional Se *in vitro* as sodium selenite (0.01-0.1 μmol/L)

showed the greatest increase in cell growth when compared to the other sources used (sodium selenate, sodium selenide, selenocystamine dichloride, seleno-DL-cystine, seleno-DL-ethionine seleno-L-methionine). Supplementation below 0.01 µmol/L resulted in very little difference between Na_2SeO_4, seleno-DL-cystine and seleno-L-methionine, while exceeding 0.1µmol/L had a suppressive effect. Finally, *in vitro* supplementation with sodium selenite elevated both GPx and TR in the cultured cells. The recovery of GPx activity was not to a comparable level to the adequate cells, while TR was similar to the adequate cells. This observation led to the conclusion that TR may play an essential role in immune cell proliferation (Ueno et al., 2008).

Continuing the focus on selenoproteins, Goldson et al. (2011) evaluated the response of selected selenoproteins (SEPS1; SEPR and SEPW1) to an influenza vaccine challenge. Humans were used as research subjects, allocated to one of 6 treatments for 12 wk. The different treatments included a placebo, Se-yeast tablets providing either 50, 100 or 200 µg Se/d, or meals made with low Se onions or Se enriched onions. At wk 10, all research subjects were vaccinated with a trivalent flu vaccine. Blood samples were collected for PBMC isolation prior to vaccination and 1 and 2 wk post vaccination. The PBMC RNA was isolated and selenoprotein expression measured using RT-PCR. SEPS1 was elevated in the Se-yeast treatment in a dose dependent manner 1 wk post vaccination compared to placebo treatment, but decreased to prior levels at the 2 wk post time point. A treatment effect was observed for SEPW1 indicating the highest Se-yeast treatment group to have lower expression of this selenoprotein compared to the other treatment groups. In addition an overall trend was observed for higher levels of these selenoproteins in the Se enriched onion group. The SEPS1 results leads to the conclusion that SEPS1

might be of interest to research defining the role of Se in the immune response (Goldson et al., 2011).

The final proposition of a potential mechanism of action is through increased T-cell receptor signal strength (Hoffmann et al., 2010). These researchers found that increased Se intake in mice resulted in an increase in early cell signaling via elevated Ca^{2+} flux and nuclear factor activated T-cells. They further reported that Se did not affect phosphorylation of extracellular kinases and had no effect on oxidative stress levels. Increased T-cell receptor signal strength can potentially result in improved proliferation and differentiation. Higher Se intake was also found to increase T-cell receptor induced oxidative burst in activated T-cells, and the upregulated levels of TrxR and GPx that they measured were thought to be a precautionary adaptation to neutralize the resulting ROS (Hoffmann et al., 2010). Finally, increased Se intake resulted in a more reduced intracellular environment, theorized to induce an immune response bias towards Th1, benefiting antiviral response and cytotoxic T-cell production (Hoffmann et al., 2010). In a follow up study from the same laboratory, it was reported that the involvement of selenoprotein K in receptor mediated Ca^{2+} flux in immune cells may be a clue to the mechanism of action (Verma et al., 2011). Selenoprotein K knockout mice were used to study Ca^{2+} flux in immune cells, which was found to be lower in the knockout mice when compared to the wild type controls. T-cell proliferation was impaired, while T-cell migration capacity was almost completely absent, and similarly neutrophil function was suppressed. Efficient Ca^{2+} flux is important for immune cell activation. The research findings from this laboratory suggest that the mechanism of action for Se and the immune

system may lie in the regulation of Ca^{2+} flux, implicating the importance of selenoprotein K in immune function (Verma et al., 2011).

Selenium as antioxidant during exercise

The generation of energy in the mitochondria of cells depends on oxygen metabolism. Although the majority of O_2 will be reduced to H_2O via the electron transport system, even under normal circumstances, this process is not always 100% complete. Incomplete reduction of O_2 results in the generation of reactive oxygen species (ROS), described as reactive O_2-containing radicals with unpaired electrons that compromise the stability of the compound. The major ROS generated are superoxide radicals (O_2^-), hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2). Although H_2O_2 does not have an unpaired electron, it is included under the term ROS based on its reactivity (Ji, 1999; Surai, 2006; Battin and Brumaghim, 2009). During strenuous physical exercise the consumption of O_2 increases dramatically. In humans it is estimated that strenuous exercise results in a 20-fold increase in O_2 demand (Ji, 1999), while the increase in O_2 demand in the horse is 30-fold (Deaton et al., 2002; Hargreaves et al., 2002). This increase in demand for O_2 also results in an increase in the amount of ROS produced (Avellini et al., 1999; Ji, 1999). In addition, other sources of ROS in the body exist such as the ROS generated by the immune system during respiratory burst. High intensity exercise can also induce inflammatory responses that contribute to ROS production in the body (Ji, 1999).

The antioxidant mechanism is comprised of multiple layers of defense. It includes both non-enzymatic antioxidants (vitamin E, vitamin C, carotenoids, ubiquinols, flavonoids, glutathione and uric acid) and antioxidant enzymes (superoxide dismutase, catalase, GPx and the thioredoxin system). When working in unison, the various components of the antioxidant system are capable of preventing as well as repairing oxidative damage (Ji, 1999; Urso and Clarkson, 2003; Surai, 2006; Battin and Brumaghim, 2009).

Under normal conditions the antioxidant mechanism is capable of neutralizing the effect of ROS production, creating a pro-/anti- oxidant balance within the body. With training the antioxidant mechanism adjusts to maintain this balance (Yur et al., 2008). However, during a strenuous exercise session, this delicate balance may be perturbed to the point that the antioxidant mechanism cannot compensate for the increased ROS. This results in oxidative stress and potentially oxidative damage to tissues, cell membranes and DNA structures (Deaton et al., 2002). In horses, exercise induced oxidative stress has been thought to contribute to muscle damage and fatigue, exercise intolerance and even poor performance (de Moffarts et al., 2005). Ogonski et al. (2008) stated that the impact of ROS on performance may be of such a nature that it should be considered an important and routine variable, such as lactate, when evaluating exercise response studies in horses. In horses, oxidative stress may be assessed by measuring malondialdehyde (MDA) concentrations in plasma or serum using the TBARS assay (Ducharme et al., 2009). MDA is the end product of lipid peroxidation when cell membranes are damaged by ROS (Ducharme et al., 2009).

Se is believed to exert its antioxidant effects through the selenoproteins such as GPx, although some *in vitro* evidence indicate that Se-Met and Se-Cys may be capable of reducing ROS DNA damage (Battin and Brumaghim, 2009). The function of GPx is to reduce peroxides such as H_2O_2 and is discussed in an earlier section of this literature review. The complexity of the antioxidant system also results in synergistic interactions between the different antioxidants (Deaton et al., 2002). As a consequence very little research exists looking at the effect of Se alone on exercise induced oxidative stress.

Akil et al. (2011) evaluated the effect of Se supplementation on exercised induced oxidative stress in adult rats. The rats were given 200 µg Se/d (intraperitoneal) for 4 wk. After 4 wk the rats completed a 30 min forced swim exercise test. Un-supplemented rats served as controls. Samples taken immediately post exercise indicated lower plasma MDA and lactate levels in supplemented rats. Serum GPx protein levels and serum Se levels were also elevated in the supplemented rats. The authors concluded that the Se supplementation contributed to maintaining the pro-/anti- oxidant balance when compared to the control group, and that Se did participate in the antioxidant mechanism during exercise induced oxidative stress (Akil et al., 2011).

Pagan et al. (1999) compared Se-yeast and sodium selenite supplementation in exercised Thoroughbreds. Although investigating oxidative stress was not one of their objectives, they did report an increase in whole blood and plasma Se post exercise. While plasma Se level returned to basal levels within 24 h in the sodium selenite group, it remained elevated in the Se-yeast supplemented group (Pagan et al., 1999). Similarly, a study that included 56 endurance horses competing in 25 to 100 mile endurance races reported an increase in whole blood Se concentrations 1 hr after completion of the

endurance race (Haggett et al., 2010). Although some variation existed in whole blood Se concentration (all within normal range with one exception), Se status did not have an effect on performance (Haggett et al., 2010). White et al. (2011) supplemented 12 untrained Thoroughbreds with sodium selenite at a rate of either 0.1 (control) or 0.3 mg Se/ kg DM for 34 d. The horses were then subjected to a prolonged exercise test at submaximal activity with sampling before and at 0, 6 and 24 h post exercise. Although creatine kinase (CK) levels increased in response to the exercise, there was no effect of treatment on CK, used as an indicator of muscle damage. Elevated total serum lipid hydroperoxides were observed post exercise in the control group compared to the supplemented group and reported to indicate improved protection against oxidative stress in the supplemented horses. RBC GPx activity did not respond similarly between treatments to exercise. Post exercise RBC GPx activity was lower in the 0.3 mg Se/kg supplemented group while RBC GPx only decreased at the 6 h post exercise time point in the control horses. In contrast, muscle GPx activity was elevated 6 h post exercise in the 0.3 mg Se/kg group compared to baseline values, while it remained unchanged in the control group (White et al., 2011). Se supplementation at 0.3 mg/kg DM was thought to provide better protection against exercise induced oxidative damage possibly via the changes observed in the GPx activity of muscle and RBC (White et al., 2011).

Brady et al. (1978) also investigated the effect of Se on exercise induced oxidative stress. They supplemented one group of horses with 0.15 mg Se/kg DM trace-mineral salt for 4 wk while an un-supplemented group served as control. Total dietary Se intake was not reported. Blood samples were obtained before and at 1 and 24 h post exercise, which consisted of a 2.2 km run in deep sand within a 10 min period. In contrast to White et al.

(2011), no effect of treatment was observed on Se status, although these horses were not supplemented at comparable levels. A decline in RBC GPx was observed post exercise, but this decline was unaffected by treatment. An increase in RBC MDA after exercise was reported, indicating that there was an increase in oxidative damage. CK was also found to increase due to the exercise, but this increase was independent of treatment. The authors concluded that the unresponsiveness of their horses to the Se supplements may be indicative of an adequate Se status of both control and supplemented group. However, they hypothesized that their study outcome may have been different if they had included a Se deficient group of horses (Brady et al., 1978). Taking a different approach, Janiak et al. (2010) were interested in evaluating the change in GPx that would potentially occur in young horses (16 to 20 mo) on an adequate Se diet (providing 3.02 mg Se/d from natural feedstuffs) in response to training. Horses were sampled before and following a 4 mo training period, as well as pre, 0 and 1 h post exercise at the end of the 4 mo period. RBC lysate GPx activity increased over the 4 mo training period, but decreased immediately post exercise, then recovered by 1 h post (Janiak et al., 2010).

Some studies have also investigated the effect of a combination of antioxidants on exercise induced oxidative stress. Avellini et al. (1999) supplemented horses for 70 d with a combination of vitamin E (40 mg/kg DM) and Se (0.02 mg/kg DM) in addition to their basal diet (total dietary vitamin E and Se intakes not reported). During this 70 d period horses were trained and completed a ridden exercise test at the end of the 70 d. The exercise test consisted of two gallop sessions with samples taken prior to, in-between and after the gallops. The MDA levels prior to the start of the trial were higher than those measured after 70 d of training and supplementation. The level of MDA increased in

response to the exercise test, but it was still lower than prior to the study. Lymphocyte GPx activity was increased as a result of the supplementation, but was unaffected by the exercise test. It was concluded that the training, in conjunction with the vitamin E and Se supplementation, resulted in improved extracellular antioxidant capacity, subsequently reducing cellular damage as measured by MDA levels (Avellini et al., 1999). In contrast, an exercise study conducted with 6 Thoroughbreds, supplemented with vitamin E, vitamin C and Se for 6 wk, found no effect of exercise or treatment on plasma MDA (Deaton et al., 2002). Measures of circulating antioxidants (glutathione, vitamin E or uric acid) were also unaffected (Deaton et al., 2002). It was noted though that the standardized exercise test performed on a treadmill did not seem to induce oxidative stress. The horses were in regular treadmill training for the 6 wk supplementation period, and may have been too adapted to the exercise. It was concluded that antioxidant supplementation may only be beneficial if a deficiency existed (Deaton et al., 2002).

de Moffarts et al. (2005) evaluated the effect of a multi-antioxidant or placebo supplement in 40 Thoroughbred race horses. The daily supplement provided a combination of vitamin C (11.5 g), vitamin A (0.5 g) and vitamin E (7 g), Zn (769 mg), Cu (187 mg) and Se (7 mg). Horses were supplemented for 12 wk while completing their training (same trainer) and racing schedule as normal. Samples were taken prior to, at 6 and 12 wk. Overall an increase was observed in both water and lipid soluble plasma antioxidants. RBC Se and GPx activity were increased over time in the supplemented group while they decreased in the placebo group as expected due to the deficient Se levels in the basal diet. However, CK levels remained comparable between the treatments and no significant performance improvement effects were observed. Focusing just on the

Se component, the decreased RBC GPx activity appeared to have no effect on the performance of these horses (de Moffarts et al., 2005). Ji et al. (1990) investigated the effect of 7 wk of vitamin E supplementation (300 mg d,1-tocopherol acetate/kg DM) on RBC enzyme activity using treadmill conditioned American Quarter Horses. Neither treatment nor exercise affected RBC antioxidant enzymes, including GPx, catalase and superoxide dismutase. Yet the treadmill test did result in an 18-fold increase in lactate. It was theorized that equine blood may have enough antioxidant reserves so that a single strenuous bout of exercise would not have affected antioxidant enzyme activity (Ji et al., 1990).

Some research studies simply evaluated the effect of exercise on the antioxidant system, although for observational types of studies it should be kept in mind that supplementation may not always be reported accurately by participants. Hargreaves et al. (2002) evaluated the effect of endurance exercise on antioxidant status and muscle damage in 35 horses competing in an official 80 or 160 km endurance race. Dietary composition was not reported. The horses that completed the 80 km race had elevated GPx activity, though it returned to baseline 1 h post exercise. The horses that completed the 160 km endurance race had elevated GPx activity at 142 and 160 km, but it returned to baseline values 1 h post exercise. The authors hypothesized that the 1 h post exercise decrease in GPx may have been the result of depleted glutathione stores. In both cases CK and AST levels were correlated with GPx activity, interpreted as indicative of adequate antioxidant protection (Hargreaves et al., 2002). Similarly, it was reported that horses exercised on the treadmill until the point of fatigue had an increase in RBC GPx activity, but at 1 and 24 h post exercise GPx activity had returned to normal (Smarsh et

al., 2010). Using trained Standardbred trotters, Ogonski et al. (2008) evaluated the effect of exposure to the extreme exertion experienced during a race on the antioxidant status of the horses. The plasma total antioxidant status of the horses was found to decrease after the race. In addition, it was noted that the greatest decrease in antioxidant status was measured in the horses that displayed the best performance as determined by heart rate and respiration recovery post exercise (Ogonski et al., 2008). In an example of extreme endurance exercise, oxidative stress by itself was evaluated in 9 Arabian type horses competing in a 210 km race over a 3 day period (Gondim et al., 2009). It was determined that apart from electrolytes, horses were not provided with any additional antioxidant supplements, and all horses were kept on a diet consisting of a performance horse level concentrate, corn oil and hay. Blood samples were obtained prior to the onset of the race and at the end of each day. Only five horses completed the 210 km race. Plasma MDA increased and then stabilized, especially in the more competitive horses. Plasma CK levels increased up to d 2, and then decreased. Interestingly, horses that were eliminated throughout the race all showed highly elevated CK levels, while those horses that finished were always within the normal biological range. The finishers also had higher glutathione reductase capacities. It was hypothesized that the eliminated horses were not able to compensate for the increase in oxidative stress (Gondim et al., 2009).

Not all equine studies reported an increase in GPx activity in response to exercise. A summary of these studies appear below in Table 2.5 for comparison. GPx has been shown to be consistent in adapting in response to training as cells respond to consistent exercise training by activating the synthesis of antioxidant enzymes. (Ji, 1999). Williams (2010) hypothesized elevated GPx activity in response exercise may be indicative of an

increase in pro-oxidant scavenging activity. Regardless a complete understanding of the changes in GPx due to exercise and the direct impact on equine athletes is still lacking (White et al., 2011).

Summary and study objectives:

Selenium is a micro nutrient that has received a lot of attention in the research world for both its antioxidant and immune modulating properties. In contrast, comparably few studies in this research area have focused on the horse. Those researchers that have evaluated the effect of Se on the equine immune system have mostly concentrated on the humoral immune response. Improving immune function and antioxidant capacity by optimizing Se status can be of benefit for all classes of horses without requiring a large financial investment on the horse owner's part. Therefore the overall objectives of this research were to evaluate immune- and antioxidant function in horses of low, adequate or high Se status for an extended period of time. We hypothesized that horses of low Se status would exhibit a suppressed immune response and experience higher levels of oxidative stress due to a compromised antioxidant mechanism. We were also interested in determining if source of Se (i.e. Se-yeast vs sodium selenite) could have an effect on the observed responses. To meet these objectives we conducted a preliminary study followed by a three-phase long term study over 18 mo period. The specific aims were:

Aim 1 (Preliminary study Chapter 3): To investigate the relationship between Se status, antioxidant status and immune function in a group of horses ranging from deficient to adequate Se status.

Aim 2 (Chapter 4): To determine if Se depletion over an extended period of time (8 mo) would affect measures of immune function as compared to horses kept on a Se adequate diet. The objectives were to evaluate the ability of the immune system of horses of low

Se status to respond to a novel vaccine challenge (keyhole limpet hemocyanin; KLH) as measured by *in vitro* lymphocyte proliferation, peripheral blood mononuclear cell (PBMC) intracellular cytokine production, PBMC cytokine mRNA expression and antigen specific antibody production. The second objective was to evaluate the ability of the immune system of horses of low Se status to mount an anamnestic respond to an equine influenza vaccine (KY02) that all horses had been vaccinated with before. Again *in vitro* lymphocyte proliferation, PBMC intracellular cytokine production, PBMC cytokine mRNA expression, flu specific IgGa, IgGb and IgG(T) production and hemagglutination titer was measured.

Aim 3 (Chapter 5): Determine the impact of repleting Se deficient horses with either organic (0.3 ppm) or inorganic Se (0.3 ppm) on immune function. The study objectives were to assess the effect of Se status on immune function in response to a novel vaccine challenge (ovalbumin) by means of *in vitro* assessment of lymphocyte proliferation, intracellular cytokine production, cytokine mRNA expression, antigen specific antibody production. The second objective was to assess the effect of Se status on memmory immune function in response to an equine influenza vaccine (KY02) challenge by means of *in vitro* assessment of lymphocyte proliferation, PBMC intracellular cytokine production, PBMC cytokine mRNA expression, whole blood cytokine expression, antigen specific IgGa, IgGb and IgG(T) production and hemagglutination titer.

Aim 4 (Chapter 6): To evaluate the effect of long term Se depletion and repletion on complete blood count analysis, glutathione peroxidase activity, total antioxidant capacity, and malondialdehyde concentration as indicator of oxidative stress.

Aim 5 (Chapter 7): To evaluate the effect of Se status on malondialdehyde concentration, pro-inflammatory cytokine expression and indicators of muscle damage in response to mild exercise.

Brief experimental design description (Aims 2 to 5):

The study was conducted in three continuous phases: A 28 wk Se depletion phase and 7 wk vaccine challenge, followed by a 22 wk Se repletion phase and 7 wk vaccine challenge (29 wk), as illustrated in Figure 2.3. The final vaccine challenge after the repletion phase was followed with an exercise test that will be defined as phase 3. In order to accommodate the repletion phase, the horses were blocked by age and gender and randomly allocated within block to one of 4 treatment groups prior to the start of the study. Each treatment group consisted of seven horses. As is evident from Figure 2.3, one group received the same adequate Se diet throughout the study and functioned as a control.

Table 2.1. Summary of Se supplementation response data: peripheral blood indicators of Se status.

Study	Study length	Se status prior to study	Source	Total Se intake (DM)	Final Plasma/serum Se (ng/mL)	Final plasma GPx activity	Final Whole blood Se	Final whole blood (WB) or red blood cell (RBC) GPx activity
Stowe, 1967	60 d	Deficient	Control	Purified diet	36.8 ± 13.1 (SD)	*	*	*
		Deficient	Sodium selenite	0.500 mg/kg	142.0 ± 12.4	*	*	*
		Deficient	Sodium selenite	1.000 mg/kg	153.0 ± 14.3	*	*	*
		Deficient	Sodium selenite	2.000 mg/kg	167.0 ± 19.5	*	*	*
Shellow et al., 1985	140 d	*	Control	0.070 mg/kg	69.0 ± 3.0 (SE)	0.690 U/mL plasma	104.0 ± 5.0 (SE) ng/mL	*
		*	Sodium selenite	0.162 mg/kg	110.0 ± 4.0	0.743 U/mL plasma	176.0 ± 12.0 ng/mL	*
		*	Sodium selenite	0.288 mg/kg	143.0 ± 4.0	0.736 U/mL plasma	208.0 ± 3.0 ng/mL	*
		*	Sodium selenite	0.460 mg/kg	140.0 ± 8.0	0.742 U/mL plasma	183.0 ± 12.0 ng/mL	*
Richardson et al., 2006	56 d	Marginal	Control	1.3 mg/d	96.0 ± 8.0 (SEM)	11.8 mU/mg protein	*	278 mU/mg Hb (RBC)
		Marginal	Sodium selenite	4.7 mg/d	151.0 ± 8.0	12.3 mU/mg protein	*	438 mU/mg Hb (RBC)
		Marginal	Zn-L-selenomethionine	5.1 mg/d	169.0 ± 8.0	10.0 mU/mg protein	*	238 mU/mg Hb (RBC)
Calamari et al., 2009	112 d	Marginal	Control	0.085 mg/kg	92.8 ± 13.9 (SED)	0.839 U/mL plasma	178 ± 22.3 (SED) ng/g	171 U/g Hb (WB)
		Marginal	Se-yeast	0.182 mg/kg	169.6 ± 13.9	0.917 U/mL plasma	348.2 ± 22.3 ng/g	244 U/g Hb (WB)
		Marginal	Se-yeast	0.290 mg/kg	180.0 ± 13.9	1.098 U/mL plasma	448.5 ± 22.3 ng/g	291 U/g Hb (WB)
		Marginal	Se-yeast	0.395 mg/kg	208.1 ± 13.9	0.878 U/mL plasma	489.0 ± 22.3 ng/g	298 U/g Hb (WB)
		Marginal	Sodium selenite	0.288 mg/kg	184.2 ± 13.9	1.070 U/mL plasma	381.0 ± 22.3 ng/g	299 U/g Hb (WB)

Table 2.1. Continued.

Karren et al., 2010	110 d	*	Pasture	0.190 mg/kg	224 ± 6.0 (SE)	6.85 mU/mg protein	*	*
		*	Pasture+Se yeast	0.490 mg/kg	255 ± 5.0	7.56 mU/mg protein	*	*
		*	Pasture+grain	0.350 mg/kg	253 ± 7.0	7.37 mU/mg protein	*	*
		*	Pasture+grain+Se yeast	0.650 mg/kg	260 ± 6.0	7.14 mU/mg protein	*	*

Table 2.2. Summary of selected cytokines and their related functions*.

Cytokine	Secreting Cells	Target cells	Functions
IL-1β	Macrophages & others	Macrophages	Activation and co-stimulation of other cytokines; pyrogenic [1]; synergistic relationship with TNFα; pro-inflammatory; tissue damage [5]
IL-2	T-cells	T-, B-cells and macrophages	Proliferation; IFNγ secretion; antibody production [1, 7]
IL-6	T-, B-cells macrophages	T-and B-cells	Pro-inflammatory and co-stimulatory; proliferation and differentiation [1]; Anti-inflammatory via inhibition of TNFα and IL-1 [4]
IL-8	Macrophages and endothelial cells	Neutrophils	Chemokine, neutrophil chemo-attractant, activates degranulation of neutrophils [5]; adherence to vascular endothelium [6]
IL-10	Th2 and T-regulatory cells; B-cells	T-, B-cells and macrophages, dendritic cells	Immune suppression, down regulates Th1 [1]; reduce suppress IL-2 and IFNγ; deactivate macrophage pro-inflammatory synthesis [4]
IL-13	Activated T-cells (Th2)	B-cells, macrophages	Regulation of CMI [1]; suppress monocyte/ macrophage TNFα; IL-8, IL-1 production [4]
TNFα	T-cells, macrophages, monocytes	Neutrophils, macrophages, monocytes	Activation of acute phase proteins [2]; synergistic relationship with IL-1; pro-inflammatory; tissue damage [5]
IFNγ	Th1, cytotoxic T-cells and NK cells	T-cells; macrophages, NK-cells, others	Activation of APC's and CMI; increased MHC II expression [3]; antiviral [5]

*[1] = Tato and Cua (2008a); [2] = Tato and Cua (2008b); [3] = Tato and Cua (2008c); [4] = Opal and DePalo (2000); [5] = Dinarello (2000); [6] = Goldsby et al. (2000); [7] = Farrar et al. (1982b).

Table 2.3. Response of humoral immune variables to selenium supplementation*.

Study	Species	Se Status	Se Source	Variable	Response
Broome et al. (2004)	Human	Supplemented	Sodium selenite	Polio-antibody	Similar to control[L*]
Beck et al. (2001)	Mouse	Deficient	*	Flu-antibody	Similar to control[L]
Li and Beck (2007)	Mouse	Deficient	*	Flu-antibody	Similar to control[L]
Baalsrud and Overnes (1986)	Horse	Supplemented	Sodium selenite	Total antibody	Similar to control[L]
Janicki (2001)	Horse	Supplemented	Sodium selenite/ Se-yeast	Serum IgG	Higher[A]
Knight and Tyznik (1990)	Horse	Supplemented	Sodium selenite	Serum IgG	Higher[L]
	Horse	Supplemented	Sodium selenite	HA titer	Higher[L]
Thorson et al. (2010)	Horse	Supplemented	Selenomethionine	Serum IgG	Similar to control[A]
Nemec et al. (1990)	Cattle	Supplemented	Elemental Se (bolus)	Salmonella antibody	Similar to control[L]
	Cattle	Supplemented	Elemental Se (bolus)	*B. abortus* antibody	Similar to control[L]
	Cattle	Supplemented	Elemental Se (bolus)	HA titer	Similar to control[L]
Nicholson et al. (1993)	Cattle	Supplemented	Se-yeast	Anti-OVA titer	Slower decline vs. control[L]
Reffett et al. (1988b)	Cattle	Supplemented	Sodium selenite	Serum IgG	Similar to control[L]
	Cattle	Supplemented	Sodium selenite	Serum IgM	Higher[L]
	Cattle	Supplemented	Sodium selenite	HA titer	Higher[L]
Kumar et al. (2009)	Sheep	Supplemented	Unspecified org source	*P. multocida* antibody	Higher[A]
Reffett et al. (1988a)	Sheep	Supplemented	Sodium selenite	Flu-antibody	Higher primary response[L]
	Sheep	Supplemented	Sodium selenite	Serum IgM	Higher[L]
	Sheep	Supplemented	Sodium selenite	Serum IgG	Similar to control[L]
Singh et al. (2006)	Chicken	Supplemented	Sodium selenite	HA titer	Similar to control[L]
	Chicken	Supplemented	Sodium selenite	Total IgG	Similar to control[L]
Biswas et al. (2006)	Quail	Supplemented	Sodium selenite	HA titer	Higher[A]

*[L] indicates control diet below adequate Se concentration; [A] indicates control diet contains adequate Se concentration

Table 2.4. Response of various cell mediated and innate immune variables to selenium supplementation*.

Study	Species	Se Status	Se source	Variable	Response
Broome et al. (2004)	Human	Supplemented	Sodium selenite	Lymphocyte proliferation	Higher[L]
	Human	Supplemented	Sodium selenite	Various cytokines (protein)	Higher[L]
	Human	Supplemented	Sodium selenite	Natural killer cell activity	Similar to control[L]
Kiremidjian-Scumacher et al. (1994)	Human	Supplemented	Sodium selenite	Cytotoxicity levels of cytoxic Tcells	Higher[L]
	Human	Supplemented	Sodium selenite	Natural killer cell activity	Higher[L]
Lee and Wan (2002)	Human	*In vitro* suppl.	Sodium selenite	Lymphocyte proliferation with mitogen	Similar to control[L]
	Human	*In vitro* suppl.	Sodium selenite	Lymphocyte proliferation no mitogen	Higher[L]
	Human	*In vitro* suppl.	Sodium selenite	Total T- & T-helper cell number (culture)	Higher[L]
Peretz et al. (1991)	Human	Supplemented	Se-yeast	Lymphocyte proliferation	Higher[A]
Roy et al. (1994)	Human	Supplemented	Sodium selenite	Lymphocyte proliferation	Higher[L]
	Human	Supplemented	Sodium selenite	T-cell IL-2 receptor expression	Higher[L]
Beck et al. (2001)	Mouse	Deficient	*	Lung IL-4, -5, -10, -13 (lymph node mRNA)	Higher[A]
	Mouse	Deficient	*	IFNγ (lymph node mRNA)	Lower[A]
Li and Beck (2007)	Mouse	Deficient	*	RANTES and MIP-1α (lung, mRNA)	Lower[A]
	Mouse	Deficient	*	IL-2, -4 (lung, mRNA)	Higher[A]
Petrie et al. (1989)	Mouse	Supplemented	Sodium selenite	Natural killer cell activity	Higher[A]
	Mouse	Supplemented	Sodium selenite	Cytotoxicity levels of cytoxic Tcells	Higher[A]
Sheridan et al. (2007)	Mouse	Se-prot KO	*	IFNγ, IFNβ, TNFα, IL-6 (lung mRNA)	Lower[A]
	Mouse	Se-prot KO	*	IFNγ (Lung mRNA) d 7 post challenge	Similar to control[A]
Ueno et al. (2008)	Mouse	*In vitro* suppl.	*	Spleen T-cell proliferation	Similar to control[L]
Vunta et al. (2008)	Mouse	Supplemented	N/A	Macrophage TNFα and COX2 expression	Lower[L]

*[L] indicates control diet below adequate Se concentration; [A] indicates control diet contains adequate Se concentration

Table 2.4. Continued.

Study	Species	Se Status	Se source	Variable	Response
Lessard et al. (1991)	Pig	Supplemented	Sodium selenite	Lymphocyte proliferation	Higher[L]
	Pig	Supplemented	Sodium selenite	Natural killer cell activity	Lower[L]
	Pig	Supplemented	Sodium selenite	Granulocyte respiratory burst	Similar to control[L]
Wuryastuti et al. (1993)	Pig	Supplemented	Sodium selenite	Lymphocyte proliferation	Similar to control[L]
	Pig	Supplemented	Sodium selenite	Neutrophil function	Higher[L]
Biswas et al. (2006)	Quail	Supplemented	Sodium selenite	Foot web thickness	Higher[A]
Cao et al. (1992)	Cattle	Deficient	*	Lymphocyte proliferation	Suppressed[A]
Azis and Klesius	Goat	Deficient	*	Lymphocyte proliferation	Similar to control[A]
(1985)	Goat	Deficient	*	IL-2 levels	Similar to control[A]
Malá et al. (2009)	Goat	Supplemented	Org/Inorg	Leukocyte numbers/phagocytic activity	Similar to control[A]

*[L] indicates control diet below adequate Se concentration; [A] indicates control diet contains adequate Se concentration

Table 2.5. Glutathione peroxidase activity response to exercise.

Study	Species	Control or treatment Se intake	Study length	Initial fitness	Type of exercise	GPx* response post exercise
Akil et al. (2011)	Rat	0 or 200 µg/d (intraperitoneal)	4 wk	Unfit	30 swim test	Higher than control (serum)
White et al. (2011)	Horse	0.1 or 0.3 mg/d	34 d	Unfit	Mild prolonged exercise	Lower than control (RBC)
White et al. (2011)	Horse	0.1 or 0.3 mg/d	35 d	Unfit	Mild prolonged exercise	Higher than control (muscle)
Brady et al. (1978)	Horse	Basal (N/A) or 0.15 mg Se/kg DM	4 wk	N/A	2.2km trot in 10 min	Similar to control (RBC)
de Moffarts et al. (2005)	Horse	0 or 7 mg/d	12 wk	Fit	Race training	Higher than control (RBC)
Janiak et al. (2010)	Horse	3.02 mg Se/d (no control)	4 mo	In training	Trotter training	Increase with training (RBC)
Hargreaves et al. (2010)	Horse	N/A	*	In training	Endurance race	Elevated (RBC)

* GPx = glutathione peroxidase

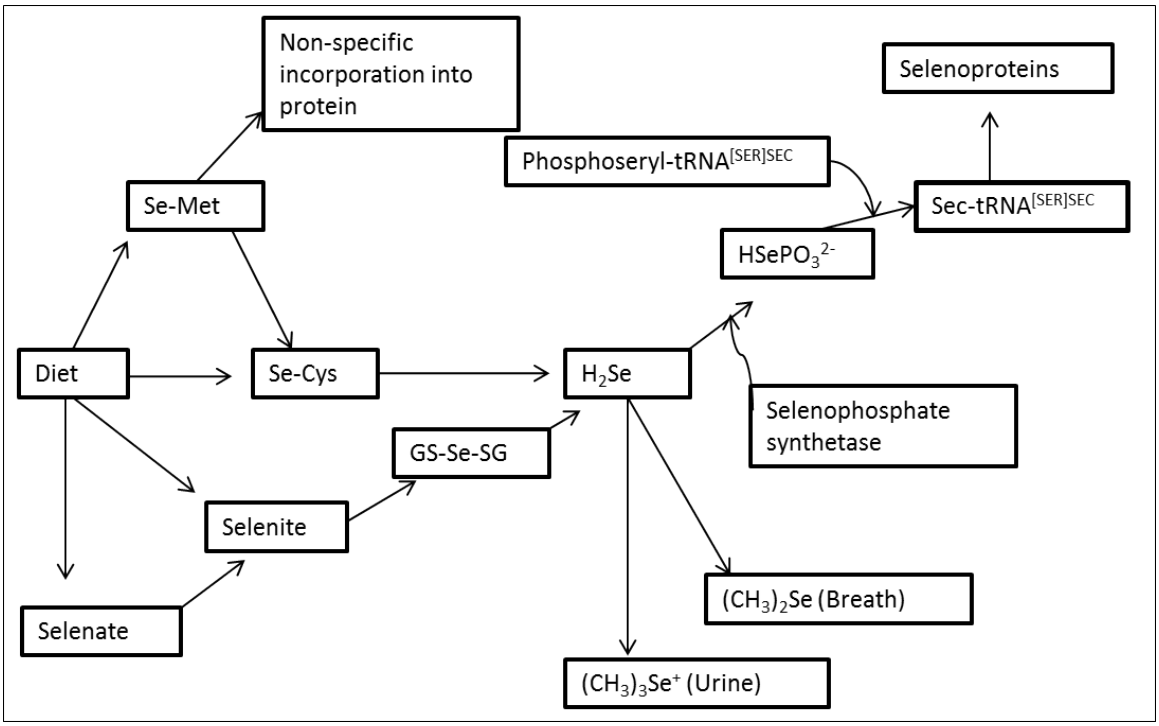

Figure 2.1. Selenium metabolism pathway as adapted from Combs (2001), Rayman (2007), Papp et al. (2007) and Hesketh (2008). Abbreviations: GS-Se-SG = selenodiglutathione; $(CH_3)_2Se$ = dimethyl selenide; $(CH_3)_3Se^+$ = trimethyl selenonium ion; H_2Se = selenide; $HSePO_3$ = selenophosphate.

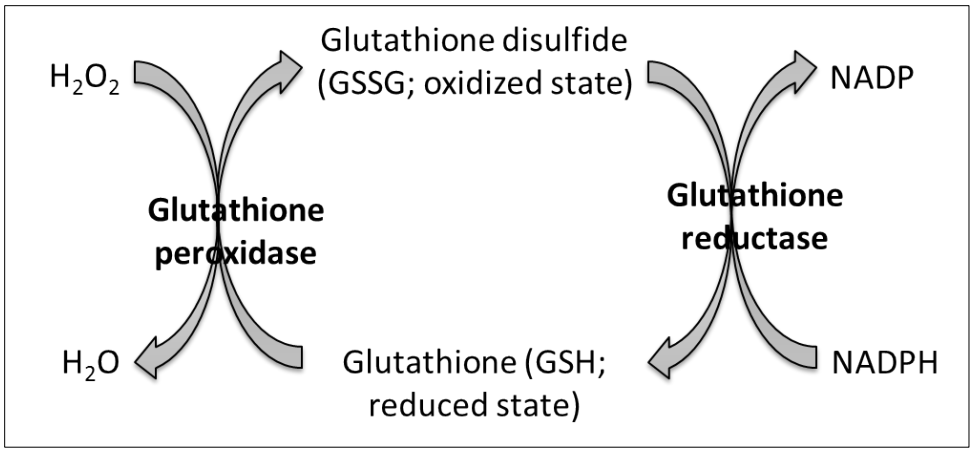

Figure 2.2. Detoxification of H_2O_2 by glutathione peroxidase via glutathione recycling. Adapted from Urso and Clarkson (2003).

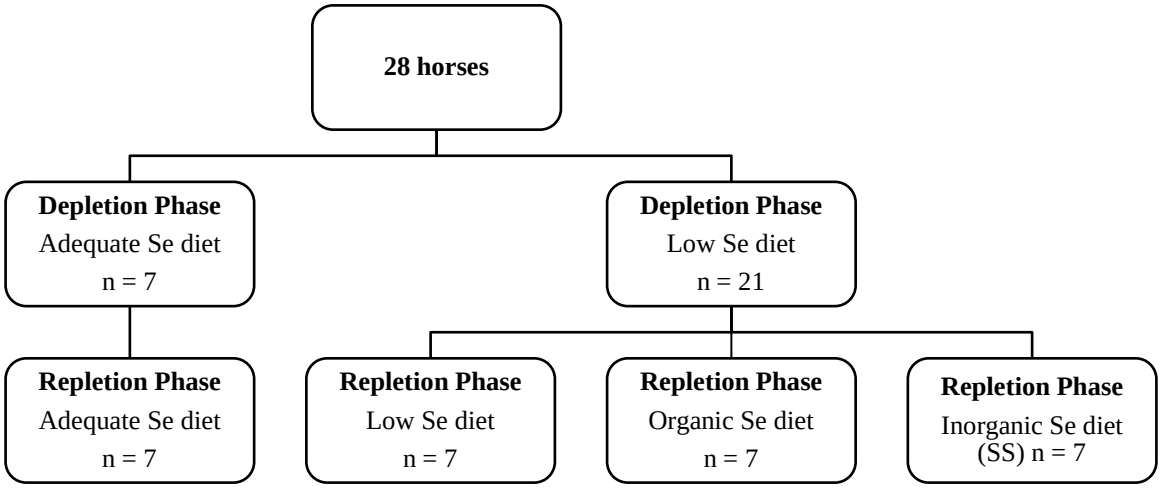

Figure 2.3. Experimental design and treatment allocation during the depletion and repletion phases.

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CHAPTER 3: The relationship between selenium status, antioxidant status and the immune system of the horse

INTRODUCTION

Only a few studies have investigated the effect of Se on the equine immune system (Knight and Tyznik, 1990; Janicki, 2001; Thorson et al., 2010) and the relationship between Se status and immunomodulating cytokines in the adult horse have not been investigated. In the horse, the majority of Se research conducted has focussed on the effect of Se supplementation on overall Se status and GPx activity (Shellow et al., 1985; Richardson et al., 2006; Calamari et al., 2009b; Karren et al., 2010). One study that investigated the effect of Se on immune function in the horse reported that ponies on a high Se diet (0.22 ppm) had higher IgG concentrations in response to an immunological challenge than ponies on a Se deficient (0.02 ppm) diet (Knight and Tyznik, 1990). Another study conducted on broodmares and foals found that foals from Se supplemented mares had greater serum IgG concentrations at 2 wk of age than foals from un-supplemented mares (Janicki, 2001). In contrast, supplementing broodmares with selenomethionine during the last trimester of pregnancy was reported to have no effect on colostrum or foal serum IgG concentrations (Thorson et al., 2010). In other species Se status has been shown to affect the mRNA expression of a selected cytokines (Li and Beck, 2007), IL-2 receptor (Roy et al., 1994) and lymphocyte proliferation (Broome et al., 2004). Understanding the effect of Se status on the equine immune system will be of benefit to the horse, especially those horses kept in areas marginal in Se. Therefore the objective of this study was to investigate the relationship between the Se status of the horse (serum Se concentration and whole blood GPx activity), indicators of cell-mediated

immune function and antioxidant status (total antioxidant capacity, TAC; malondialdehyde concentration, MDA). We investigated *in vivo* cytokine gene expression in whole blood and *in vitro* cytokine gene expression in stimulated PBMC using RT-PCR. The cytokines investigated included IFNγ, TNFα, IL-2, IL-8, and IL-10. Lymphocyte proliferation was also investigated. We hypothesized that horses of lower Se status would have lower lymphocyte proliferation and that Se status would affect intracellular cytokine production and cytokine gene expression.

MATERIALS AND METHODS

Animals and Sampling:

This research project was approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

The aim was to sample horses that would be diverse with regard to Se status. To achieve this, 24 horses that had been on pasture and fed various amounts of concentrate for 4 mo (May to September) were used. The pastures had been sampled and determined to be deficient in Se content (0.04 ppm Se on dry matter (DM) basis). Sixteen horses, including 8 mares and 8 geldings (mean age ± SD; 11.4 yr ± 5.24), had been kept on pastures with sufficient forage availability to maintain body weight. These horses received no dietary supplementation except for access to an iodized salt block. The remaining 8 horses, which included 5 mares and 3 geldings (10.1 yr ± 5.96), were kept on pastures with more limited pasture availability, but similar Se content, and were supplemented with a pelleted grain-based concentrate containing at least 0.3 ppm Se (as

fed basis, McCauley Bros Inc., Versailles, KY). Horses receiving additional concentrate were provided with a quantity sufficient to maintain body condition (on average 2.7 kg of the concentrate per day). Each horse was sampled once only.

Se and antioxidant status:

Whole blood (7 mL) was collected in lithium-heparin tubes (Becton Dickson, Franklin Lakes, NJ) for GPx activity analysis. Blood was also collected in tubes with no additive (Becton Dickson) for serum separation. Whole blood and serum were stored at -80˚C until analysis. Whole blood GPx activity was measured using the method of Paglia and Valentine (1967). Final GPx activity was then expressed as μmol NADPH oxidized·mL^{-1}·min^{-1}·g protein (μmol EU/g protein). Serum was analyzed for Se content by means of inductively coupled plasma-mass spectroscopy (DCPAH, Michigan State University, Lansing, MI). Serum TAC was determined using a method that compared the ability of the antioxidants in the serum sample to inhibit the oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] to that of Trolox, a water soluble tocopherol analogue (Caymenchemical; Ann Arbor, MI). Serum MDA concentration was measured using a thiobarbituric reactive substances (TBARS) method (Caymenchemical). This method was based on the reaction of MDA with thiobarbituric acid under acidic, high temperature conditions, using MDA to create a standard curve. Serum vitamin E analysis was conducted by the Veterinary Diagnostic Laboratory, Iowa State University by means of HPLC analysis with UV detection.

Complete Blood Count (CBC):

A CBC analysis was performed for each horse by a commercial equine hospital (Rood & Riddle, Lexington, KY). The number of neutrophils and lymphocytes were calculated for each horse using the CBC data.

Peripheral blood mononuclear cell (PBMC) isolation:

For PBMC isolation, venous blood (30 mL) was collected into heparinized tubes (Kendall, Monoject blood collection tubes, Mansfield, MA). The PBMC were isolated using Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) gradient centrifugation. Approximately 15 mL of plasma was layered on top of 10 mL Ficoll-Paque Plus in a 50 mL centrifuge tube (Fisher) and centrifuged for 30 min at 500 x g. The interface cells were collected, washed 3 times with phosphate buffered saline (Sigma), counted and re-suspended at the required concentrations in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2.5% fetal equine serum (Sigma), 2mM L-glutamine (Sigma), 100 U/mL penicillin/streptomycin (Sigma) and 55 μM 2-mercaptoethanol (Gibco), as described by (Adams et al., 2008).

IFNγ and TNFα Intracellular Staining:

The PBMC were plated (4 x 10^6 cells per mL) in 3.5 mL culture wells (TPP, Trasadingen, Switzerland), and stimulated for 4 h with PMA (25 ng/mL; Sigma) and ionomycin (1μM; Sigma) as described in Adams et al. (2008). Un-stimulated PBMC cultures incubated with media alone served as the control. To assess IFNγ and TNFα production, intracellular staining was performed, as previously described (Breathnach et al., 2006; Adams et al., 2009). The stained cells were analyzed by means of flow

cytometry (FACSCalibur, Becton Dickson, San Jose, CA). The percentage of lymphocytes producing IFNγ and TNFα and the mean fluorescence intensity (MFI) of the stained cells were determined using Cell Quest software (Becton Dickson, San Jose, CA). The percentage gated refers to the percentage of lymphocytes, identified and gated based on the forward and side scatter parameters, producing IFNγ or TNFα, while MFI refers to the intensity of the IFNγ or TNFα signal in the cells producing IFNγ or TNFα (Breathnach et al., 2006). Using the computer software, markers were placed on the isotype control antibody labelled stimulated cells so that 1% of cells were IFNγ or TNFα positive. The MFI and percentage gated values reported are values corrected for each individual sample subtracting the isotype control MFI and % gated values from the stimulated values. Some of the intracellular staining results did not reflect proper staining and were therefore excluded from the results. However the results that were included (n = 17) represented a range of Se status.

Relative expression of cytokine mRNA

Stimulated PBMC, as described for intracellular staining, were used to assess the *in vitro* gene expression of IFNγ, TNFα, IL-2 and IL-10. The stimulated PBMC were lysed by the addition of 1mL RNA-STAT 60 (Tel-Test, Isotex Diagnostics Inc., Friendswood, TX) and stored at -80ºC until total RNA was extracted according to manufacturer's protocol.

In vivo cytokine gene expression of IFNγ, TNFα, IL-10 and IL-8 was assessed by collecting 2.5 mL blood via jugular venipuncture into PAXgene blood RNA tubes

(PreAnalytiX, Valencia, CA). Total RNA was extracted using the PAXgene RNA extraction kit (Qiagen, Valencia, CA), according to the manufacturer's protocol.

Reverse transcription was conducted as described by Breathnach et al. (2006). Cytokine gene expression was measured by means of RT-PCR (7900 HT Fast Real-Time PCR system, Applied Biosystems, Foster City, CA) using equine specific intron spanning primer/probe sets (Breathnach et al., 2006; Adams et al., 2009). Relative change in gene expression was calculated as described in (Livak and Schmittgen, 2001). Mean of the un-stimulated cells served as calibrator and results were expressed as relative quantity (RQ) calculated as $2^{-\Delta\Delta CT}$. Beta-glucuronidase was used as the internal control (Breathnach et al., 2006). *In vivo* cytokine gene expression in un-stimulated whole blood analysis was completed for 16 horses, representing a range of Se status.

Lymphocyte proliferation:

Isolated PBMC were plated ($2x10^6$ cells per mL) in 96 well flat-bottom plates (TPP, Trasadingen, Switzerland) and incubated with ConA (10 μg/mL). Following 72 h incubation in a 37ºC, 5% CO_2 humidified incubator, PBMC were pulsed with 0.5 μCi ^{3}H-thymidine, incubated for an additional 18 h, and frozen (-20ºC) until analysis. DNA was extracted from the PBMC onto fiber filter pads using a Tomtec harvester (J/B Industries Inc, IL) and liquid scintillation counting performed (Wallac Inc., Gaitheisburg, MD). Corrected counts per minute were used for statistical analysis.

Statistical analysis:

Statistical analysis was conducted using SAS 9.1 (SAS Institute Inc., Cary, NC). The PROC CORR procedure was used to evaluate relationships between immune

variables and indicators of Se and antioxidant status. Significance was set at $p < 0.05$ and a trend for significance at $p < 0.1$.

RESULTS AND DISCUSSION

The serum Se concentration of the horses on this study ranged from 69 to 193 ng/mL. The adequate range for serum Se in the adult horse is 130 to 160 ng/mL (Stowe and Herdt, 1992). To investigate the relationship between the Se status and the chosen immune variables, it was important to sample a group of horses that represented a range in Se status. The individuals on this study met that requirement as there were individuals with low, moderate and high serum Se concentrations. Whole blood GPx activity ranged from 25.43 to 46.75 μmol EU/g protein and was correlated with serum Se concentration ($r = 0.71$, $P = 0.0001$). A similar correlation ($r = 0.75$; $P < 0.001$) was reported for plasma Se concentration and whole blood GPx activity in horses fed various levels of Se-yeast or sodium selenite (Calamari et al., 2009b). Although serum Se concentration is used for clinical interpretation of Se status (Stowe and Herdt, 1992), selenoproteins such as GPx are thought to provide a more functional measure of Se status (Gromadzinska et al., 2008). Both indicators of Se status confirmed that the horses included in this study represented a range in Se status.

A positive correlation was found between the amount of IFNγ produced by stimulated lymphocytes (MFI) and whole blood GPx ($r = 0.78$; $P = 0.0002$; Figure 3.1). However, no correlation was observed between the amount of IFNγ produced and serum Se (Table 3.1). Relative gene expression for IFNγ by *in vitro* stimulated PBMC was not

correlated with any of the indicators of Se status. A lack of relationship also existed between Se status and *in vivo* gene expression of IFNγ. The cytokine IFNγ is considered to be pro-inflammatory as it potentiates the actions of the tumor necrosis factor cytokines that are known to stimulate pro-inflammatory cascades. At the same time, IFNγ has essential anti-viral properties and activates the pathway for cytotoxic T-cell activation (Dinarello, 2000). Other studies have also indicated a relationship between Se status and IFNγ. Broome et al. (2004) reported that greater amounts of IFNγ were produced by polio virus-stimulated whole blood cultures (*in vitro*) from Se supplemented humans, 7 d after challenge with a polio virus vaccine. Another study reported that Se deficient mice challenged with a mild strain of influenza virus (A/Bangkok/1/79) had a decrease in mediastinal lymph node IFNγ mRNA levels compared to Se adequate mice (Beck et al., 2001). Se deficient mice were also found to be more susceptible to infection and developed more severe inflammation in their lungs than the mice that were of adequate Se status (Beck et al., 2001). Therefore the observed relationship between the amount of IFNγ produced and GPx activity is consistent with previous work, although no correlation existed for IFNγ gene expression in stimulated PBMC in this study.

A correlation existed between serum Se concentration and the relative gene expression of IL-10 ($r = 0.42$; $P = 0.0414$) by PBMC stimulated *in vitro*. IL-10 is an anti-inflammatory cytokine that functions as an inhibitor of pro-inflammatory cytokines such as TNFα and IFNγ, as well as IL-2 (Opal and DePalo, 2000). Dunstan et al. (2006) found no correlation between plasma Se levels and IL-10 production by lymphocytes from human subjects stimulated *in vitro*, but Beck et al. (2001) reported Se deficient mice to have higher IL-10 expression in mediastinal lymph node tissue than Se adequate mice in

response to an immune challenge. Even though IL-10 is expected to suppress pro-inflammatory cytokines such as IFNγ, positive correlations with Se status were found for both of these cytokines. In addition a trend was observed for a correlation between serum Se and gene expression of TNFα (r = 0.38; P = 0.0660) in *in vitro* stimulated PBMC. Similarly, a trend was observed between serum Se concentration and the percentage of lymphocytes, stimulated *in vitro*, producing TNFα (r = 0.45; P = 0.0731). From this preliminary study it would appear that higher Se status may be associated with an increase in both pro- and anti-inflammatory cytokine expression in the horse. The possibility exists that the increased IL-10 mRNA was in response to elevated levels of the pro-inflammatory cytokines.

Although previous studies have found a relationship between lymphocyte proliferation and Se status (Roy et al., 1994; Broome et al., 2004) there was no correlation between the Se status of the horses and lymphocyte proliferation in this study. Similarly there was no relationship between Se status and IL-2, a cytokine associated with proliferation (Farrar et al., 1982a). In addition, no relationship existed between Se status and neutrophil or lymphocyte numbers in this study. Although Se status has been reported to affect IgG concentrations in horses, the absence of an effect on lymphocyte proliferation and cell numbers suggest that the effect is not mediated through lymphocyte number. Wuryastuti et al. (1993) also reported a lack of effect of Se intake on lymphocyte proliferation.

The mean serum TAC was 0.99 ± 0.02 mM. No relationship existed between TAC and serum Se concentration or GPx activity. Similarly MDA concentration (mean = 5.39 ± 0.35 μM) was not correlated with serum Se or GPx. Dunstan et al. (2006) reported

a positive correlation between IL-10 and TAC in humans, but noted that animal models tend to report negative correlations between these two variables. Indeed, our study found a negative correlation (P = 0.025) between the gene expression of IL-10 and TAC (Table 3.2). Similarly, negative correlations existed between TAC, the amount of IFNγ produced (r = - 0.64; P = 0.0054), and the gene expression for IFNγ (r = - 0.40; P = 0.0505) and TNFα (r = - 0.45; P = 0.0283) by PBMC stimulated *in vitro*. Given the pro-inflammatory nature of both IFNγ and TNFα, the negative association with antioxidant capacity is an interesting finding, and may indicate that additional components of the antioxidant mechanism, other than Se, may have an effect on the equine immune system.

In elderly human patients vitamin E has been shown to enhance the immune system by affecting variables such as lymphocyte proliferation (Meydani et al., 1990). Vitamin E concentrations of the horses on this study indicated that they were of adequate vitamin E status (mean = 3.8 ± 0.24 µg/mL), as indicated by vitamin E concentrations above 2 ug/mL (NRC, 2007). Vitamin E status was not correlated with TAC, MDA or indicators of Se status. It is possible that the lack of correlation between Se status and some of the immune variables such as lymphocyte proliferation could have been the result of adequate vitamin E status and other components of TAC that may be interesting to investigate. A stronger relationship between Se status and the immune variables might have been evident if the horses also had a low vitamin E status.

CONCLUSION

In conclusion, horses with low Se status were able to sustain most aspects of their immune function, but IFNγ production by stimulated lymphocytes was compromised.

The significant role of IFNγ in viral immune defence of the body suggests that adequate Se nutrition should be an important consideration for horses kept in Se marginal regions.

Table 3.1. Correlations (r) between serum Se, whole blood GPx and the immune variables investigated.

	Number of observations*	Serum selenium		Whole blood GPx	
		r	*P*-value	r	*P*-value
Amount IFNγ produced	17	0.34	0.1768	0.78	0.0002
IFNγ producing lymphocytes	17	0.32	0.2180	0.06	0.8274
RQ IFNγ *In vitro*	24	0.15	0.4844	0.09	0.3621
RQ IFNγ *In vivo*	16	0.43	0.1119	0.33	0.2199
Amount TNFα produced	17	0.35	0.1650	0.43	0.0825
TNFα producing lymphocytes	17	0.45	0.0731	0.29	0.2585
RQ TNFα *In vitro*	24	0.38	0.0660	-0.06	0.7650
RQ TNFα *In vivo*	16	0.43	0.0950	0.49	0.0556
RQ IL-10 *In vitro*	24	0.42	0.0414	-0.01	0.9667
RQ IL-10 *In vivo*	16	0.29	0.2770	-0.04	0.8881
RQ IL-8 *In vivo*	16	-0.21	0.4314	0.27	0.3077
RQ IL-2 *In vitro*	24	0.07	0.7433	0.13	0.5594
Proliferation	24	0.12	0.5727	0.04	0.8401
Number of Neutrophils	24	-0.13	0.5590	-0.06	0.7870
Number of Lymphocytes	24	0.33	0.1101	0.18	0.3909

*Upon closer evaluation some of the staining and mRNA expression data were omitted. Although the number of observations varies they are representative of the range of Se status.

Table 3.2. Correlations (r) between indicators of antioxidant status and immune variables.

	Number of observations	TAC		MDA	
		r	*P*-value	r	*P* -value
Amount IFNγ produced	17	-0.6428	0.0054	0.0249	0.9243
IFNγ producing lymphocytes	17	-0.3251	0.2030	0.3277	0.1991
RQ IFNγ *In vitro*	24	-0.4036	0.0505	-0.2381	0.2624
RQ IFNγ *In vivo*	16	0.1598	0.5693	0.3200	0.2449
Amount TNFα produced	17	-0.4401	0.0770	0.0230	0.9301
TNFα producing lymphocytes	17	-0.4285	0.0862	0.2121	0.4138
RQ TNFα *In vitro*	24	-0.4480	0.0283	-0.3127	0.1368
RQ TNFα *In vivo*	16	0.0526	0.8467	0.1515	0.5753
RQ IL-10 *In vitro*	24	-0.4560	0.0250	-0.0402	0.8521
RQ IL-10 *In vivo*	16	0.2210	0.4107	-0.0945	0.7275
RQ IL-8 *In vivo*	16	-0.1814	0.5012	-0.1207	0.6561
RQ IL-2 *In vitro*	24	-0.0948	0.6596	0.0077	0.9714
Proliferation	24	0.1947	0.3620	-0.2927	0.1651
Number of Neutrophils	24	-0.0408	0.8496	-0.1535	0.4741
Number of Lymphocytes	24	-0.0496	0.8179	0.2975	0.1579

*Upon closer evaluation some of the staining and mRNA expression data were omitted. Although the number of observations varies they are representative of the range of Se status.

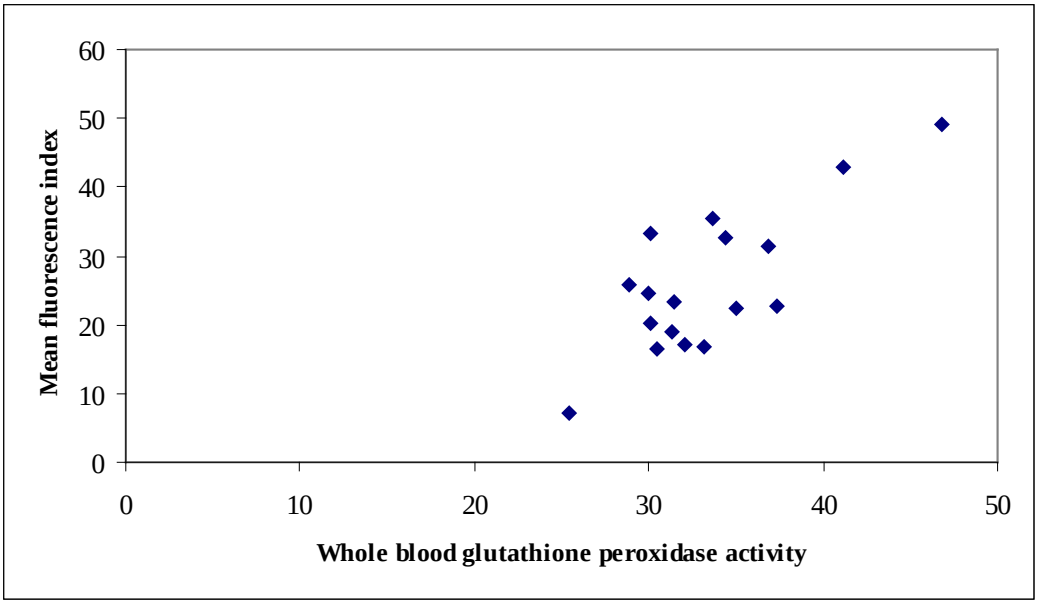

Figure 3.1. The correlation between the amounts of IFNγ produced by stimulated lymphocytes and whole blood glutathione peroxidase activity (µmol NADPH oxidized·mL^{-1}·min^{-1}·g protein) (r = 0.78; *P* = 0.0002; n=17).

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CHAPTER 4: Effect of Selenium Deficiency on the Vaccination Response and Immune Function in the Adult Horse

INTRODUCTION

Selenium (Se) is a trace mineral that has been shown to affect the humoral and cell-mediated components of the immune system in many different species (Roy et al., 1994; Finch and Turner, 1996; McKenzie et al., 1998). However, limited research has focused on the effect of Se on the equine immune system. Knight and Tyznik (1990) found ponies fed a diet containing 0.22 ppm Se had higher serum IgG levels and antibody titers in response to a sheep red blood cell vaccination than ponies fed a diet containing 0.02 ppm Se. In contrast, Baalsrud and Overnes (1986) reported no effect of Se supplementation on antibody titers unless combined with vitamin E, in comparison to an un-supplemented control group of low Se and vitamin E status. These studies focused on the effect of Se on humoral immunity, and cell-mediated immune function was not investigated. Se supplementation has been shown to affect the cell-mediated immune response in other species (Broome et al., 2004) and Se deficiency has also been reported to have an impact on cell-mediated immune function in response to an immune challenge (Beck et al., 2001; Li and Beck, 2007). Therefore, the aim of this study was to evaluate the effect of long term Se depletion on vaccine response and immune function as compared to horses fed an adequate Se (NRC, 2007) diet. The first objective was to evaluate primary and memory vaccine responses, evaluating both humoral and cell-mediated variables of immune response. The second objective was to assess cell-mediated immune function to mitogen stimulation *in vitro*. It was hypothesized that cell-mediated immune function as measured by mitogen stimulation would be suppressed in

horses of low Se status, and consequently low Se horses would have a poor vaccine response.

MATERIALS AND METHODS

Animals:

This research project was approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Twenty eight mature horses, aged 5 to 23 yr, were used in this study. The 28 horses included 8 geldings and 20 mares. The geldings consisted of 6 Thoroughbreds (TB), 1 American Quarter Horse (AQH) and 1 Standardbred, while the mares consisted of 19 TB and 1 AQH. All horses were kept at the University of Kentucky equine research farm, Maine Chance, Lexington, KY, for at least 60 d prior to the start of this study.

Experimental design:

The aim of this experiment was to compare the immune response of Se adequate and Se deficient horses. Therefore it was necessary to deplete a group of horses of Se. A diet low in Se (LS) was fed to 21 horses while 7 horses were fed an adequate Se (AS) diet and served as the control group. This resulted in an unbalanced design, but was necessary to accommodate a subsequent experiment that was to follow immediately upon completion of this experiment. One TB mare in LS was removed from the study following colic. Therefore LS consisted of 20 horses, while AS consisted of 7 horses.

Diets and treatments:

Horses were kept on pasture in groups of 4 to 8 for the duration of the study. Horses were allocated to pastures so that all treatments were equally represented. Pastures were sampled prior to the start of the study to establish Se content, and on a monthly basis thereafter while pasture availability was sufficient to sample. Although the Se content of the pastures fluctuated, it remained marginal (Table 4.1; 0.03 - 0.07 mg Se/kg DM) at all times. When pasture availability declined, horses were fed hay that was produced on the same research farm (< 0.06 mg Se/kg DM), and cracked corn (< 0.04 mg Se/kg DM). Horses had *ad libitum* access to water and an iodized salt block.

Custom formulated (Table 4.1) adequate Se (2.52 mg Se/kg DM) or low Se (0.48 mg Se/kg DM) supplements (McCauley Bros, Inc., Versailles, KY) were fed to meet the horses' nutritional requirements (NRC, 2007) above that provided by pasture, hay and corn. The composition of the balancer pellets appear in Appendix A Table 4.a. Horses were weighed on a monthly basis for the duration of the study by means of a portable, large animal scale (Trancell Technology TI-500BWL, Buffalo Grove, IL) and body weight (BW) was used to calculate (Appendix A Table 4.b.) the amount of supplement needed by each horse. The supplement was fed to the horses using individual feeding pens constructed for this purpose. The amount of supplement fed to each horse resulted in a total dietary Se intake 0.06 ppm Se on a dry matter basis for LS and 0.12 ppm Se on a DM basis for AS. The recommended Se intake for a mature horse is 0.0002 mg/kg BW/d (NRC, 2007). Horses were kept on their respective diets for a period of 28 wk prior to the vaccine challenge.

Blood sampling procedures:

Blood samples were taken prior to the start of the study and every 4 wk thereafter during the depletion period. All blood samples were obtained via jugular venipuncture. Blood was collected in 7 mL lithium heparin blood collection tubes (Becton Dickson, Franklin Lakes, NJ) for analysis of whole blood selenium and whole blood glutathione peroxidase activity (GPx). Blood was also collected in untreated blood collection tubes for serum separation (Becton Dickson) and EDTA containing tubes (Becton Dickson) for CBC analysis.

A vaccine challenge was conducted following the 28th wk of the depletion period. During the vaccine challenge additional blood samples were taken to isolate PBMC. For the isolation of PBMC, blood was collected in sodium heparin (Wickliff Veterinary Pharmacy, Lexington, KY) treated (100 µL per tube) 15 mL vacutainer tubes (Kendall, Monoject blood collection tubes, Mansfield, MA).

Vaccine challenge:

At the end of the 28 wk depletion period a vaccine challenge was used to evaluate the effect of the Se status on immune response. Vaccination and sampling time points are described in Figure 4.1. The selected vaccination and sampling time points, as well as the methods developed to evaluate the immune response to the novel vaccine, were tested prior to the initiation of the vaccine challenge in this study.

Keyhole limpet hemocyanin (Sigma, Saint Louis, MO; KLH) vaccines were prepared under sterile conditions in a laminar flow hood (NuAire Biological safety cabinets, Plymouth, MN). The KLH was dissolved in sterile physiological saline (Butler;

Lexington, KY) at a concentration appropriate to allow the final vaccine volume to be 1 mL (i.e. 10 mg KLH/mL for the first vaccine, 5 mg KLH/mL for the second vaccine). The vaccine was administered in the pectoral muscle after appropriate cleaning of the vaccination area with ethanol.

Pre-vaccination blood samples were taken immediately prior to administration of the first KLH vaccine. The 3 wk post vaccination blood samples were also collected immediately prior to the administration of the second KLH vaccine (5 mg KLH). At this time horses were also vaccinated using a combination vaccine containing equine influenza strain KY 02 (Prestige V; Intervet, Summit, NJ) to evaluate memory immune response. This vaccine was selected as all horses had received this vaccine as part of the routine vaccination protocol followed on the research farm prior to the start of the study. All horses were therefore expected to display a memory response to this KY 02 influenza strain.

Laboratory procedures:

Peripheral blood mononuclear cell isolation:

The PBMC were isolated at the pre (0 wk), 3 and 5 wk time points using Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) gradient centrifugation. Approximately 30 mL of plasma was layered on top of 10 mL Ficoll-Paque Plus in a 50 mL centrifuge tube (Fisher, Florence KY) and centrifuged for 30 min at 500 x g. The interface cells were collected, washed 3 times with phosphate buffered saline (Sigma), counted (Vi-cell counter; Beckman Coulter, Miami, FL) and re-suspended at the required concentrations for each assay in RPMI 1640 (Gibco, Grand Island, NY). The RPMI 1640

was supplemented with 2mM L-glutamine (Sigma), 100 U/mL penicillin/streptomycin (Sigma) and 55 μM 2-mercaptoethanol (Gibco), as described by Adams et al. (2008), and 2.5% heat inactivated autologous serum. Autologous serum was used so that each horse's PBMC's were exposed *in vitro* to its own serum, mimicking the amount of Se that PBMC would be exposed to *in vivo*. The autologous serum was heat inactivated by incubating the serum sample, collected as for serum separation, from each horse at the same time when blood was collected for PBMC isolation, and placing it in a 56˚C water bath for 30 min (Lessard et al., 1991). The sample was then centrifuged at 800 x g for 10 min, the serum aspirated and used to enrich the RPMI 1640 as stated above.

In vitro cell cultures:

Isolated PBMC were plated in 3.5 mL culture wells (TPP, Trasadingen, Switzerland), at a concentration of 4 x 10^6 cells per mL. Four culture wells were set up per horse. The cells were then stimulated in separate culture wells with either media alone (control), PMA (25ng/mL; Sigma) and ionomycin (1μM; Sigma), KLH (200 μg/mL; Sigma) or equine influenza virus (A/equine/KY/5/02). The PMA/ionomycin was only added for the last 4 h of the 24 h incubation period. Influenza virus for *in vitro* cell culture stimulations was prepared using 10-d-old embryonated chicken eggs as described by Adams et al. (2011). Influenza stimulation was conducted as follows: 2x10^7 PBMC's were first transferred to a microcentrifuge tube (Fisher). Equine influenza virus was then added to the cells at a multiplicity of infection (MOI) of 1. The cells and virus were incubated for 45 min in a 37˚C water bath. The cells were then centrifuged, re-suspended in autologous serum containing media, and plated in the culture wells at 4 x 10^6 cells per mL. Once plated, cell cultures were placed in a 37˚C, 5% CO_2 humidified incubator.

After 20 h Brefeldin A (2µL/well; Sigma) was added to all cell cultures. At this time 10 µL PMA (Sigma) and ionomycin (Sigma) was also added to the PMA designated wells. Cells were then incubated for an additional 4 h.

After a total incubation period of 24 h, 2 x 10^6 PBMC's from each culture well were removed for intracellular staining for the cytokines IFNγ and TNFα using a flow cytometer, while the remaining stimulated PBMC's were used to evaluate *in vitro* mRNA expression of cytokines using RT-PCR as described by Breathnach et al (2006).

Intracellular staining for IFNγ and TNFα:

To assess IFNγ and TNFα production, intracellular staining was performed, as previously described (Breathnach et al., 2006; Adams et al., 2009). The stained cells were analyzed using a flow cytometer (FACSCalibur, Becton Dickson, San Jose, CA). Using Cell Quest software (Becton Dickson, San Jose, CA) the percentage of lymphocytes, identified and gated based on the forward and side scatter parameters, producing IFNγ or TNFα was calculated. The mean fluorescence intensity (MFI) was determined as a measure of the amount of cytokine produced by means of the intensity of the IFNγ or TNFα signal (Breathnach et al., 2006). Using the computer software, markers were placed on the antibody labelled un-stimulated cells so that 1% of cells were IFNγ or TNFα positive. This marker was then set and used as the marker on the stimulated cells.

Cytokine mRNA expression in stimulated PBMC:

The PBMC stimulated as described above were used to assess the relative quantity of *in vitro* mRNA expression of IFNγ, TNFα, IL – 1, IL-2, IL-6, IL-8, IL-10, IL-13, as well as transcription factors GATA-3 and T-bet. Primer probe set sequences for the

detection of these equine cytokines have been published elsewhere (Saulez et al., 2010; Adams et al., 2011; Soboll Hussey et al., 2011).

After *in vitro* cell culture, $2x10^6$ cells were lysed by the addition of 1mL RNA-STAT 60 (Tel-Test, Isotex Diagnostics Inc., Friendswood, TX) and stored at -80˚C until total RNA was extracted according to manufacturer's protocol. Reverse transcription was conducted as described by Breathnach et al. (2006).

Cytokine gene expression was measured by means of RT-PCR (7900 HT Fast Real-Time PCR system, Applied Biosystems, Foster City, CA) using equine specific intron spanning primer/probe sets (Breathnach et al., 2006; Adams et al., 2009). Beta-glucuronidase (BGUS) was used as the internal control (Breathnach et al., 2006). Relative change in gene expression was calculated as $2^{-\Delta\Delta CT}$ where $\Delta\Delta C_T$ = [(average gene of interest C_T − average BGUS $C_T)_{horse}$ − (average gene of interest C_T − average BGUS $C_T)_{calibrator}$] as described in Livak and Schmittgen (2001). The average of the un-stimulated PBMC cultures was used as the calibrator.

Lymphocyte proliferation:

The ^{3}H-Thymidine incorporation method was used to evaluate lymphocyte proliferation. Isolated PBMC were plated at a concentration of $2x10^6$ cells/mL in 96 well flat-bottom plates (TPP, Trasadingen, Switzerland). Again 4 cultures were set up in triplicate for each horse containing either media alone, ConA (10 µg/mL; Sigma) or KLH (150 µg/mL Sigma). Equine influenza stimulated proliferation was evaluated utilizing PBMC stimulated with equine influenza as described above. These influenza simulated cells were then plated at $2x10^6$ cells/mL. After a 72 h incubation period in a 37˚C, 5%

CO_2 humidified incubator, PBMC were pulsed with 0.5μCi ^{3}H-thymidine and incubated for an additional 18 h period, and frozen to lyse cells (-20ºC). DNA was extracted from the cells onto fiber filter pads (Perklin Elmer Inc., Waltham, MA) using a Tomtec harvester (J/B Industries Inc, IL) and liquid scintillation counting performed (Beta counter; Wallac Inc., Gaitheisburg, MD). The results are reported as the stimulation index of corrected counts per minute of stimulated cells, divided by the corrected counts per minute for the media alone cells.

KLH specific IgG production:

Serum KLH specific antibody levels were measured by means of an ELISA assay. Briefly, Immunolon I - B microtiter plates (Fisher Scientific, Hanover Park, IL) were coated overnight at 4˙C with 300 μg KLH per well. Coated plates were then washed with PBS/0.05% TWEEN®-20 (PBS-T; Sigma) and blocked with PBS containing 0.5% polyvinyl alcohol (Sigma) for 1 h. The serum samples were diluted 1:300 in PBS-T. Plates were washed with PBS-T and serum was added to wells in triplicate. Plates were incubated at 37˙C for 1 h. Plates were washed with PBS-T and secondary antibody (Peroxidase–conjugated AffiniPure Goat Anti-Horse IgG; Jackson Immuno Research, West Grove, PA) was added to each well at 1:2500 dilution. Again plates were incubated at 37˙C for 1 h. After washing plates with PBS-T, substrate (3,3´ 5,5´,-tetramethylbenzidine; Sureblue TMB 1, KPL, Gaithersburg, MD) was added and color developed for 30 s before the reaction was stopped (TMB Stop Solution, KPL, Gaithersburg, MD). Optical density was measured at 450 nm (Bio-Rad, Hercules, CA). A standard curve developed from the serum of a previously vaccinated animal shown to

have a strong response to the vaccine was used to create a standard curve on each plate for the determination of relative ELISA units.

Equine influenza (A/equine/KY/5/02) antibody production:

Influenza specific antibody production was evaluated by means of a hemagglutination inhibition (HI) assay as well as an ELISA assay. Influenza virus (A/equine/KY/5/02) for the HI assay was prepared using 10-d-old embryonated chicken eggs, but the virus was concentrated from the collected allantoic fluid by polyethylene glycol precipitation and purified by sucrose gradient centrifugation as described by Adams et al. (2011).

Equine influenza (A/equine/KY/5/02) antibody (IgGa, IgGb, IgG(T)) levels were determined using the ELISA method. The 96-well Immunolon I - B microtiter plates (Fisher Scientific, Hanover Park, IL) were coated overnight at 4ºC with 10 hemagglutination units per well of purified influenza virus. Coated plates were then washed with PBS/0.05% TWEEN®-20 (PBS-T; Sigma, St. Louis, MO) and blocked with 2% non-fat dried milk powder in PBS-T (Sigma) for 1 h at 37˚C. A standard curve was constructed using serial dilutions of a serum sample from an influenza virus hyper immune horse. This allowed for the calculation of relative antigen-specific antibody concentrations in all experimental serum samples. Serum samples were diluted in PBS-T, so that OD values fell within the standard curve (IgGa, IgGb and IgG(T) at 1:100). Plates were incubated for 1 h at 37˚C. Plates were washed with PBS-T and incubated for 1 h at 37˚C with monoclonal antibodies specific for IgGa (CVS 40), IgGb (CVS 39) and IgG(T) (CVS 48). Following washes with PBS-T, plates were incubated with the

secondary antibody, horseradish peroxidase-conjugated goat-anti-mouse IgG antibody (Jackson Laboratories Inc., West Grove, PA) for 1 h at 37˚C. Again, plates were washed and substrate (3,3´ 5,5´,-tetramethylbenzidine; Sureblue TMB 1, KPL) was added and color developed. The reaction was stopped (TMB Stop Solution, KPL) and the optical density determined at 450 nm using a 96 well plate reader (BioRAD, Hercules, CA). Relative serum antibody concentrations expressed as ELISA units were calculated by comparison with the standard curve.

To conduct the HI titer assay each serum sample was trypsin-periodate treated to remove non-specific inhibitors of virus hemagglutination which are present in serum samples. This was done by adding 100 µL trypsin to 100 µL serum followed by 30 min incubation in a 56˚C water bath. Then 300 µL periodate was added and samples incubated at room temperature for 15 min, followed by the addition of 500 µL 0.6% glycerol in saline. This yielded a final serum dilution of 1:10. Next 25 µL PBS was added to all the wells of a round bottom 96 well plate (Thermo Fisher U-bottom micro titer vinyl plate). Then 50 µL of the treated serum was added to the first well on the plate, and 25 µL was then titrated across the row. Finally 25 µL virus (1:8 dilution) was then added to all wells. Plates were incubated for 30 min at room temperature. Buttons of non-agglutination were then read, and the titer allocated to the last non agglutinating dilution for each sample.

Complete blood count analysis:

Complete blood count analyses were performed by a local commercial equine hospital (Rood and Riddle Equine Hospital, Lexington, KY).

Selenium Status: Whole blood selenium

Whole blood Se concentration was determined by the Diagnostic Center for Population and Animal Health (Michigan State University; Lansing, MI) by means of inductively coupled plasma-mass spectroscopy.

Selenium Status: Whole blood glutathione peroxidase activity

Whole blood GPx activity was determined using the Bioxytech GPx-340 assay kit (OXIS research, Portland, OR), based on the method developed by Paglia and Valentine (1967). The kit was adapted for use with a microplate reader, as recommended by the manufacturer. Briefly, whole blood samples were diluted 1:40 in the buffer provided by the kit and kept on ice. Each sample was then added to a solution containing glutathione, glutathione reductase, and nicotinamide adenine dinucleotide phosphate (NADPH). Tert-butyl hydroperoxide was added to initiate the reaction, and the change in absorbance was measured at 340 nm over a 5 min period using a microplate reader with kinetic test capabilities (Versamax tuneable microplate reader, Molecular Devices, Sunnyvale, Ca). The GPx activity of each sample was calculated from the change in absorbance and expressed as units of enzyme activity per mg hemoglobin (mU/mg Hb).

Statistical analysis

Data were analyzed as a repeated measures design using the Proc Mixed function of SAS 9.1 (SAS Institute Inc., Cary, NC) with least square means separation procedure. The model included time, treatment and block as fixed effects, while horse was included as a random effect. Data were tested for normality, and log transformed when required

for statistical analysis. Data were back transformed and are presented in tables and figures as least squares means.

RESULTS

Selenium and vitamin E status:

At the time of vaccination the whole blood Se concentration and GPx activity of LS was lower than the Se concentration ($P < 0.0001$) and GPx activity ($P = 0.011$) of AS (Table 4.2). Serum vitamin E concentration did not differ between treatments ($P = 0.4538$) and ranged from 3.1 to 7.4 µg/mL in individual horses.

Complete blood count data:

During the vaccine challenge horses receiving the LS diet had an overall higher neutrophil count (effect of treatment; $P = 0.010$), but there was no effect of time or treatment x time ($P > 0.05$). There was no effect of treatment, time or treatment x time interaction for lymphocyte numbers or lymphocyte to neutrophil ratio (Table 4.3).

KLH vaccination response:

The antibody response to KLH vaccination (Figure 4.2) was not affected by treatment ($P = 0.2082$) but there was an effect of time ($P < 0.0001$) and a treatment x time interaction ($P = 0.0464$). Separation of means indicated AS produced more KLH antibodies at the 3 wk post vaccination time point compared to LS ($P < 0.05$). However, KLH antibody production at subsequent time points was similar between treatments. Lymphocyte proliferation in response to KLH stimulation was unaffected ($P > 0.05$) by

Se status (Table 4.4). The mRNA expression of selected cytokines for PBMC stimulated *in vitro* with KLH is presented in Table 4.5. The mRNA expression of the transcription factor T-bet was affected by treatment (P = 0.025) and time (P = 0.046). Overall T-bet expression was higher for AS than LS. In addition a trend existed for higher IL-1 (treatment, P = 0.070) in AS compared to LS. The mRNA expression of the remaining cytokines were unaffected by Se status. Similarly intracellular IFNγ and TNFα production by KLH stimulated lymphocytes were unaffected by Se status (Appendix A Table 4 c and d).

Equine influenza vaccination response:

Overall Se status did not have an effect on IgGa, IgGb, IgGT or influenza titers (Table 4.6). Lymphocyte proliferation (Table 4.6) in response to influenza stimulation was also similar between AS and LS. Lymphocyte intracellular IFNγ and TNFα production in response to *in vitro* influenza stimulation was unaffected by Se status (Appendix A Table 4 c and d). The mRNA expression of cytokines stimulated with influenza *in vitro* was also not affected by Se status (Appendix A Table 4 e).

Mitogen stimulation:

Stimulation of lymphocytes with ConA indicated that the ability of the cells to proliferate was comparable between AS and LS (Table 4.7). Similarly PBMC intracellular IFNγ and TNFα production and cytokine mRNA expression in response to PMA stimulation were not affected by Se status (Appendix A Table 4 c and d).

DISCUSSION

The onset of the vaccine challenge followed a 28 wk feeding period. At that time Se status was lower for LS compared to AS, as indicated by whole blood Se and GPx activity. Mean whole blood Se concentration of LS fell below the adequate reference range for mature horses (180 to 240 ng/mL) as reported by Stowe (1998). The GPx activity for LS was also at the lower end of the adequate GPx activity range (40 to 160 EU/g hb) (Stowe, 1998). The selenoenzyme GPx was one of the first selenoproteins identified, and has subsequently been used as an indicator of Se status (Brown and Arthur, 2001). The current Se requirements (NRC, 2007) of the horse is based on research studies that used GPx as an indicator of Se status (Stowe, 1967; Shellow et al., 1985). Therefore, at the time of vaccination horses in LS were considered to be of low Se status, while horses in AS were in adequate Se status. Vitamin E and Se have a synergistic relationship and vitamin E status has been shown to affect immune function across different species (Finch and Turner, 1996), including the horse (Baalsrud and Overnes, 1986; Petersson et al., 2010). Vitamin E status was assessed at the initiation of the vaccine challenge, and found to be adequate (> 2 µg/mL; NRC (2007)) and similar across treatments. Because micronutrient status may be important when evaluating lymphocyte function *in vitro* (Lee and Wan, 2002) the supplements fed to the horses were formulated to meet their micronutrient requirements (with the exception of Se in the supplement used for the LS group). Therefore any differences in immune function observed between treatments can be attributed to the differences in Se status between groups.

Calamari et al. (2009a) reported no effect of Se supplementation on neutrophil numbers in horses, although a trend for a linear increase in lymphocyte numbers with an

increase in Se supplementation was observed. In this study, the number of lymphocytes and lymphocyte to neutrophil ratio did not differ between AS and LS. However, LS did have a higher number of neutrophils. The reason for the differences between the two studies is not clear, although the experimental designs are different in that a longer feeding period was accommodated in the current study compared to Calamari et al. (2010) (196 d vs 112 d) and these researchers also incorporated a light exercise regime in their study.

A novel antigen (KLH) was used to assess the primary immune response, evaluating both humoral and cell-mediated components. The antigen KLH has previously been used to assess vaccination response in horses (Edmonds et al., 2001; Sturgill and Horohov, 2010). Prior to participation in this study all horses were vaccinated with a combination vaccine that contained the KY02 equine influenza strain. Therefore memory response was evaluated using this same vaccine.

The KLH-specific IgG levels increased over time in both groups. However, AS responded quicker to the first vaccine and had higher antibody levels at 3 wk than LS. The response to the first vaccination can be regarded as the true primary response, indicating a quicker humoral response for the adequate horses. Antibody production was comparable between LS and AS after the second vaccination. Similarly, Knight and Tyznik (1990) reported ponies fed a diet containing 0.22 ppm Se had higher serum IgG levels and antibody titers in response to a sheep red blood cell vaccination compared to ponies fed a diet low in Se (0.02 ppm Se). However, in contrast to our results, the ponies on the higher Se diet maintained higher antibody levels in response to a second vaccination at 2 wk (Knight and Tyznik, 1990). Baalsrud and Overnes (1986) evaluated

antibody production in horses supplemented with vitamin E (80 ppm α-tocopheryl acetate), Se (0.5 ppm as sodium selenite) or a combination of the two. Horses on an un-supplemented Se (0.03 ppm) and vitamin E (18 mg α-tocopheryl acetate/kg) diet served as the control group. Horses were kept on this diet for 12 wk prior to the vaccine challenge. The reported serum Se values suggested that the control group was of low Se status. Following vaccination, at the start and wk 6 of the vaccination period, antibody titres against *Clostridium tetani* and equine influenza strains were evaluated. It was reported that horses had not been vaccinated against these antigens before. In contrast to our study no difference was detected between the Se only and control group (Baalsrud and Overnes, 1986), although the vitamin E alone and vitamin E and Se combination treatments did respond better than the control group.

In addition Baalsrud and Overnes (1986) evaluated antibody titres against *E. coli* O-antigens. The horses were exposed to *E. coli* at a prior occasion so their response to this exposure may be viewed as a memory type of response. It was reported that antibody titres against *E. coli* were comparable between treatments (Baalsrud and Overnes, 1986). Influenza-specific antibody production and influenza HA titres were unaffected by Se status in our study. These results suggest that Se status may not affect the anamnestic humoral immune response.

Lessard et al. (1991) reported that the effect of Se status on lymphocyte proliferation was dependent on the use of autologous serum in culturing media. In this study antigen specific lymphocyte proliferation in response to KLH and influenza stimulation *in vitro* was not affected by Se status even though autologous serum was used. This is in contrast to research conducted by Broome et al. (2004) in humans that

reported peak lymphocyte proliferation in response to polio virus stimulation to occur earlier (d 7) in Se supplemented groups compared to the placebo treatment group (d 14). It should be noted that in our study proliferation in response to KLH did increase over time, indicating that the KLH vaccination itself did have an effect on the ability of cells to proliferate in response to *in vitro* stimulation with KLH.

T-bet is a transcription factor associated with the activation of the Th1 response, associated with intracellular pathogen defense, while GATA-3 is associated with a Th2 response related to extracellular pathogen defense (Chinen et al., 2006). In addition, the specific cytokines produced by either Th1 or Th2 subset also play a role in inhibiting the other subset (Yates et al., 2004). Higher expression of the transcription factor T-bet was found for the KLH stimulated PBMC from the AS horses which may suggest a Th1 biased response in the AS horses. However, we did not observe a corresponding increase in expression of IFNγ in PBMC from the AS horses. Beck et al. (2001) evaluated the immune response in mice, fed a low or an adequate Se diet, upon exposure to influenza virus strain A/Bangkok/1/79; H3N2. Based on the cytokine responses measured these researchers concluded that the immune response observed in the lungs of Se-deficient mice were skewed towards a Th2 response, the opposite of what would be expected after a viral challenge (Beck et al., 2001). Intracellular production of IFNγ and TNFα, following stimulation with KLH and influenza *in vitro* was also not affected by Se status. Broome et al. (2004) reported higher IFNγ production (pg/mL) by polio virus stimulated whole blood on d 7 in Se supplemented human subjects compared to the placebo group. These researchers also noted that the overall pattern of Th1 and Th2 cytokine release was not affected by Se status.

The relative mRNA cytokine expression measured in KLH and influenza stimulated PBMC indicated no difference between LS and AS for any of the cytokines measured, with the exception of a trend for IL-1 (KLH stimulated PBMC) to be higher in horses of adequate Se status. This cytokine is regarded as a pro-inflammatory cytokine that targets macrophages, and has a synergistic relationship with TNFα (Dinarello, 2000). In addition IL-1 is reported to be involved in the activation and stimulation of other cytokines (Tato and Cua, 2008a). However, in our study all other cytokines were present at similar mRNA levels, including TNFα in both groups.

In addition to vaccine response, we also investigated immune function in response to stimulation with the mitogens, ConA and PMA. Our results indicated lymphoproliferative response to ConA was not affected by Se status. Lack of effect of Se status on lymphocyte proliferation has been reported by other researchers (Aziz and Klesius, 1985; Wuryastuti et al., 1993). In contrast, Cao et al. (1992) reported Se deficient bovine cells were not able to proliferate to the same extent as Se adequate cells. However, the blood Se and GPx activity reported for the cattle suggests that the Se deficient cattle were of lower Se status and the Se adequate cattle of higher Se status than the horses in this study. Research has also indicated that the *in vitro* addition of Se to cell cultures obtained from deficient animals improved lymphocyte response (Finch and Turner, 1996) while the addition of H_2O_2 *in vitro* has been shown to suppress lymphocyte proliferation in a dose dependent manner, implicating the importance of adequate Se status in the neutralization of H_2O_2 for optimal lymphocyte proliferation (Lee and Wan, 2002). In our study a difference existed in GPx activity between AS and LS. Therefore, if low GPx activity resulted in higher, proliferation inhibiting levels of H_2O_2, we would

have noted a difference in the lymphoproliferative responses of LS and AS. Supplementation of human subjects with vitamin E have been reported to increase lymphocyte proliferation (Meydani et al., 1990). Adequate vitamin E status of the horses on this trial may therefore have contributed to the lack of difference in proliferation observed between LS and AS.

Intracellular IFNγ and TNFα production as well as mRNA expression of cytokines and transcription factors in PBMC stimulated with PMA were similar across all treatments, indicating of a lack of effect of Se status on the ability of the cells to express these cytokines. It has been proposed that the ability of Se to affect immune response lies in its antioxidant properties. Adequate Se status is thought to control reactive oxygen species levels, leading to lower levels of oxidative stress, which in turn prevents the over-activation of NF-κβ (Beck et al., 2001). Activation of NF-κβ stimulates the production of pro-inflammatory proteins associated with immune response such as IL-6 and TNFα (Duntas, 2009). Vitamin E forms a part of the antioxidant mechanism of the body, preventing oxidative stress (Surai, 2006), therefore adequate vitamin E status may also have contributed to the lack of Se status on cell-mediated immune response to non-specific stimulation.

CONCLUSION

Overall Se status did affect the humoral and cell-mediated immune response observed to vaccination with a novel antigen. The AS horses responded faster to the initial vaccine by producing greater amounts of KLH-specific antibodies in response to the first vaccine, and more effectively priming a cellular immune response, as evidenced by the overall increased expression of T-bet in the AS horses. Although vaccination

response was diminished in LS, cell-mediated immune function as measured by non-specific stimulation *in vitro* was unaffected by Se status. This study outcome suggests that low Se status could affect immune response to primary vaccination in horses kept in Se deficient areas of the U.S.

Table 4.1. Nutrient composition (DM basis)[1] of pasture, hay and the supplements fed to mature horses during the experimental period.

Item		Pasture	Grass Hay	Alfalfa Hay	Adequate Se supplement[2]	Low Se supplement[2]
DM	%	92.17	89.70	89.7	89.2	90.1
DE	Mcal/kg	2.11	2.10	2.22	3.35	3.38
CP	%	13.27	16.30	16.2	36.5	34.8
ADF	%	41.83	36.10	34.2	9.2	8.5
NDF	%	59.90	59.70	50.6	15.4	14.9
Starch	%	0.47	1.50	1.9	5.1	7.8
Calcium	%	1.06	1.10	1.06	4.07	4.16
Phosphorus	%	0.36	0.47	0.4	2.16	2.07
Magnesium	%	0.22	0.29	0.24	0.45	0.47
Potassium	%	1.59	1.77	2.15	2.05	1.98
Sodium	%	0.01	0.02	0.03	0.85	0.88
Iron	ppm	728.33	570.00	376	806	802
Zinc	ppm	34.67	33.00	32	448	427
Copper	ppm	8.00	9.00	7	160	154
Selenium	ppm	0.07	0.07	0.06	2.52	0.48

[1] Equi-analytical laboratories; Ithaca, NY
[2] McCauley Bros, Inc., Versailles, KY

Table 4.2. Indicators of Se and vitamin E status evaluated at the start of the vaccine challenge (week 28).

Variable	AS[1]	LS[1]	*P*-value
Whole blood Se (ng/mL)	211.14[a]	164.70[b]	<0.0001
Whole blood GPx (mU/mg hb)	55.00[a]	42.72[b]	0.0011
Serum vitamin E (µg/mL)	4.70[a]	5.11[a]	0.4538

[a,b] Means within row lacking a common superscript differ, $P < 0.05$

Table 4.3. Complete blood count[1] analysis of horses on different dietary Se treatments at 0, 3 and 5 wk of the vaccine challenge period[2].

	Week				Main effect (*P*-value)		
Item	0	3	5	SEM	Treatment	Time	Treatment x time
Lymphocyte (x 10^3)							
AS	2.48	2.53	2.53	0.38	0.192	0.836	0.904
LS	2.83	3.08	2.88	0.23			
Neutrophil (x 10^3)							
AS	4.46	4.24	4.26	0.33	0.010	0.676	0.608
LS	5.22	5.20	5.67	0.20			
Ratio (N/L)							
AS	2.00	1.76	1.82	0.41	0.391	0.694	0.733
LS	2.06	1.90	2.37	0.24			

[1]Complete blood count analysis conducted by Rood and Riddle Equine Hospital (Lexington, KY)
[2]Vaccinations administered at wk 0 and wk 3

Table 4.4. Lymphocyte proliferation for horses on different dietary treatments[1] in response to *in vitro* stimulation with keyhole limpet hemocyanin[2] (presented as stimulation index[3]; LS means).

	Week				Main effect (*P*-value)		
Item	0	3	5	SEM	Treatment	Time	Treatment x time
AS[1]	1.41	1.77	3.60	0.49	0.182	0.001	0.131
LS[1]	1.31	1.70	2.18	0.29			

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM)
[2]Vaccinations administered at wk 0 and wk 3
[3]Stimulation index: Lymphocyte proliferation in response to *in vitro* stimulation keyhole limpet hemocyanin

Table 4.5. The mRNA expression as relative quantity of selected cytokines in peripheral blood mononuclear cells stimulated with keyhole limpet hemocyanin *in vitro* in mature horses fed a selenium adequate (AS) or a low selenium (LS) diet[1].

	Week				Main effect (*P*-value)		
Item[2]	0	3	5	SEM	Treatment	Time	Treatment x time
T-bet							
AS[3] (n=4)	1.91	1.27	2.78	0.39	0.025	0.046	0.268
LS[3] (n=8)	0.11	1.38	0.58	0.34			
GATA 3							
AS (n=4)	1.54	2.60	1.04	0.56	0.498	<0.0001	0.476
LS (n=10)	1.22	2.76	0.64	0.32			
IL-1							
AS (n=4)	25.34	9.48	6.13	4.16	0.070	0.016	0.123
LS (n=10)	5.07	11.11	2.25	2.49			
IL-6							
AS (n=4)	7.93	3.69	3.52	2.98	0.406	0.344	0.126
LS (n=10)	1.24	5.19	1.63	1.75			
IL-8							
AS (n=4)	1.14	0.72	0.72	0.27	0.728	0.001	0.372
LS (n=10)	1.37	0.92	0.58	0.15			
IL-10							
AS (n=4)	11.14	8.49	6.42	3.22	0.242	0.046	0.342
LS (n=10)	3.63	8.27	3.03	1.93			
IL-13							
AS (n=4)	2.17	1.50	1.97	1.38	0.740	0.331	0.240
LS (n=9)	0.65	1.27	1.41	0.87			

[1] Vaccinations administered at wk 0 and wk 3
[2]Upon analysis datasets from some horses were omitted as indicated in the table
[3]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM)

Table 4.6. The humoral immune response and lymphocyte proliferation in horses fed different dietary treatments following vaccination with equine influenza.

	Week[2]				Main effect (*P*-value)		
Item	0	5	7	SEM	Treatment	Time	Treatment x time
IgGa[1]							
AS[3]	105	336	167	117	0.389	<0.0001	0.261
LS[3]	174	392	296	69			
IgGb[1]							
AS	3763	4460	3873	3515	0.464	0.697	0.775
LS	3972	8629	7634	2084			
IgGT[1]							
AS	4397	4711	4794	1774	0.200	0.016	0.858
LS	6564	7675	7500	1053			
Flu titer[4]							
AS	156	208	208	36	0.222	0.016	0.943
LS	87	148	143	22			
SI[5]							
AS	4.62	4.31	*	3.9	0.794	0.395	0.479
LS	6.84	3.46	*	2.4			

[1]IgGa, IgGb and IgGT measured as ELISA units relative to a standard curve produced from a high responding horse
[2]0 = pre vaccination, 5 = 2 wk post vaccination, 7 = 4 wk post vaccination for equine influenza
[3]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM)
[4]Hemagglutination inhibition assay using equine influenza A/equine/KY/5/02
[5]SI = stimulation index: Lymphocyte proliferation in response to stimulation with equine influenza strain A/equine/KY/5/02

Table 4.7. Lymphocyte proliferation for horses on different dietary treatments in response to stimulation with concanavalin A (ConA) (presented as stimulation index[2] LS means).

	Week				Main effect (*P*-value)		
Item	0	3	5	SEM	Treatment	Time	Treatment x time
AS	16.44	8.81	6.25	5.05	0.994	0.099	0.397
LS	12.24	12.01	7.36	3.02			

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM)
[2]SI = stimulation index: Lymphocyte proliferation in response to *in vitro* stimulation with ConA

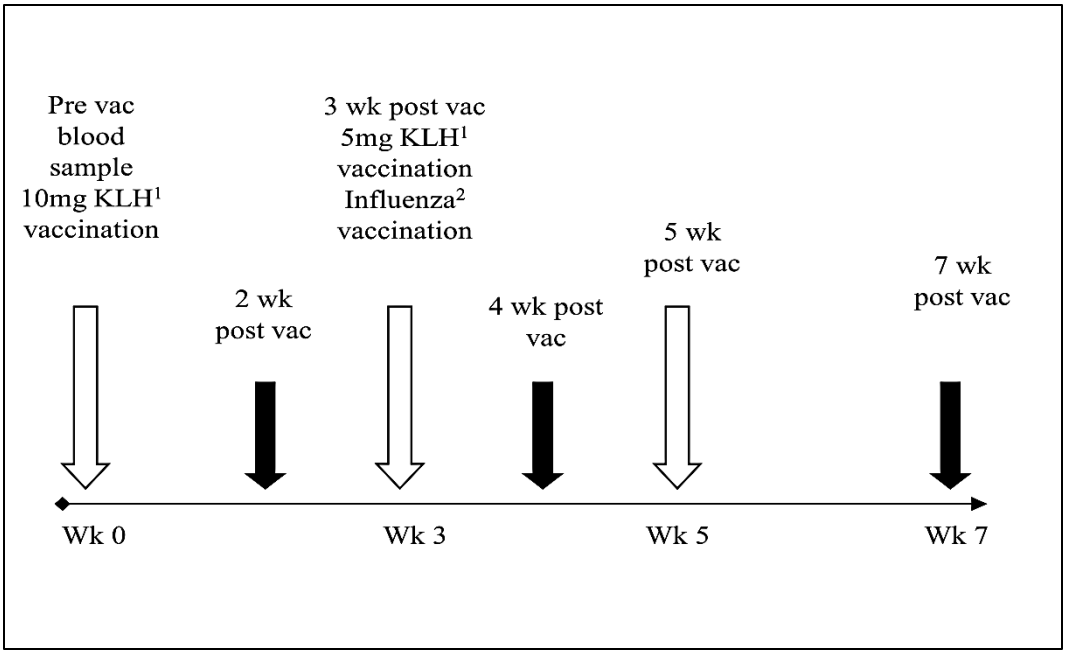

Figure 4.1. Sampling and vaccination time points for KLH vaccination. White arrows denote blood collection for determination of Se status, antibody production and peripheral blood mononuclear cell isolation. Black arrows denote blood collection for antibody production determination only.

[1]Keyhole limpet hemocyanin (KLH), Sigma Saint Louis, MO.
[2]Prestige V, Intervet, Summit, NJ

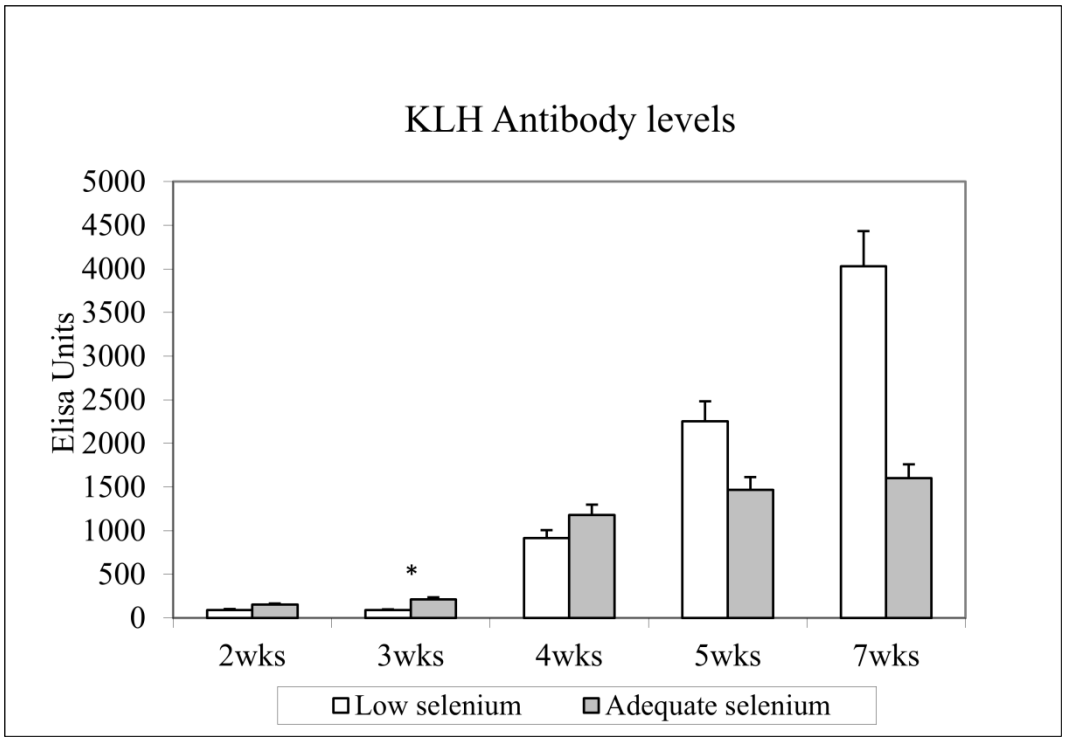

Figure 4.2. Anti-KLH-IgG response[1] to KLH vaccination[2] in mature horses[3].

[1]Treatment P = 0.2082; time P = <0.0001; treatment x time P = 0.0464
[2]Vaccinations administered at wk 0 and wk 3 of the vaccine challenge period
[3]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM)
* Treatment differences within time point ($P < 0.05$)

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CHAPTER 5: The Effect of Selenium Supplementation on Vaccination Response and Immune Function in Adult Horses

INTRODUCTION

Selenium has been shown to affect immune function, although the exact mechanism through which Se and the immune system interact remains unknown. In studies with mice, Se status has been shown to affect the mRNA expression of some immune modulating cytokines (Li and Beck, 2007) and cytokine receptor, e.g. interleukin-2 receptor (Roy et al., 1994). Alteration in lymphocyte proliferation has also been linked to Se status (Roy et al., 1994). However, studies investigating the effect of Se supplementation on the equine immune system are limited (Knight and Tyznik, 1990; Janicki, 2001; Thorson et al., 2010; Montgomery et al., 2012) and the relationship of Se status to immune related cytokines in the adult horse has not been investigated. Therefore the aim of this study was to evaluate the effect of Se status and source of supplementation on the vaccination response and immune function of the horse. The first objective was to determine if Se status affected the primary response to vaccination with a novel antigen (ovalbumin) and memory response to an equine influenza vaccine. Measures of vaccine response included both humoral and cell-mediated components of the immune system: antigen-specific antibody production, lymphocyte proliferation, intracellular cytokine production and cytokine mRNA expression in response to antigen stimulation *in vitro*. The second objective was to determine if cell-mediated immune function was affected by Se status using *in vitro* cell cultures stimulated with non-specific mitogens. Immune function was assessed using lymphocyte proliferation, intracellular cytokine production and cytokine mRNA expression by PBMC. Un-stimulated whole blood mRNA cytokine

expression was used to assess *in vivo* cytokine expression. We hypothesized that horses of low Se status would have diminished immune function, contributing to a poor vaccine response.

MATERIALS AND METHODS

Animals:

This research project was approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Twenty eight mature horses, aged 5 to 23 yr, were used in this study and included 8 geldings and 20 mares. The geldings consisted of 6 Thoroughbreds, 1 American Quarter Horse and 1 Standardbred, while the mares consisted of 19 Thoroughbreds and 1 American Quarter Horse.

Experimental design, diets and treatments:

In preparation for this study the 28 horses were blocked by age and gender and randomly allocated within block to one of 4 dietary Se treatments: a low Se group (LS), adequate Se group (AS) an organic Se group (Sel-Plex®, Alltech Inc., Nicholasville, KY; SP) and an inorganic Se group (sodium selenite; SS). For 35 wk prior to the onset of this experiment horses assigned to the LS, SP and SS treatments were kept on a low Se diet (0.06 ppm) to allow for the depletion of their Se stores while AS was provided with an adequate Se diet (0.12 ppm). The results of the depletion phase were reported previously

(Chapter 4). The depletion phase was immediately followed by the repletion (supplementation) phase.

Throughout the repletion phase the horses were kept out on pasture, but were allocated to pasture so that all treatments were represented in each pasture. Pastures were sampled on a monthly basis until pasture availability was too low to sample. The Se content of the pastures fluctuated but remained marginal in Se (0.03 - 0.07 ppm DM; Table 5.1). When pasture availability declined in winter, horses were fed hay that was produced on the same farm (0.06 ppm DM), and cracked corn (< 0.16 ppm DM) to maintain an adequate calorie intake. Horses had *ad libitum* access to water and an iodized salt block. Horses were weighed on a monthly basis by means of a portable, large animal scale (Trancell Technology TI-500BWL; Buffalo Grove, IL) and BW was used to calculate supplementation rates. The Se intake as well as other nutrient intakes from pasture was calculated based on estimated pasture intake of 2.25% of BW (Table 5.2). Custom formulated adequate Se (2.52 ppm DM) or low Se (0.53 ppm DM) protein–vitamin-mineral balancer pellets (McCauley's; Versailles, KY) were fed to meet each horse's trace mineral requirements above that provided by pasture. The composition of the balancer pellets appear in Appendix A Table 4.a. The balancer pellet was fed on an individual basis, using feeding pens constructed for this purpose.

The 29 wk repletion phase followed the depletion phase. Horses originally assigned to AS and LS remained on their respective diets, while SP and SS were provided with their assigned supplements. The respective Se supplements were top-dressed on the balancer pellet. To account for the yeast component in the Se-yeast supplement, the same strain of brewer's yeast was used as carrier for the sodium selenite supplement. Horses on

the AS and LS treatments received the brewer's yeast carrier only. This allowed for a similar brewer's yeast intake between the four treatment groups. A small amount of water was added to the balancer pellet and top-dressed supplement prior to feeding to improve the palatability and texture. The horses were closely monitored to ensure that the allotted supplement and balancer pellet was completely consumed.

Total dietary Se intake for the LS group was calculated to be 60% of the NRC (2007) recommended amount of 1 mg Se per day for a 500 kg horse (or 0.1 mg Se/kg DM), while the AS group received a total of 120% of the recommended amount of Se. Horses on the SP and SS treatments were calculated to receive a total dietary Se intake of 340% (0.3 mg Se/kg DM) of the recommended Se intake. To achieve this 2.74 mg supplemental Se (SP or SS respectively) was added to the low Se balancer pellet as indicated in Table 5.2. Total dietary Se intake was calculated to be equal among horses within treatment on a per kg BW basis. The horses were kept on their respective diets for a period of 22 wk prior to the onset of the vaccine challenge. This period was chosen to ensure that the turnover of circulating red blood cells would be sufficient to identify change in Se status through measurements of whole blood Se and GPx activity. Horses remained on the same diets throughout the 7 wk vaccine challenge. Prior to the vaccine challenge two Thoroughbred mares and one gelding had to be removed from the study due to unrelated medical reasons. Therefore the AS, LS and SS groups consisted of 6 horses each, while the SP group consisted of 7 horses.

Blood sampling:

Blood samples were taken prior to the start of the repletion phase and every 4 wk thereafter, to monitor Se status and during the vaccine challenge as indicated in Figure 5.1. All blood samples were obtained via jugular venipuncture. Blood was collected in 7 mL lithium heparin blood collection tubes (Becton Dickson, Franklin Lakes, NJ) for analysis of whole blood Se and GPx activity. Blood was also collected in untreated blood collection tubes for serum separation (Becton Dickson) and EDTA containing tubes (Becton Dickson) for CBC analysis. During the vaccine challenge, additional blood samples were taken to isolate peripheral blood mononuclear cells (PBMC). For the isolation of PBMC, blood was collected in 15 mL, sodium heparin (Wickliff Veterinary Pharmacy, Lexington, KY) treated (100 µL per tube), vacutainer tubes (Kendall, Monoject blood collection tubes, Mansfield, MA).

Vaccine challenge:

Upon completion of the 22 wk repletion period a novel vaccine challenge was used to assess immune response. The vaccine challenge was completed over 7 wk. Vaccination and sampling time points for the vaccine challenge are described in Figure 5.1. The ovalbumin (Sigma, Saint Louis, MO; OVA) vaccines were prepared under sterile conditions in a laminar flow hood (NuAire Biological safety cabinets, Plymouth, MN). The OVA was dissolved in sterile physiological saline (Butler; Lexington, KY) at a concentration appropriate to allow the final vaccine volume to be 1 mL (i.e. 10 mg OVA/mL). The vaccine was administered i.m. in the pectoral muscle following thorough cleaning of the area with ethanol.

Pre-vaccination blood samples were taken immediately prior to the first vaccination. Horses were then injected with 10 mg OVA (Sigma) followed by a second 10 mg OVA injection 3 wk later (wk 25). Horses were also vaccinated against equine influenza strain KY 02 (Prestige V; Intervet, Summit, NJ) at the time of the second OVA vaccine i.e. 3 wk post the initial OVA vaccine (wk 25), to evaluate memory immune response. This vaccine was selected as all horses had been vaccinated with this vaccine 8 mo prior. All horses were therefore expected to display a memory response to the KY 02 influenza strain. The influenza vaccine was administered intramuscular in the neck, again following thorough cleaning of the vaccination area with ethanol.

Laboratory procedures:

Selenium Status: Whole blood selenium

Whole blood Se concentration was determined by the Diagnostic Center for Population and Animal Health (Michigan State University; Lansing, MI) by means of inductively coupled plasma-mass spectroscopy.

Selenium Status: Whole blood glutathione peroxidase (GPx) activity

Whole blood GPx activity was determined using the Bioxytech GPx-340 assay kit (OXIS research, Portland, OR) (Richardson et al., 2006), based on the method developed by Paglia and Valentine (1967). The GPx activity of each sample was calculated from the change in absorbance and expressed as units of enzyme activity per mg hemoglobin (mU/mg Hb).

Complete blood count analysis (CBC):

The CBC analyses were performed by a local commercial equine hospital (Rood and Riddle Equine Hospital, Lexington, KY). The number of lymphocytes and neutrophils were then calculated from this analysis.

Peripheral blood mononuclear cells (PBMC):

The PBMC were isolated using Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) gradient centrifugation. Approximately 30 mL of plasma was layered on top of 10 mL Ficoll-Paque Plus and centrifuged for 30 min at 500 x g. Interface cells were collected, washed 3 times with phosphate buffered saline (Sigma), counted (Vi-cell counter; Beckman Coulter, Miami FL) and re-suspended at the required concentrations for each assay in RPMI 1640 (Gibco, Grand Island, NY). The RPMI 1640 (Gibco) was supplemented with 2mM L-glutamine (Sigma), 100 U/mL penicillin/streptomycin (Sigma), 55 μM 2-mercaptoethanol (Gibco) and 2.5% heat inactivated autologous serum. Autologous serum was used so that the PBMC from each horse were incubated in media containing its own serum, especially as serum Se would vary between horses on different dietary treatments. Autologous serum was heat inactivated as described by Lessard et al. (1991).

In vitro cell cultures:

The PBMC were plated in 3.5 mL culture wells (TPP, Trasadingen, Switzerland) at a final concentration of 4 x 10^6 cells per mL. Four culture wells were prepared per horse for *in vitro* stimulation of cells: media alone (control); phorbol 12-myristate 13-acetate (PMA; 25 ng/mL) and ionomycin (1μM), added for the last 4 h of incubation;

OVA (5 μg/mL) and equine influenza virus. Prior to plating, PBMC's intended to be stimulated with equine influenza virus (KY/5/02) were transferred to microcentrifuge tubes and incubated in a 37˚C water bath for 45 min with KY/5/02 virus at a multiplicity of infection (MOI) of 1. Following incubation the influenza stimulated PBMC were centrifuged (300 x g for 5 min) and the PBMC pellet suspended in the supplemented media. After adding PBMC to the cell culture wells the plates were placed in a 37˚C, 5% CO_2 humidified incubator for 20 h. After 20 h Brefeldin A (2 μL/well) was added to all cell cultures. At this time the PMA and ionomycin (10 μL/well) was added to the PMA wells and all PBMC incubated for an additional 4 h. After a total incubation period of 24 h, 2 x 10^6 PBMC's from each culture were used for intracellular staining for interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) using a flow cytometer, while *in vitro* mRNA expression of cytokines was measured on the remaining stimulated PBMC's using RT-PCR as described by Breathnach et al (2006).

Intracellular staining for IFNγ and TNFα:

Intracellular staining was performed to assess IFNγ and TNFα production, as previously described (Breathnach et al., 2006; Adams et al., 2009). The stained cells were analysed using a flow cytometer (FACSCalibur, Becton Dickson, San Jose, CA). Using Cell Quest software (Becton Dickson, San Jose, CA) markers were placed on the unstimulated (media alone) cells so that 1%of the cells were IFNγ or TNFα positive. These markers were then used to analyze the stimulated cells. The percentage of lymphocytes, identified and gated based on the forward and side scatter parameters, producing IFNγ or TNFα was calculated. The mean fluorescence intensity (MFI) was

determined as a measure of the amount of cytokine produced as indicated by the intensity of the IFNγ or TNFα signal (Breathnach et al., 2006).

Cytokine mRNA expression in stimulated PBMC

The PBMC stimulated as described above were used to assess the *in vitro* gene expression of IFNγ, TNFα, Granzyme B (GrzB), IL (interleukin) -1, IL-2, IL-6, IL-8, IL-10, IL-13, GATA-3 and T-bet. After stimulation, 2 x 10^6 cells were lysed by the addition of 1 mL RNA-STAT 60 (Tel-Test, Isotex Diagnostics Inc., Friendswood, TX) and stored at -80˚C until total RNA was extracted following the manufacturer's protocol. Relative quantity of cytokine gene expression was determined by means of RT-PCR (Applied Biosystems, Foster City, CA) using equine specific intron spanning primer/probe sets (Applied Biosystems, Foster City, CA) as previously described (Breathnach et al., 2006; Adams et al., 2009). Relative change in gene expression was calculated as described in Livak and Schmittgen (2001), and results were expressed as relative quantity (RQ) calculated as $2^{-\Delta\Delta CT}$ with the un-stimulated PBMC cultures of each treatment group serving as calibrators for stimulated samples within the treatment as no statistical differences were detected between the un-stimulated samples. Beta-glucuronidase was used as the internal control (Breathnach et al., 2006).

In vivo cytokine expression: Paxgene analysis

In vivo mRNA expression of IFNγ, TNFα, IL-6, IL-8 and IL-10 in unstimulated whole blood was assessed by collecting 2.5 mL blood via jugular venipuncture into PAXgene blood RNA tubes (PreAnalytiX, Valencia, CA). Total RNA was extracted using the PAXgene RNA extraction kit (Qiagen, Valencia, CA), according to the

manufacturer's protocol. Reverse transcription was conducted as described by Breathnach et al. (2006). RT-PCR was performed and relative quantity of mRNA calculated as described above for the stimulated PBMC. Samples taken prior to the onset of the dietary supplementation period were analysed and the mean for each cytokine calculated and used as the calibrator.

Lymphocyte proliferation:

The ^{3}H-Thymidine incorporation method was used to evaluate lymphocyte proliferation. Isolated PBMC were plated at a concentration of 2 x 10^{6} cells per mL in 96 well flat-bottom plates (TPP, Trasadingen, Switzerland). Again 4 cultures were set up in triplicate for each horse: media alone (control), concanavalin A (ConA; 10 μg/mL) as mitogen to stimulate proliferation (Lis and Sharon, 1998), OVA (25 μg/mL), and equine influenza. To evaluate proliferation in response to influenza virus, PBMC stimulated with the purified equine influenza virus KY/5/02 as described above were added to wells containing media alone. After a 72 h incubation period in a 37ºC, 5% CO_2 humidified incubator, PBMC were pulsed with 0.5μCi ^{3}H-thymidine, incubated for an additional 18 h period, and frozen (-20ºC). DNA was extracted from the cells onto fiber filter pads (Perklin Elmer Inc., Waltham, MA) using a Tomtec harvester (J/B Industries Inc, IL) and liquid scintillation counting performed (Wallac Inc., Gaitheisburg, MD). The results are reported as the stimulation index of corrected counts per minute of stimulated cells, divided by the corrected counts per minute for control cells.

Antibody production: OVA specific ELISA

Serum OVA specific antibody levels were measured by means of an ELISA assay. Briefly, Immunolon I-B microtiter plates (Fisher Scientific, Hanover Park, IL) were coated overnight at 4˙C with 300 µg OVA per mL. The following day plates were washed with PBS/0.05% TWEEN®-20 (PBS-T; Sigma, St. Louis, MO) and blocked with PBS containing 0.5 % polyvinyl alcohol (Sigma). Serum samples were diluted 1:300 in PBS-T and added to wells in triplicate. Plates were incubated at 37˙C for 1 h. After washing plates with PBS-T the secondary antibody, Peroxidase – conjugated AffiniPure Goat Anti-Horse IgG (Jackson Immuno Research, West Grove, PA), was added to each well at a dilution of 1:2500. Following incubation at 37˙C for 1 h plates were washed again using PBS-T, and substrate (3,3´ 5,5´,-tetramethylbenzidine; Sureblue TMB 1, KPL, Gaithersburg, MD) was added. Color was developed for 30 s before the reaction was stopped (TMB Stop Solution, KPL, Gaithersburg, MD). Optical density was measured at 450 nm (Bio-Rad Laboratories, Hercules, CA). A standard curve developed from the serum of a previously vaccinated animal shown to have a strong response to the vaccine was used for the determination of relative ELISA units. The standard curve was constructed on every plate and used to calculate the relative serum antibody concentrations (expressed as ELISA units) by comparison with the standard curve constructed using the dilutions of the positive control serum.

Equine influenza (KY/5/02) antibody production:

Influenza specific antibody production was evaluated by means of a hemagglutination inhibition (HI) assay as well as an ELISA assay.

Equine influenza (KY 02) antibodies (IgGa, IgGb, IgG(T)) were measured using an ELISA assay. The 96-well Immunolon I - B microtiter plates (Fisher Scientific, Hanover Park, IL) were coated overnight at 4˚C with 10 hemagglutination units per well of purified influenza virus (KY/5/02). The coated plates were then washed with PBS-T (Sigma) and blocked with 2% non-fat dried milk powder dissolved in PBS-T (Sigma) for 1 h at 37˚C. Serum samples (diluted in PBS-T at 1:100) were added following the blocking and washing step. Plates were incubated for 1 h at 37˚C. Plates were then washed with PBS-T and incubated for 1 h at 37˚C with monoclonal antibodies specific for IgGa (CVS 40), IgGb (CVS 39) and IgG(T) (CVS 48). Following another wash cycle with PBS-T (Sigma), plates were again incubated with the secondary antibody (horseradish peroxidase-conjugated goat-anti-mouse IgG antibody; Jackson Laboratories Inc., West Grove, PA) for 1 h at 37˚C. Again, plates were washed, substrate (3,3´ 5,5´,-tetramethylbenzidine; Sureblue TMB 1, KPL) was added and color developed. The reaction was stopped (TMB Stop Solution, KPL) and the optical density determined at 450 nm using a 96 well plate reader (BioRAD, Hercules, CA). A standard curve was constructed using serial dilutions of a serum sample from an influenza virus hyper immune horse. This allowed for the calculation of relative antigen-specific antibody concentrations in all experimental serum samples. Relative serum antibody concentrations expressed as ELISA units were calculated by comparison with the standard curve.

For equine influenza HI assay each serum sample was trypsin-periodate treated to remove non-specific inhibitors of virus hemagglutination which are present in serum samples. This was done by adding 100 µL trypsin to 100 µL serum followed by 30 min

incubation in a 56˚C water bath. Then 300 µL periodate was added and samples incubated at room temperature for 15 min, followed by the addition of 500 µL 0.6 % glycerol in saline. This yielded a final serum dilution of 1:10.

Next 25 µL PBS was added to all the wells of a round bottom 96 well plate (Thermo Fisher U-bottom micro titer vinyl plate). Then 50 µL of the treated serum was added to the first well on the plate, and 25 µL was then titrated across the row. Finally 25 µL virus (1:8 dilution) was then added to all wells. Plates were incubated for 30 min at room temperature. Buttons of non-agglutination were then read, and the titer allocated to the last non agglutinating dilution for each sample.

Statistical analysis:

Data were analyzed as a repeated measures design using the Proc Mixed function of SAS 9.2 (SAS Institute Inc., Cary, NC) with least square means separation procedure. The model included time, treatment and block as fixed effects, while horse was included as a random effect. Data were tested for normality, and log transformed when required for statistical analysis. Data were back transformed and are presented as least squares means. Significance was set at $P < 0.05$ and a trend at $P < 0.1$.

RESULTS

Selenium and vitamin E status:

At the initiation of the vaccine challenge whole blood Se concentration and whole blood GPx activity (Table 5.3) were higher for SP and SS when compared to AS, which

in turn exceeded whole blood Se but not GPx activity of LS ($P < 0.0001$). Serum vitamin E concentration (Table 5.3) was similar across all treatment groups ($P = 0.9876$) and indicated adequate vitamin E status.

Complete blood count data:

The CBC data (Table 5.4) collected during the vaccination period indicated no effect of treatment or treatment x time ($P > 0.05$) on lymphocyte numbers, neutrophil numbers or the ratio of lymphocytes to neutrophils. However, there was an overall effect of time ($P < 0.05$) on both lymphocyte and neutrophil numbers.

OVA vaccination response:

The anti-OVA-IgG values (Figure 5.2) indicated a trend for an effect of treatment ($P = 0.0655$), an effect of time ($P < 0.0001$) and a treatment x time interaction ($P = 0.0065$). Overall there was a trend for antibody production to be lower for AS compared to the other dietary treatments, but separation of means showed no difference among treatments in response to the initial vaccine (wk 2 and 3). The second vaccine elicited a stronger antibody response (wk 4, 5, 6 and 7). At wk 4 LS and SS antibody levels were similar but higher than AS and SP. At wk 5 and 6 antibody production was comparable between LS, SP and SS, but LS and SS had higher antibody levels than AS. At the final time point SS antibody production was higher compared to AS and SP, but similar to LS. Lymphocyte proliferation in response to OVA stimulation was similar between the different treatments (Table 5.5). The intracellular production of IFNγ and TNFα in response to *in vitro* OVA stimulation, measured by flow cytometry, indicated no effect of Se status on the percentage of lymphocytes producing IFNγ or TNFα. However, a higher

(treatment, $P = 0.031$) mean fluorescence index (MFI) was observed for IFNγ in LS when compared to AS and SP, but MFI was comparable between LS and SS (Appendix A Table 5 a and b). Similarly the PBMC mRNA expression of cytokines in response to OVA stimulation (Appendix A Table 5 c) indicated no effect of Se status on any of the cytokines evaluated.

Flu vaccination response:

The humoral immune response to the flu vaccination is presented in Table 5.6. Although there was an effect of time on IgGa, IgGb, IgGT and the influenza titer, no effects of treatment or treatment x time interactions existed. Lymphocyte proliferation in response to *in vitro* flu stimulation was also not affected by treatment. Intracellular production of IFNγ and TNFα in PBMC stimulated *in vitro* with flu was unaffected by treatment (Appendix A Table 5 a, Table 5 b, Table 5d). The mRNA cytokine expression of PBMC stimulated *in vitro* with flu (Appendix A, Table 5d) indicated higher IL-6 expression in AS pre-vaccination compared to SS, and higher IL-6 expression in AS compared to LS at 2 wks following vaccination with influenza (treatment x time P = 0.026). Similarly, mRNA expression of IL-8 increased following the influenza vaccination in SP, while it decreased in the other 3 dietary treatment groups (treatment x time, P = 0.045).

General immune response:

Lymphocyte proliferation in response to ConA stimulation (Table 5.5) indicated no difference in the ability of the lymphocytes to proliferate, regardless of dietary treatment. Intracellular production of IFNγ and TNFα in lymphocytes stimulated with

PMA was also unaffected by treatment (Appendix A Table 5 a and b). In contrast, the PBMC mRNA expression of several cytokines stimulated *in vitro* with PMA (Table 5.7) indicated an overall suppression of several cytokines in LS cells. LS had a lower expression of IL-8 (P = 0.024) and IL-13 (P = 0.008) compared to AS, SP and SS. LS also had lower expression of IL-6 (P = 0.002) and IL-10 (P = 0.026) when compared to AS and SP, and lower expression of IL-1 compared to AS and SS. The mRNA expression of IL-6 and IL-10 was higher in SP than SS but comparable to AS. Un-stimulated whole blood mRNA expression of selected cytokines (Table 5.8) indicated an overall higher expression of IL-10 for SS compared to other treatment groups (P = 0.043). A trend for treatment x time interaction (P = 0.057) existed for IL-6. Separation of means revealed IL-6 expression of SP to be lower at wk 0 compared to LS, but similar to AS and SS. In addition IL-6 mRNA expression remained similar across all time points for LS and AS, but increased from wk 0 to wk 3 in SP and SS ($P < 0.05$).

DISCUSSION

Supplementation with Se has been reported to affect immune function across different species (Finch and Turner, 1996; McKenzie et al., 1998; Broome et al., 2004). Preliminary work conducted in our laboratory indicated that low Se status did affect some measures of immune function (Brummer et al., 2009; Brummer et al., 2011). Therefore, we were interested in evaluating the effect of Se supplementation on immune function in horses that were previously of low Se status. After 22 wk on the supplemented diets the whole blood Se concentrations and GPx activity between treatments were different, reflecting the different dietary Se intakes between treatments. The reference range for

adequate whole blood Se concentration is 180 to 240 ng/mL (Stowe, 1998). At the onset of the vaccine challenge the range of Se concentrations between dietary treatments were as follow: LS, 101 to 149 ng/mL; AS, 168 to 202 ng/mL; SP, 232 to 278 ng/mL; SS, 232 to 281 ng/mL. Therefore, horses in the LS group had whole blood Se concentration below the reference range and all the other horses in SP and SS had values above or at the upper end of the reference range. Whole blood GPx activity was measured as a biological indicator of Se status and was reflective of whole blood Se concentrations. In addition, serum vitamin E concentrations were similar between treatments and indicative of adequate vitamin E status. Vitamin E status has been linked to immune function (Meydani et al., 1990; Beck, 2007). Therefore a similar vitamin E status across treatments was important to ensure correct interpretation of the immune response results. Based on the above variables our experimental model responded as hypothesized, and therefore the immune variable response anticipated to present an accurate representation of the effect of Se status on immune function.

The CBC analysis conducted throughout the vaccination period served to monitor the overall health of the horses, and to evaluate lymphocyte and neutrophil numbers. Change in lymphocyte numbers in response to Se supplementation in the horse have been reported (Calamari et al., 2010). However, in this study Se status did not affect any of these variables. Therefore differences observed in immune response would likely not be due to altered lymphocyte or neutrophil numbers *in vivo*.

Vaccination response was assessed using OVA and equine influenza as antigens. These antigens allowed the evaluation of both primary and memory vaccination response on an *in vivo* (humoral response) and *in vitro* (antigen specific cell-mediated response)

basis. The OVA-specific antibody response followed the expected pattern of response as a small increase in antibody production was observed in response to the first vaccine, followed by a much stronger antibody response after administration of the second vaccine at 3 wk. Lower antibody levels in response to vaccination as a result of Se deficiency has been reported before (Reffett et al., 1988b; Finch and Turner, 1996). Similarly higher antibody levels have been associated with Se supplementation (Reffett et al., 1988a; Knight and Tyznik, 1990; Biswas et al., 2006). In our study, the response to the first OVA vaccination resulted in a similar primary antibody response across treatments. This was in contrast to previous work from our laboratory that indicated lower primary antibody response in horses of low Se status compared to horses of adequate Se status (Brummer et al., 2011). Knight and Tyznik (1990) reported higher antibody production in Se supplemented ponies (0.2 ppm) compared to un-supplemented ponies (0.02 ppm) in response to two sheep red blood cell vaccinations, 2 wk apart. In our study, the response to the second vaccine indicated that low Se status did not affect the ability of the horses to produce antibodies, as LS antibody levels were similar to SS throughout the 7 wk vaccination period. This is in agreement with Baalsrud and Overnes (1986) who reported similar antibody production in response to vaccination between horses on Se supplemented or un-supplemented diets. Horses receiving a combination of Se and vitamin E did have improved antibody production compared to the control group (Baalsrud and Overnes, 1986). Therefore it is likely that the adequate vitamin E status of our horses allowed for a normal response antibody response to vaccination even in horses with low Se status.

In this study, SS antibody production exceeded that of SP and AS at the final time point. Comparing SP and SS it would appear as if the antibody response was greater for SS, regardless of similar Se intakes. It has been hypothesized that Se improves humoral immune response by protecting immune cells from excess H_2O_2 allowing for optimal functioning (Baalsrud and Overnes, 1986; Knight and Tyznik, 1990). However, in this study differences in antibody response were observed regardless of similar GPx activity. It is also noteworthy that the AS treatment group did not have a very strong antibody response to the OVA vaccination, as indicated by the trend for overall lower antibody production by AS. Because Se deficiency has been associated with lower antibody production, it was interesting to note that antibody production in this study was unaffected for LS. Overall, the primary humoral response was unaffected by Se status, although there was an effect of source of Se on antibody production following the second vaccination with OVA at specific time points. Influenza specific antibody production as well as HA titers were unaffected by Se status.

Antigen specific cell-mediated measures of immune function were evaluated by means of *in vitro* stimulation of PBMC with OVA and equine influenza. When using *in vitro* methods to assess the effect of a nutrient on immune function the nutrient composition of the culture media is of utmost importance (Fraker, 1994). To better reflect the *in vivo* environment in such studies the use of autologous serum is thought to be more appropriate (Fraker, 1994) and has indeed been shown to affect lymphocyte proliferation results in pigs deficient in either Se or vitamin E (Lessard et al., 1991). Therefore heat inactivated autologous serum was used in the culture media for all the *in vitro* stimulation assays.

Lymphocyte proliferation in response to OVA stimulation did not exhibit an effect of Se status on observed response. In addition the pre-vaccination proliferation response was similar to the post vaccination proliferative response, indicating no effect of OVA vaccination on *in vitro* measures of OVA stimulated lymphocyte proliferation. Lymphocyte proliferation in response to influenza stimulation was also unaffected by Se status. In contrast, enhanced proliferation in response to *in vitro* stimulation with polio virus has been reported in human subjects supplemented with Se compared to placebo controls (Broome et al., 2004). Intracellular production of IFNγ and TNFα indicated that although the number of IFNγ producing cells was not affected by Se status, the amount of IFNγ produced by these lymphocytes (mean fluorescence index) was higher for the LS group. Yet, Se status did not affect the relative mRNA expression of IFNγ. Similarly Se status had no effect on the relative expression of any of the other cytokines and transcription factors in OVA or influenza stimulated PBMC. These results suggest that Se status only had limited effects on the cell-mediated response to vaccination, whether a novel or previously administered vaccine was used. However, together with the lymphocyte proliferation results, comparing pre-vaccination response to post-vaccination response data suggests that the OVA vaccination in general did not have a strong effect on cell-mediated measures. It is possible that the previously tested *in vitro* stimulation protocol was not sufficient in this case, that there was a failure of cells to recognize the antigen *in vitro,* or there was a stronger stimulation of the humoral component of the immune system by the OVA vaccination. In addition, previous work conducted indicated lower expression of the transcription factor T-bet in horses of low Se status compared to

horses of adequate Se status in response to vaccination with keyhole limpet hemocyanin (Brummer et al., 2011), but that response was not observed here.

Along with vaccination response, we also evaluated cell-mediated immune function by assessing response to non-specific mitogen stimulation *in vitro*. Mitogen stimulation is non-specific and targets a large proportion of the cells in culture, as opposed to highly specific antigen stimulation (Calder, 2007). The use of mitogens also provides a measure of the functional capacity of cells (Touraine et al., 1977; Vance et al., 2004).

Lymphocyte proliferation in response to ConA stimulation was unaffected by Se status. This is in contrast to studies showing an increase in proliferation in response to Se supplementation (Lessard et al., 1991; Peretz et al., 1991; Roy et al., 1994). Previous research from our laboratory also indicated no effect of Se status on lymphocyte proliferation (Chapter 3 and Chapter 4). Recently, lymphocyte proliferation was reported to be similar between foals of 1 mo of age, regardless of the source of Se (sodium selenite or Se-yeast) fed to their dams (Montgomery et al., 2012). It has been suggested that lymphocyte proliferation is enhanced by Se supplementation due to a role of Se in the modulation of the arachidonic acid metabolism (Cao et al., 1992). Using *in vitro* assays with bovine cells the authors reported that low Se status was associated with a reduction in the metabolites of the 5-lipoxygenase pathway, which in turn was linked to lower lymphocyte proliferation. The suppressed proliferation response was reportedly reversed by the addition of specific lipoxygenase pathway products (Cao et al., 1992). Collectively, our results suggest that lymphocyte proliferation in the horse may not be as

sensitive to Se status as reported in other species or that differences in proliferation may be too small to detect or responses among horses too variable.

Intracellular production of IFNγ and TNFα in response to non-specific stimulation indicated no differences between dietary treatment groups. However, differences were observed in the relative mRNA expression of several cytokines. Overall LS had lower relative expression of IL-8 and IL-13 compared to AS, SP and SS. IL-8 is a cytokine that functions as a neutrophil chemo-attractant and activator of the degranulation of neutrophils (Dinarello, 2000). Wuryastuti et al. (1993) reported reduced phagocytic and microbicidal ability of neutrophils isolated from Se deficient sows, while Aziz and Klesius (1985) reported Se deficiency affected the ability of lymphocytes to modulate neutrophil migration. Therefore the lower expression of IL-8 upon stimulation with PMA in the LS group was an interesting observation. IL-13 is associated with the regulation of cell-mediated responses (Tato and Cua, 2008a). In addition, IL-6 and IL-10 expression was also lower in LS compared to AS and SP. In contrast, Beck et al. (2001) reported mRNA levels of IL-10 and IL-13 were elevated in Se deficient mice by d 6 post vaccination with an influenza virus strain when compared to adequate mice. These authors also reported elevated IL-4, IL-5, IL-10 and IL-13 from d 14 onwards, the opposite of what was expected following a viral challenge (Beck et al., 2001).

Relative mRNA expression of cytokines in un-stimulated whole blood provides an *in vivo* measure of cytokine production in the horse. Here the relative expression of IL-10 was higher in SS compared to LS, AS and SS. The cytokine IL-10 is described as one of the most important anti-inflammatory cytokines (Opal and DePalo, 2000). IL-10 is produced by the B-cells and T-helper (Th) cells involved in the humoral response (Th2)

(Goldsby et al., 2000). IL-10 also down regulates the Th1 cells that provide defense against intracellular pathogen response via IL-2 and IFNγ production (Opal and DePalo, 2000). Although IFNγ expression in the un-stimulated whole blood samples was similar across treatments, the SS treatment group did have a strong humoral (Th2) response to the OVA vaccination. A trend also existed for the relative expression of IL-6 to increase from wk 0 to wk 3 in SP and SS. Although IL-6 is a cytokine with both pro and anti-inflammatory properties, it can be regarded as anti-inflammatory due to its ability to down regulate TNFα and IL-1. In our study un-stimulated whole blood expression of TNFα was unaffected by Se status. In mice, TNFα expression in macrophages from LPS stimulated lung cells were found to be lower in the Se adequate mice compared to mice of low Se status (Vunta et al., 2008).

Comparing the effect of source of Se supplementation, the SP group had a higher mRNA expression of IFNγ and IL-2 at wk 3 of the vaccination period in response to PMA stimulation compared to SS, while SS elicited a stronger humoral response to OVA immediately following this time point. Although OVA specific stimulation at this time indicated no difference between IFNγ and IL-2 for SS and SP, PMA stimulation suggests a stronger Th1 type of response in SP at wk 3, while the higher antibody production following this time point in SS may suggest a stronger humoral (Th2) response in SS. At wk 3 *in vivo* IL-10 mRNA expression was also higher for SS compared to SP. Similarly Broome et al. (2004) reported higher levels of IFNγ and IL-10 at day 7 post vaccination with a polio virus in stimulated whole blood cultures from Se supplemented groups compared to an un-supplemented group of marginal Se status, using humans as research subjects.

The mechanism of action of Se on the immune system is a subject that has received a lot of interest, yet the exact mechanism remains elusive. The role of NF-κβ has been considered. NF-κβ is a transcription factor that initiates the transcription of cytokines, specifically the pro-inflammatory cytokines (Tosi, 2005) including the production of pro-inflammatory proteins such as TNFα and IL-6 (Duntas, 2009). It has been hypothesized that the antioxidant properties of Se prevent the over-activation of NF-κβ by high levels of ROS (Beck et al., 2001; Zeng and Combs Jr, 2008). In our study LS had lower GPx activity which could potentially result in higher ROS levels, but TNFα and IL-6 were not elevated in LS. Another proposed mechanism suggests that a higher Se intake may improve T-cell receptor strength signaling via elevated Ca^{2+} flux (Hoffmann et al., 2010). Recently, a study using selenoprotein K knockout mice also suggested that the mechanism of action for Se and the immune system may lie in the regulation of Ca^{2+} flux, implicating the importance of selenoprotein K in immune function (Verma et al., 2011). In our study the PMA/ionomycin (calcium ionophore) stimulation protocol results in the activation of PBMC's via activation of the Protein Kinase C as well as calcium signaling pathways. The overall suppressed cytokine mRNA expression observed for the low Se group despite the addition of ionomycin to the cultures may support these proposed mechanisms of action.

CONCLUSION

In conclusion, our study indicated that Se status, either high or low, did not affect the ability of the horses to mount an immune response to a novel antigen, or to a previously administered antigen as measured by antigen specific antibody production and

antigen specific cytokine production. However, general cell-mediated immunity appeared suppressed in the horses of low Se status, as measured by relative expression of cytokines in *in vitro* stimulated PBMC. In addition, *in vivo* relative cytokine gene expression was also affected by Se status. However, no clear advantage in immune function was observed comparing supplementation at 0.12 ppm with 0.3 ppm. Collectively these results suggest that adequate Se supplementation is important to maintain immune function in the horse.

Table 5.1. Nutrient composition[1] (DM basis) of pasture and hay sampled throughout the experimental period and balancer pellet[2] fed during the Se repletion phase.

Item		Pasture	Grass Hay	Alfalfa Hay	Adequate Se Balancer Pellet[2]	Low Se Balancer Pellet[2]
DM	%	87.60	92.10	91.2	89.2	90.9
DE	Mcal/kg	2.33	2.24	2.22	3.35	3.37
CP	%	24.15	21.60	15.8	36.5	36.9
ADF	%	31.85	34.60	38.6	9.2	7.7
NDF	%	49.25	53.60	49.6	15.4	14.7
Starch	%	1.60	2.80	1.40	5.10	3.60
Calcium	%	0.91	0.97	1.31	4.07	3.75
Phosphorus	%	0.52	0.53	0.40	2.16	2.41
Magnesium	%	0.33	0.40	0.20	0.45	0.44
Potassium	%	2.84	2.75	2.97	2.05	2.14
Sodium	%	0.14	0.26	0.046	0.85	0.84
Iron	ppm	856	673	217	806	808
Zinc	ppm	33.0	35.0	23.0	448	403
Copper	ppm	9.00	9.00	8.00	160	144
Selenium	ppm	0.06	0.06	0.02	2.52	0.53

[1] Equi-analytical laboratories, Ithaca, NY
[2] McCauley Bros, Inc., Versailles, KY

Table 5.2. Example of calculated dietary Se intake[1] for a 500 kg horse.

Se source	Low Se (mg)	Adequate Se (mg)	Se-Yeast (mg)	Sodium selenite (mg)
Pasture	0.56	0.56	0.56	0.56
Balancer pellet	0.16	0.86	0.16	0.16
Supplement	*	*	2.74	2.74
Total (mg/d)	0.72	1.42	3.46	3.46
Total (mg/kg DM)	0.062	0.12	0.30	0.30

[1] Total DM intake estimated at 2.25% of BW or 11.6 kg DM per day

Table 5.3. Indicators of Se and vitamin E status at the onset of the vaccine challenge (LS Means ±SE).

Variable	LS[1]	AS[1]	SP[1]	SS[1]	*P*-value
Whole blood Se (ng/mL)	116.5±6.3[a]	185.4±6.8[b]	256.3±6.8[c]	250.3±6.8[c]	<0.0001
Whole blood GPx (mU/mg hb)	37.4±3.6[a]	52.1±4.0[a]	69.3±3.4[b]	72.9±3.6[b]	<0.0001
Serum vitamin E (µg/mL)	3.6±0.47[a]	3.8±0.51[a]	3.8±0.47[a]	3.9±0.50[a]	0.9876

[a,b,c] Means within row lacking a common superscript differ, $P < 0.05$

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)

Table 5.4. Complete blood count[1] analysis of horses on different dietary treatments[2] during the vaccination challenge[3].

	Week				Main effect (*P*-value)		
Item	0	3	5	SEM	Treatment	Time	Treatment x time
Lymphocyte ($x10^3$)							
LS	2.35	2.50	2.70	0.26	0.479	0.008	0.440
AS	2.23	2.15	2.98	0.26			
SP	2.54	2.44	2.61	0.24			
SS	2.93	2.57	3.10	0.26			
Neutrophil ($x10^3$)							
LS	3.73	3.60	4.18	0.31	0.480	0.0003	0.898
AS	3.23	3.50	3.78	0.32			
SP	3.29	3.47	3.97	0.31			
SS	3.73	4.08	4.38	0.31			
Ratio							
LS	1.67	1.56	1.63	0.29	0.670	0.682	0.375
AS	1.45	1.63	1.24	0.29			
SP	1.38	1.50	2.25	0.27			
SS	1.42	1.60	1.50	0.29			

[1]Complete blood count analysis conducted by Rood and Riddle Equine Hospital (Lexington, KY)
[2]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)
[3]Vaccinations were administered at wk 0 and wk 3

Table 5.5. Lymphocyte proliferation in response to different stimulations[1] in horses on different dietary treatments[2] (presented as stimulation index LS means).

	Week[3]				Main effect (*P*-value)		
Item	0	3	5	SEM	Treatment	Time	Treatment x time
ConA							
LS	25.7	11.0	12.7	4.75	0.443	<0.0001	0.998
AS	24.5	7.67	9.31	4.78			
SP	28.7	11.1	13.1	4.41			
SS	22.3	8.26	10.4	4.75			
OVA							
LS	0.97	1.06	1.14	0.092	0.428	0.781	0.225
AS	0.98	0.86	0.95	0.094			
SP	0.97	0.91	0.92	0.085			
SS	0.84	1.07	0.88	0.092			
Influenza							
LS	2.90	*	2.95	0.77	0.424	0.305	0.767
AS	1.39	*	1.29	0.80			
SP	1.71	*	1.39	0.72			
SS	2.65	*	1.97	0.77			

[1]ConA (concanavalin A), OVA (ovalbumin) and Influenza (Equine influenza strain KY 2002)
[2]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)
[3]Vaccinations were administered at wk 0 and wk 3

Table 5.6. Humoral immune response variables in response to vaccination of mature horses on different dietary treatments[2] with equine influenza (KY02).

	Week				Main effect (*P*-value)		
Item	0	5	7	SEM	Treatment	Time	Treatment x time
IgGa[3]							
LS	66	1277	356	393	0.7279	<0.0001	0.7778
AS	96	579	291	408			
SP	42	709	276	370			
SS	170	626	410	397			
IgGb[3]							
LS	839	2817	4365	2599	0.285	<0.0001	0.447
AS	716	1756	3217	2664			
SP	2338	8009	9252	2422			
SS	2389	7690	10562	2599			
IgGT[3]							
LS	5344	6628	7030	2018	0.325	0.025	0.561
AS	2191	2632	2613	2079			
SP	4490	7983	8445	1883			
SS	5031	5089	5458	2019			
Influenza titer[4]							
LS	82	167	133	53	0.430	<0.0001	0.289
AS	75	195	169	55			
SP	105	232	226	53			
SS	147	267	267	50			

[1]0 = pre vaccination, 5 = 2 wk post vaccination, 7 = 4 wk post vaccination for equine influenza
[2]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)
[3]IgGa, IgGb and IgGT measured as ELISA units relative to a standard curve produced from a high responding horse
[4]Hemagglutination inhibition

Table 5.7. mRNA expression as relative quantity of cytokines[1] and transcription factors in peripheral mononuclear cells stimulated with PMA from horses on different dietary treatments[2] during the vaccine challenge[3].

		Week				Main effect (*P*-value)		
Item	Diet[1]	0	3	5	SEM	Treatment	Time	Treatment x time
Tbet	LS	52.31	51.47	41.28	53.3	0.734	0.020	0.539
	AS	167.36	127.63	143.21	55.4			
	SP	25.21	49.09	44.07	49.8			
	SS	25.80	37.08	57.20	53.3			
GATA 3	LS	4.62	6.53	6.50	1.02	0.913	0.049	0.152
	AS	5.93	5.87	4.71	0.99			
	SP	4.80	5.87	5.90	0.92			
	SS	4.46	4.79	5.74	1.01			
GranzymeB	LS	222.50	158.93	229.81	75.0	0.581	0.338	0.612
	AS	247.18	241.30	288.97	78.0			
	SP	140.21	123.91	152.47	70.2			
	SS	98.10	94.13	103.73	75.0			
IFNγ	LS	391036	99935[a]	392527	91506	0.876	< 0.01	< 0.0001
	AS	548946	225988[ab]	292075	91280			
	SP	323839	298318[b]	242302	82354			
	SS	448286	118959[a]	205065	88132			
TNFα	LS	3743[b]	807[a]	1148[b]	1964	0.037	< 0.01	0.001
	AS	12639[a]	4541[b]	4257[a]	1923			
	SP	2690[b]	1427[ab]	2871[ab]	1740			
	SS	3333[b]	1082[a]	4717[ab]	1864			
IL-1	LS	5.29	1.52	1.69	2.53	0.002	< 0.01	0.079
	AS	21.24	3.49	6.59	2.33			
	SP	3.77	2.07	3.25	2.12			
	SS	5.89	3.68	5.79	2.29			
IL-2	LS	243704	125236[a]	188408	53000	0.677	< 0.01	0.003
	AS	191891	133682[a]	125565	54958			
	SP	187074	190925[a]	242967	49540			
	SS	194951	72421[b]	99365	45722			

Table 5.7. Continued.

		Week				Main effect (*P*-value)		
Item	Diet[1]	0	3	5	SEM	Treatment	Time	Treatment x time
IL-6	LS	13.27[a]	2.27[a]	3.43[a]	5.47	0.002	< 0.01	< 0.0001
	AS	59.24[b]	15.95[b]	13.64[b]	5.62			
	SP	32.24[c]	8.33[bc]	15.63[b]	5.10			
	SS	9.14[a]	4.95[ac]	9.94[b]	5.47			
IL-8	LS	11.3	3.30	2.18	3.10	0.024	< 0.01	0.489
	AS	25.3	8.72	4.50	3.17			
	SP	11.5	5.08	6.12	2.89			
	SS	13.6	8.34	7.21	3.10			
IL-10	LS	1710[a]	604[b]	538[a]	448.3	0.026	< 0.01	< 0.0001
	AS	4867[b]	1811[a]	1315[b]	420.1			
	SP	2214[a]	614[b]	1397[b]	430.5			
	SS	2363[ab]	397[c]	1362[b]	472.7			
IL-13	LS	1673	1604	1173	1971	0.008	0.481	0.315
	AS	3415	9968	4229	2024			
	SP	4030	6583	7690	1838			
	SS	7179	4964	6143	1971			

[1]IL = Interleukin

[2]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)

[3]Vaccinations were administered at wk 0 and wk 3

Table 5.8. Relative quantity of mRNA expression of cytokines[1] in un-stimulated whole blood from horses on different dietary treatments[2] during the vaccine challenge[3].

		Week				Main effect (*P*-value)		
Item	Diet	0	3	5	SEM	Treatment	Time	Treatment x time
IFNγ	LS	0.592	0.444	0.615	0.140	0.336	0.367	0.235
	AS	0.608	0.636	0.713	0.145			
	SP	0.596	0.685	0.528	0.131			
	SS	1.038	0.789	0.809	0.140			
TNFα	LS	1.002	0.926	0.944	0.148	0.603	0.329	0.639
	AS	1.166	1.020	0.924	0.153			
	SP	1.196	1.172	1.121	0.138			
	SS	1.077	1.128	1.134	0.148			
IL-6	LS	0.922	1.040	1.180	0.287	0.422	0.030	0.057
	AS	0.635	0.522	0.720	0.294			
	SP	0.353	0.819	1.176	0.267			
	SS	0.477	0.713	0.753	0.287			
IL-8	LS	1.243	1.216	1.276	0.257	0.696	0.759	0.788
	AS	1.104	1.003	0.843	0.263			
	SP	0.933	0.781	1.018	0.239			
	SS	0.851	0.994	1.071	0.257			
IL-10	LS	0.738	0.793	0.762	0.210	0.043	0.655	0.598
	AS	0.823	0.889	0.704	0.218			
	SP	0.730	0.828	0.630	0.198			
	SS	1.774	1.316	1.555	0.210			

[1]IL=Interleukin

[2]Dietary treatments (total dietary Se): LS = low Se (0.07 mg Se/kg DM); AS = Adequate Se (0.14 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)

[3]Vaccinations were administered at wk 0 and wk 3

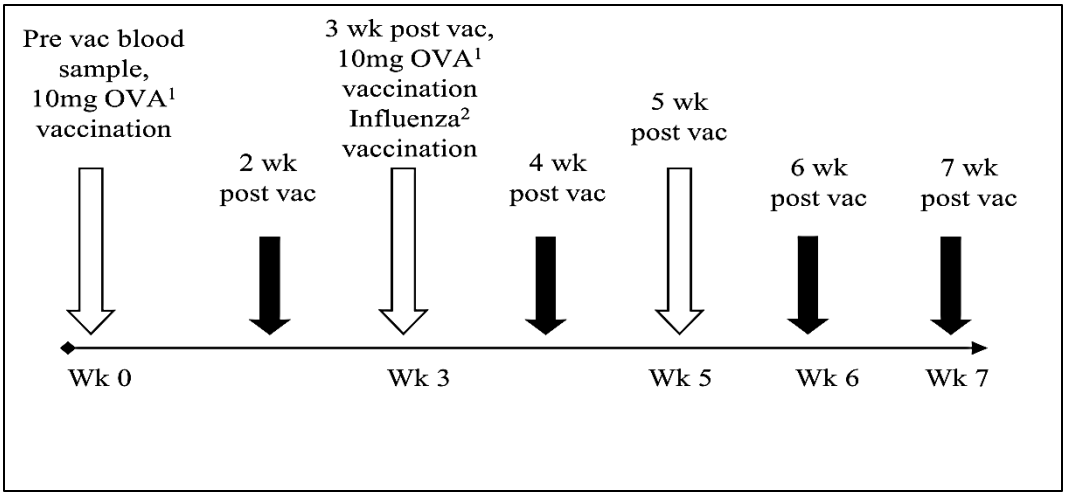

Figure 5.1. Sampling and vaccination time points for OVA vaccination. White arrows denote blood collection for determination of Se status, antibody production and peripheral blood mononuclear cell isolation. Black arrows denote blood collection for antibody production determination only.

[1]Ovalbumin (OVA), Sigma Saint Louis, MO.
[2]Prestige V, Intervet, Summit, NJ

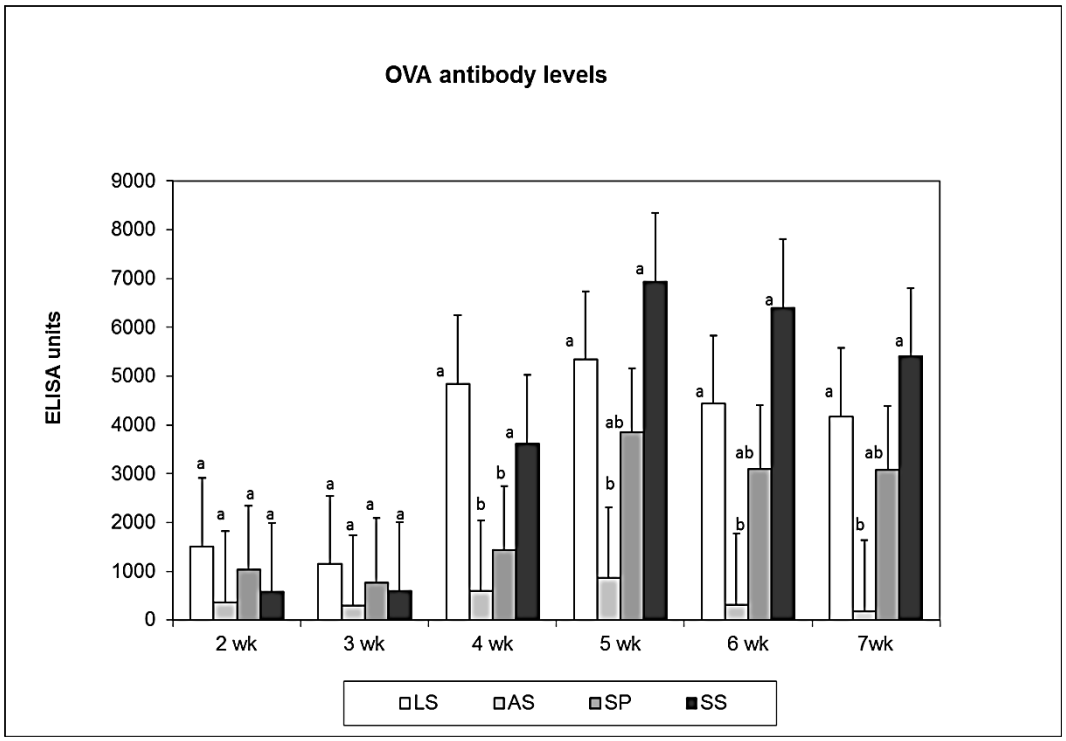

Figure 5.2. Anti-OVA-IgG levels in response[1] to OVA vaccination[2] of mature horse[3].

[1]Treatment $P = 0.0655$; time $P = <0.0001$; treatment x time $P = 0.0065$.
[2]The 10 mg ovalbumin vaccinations were administered at wk 0 and wk 3, immediately after obtaining blood samples at those time points
[3]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)
[a,b] means within time point lacking common superscripts differ, $P < 0.0.5$

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CHAPTER 6: Measures of Antioxidant Status of the Horse in Response to Selenium Depletion and Repletion

INTRODUCTION

The antioxidant system is complex and consists of many different antioxidant components. In addition, synergistic interactions are known to exist between some of the antioxidants (Deaton et al., 2002; Surai, 2006), further complicating the assessment of the antioxidant system. Glutathione peroxidase (GPx) was one of the first identified selenoenzymes, and its activity is influenced by the selenium (Se) status of the body (Brown and Arthur, 2001). GPx is a component of the antioxidant system as it regulates hydrogen peroxide levels inside the cell (Arthur, 1997; Ferguson and Karunasinghe, 2011). Therefore it seems likely that total antioxidant status might decline when Se status declines. Serum malondialdehyde (MDA) is an end product of lipid peroxidation that occurs when cell membranes are damaged by reactive oxygen species (Ducharme et al., 2009) such as hydrogen peroxide (Surai, 2006). Serum MDA has therefore been used as a measure of oxidative stress in the horse. Although some studies have evaluated the relationship between dietary Se intake and GPx activity in horses, few studies have investigated the relationship between Se status, GPx activity and antioxidant status in response to Se depletion and repletion.

Hence, the objectives of this study were to evaluate the impact of Se depletion, followed by repletion, on GPx activity, antioxidant status and oxidative stress in the horse. Secondary objectives were to determine if Se supplementation above NRC recommended levels (1 mg/d for a 500 kg horse or 0.1 ppm) at 0.3 ppm fed as either sodium selenite or Se-yeast for a 154 d would affect Se status as indicated by whole

blood Se and GPx activity. In addition serum vitamin E concentration, triiodothyronine (T3), thyroxine (T4), lymphocyte and neutrophil numbers were evaluated throughout the Se depletion and repletion phases. Iodothyronine deiodinase is a selenoenzyme responsible for the conversion of T4 to T3 (Brown and Arthur, 2001).

We hypothesized that depletion of Se would result in a decrease in TAC and an increase in MDA. Upon supplementation with 0.3 ppm we hypothesized that whole blood Se and GPx activity would increase which would result in an increase in TAC, T3/T4 ratio and a decrease in MDA. Low Se status was also expected to result in low lymphocyte numbers.

MATERIALS AND METHODS

Animals:

This research project was approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Twenty eight mature horses, aged 5 to 23 yr, were used in this study. The 28 horses included 8 geldings and 20 mares. The geldings consisted of 6 Thoroughbreds, 1 American Quarter Horse and 1 Standardbred, while the mares consisted of 19 Thoroughbreds and 1 American Quarter Horse.

Experimental design, diets and treatments:

The study was conducted in two phases as depicted in Figure 6.1. The first phase was a Se depletion phase (196 d), immediately followed by a Se repletion phase (189 d).

Preceding the onset of the depletion phase, horses were blocked by age and gender and randomly assigned within block to one of four treatment groups: LS, AS, SP or SS which are explained later. Throughout the study horses were kept out on pasture. The horses were allocated to pastures so that all treatment groups were represented within each pasture. Prior to and throughout the study pastures were sampled periodically when pasture availability was sufficient. Although the Se content of the pastures fluctuated, it remained marginal in Se (range 0.03 to 0.08 ppm DM) at all times. When pasture availability declined in winter, horses were fed hay that was produced on the same farm (Se < 0.05 ppm DM), and cracked corn (Se < 0.16 ppm DM) to provide additional calories. Horses had *ad libitum* access to water and an iodized salt block. The horses were weighed on a monthly basis.

Depletion phase:

During the depletion phase, AS received an adequate Se diet and served as a control, while the remaining three groups (LS, SP, and SS) received a low Se diet with the goal of depleting Se stores. The three depleting groups were statistically grouped together so that only 2 treatment groups existed: AS (n = 7) and LS (n = 21). One TB mare had to be removed from the depleting group during the depletion phase, therefore LS treatment group consisted of n = 20.

To ensure that nutrients other than Se were provided, a custom formulated low Se (0.48 ppm DM) protein–vitamin-mineral balancer pellet was fed to the Se depleting horses (Table 6.1, McCauley Bros, Inc., Versailles, KY). The composition of the ballancer pellets appear in Appendix A Table 4.a. The horses assigned to AS were fed a

similar pellet that contained 2.52 ppm Se on a DM basis. The adequate and low Se balancer pellets allowed for the manipulation of dietary Se intake. Individual feeding pens were constructed to feed the balancer pellets on an individual BW basis. The calculated (Table 6.2) total Se intake for the horses on the low Se diet (LS) was 60 % of the NRC (2007) recommended amount of 1 mg Se per day for a 500 kg horse or 0.1 ppm DM, while horses on the adequate Se diet (AS) received a calculated total Se intake of 120 % of the recommended amount of Se. Horses were kept on their respective diets for a period of 196 d to allow for Se depletion.

Repletion phase:

During the repletion phase the horses in the AS group remained on the same adequate diet, while two of the depleted groups were now supplemented with either an organic (SP, Se-yeast; Sel-Plex®, Alltech Inc. Nicholasville, KY) or inorganic (SS, sodium selenite) Se supplement (Figure 6.1). The 7 horses initially allocated to the LS group remained on the low Se diet. One TB mare was removed from the repletion period due to an eye injury, and consequently her entire data set was also removed from the repletion phase. Therefore the number of experimental units per dietary treatment for the repletion phase was as follows: repletion phase LS and SS consisted of n = 6 horses each, while AS and SP consisted of n = 7 horses.

Throughout the repletion phase horses had access to pasture, and to hay when pasture availability declined. Similar to the depletion phase custom formulated adequate Se (2.52 ppm DM) or low Se (0.53 ppm DM) protein–vitamin-mineral balancer pellets (Table 6.3, McCauley's; Versailles, KY) were fed. The same individual feeding protocol

from the depletion phase was followed during the repletion phase, except that SP and SS supplements were top-dressed on the balancer pellet. The AS and LS horses received brewer's yeast to account for the yeast component in the Se-yeast supplement and brewer's yeast was also used as carrier for the sodium selenite supplement. A small amount of water was added to the balancer pellet and top-dressed supplement prior to feeding to improve the palatability and texture. The horses were monitored to ensure that allotted supplement and balancer pellet was entirely consumed.

During the repletion period the total dietary Se intakes were calculated (Table 6.2) to be as follows: LS received 60 % of the NRC (2007) recommendation. The AS group received a total of 120 % of the recommended amount of Se. Horses on the SP and SS treatments received 300 % (0.3 ppm DM) of the recommended Se intake. Total dietary Se intake was calculated to be equal between horses within treatment on a BW basis. The horses were kept on their respective diets for a period of 189 d. This supplementation period was selected to ensure that the turnover of red blood cells would be sufficient for the identification of change in indicators of Se status (whole blood Se concentration and GPx activity).

Blood sampling procedures:

Baseline blood samples were taken at the start of each phase, and every 4 wk thereafter throughout the Se depletion and repletion phases. Blood was collected in 7 mL lithium heparin blood collection tubes (Becton Dickson, Franklin Lakes, NJ) for analysis of whole blood Se and whole blood GPx activity. Blood was also collected in untreated blood collection tubes for serum separation (Becton Dickson) and EDTA containing

tubes (Becton Dickson) for complete blood count (CBC) analysis. Whole blood was transferred to storage vials and kept at -80˚C until analysis. Serum separation was conducted by allowing blood samples to clot for approximately 60 min at room temperature. Samples were then centrifuged at 2500 x g for 20 min at 4˚C. Serum was aspirated, transferred to storage vials and stored at - 80˚C until analysis.

Laboratory procedures:

Selenium Status: Whole blood selenium

Whole blood Se concentration was analyzed by the Diagnostic Center for Population and Animal Health (Michigan State University; Lansing, MI) by means of inductively coupled plasma-mass spectroscopy.

Selenium Status: Whole blood GPx activity

Whole blood GPx activity was determined using the Bioxytech GPx-340 assay kit (OXIS research, Portland, OR) (Richardson et al., 2006). This assay is based on the method developed by Paglia and Valentine (1967). The GPx activity of each sample was calculated from the change in absorbance and expressed as units of enzyme activity per mg hemoglobin (mU/mg Hb). Hemoglobin values were obtained from the complete blood count analysis described below.

Complete blood count analysis (CBC):

The CBC analyses were performed by a local commercial equine hospital (Rood and Riddle Equine Hospital, Lexington, KY). Lymphocyte and neutrophil numbers were then calculated from the CBC analysis.

Serum total antioxidant capacity (TAC):

Serum TAC was determined using a method that compares the ability of the antioxidants in the serum sample to inhibit the oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] to that of Trolox, a water soluble tocopherol analogue. (Antioxidant assay kit; Caymenchemical; Ann Arbor, MI). Samples were analyzed in triplicate according to the manufacturer protocol (Caymenchemical). The TAC was determined for the baseline and d 154 samples collected for each phase. The inter-assay coefficient of variation was 3.82%.

Serum malondialdehyde (MDA) concentration:

Serum MDA concentration was measured using a thiobarbituric acid reactive substances (TBARS) method (Ducharme et al., 2009). This method was based on the reaction of MDA with thiobarbituric acid under acidic, high temperature conditions, using MDA as standard. Samples were analyzed in triplicate according to the directions supplied by the kit manufacturer (Caymenchemical). Serum MDA was determined on the baseline and endpoint samples collected for each phase. The inter-assay coefficient of variation for MDA concentration was 3.68 %.

Triiodothyronine (T3) and thyroxine (T4):

A solid phase ^{125}I radio immunoassay (Coat-a-count; Siemens) was used to measure T3 and T4. The T3/T4 ratio was then calculated. T3/T4 was determined for the baseline and endpoint samples collected for both phases. The inter-assay coefficient of variation for T3 and T4 was 2.47 % and 3.14 % respectively.

Statistical analysis:

Data were analyzed as a repeated measures design using the Proc Mixed function of SAS 9.2 (SAS Institute Inc., Cary, NC) with least square means separation procedure. Each horse served as experimental unit. The model included time, treatment and block as fixed effects, while horse was included as a random effect. Data are presented as least squares means.

RESULTS

Depletion phase:

Whole blood Se concentration and GPx activity for the depletion phase is presented in Table 6.4. Whole blood Se concentration was similar between AS and LS at the onset of the depletion phase ($P > 0.05$). Whole blood Se concentration data during the depletion phase were affected by treatment ($P < 0.0001$), time ($P < 0.0001$) and a treatment x time interaction existed ($P = 0.0073$). The whole blood Se concentration of LS decreased until d 140 before it stabilized, at which time it was lower than in AS. Whole blood Se concentration at the end of the depletion period (d 196) was different

between the two treatment groups ($P < 0.05$). The Se concentration of AS did decrease within the first 84 d, likely due to adaptation from a higher Se diet, but then stabilized. Whole blood GPx activity for the depletion phase was affected by treatment ($P < 0.0001$) and time ($P < 0.0001$) but there was no treatment x time interaction ($P = 0.9160$). Similar to Se, whole blood GPx activity in AS initially decreased and then stabilized while GPx activity decreased in LS. Final GPx activity was lower in LS compared to AS at d 196 ($P < 0.05$). A positive correlation existed between whole blood Se and GPx activity ($r = 0.63$; $P < 0.0001$).

The T3/T4 ratio decreased over time during the depletion period ($P = 0.0179$), this decrease was not affected by Se status ($P > 0.05$; Appendix A Table 6.a).

Serum TAC (Table 6.5) was not affected by Se status during the depletion phase. However there was an effect of time ($P <0.0001$) during the depletion phase due to the decrease in TAC from d 0 to 196 in both groups. Serum MDA (Table 6.5) concentrations were higher in AS (treatment, $P < 0.0226$) and an increase in MDA occurred for both AS and LS from d 0 to d 196 (time, $P = 0.0408$) during the depletion phase.

The CBC data collected during the depletion phase (Table 6.6) indicated a trend for higher lymphocyte numbers for LS compared to AS. The number of neutrophils and the ratio of lymphocytes to neutrophils were similar between LS and AS.

Repletion phase:

At the start of the repletion phase whole blood Se concentration was similar between LS, SP and SS. Whole blood Se concentration during the repletion phase (Table 6.7) was affected by treatment ($P < 0.0001$), time ($P < 0.0001$), and a treatment x time

interaction ($P < 0.0001$). Within 28 d of starting the repletion phase whole blood Se was comparable between AS, SP and SS but higher than LS ($P < 0.05$). On d 154 whole blood Se concentrations in SP and SS were higher than in AS ($P < 0.05$), and by d 189 SP tended ($P = 0.08$) to have a higher whole blood Se concentration than SS. As expected, LS and AS maintained their respective low and adequate Se concentrations over time. Whole blood GPx activity (Table 6.8) during the repletion phase also had an effect of treatment ($P < 0.0001$), time ($P < 0.0001$), and treatment x time interaction ($P < 0.0001$). Whole blood GPx activity followed a similar but delayed response to the Se concentration data. At the beginning of the repletion phase GPx activity was higher for AS in comparison to LS, SP and SS. At d 154 the GPx activity of SS was comparable to SP but higher than AS. At the final time point (d 189) the GPx activity of SP and SS were similar but higher when compared to AS and LS. Between d 154 and 189 GPx activity plateaued within SS but continued to increase in SP. A strong, positive correlation existed between whole blood Se and GPx activity (r = 0.82; $P < 0.0001$). However, within treatment the correlations differed for these two variables: LS (r = 0.81, $P < 0.0001$), SP (r = 0.85; $P < 0.0001$) and SS (r = 0.87; $P < 0.0001$) had similar correlations, however, this correlation was much weaker for AS (r = 0.48; $P = 0.0018$).

The aim of the repletion phase of this study was to evaluate the effect of Se supplementation over a period long enough to allow for turnover of the red blood cell population (140 - 150 d). Therefore the variables discussed below compare change in samples taken at d 0 and d 154.

Although the T3/T4 ratio increased over time during the repletion phase ($P = 0.0726$) the increase was unaffected by Se status ($P > 0.05$; data not shown). This ratio

was expected to be affected by low Se status. However, the change within LS over both phases alone also indicated no effect of depletion on T3/T4 (Appendix A Table 6 a).

Serum TAC (Table 6.9) was not affected by Se status, and remained similar throughout the repletion phase. Similarly serum MDA concentration (Table 6.9) was unaffected by treatment and time during the repletion phase ($P > 0.05$). Serum vitamin E concentration (Figure 6.2) was monitored across both phases. Although vitamin E concentrations fluctuated over time ($P = 0.0004$) there was no effect of treatment or treatment x time ($P > 0.05$).

Lymphocyte and neutrophil numbers were similar between treatment groups throughout the repletion phase. The lymphocyte to neutrophil ratio was also unaffected by treatment (Appendix A Table 6 b).

DISCUSSION:

Studies showing an increase in the activity of GPx in response to Se supplementation introduced the concept of improved functional Se status through dietary Se supplementation (Brown and Arthur, 2001). In this study, the change in whole blood Se and GPx activity throughout the depletion and repletion phases followed the expected response, indicating that the Se status of the horse can be manipulated by dietary Se intake if enough time is allowed for the variables to adjust.

The reference range for whole blood Se in a mature horse is estimated at 180 to 240 ng/mL (Stowe, 1998). Based on this reference range the horses included in this study were of high Se status at the start of the study; possibly because of the Se content of the

commercial feed that the horses were fed prior to the onset of the depletion phase. Consequently the initial decrease in Se concentration observed for AS from high Se status to within the adequate range at d 84 was interpreted as a normal response. The decrease in whole blood Se concentration of LS was faster than that of AS. Within 140 d the Se concentration of LS was below the adequate reference range, and remained so for the duration of the study. Yet the diet fed to the LS horses were still providing 70% of their estimated requirement (NRC, 2007). The observed decrease to below the reference range within 140 d suggests that the current Se recommendation must close to the minimum Se requirement for mature idle horses.

Similar to whole blood Se, GPx activity decreased in both AS and LS during the depletion phase. This decrease was anticipated for LS, but unexpected for AS. It has been stated that erythrocyte GPx activity will plateau when a whole blood Se concentration of 160 ng/mL is reached (Blackmore et al., 1982). Whole blood Se concentration remained above this level during the depletion phase. Therefore whole blood GPx activity of AS was expected to remain similar throughout the depletion period. In contrast it declined, regardless of adequate whole blood Se concentration and a Se intake of 140% of the recommended daily Se intake (NRC, 2007). The whole blood GPx activity reference range is 40 to 160 enzyme units per g hemoglobin (Stowe, 1998), though the sensitivity of GPx activity to storage times and assay conditions makes it difficult to compare absolute GPx activity values across studies. In this study GPx activity of LS approached the lower end of this reference range, while AS remained within the reference range.

The correlation between whole blood Se and GPx during the depletion phase was not very strong. Calamari et al. (2007) reported a slow decline in GPx activity upon

withdrawal of a Se supplement and hypothesized it was due to the slow red blood cell turnover (140 to 150 d) of the horse, maintaining GPx activity for longer. A slower change in GPx activity in comparison to Se concentration may explain the weak correlation found between whole blood Se and GPx activity during the depletion period. This weak correlation also suggests that whole blood Se may be a more accurate indicator of Se status at a particular point in time if Se deficiency or a too low Se intake is suspected. Similarly Ludvíková et al. (2005) found that when identifying low Se status in the horse, the use of only GPx activity as an indicator resulted in the overestimation Se status. However, blood GPx activity still remains an important indicator of long term Se status (Lee et al., 1995).

At the start of the repletion phase the whole blood Se concentration of LS, SP and SS fell well below the adequate Se range, while AS fell within the adequate range. Throughout the repletion period the whole blood Se concentration of AS was slightly higher but still comparable to the whole blood Se concentration reported by Shellow et al. (1985) of 176 ± 12.0 ng/mL. However, these whole blood concentrations were for horses fed at a slightly higher rate of 0.162 ppm Se for 140 d (Shellow et al., 1985) than the AS horses in our study. Within the relatively short supplementation period of 28 d the whole blood Se concentration of SP and SS increased above LS and became similar to AS. This rapid response to supplementation has been described before in whole blood (Calamari et al., 2009b) and plasma (Richardson et al., 2006). Calamari et al. (2009b) reported that although plasma Se plateaued between d 75 and 90, whole blood Se concentration did not plateau over a 112 d Se supplementation study that was conducted with lightly exercised horses. In our study whole blood Se concentrations for SP and SS did reach a plateau

between d 154 and 189, observed by lack of change in Se concentrations between these time points. The Se concentration results for SS and SP suggest that whole blood Se concentrations would range from 225 to 278 ng/mL when horses are fed diets containing 0.3 ppm Se. In addition there was a trend for higher whole blood Se concentration in SP horses compared to SS at d 189. These treatment groups were supplemented at a similar rate. In a study comparing Se-yeast to sodium selenite, Se-yeast was found to have a higher digestibility (Pagan et al., 1999), which could explain the observed difference in Se concentrations between SP and SS at d 189.

Whole blood GPx activity required 56 d to respond to supplementation in comparison to the 28 d for whole blood Se. This delay is likely due to the time required for the incorporation of GPx in recently formed red blood cells, which is dependent on red blood cell turnover (Knight and Tyznik, 1990). At d 189 GPx activity was higher for both SP and SS compared to AS. These results are in agreement with those of Calamari et al. (2009b) who reported whole blood GPx activity of horses supplemented at 0.29 or 0.39 ppm to be higher when compared to horses receiving 0.085 or 0.182 ppm for 112 d. At this time horses receiving 0.29 ppm as Se-yeast also had higher GPx activity than horses receiving 0.29 ppm as sodium selenite. However, it was reported that a plateau was not reached for GPx activity within the 112 d feeding period (Calamari et al., 2009b). In the current study, a plateau was reached in GPx activity for the SS group between d 154 and 189, but not for the SP treatment which had higher GPx activity at d 189 compared to d 154. This suggests that maximum GPx activity was reached for SS, but not SP, even though the supplementation rate was similar between these groups. As SP whole blood Se concentration did reach a plateau one may hypothesize as to whether SP GPx

activity would have plateaued at a later time point or exceeded that of SS if the feeding period was extended.

Whole blood GPx activity appeared to increase faster in SS than SP. The GPx activity of SS was higher compared to AS at d 154, but similar between AS and SP. Sodium selenite supplementation has been reported to be more effectively incorporated into GPx than organic Se in finishing pigs as observed by a faster increase in GPx activity (Mahan et al., 1999). However, the authors reported that this effect was more prominent at lower levels of Se inclusion (0.05 and 0.10 ppm).

A strong correlation existed between whole blood Se concentration and GPx activity during the repletion period (r = 0.82), similar to the correlation (r = 0.86) reported by Calamari et al. (2009b). Within treatment the correlation between Se and GPx for AS was weak and similar to the correlation observed during the depletion phase. Throughout the study AS maintained an adequate Se status, while the other groups went through a depletion phase, lowering Se stores as indicated by lower GPx activity. Therefore, the lack of a strong correlation for AS may simply be the result of adequate Se stores capable of buffering any change in whole blood Se or a narrow range of whole blood Se and GPx values. The strong correlations observed for LS, SP and SS may be the result of a lack of Se stores that can be used as alternate source of Se (e.g. muscle Se stores) for GPx synthesis. Consequently a stronger correlation was observed between whole blood Se and GPx activity.

The current Se requirement for horses is estimated at 1 mg Se per day for a 500 kg adult horse (NRC, 2007) or a dietary concentration of 0.1 ppm (assuming a dry matter

intake of 2% of BW). This requirement is estimated based on the studies conducted by Stowe (1967) and Shellow et al. (1985), indicating no additional benefit to feeding higher amounts of Se, based on plasma GPx response. Richardson et al. (2006) stated that a 56 d supplementation period, comparing Zn-L-selenomethionine (total Se intake 5.1 mg Se/d) and sodium selenite (total Se intake 4.7 mg/d) to a control (total Se intake 1.3 mg Se/d) did not affect plasma, red blood cell or muscle GPx activity. The authors reported a trend for the organic supplementation group to have a faster red blood cell GPx response within the first 28 d but this was attributed to the response of a single horse within that treatment. Similarly Karren et al. (2010) reported no difference in plasma GPx between horses provided a total Se intake of 0.19 mg/kg (pasture) and intakes of 0.35 mg/kg DM (pasture and grain), 0.49 mg/kg DM (pasture and Se-yeast) or 0.65 mg/kg DM (pasture, grain and Se-yeast) for 110 d. In other species such as growing/finishing pigs a higher serum GPx was reported when comparing Se intake of 0.15 or 0.3 ppm over 90 d, even though the difference was small (Mahan and Peters, 2004). More recently it has been suggested that the lack of detectable change in GPx activity in response to supplementation levels above 0.1 ppm could be due to the shorter experimental periods used in research trials, relative to the length of red blood cell turnover of the horse (Richardson et al., 2006; Calamari et al., 2009b). In this current study, both depletion and repletion phases exceeded red blood cell turnover (~150 d), and it was demonstrated that a dietary Se intake of 0.3 ppm resulted in higher GPx activity, regardless of Se source, when compared to a dietary Se intake of 0.12 ppm. In addition, maximum GPx activity was achieved for SS between d 154 and 189. These results are in agreement with Calamari et al. (2009b) who also reported higher GPx activity with higher Se intakes.

Although the current Se requirement of the horse is estimated at 0.1 mg/kg DM or 0.1 ppm, based on whole blood GPx activity in this study, horses may benefit from higher dietary Se levels, especially horses in geographically low Se areas.

As stated warlier GPx is a component of the antioxidant mechanism of the body. The antioxidant mechanism is complex, consisting of a range of different non-enzymatic (vitamin E, vitamin C, carotenoids, ubiquinols, flavonoids, glutathione and uric acid) and enzymatic (superoxide dismutase, catalase, GPx, thioredoxin system) antioxidants. When working in unison the various components of the antioxidant system are capable of preventing as well as repairing oxidative damage (Ji, 1999; Urso and Clarkson, 2003; Surai, 2006; Battin and Brumaghim, 2009). A variety of different measures of antioxidant capacity exist including oxygen radical absorbance capacity, trolox equivalent antioxidant capacity assay and ferric reducing ability assay (Cao and Prior, 1998). Serum or plasma antioxidant capacity is a variable that is frequently included in equine exercise studies (Avellini et al., 1999; de Moffarts et al., 2005; Ogonski et al., 2008). Although the horses were not exercised during this study, we were interested in determining if the overall antioxidant capacity of the horses would be affected by low or high Se status. Because of expected changes in GPx activity, we hypothesized that antioxidant status and oxidative stress would be altered in response to this change in Se status. The lack of response in TAC to change in Se status may suggest a lack of specificity of the assay to account for the GPx - associated antioxidant capacity. However, it has been suggested that GPx may only play a small role in the total cellular antioxidant system (Ho et al., 1997), and based on these results the same may be true for the extracellular total antioxidant system. Calamari et al. (2009a) measured the total plasma antioxidants in

lightly exercised horses on different dietary Se treatments using an assay that tests antioxidant capacity via the addition of hypochlorous acid. In contrast to our studies they reported lower total plasma antioxidants for the Se – yeast treatments compared to sodium selenite, with a linear decrease as Se – yeast supplementation increased. The authors hypothesized that this decrease in total plasma antioxidants was indicative of a decrease in free radical formation likely due to an increase in chain breaking antioxidants (e.g. thioredoxin system or phospholipid hydroperoxide GPx) not detected by their assay. The difference in methods used to evaluate TAC between the current study and Calamari et al. (2009a) makes it difficult to draw a direct comparison between the two studies.

Malondialdehyde is an end product of lipid peroxidation in biological membranes (Urso and Clarkson, 2003; Ducharme et al., 2009). Therefore, serum MDA concentration is frequently used as an indicator of oxidative stress, most commonly measured using the TBARS assay, but also via HPLC and spectrophotometry (Urso and Clarkson, 2003). The TBARS assay has been used to evaluate MDA as an indicator of oxidative stress in the horse (Ducharme et al., 2009). Oxidative stress is normally a variable of interest in exercise related studies because exercise has been shown to increase free radical production (Ji, 1999). However, we were interested in evaluating oxidative stress in these idle horses with low and high Se status to determine if low levels of GPx would impact oxidative stress in non-exercising horses. The results indicated a higher MDA concentration for the AS horses compared to the LS horses during the depletion phase with an increase in MDA for both treatments over time. However, this MDA increase was similar for both treatments and likely due to factors other than Se status. The MDA concentrations measured during the repletion phase were higher than those measured

during the depletion phase, while the depletion values were similar to MDA concentrations measured in exercising horses (Ducharme et al., 2009). The TBARS assay can reportedly cross react with other saturated and unsaturated nonfunctional aldehydes and carbohydrates, which may explain the overall higher values observed during the repletion phase. Regardless though, Se status did not affect MDA concentration. Similar to the TAC results, this lack of difference may indicate that the antioxidant mechanism adjusted to account for the change in GPx activity.

It has been proposed that GPx prevents damage to phagocytic cells by neutralizing high levels of H_2O_2 (Knight and Tyznik, 1990). Therefore we were interested in determining the effect of Se status on lymphocyte and neutrophil numbers. A preliminary study conducted in our laboratory found a higher number of lymphocytes in horses of higher Se status (see Chapter 3). Similarly, Calamari et al. (2009a) reported a trend for an effect of Se intake on lymphocyte numbers as a trend for a linear dose effect on lymphocyte numbers. In contrast the current study indicated a trend for higher lymphocyte numbers in the depleting LS group during the depletion phase. However, this was not observed for the repletion period. Neutrophil numbers were not affected by Se status during the depletion or repletion phase.

Vitamin E acts as a lipid soluble antioxidant, protecting cell membranes and preventing lipid peroxidation (Ronéus et al., 1986). It has been suggested that Se may have a sparing effect on vitamin E requirement, and as a consequence many studies have been conducted in other species supplementing both vitamin E and Se (MacDonald et al., 1976; Whanger et al., 1977; Scheideler et al., 2010; Tahmasbi et al., 2012). In horses, serum vitamin E concentration is used as an indicator of vitamin E status. A vitamin E

concentration above 2 µg/mL is considered adequate, while 1.5 to 2 µg/mL is a marginal status (NRC, 2007). Across the depletion and repletion period serum vitamin E ranged from 1.9 to 8.1 µg/mL for samples from individual horses. The 1.9 µg/mL was the only marginal value that was observed throughout the entire study. Across all other time points the serum vitamin concentration for this specific horse ranged from 2.6 to 5.8 µg/mL. Vitamin E supplementation has been reported to alleviate "selenium responsive disorders" such as myopathies in horses and cattle or exudative diathesis in poultry (Finch and Turner, 1996). Therefore, this synergistic relationship may explain the lack of effect of Se status on the TAC, MDA and immune cell numbers. At the same time high Se status did not improve MDA or TAC measurements over that of horses on adequate Se diet.

Iodothyronine deiodinase (ID) is a selenoenzyme responsible for the conversion of the pro-hormone thyroxine (T4) to its active form, triiodothyronine (T3) (Brown and Arthur, 2001; Calamari et al., 2009b; Muirhead et al., 2010). Calculating the ratio between T3 and T4 is thought to be a functional indicator of ID (Brown and Arthur, 2001; Calamari et al., 2009b). The current study found that the ratio of T3 to T4 was unaffected by Se status although it did increase from the baseline to final time points of both phases across all treatments. Thyroid hormones are essential to health as they are involved in metabolism, growth and development. Based on *in vitro* studies, ID seems to be more protected from Se deficiency than GPx (Köhrle, 2000). Calamari et al. (2009b) reported a lack of effect of Se source or Se dose on plasma T3, T4 and the ratio of T3 to T4. Muirhead et al. (2010) evaluated the Se, T3 and T4 levels in a group of horses in Prince Edward Island and reported a correlation between T4 and Se levels, but not T3. In

contrast, Dalir-Naghadeh and Rezaei (2008) reported a lower T3 to T4 ratio in lambs diagnosed with Se deficiency myopathy compared to healthy lambs. Although the LS horses were of low Se status according to whole blood Se and GPx activity, no physical signs of deficiency were observed. The lack of effect of Se status on this selenoenzyme in our study may also suggest a higher priority of ID for available Se in the horse.

CONCLUSION

In conclusion, a higher Se intake (0.3 ppm of diet DM) provided over a longer period of time does have an effect on GPx activity, increasing it above that of horses fed just above the NRC requirement. This response should be a consideration when formulating diets, especially for horses kept in geographically low Se areas as Se intake from forage may be lower than anticipated. However, antioxidant status and serum MDA were unaffected by change in GPx activity. Adequate vitamin E status may have played a role in the lack of effect of low Se status on MDA and TAC variables. The importance of high GPx levels in the antioxidant system may need to be further defined.

Table 6.1. Nutrient composition[1] (DM basis) of pasture and hay sampled throughout the experimental period and balancer pellet[2] fed during the Se depletion phase.

Item		Pasture	Grass Hay	Alfalfa Hay	Adequate Se Balancer Pellet[2]	Low Se Balancer Pellet[2]
DM	%	92.2	89.7	89.7	89.2	90.1
DE	Mcal/kg	2.11	2.10	2.22	3.35	3.38
CP	%	13.3	16.3	16.2	36.5	34.8
ADF	%	41.8	36.1	34.2	9.2	8.5
NDF	%	59.9	59.7	50.6	15.4	14.9
Starch	%	0.47	1.50	1.9	5.1	7.8
Calcium	%	1.06	1.10	1.06	4.07	4.16
Phosphorus	%	0.36	0.47	0.4	2.16	2.07
Magnesium	%	0.22	0.29	0.24	0.45	0.47
Potassium	%	1.59	1.77	2.15	2.05	1.98
Sodium	%	0.01	0.02	0.03	0.85	0.88
Iron	ppm	728	570	376	806	802
Zinc	ppm	34.7	33.0	32.0	448	427
Copper	ppm	8.00	9.00	7.00	160	154
Selenium	ppm	0.07	0.07	0.06	2.52	0.48

[1] Equi-analytical laboratories; Ithaca, NY
[2] McCauley Bros, Inc., Versailles, KY

Table 6.2. Example of the calculated total dietary Se intake[1] for a 500 kg horse during the depletion and repletion phase.

	Depletion phase		Repletion phase			
Se source	LS	AS	LS	AS	SP	SS
Pasture (mg)	0.56	0.56	0.56	0.56	0.56	0.56
Balancer pellet (mg)	0.17	0.86	0.16	0.86	0.16	0.16
Supplement (mg)	*	*	*	*	2.74	2.74
Total (mg)	0.73	1.42	0.72	1.42	3.46	3.46
Total (mg/kg DM)	0.063	0.12	0.062	0.12	0.30	0.30

[1]Total intake estimated at 2.25% of BW or 11.6 kg DM per day

[2]Dietary treatments: LS = low Se, AS = Adequate Se, SP = organic Se (Sel-Plex, Alltech Inc., Nicholasville, KY), SS = inorganic Se (sodium selenite)

Table 6.3. Nutrient composition[1] (DM basis) of pasture and hay sampled throughout the experimental period and balancer pellet[2] fed during the Se repletion phase.

Item		Pasture	Grass Hay	Alfalfa Hay	Adequate Se Balancer Pellet[2]	Low Se Balancer Pellet[2]
DM	%	87.6	92.1	91.2	89.2	90.9
DE	Mcal/kg	2.33	2.24	2.22	3.35	3.37
CP	%	24.2	21.6	15.8	36.5	36.9
ADF	%	31.9	34.6	38.6	9.2	7.7
NDF	%	49.3	53.6	49.6	15.4	14.7
Starch	%	1.60	2.80	1.40	5.10	3.60
Calcium	%	0.91	0.97	1.31	4.07	3.75
Phosphorus	%	0.52	0.53	0.40	2.16	2.41
Magnesium	%	0.33	0.40	0.20	0.45	0.44
Potassium	%	2.84	2.75	2.97	2.05	2.14
Sodium	%	0.14	0.26	0.05	0.85	0.84
Iron	ppm	856	673	217	806	808
Zinc	ppm	33.0	35.0	23.0	448	403
Copper	ppm	9.00	9.00	8.00	160	144
Selenium	ppm	0.06	0.06	0.02	2.52	0.53

[1] Equi-analytical laboratories, Ithaca, NY
[2] McCauley Bros, Inc., Versailles, KY

Table 6.4. Whole blood Se concentration (ng/mL) and glutathione peroxidase activity (mU/mg Hb) of mature horses during the depletion phase (LS Means).

	Selenium		Glutathione peroxidase activity	
Time (d)	LS[1]	AS[1]	LS	AS
0	251.7^{aw}	261.8^{aw}	64.5^{aw}	75.9^{bw}
84	202.1^{ax}	228.2^{ax}	54.4^{ax}	62.8^{bxy}
140	173.5^{ay}	205.2^{bxy}	52.7^{ax}	65.5^{bx}
168	160.7^{ay}	182.9^{ay}	46.7^{ay}	57.4^{bxy}
196	165.1^{ay}	211.8^{bx}	43.1^{ay}	55.5^{by}
SEM	4.42	7.47	2.04	3.44
Main effect (*P*-values)				
Treatment		<0.0001		<0.0001
Time		<0.0001		<0.0001
Treatment x time		0.0073		0.916

[a,b] means within row and variable lacking common superscripts differ, $P < 0.0.5$
[w,x,y] means within column lacking common superscripts differ, $P < 0.05$
[1]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM)

Table 6.5. Change in total antioxidant capacity (TAC) and malondialdehyde (MDA) concentration of horses during Se depletion phase (LS Means).

	TAC (mM)[1]			MDA (µM)[2]		
Treatment[1]	0 d	196 d	SEM	0 d	154 d	SEM
LS	1.181	0.703	0.020	3.480	4.110	0.230
AS	1.118	0.729	0.034	4.520	4.940	0.397
	Main effects (*P*-values)			Main effects (*P*-values)		
	Treatment		0.7417	Treatment		0.0226
	Time		<0.0001	Time		0.0408
	Treatment x time		0.0740	Treatment x time		0.8307

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM)

Table 6.6. Change in lymphocyte and neutrophil numbers in horses during the depletion phase[1] (LS Means).

	Lymphocyte (10^3)		Neutrophil (10^3)		Ratio	
Time (d)	AS	LS	AS	LS	AS	LS
0	2.82	3.53	4.13	4.94	1.48	1.503
56	2.83	2.97	4.66	4.85	1.73	1.716
84	2.32	2.74	4.34	4.51	1.94	1.754
112	2.52	3.00	4.02	4.32	1.65	1.545
140	2.46	3.00	3.81	3.93	1.65	1.377
168	2.41	3.02	4.01	4.61	1.70	1.594
196	2.33	2.84	5.63	5.32	2.47	2.043
SEM	0.27	0.16	0.39	0.23	0.220	0.130
Main effect (*P*-value)						
Treatment	0.0573		0.2917		0.2736	
Time	0.0005		<0.0001		<0.0001	
Treatment x time	0.7527		0.3342		0.8379	

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM)

Table 6.7. Whole blood Se concentration (ng/mL) measured throughout the repletion phase (LS Means).

	Time (d)					
Diet	0	28	56	154	189	SEM
LS	122.0[aw]	118.0[aw]	121.6[aw]	116.5[aw]	125.5[aw]	7.2
AS	185.9[bw]	179.4[bx]	182.9[bw]	184.5[bw]	194.5[bw]	6.8
SP	121.9[aw]	168.7[bx]	189.1[by]	255.4[cz]	254.8[cz*]	7.1
SS	127.0[aw]	175.3[bx]	195.0[by]	250.3[cz]	240.0[cz*]	7.2
Main effect (*P*-values)						
Treatment		<0.0001				
Time		<0.0001				
Treatment x time		<0.0001				

[a,b] means within column lacking common superscripts differ, $P < 0.0.5$
[w,x,y] means within row lacking common superscripts differ, $P < 0.05$
* trend to differ, $P < 0.1$
[1]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM)
[2]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM); SP = organic Se (0.3 ppm DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 ppm DM, sodium selenite)

Table 6.8. Whole blood GPx activity (mU/mg Hb) throughout the repletion phase (LS Means).

	Time (d)					
Diet	0	28	56	154	189	SEM
LS	30.7^{aw}	35.8^{aw}	32.7^{aw}	34.2^{aw}	33.8^{aw}	3.8
AS	47.7^{bw}	64.4^{bx}	61.4^{bx}	61.4^{bx}	62.8^{bx}	3.8
SP	29.2^{aw}	33.9^{aw}	47.4^{ax}	69.6^{bcy}	78.9^{cz}	3.8
SS	29.5^{aw}	36.3^{aw}	50.1^{abx}	72.9^{cy}	76.2^{cy}	4.1
Main effect (*P*-values)						
Treatment		<0.0001				
Time		<0.0001				
Treatment x time		<0.0001				

^{a,b} means within column lacking common superscripts differ, $P < 0.0.5$
^{w,x,y} means within row lacking common superscripts differ, $P < 0.05$
[1]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM)
[2]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM); SP = organic Se (0.3 ppm DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 ppm DM, sodium selenite)

Table 6.9. Serum total antioxidant capacity (TAC) and malondialdehyde (MDA) concentration of horses during the Se repletion phase (LS Means).

	TAC (mM)			MDA (µM)		
Treatment[1]	0 d	154 d	SEM	0 d	154 d	SEM
LS	0.557	0.568	0.014	8.060	8.920	0.93
AS	0.528	0.550	0.013	8.160	8.150	0.87
SP	0.551	0.562	0.013	7.690	6.870	0.87
SS	0.553	0.573	0.014	8.000	7.925	0.93
Main effects				Main effects		
	Treatment		0.1651	Treatment		0.7482
	Time		0.1404	Time		0.9795
	Treatment x time		0.7349	Treatment x time		0.6177

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM); SP = organic Se (0.3 ppm DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 ppm DM, sodium selenite)

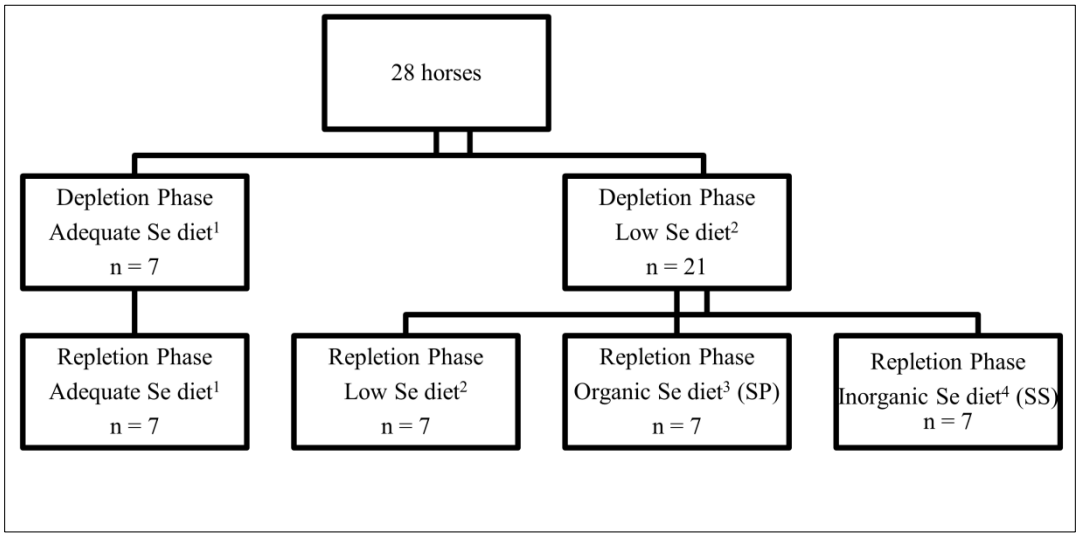

Figure 6.1. Experimental design and treatment allocation during the depletion (196 d) and repletion phase (189 d).

[1] Se source: sodium selenite. Total calculated dietary Se intake: 0.12 ppm DM
[2] Total calculated dietary Se intake: 0.06 ppm DM
[3] Selplex®, Alltech Inc., Nicholasville, KY. Total calculated dietary Se intake: 0.3 ppm DM
[4] Sodium selenite. Total calculated dietary Se intake: 0.3 ppm DM

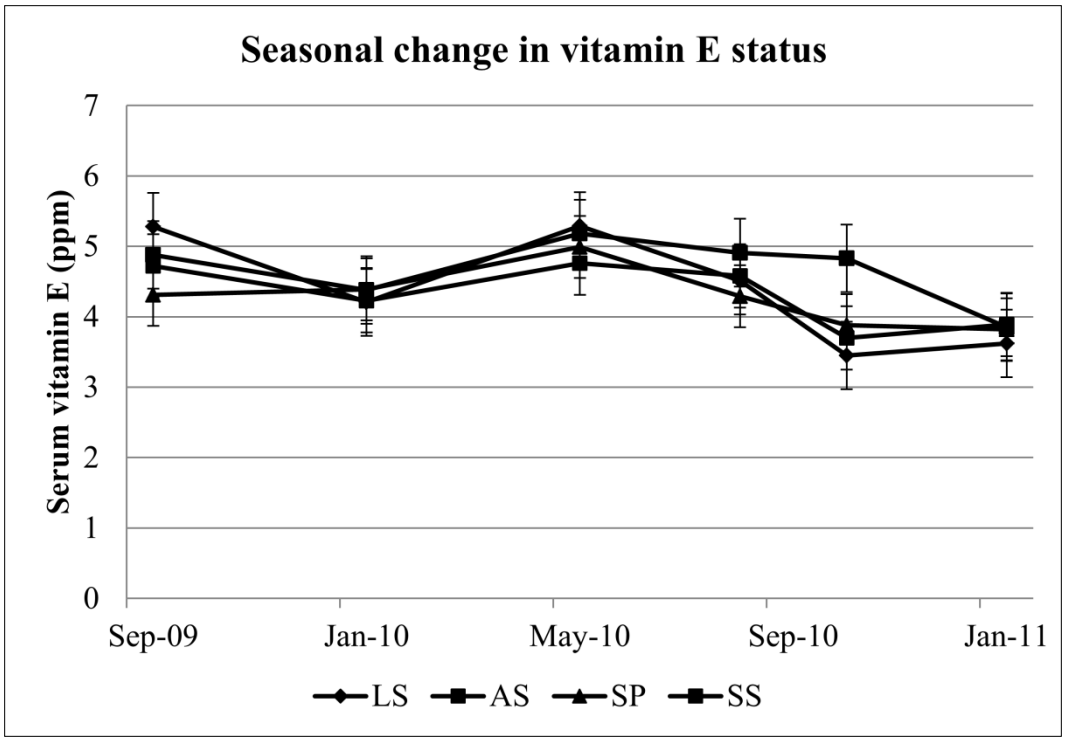

Figure 6.2. Serum vitamin E concentration (µg/mL)[1] throughout the depletion[2] and repletion[3] phases

[1]Treatment *P* = 0.7455; time *P* = 0.0004; treatment x time *P*=0.8853.
[2]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM)
[3]Dietary treatments (total dietary Se): LS = low Se (0.06 ppm DM); AS = Adequate Se (0.12 ppm DM); SP = organic Se (0.3 ppm DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 ppm DM, sodium selenite)

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CHAPTER 7: Effect of Selenium Status on the Response of Unfit Horses to Exercise

INTRODUCTION

Glutathione peroxidase (GPx) is a selenoenzyme associated with the antioxidant mechanism. It regulates hydro peroxide levels inside the cell (Arthur, 1997; Ferguson and Karunasinghe, 2011) by reducing hydrogen peroxide to water using glutathione as a donor of reducing equivalents (Urso and Clarkson, 2003). Hydrogen peroxide is very reactive and can also generate hydroxyl radicals, regarded as a harmful reactive oxygen species (ROS) (Surai, 2006). During strenuous physical exercise the consumption of O_2 increases dramatically. This increase in demand for O_2 results in an increase in the amount of ROS produced (Avellini et al., 1999; Ji, 1999). Under normal conditions the antioxidant mechanism is capable of neutralizing the effect of ROS production, creating a pro-/anti- oxidant balance within the body. With training the antioxidant mechanism adjusts to maintain this balance (Yur et al., 2008). Vitamin E is another important component of the antioxidant mechanism and is known to function in a synergistic manner with Se. Vitamin E is lipophilic, and located in the cell membrane, where it acts to protect against ROS damage (Ji, 1999), while Se contributes to the protection of cell membranes from ROS damage via GPx (Surai, 2006).

The objective of this study was to evaluate the effect of selenium (Se) status and low intensity exercise on unfit horses. We hypothesized that horses with low Se status would not be able to recover as rapidly post exercise and that low Se status would lead to higher ROS levels during and following exercise, resulting in an increase in serum malondialdehyde (MDA) concentration, serum creatine kinase (CK), serum aspartate aminotransferase (AST) and pro-inflammatory cytokine and granzyme B gene expression

in whole blood. Using these same indicators we hypothesized that the exercise would have less of an impact on the horses of high Se status.

MATERIALS AND METHODS

Animals:

Twenty five horses, aged 7 to 23 yr, were used in this study and consisted of 7 geldings and 18 mares. The geldings included of 5 Thoroughbreds, 1 American Quarter Horse and 1 Standardbred. The mares consisted of 17 Thoroughbreds and 1 American Quarter Horse.

Experimental design, diets and housing:

The horses were blocked by age and gender, and randomly allocated within block to one of 4 treatment groups so that each treatment group. The 4 treatment groups included an adequate Se group (AS, n = 6; 0.12 ppm Se), a low Se group (LS, n = 6; 0.06 ppm Se), a high organic Se group (SP, Sel-Plex®, Alltech Inc., Nicholasville, KY; n = 7; 0.3 ppm Se) and a high inorganic Se group (SS, sodium selenite; n = 6; 0.3 ppm Se).

The horses received their respective dietary treatments for at least 29 wk prior to the onset of the exercise test. During this period all horses were kept on pasture. The horses allocated to LS, SP and SS received a custom formulated low Se balancer pellet (0.53 ppm; LS, SP, SS; McCauley Bros Inc., Versailles, KY) while the horses allocated to AS received an adequate Se balancer pellet (2.52 ppm; AS). The balancer pellets were fed in individual feeding pens constructed for this purpose. The ingredient compositions of the balancer pellets appear in Appendix A Table 4.a. while the nutrient compositions

appear in Table 7.1. In addition to the balancer pellet, the SP and SS horses received their additional Se supplements top-dressed on the balancer pellets. As the Sel-plex product is a yeast based product, and brewer's yeast was used as carrier for the sodium selenite supplement, brewer's yeast alone was fed to the LS and AS horses to allow for a similar brewer's yeast intake between treatments, During the exercise test horses were kept on the same balancer pellet/supplement feeding regime as the 29 wk before.

Total dietary Se intake was calculated (Table 7.2) and balanced on a per kg BW basis for each horse. The total calculated Se intake for the LS group was 60% of the NRC (2007) recommended amount of 1 mg Se per day for a 500 kg horse (approximately 0.1 ppm Se; DM basis), while horses in the AS group received a calculated total of 120% of the recommended amount of Se. Horses in the SP and SS groups received 300% of the NRC recommended amount of Se.

To accommodate all the horses on the study for the exercise test, and to maintain accurate sampling times, the horses were divided into three groups and tested over a 3 wk period (Table 7.3). All treatments were represented within each group. Five d prior to the exercise test, the horses were moved to individual, partially covered pens (3 x 15 m) that were more conveniently located with respect to the exercise equipment. The horses were provided with the same grass hay that was fed during the preceding 29 wk on an *ad libitum* basis. During the day (0800 h to 1600 h) horses were turned out on pasture. Due to limited pasture availability hay was also provided *ad libitum* in the pasture.

Exercise test:

Prior to the exercise test horses were familiarized with the 6 horse exerciser (Stratton Equine Enterprise Inc., Lexington, KY) to ensure that horses were able to transition from walk to trot, trot to walk and reverse when asked to work in the opposite direction. For the adaptation a light exercise protocol, consisting of a total of 8 min walk and 2 min trot was used over a period of 3 days. Horses were not worked on the day preceding the exercise test.

The exercise test consisted of 4 min walk (1.4 m/s); 4 min trot (2.9 m/s); reverse; 4 min walk (1.4 m/s); 4 min trot (2.9 m/s); reverse; 4 min walk (1.4 m/s); 4 min trot (2.9 m/s); reverse; 4 min walk (1.4 m/s); 4 min trot (2.9 m/s); 4 min walk (1.4 m/s). This protocol allowed for a total of 20 min at a walk, covering 1.67 km, and 16 min at a trot covering 2.74 km for a total distance of 4.41 km in 36 min.

Sampling protocol:

On the day before the exercise test all hay and feed was removed from the individual pens at 1900 h. At 0700 h the next morning, pre-exercise blood samples were collected. These samples included whole blood samples collected in lithium heparin blood collection tubes (Becton Dickson, Franklin Lakes, NJ) for GPx analysis, blood samples collected in untreated vacutainer tubes (Becton Dickson) for serum separation and sodium fluoride (15 mg) potassium oxalate (12 mg) treated vacutainer tubes (Becton Dickson) for plasma separation, and whole blood collected in PAXgene blood RNA tubes (PreAnalytiX, Valencia, CA) for cytokine and granzyme B mRNA expression. After the pre-exercise sample collection horses were provided with their balancer pellet and

supplements. The horses started the exercise test exactly 1 h after receiving their ration. Horses were sampled immediately after the exercise test (as they left the exerciser; 0 h) and at 4 and 24 h post exercise. Whole blood, serum and plasma samples were kept at -80˚C until analysis while PAXgene blood samples were stored at -20˚C until analysis.

Selenium Status: Whole blood selenium and GPx activity

Whole blood Se concentration was determined by the Diagnostic Center for Population and Animal Health (Michigan State University; Lansing, MI) by means of inductively coupled plasma-mass spectroscopy.

Whole blood GPx activity was determined using the Bioxytech GPx-340 assay kit (OXIS research, Portland, OR), based on the method developed by Paglia and Valentine (1967). The GPx activity of each sample was calculated from the change in absorbance and expressed as units of enzyme activity per mg haemoglobin (mU/mg Hb). Haemoglobin was measured using a haemoglobin kit (Sigma, Saint Louis, MO).

Serum malondialdehyde concentration:

Serum malondialdehyde (MDA) concentration was measured using a thiobarbituric acid reactive substances (TBARS) method (Caymenchemical; Ann Arbor, MI). The method is based on the reaction of MDA with thiobarbituric acid under acidic, high temperature conditions, using a MDA standard.

Whole blood cytokine mRNA expression:

In vivo mRNA expression of interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), interleukin (IL) -1, IL-6 and granzyme B were assessed at pre-, 0 h post-,

4 h post- and 24 h post exercise. Total RNA was extracted using the PAXgene RNA extraction kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. Reverse transcription was conducted as described by Breathnach et al. (2006). Cytokine gene expression was measured by means of RT-PCR (7900 HT Fast Real-Time PCR system, Applied Biosystems, Foster City, CA) using equine specific intron spanning primer/probe sets (Adams et al., 2009; Liburt et al., 2010; Saulez et al., 2010). Relative change in gene expression was calculated as $2^{-\Delta\Delta CT}$ using the method described by Livak and Schmittgen (2001). The average of the pre exercise time point served as calibrator. Beta-glucuronidase was used as the internal control (Breathnach et al., 2006).

Serum creatine kinase (CK) and aspartate aminotransferase (AST):

Serum CK and AST was determined by a local equine hospital (Rood and Riddle Equine Hospital, Lexington, KY). All 4 time points (pre-, 0 h post-, 4 h post- and 24 h post exercise) were analysed for these two indicators of muscle damage.

Fasting insulin and glucose:

A solid phase ^{125}I radio immunoassay (Coat-a-count; Siemens) was used to determine insulin levels on the fasted pre-exercise time point. Glucose was analysed using an automated method (Konelab Arena 20XTi; ThermoScientific, West Palm Beach, FL; Thermotrace glucose hexokinase infinity reagent Konelab 301). A 1000 mg/dL glucose stock (Fisher) was used to prepare the standard curve.

Statistical analysis:

Data were analysed as a repeated measures design using the Proc Mixed function of SAS 9.2 (SAS Institute Inc., Cary, NC) with least square means separation procedure. The model included time, treatment and group as fixed effects, while horse was included as a random effect. Data were tested for normality, and log transformed when required for statistical analysis. When applicable, ANOVA procedure was used to evaluate data at a single time point (insulin and glucose). Data were back transformed and are presented as least squares means.

RESULTS

Prior to the exercise test whole blood Se concentration was higher for SP and SS compared to AS, and higher for AS than LS ($P < 0.0001$). Serum vitamin E concentration was similar between treatments ($P = 0.9876$). Whole blood Se and vitamin E data are presented in Table 7.4.

Whole blood GPx activity (Figure 7.1) was affected by treatment ($P < 0.0001$) and a treatment x time interaction existed ($P = 0.0318$), but not by time ($P > 0.05$). Post exercise GPx activity decreased for LS, and did not recover by 24 h. The GPx activity of AS did not change post exercise. In response to exercise GPx activity increased in SP at 4 h, but returned to pre values by 24 h. In contrast, GPx activity decreased in SS, recovering by 24 h.

Serum MDA concentration (Table 7.5) was not affected by treatment ($P > 0.05$) but was affected by time ($P < 0.0001$) and a treatment x time ($P = 0.0187$) interaction

existed. At 24 h MDA concentration was higher compared to the pre exercise time point. Upon separation of the means the only difference existed between AS and SP at the 4 h time point with a lower MDA concentration for SP. In addition a positive correlation existed between GPx activity and MDA at the 0 h time point (r = 0.46; P = 0.0198). This correlation did not exist at the other time points.

Whole blood mRNA expression of granzyme B and TNFα were not affected by Se status, but did change in response to exercise (Table 7.6). The expression of granzyme B increased immediately following exercise (0 h) followed by a decrease to below pre-exercise values at 4 and 24 h post exercise. Immediately post exercise (0 h) TNFα expression decreased to below the pre exercise value. At 4 h TNFα expression was higher compared to pre and 0 h, however, it was comparable to the pre-exercise time point at 24 h post exercise. Whole blood mRNA expression of IL-1 indicated a trend (treatment, P = 0.0629) for higher expression in SP compared to AS and LS. A trend also existed for IL-1 expression of SS to be higher than AS. The mRNA expression of IFNγ and IL-6 was not affected by Se status or exercise.

Serum CK (Table 7.7) and AST (Table 7.8) were affected by time ($P < 0.05$). Serum CK was higher immediately post exercise, compared to pre, 4 and 24 h post exercise. Serum AST was decreased at 24 h compared to pre, 0 and 4 h post exercise. No correlations existed between MDA and these indicators of muscle damage.

Fasted glucose and insulin levels (Table 7.9) were not affected by Se status ($P > 0.05$).

DISCUSSION

The exercise test was conducted following a 29 wk feeding period. Throughout this period the LS group received a diet that was lower in Se than the current recommendation of 1 mg Se/d for a 500 kg horse (NRC, 2007) with the intent to lower the Se status of this group, while the dietary Se concentrations of the other treatment groups were similar to or exceeded, this recommendation. Therefore, at the end of this feeding period, the Se concentrations of LS fell below the whole blood Se reference range of 180 to 240 ng/mL (Stowe, 1998), while the Se concentrations of AS fell within this range. The Se concentration for SS was at the upper end of this reference range while SP fell above this range. Serum samples taken at the same time for vitamin E analysis indicated that all horses were of adequate vitamin E status, and no difference in serum vitamin E existed among treatments. Because of the synergistic relationship between Se and vitamin E (Ji, 1999) it was important to establish and consider vitamin E status of the horses in this study for a more accurate assessment of the effect of Se status on the variables of interest.

The response of GPx activity to exercise in the horse has been inconsistent. Some studies report an increase in GPx activity (White et al., 2011), while others report a lack of response (Brady et al., 1978). This inconsistent response of GPx to exercise has also been reported in other species (Ji, 1999). When interpreting the change in GPx activity for this current study it is important to keep in mind that, although statistically significant, the numerical changes in GPx activity within each of the treatments were not large, therefore the biological impact of these changes remain unclear. Regardless, the AS group that served as control did not show any change in GPx activity at any time post exercise. However, GPx activity decreased in LS without recovering over the 24 h period.

The inability of GPx to recover within the 24 h period may be reflective of depleted Se stores, unable to restore GPx. In addition, GPx also acts as a storage pool of Se as each GPx enzyme contains 4 Se-Cys residues (Brown and Arthur, 2001). Therefore if the exercise increased the Se requirement for a different selenoprotein of higher importance than GPx it may also impede the recovery of GPx activity post exercise. In that sense it would have been interesting to assess the impact of repeated exercise bouts in this group of low Se horses.

Although supplemented at a similar rate, the SP and SS groups had different post exercise GPx activity responses. An increase in post exercise GPx activity was observed for the SP group, peaking at 4 h and returning to baseline levels by 24 h. In contrast, SS had a decrease in GPx activity, with the lowest activity value at 4 h, also returning to baseline levels at 24 h. At the 4 h time point GPx in SS was still higher compared to AS. Elevated GPx activity post exercise has been hypothesized to be indicative of an increase in pro-oxidant scavenging activity (Hargreaves et al., 2002; Williams, 2010), while a decrease in GPx activity post exercise is thought to be indicative of the saturation of enzyme activity (Janiak et al., 2010). Exercise is associated with ROS production, therefore GPx activity was expected to increase post exercise in response to elevated ROS levels in an attempt to neutralise hydrogen peroxides, but to a lesser degree in the low Se horses, due to lower Se stores. The increase in GPx in the SP group alone was unexpected, however it is likely the desired response to prevent oxidative damge.

Some research has been done to evaluate the effect of higher Se intake levels in response to exercise. White et al. (2011) supplemented 12 untrained Thoroughbreds with sodium selenite at a rate of 0.1 (control) or 0.3 mg Se/kg DM for 34 d. The horses were

then subjected to a prolonged exercise test at submaximal activity with sampling points at pre, 0, 6 and 24 h post exercise. Similar to our SS group, post exercise RBC GPx activity was decreased in the 0.3 mg Se/kg group while RBC GPx of the control group only decreased at the 6 h post exercise time point. Muscle GPx activity was elevated 6 h post exercise in the 0.3 mg Se/kg group compared to baseline values, while it remained unchanged in the control group.

The return of whole blood GPx to basal levels at 24 h in SS was potentially due to restoring of GPx. The source of Se in the balancer pellet fed to the AS horses was also sodium selenite, but the same changes was not observed for this group, indicating that either the changes were too small to detect, or the observed changes for SS was the result of the higher Se intake. Overall the whole blood GPx response observed for the SP group was more comparable to the muscle GPx response observed by White et al. (2011) in horses supplemented similar amounts of Se as sodium selenite. If the increase in GPx activity post exercise does indicate an increase in ROS scavenging, this may be the most desired response post exercise for increased protection against oxidative damage, while the decrease in activity observed for SS may be less desired.

MDA is the end product of lipid peroxidation, initiated by cell membrane damage, and may be used as an indicator of oxidative stress (Urso and Clarkson, 2003; Ducharme et al., 2009). In the current study MDA increased over time and the 24 h MDA concentration was higher compared to the pre exercise values, indicating that the exercise test did result in increased oxidative stress. At 4 h the MDA concentration was lower for SP compared to AS. This response coincided with the highest GPx activity for SP, which, similarly, was also higher than the GPx activity of AS at 4 h. However, the only

correlation between GPx activity and MDA existed immediately post exercise. Ducharme et al. (2009) measured plasma MDA in horses subjected to a stepwise exercise test and found an increase in MDA in response to exercise, but the increase was noted during the exercise phase and did not change during the recovery period.

In response to our mild exercise test both TNFα and granzyme B mRNA expression were affected. Lymphocyte populations are reported to move into peripheral blood in response to acute exercise, while intense exercise over a long period of time results in a decrease in lymphocytes and increase in neutrophils. Similarly cytokine levels are also affected by exercise (Pedersen and Toft, 2000). Exercise is reported to increase ROS production which in turn activates nuclear factor kappa beta (NFκβ) (Peake et al., 2007). The immune system requires NF-κβ to bind to DNA and activate the expression of the genes that encode the proteins that are involved in the immune response (Maggini et al., 2007) including the production of pro-inflammatory proteins such as TNFα and IL-6 (Duntas, 2009). NFκβ is therefore thought to regulate exercise induced cytokine production (Peake et al., 2007). It is of interest that this is also one of the proposed mechanisms through which Se is thought to affect the immune system. The selenoenzyme GPx is hypothesized to reduce intracellular ROS levels which limits the activation of NFκβ, and thereby regulates inflammatory protein production (Beck et al., 2001; Zeng and Combs Jr, 2008; Duntas, 2009).

Colahan et al. (2002) reported no change in IL-1, IL-6 and TNFα expression in leukocytes in response to exercise and attributed this to the exercise conditioned state of the horses. Liburt et al. (2010) evaluated cytokine expression in muscle and whole blood of unfit horses subjected to an incremental exercise test on a treadmill. They reported

blood IFNγ, TNFα, IL-1 to increase at 0 h, 6 h and 2 and 6 h respectively post exercise, while IL-6 expression was only elevated in muscle. It was concluded that IL-6 response may relate more to change in muscle metabolism than muscle damage itself (Liburt et al., 2010). Similarly we did not observe a change in IL-6 expression. We did observe an increase in TNFα at 4 h which may relate to the 6 h increase in TNFα observed by Liburt et al. (2010). The expression of IL-1 was not affected by exercise but there was a trend for it to be affected by treatment, being the highest in SP horses compared to LS and AS and similar to SS. The cytokine IL-1 is associated with the inflammatory cascade, although the impact of pro-inflammatory cytokines depends on the presence of anti-inflammatory cytokines which suppress the pro-inflammatory cascade (Dinarello, 2000). IL-1 also stimulates the expression of other cytokines such as IL-2 and IL-6, although here IL-6 was similar across all treatments. In addition, some degree of inflammation has been reported to contribute to exercise adaptation and protein synthesis (Liburt et al., 2010).

Granzyme B is one of the mediators involved in the death of target cells, released by cytotoxic cells such as lymphokine-activated killer (LAK) cells (Liu et al., 2011). Horohov et al. (1996) reported an increase in LAK cell activity in horses following a strenuous bout of exercise. The LAK cells are involved in immunological surveillance and it was noted that this increase may provide additional protection from immune insult (Horohov et al., 1996). Granzyme B has been shown to be associated with LAK cell activity, and mRNA expression of granzyme B has been reported to be a useful indicator of LAK cell activity (Liu et al., 2011). Our results showed that granzyme B expression changed in response to the mild exercise test. Although our horses were unfit, the

changes observed in response to exercise, regardless of how mild it was, may be indicative of the sensitivity of whole blood granzyme B expression to exercise stress.

Collectively the changes observed would suggest that even this mild exercise test did result in a pro-inflammatory response, although this may in part be the result of the unfit status of the horses.

Exercise induced oxidative stress has been thought to contribute to muscle damage (de Moffarts et al., 2005). Therefore we also evaluated serum muscle enzymes (CK and AST) as indicators of muscle damage. The Se status of the horses on our study did not affect CK or AST. Both indicators indicated an overall change over time, although it should be considered that these values still within or close to the reference range used by Rood and Riddle Equine Hospital (Lexington, KY) of 50 to 250 U/L for CK and 80 to 250 U/L for AST. Serum CK was elevated immediately post exercise, but returned to baseline levels at 4 and 24 h. White et al. (2011) also reported an increase in CK levels in response to the exercise but also observed no effect of Se treatment on CK. In contrast, AST did not increase in response to exercise. A faster increase and decline in CK, followed by a later peak in AST in response to exercise has been reported (Siciliano et al., 1997), however, our results only conform to the CK response. In our study there was no correlation between CK and MDA. A correlation between these variables has been reported in human studies (Urso and Clarkson, 2003). Williams et al. (2004) used plasma lipid hydro peroxide as an indicator of oxidative stress in endurance horses, and reported a weak but positive correlation between lipid hydro peroxide and both CK and AST. The lack of correlation observed for our study may simply relate to the small

increases observed for these indicators of muscle damage and the mild exercise that was imposed.

Some reports have indicated that selenate had the ability to act as an insulin mimic (Stapleton, 2000). In addition, a recent *in vitro* study using rat skeletal muscle cells indicated that while sodium selenite delayed insulin signalling, the same effect was not observed for the organic source of Se, selenomethionine (Pinto et al., 2011). Therefore we were interested in determining if fasted glucose and insulin would differ between the different Se treatments. However, no differences were detected for glucose or insulin levels between treatments.

CONCLUSION

In conclusion, it appears that the source and level of Se intake may affect the post exercise GPx response observed in unfit horses. Low Se status resulted in suppressed GPx activity post exercise. Because most exercise studies are conducted using fit or trained horses, the different responses observed in this study may be related to the level of fitness of these horses, and the intensity of the exercise test. Yet, it may be more representative of the non-competitive horse kept for recreational purposes who might graze Se deficient pasture and receive variable levels of Se supplementation. In addition, changes in MDA, TNFα and granzyme B indicate that the exercise did induce some physiological changes. Based on the changes in whole blood GPx post exercise, organic Se supplementation as Se-yeast may provide better protection against oxidative damage. However, the limited changes observed for MDA, pro-inflammatory cytokines, CK and AST suggest that overall Se status did not have a substantial impact on the ability of these unfit horses to recover from mild exercise.

Table 7.1. Nutrient composition of the balancer pellet[1] (DM basis) fed to mature horses on a BW basis and hay[1], provided ad libitum.

Item		Adequate Se Balancer Pellet[2]	Low Se Balancer Pellet[2]	Hay
DM	%	89.2	90.9	92.1
DE	Mcal/kg	3.35	3.37	2.24
CP	%	36.5	36.9	21.6
ADF	%	9.2	7.7	34.6
NDF	%	15.4	14.7	53.6
WSC	%	13.8	13.9	6.3
ESC	%	13.6	11.6	2.7
Starch	%	5.1	3.6	2.8
NFC	%	38.5	38.8	15.6
Calcium	%	4.07	3.75	0.97
Phosphorus	%	2.16	2.41	0.53
Magnesium	%	0.45	0.44	0.4
Potassium	%	2.05	2.14	2.75
Sodium	%	0.85	0.84	0.26
Iron	ppm	806	808	673
Zinc	ppm	448	403	35
Copper	ppm	160	144	9
Manganese	ppm	389	383	108
Molybdenum	ppm	5.3	4.4	0.4
Selenium	ppm	2.52	0.53	0.06

[1] Equi-analytical laboratories; Ithaca, NY
[2] McCauley Bros, Inc., Versailles, KY

Table 7.2: Example of calculated dietary Se intake[1] for a 500 kg horse.

Se source	Low Se (mg)	Adequate Se (mg)	Se-Yeast (mg)	Sodium Selenite (mg)
Pasture	0.56	0.56	0.56	0.56
Balancer pellet	0.16	0.86	0.16	0.16
Supplement	*	*	2.74	2.74
Total (mg)	0.72	1.42	3.46	3.46
Total (mg/kg DM)	0.062	0.12	0.30	0.30

[1]Total DM intake estimated at 2.25% of BW or 11.6 kg DM per day

Table 7.3: Exercise test groups[1] staggered over a 3 week period.

	Time	# Horses	Gender	Age range
Group 1	Wk 1	7	Geldings	13-18 yr
Group 2	Wk 2	12	Mares	7-14 yr
Group 3	Wk 3	6	Mares	14-23 yr

[1]All treatments were represented in each group

Table 7.4. Whole blood Se and serum vitamin E concentrations in mature horses prior to the exercise test.

	Dietary treatments[1]				
Variable	LS	AS	SP	SS	*P*-value
Selenium (ng/mL)	125.5±7.6[a]	195.4±7.0[b]	255.7±7.0[c]	240±7.5[c]	<0.0001
Vitamin E (µg/mL)	3.62±0.5[a]	3.83±0.47[a]	3.76±0.47[a]	3.85±0.51[a]	0.9876

[a,b,c]Means within row lacking a common superscript differ, $P < 0.05$

[1]Dietary treatments (total dietary Se concentration): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)

Table 7.5. Serum malondialdehyde (MDA, µM) concentrations in response to exercise in horses of different Se status.

	Time (h) post exercise					Main effect (*P*-values)		
Diet[1]	Pre	0	4	24	SEM	Treatment	Time	Treatment x time
LS	6.7[a]	6.4[a]	5.8[ab]	8.9[a]	0.55	0.7813	<0.0001	0.0187
AS	6.2[a]	7.9[ab]	6.5[a]	9.1[a]	0.56			
SP	6.2[a]	9.2[b]	4.6[b]	8.8[a]	0.51			
SS	6.8[a]	7.6[ab]	5.6[ab]	8.9[a]	0.55			

[a,b] means within column lacking a common superscript differ $P < 0.05$

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)

Table 7.6. Whole blood mRNA cytokine expression in response to exercise.

		Time (post exercise)					Main effect (*P*-value)		
Item	Diet[1]	Pre	0 h	4 h	24 h	SEM	Treatment	Time	Treatment x Time
GRZB[2]	LS	0.83	0.95	0.63	0.70	0.20	0.169	<0.0001	0.503
	AS	1.12	1.39	0.83	0.83	0.20			
	SP	1.06	1.28	0.76	0.85	0.18			
	SS	1.47	1.85	1.06	1.12	0.20			
TNFα	LS	1.24	1.35	2.01	1.50	0.51	0.278	0.0004	0.256
	AS	0.57	0.29	1.02	0.80	0.53			
	SP	1.87	1.57	2.16	1.99	0.48			
	SS	1.81	1.56	1.99	2.07	0.51			
IFNγ	LS	0.97	0.97	1.08	0.88	0.21	0.614	0.259	0.584
	AS	1.10	0.77	0.80	0.81	0.22			
	SP	1.24	1.04	1.04	0.94	0.20			
	SS	1.16	1.30	1.22	1.13	0.21			
IL-1	LS	0.94	0.91	1.03	0.95	0.14	0.063	0.209	0.993
	AS	0.85	0.82	0.88	0.86	0.15			
	SP	1.24	1.18	1.29	1.19	0.13			
	SS	1.05	0.97	1.17	0.92	0.14			
IL-6	LS	1.68	1.43	1.54	1.65	0.35	0.638	0.908	0.248
	AS	1.05	1.08	0.86	0.99	0.36			
	SP	1.35	0.90	1.11	0.97	0.33			
	SS	0.91	1.26	1.62	1.17	0.35			

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)
[2]GRZB = granzyme B

Table 7.7. Serum creatine kinase (CK, U/L) concentration in response to exercise.

	Time (h) post exercise					Main effect (*P*-values)		
Diet[1]	Pre	0	4	24	SEM	Treatment	Time	Treatment x time
LS	221	229	231	217	34	0.675	0.0281	0.1635
AS	262	274	233	245	34			
SP	244	328	247	215	32			
SS	274	281	283	273	34			

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)

Table 7.8. Change in serum aspartate aminotransferase (AST, U/L) in response to exercise in mature horses.

	Time (h) post exercise					Main effect (*P*-values)		
Diet[1]	Pre	0	4	24	SEM	Treatment	Time	Treatment x time
LS	260	271	270	267	19.7	0.5927	0.0311	0.759
AS	297	303	286	279	20.3			
SP	294	295	291	262	18.4			
SS	303	307	308	284	19.7			

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)

Table 7.9. Fasted insulin and glucose concentrations prior to the onset of the exercise test.

Variable	LS[1]	AS[1]	SP[1]	SS[1]	*P*-value
Glucose (mg/dL)	89.1±1.3	88.59±1.3	88.22±1.2	86.1±1.3	0.3646
Insulin (uIU/mL)	7.15±1.7	8.47±1.7	6.56±1.6	7.9±1.7	0.8593

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)

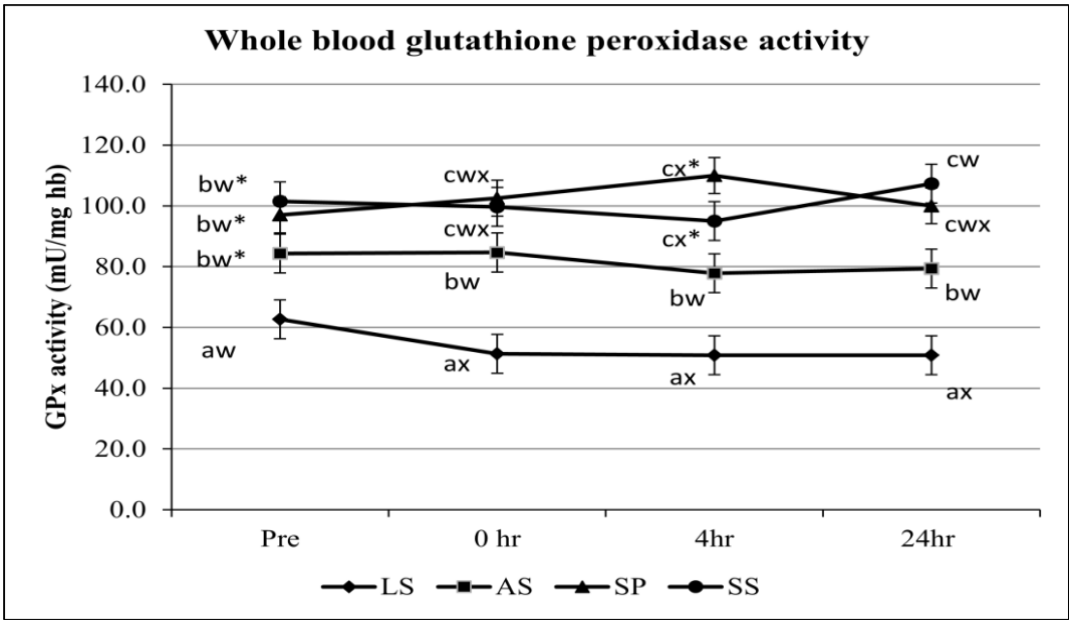

Figure 7.1. Change[1] in whole blood glutathione peroxidase activity (mU/mg hb) in response to exercise in unfit horses on different dietary treatments[2].

[1]Treatment $P < 0.0001$; time $P = 0.7496$; treatment x time $P = 0.0318$.
[2]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)

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CHAPTER 8: Conclusions and Implications

The results from this research indicate that the Se status of the horse can be manipulated through supplementation, affecting the traditional indicators of Se status, whole blood Se concentration and GPx activity. In addition, Se supplementation at 0.3 ppm DM was demonstrated to increase GPx activity compared to horses fed 0.12 ppm which is just above the NRC recommended level of 0.1 ppm DM. Similarly, the difference in GPx activity between horses receiving 0.3 or 0.12 ppm DM was also detectable post exercise. This suggests that both idle and exercising horses may benefit from a higher level of supplementation than currently recommended. In terms of source of Se, a trend existed for higher whole blood Se in the Se-yeast supplemented group after a 189 d feeding period. The outcome of the exercise study also indicated that Se-yeast resulted in a more favorable GPx response in comparison to sodium selenite at a similar supplementation rate, although the physiological impact requires further investigation. Change in Se status over the 18 mo study did not have an impact on TAC or oxidative stress as measured by MDA concentration. The adequate vitamin E status of the horses may have played a role in this finding, adjusting to account for Se deficiency.

The immune related results of our study indicated that initial humoral response and some indicators of cell-mediated immunity were affected by low Se status. At the time of the first vaccine challenge horses of low Se status exhibited a slower antibody response to the first KLH vaccine and lower mRNA expression of transcription factor T-bet in response to KLH stimulation compared to adequate Se horses. The humoral and cell-mediated response to the OVA vaccine challenge was unaffected by low Se status. In

addition OVA did not result in a strong cell-mediated response. However, the non-specific cell-mediated response to PMA stimulation was affected by Se status as the expression of selected cytokines was suppressed in the low Se horses. This may imply that the duration of Se deficiency affects the measured immune response. The humoral response to OVA vaccination for horses receiving 0.3 ppm Se exceeded that of horses receiving the NRC adequate Se diet although general cell mediated response was similar. Therefore, it is not clear from our results whether immune function is improved by feeding Se at levels above 0.12 ppm.

Collectively, it appears that the immune function of the horse is affected by Se status, however, more so by low Se status than high Se status. Whole blood GPx activity did indicate an advantage to feeding 0.3 ppm DM over lower levels of Se, and may be especially important when formulating diets for horses in geographically low Se areas.

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APPENDIX A: ADDITIONAL DATA TABLES

Chapter 4:

Appendix Table 4.a. Composition of the adequate and low Se balancer pellet[1] fed during the depletion and repletion phase.

Item	Adequate Se pellet (%)	Low Se pellet[2] (%)
Soybean meal (dehulled)	60.0	60.0
Ground oats	8.25	8.25
Wheat bran	7.50	7.50
Defluorinated phosphate	7.50	7.50
Molasses	6.60	6.60
Dehydrated alfalfa meal	5.00	5.00
Calcium carbonate	2.50	2.50
Salt	1.25	1.25
Mineral pre-mix*	0.50	0.50
Vitamin pre-mix**	0.50	0.50
Soybean oil	0.30	0.30
Mold inhibitor (buffered propionic and sorbic acid)	0.10	0.10

[1]McCauley Bros, Inc., Versailles, KY
[2] Mineral pre-mix used for low Se balancer pellet did not contain any Se.
*Mineral pre-mix proprietary to McCauley Bros, Inc., Versailles, KY. Pre-mix used for adequate Se balancer pellet contained Se, added as sodium selenite.
**Vitamin pre-mix proprietary to McCauley Bros, Inc., Versailles, KY.

Appendix Table 4.b. Example of the calculated total dietary Se intake* for a 500 kg horse.

Se source	Adequate Se diet	Low Se diet
Pasture/Hay	0.56	0.56
Balancer pellet	0.86	0.17
Total (mg/d)	1.42	0.73
Total (mg/kg DM)	0.12	0.063

* Total DM intake estimated at 2.25% of BW or 11.6 kg DM per day

Appendix Table 4.c. Intracellular production of IFNγ in response to *in vitro* stimulation with different mitogens[1].

			Week				Main effect (*P*-value)		
Item		Diet	0	3	5	SEM	Treatment	Time	Treatment x time
IFNγ									
KLH	% gated	AS[2]	0.59	0.69	1.22	0.23	0.085	0.069	0.027
		LS[2]	0.50	0.99	1.09	0.13			
KLH	MFI	AS	11.79	6.76	6.02	1.25	0.114	0.001	0.071
		LS	7.93	5.95	6.12	0.74			
PMA	% gated	AS	30.41	24.3	21.5	3.21	0.265	0.005	0.650
		LS	25.74	23.5	19.3	1.90			
PMA	MFI	AS	37.02	25.1	27.0	1.76	0.361	0.026	0.714
		LS	30.38	24.6	23.7	1.04			
FLU	% gated	AS	1.69	*	1.46	1.08	0.968	0.042	0.746
		LS	2.00	*	2.16	0.64			
FLU	MFI	AS	18.01	*	10.1	4.02	0.903	0.235	0.748
		LS	13.14	*	11.1	2.37			

[1]Mitogens include KLH (keyhole limpet hemocyanin); PMA (phorbol 12-myristate 13-acetate); FLU (equine influenza strain KY02)
[2]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM)

Appendix Table 4.d. Intracellular production of TNFα in response to *in vitro* stimulation with different mitogens[1].

			Week				Main effect (*P*-value)		
Item		Diet	0	3	5	SEM	Treatment	Time	Treatment x time
TNFα									
KLH	% gated	AS[2]	0.43	0.82	0.77	0.24	0.734	0.790	0.562
		LS[2]	0.73	1.12	1.33	0.14			
KLH	MFI	AS	15.08	12.2	11.1	1.85	0.166	0.531	0.289
		LS	16.77	12.7	11.5	1.10			
PMA	% gated	AS	58.83	57.1	53.7	3.61	0.941	0.033	0.957
		LS	59.13	58.5	54.5	2.13			
PMA	MFI	AS	53.74	44.8	47.5	3.98	0.908	0.007	0.610
		LS	52.18	46.9	45.7	2.35			
FLU	% gated	AS	0.52	*	1.30	0.35	0.691	0.718	0.504
		LS	0.82	*	1.31	1.86			
FLU	MFI	AS	12.56	*	8.90	1.68	0.899	0.139	0.924
		LS	10.94	*	8.86	1.00			

[1]Mitogens include KLH (keyhole limpet hemocyanin); PMA (phorbol 12-myristate 13-acetate); FLU (equine influenza strain KY02)

[2]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM)

Appendix Table 4.e. Relative quantity of mRNA expression of transcription factors and cytokines in response to *in vitro* stimulation with equine influenza KY02.

	Week			Main effect (*P*-value)		
Item	0	5	SEM	Treatment	Time	Treatment x time
T-bet						
AS[1]	2.62	3.31	1.10	0.589	0.615	0.646
LS[1]	2.56	1.74	0.55			
GATA 3						
AS	7.23	6.95	8.42	0.563	0.146	0.866
LS	8.02	7.64	3.90			
IFNγ						
AS	7.23	6.95	8.40	0.599	0.496	0.510
LS	8.02	7.64	3.90			
TNFα						
AS	2.13	4.18	1.25	0.542	0.943	0.360
LS	2.66	2.25	0.59			
IL-1						
AS	7.23	6.95	8.42	0.994	0.039	0.960
LS	8.02	7.65	3.91			
IL-2						
AS	1.19	2.42	0.76	0.506	0.095	0.377
LS	1.18	1.94	0.34			
IL-6						
AS	2.84	5.28	1.23	0.377	0.012	0.678
LS	1.43	2.48	0.52			
IL-8						
AS	1.85	2.2	0.78	0.833	0.743	0.663
LS	1.72	1.94	0.35			
IL-10						
AS	2.39	3.7	0.84	0.275	0.872	0.179
LS	2.96	2.02	0.41			
IL-13						
AS	1.32	1.4	1.18	0.601	0.637	0.837
LS	0.58	1.89	0.68			

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM)

Chapter 5:

Appendix Table 5.a. Intracellular production of IFNγ in response to *in vitro* stimulation with different mitogens[1].

			Week				Main effect (*P*-value)		
Item		Diet[2]	0	3	5	SEM	Treatment	Time	Treatment x time
IFNγ									
OVA	% gated	LS	1.46	0.83	1.12	0.15	0.801	0.605	0.490
		AS	0.91	0.87	1.27	0.15			
		SP	1.24	0.90	1.11	0.14			
		SS	0.87	1.16	1.33	0.15			
OVA	MFI	LS	7.72	10.4	6.17	1.09	0.031	0.118	0.433
		AS	4.88	6.19	5.09	1.11			
		SP	4.59	6.81	6.93	1.01			
		SS	6.17	6.40	6.41	1.09			
PMA	% gated	LS	36.79	40.6	38.0	3.23	0.180	0.041	0.422
		AS	37.07	40.2	38.5	3.36			
		SP	28.40	32.1	32.3	3.03			
		SS	37.86	36.9	38.7	3.24			
PMA	MFI	LS	34.22	35.5	35.7	2.28	0.443	0.001	0.415
		AS	31.73	37.5	35.8	2.35			
		SP	30.86	35.6	31.2	2.13			
		SS	28.52	33.1	34.1	2.28			
FLU (KY02)	% gated	LS	4.26	*	2.82	1.09	0.721	0.849	0.451
		AS	2.60	*	2.71	1.27			
		SP	1.65	*	1.92	1.08			
		SS	3.85	*	3.50	1.12			
FLU (KY02)	MFI	LS	19.09	*	12.16	4.70	0.694	0.008	0.804
		AS	15.26	*	7.99	5.47			
		SP	8.68	*	6.33	4.86			
		SS	15.05	*	10.37	4.69			

[1]Cell stimulated with KLH (keyhole limpet hemocyanin); PMA (phorbol 12-myristate 13-acetate) or FLU (equine influenza strain KY02)

[2]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = Sel-Plex (0.3 mg Se/kg DM); SS = Sodium Selenite (0.3 mg Se/kg DM)

Appendix Table 5.b. Intracellular production of TNFα in response to *in vitro* stimulation with different mitogens[1].

			Week				Main effect (*P*-value)		
Item		Diet[2]	0	3	5	SEM	Treatment	Time	Treatment x time
TNFα									
OVA	% gated	LS	0.97	1.32	1.45	0.17	0.441	0.009	0.424
		AS	1.20	1.23	1.08	0.17			
		SP	1.18	1.57	1.43	0.15			
		SS	0.99	1.65	1.41	0.17			
OVA	MFI	LS	12.03	13.98	9.15	2.59	0.243	0.257	0.360
		AS	11.83	13.59	7.65	2.59			
		SP	13.05	10.49	9.21	2.37			
		SS	13.23	13.43	17.9	2.56			
PMA	% gated	LS	69.56	66.56	66.7	2.99	0.705	0.019	0.943
		AS	70.50	66.83	70.2	3.09			
		SP	68.05	65.62	67.9	2.79			
		SS	72.50	68.28	72.2	2.99			
PMA	MFI	LS	76.99	74.77	54.26	5.11	0.921	0.001	0.1911
		AS	65.85	72.26	66.3	5.24			
		SP	66.72	70.68	58.6	4.76			
		SS	70.38	72.06	63.9	5.11			
FLU (KY02)	% gated	LS	3.04	*	2.30	0.74	0.916	0.007	0.8779
		AS	1.62	*	2.09	0.85			
		SP	1.80	*	2.51	0.76			
		SS	1.56	*	2.41	0.74			
FLU (KY02)	MFI	LS	8.35	*	5.79	0.78	0.970	<0.001	0.1044
		AS	7.42	*	6.84	0.90			
		SP	7.25	*	6.27	0.80			
		SS	8.64	*	5.87	0.78			

[1]Cell stimulated with KLH (keyhole limpet hemocyanin); PMA (phorbol 12-myristate 13-acetate) or FLU (equine influenza strain KY02)

[2]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = Sel-Plex (0.3 mg Se/kg DM); SS = Sodium Selenite (0.3 mg Se/kg DM)

Appendix Table 5.c. The mRNA expression as relative quantity of cytokines[2] in peripheral blood mononuclear cells stimulated with ovalbumin *in vitro*.

		Week				Main effect (*P*-value)		
Item	Diet[1]	0	3	5	SEM	Treatment	Time	Treatment x time
Tbet	LS	1.97	1.26	1.07	0.58	0.532	0.355	0.108
	AS	1.95	0.98	1.78	0.59			
	SP	0.59	0.85	0.91	0.54			
	SS	1.01	2.65	1.54	0.58			
GATA 3	LS	0.90	0.98	0.87	0.17	0.541	0.258	0.095
	AS	1.24	0.76	0.98	0.17			
	SP	0.63	0.85	0.89	0.15			
	SS	0.80	1.03	1.11	0.17			
Granzyme B	LS	1.62	1.05	1.03	0.68	0.973	0.807	0.120
	AS	2.27	1.50	1.84	0.71			
	SP	2.61	2.20	2.53	0.63			
	SS	0.75	1.61	0.88	0.68			
IL-1	LS	1.34	0.65	1.69	0.78	0.819	0.022	0.873
	AS	2.76	0.65	1.07	0.79			
	SP	1.75	1.25	1.33	0.72			
	SS	2.43	0.74	1.71	0.78			
IL-6	LS	0.63	0.25	0.32	0.43	0.166	0.002	0.758
	AS	1.14	0.95	1.34	0.43			
	SP	1.13	0.25	0.83	0.40			
	SS	0.71	0.25	0.55	0.43			
IL-8	LS	1.84	0.91	1.57	0.68	0.718	0.044	0.925
	AS	2.05	0.83	0.97	0.69			
	SP	1.80	1.67	1.56	0.63			
	SS	2.52	1.64	1.77	0.68			
IL-10*	LS (n=5)	1.38	0.85	0.75	0.41	0.240	0.005	0.529
	AS (n=6)	2.02	1.27	1.02	0.38			
	SP (n=5)	2.71	1.01	1.61	0.42			
	SS (n=3)	1.86	1.09	1.70	0.55			

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)
[2]IL = Interleukin
*Upon analysis of IL-10 datasets from some horses were omitted as indicated in the table

Appendix Table 5.d. Relative quantity of mRNA expression of transcription factors and cytokines in response to *in vitro* stimulation with equine influenza KY02.

		Week			Main effect (*P*-value)		
Item	Diet[1]	0	5**	SEM	Treatment	Time	Treatment x time
Tbet	LS	2.27	1.65	0.74	0.632	0.653	0.960
	AS	3.14	2.02	0.81			
	SP	1.70	1.65	0.72			
	SS	2.26	2.16	0.74			
GATA 3	LS	1.25	1.37	0.21	0.993	0.543	0.485
	AS	1.51	1.00	0.21			
	SP	1.15	1.30	0.19			
	SS	1.24	1.29	0.21			
GranzymeB	LS	12.54	11.79	4.94	0.899	0.265	0.512
	AS	7.89	1.58	5.30			
	SP	3.87	4.49	4.80			
	SS	2.46	5.69	5.16			
TNFα	LS	5.69	0.40	2.46	0.675	0.010	0.232
	AS	7.97	0.31	2.83			
	SP	2.50	1.80	1.29			
	SS	2.21	1.33	2.76			
IL-1	LS	1.04	0.16	2.24	0.702	0.007	0.076
	AS	7.93	0.97	2.36			
	SP	0.89	1.79	2.09			
	SS	0.60	0.62	2.50			
IL-2	LS	4.90	2.14	1.06	0.399	0.077	0.183
	AS	1.29	0.62	1.02			
	SP	1.04	1.41	1.00			
	SS	1.27	1.07	1.15			
IL-6	LS	2.74^{ab}	0.31^{a}	1.62	0.057	0.0004	0.026
	AS	7.57^{a}	3.89^{b}	1.87			
	SP	2.84^{ab}	0.91^{ab}	1.51			
	SS	1.16^{b}	1.11^{ab}	1.83			
IL-8	LS	1.92^{w}	0.31^{x}	1.52	0.812	<0.001	0.045
	AS	5.86^{w}	1.42^{x}	1.56			
	SP	1.42^{w}	1.93^{w}	1.41			
	SS	1.91^{w}	0.84^{x}	1.70			
IL-10	LS	1.04	0.40	0.79	0.149	0.8582	0.5987
	AS	2.60	1.32	0.80			
	SP	1.82	2.42	0.70			
	SS	2.28	1.25	0.97			

Appendix Table 5.d. Continued.

		Week			Main effect (*P*-value)		
Item	Diet[1]	0	5**	SEM	Treatment	Time	Treatment x time
IL-13	LS	1.20	0.46	0.93	0.440	0.001	0.280
	AS	2.21	2.08	0.96			
	SP	1.26	0.83	0.87			
	SS	4.40	1.00	1.03			

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = Sel-Plex (0.3 mg Se/kg DM); SS = Sodium Selenite (0.3 mg Se/kg DM)
** 2 wks following equine influenza vaccination

Chapter 6:

Appendix Table 6.a. The thyroxine/triiodothyronine ratio evaluated in response to Se depletion and Se repletion.

	Depletion[1] (d)			Repletion[2] (d)		
Treatment	0	196	SEM	0	154	SEM
LS	0.068	0.159	0.040	0.048	0.109	0.018
AS	0.036	0.055	0.067	0.035	0.051	0.017
SP	*	*	*	0.035	0.052	0.017
SS	*	*	*	0.097	0.087	0.018
	Main effects		P-value	Main effects		P-value
	Treatment		0.2740	Treatment		0.1299
	Time		0.0179	Time		0.0726
	Treatment x time		0.3604	Treatment x time		0.5191

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM)
[2]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)

Appendix Table 6.b. Lymphocyte and neutrophil numbers in horses on different dietary Se treatments during the repletion phase[1].

	Lymphocyte (10^3)				Neutrophil (10^3)			
Time (d)	LS	AS	SP	SS	LS	AS	SP	SS
0	2.38	2.30	2.30	2.77	4.78	4.38	4.52	5.00
28	3.05	2.07	2.40	2.63	4.30	4.34	4.25	4.68
56	2.87	2.39	2.58	2.70	4.00	4.12	3.69	4.84
84	2.89	2.86	3.34	3.16	4.24	4.46	3.80	4.77
121	3.29	2.71	2.85	2.99	4.34	3.99	3.91	4.05
154	2.35	2.22	2.56	2.93	3.73	3.41	3.28	3.73
SEM	0.29	0.26	0.26	0.29	0.40	0.37	0.37	0.40
	Main effect (*P*-value)				Main effect (*P*-value)			
	Treatment		0.4678		Treatment		0.6057	
	Time		<0.0001		Time		<0.0001	
	Treatment x time		0.2964		Treatment x time		0.7451	

[1]Dietary treatments (total dietary Se): LS = low Se (0.06 mg Se/kg DM); AS = Adequate Se (0.12 mg Se/kg DM); SP = organic Se (0.3 mg Se/kg DM, Sel-Plex, Alltech Inc., Nicholasville, KY); SS = inorganic Se (0.3 mg Se/kg DM, sodium selenite)

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APPENDIX B: Laboratory protocols

Methods relating to selenium and antioxidant status:

Whole blood glutathione peroxidase activity:

Oxis Research Bioxytech GPx – 340 kit
(www.percibio.com; Cat No 21017)

Method for analysis of whole blood samples:

Reagent preparation

1) NADPH

- Add 7.5 mL Assay buffer to vial. Vortex well.
- Allow the reagent to reach room temperature before use in the assay.
- Keep shielded from light.
- Will remain stable at room temperature for 9hrs.

2) Assay Buffer

- Calculate and remove the amount of assay buffer that will be needed to run all the samples for one day (about 30mL/Plate). Refrigerate remaining assay buffer.
- Allow buffer to reach room temp before use in assay.

3) Tert-Butyl Hydroperoxide

- First make up a stock dilution: 50uL tert – Butyl Hydroperoxide into 4950uL nano pure water
- Then make up working solution: 200uL stock + 19.8mL nano pure water.
- Working solution will remain stable for entire working day at room temp.

Standard and samples preparation:

Standard: Run Std at 1:30 (16.5 uL std + 484.5 uL standard diluent)
Keep diluent frozen and std refrigerated after thawing

Samples: Dilute samples at 1:40:
i.e. 12.5 uL whole blood + 487.5 uL Assay Buffer
Thaw samples out as close to when it has to be analyzed as possible, then keep on ice while diluting out.
Run assay in triplicate.

NB: Remember to include water blank to subtract background activity.

Running Assay:

Turn microplate on, open correct program (kinetic; 340nm; 30s interval for 5 min), set temperature at 25º C and make sure it starts to warm up.

To each well add:

1) 75 uL assay buffer
2) 15 uL sample (triplicate)
3) 75 uL NADPH
4) 75 uL peroxide for activation.

Read immediately

Data analysis:

1) Average replicates.
2) Absorbance(30s) – Abs (300s)
3) Change in Abs / 4.5min
4) Change/min – Blank change/min
5) Net change/min / 0.004043
6) x DF (16 x 40 = 640 / 480 for Std)
7) Express as activity/gHb

Total Hemoglobin Assay:

(Pointe Scientific Inc.; assay kit number H7504)

1) Add 2 mL reagent to test tube
2) Add 10 µL Standard, low control, high control or sample to test tube
3) Allow to stand at room temperature for atleast 3min
4) Set spec at 540 nm
5) Zero spec using reagent only as blank
6) Read an record absorbance (Must be read within 1 hr)
7) Calculate values

Blank – reagent only
Std – 0 µL reagent
Std – 5 µL reagent
Std – 10 µL reagent
Std – 20 µL reagent

Total Antioxidant Assay:
(Cayman chemicals; assay kit number: 709001)

Pre-assay preparation:

Set up plate plan

Reagent preparation:

SEE INSTRUCTION SHEET

Standard preparation:

Tube	Trolox	Assay buffer (uL)	Trolox concentration (in mM)
A	0	1000	0
B	30	970	0.045
C	60	940	0.090
D	90	910	0.135
E	120	880	0.18
F	150	850	0.225
G	220	780	0.330

***Samples can be diluted with Assay buffer to fall within the range of the STD curve.

Assay:

1) To each well add 10 uL sample/STD
10 uL Metmyoglobin
150 uL Chromogen
2) Initiate reaction by adding 40 uL hydrogen peroxide working solution within 1 min
3) Cover plate and incubate for 5 min while shaking gently.
4) Read absorbance at 750 nm.

Thiobarbituric Acid Reactive Substances Assay:
(Cayman chemicals; assay kit number: 10009055)

Pre assay preparation:

Serum should not be diluted for this assay
All reagents must be at room temperature before use (sit at least 2 hrs on bench)

1) Set up plate plan
2) Prepare reagents
    a) Thiobarbituric Acid – use to prep "f"
    b) TBA Acetic Acid
        add 40 mL + concentrated TBA acetic acid to 160mL nano pure water
        Stable for 3 months at room temperature
    c) TBA sodium hydroxide
        add 20mL concentrated TBA sodium hydroxide to 180mL nano pure water
        Stable for 3 months at room temperature
    d) TBA Malondialdehyde standard
        Ready for use to prepare standard curve
    e) TBA SDS solution
        Ready for use
    f) Color reagent – prepare in 250mL beaker (Need 150mL / plate)
        for 25 samples + 8 STD: Weigh out 795mg Thiobarbituric Acid
        add 75mL TBA Acetic acid solution
add 75mL TBA sodium hydroxide solution
        mix until completely dissolved
        Stable for 24 hours

Standard preparation
Prepare a stock solution: 250uL MDA standard + 750uL nano pure water
Label 8 test tubes and label A-H
Prepare std curve as follows from the stock solution:

Tube	MDA	Water (uL)	MDA concentration (in uM)
A	0	1000	0
B	5	995	0.625
C	10	990	1.25
D	20	980	2.5
E	40	960	5
F	80	920	10
G	200	800	25
H	400	600	50

Performing the assay

1) Label vial cups
2) Add 100 uL sample or STD to labeled 5mL vial
3) Add 100 uL SDS solution to vial and swirl to mix
4) Add 4 mL colour reagent forcefully down side of each vial
5) Cap vials, place in holder to keep upright while boiling
6) Boil for 1 hr
7) Remove vials and immediately place on ice. Keep on ice for 10 mins.
8) Centrifuge vials for 10 mins at 1600xg at 4˚C
9) Load 150uL in duplicate from each vial to plate
10) Read absorbance at 530 nm

Methods relating to Immunology:

PBMC isolation and antigen specific stimulation:

1) Let heparinized tubes settle for about 20 min. If the cells have settled on top of the red blood cell layer remember to collect from just below the cells to make sure you collect all the cells.
2) Set up 50 mL centrifuge tubes with approx 10 mL ficoll. 1 tube per horse/2 blood tubes.
3) SLOWLY transfer the top layer of the blood (white cells) to the ficoll tubes. Hold the ficoll tube at an angle and add the blood component so that it forms a layer on top of the ficoll.
4) Centrifuge for 30 mins at 500xg (slow brake) [Program 1]
5) Transfer cell layer to new 50 mL tube. The hazy layer is the cell layer. It is very hard to remove only that portion, so best to remove more than that i.e. remove some of the ficoll layer too. Remember to set pipette on "Slow".
6) Top off tube with warm PBS (+/- 50 mL).
7) Centrifuge for 10 mins at 500xg (fast break) [Program 3]
8) Dump supernatant; Flick pellets.
9) Top off tube with PBS (+/- 50 mL).
10) Centrifuge for 10 mins at 300xg (fast break) [Program 4]
11) Dump supernatant; Flick pellets.
12) Top off tube with PBS (+/- 35 mL).
13) Centrifuge for 10 mins at 300xg (fast break) [Program 4]
14) Dump supernatant; Flick pellets.
15) Re-suspend in 10 mL PBS
16) Label count cups, 1 cup per sample.

You want 100uL cell solution and 900 uL PBS in each cup. Start by adding 900 uL PBS to each cup (you can do this while the last centrifuge step is running). Then add 100 uL of the re-suspended cells to each cup. Count.

17) Want $3x10^6$ cells/well in cRPMI+2.5%
18) Transfer 0.5mL to 24 well plate – Already containing 0.5mL Med / med+antigen
19) Incubate 20 hrs
20) Add 2uL Brefeldin A to all wells
21) Add 10uL PMA/IONO to PMA wells.
22) Incubate 4 hrs in 37˙, 5% CO_2 incubator
23) Withdraw 0.5 mL for RNA stat – follow RNA stat method
24) Transfer cells to 96 well V bottomed plate (200uL per well in triplicate
25) Spin plate at 500xg for 5min.
26) Flick once.
27) Re-suspend cells in 100 uL paraformaldehyde.
28) Cover with parafilm and refrigerate.

Autologous serum

Serum must be heat inactivated by incubating for a 30 min period in a 56˚C water bath. Centrifuge serum tubes at 800xg for 10 min and aspirate serum as usual.

<u>Prepare autologous media:</u>

250 mL RPMI
Remove 6.25 mL
Add 2.5 mL PSG
Add 250 uL mercapto-ethanol.

Add 10 mL of above media to separate 15 mL tubes – 1 per horse.
Add 500 uL autologous serum for each individual horse.

PS: 500 uL is twice as much as usual but your final cell culture will have half of this media and half of the un-supplemented media.

Lymphocyte proliferation assay:

Per well the final assay volume:
100 μL PBMC
100 μL mitogen solution
50 μL ^{3}H-Thymidine

Mitogen solutions:
Concanavalin A
Amersham stock at 1mg/mL
Want 4 mL at 10 μg/mL
80 μL Con A + 3920 μL cRPMI

1) Prepare stock solutions day before, plate at 100 μL per well and place in incubator. Use cRPMI as control wells.
2) Isolate cells and wash as described previously
3) Re-suspend PBMC at $2x10^{6}$ cells/mL
4) Add 100 μL of PBMC per well to already prepared plate
5) Incubate for 72 hr in 5 % CO_2 incubator at 37˚C
6) Pulse cells with ^{3}H-Thymidine solution – 50 μL per well

   ^{3}H-Thymidine solution:
   50 μL concentrated ^{3}H-Thymidine + 4950 μL cRPMI

7) Incubate an additional 18 hr
8) Place in Freezer until DNA extraction.

IFN γ / TNF α Staining (Intracellular Staining)

1) Centrifuge plate (500xg for 5 min).
2) Flick excess and press on paper towel
3) Add 200 uL PBS – Saponin buffer to each well, mix by pipetting repetitively.
4) Spin plate (500xg for 5 min)
5) Check for cells, flick off excess
6) Add 100uL of stain solution: for 10 wells prepare 1.5mL stain

FITC 1:100 [For 1 mL add 2uL Ab to 998uL saponin buffer; for 1.5 mL add 3 uL to 1497uL saponin buffer]

IFN γ 1:100 [For 1 mL add 10uL Ab to 990uL saponin buffer; for 1.5 mL add 15 uL to 1485uL saponin buffer]

MOPC-21 Sigma mouse IgG1κ (isot. Cntl) 1:100 [For 1 mL add 10 uL Ab to 990 uL saponin buffer; for 1.5 mL add 15 uL to 1485 uL saponin buffer]

HL801 (TNF α; refrigerate) 1:10 [For 1 mL add 100 uL Ab to 900 uL saponin buffer; for 1.5 mL add 150uL to 1350uL saponin buffer]

7) Incubate in an ice cooler with the lid on for a minimum of 30 min. Make 4mL secondary.
8) After incubation, centrifuge plate (500xg for 5 min); Check for cells; Flick off excess
9) Wash with FACS buffer (200 uL in each well, pipette up and down)
10) Centrifuge (500xg for 5 min); check for cells, flick.
11) Wash with FACS buffer (200 uL in each well, pipette up and down)
12) Centrifuge (500xg for 5 min); check for cells, flick.
13) Add 100uL Saponin buffer to IFN γ and IFN γ control wells***
14) Add 100uL of the secondary Ab (Caltag) Goat F(Ab') 2 anti mouse IgG (H&L) FITC (light green top) @ 1:400 [for 1 mL add 2.5 uL Ab to 997.5 uL saponin buffer; for 1.5 mL add 3.75 uL Ab to 1496.25 uL saponin buffer
15) Incubate in ice cooler with lid on for 30 min
16) After incubation spin plate, Centrifuge (500xg for 5 min)
17) Wash with FACS buffer (200 uL in each well, pipette up and down)
18) Centrifuge (500xg for 5 min); check for cells, flick.
19) Wash with FACS buffer (200 uL in each well, pipette up and down)
20) Centrifuge (500xg for 5 min); check for cells, flick.
21) Put 100 uL FACS Flow into each well (pipette up and down)
22) Label FACS FLOW tubes, add 200uL FACS buffer to each tube. You need 1 tube per well.

23) Transfer contents of each well into a FACS tube, so that total contents of the tube = 300 uL. Remember to swivel tip on bottom of the well to pick up the cells, and go back at least once for maximum cell extraction from well.

****If you use separate plates for IFNg and TNFa you can start process of removing IFNg cells at this point, while TNFa is incubating in secondary*

Total RNA purification: Paxgene Kit protocol

1) Centrifuge paxgene blood samples 10min @ 2800 x g
2) Discard lid and pour off supernatant
3) Add 4mL of RNAse – free water to pellet
4) Place new lids on tubes; vortex until resuspended
5) Centrifuge 10 min @ 2800 x g
6) Pour off supernatant
7) Add 350µL Buffer 1 to pellet
8) Vortex until pellet is completely resuspended
9) Transfer to labeled 1.5mL eppendorf tubes (aprox. 700 µL)
10) Add 300 µL Buffer 2
11) Add 40 µL Proteinase K
12) Vortex, incubate at room temperature for 5 min
13) Incubate for 10 min in 55˚C water bath
14) Vortex briefly
15) Centrifuge 10 min at 20000xg
16) Transfer supernatant to new labeled 1.5mL Eppendorf tube. Avoid pellet!
17) Add 350µL 100% ethanol
18) Vortex
19) Label spin columns and place in 2mL collection tube
20) Add 700µL sample to appropriate spin column
21) Centrifuge for 1 min @ 8000 x g
22) Place spin columns into new 2mL collection tubes
23) Add any remaining sample volumes to appropriate spin column
24) Centrifuge for 1 min @ 8000 x g
25) Transfer spin columns into new 2mL collection tubes
26) Add 700µL Buffer 3 to spin columns
27) Centrifuge for 1 min @ 8000 x g
28) Transfer spin columns into new 2mL collection tubes
29) Add 500µL Buffer 4 to spin columns (1st wash)
30) Centrifuge for 1 min @ 8000 x g
31) Transfer spin columns into new 2mL collection tubes
32) Add 500µL Buffer 4 to spin columns (2nd wash)
33) Centrifuge 3 min at max speed (20000xg)
34) Transfer spin columns into new 2mL collection tubes
35) Centrifuge 1 min at max speed (20000xg)
36) Transfer spin columns into new fully labeled 1.5 mL eppendorf tubes
37) Add 40µL Buffer 5 to spin columns (1st elution)
38) Centrifuge for 1 min @ 8000 x g
39) Add 40µL Buffer 5 to spin columns (2nd elution)
40) Centrifuge for 1 min @ 8000 x g
41) Incubate 5 min in 65˚C water bath
42) Immediately place samples on ice if proceeding to second phase or store at -80˚C

Total RNA purification: RNAstat samples

NB: DO NOT PROCESS MORE THAN 11 SAMPLES AT A TIME

1) Prepare workbench by wiping it down with RNAse away
2) Also wipe off all tip boxes, pipettes and test tube racks
3) Remove samples from -80˚C freezer to thaw
4) Label one 1.5 mL eppendorf tube per sample
5) Start microcentrifuge on fast cool at 4˚C (20 min)
6) Vortex thawed samples to homogenize samples
7) Add 200 µL chloroform to each sample, vortex each sample for 20 s
8) Let samples incubate for 3 min at room temperature
9) Centrifuge samples at 12000g for 15 min at 4˚C
10) Transfer 420 µL of the upper aqueous phase into new eppendorf tubes
11) Add 320 µL pure isopropanol to each sample
12) Pulse vortex each sample 5 times
13) Incubate at -20˚C for atleast 30 min
14) Centrifuge at 20000g for 10 min at 4˚C
15) Set the waterbath at 60˚C
16) Decant supernatants, work quickly to ensure that pellet don't move
17) Add 800 µL 75% ethanol and pulse vortex
18) Centrifuge at 15000g for 10 min at 4˚C
19) Remove and discard 700µL
20) Centrifuge at 15000g for 10 min at 4˚C
21) Remove as much supernatant as possible, leave pellet untouched
22) Place tubes in hood to dry
23) Add 60 µL RNAse free water per tube
24) Place in the water bath (60˚C) for 10 min
25) Pulsevortex and store at -80 ˚C

Reverse transcription of RNA

A) First step is to determine the quality of the RNA using a bioPhotometer

1) One "uvette" (cuvette) required per sample, and one for a blank
2) To each cuvette add 95µLRNAse free water; take care to place the water in the cuvette well
3) To the blank cuvette, add 5µL Buffer 5 from kit. Place tip in well, pipette the liquid and mix by moving tip from side to side
4) Add 5µL RNA to the appropriate cuvette and take reading.
5) Use reading to calculate amount of RNA and amount of RNAse free water to be used for transcription process. (Use computer program Rutgers samples #2 version 1.0) A total volume of 41.5µL required.

B) Prepare master mix of reagents for transcription process:

Reagents used:

1) ANIV RT 5X buffer (16µL/sample)
2) $MgCl_2$ (16µL/sample)
3) dNTP (4µL/sample)
4) RNAsin (1µL/sample)
5) Oligo primer (1µL/sample)
6) AMV RT reverse transcriptors (0.5µL/sample)

Reagents can be combined in a 1.5mL eppendorf tube in correct ratios according to number of samples. Prepare enough for # samples + 1. Vortex well.

C) Transcription process:

1) Set out required number of thermowell PCR tubes
2) Label tubes fully
3) Add calculated quantity of RNAse free water to respective labeled tube
4) Add 38.5µL of the master mix to each tube
5) Add calculated volume of RNA to correct tube.
6) Vortex each sample. Should have total volume of 80µL per sample
7) Place samples in BioRad My IQ single color Real Time PCR detection system and follow the instrument protocol for the transcription process.
8) Place cDNA in -20˚ C feezer, and left over RNA in the -80˚ C freezer

RealTime-PCR

cDNA dilution:
Dilute cDNA by adding 80 µL RNAse free water to the cDNA.
Transfer 135 µL of the diluted cDNA to a 96 well plate (Note which sample is transferred to which well).

Plate loading:

Eppendorf ep Motion 5070 robot used for loading the 384 well plate

Each well will contain 10µL for the reaction, made up of :

1. 0.5 µL primer probe
2. 5 µL TAC
3. 4.5 µL cDNA

TAC and Primer probe is mixed separately in containers placed onto the robot platform

96 well plate containing samples placed on robot platform as well as the 384 well plate. Note instructions for placement.

Select program. Note plate barcode.

Plate reading:

Instrument: 7900 HT Fast Real-Time PCR system, Applied Biosystems

Plate scanned for identification and added to the computer program.

Run over night.

OVA/KLH ELISA PROTOCOL

Preparation steps

Coat:
OVA Coating concentration = 300 µg/mL (i.e. 30 µg /well)
KLH Coating concentration = 300 µg/mL (i.e. 30 µg /well)

(OVA stock = 2500 µg /mL; So dilute 1.2mL OVA stock in 8.8mL Coating buffer)

Samples:
Dilute samples at 1:300
i.e. 3.4uL sample into 996.6uL Elisa wash

Standard:
Horse "A3", 2 wk post vac 2 for KLH
Horse "I10", 2 wk post vac 2

Dilute standard at 1:40
i.e. 250uL into 9750uL Elisa wash

(Add 200uL to top wells; do 3 fold dilutions down the rows i.e.:
Add 100 uL Elisa wash to all rows below
Transfer 100uL Std from top well to row below; mix by pipetting up and down, transfer 100uL to row below etc etc so the all wells contain 100uL std)

Block:
Prepare 1% PVA block by mixing 1g PVA and 100mL ddH2O; heat and stir until dissolved (about 1 hr)

Just before use dilute 1% PVA with PBS (1:1)

Secondary antibody:
Add 4uL goat-anti-horse IgG in 10mL Elisa wash (Peroxidase – conjugated AffiniPure Goat Anti-Horse IgG; Jackson Immuno Research, West Grove, PA)

Procedure:

1) Add 100uL OVA/coat/well. Refrigerate plate over-night.
2) Wash plate 3 times.
3) Add 200uL PVA per well. Incubate 1 hr at room temperature on bench top
4) Wash plate 3 times.
5) Add 100uL diluted serum samples or standard as described above (triplicate). Incubate 1 hr at 37'C.
6) Wash 3 times
7) Add 100 uL secondary antibody. Incubate for 1 hr at 37'C.

8) Wash 3 times
9) Add 100uL SureBlue. Watch colour development – usually very quick < 1 min
10) Add 100uL Stop solution.
11) Read immediately at 450 nm

Influenza Specific IgGa, IgGb, IgG(T) ELISA procedure

Antibodies:

Monoclonal antibodies IgGa (CVS48) 1:100, IgGb (CVS39) 1:10, IgGT (CVS40) 1:10.
Goat anti-mouse (Bethyl Ab Cat No: A90-216P, 0.5mg) 1:5000

Procedure

1) Coat ELISA plates the night before ready to run the ELISA, cover with parafilm/foil and place in the 4˚C overnight. For Influenza ELISAs coat using 10HA/well (example: 1:32=640HA/ml, thus 10HA/well do 1:6.4 dilution in coating buffer). NOTE: Make sure the Coating Buffer pH = 9.6. Coat using 100ul/well.
2) Next morning, wash the plates 3X with ELISA Wash (1 cycle with washer).
3) Block using 2% non-fat dry milk (2 grams + 100 mls—warm 37C water bath to dissolve) made in ELISA Wash. Add 200ul/well of block to the plate. Incubate 30 mins to 1 hour at Room Temperature (using ELISA incubation box).
4) Wash plates 3X using ELISA washer (1 cycle with washer).
5) Add Serum samples in triplicate @ 100ul/well at appropriate concentration/dilution. Incubate at room temperature for 1 hour.
6) Wash plates 3X using ELISA washer (1 cycle with washer).
7) Add Primary Antibody (Monoclonal antibody-mAb) to appropriate wells using the above dilutions @ 100ul/well. Incubate for 1 hour at room temp.
8) Wash plates 3X using ELISA washer (1 cycle with washer).
9) Add Secondary Antibody (Goat anti-mouse-HRP) using 1:5000 dilution to ALL wells @ 100ul/well. Incubate for 1 hour at room temp
10) Wash plates 3X using ELISA washer (1 cycle with washer).
11) Add substrate (TMB-KPL) to ALL wells @ 50ul/well. Incubation time varies depending on which antigen using to coat plates with. For Influenza ELISAs incubation time ~ 5.0 minutes.
12) Add Stop Solution (KPL) when wells/substrate reaction has reached appropriate color.
13) Using the ELISA plate reader, measure Optical Density (OD) at 450nm absorbance.

Influenza hemagglutination inhibition protocol

Each serum sample must be trypsin-periodate treated to remove non-specific inhibitors of virus hemagglutination which are present in serum samples:

1) Add 100 µL trypsin to 100 µL serum
2) Incubate 30 min in a 56˚C water bath.
3) Add 300 µL periodate
4) Incubated at room temperature for 15 min
5) Add 500 µL 0.6% glycerol in saline

This yielded a final serum dilution of 1:10.

1) Next add 25 µL PBS added to all the wells of a round bottom 96 well plate (Thermo Fisher U-bottom micro titer vinyl plate)
2) Add 50 µL of the treated serum to the first well on the plate
3) Titrate 25 µL across the row.
4) Add 25 µL virus (1:8 dilution) all wells.
5) Incubated for 30 min at room temperature
6) Read buttons of non-agglutination
7) The titer is allocated to the last non agglutinating dilution for each sample

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VITA

Mieke Brümmer was born in Pretoria, South Africa on 20th of February 1981. Mieke obtained her B.Sc. (Agric) Animal Science from the Department of Animal and Wildlife Sciences, University of Pretoria. In 2007 Mieke completed her M.Sc. (Agric) Animal Science, Nutrition, also from the University of Pretoria titled**:** The effect of yeast cell wall preparations on salmonella colonisation, gastrointestinal health and performance of broiler chickens. During this time Mieke completed a 10 month research internship at Alltech Biosciences Center, Nicholasville, KY. In Fall 2007 Mieke started her Ph.D. with Dr Lawrence at the University of Kentucky.

Throughout her undergraduate and graduate studies Mieke received several awards: 2003: Best third year student in Animal Science from the South African Society of Animal Science (Transvaal Branch). 2004: Completion B.Sc. Animal Science with a total of 19 distinctions, 7 of which in final year. 2005 and 2006: University of Pretoria Master's student academic achievement bursary. 2006: Professor J.C. Bonsma Achievement Bursary. 2010: Invited member in the Honor Society of Agriculture Gamma Sigma Delta, University of Kentucky Chapter, 2010. 2010: International Ingredient Corporation Pinnacle award. 2011 and 2012: Award recipient at the Animal and Food Sciences Graduate Association poster symposium, Ph.D. section.

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