THE EFFECTS OF ORGANIC AND INORGANIC DIETARY SELENIUM ON SELENIUM STATUS AND IMMUNE FUNCTION OF HORSES

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A Thesis Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Selenium (Se) is a trace element essential for animal and human cell biology. Incorporated into selenoproteins, Se is an integral part of several antioxidant enzymes, including glutathione peroxidase and thioredoxin reductase. In addition to other well recognized roles of Se, such as in thyroid metabolism, an important research focus has been Se and its role in immune function. The selenium status of horses is determined by geographical location and management practices.

In the first study, the effects of Se supplementation and source of dietary Se on blood Se status and measures of immune function were investigated in adult, non-pregnant horses. Plasma and red blood cell Se concentrations were highest in horses in the organic Se group when compared to horses in the inorganic Se group or control group. Dietary Se content and source led to alterations in relative lymphocyte expression of IL-5, and relative neutrophil expression of IL-1 and IL-8.

In the second study, different methods of Se supplementation were compared in adult, non-pregnant horses. Horses received either no supplementary Se, access to Se-containing trace mineral block, or Se-enriched hay, obtained from fields after application of Se-containing fertilizer. The concentration of Se in fresh forage and hay was increased from concentrations considered inadequate to meet dietary requirements to those considered adequate. Both Se-enriched hay and the Se-containing trace mineral block significantly increased the plasma and red blood cell Se concentrations of adult horses. However, horses fed Se-enriched hay had significantly higher plasma Se concentrations when compared to horses with access to the Se-containing trace mineral block.

The third study investigated the effects of dietary Se on the Se status of broodmares and their foals, as well as, measures of immune function in foals. Se source fed to mares during late pregnancy and early lactation influenced the red blood cell Se concentrations of their foals at one month of age with organic Se leading to higher red blood cell Se when compared to inorganic Se. Mare blood and milk Se as well as foal blood Se at birth were not affected by Se source. Furthermore, the blood Se status of mares during late pregnancy was correlated with their colostrum Se and early lactation milk Se. Selenium source influenced relative gene expression of foal lymphocyte cytokines, namely IL-2, TNFα, and IFNγ; however, no Se source consistently led to alteration in expression.

In conclusion, different methods of Se supplementation, including Se-fortified forage, can be used to improve the Se status of horses in Se-deficient regions. In adult, non-pregnant horses, organic Se is consistently superior to inorganic Se in raising blood Se; however, this effect of dietary Se source could not be demonstrated as clearly in broodmares and their foals. Selenium may contribute to alterations in immune function of horses through changes of relative cytokine gene expression, however, the significance of these observations remains to be clarified.
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TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... v
ACKNOWLEDGEMENTS ...................................................................................................... vi
LIST OF TABLES .................................................................................................................. xii
LIST OF FIGURES .............................................................................................................. xiii
LIST OF ABBREVIATIONS .................................................................................................. xv
CHAPTER 1: THE ROLE OF SELENIUM IN HEALTH AND NUTRITION AND SELENIUM-
RELATED RESEARCH IN HORSES .................................................................................. 1
   Introduction ........................................................................................................................ 1
   Selenium in Health and Disease ........................................................................................ 2
      Selenoaminoacids and selenoproteins .......................................................................... 2
      Selenium metabolism .................................................................................................... 3
      Antioxidant defense ...................................................................................................... 5
      Thyroid hormone metabolism ....................................................................................... 7
   Other selenoproteins ........................................................................................................ 9
   Selenium and vitamin E interactions ............................................................................... 10
   Selenium research related to human health ...................................................................... 10
   Selenium research related to animal health ...................................................................... 13
   Selenium and the Immune Response .............................................................................. 15
      Neutrophil function ..................................................................................................... 16
      Macrophage function ................................................................................................. 17
      Lymphocyte function and antibody production .......................................................... 17
      Inflammation, infection, allergy, and aging .................................................................. 20
   Selenium and the neonatal immune system ................................................................... 24
   Selenium Requirements and Supplementation of Livestock .......................................... 26
      Assessment of selenium status .................................................................................... 26
LIST OF TABLES

Table 1.1 Selenoproteins with known functions.......................................................... 46

Table 2.1 Summary of relevant functions of cytokines measured during lymphocyte and neutrophil gene expression ............................................................................ 89

Table 4.1 Correlations coefficients, with accompanying p-values, for the relationship between colostrum or milk selenium (Se) concentration and plasma or red blood cell (RBC) selenium concentration in broodmares. Value in blue indicates a significant relationship......................... 157
LIST OF FIGURES

Figure 2.1  Mean (± SE) plasma Se concentration in adult horses receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se .............................................. 90

Figure 2.2  Mean (± SE) red blood cell Se concentration in adult horses receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se ................................................................. 91

Figure 2.3  Mean (± SE) blood glutathione peroxidase activity in adult horses receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se ..................................... 92

Figure 2.4  Percent inhibition of serum anti-rabies antibodies prior to vaccination, prior to booster vaccination, and at the end of the study in adult horses receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se ...................................... 93

Figure 2.5  Mean stimulation index (SI) for expression of IL-5 in lymphocytes from adult horses receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se ................... 94

Figure 2.6  Mean relative quantification of IL-1 expression in stimulated neutrophils from adult horses receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se ... 95

Figure 2.7  Mean relative quantification of IL-8 expression in stimulated neutrophils from adult horses receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se .... 96

Figure 3.1  Schematic showing the two experimental fields in relation to each other as well as the division into untreated and selenium-treated plots within each field .......................................... 122

Figure 3.2  Forage selenium concentration (means ± SE, DM basis) prior to application of nitrogenous fertilizer either with or without added selenium, 1 and 2 months after fertilizer application, and in hay one month after harvest ..................................................................................... 123

Figure 3.3  Mean (± SE) plasma selenium concentration for horses provided with a selenium-containing trace mineral block, fed selenium-fortified hay, or left unsupplemented ...................... 124

Figure 3.4  Mean (± SE) red blood cell selenium concentration for horses provided with a selenium-containing trace mineral block, fed selenium-fortified hay, or left unsupplemented... 125

Figure 3.5  Mean (± SE) blood glutathione peroxidase activity for horses provided with a selenium-containing trace mineral block, fed selenium-fortified hay, or left unsupplemented... 126

Figure 4.1  The effect of dietary selenium source on plasma selenium concentration in broodmares. Inorganic selenium (sodium selenite) or organic selenium (selenium yeast) was fed from two months before foaling to one month after foaling, to deliver 0.3 ppm supplementary selenium on a ration drymatter basis ........................................................................................................ 159

Figure 4.2  The effect of dietary selenium source on red blood cell selenium concentration in broodmares. Inorganic selenium (sodium selenite) or organic selenium (selenium yeast) was fed
from two months before foaling to one month after foaling, to deliver 0.3 ppm supplementary selenium on a ration drymatter basis................................................................. 160

Figure 4.3 The effect of dietary selenium source (inorganic vs. organic) on plasma selenium concentration in their foals. Supplementary selenium was fed to broodmares from two months before foaling to one month after foaling................................................................. 161

Figure 4.4 The effect of dietary selenium source (inorganic vs. organic) on red blood cell selenium concentration of foals. Supplementary selenium was fed to broodmares from two months before foaling to one month after foaling ..................................................................................162

Figure 4.5 The effect of dietary selenium source on colostrum and milk selenium concentrations in broodmares. Inorganic selenium (sodium selenite) or organic selenium (selenium yeast) was fed from two months before foaling to one month after foaling, to deliver 0.3 ppm supplementary selenium on a ration drymatter basis..........................................................................................................163

Figure 4.6 Mean relative quantification of IL-2 expression of ConA stimulated lymphocytes of one month old foals born to mares receiving either inorganic or organic Se ..................................164

Figure 4.7 Mean relative quantification of IFNγ expression of ConA stimulated foal lymphocytes from foals born to mares receiving either inorganic or organic Se at a) one day and b) 30 days post partum........................................................................................................165

Figure 4.8 Mean relative quantification of TNFα expression of PMA stimulated foal lymphocytes (previously cryopreserved) from one month old foals born to mares receiving either inorganic or organic Se ..........................................................................................................................166

xiv
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AVC</td>
<td>Atlantic Veterinary College</td>
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<tr>
<td>B-GUS</td>
<td>Beta Glucuronidase</td>
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<td>CBC</td>
<td>Complete blood count</td>
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<td>CD</td>
<td>Clusters of differentiation</td>
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<td>ConA</td>
<td>Concanavalin A</td>
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<td>COX</td>
<td>Cyclooxygenase</td>
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<td>DM</td>
<td>Dry matter</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>GPx</td>
<td>Glutathione peroxidase</td>
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<td>Interferon gamma</td>
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<td>Interleukin</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<td>NRC</td>
<td>National Research Council</td>
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<td>PEI</td>
<td>Price Edward Island</td>
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<td>PMA</td>
<td>Phorbol myristate acetate</td>
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<td>ppm</td>
<td>parts per million</td>
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<td>RID</td>
<td>Radial immunodiffusion</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RQ</td>
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<td>Se</td>
<td>Selenium</td>
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<td>SE</td>
<td>Standard error</td>
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<td>Stimulation index</td>
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<td>T3</td>
<td>Triiodothyronine</td>
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<td>T4</td>
<td>Thyroxine</td>
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<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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<td>WMD</td>
<td>White muscle disease</td>
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CHAPTER 1: THE ROLE OF SELENIUM IN HEALTH AND NUTRITION AND SELENIUM-RELATED RESEARCH IN HORSES

Introduction

Selenium (Se) is a trace element essential for animal and human cell biology, where it is involved in a number of key metabolic activities. Selenium enters the food chain through plants, which take it up from the soil.

Selenium (Greek selene meaning “moon”), a nonmetal with chemical similarity to sulfur, was discovered in 1817 by the Swedish chemist Jöns Jacob Berzelius and its essentiality as a micronutrient was recognized in 1957(1). This was followed, in 1973, by the discovery that the enzyme glutathione peroxidase is a selenoprotein (2). More recently, other selenoproteins have been identified (Table 1.1) and research investigating their biological functions is ongoing.

The importance of Se has classically been ascribed to its role in antioxidant defense, as an integral part of several antioxidant enzymes such as glutathione peroxidase and thioredoxin reductase. This is still considered an essential function of the trace element; however, newer research indicates a more complex role of Se in animal and human metabolism. In addition to other well recognized roles of Se, such as in thyroid metabolism, an important research focus has been Se and its role in immune function, reviewed recently by Hoffmann and Berry (3).

To date, the majority of Se research has focused on humans and domestic farm animal species, both for investigating nutritional strategies to ensure adequacy, and the influence of Se on immune function. Comprehensive research in horses is lacking. Therefore, the objectives of this thesis were firstly to investigate the effects of Se
supplementation, various methods of Se supplementation, and the form of dietary Se, on the Se status of horses, and secondly to examine the effects of dietary Se source on measures of innate and adaptive immunity. To reach these research objectives, three separate studies were performed, examining different methods of Se supplementation (mineral supplement, Se-containing trace mineral block, Se-fortified forage), different dietary sources of Se (inorganic, organic), and different groups of horses (adult non-pregnant and non-lactating, broodmares, and foals).

This chapter summarizes the following topics: our current state of knowledge regarding the role of Se in mammalian health and its involvement in disease processes in humans and animals; the known and suggested relationships between Se status and the immune response; the currently recommended Se requirements of livestock, including horses, how they were derived, and current recommendations for supplementation to ensure adequacy, and finally an overview of Se research specifically in the horse.

**Selenium in Health and Disease**

**Selenoaminoacids and selenoproteins**

Selenium can replace sulfur in the amino acids cysteine and methionine, forming selenocysteine and selenomethionine, respectively. Mammalian form Se-containing proteins from two classes: those that incorporate Se in a non-specific manner, and those that contain and require Se at an active site, in the form of selenocysteine, for functional activity (4). Selenocysteine is important for the function of many proteins. It is considered to be the 21st amino acid and is encoded by TGA in DNA (5). Research has
shown that during translation, the UGA codon, which normally functions as a stop codon leading to translation termination, also encodes for selenocysteine (6, 7).

In mammals, the Se-containing enzymes glutathione peroxidase and thioredoxin reductase are the most prevalent and well known selenoproteins. The role of selenoproteins in human disease processes has been recently reviewed by Bellinger et al. (8). To date, at least 25 selenoproteins have been identified; however not in all cases are their functions known (4). Those with known function are presented in Table 1.1.

Selenium metabolism
Selenium metabolism in humans and animals is dependent on the chemical form (organic or inorganic) of the Se absorbed. In mammals, selenomethionine, the major component of Se yeast, is absorbed and metabolized in a similar way as the amino acid methionine, utilizing active transport mechanisms across intestinal membranes (sodium-dependent neutral amino acid transport system) (9). After absorption, selenomethionine can be further metabolized to selenocysteine, becoming available for insertion into selenoproteins or for passive storage, preferentially in organs possessing a high rate of protein synthesis such as red blood cells, liver, kidneys, pancreas, gastrointestinal mucosa, and skeletal muscle (9, 10). In contrast, inorganic Se selenite, like other minerals, is absorbed through passive diffusion and a relatively small proportion is incorporated in proteins and retained; the remainder is excreted through the urine and feces (11). Pagan et al. (12) reported that horses supplemented with inorganic Se (as selenite) excreted more Se in the feces than horses receiving organic Se (as Se yeast).
Mammalian Se metabolism is further influenced by the dietary mineral and vitamin balance. Copper excess, as well as zinc and magnesium deficiency, have been associated with reduced Se availability. The effect of copper excess on Se status may be a result of alterations in liver function (11). Furthermore, copper deficiency has been associated with a decrease in the activity of hepatic glutathione peroxidase in rats (13). At normal dietary concentrations, copper, cadmium, iron, molybdenum, and manganese did not affect Se metabolism in another study in rats (14). In cattle, the interactions between heavy metals (arsenic, cadmium, mercury, lead), minerals, and trace elements, including Se, have been studied (15). The animals (tissue samples from 120 animals collected at time of slaughter) in the study by Lopez et al. (15) had mineral and trace element concentrations within the reference range and low tissue concentrations of heavy metals. Results relevant to Se included a positive correlation between the tissue concentrations of calcium and Se in the kidneys and tissue Se and copper concentrations in the liver.

Interestingly, for plants, Se in the form of inorganic selenate is most bioavailable to the plant, due to selenate’s resemblance to sulfate. Using the sulfate assimilation pathway, selenate is taken up from the soil through the roots of the plant and transported to the leaves where it is reduced to selenide which is then used for synthesis of selenocysteine. Selenocysteine can further be metabolized to selenomethionine and both are incorporated into plant proteins, thereby providing an organic source of Se for animals ingesting plant material (16, 17).
Antioxidant defense

Selenium, together with vitamin E, plays an important role in the defense against oxidation-induced damage of cellular components. The metabolic process of oxidation of lipids, carbohydrates, and proteins results in the production of carbon dioxide, water and energy. During this process, reactive oxygen species are formed. These powerful oxidizing agents can damage living cells, especially their proteins and lipids. Unsaturated fatty acids, the major component of all cell membranes, are particularly susceptible to oxidation-induced damage (18).

Specific antioxidant protective mechanisms have evolved to prevent damage from oxidation. These mechanisms are often described by the general terms “antioxidant system” or “antioxidant defense”, which includes fat-soluble antioxidants (e.g. vitamins A, E, carotenoids, ubiquinones), water-soluble antioxidants (e.g. ascorbic acid, uric acid, taurine), and antioxidant enzymes (glutathione peroxidase, catalase, superoxide dismutase).

The antioxidant system of the living cell includes three “levels of defense”. The first level is the prevention of free radical formation through the removal of free radical precursors or inactivation of catalysts. The antioxidant enzymes glutathione peroxidase, catalase, and superoxide dismutase are involved in this aspect of antioxidant defense. The next step includes the prevention and restriction of chain formation and propagation, which is facilitated by the vitamins A, E, C, carotenoids, ubiquinols, glutathione, and uric acid. Lastly, excision and repair of damaged parts of molecules is attempted with the help of lipases, peptidases, proteases, transferases, and DNA-repair enzymes (19).

Antioxidant-prooxidant balance in the cell is an important determinant of many physiological functions. Oxidative stress results when reactive oxygen species are in
excess of antioxidant defense mechanisms. This can be a consequence of an excess of reactive oxygen species, a reduction in antioxidants, or both. Damage due to oxidative stress is considered a major component of the normal aging process, as well as several disease processes, including neoplasia, heart disease, trauma, and burns (19, 20).

Glutathione peroxidases are found in all mammalian tissues in which oxidative processes occur. At least six Se-dependent glutathione peroxidases have been described: cytosolic (GPx1), gastrointestinal (GPx2), extracellular (plasma) (GPx3), phospholipid hydroperoxide (GPx4), epididymal (GPx5), and non-Se (GPx6). These enzymes provide an extensive system of antioxidant activity at both intracellular and extracellular sites, as well as in the cell membrane itself (4, 6, 21, 22). Glutathione peroxidase-6 has only recently been identified and seems to be expressed only during development and in the olfactory epithelium. This enzyme contains Se in humans, but not in mice (10).

Glutathione peroxidases are antioxidant enzymes that metabolize lipid hydroperoxides and hydrogen peroxide at their respective site of actions: GPx1 and GPx2 in the cytosol and GPx4 in the cell organelles. During this process, reduced glutathione donates an electron resulting in reduction of hydroperoxides, using glutathione peroxidase as a catalyst. Glutathione peroxidases are further involved in maintenance of the cellular redox state, eicosanoid metabolism, and cell-signaling mechanisms through their influence on reactive oxygen species concentrations within the cell. Participation of glutathione peroxidase enzymes in the regulation of biosynthesis of leukotrienes, thromboxanes, and prostaglandins has been associated with the modulation of inflammatory reactions (23, 24). Additionally, glutathione is important for the recycling of vitamin E. During this process, oxidized vitamin E can be converted back into the
active reduced form by redox reaction with other antioxidants (glutathione, ascorbic acid, ubiquinols, carotenoids) (19, 22).

Thioredoxin reductase is another widely prevalent Se-containing antioxidant enzyme. It participates in the regulation of the redox status of the cell and is involved in vitamin E recycling. Known functions of thioredoxin reductase include antioxidant defense, redox regulation, gene regulation, modulation of protein phosphorylation, regulation of apoptosis, DNA synthesis and repair, involvement in hormone action and cytokine function, protein biosynthesis, and recycling of vitamin C (4, 25, 26).

**Thyroid hormone metabolism**

Thyroid hormones are of vital importance for a number of normal metabolic functions, including growth and development. Selenium is an essential component of thyroid hormone metabolism, playing a role in synthesis and activation of thyroid hormones. The iodothyronine deiodinase isoenzymes (types I, II, III) are selenoenzymes, which link Se biology to thyroid hormone metabolism, through their role in activation and termination of thyroid hormone signaling. Types I and II are responsible for the deiodination of biologically inactive thyroxine (T4) to the biologically active triiodothyronine (T3) whereas inactivation of T4 and T3 by 5'-deiodination is mainly a result of the activity of the type III enzyme (10, 27).

The role of Se in thyroid hormone metabolism has recently been reviewed by Schomburg and Köhrle (28). An interesting finding in healthy humans with mild Se deficiency was that it does not affect measurable thyroid hormone concentrations. Instead, mild Se deficiency seemed to result in decreased activity of the Se-dependent
deiodinase isoenzymes in Se-deficient peripheral tissues which reduced turnover of thyroid hormones. This is achieved by prolonging the biological half-lives of thyroid hormones and enhancing the enterohepatic recycling of iodothyronines and their conjugates. The result is an iodine sparing effect, which relieves the pressure on the thyroid gland to produce more hormones. More severe and chronically reduced Se deficiency states, however, can affect deiodinase isoenzyme activity in people (28).

The relationship between Se status and thyroid hormone concentrations has been researched in domestic animals. One study in cows grazing low-Se pasture revealed low serum T3 concentrations with no effect on serum T4 concentrations, compared to cows supplemented with barium selenate (1 mg Se/kg subcutaneously) (29). In another study, Se supplementation of either selenite or Se yeast (60 ppm in salt mix offered free choice) increased the concentration of T3 and the ratio of T3:T4 in plasma of cows compared to cows with free choice access to 20 ppm selenite in salt mix (30). In lambs with myopathy as a result of Se deficiency, serum T3 concentrations and T3:T4 ratio were significantly decreased when compared to healthy lambs (31, 32). A recent study in adult horses, however, found no effect of Se source (inorganic vs. organic) or dose on plasma T3 or T4 concentrations or the T3:T4 ratio, compared to an unsupplemented control group (33). The role of Se supplementation on thyroid hormone metabolism in domestic animals requires further study.
Other selenoproteins
To date, at least 25 selenoproteins have been identified in humans; however, their functions have not been established in all cases. A few examples of selenoproteins with known functions are given in this section, and in Table 1.1.

Selenoprotein W is present in muscle, spleen, testis, and brain and is involved in skeletal and cardiac muscle metabolism. Similar to other selenoproteins, the selenoprotein W concentration is dependent on the amount of dietary Se available and there appear to be species-specific differences in the structure and amino acid composition. Selenoprotein W plays a role in antioxidant defense and is up-regulated in muscle cells in response to exogenous oxidants; however, its resistance to oxidative stress is dependent on availability of glutathione (4, 8, 34). Selenoprotein W has been identified in the developing nervous system, skeletal muscle, and heart in mice (35). Roles for selenoprotein W in muscle growth and differentiation, protecting the developing myoblasts from oxidative stress, were suggested by the authors.

Selenoprotein P is a plasma selenoprotein, like extracellular glutathione peroxidase (GPx3). Plasma concentration can be used as a marker of Se nutritional status because of its close relationship to Se intake. This selenoprotein is mainly synthesized by the liver, but can be also synthesized and secreted by various other tissues. Selenoprotein P plays a role in Se homeostasis as well as antioxidant defense. It is important for Se transport and distribution from the liver to peripheral tissues. The brain is another site of selenoprotein P biosynthesis and it has been suggested that it is a site of extracellular Se storage in the brain as well as providing Se to neurons for selenoprotein synthesis (36-40). Selenoprotein P binds to endothelial cells and has been shown to prevent oxidative damage of human endothelial cells (41).
Selenoprotein R (methionine sulfoxide reductase B) is involved in antioxidant defense through prevention and repair of protein oxidation (4).

Selenoprotein K has only recently been identified as an antioxidant in cardiomyocytes, protecting them from oxidative stress-induced toxicity (42).

**Selenium and vitamin E interactions**

As part of antioxidant defense, selenoproteins function together with other antioxidants, particularly vitamin E. Vitamin E, a fat-soluble vitamin, functions as a free radical scavenger and is the main chain breaking antioxidant in the cell. During the reaction of vitamin E with a peroxyl radical, hydroperoxides are produced, which are then converted to non-reactive compounds by Se-containing glutathione peroxidase. Glutathione, together with vitamin C, is involved in the recycling of vitamin E to its active reduced form (19). Therefore, a depletion of reduced glutathione as a result of increased glutathione peroxidase activity, during which glutathione is oxidized, has an effect on vitamin E metabolism. Even though Se and vitamin E function together in antioxidant defense, reduced cytosolic glutathione peroxidase activity cannot be compensated for with an increase in dietary vitamin E leading researchers to suspect a specialized role of the enzyme in the regulation of tissue peroxide levels (22).

**Selenium research related to human health**

Selenium deficiency syndromes in humans generally have a geographical distribution which reflects the concentration of Se in the soil. Severe Se deficiency in people is associated with cardiomyopathy whereas moderate deficiency generally results in less
severe myodegenerative syndromes accompanied by muscular weakness. Selenium deficiency in the human population is associated with two specific diseases: Keshan disease (cardiomyopathy of children and adolescents; China) and Kaschin-Beck disease (osteoarthropathy; Tibet, China, Siberia, North Korea) (43).

In recent years, the influence of Se supplementation on certain metabolic functions and disease processes as well as disease prevention has been extensively studied. Selenium deficiency has been linked to thyroid gland disease (myxedematous cretinism), and both Se deficiency and toxicity have been linked to endocrine disturbances (44). Selenium supplementation in patients with autoimmune thyroiditis can modify the immune response, which is assumed to be due to an increase in plasma glutathione peroxidase activity resulting in decreasing levels of hydrogen peroxide (45). In several species, Se accumulates in the pituitary gland and both excess and deficiency of Se can lead to impaired growth (44). One study demonstrated a marked decrease in glutathione peroxidase activity in an adrenal cell line associated with decreased steroid hormone production as a consequence of Se deficiency (46). Several studies have investigated the effect of Se on pancreatic β-cell function. Results from a recent study indicate that Se may contribute to an improvement of islet function (47). Specifically, the study by Campbell et al. (47) showed that Se up-regulated \(lpf1\) (insulin promoter factor 1) gene expression and increased insulin mRNA in a glucose-responsive mouse beta-cell line. Selenium stimulated increases in both insulin content and insulin secretion in isolated rat islets of Langerhans. Since Se is a component of several antioxidant enzymes, the effect of Se supplementation on different inflammatory processes is another research focus of
interest. According to one study, there is evidence that high doses of Se may be useful as a therapeutic agent in cases of inflammatory bowel disease (48).

A close relationship between Se nutrition and cardiovascular disorders has been demonstrated, focusing on disorders in which oxidative stress or inflammation are implicated. A recent study showed that Se supplementation can reduce the expression of intracellular adhesion molecule 1 (ICAM-1) in the vascular endothelium that contributes to the development of atherosclerotic lesions (49). Selenium supplementation may increase antioxidant protection in aged individuals, particularly those at risk of ischemic heart disease or those undergoing clinical procedures involving transient periods of myocardial hypoxia (50, 51). Regarding the association between Se status and cardiovascular disorders, the effect of Se supplementation on lipid metabolism has been evaluated (52); Se supplementation led to up-regulation of low density lipoprotein receptor activity and mRNA expression during hypercholesterolemia.

There is strong evidence that supranutritional dietary levels of Se can help to prevent the development of some types of cancer, including cancer of the colon, prostrate, breast, ovary, lung, and hematopoetic cancers (54). Selenium appears to influence different stages of carcinogenesis; it can inhibit the initiation phase induced by various carcinogens, inhibit cell growth and induce tumor cell apoptosis (53-56). However, the positive effect of Se supplementation on cancer prevention appears to be dose-dependent. High concentrations of Se can be cytotoxic and have been shown to cause DNA damage, particularly DNA strand breaks and base damage (54).

The brain is a privileged organ with respect to Se metabolism: Se is preferentially retained in brain tissue during periods of Se deficiency (57). Selenium may attenuate or
prevent secondary pathologic changes occurring after oxidative brain damage, traumatic brain injury, and spinal cord injury (57-60). Selenium, among other micronutrients, also plays an important role in brain development (61).

The effect of Se deficiency and supplementation on viral infectious diseases has been investigated and there is evidence that the host nutritional status may influence the pathogenicity and mutation rate of viruses (62-64). Selenium deficiency of the host can increase viral pathogenicity and result in specific viral mutations, making relatively benign viruses more virulent. Selenium supplementation has resulted in decreased viral load and increased CD4 T-lymphocyte count in HIV-infected patients (65). Conversely, an increase in reactive oxygen species was demonstrated to enhance HIV replication in T-lymphocytes and monocytes (66-68). The specific mechanisms responsible for disease severity and virus mutations in Se-deficient individuals are currently unknown. A recent study, which investigated the in vitro effects of Se deficiency on West Nile Virus replication and cytopathogenicity, found an increase in virus-induced apoptosis. The authors concluded that an adequate Se status may be important for the protection of West Nile Virus infected cells against virus-induced cell death (69).

**Selenium research related to animal health**

The majority of Se-related research in animals to date has been focusing on the effect of Se supplementation on health and production in domestic food animal species (cattle, pigs, and poultry). There are several equine studies looking at the antioxidant status and effects of antioxidant supplementation on health and performance (70-73), however, only a few studies specifically evaluated the effects of Se supplementation and dietary Se
source on equine health. Selenium-responsive diseases as well as Se-related research in horses are reviewed separately in this chapter.

In food animals, health and productivity have been associated with Se status in numerous studies. Selenium deficiency in cows has been associated with an increased risk of retained placenta, delayed conception, abortion, and perinatal mortality (74). Muscular degeneration of skeletal and cardiac muscle in calves and lambs, known as white muscle disease (WMD), is the most specific manifestation of Se and vitamin E deficiency. The association of WMD with vitamin E status was initially established in lambs in 1945 (75). In 1958, selenium was first reported to be effective in preventing WMD in lambs (76).

Many studies have investigated the relationship between Se status, milk somatic cell count, and mastitis in cows. Selenium status has been shown to influence the activity and expression of glutathione peroxidase and thioredoxin reductase in bovine mammary tissue (77). Vitamin E and Se status may affect udder health through influencing leukocyte function (78, 79). In a recent report from South America, Se supplementation of deficient cows decreased milk somatic cell count (80), confirming earlier work from Ohio (81, 82).

The effect of maternal Se status on the Se status of offspring has been another research focus. It appears that prenatal as well as postnatal Se supplementation is essential for antioxidant function of the offspring and that both trans-placental Se transfer as well as transfer through colostrum and milk can influence the Se status of the fetus and neonate. Calves from cows supplemented with Se have greater whole blood, plasma, and liver Se concentrations than calves from cows receiving no Se (83, 84). In pigs, a low-Se
diet fed during gestation led to an increase in fetal oxidative stress, measured as fetal liver hydrogen peroxide and malondialdehyde (MDA), both indicators of lipid peroxidation (85). Selenium intake during gestation can affect levels of mRNA encoding for glutathione peroxidase-1 during porcine fetal development (86). The finding that selenomethionine influenced the expression of genes involved with DNA damage, oxidative stress, and detoxification in the intestine of the mouse (87) led to a study in chickens where it was found that maternal supplementation with vitamins and trace minerals, including Se, influenced intestinal development of the offspring through similar alterations of gene expression in the intestine (88). The study in mice indicated that low Se status up-regulated genes associated with DNA damage and oxidative stress and down-regulated the expression of genes involved in detoxification (87).

Colostrum and milk Se concentration appear to depend on the maternal Se status; however, there is evidence for differences in effectiveness between inorganic and organic dietary Se sources for increasing milk Se concentration. In cows and pigs, at similar Se intakes, supplementation with organic Se led to higher Se concentration in milk compared to supplementation with inorganic Se (83, 84, 89-91).

**Selenium and the Immune Response**

Selenium deficiency has been shown to alter both innate and adaptive immunity, including neutrophil and macrophage function, lymphocyte function, and antibody production. The role of Se deficiency in inflammation, infection, allergy, cancer, and ageing is currently an active area of research, and immunological processes are an important aspect of the pathophysiology of these conditions (3, 92).
As previously discussed, Se deficiency may affect thyroid function, and impairment of thyroid function can negatively influence immune function. This may include decreased neutrophil function and reduced development and function of thymic cells, as the thymus contains the selenoprotein-containing enzyme type 2 deiodinase (92).

**Neutrophil function**
The effect of Se on neutrophil function has been investigated in both humans and animals. Neutrophils are part of the innate immune response, during which they produce superoxide-derived free radicals in a process known as respiratory (or oxidative) burst. Through this mechanism, neutrophils participate in host defense against invading microorganisms (93). In cases of Se adequacy, glutathione peroxidase activity in neutrophils helps to protect the cell from the free radicals they produce. During Se deficiency, however, if cytosolic glutathione peroxidase (GPx1) activity is reduced, the free radicals produced during oxidative burst can be directed against the neutrophils. Both the phagocytic and microbicidal ability of neutrophils have been studied in relation to Se status of the host and have been reviewed in mice (92) and domestic animals (94). Studies with neutrophils from goats have suggested that Se deficiency may lead to a decrease in phagocytic activity as well as production of and response to chemotactic factors (95, 96), both of which play important roles in recruiting immune cells to the site of infection. The decrease in neutrophil chemotaxis in Se deficiency may be partly due to a decrease in leukotriene B4 production, according to one study in goats (97).

The effect of Se status on neutrophil function has been extensively studied in cattle with special emphasis on dairy cows. Sequelae of impaired immune responses can
frequently be observed in periparturient dairy cows where the high metabolic demands of late pregnancy, parturition, and onset of lactation are believed to contribute to an increase in production of reactive oxygen species (98). One study in postparturient Holstein cows supplemented with Se showed that animals with higher blood Se concentrations (>300 ng/ml) had greater neutrophil function, measured as neutrophil adhesion to nylon fibers and superoxide production (99).

Macrophage function
Like neutrophils, macrophages produce reactive oxygen species. These cells play an important role in the immune response as antigen-presenting cells, presenting antigen to T-lymphocytes. Macrophages are essential effector cells in inflammatory reactions during which they ingest and kill invading microorganisms (93).

Recent research has shown that Se decreases expression of the pro-inflammatory genes cyclooxygenase (COX) and tumor necrosis factor alpha (TNFα) in macrophages and therefore has been proposed as an important anti-inflammatory agent. These anti-inflammatory effects of Se demonstrated by Vunta et al. (100) may be due to an increase in 15-deoxy-\(\Delta^{12,14}\)-prostaglandin \(J_2\), a metabolite of prostaglandin \(D_2\) (101).

Lymphocyte function and antibody production
The effect of Se status on lymphocyte function has been studied in humans and several domestic animal species including dogs, pigs, and cattle. Lymphocyte function has mainly been studied by investigating \textit{in vitro} blastogenesis of lymphocytes after mitogen stimulation. Selenium deficiency leads to a decrease in lymphocyte proliferation in
response to mitogens (102-106). A study in mice showed that different dietary sources of Se can increase the blastogenesis of T-lymphocytes after stimulation with ConA; however, this effect was only observed at lower doses of Se (107). Ueno et al. have further investigated the mechanism as to how Se influences lymphocyte blastogenesis in mice (108). Their results showed an increase in thioredoxin reductase and nuclear factor kappa B (NF-κB)-DNA binding activity after supplementation with nutritional levels of sodium selenite in wild-type mice when compared to human thioredoxin-1-transgenic mice. Based on their findings, Ueno et al. (108) proposed that T-lymphocyte blastogenesis in response to supplementation with sodium selenite results from an increased thioredoxin reductase activity causing a reduction in thioredoxin-1, which, in turn, has been associated with alterations of NF-κB activity. Studies investigating the effects of Se and vitamin E deficiency and Se and vitamin E supplementation on lymphocyte function in different animal species have been reviewed by Finch and Turner (94).

Research concerning the relationship between Se supplementation and human immune cell function suggested an increased expression of the interleukin-2 receptor in T-lymphocytes following Se supplementation (105). The production of interleukin-2 by lymphocytes, however, did not appear to be related to Se status (109, 110). In goats, Se deficiency resulted in decreased production of leukocyte migration inhibitory factor by lymphocytes. Based on these findings, the ability of lymphocytes to modulate neutrophil migration may be impaired in states of Se deficiency (109).

More recently, lymphocyte dysfunction during Se deficiency has been associated with altered formation of eicosanoids and signal transduction during arachidonic acid
metabolism in rat lymphocytes (24). Based on results from the study by Cao et al. (24), Se status can influence the lipoxygenase and cyclooxygenase pathways of arachidonic acid metabolism, as well as the activation of phospholipase D. The authors suggested that lymphocyte dysfunction may be a consequence of reduced production of cyclooxygenase pathway metabolites that may contribute to diminished phospholipase D activation in Se-deficient rats.

To investigate the molecular mechanism connecting Se status and T-lymphocyte immunity, researchers recently generated mutant mice with selenoprotein-deficient T-lymphocytes (111). These mice had decreased pools of mature T-lymphocytes and a defect in T-lymphocyte-dependent antibody responses. The study demonstrated that selenoprotein deficiency in T-lymphocytes led to an overproduction of oxidants in these cells and thereby suppressed T-lymphocyte proliferation in response to T-lymphocyte receptor stimulation.

In a study of healthy humans, increased expression of lymphocyte protein biosynthesis genes could be observed after Se supplementation (112). The authors hypothesized that the up-regulation of ribosomal protein and translation factor genes in response to increased Se intake was linked to increased selenoprotein production and enhanced lymphocyte function. Cytokine profiles of lymphocytes from Se-deficient mice were compared to those from Se-adequate mice after infection with *Cryptosporidium parvum* (113). Lymphocytes from Se-adequate mice produced higher levels of IFNγ, IL-2, and IL-4 throughout the course of infection when compared to lymphocytes from Se-deficient mice.
Besides the cellular immune response, Se status can affect humoral immune function. The effect of dietary Se on immunoglobulin production has been investigated in humans, horses, cattle, sheep, and pigs (106, 114-117); generally speaking, Se-supplemented individuals show an increased production of immunoglobulins following antigenic stimulation compared to Se-deficient individuals. An in vitro study of bovine B-lymphocyte function demonstrated an increase in mitogen-induced immunoglobulin M synthesis after Se supplementation (118). In contrast, Reis et al. (119) found that supplementation of Se-deficient cattle (12 months old) with different doses of Se, or no Se, failed to affect rabies antibody titers after vaccination, but did affect the persistence of titers. Only animals receiving 3-6 mg Se daily had antibody titers considered protective against rabies for the entire study period of 120 days.

**Inflammation, infection, allergy, and aging**

Selenium can attenuate pro-inflammatory gene expression in macrophages of mice, suggesting that Se may have an anti-inflammatory effect in those cells (100). This study in mice demonstrated a significant decrease in LPS-induced expression of two pro-inflammatory genes, cyclooxygenase-2 (COX-2) and tumor necrosis factor-alpha (TNFα) via the inhibition of mitogen-activated protein (MAP) kinase pathways. The relationship between Se status and COX-2 expression has been previously investigated (120). The expression of COX-2 is regulated by the transcription factor NF-κB, which is involved in the induction of many inflammatory responses. This transcription factor can be induced by pro-inflammatory stimuli such as LPS, TNFα, and ovalbumin. Research has shown
that Se status can modulate the activity of NF-κB in murine monocytes and macrophages (120-122).

Selenoprotein S has been shown to be anti-apoptotic and to reduce stress in the endoplasmic reticulum in macrophages and astrocytes. Selenoprotein S polymorphism has been associated with increased plasma levels of inflammatory cytokines (TNFα and IL-1β) in people. This appears to be a consequence of decreased expression of selenoprotein S, which in turn increases stress to the endoplasmic reticulum resulting in NF-κB induction and inflammatory cytokine expression (8).

Besides its role in inflammatory processes, the role of Se in several infectious diseases has been studied. There is mounting evidence that the nutritional status can influence the immune response of an individual during infectious disease and that nutritionally deficient humans and animals are more susceptible to infection, probably due to an impaired host immune response as a result of a deficient diet (123-129). As previously discussed, Se deficiency can result in impairment of different aspects of the immune response. It is, however, not only the immune response that can be affected by Se deficiency; the pathogenicity of invading organisms themselves may be altered based on Se status. This phenomenon has been investigated in regards to viral pathogenicity (63, 64, 130).

Keshan disease may have a viral component to its etiology (131, 132). A coxsackievirus has been isolated from blood and tissue samples, including cardiac tissue, from Keshan disease patients. Investigations in mice have revealed that an amyocardic strain of coxsackievirus can be converted to virulence after inoculation into Se-deficient mice (130). The conversion was accompanied by changes in the genetic structure of the
virus and the genome of the altered virus closely resembled that of other known virulent strains. Similar changes of virulence and genomic composition could be observed in glutathione peroxidase knockout mice fed Se-containing diets (130). Based on these findings the authors proposed that the antioxidant function of glutathione peroxidase contributes to the protection against coxsackievirus-induced myocarditis.

Selenium deficiency has been shown to alter the immune response, increase the severity of lung pathology (130), and decreased the activity of glutathione peroxidase-1 and thioredoxin reductase in the lung (133) during influenza infection. These studies indicate that reactive oxygen and nitrogen species play a role in the influenza-induced lung pathology. Therefore, it has been suggested that the decreased expression of these antioxidant enzymes in Se-deficient individuals may result in more severe pulmonary changes. A more recent study indicated, however, that there was a threshold of glutathione peroxidase-1 activity that could prevent an increase in lung pathology (134). In this study there was no difference in the severity of lung pathology between normal mice and transgenic mice that had decreased levels of selenoproteins, even though the selenoprotein-deficient mice had decreased activities of glutathione peroxidase-1 and thioredoxin reductase in their lungs. An in vitro study using differentiated human bronchial epithelial cells found a difference in immune response and cell morphology when comparing Se-adequate and Se-deficient states (135). Results from this study indicated an increase in IL-6 production and influenza virus-induced apoptosis in Se-deficient cells. Contradicting results in another study in mice found no viral genome mutations during influenza virus infection of Se-adequate and Se-deficient mice (136). The study further showed an altered immune response in Se-deficient mice with a
difference in cytokine expression. Interestingly, the altered immune response in the Se-
deficient group appeared to be beneficial for protecting the mice from mortality as a result of influenza virus infection.

One recent study investigating the effect of Se on porcine circovirus type 2 replication \textit{in vitro}, found that selenomethionine supplementation resulted in inhibition of virus replication (137). Less is known about the relationship between Se status of the host and immunity to nonviral pathogens and research data suggests that it differs depending on the microorganism involved (3).

The host antioxidant status has been extensively studied in regards to immune response during allergic diseases such as asthma, with contradicting results. As an antioxidant, Se plays a role in reducing oxidative stress in the lower airways during asthma, most likely through up-regulation of glutathione peroxidase (138), but it also appears to have an enhancing effect on the immune response, mainly T helper 2 response, that is part of the pathophysiology of asthma (139, 140). In one study, antioxidant supplementation of asthmatic patients resulted in an increase in disease severity with a positive correlation between antioxidant (vitamin C and Se) levels and allergen responses to house dust mites (139). A study in mice found that Se intake and airway inflammation did not appear to interact in a dose-dependent manner, but that glutathione peroxidase-1 and selenoprotein P were up-regulated within the lung after antigen challenge (141).

Aging is accompanied by chronic inflammation and oxidative stress, and has been associated with impairment of immune function. During aging an imbalance develops between prooxidants and antioxidants with a shift towards prooxidants. The relationship between host antioxidant status and immune function during aging has been examined in
several studies. Based on research in prematurely aging mice, supplementation with adequate levels of antioxidants may help to improve leukocyte function and restore redox balance (142). These findings are supported by research in people which indicated that supplementation of Se and other antioxidants improved innate as well as acquired immune function (3).

Selenium and the neonatal immune system
As in adults, Se status can influence the immune response of neonates. A study of maternal Se nutrition and neonatal immune system development in rats found that thymocytes from neonates receiving low-Se milk showed impaired activation in vitro. Further, the percentages of CD8 cytotoxic T-lymphocytes, CD2 T-lymphocytes, B-lymphocytes, and natural killer cells were all decreased in neonates nursed by mothers receiving a diet low in Se (143).

The relationship between Se status and immunity has to date only been investigated in one equine study; this study found greater influenza antibody titers in foals from mares supplemented with Se at 3 mg/d compared to mares receiving 1 mg Se/d (144). Selenium supplementation of the same mares was associated with a higher concentration of serum IgG in foals at two weeks of age (145).

Selenium supplementation of pregnant cows and ewes led to higher colostral immunoglobulin concentrations when compared to Se-deficient animals (146, 147). In a recent study, Se fortification of colostrum led to an increase in serum IgG of newborn calves (148); however, higher concentrations of Se in colostrum (5 ppm) appeared to inhibit IgG absorption. Research in lambs demonstrated similar dose-dependent effects
with an increase in lymphocyte proliferation in response to mitogens at lower doses of Se whereas high doses of Se led to a decrease in mitogen response (149). In another study, lambs with low glutathione peroxidase activity and signs of WMD showed decreased lymphocyte blastogenesis in response to mitogen stimulation (150).

Functional differences between reactivity of the neonatal and adult immune system must be considered when measuring the neonatal immune response. In human neonates, a decreased bone marrow reserve, reduced adherence and chemotaxis, and lower enzymatic activity and signaling of neonatal neutrophils have been described (151). Further limitations occur in regards to monocytes and macrophages, the complement system, natural killer cells, and lymphocyte-activated cell cytotoxicity. Generally, neonatal T-lymphocytes are immunocompetent, but their differentiation appears to be biased towards a T helper 2 profile (151, 152). There are several theories as to why this may be the case, including a preferential T helper 2 cytokine milieu during pregnancy resulting in limited induction of a T helper 1 response in early life, initial low exposure to pathogens and microbial stimulation, and/or suboptimal antigen presenting cell-T-lymphocyte interactions (153).

In horses, the difference in immune response between neonatal foals and adult horses is under investigation. In one study, newborn foals showed decreased expression of the interferon gamma gene and interferon gamma protein (154). This deficiency was observed in lymphocytes obtained from the peripheral blood as well as from the lung. Interferon gamma gene expression and protein production increased steadily throughout the first six months of life, reaching adult levels within the first year of life. The authors concluded that their findings suggest that foals are born with a decreased ability to mount
a T helper 1-based cell-mediated immune response. These age-related differences may help to explain the apparent increased susceptibility of foals to intracellular pathogens such as *Rhodococcus equi*, a bacterial organism that does not cause disease in healthy adults. Flaminio et al. (155) investigated different aspects of the immune response of foals and demonstrated age-dependent changes in immunoglobulin class concentrations and lymphocyte subsets. Mitogenic responses of lymphocytes, phagocytosis, and oxidative burst activity of neutrophils from newborn foals were similar to those of adult horses. The authors concluded that the total number of immune cells, not their function, was the cause for the difference in the immune response of equine neonates compared to adult horses.

The influence of age on equine neutrophil function has been studied with the consensus that foal neutrophil function appears to be affected by age as well. Peripheral blood neutrophils have a decreased phagocytic ability and show down-regulation of oxidative burst activity (156-158). The decrease in phagocytic activity appeared to be present for the first one to three weeks of life (158, 159).

### Selenium Requirements and Supplementation of Livestock

**Assessment of selenium status**

Measurements of the Se status of an individual include determination of Se concentration in plasma/serum and whole blood, as well as calculation of red blood cell Se:

\[
\text{Red blood cell Se} = \text{whole blood Se} - \left(1 - \text{hematocrit}\right) \times \text{plasma Se}\] / hematocrit,

as previously described (160). Long-term and short-term effects of Se supplementation can be measured by determination of either whole blood (red blood cell) or plasma/serum
Se, respectively (161). Waldner et al. (161) compared three methods of Se status assessment in cattle. Their results showed that serum (and plasma) Se concentration was highly correlated with the rate of Se administration and responded quickly to changes in Se intake whereas Se in whole blood responded more slowly to alterations in Se availability.

Most of the measurable Se in blood is present in red blood cells where it is incorporated during erythropoiesis. Therefore, a period equal to the average life span of red blood cells (approximately 90 days [cattle]-120 days [horses]) is required for the red blood cell Se concentration to reach a new plateau following any change in daily Se intake. Consequently, whole blood concentrations of Se reflect the efficacy of long-term Se intake, whereas, plasma/serum Se measurements reflect immediate or short-term responses to changes in Se intake.

Another method to assess Se status is indirectly through measurement of blood glutathione peroxidase activity. As mentioned previously, glutathione peroxidase is a selenoprotein containing enzyme and its activity is dependent on the Se status of the individual (161, 162). Being an enzyme assay, it is highly susceptible to assay conditions and techniques resulting in considerable within- and between-laboratory variation. Similarly, selenoprotein P, a plasma selenoprotein like extracellular glutathione peroxidase (GPx3), can also be used as a marker of Se status because Se deficiency leads to a decrease in the selenoprotein P concentration. Selenoprotein P is used to assess Se status in people, but is not used routinely for the assessment of Se status in livestock.
Selenium requirements
Reference ranges for equine serum/plasma and whole blood Se concentrations, and glutathione peroxidase activity, have been published (163). The cut-off values for these reference ranges have been based primarily on values associated with clinical signs of muscular degeneration, for determination of “deficiency”, and on the distribution of values from normal populations, for the determination of “adequacy”. A marginal range is sometimes used for values falling between these. In the adult horse, serum Se concentrations of 0.14 parts per million (ppm) or less are considered to be below normal (marginal), and signs of deficiency (WMD) can occur at 0.053 ppm or less, with foals being more susceptible than adult horses. The published reference range for plasma Se concentrations in neonatal foals is 0.06 to 0.1 ppm (163, 164). A gradual increase of serum Se with age can be observed and values for serum Se of cattle, horses, swine, and sheep of different ages have been published by Stowe and Herdt (164). These authors reported “expected” or “normal” values for adult horses, similar to those reported by Puls (163, 164). In horses, Shellow et al. found that plasma Se reached a plateau of 0.12 to 0.14 ppm and 0.13 to 0.14 ppm after sodium selenite supplementation delivering 0.1 and 0.2 ppm of Se, respectively (165).

To prevent Se deficiency of livestock in deficient areas, Se supplementation is common practice. The Food and Drug Administration (FDA) has limited Se supplementation in food-producing animals to 0.3 ppm (mg/kg) of diet dry matter in complete feeds (166). For Se supplementation of equine feeds, no regulations exist, but general guidelines are provided by the National Research Council (NRC) (167). The Se requirement for mature idle horses was estimated by the NRC to be 0.1 ppm of diet dry matter; however, it has been suggested that 0.3 ppm would be a more appropriate
recommendation for exercising horses (12). Calamari et al. evaluated different doses of Se yeast (0.2, 0.3, and 0.4 mg/kg dry matter) in their study in adult horses and found a linear dose effect for blood Se concentration; all three doses resulted in plasma Se concentrations within the “adequate” range (33).

Current reference ranges for Se status and suggested Se requirements in horses are tentative at best, as they are based on limited amounts of data. The challenge in determining reliable reference ranges in horses is that the effects of sub-clinical Se deficiency are difficult to identify. Prevention of clinical myodegeneration, the basis for the current definition of a “deficient” state, is not our only (or perhaps our most serious) concern. Considerations should also be given to levels of Se required which will ensure optimal antioxidant defense, thyroid function, and immunity, amongst others. Since the optimal blood Se concentrations associated with protection of horse populations from sub-clinical Se-responsive disorders have yet to be established, the optimal Se requirements have yet to be determined for this species. This situation differs from cattle and sheep, where large, repeated, multi-site production-response trials have been performed using liveweight gain, fleece growth or milk production as outcomes; as a result, a solid basis exists for defining reference ranges in these species (168).

Keeping these considerations in mind, we decided to use the reference range supplied by Puls (163) because it is a well-researched synthesis of various recommendations published in the literature, and is the most widely cited source of equine Se reference ranges. The Se supplementation at 0.3 ppm of ration dry matter, as used in our trials, was chosen because it is consistent with upper-end values suggested by other researchers studying Se metabolism in horses (12, 33, 144) and by researchers studying Se
requirements of cattle who have recommended at least 0.3 ppm as a minimum feed concentration for promotion of optimal resistance to mastitis (169).

Selenium availability
Herbivores obtain Se from plants which take it up from the soil. The amount of Se incorporated into the plant is influenced by the soil Se concentration as well as the amount of precipitation and the soil pH. Selenium is taken up by plants more readily in geographical areas with arid climate and alkaline soil; such conditions are typical of the mid-western regions of North America. The coastal regions of North America, including Eastern Canada, and the areas around the Great Lakes have lower soil Se concentrations as they tend to have relatively acidic soils and higher rainfall. Under acidic soil conditions, Se more readily forms complexes with iron (iron hydroxides) which have low solubility, making it less available to plants; furthermore, as a consequence of higher rainfall, Se tends to leach from the soil (18, 170, 171).

Plants either accumulate Se in direct relationship to the soil Se concentration or they accumulate Se in much higher amounts (Se-accumulating plants) (172-174). Selenium-accumulating plants can be found in the arid mid-west of North America and include two-grooved milkvetch [Astragalus bisulcatus], prince’s plume [Stamleya pinnata], and woody asters [Xylorhiza glabriuscula]. Selenium metabolism differs between Se-accumulators and non-accumulators with Se-accumulators mainly synthesizing non-protein amino acids such as methylselenocysteine and selenocystathionine while the predominant form of Se in non-accumulators is selenomethionine (17, 174).
In the soil, selenate is most bioavailable to the plant and transported more rapidly from the roots to the leaves than selenite or selenomethionine. Selenite, on the other hand, is quickly converted to organic forms of Se, such as selenomethionine, which are mainly retained in the roots. Due to the chemical resemblance of Se and sulfur, selenate uptake is mediated by sulfate transporters in the roots of the plants, using an active transport mechanism within the plasma membrane of root epidermal cells. Organic forms of Se are taken up actively whereas selenite accumulates in plant roots through passive diffusion (16, 17). Once it reaches the leaves, selenate is taken up by the chloroplasts and is metabolized by enzymes utilized in sulfate assimilation. During this process, selenate is reduced to selenide, which is then used for synthesis of selenocysteine. Selenocysteine can be further metabolized to selenomethionine and both are incorporated into proteins through nonspecific substitution of selenocysteine and selenomethionine with cysteine and methionine, respectively (16).

Since selenate and sulfate utilize the same metabolic pathway in plants, sulfate can interfere with Se assimilation of the plant. In non-accumulating plants the presence of sulfate inhibits selenate uptake whereas Se-accumulators take up selenate preferentially over sulfate (16, 17).

**Selenium supplementation**
Selenium can be added to concentrate feed, using either an inorganic or organic form, or offered by means of a Se-containing trace mineral block. Available inorganic dietary Se sources include sodium selenite and sodium selenate. A study, which compared the bioavailability of selenite and selenate in animals, found that they were equally effective
in increasing serum Se concentration and glutathione peroxidase activity (175). Organic Se is commonly supplemented in the diet as Se-fortified yeast. The predominant form of Se in Se yeast is selenomethionine (10, 176, 177).

Studies in pigs demonstrated that supplementation with Se, using inorganic and organic dietary sources, led to differences in Se retention, tissue Se concentration, serum Se concentration and glutathione peroxidase activity, as well as colostrum and milk Se concentration, with organic Se being more effective in improving measures of Se status overall (178-180). Researchers studying Se nutrition in cattle have shown that organic Se is more effective than inorganic Se in increasing the concentration of Se in colostrum, milk, whole blood and serum (90, 181, 182). To date, research in horses comparing dietary sources of Se is sparse. One study reported a tendency for greater plasma Se concentrations in horses receiving organic Se compared to horses receiving inorganic Se, but the difference was not statistically significant (183). Recently, another study found that Se yeast was more effective than sodium selenite in increasing total Se in blood; no differences, however, were found for glutathione peroxidase-1 activity (33).

**Fortification of plants with selenium**

Selenium-fortification of food crops for human consumption is common practice in some areas with Se-deficient soils, including Finland and the United Kingdom, where Se-containing fertilizer is used to increase the Se content of plants for human consumption (173, 184). Selenium has been added to fertilizer to increase the Se concentration of forage offered to cattle and sheep (185, 186); however, this method is not widely used as a means of Se supplementation. An earlier study in dairy cattle demonstrated a slight
increase in blood Se and a significant increase in milk Se after feeding hay from selenized soil (185). The same study found that the Se-fortified hay was superior to sodium selenite in raising the Se levels in whole blood and organs of fattening bulls and sheep.

A study performed on Prince Edward Island (PEI) in 1989 and 1990 investigated the use of Se fertilizer on beef cow/calf farms and its effects on calves from birth to weaning (186). Results indicated that Se fertilizer was a successful method of Se supplementation, leading to higher pasture and animal blood Se levels on treated farms when compared to control farms; however, no significant differences could be observed in average daily gain or adjusted weaning weights on treated compared to untreated farms. For practical reasons, the treatment allocations were not performed at the animal level, but instead at the farm level; because of this, between-farm differences may have obscured the relationships examined in this study.

In a more recent study presented as an abstract, Se-enriched fertilizer was spread on pasture, barley and maize fields (187). Cows grazing the fortified pasture and fed silage made from the fortified grass and maize, as well as fortified winter barley, had increased blood Se concentrations when compared to animals in the control group. Only one study has been published concerning the use of Se-enriched fertilizer on feedstuffs for horses (188). The fertilizer used in the study by Hornick et al. contained Se in the form of selenate, and was applied at a rate of 4g Se/ha on winter barley and spelt fields as well as 3g Se/ha on fields intended for hay production (188). These researchers found it to be an effective method for increasing the Se content of barley, straw, and hay; however, the time between fertilizer application and harvest was not specified. Hornick et al. found an
increase in plasma Se in horses receiving Se-fortified feedstuffs when compared to unsupplemented controls; however all horses had adequate plasma Se levels at the beginning of the study.

**Selenium-related Research in Horses**

**Selenium-responsive diseases in horses**
As a result of its association with Se deficiency, cases of equine WMD can be seen in geographic areas with low soil Se concentrations. The association between Se status and the occurrence of WMD was first shown in lambs in 1958 (76). White muscle disease can affect skeletal and cardiac muscle in foals, with foals from birth to eleven months of age being most susceptible to disease. Involvement of myocardium, diaphragm and respiratory muscles can result in heart failure, dyspnea, and pulmonary edema. White muscle disease can be acute, with cardiovascular collapse and death within hours or days, or subacute with profound muscular weakness, stiffness, lethargy, and dysphagia - often one of the early clinical signs. Most affected foals have markedly elevated creatine phosphokinase and aspartate aminotransferase enzyme activities. Histologically, extensive, floccular, granular, and severe hyaline degeneration of myofibers occurs during the first few days of the disease. After about a week, should the foal survive, phagocytosis of necrotic tissue occurs with endomysial thickening caused by edema, mononuclear cell infiltration, and proliferation of fibroblasts. Early regenerating fibers are found to coexist with signs of myodegeneration. Foals may respond to treatment with Se and vitamin E, but generally the prognosis is guarded to poor; as a consequence, prevention through supplementation is preferred (189, 190).
Other manifestations of selenium deficiency

Selenium deficiency of adult horses does not typically present with overt clinical signs. Generalized muscular degeneration is rare; however, focal myonecrosis has been described (191). As previously discussed, Se deficiency has hypothesized links to several sub-clinical disorders, through its roles in antioxidant defense, thyroid metabolism, and immune function. Research in these areas is currently lacking in horses.

Selenium toxicity

In areas with arid, alkaline soil, as is commonly found in the North American mid-west, Se can accumulate in various plants (alfalfa, Asteraceae, Castilleja, Atriplex) to toxic levels. In these areas, signs of Se toxicity, including hair loss and lesions around the coronary bands, have been observed in horses (164). Selenium indicator plants (prince’s plume, two-grooved milk vetch, woody aster) may be helpful to recognize areas with high soil-Se. Furthermore, Se toxicosis can result from over-supplementation of Se administered through feed or via injection; it can be peracute or chronic, based on the dose, form and frequency of Se administered. As mentioned previously, organic Se is more bioavailable to mammals, increasing the risk of toxicity if errors in dosing occur. Fortification of non-Se-accumulating plants through application of Se-containing fertilizer should decrease the risk of Se toxicity based on the plant metabolism as described earlier in this chapter. Acute Se poisoning can result from doses of 25 to 50 ppm (192, 193). The relevance of considering the risk of Se toxicity was recently brought to attention when several polo ponies in Florida succumbed to sequelae of acute Se poisoning.
toxicosis after intravenous administration of a compounded mineral supplement containing Se at toxic levels (194).

The mechanism of acute Se toxicity is unknown and may involve direct effects of organoselenium compounds on cell metabolism. During chronic Se poisoning Se is replaced or interacts with sulfur in sulfur-containing structural amino acids needed for cross-linking of glycoproteins and polypeptides. Consequently, disulfide bonds are substituted with selenosulfides. Due to the antagonizing effect of Se on sulfur in the sulfur-containing amino acids methionine and cysteine, high doses of Se can affect cell division and growth. Clinical signs of acute Se toxicosis include weakness, dyspnea, abdominal pain with diarrhea, shock, and death from respiratory failure. Chronic Se toxicosis (alkali disease) may present as ill-thrift, anemia, stiffness, lameness, loss of mane and tail, and deformation and sloughing of hooves. Hooves and hair are predominantly affected because they contain high concentrations of cysteine and methionine (192, 193).

To avoid toxicosis, Se in feed should not exceed 1-2 ppm of dry weight. Chronic Se toxicosis can be observed in livestock if the Se concentration in the total diet exceeds 5 ppm. No specific treatment is available for chronic selenosis. Removal from the source is essential and feeding of a protein-rich diet high in the sulfur-containing amino acids methionine and cysteine has been proposed to be protective (192, 193).

The toxicity of Se depends on its chemical form (195), with selenite and selenocysteine being more toxic than selenate and selenomethionine. The toxicity can further be affected by arsenic, cadmium, copper, lead, mercury, silver, and zinc.
Selenium can form complexes with other metals, which generally reduces its toxicity (195).

**Selenium status and supplementation**

Early equine studies identified that blood Se concentration correlates well with glutathione peroxidase activity, as in other domestic species, and therefore both blood concentrations of Se and glutathione peroxidase enzyme activity can be useful for the assessment of Se status of horses (196, 197). One study showed that plasma and whole blood Se of unsupplemented horses increased linearly for the first five to six weeks, respectively, after supplementation with sodium selenite in all groups (0.05 ppm, 0.1 ppm, 0.2 ppm) (165). For the remainder of the 12 week feeding trial, Shellow et al. (165) found no further increase or significant changes in plasma and blood Se. Whole blood Se concentrations were measured in a study of 346 horses in Virginia and Maryland (198). Factors significantly affecting blood Se status included feeding of supplements, origin of hay, and amount of time spent on pasture. Crisman et al. (198) found that horses receiving feed supplements had significantly higher blood Se when compared to horses not receiving supplements. Furthermore, feeding commercially-grown hay resulted in higher blood Se than feeding of home-grown hay. Finally, horses that spent less than 50% of their time on pasture had a higher blood Se concentration than horses that spent more than 50% of their time on pasture. Crisman et al. found that pleasure horses had significantly lower blood Se than horses used for breeding or working/eventing (198). In New York, horses receiving commercial feeds similarly had higher whole blood Se levels.
compared to horses eating locally produced feeds (196). That study by Maylin et al., was a survey of horses on four breeding farms.

The Se status of horses not receiving Se supplementation is largely dependent on the geographical area in which the horses live. Areas with low soil-Se have a low Se concentration in forage and grains grown on these soils. This is particularly the case in most coastal areas of North America, including PEI, Canada (199). Results from a recent study indicate that out of 201 horses tested for Se status on PEI, 79 percent had inadequate Se concentrations (deficient or marginally deficient) (200; unpublished observations). Aged and young adult pleasure horses had the highest prevalence of low Se concentrations, whereas broodmares and racehorses had significantly higher blood Se levels than the pleasure horse groups, probably because of feeding practices, as previously suggested by Maylin (196).

In Se-deficient areas, Se can be provided to horses through dietary supplementation or parenterally. A study of horses in New Zealand showed that a single injection of barium selenate (0.5 mg Se/kg) was effective in maintaining the whole blood Se of horses in the adequate range for the study period of one year (201). The effects of dietary supplementation with Se and other antioxidants have been studied in exercising horses (70, 72). Most studies, however, used a “cocktail” of different antioxidants (such as vitamins E and C, β-carotene, copper, zinc, Se), which makes it difficult to assess the effects of the individual antioxidant supplied. One study looking at just the effect of Se and vitamin E in exercising horses found a significant increase in measures of antioxidant defense after supplementation with these antioxidants when compared to measures in the same horses prior to supplementation (202). In California, a study in endurance horses
(n=56), a population group predisposed to an imbalance between oxidants and antioxidants as a result of extended periods of aerobic exercise, found that Se deficiency amongst the horses tested was uncommon (2%) (203). Furthermore, the authors found no association of blood Se concentration with either improved or decreased performance based on ride time and completion of the ride. In the Study by Haggett et al. (203), the horses that received Se supplementation (23%) did, however, have significantly higher blood Se concentrations when compared to unsupplemented horses in the study. Haggett et al. (203) also found an increase in blood Se shortly after endurance exercise. Increase in blood glutathione peroxidase activity in endurance horses after exercise has been demonstrated previously (204), however, the reason for this finding remains unclear; relative polycythemia associated with exercise has been proposed as an explanation for this finding, but this remains unproven.

Research in cattle and pigs indicated that organic Se was more effective than inorganic Se to improve the Se status in these species. Only a few studies have thus far compared the effect of organic and inorganic Se on the Se status of horses. One study reported a tendency for greater plasma Se concentrations in horses receiving organic Se compared to horses receiving inorganic Se, but the difference was not statistically significant (183). Recently, another study found that Se yeast was more effective than sodium selenite in increasing total Se in blood, but this difference was not observed for glutathione peroxidase-1 activity (33). The same study investigated the effect of Se source and dose on plasma T3 and T4 as well as T3:T4 ratio, but found no effect of Se supplementation on these measures of thyroid metabolism. Research in Thoroughbreds demonstrated that organic Se had greater bioavailability compared to inorganic Se.
leading to a greater positive Se balance in the horses studied (12); with the greatest
difference in measures of Se status between horses receiving inorganic or organic Se
observed after exercise. The authors concluded, based on the results of their study, that
the Se requirements of exercised horses may be dependent on the source of Se as well as
the exercise frequency.

To date, there are only a few studies looking at the Se status and Se supplementation
of mares and their foals, even though foals are most susceptible to problems associated
with Se deficiency such as WMD. One study found that foals born to Se-adequate mares
had higher blood Se concentrations when compared to foals born to mares with low Se
status (205). The same study found that the Se concentration of milk was one fourth of
the Se concentration of colostrum one day post partum. The authors concluded from this
finding that milk appears to be only a minor source of Se. Results from another study
indicated that parenteral Se supplementation during gestation and lactation as well as
parenteral Se supplementation of foals, beginning at birth, helped to prevent WMD (196).
A study by Breedveld et al. studied serum copper and zinc concentrations of mares and
foals after supplementation of mares with copper and zinc at different dietary
concentrations (206). The concentrate offered to all mares also contained 0.062 ppm of
Se. Plasma and milk Se was also measured; results showed that mare and foal plasma Se
was highest at parturition and colostrum Se was higher than milk Se. Mare and foal
plasma Se remained below the adequate range during the entire study by Breedveld et al.
(206), based on published reference ranges (163, 164).

On PEI, many broodmares have blood Se concentrations below the level considered
adequate (200). The majority of Se-deficient foals in that study were born to mares that
had either deficient or marginal blood Se concentrations. The prevalence of inadequate serum Se was considerably higher in foals than in their dams (96% versus 72%).

Only one study has investigated the effect of Se source on the Se status of mares and their foals to date (144). Results indicated that serum Se concentrations were greater in mares receiving organic Se when compared to mares receiving inorganic Se, and foals from mares receiving organic Se had higher serum Se concentrations as well. Colostrum and milk Se were also higher in mares receiving the organic Se supplement (144).

**Selenium status and immunity**
The effect of Se on the humoral immune response has been investigated in two equine studies, both using an inorganic Se supplement. One study showed an increase in humoral immune function in horses supplemented with either vitamin E or vitamin E and Se compared to unsupplemented horses or horses receiving just Se (115). Another study in ponies found a higher concentration of immunoglobulins after antigen challenge in ponies supplemented with Se compared to unsupplemented ponies (117).

Baalsrud and Ovemes (115) studied 15 horses which were divided into 4 groups receiving either no supplementation (n=4), 600mg vitamin E/d (n=4), 5mg sodium selenite/d (n=3), or vitamin E and sodium selenite (n=4). The animals were studied for 24 weeks. Twelve weeks into the feeding trial, the horses were vaccinated against tetanus and influenza; they received a booster six weeks later. The Se and vitamin E concentrations at the beginning of the feeding trial were not specified. Results showed a significantly higher humoral immune response to influenza equi A 1 antigen in horses receiving Se and vitamin E (measured as haemagglutination inhibition titer). There was
no difference in immunoglobulin G (IgG) levels between any of the groups. One confounding factor in this study may have been that the same horses were used for the production of hyperimmune sera against enteropathogenic Escherichia coli. For this purpose the animals received live Escherichia coli culture intravenously every two weeks which may have affected their overall immune response and made them a less than ideal study population.

Knight and Tynik (117) studied 15 ponies receiving either a low (0.02 ppm; n=7) or high (0.22 ppm; n=8) Se diet containing sodium selenite for seven weeks. All ponies were previously depleted of Se and shown to be deficient at the beginning of the feeding trial based on plasma Se and glutathione peroxidase activity. To test the humoral immune response, each pony received packed sheep red blood cells intramuscularly at week 0 and week 2 of the study. Immune response was measured as serum IgG concentration and hemagglutination titers to sheep red blood cells. Analysis of results obtained showed significantly higher IgG concentrations as well as hemagglutination titers in Se-supplemented ponies.

In foals, higher influenza antibody titers were found when foals were born to mares supplemented with Se at 3mg per day when compared to foals born to mares receiving 1mg of Se daily (144). Both inorganic and organic Se were used and compared at the dose of 3mg per day in the study by Janicki et al. (144), but no difference between the two dietary Se sources was found. The same researchers found that that Se source did not affect colostrum IgG concentration or foal serum IgG at 12 hours (results for IgG concentrations not reported); however, Se supplementation of mares with either an
inorganic or organic source at a rate of 3 mg of Se per day was associated with a higher concentration of serum IgG in foals at two weeks of age (145).
Overview of Research Presented in this Thesis

The goals of our research were to provide practical information on the effects of Se supplementation, various modes of supplementation, and the form of supplementary Se (organic vs. inorganic) on the Se status of horses, and to examine the effects of dietary Se source on measures of innate and adaptive immunity. Three separate studies were performed, examining different methods of Se supplementation (mineral supplement, Se-containing trace mineral block, Se-fortified forage), different dietary sources of Se (inorganic, organic), and different groups of horses (adult non-pregnant and non-lactating, broodmares, and foals).

In the first study (Chapter 2), the effects of Se supplementation and source on measures of Se status and immune function in adult horses were investigated. The working hypotheses for this study were as follows: supplementary Se leads to higher blood Se concentrations and GPx activity and to changes in measures of immune response, when compared to no supplementation; and that supplementation of horses with an organic form of Se affects measures of Se status and immune response differently when compared to horses receiving inorganic supplementary Se, with organic Se being superior to inorganic Se in enhancing the immune response.

In the second study (Chapter 3), Se-enriched forage was examined as a potential source of organic Se for horses. This method of Se supplementation was compared to a more conventional method of Se supplementation of horses (Se-containing trace mineral block), that is commonly used in pleasure horses which may not receive concentrate feed on a regular basis. The specific hypothesis to be tested was that the Se status of Se-deficient adult horses can be enhanced when they are fed Se-treated forage, as compared
to horses receiving inorganic supplementary Se in the form of a Se-containing trace mineral block, or no supplementary Se.

The third study (Chapter 4), a field study, was designed to investigate the effects of dietary Se source (inorganic vs. organic) on broodmares and their foals, including blood Se status of mares and their foals, colostrum and milk Se concentrations, and measures of immune function of their foals. This study builds on the results obtained in Chapter 2, in which changes in immune function in adult horses were observed in response to Se supplementation, with the nature of these changes depending on the form of Se fed. We wished to extend this research to mares and foals, a group considered at high risk for becoming Se deficient. The hypotheses were, firstly, that supplementation with organic Se increases blood, colostrum, and milk Se concentration of mares as well as blood Se concentration in their foals to a greater degree than supplementation with inorganic Se. Secondly, we hypothesized that organic Se alters measures of innate and adaptive immunity in a way that differs from inorganic Se, with organic Se being superior in enhancing the immune response.

A summary of the results from the three studies as well as common conclusions and problems encountered are discussed in the final chapter (Chapter 5). Implications of this research for horse owners, veterinarians and nutritional advisors are provided. Areas of further research directions are discussed.
Table 1.1 Selenoproteins with known functions (4, 207)

<table>
<thead>
<tr>
<th>Selenoprotein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione Peroxidase (GPx); GPx1-6</td>
<td>Antioxidant enzyme, detoxification of lipid hydroperoxides and hydrogen peroxide</td>
</tr>
<tr>
<td>Thioredoxin reductase; Type I, II, III</td>
<td>Antioxidant enzyme, regulation of cell redox status, vitamin E &amp; C recycling</td>
</tr>
<tr>
<td>Iodothyronine deiodinase; type I, II, III</td>
<td>Thyroid hormone metabolism: type I &amp; II – deiodination of T4 to T3; type III – inactivation of T4 &amp; T3</td>
</tr>
<tr>
<td>Selenoprotein W</td>
<td>Skeletal and cardiac muscle metabolism; muscle growth and differentiation; antioxidant defense</td>
</tr>
<tr>
<td>Selenoprotein P10 and P12</td>
<td>Selenium homeostasis and antioxidant defense; selenium transport and distribution from the liver (P10) to peripheral tissues; P12 may be responsible for selenium transport in the brain</td>
</tr>
<tr>
<td>Selenophosphate synthase-2 (SPS)</td>
<td>Selenocysteine biosynthesis and incorporation into selenoproteins</td>
</tr>
<tr>
<td>15-kDa selenoprotein</td>
<td>Role not clearly defined, may be responsible for beneficial effect of selenium in certain human cancers; role in cell apoptosis</td>
</tr>
<tr>
<td>Mitochondrial capsular selenoprotein (MCS)</td>
<td>Different name for phospholipid GPx</td>
</tr>
<tr>
<td>Selenoprotein N</td>
<td>Suggested role in cell proliferation or regeneration; high expression in human fetuses, low expression in adults; gene mutation associated with congenital muscular dystrophy in humans</td>
</tr>
<tr>
<td>Selenoprotein R</td>
<td>Antioxidant defense, reduction of oxidized methionine residues in damaged proteins</td>
</tr>
<tr>
<td>Selenoprotein S</td>
<td>Regulation of cell redox balance</td>
</tr>
<tr>
<td>Selenoprotein K</td>
<td>Regulation of cell redox balance in cardiomyocytes</td>
</tr>
</tbody>
</table>

46
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Abstract
Selenium (Se) status influences both the innate and adaptive immune response, but the mechanisms are not well understood. Further, the effects of Se supplementation and Se source (inorganic vs. organic) on measures of immune function of horses have not been extensively studied. The purpose of this research was to examine the effects of Se supplementation and source on aspects of innate and adaptive immune function in adult horses. Fifteen adult Standardbred horses were assigned to one of three dietary groups: control group (Se-deficient diet); inorganic Se group (sodium selenite supplement); organic Se group (Se yeast supplement). Over the four-month experimental feeding period, the following assays of immune function were performed: lymphocyte proliferation in response to the mitogen concanavalin A, neutrophil phagocytosis, antibody production in response to rabies vaccination, and relative gene expression of cytokines in stimulated lymphocytes (IFNγ, IL-2, IL-5, IL-10, TNFα) and neutrophils (IL-1, IL-6, IL-8, IL-12, TNFα). Plasma and red blood cell Se concentrations, and blood glutathione peroxidase activity, were also measured. Plasma and red blood cell Se concentrations were highest in horses in the organic Se group when compared to horses in the inorganic Se group or control group. Organic Se supplementation increased the stimulation index for expression of IL-5 in lymphocytes, when compared to inorganic Se supplementation or no Se supplementation. Selenium supplementation also increased relative gene expression of IL-1 and IL-8 in neutrophils. Other measures of immune function were not affected by Se supplementation or source of dietary Se. In summary,
dietary Se content and source appear to influence immune function in horses and these effects may be mediated in part by alterations in lymphocyte expression of IL-5, and neutrophil expression of IL-1 and IL-8.
Introduction
Selenium is an essential micronutrient that has diverse roles in mammalian metabolism, but is best known as a component of antioxidant enzymes such as glutathione peroxidase and thioredoxin reductase (1, 2). Selenoproteins act in concert with other antioxidants such as vitamin E to prevent oxidative damage to cell membranes and intracellular structures. The classical example of a Se and vitamin E-responsive disease in large animals is white muscle disease (WMD), known also as nutritional muscular dystrophy, which mainly affects neonates and young animals (3-6). Selenium deficiency of adult horses does not usually present with generalized muscular degeneration, however, localized myonecrosis has been described (7).

In addition to its role in antioxidant defense, Se has been associated with changes in immune function in humans and many animal species; thus it appears to play a role in inflammatory processes and susceptibility to infectious disease. Selenium deficiency has been shown to alter both innate and adaptive immunity, including neutrophil and macrophage function, lymphocyte function, and antibody production in humans and animals (8-12). A recent study showed that lymphocytes from mice with an adequate Se status produced higher levels of interferon gamma (IFNγ), interleukin 2 (IL-2), and IL-4 throughout the course of infection with Cryptosporidium parvum when compared to Se-deficient mice (13). The effect of Se supplementation on the humoral immune response has been investigated in two equine studies (8, 9). Both studies found an increase in the humoral immune response after supplementation with Se compared to unsupplemented controls.
Forages and grains grown in Eastern Canada and many other parts of North America contain insufficient amounts of Se to meet the dietary requirements of livestock (14-16). Animals receiving feedstuffs grown in Se-deficient areas require Se supplementation. For Se supplementation of equine feeds, no regulation exists; however, guidelines are provided by the National Research Council (NRC) (17). The Se requirement for mature idle horses was estimated by the NRC to be 0.1 ppm of diet dry matter, but it has been suggested that 0.3 ppm would be a more appropriate recommendation for exercising horses (18). Selenium requirements for optimal immune function are currently unknown and may also be higher than present NRC recommendations (17), but a feeding regimen has not been established to date.

Selenium can be supplemented using either inorganic or organic forms. Available inorganic compounds include sodium selenite and sodium selenate. These compounds appear to be equally bioavailable and capable of increasing and maintaining serum Se concentration and glutathione peroxidase activity (19). Organic Se can be added to the diet as Se yeast. The predominant form of Se in Se yeast is selenomethionine (1, 20, 21). Several studies in domestic animal species have examined differences in the effect of inorganic versus organic Se supplementation. Bovine and porcine studies have demonstrated organic Se to be overall more effective in increasing common measures of Se adequacy (further referred to in this text as “Se status”), specifically plasma/serum Se concentration and whole blood Se concentration when compared to inorganic Se (22-24). One study reported a tendency for greater plasma Se concentrations in horses receiving organic Se compared to horses receiving inorganic Se, but did not find this difference to be statistically significant (25). Recently, another study found that Se yeast was more
effective than sodium selenite at increasing total Se in blood, but it did not modify glutathione peroxidase-1 activity (26).

To date, the effects of Se supplementation and Se source on immune function have not been extensively studied in horses. Therefore, it was the purpose of this study to examine the effects of inorganic and organic Se on aspects of innate and adaptive immune function in adult horses, including lymphocyte proliferation in response to the mitogen concanavalin A, neutrophil phagocytosis, antibody production in response to rabies vaccination, and relative cytokine gene expression of lymphocytes and neutrophils. The effects of Se supplementation and source on measures of Se status in blood were also investigated.

Materials and Methods

Experimental animals and study design

Fifteen adult, non-pregnant, non-lactating Standardbred horses (13 mares and two geldings) were used in this study. The experimental animals had been kept as a herd for several years, with minimal contact with other horses. All horses were vaccinated annually against tetanus, influenza, and equine herpes viruses 1 and 4. The use of animals in this study was approved by the Animal Care Committee of the University of Prince Edward Island.

Selenium supplementation was withheld for three months prior to beginning the feeding trial. During this period the horses were turned out onto pasture, determined to be Se-deficient (< 0.05 ppm), with continuous access to non-Se-containing trace mineral blocks (Sifto® Canadian Stockman, Mississauga, ON).
Prior to the beginning of the experimental period, horses were randomly assigned to one of three experimental groups, with five horses per group, and were housed in individual stalls. All horses were fed a basal diet of timothy hay and locally-grown whole oats. Pasture samples, core samples of the hay, and composite samples of the oats were analyzed for Se content; analyses were performed using fluorimetric determination of Se (28) (Appendix A). The Se concentrations in the hay and oats were 0.047 and 0.05 ppm DM, respectively. Amounts fed to horses were based on liveweight calculated to meet NRC maintenance requirements for energy (27) (Appendix B). Continuous access to a non-Se-containing trace mineral block (Sifto® Canadian Stockman, Mississauga, ON) was provided during the study period. Dietary treatments were assigned as follows: control group: Se-deficient diet; inorganic Se group: sodium selenite supplement; organic Se group: Se yeast supplement. A small amount of barley was used as a carrier to deliver the respective Se supplements. The control group received an equal amount of barley without the addition of a Se supplement. Selenium was added to deliver 0.3 ppm (mg/kg) Se on a dry matter basis in the total ration.

Blood samples were obtained from the jugular vein of each horse using EDTA and heparinized vacuum tubes, prior to onset of treatments, and one, two, three, and four months after beginning of treatments. Plasma and whole blood Se concentrations were determined at all of these time points (EDTA vacuum tubes). Plasma and whole blood samples were frozen and maintained at -20°C until analyses could be performed. Whole blood glutathione peroxidase activity was also determined at all time points (heparinized vacuum tubes). After collection, the blood was transferred into several aliquots of 2ml Eppendorf® microfuge tubes and frozen at -20°C until analysis. For lymphocyte and
neutrophil isolation, additional heparinized blood samples were collected from each horse (two 7ml tubes per horse) before beginning the feeding trial, one, and three months into the experiment. Rabies titers for each horse were measured on three occasions (serum vacuum tubes): prior to initial rabies (IMrab®, Merial, Baie d’Urfé, QC, Canada) vaccination (one month after the beginning of the feeding trial), prior to booster vaccination (eight weeks later), and at the end of the trial (one month after booster). Serum harvested from blood was frozen and maintained at -80°C until analyses could be performed. Records for the experimental horses indicated that they had not been vaccinated for rabies since they had entered the herd (at least two years in all cases; for most horses, several years).

Blood selenium concentrations and blood glutathione peroxidase activity
Plasma and whole blood Se concentrations were measured using atomic absorption spectrophotometry (29; Appendix A) and erythrocyte Se concentration was estimated using the following formula after sub-sampling for estimation of hematocrit:

\[
\text{Red blood cell Se} = \text{whole blood Se} - \frac{[(1 - \text{hematocrit}) \times \text{plasma Se}]}{\text{hematocrit}},
\]

as previously described (30).

Glutathione peroxidase activity in blood was measured using a commercial assay (Ransel® by Randox laboratories, Mississauga, ON, Canada) based on the method by Paglia and Valentine (31). Specifically, this is a UV method which measures the decrease in absorbance of light at 340 nm when glutathione is oxidized by cumene hydroperoxide catalyzed by glutathione peroxidase. Results were reported in U/g hemoglobin (Hb). All
measurements were performed at the Atlantic Veterinary College at the University of Prince Edward Island.

**Lymphocyte blastogenesis in response to the mitogen Concanavalin A (ConA)**

Preparations for the lymphocyte blastogenesis were started immediately after the blood was collected. During the first step, the blood was mixed with an equal volume of RPMI-1640 (Sigma, Oakville, ON, Canada). The blood-RPMI mixture was layered onto Ficoll-Paque Plus (Amersham Biosciences, Baie d'Urfé, QC, Canada) and centrifuged at 400g for 30 minutes at 4°C to separate the lymphocyte fraction from the whole blood. After separation, the lymphocyte layer was harvested and the cells were washed twice with sterile saline (700g for 5 minutes) and the cell pellet was resuspended in 1ml of RPMI+ (RPMI-1640 with 100 Units/ml of Penicillin and 100 μg/ml of Streptomycin, 2 mM glutamine, and 10% heat inactivated fetal calf serum). The cells were counted and cell viability was evaluated using the trypan blue exclusion method (32).

The cell number was adjusted to 2x10⁶/ml and the cells were dispensed at 100 μl/well in a 96 well plate (BD Falcon, VWR, Mississauga, ON, Canada) to which an equal volume of ConA (Sigma, Oakville, ON, Canada) diluted in RPMI+ was added to give a final concentration of 5, 2, and 0 μg/ml of ConA. All samples were run in quadruplicate. Cellular proliferation in response to mitogen stimulation was determined using [³H]thymidine incorporation into cellular DNA. The cells were incubated at 37°C for 48 hours in a 5% CO₂ air humidified atmosphere, after which 1 μCi of [³H]thymidine (GE Healthcare, Baie d’Urfé, QC, Canada) was added to each well. The cells were incubated for 18 hours with the [³H]thymidine and then harvested onto glass fiber filters.
(Skatron Instruments Inc., Sterling, VA, USA) using a Skatron semi-automatic cell harvester (Sterling, VA, USA). Finally, the amount of radioactivity (counts per minute, CPM) incorporated into cellular DNA was detected with a Wallac Microbeta Trilux 11450 (Perkin Elmer, Woodbridge, ON, Canada) liquid scintillation counter. Stimulation indices (SI) were calculated using the following formula: the CPM of the stimulated cells divided by the CPM of the unstimulated cells. The optimal number of cells per well and concentration of ConA used was established in a pilot trial preceding this study (Appendix C).

**Neutrophil phagocytosis of fluorescent beads measured by flow cytometry**

Neutrophils were isolated from the red blood cell fraction after Ficoll-Paque separation of lymphocytes. First, the red blood cells were washed twice with saline at 700g for five minutes. This was followed by red blood cell lysis modified from Raidal et al. (33). To lyse the red blood cells, 19 ml of lysis solution (0.8% ammonium chloride, 0.08% sodium EDTA, 0.08% sodium carbonate) were added to 1 ml of blood at room temperature. After ten minutes, 19 ml of saline were added and the mixture was pipetted through a 50 micron nylon mesh to eliminate remaining cellular debris. This was then centrifuged at 700g for five minutes. The cell pellet was resuspended in 10 ml of lysis solution, after which 19 ml of saline were added. This mixture was again centrifuged at 700g for five minutes and the cell pellet was then washed twice with saline. Finally, the cell pellet was resuspended in 1 ml of saline. After isolating the neutrophils, the cells were counted and their viability was assessed using the trypan blue exclusion method (32).
To evaluate phagocytosis, neutrophils were incubated with 2µm polystyrene fluoresce (FITC) labeled beads (Polysciences, Warrington, PA, USA). Incubation lasted for 30 minutes at 37°C in the dark on a shaker. To differentiate beads that were attached to the outside of the cell from engulfed beads (evidence of phagocytosis activity), a negative control was included for each sample. This control was prepared on ice and 0.2% PBS-EDTA (0.2g EDTA in 100ml PBS) was added to inhibit phagocytosis. Fluorescence of each sample was measured using flow cytometry (FACSCalibur, Becton-Dickinson, San Jose, CA, USA) and the phagocytic activity was reported as the difference between the sample and the control (% gated cells). Before the study, a pilot trial was performed to assess different methods to inhibit phagocytosis (Appendix D).

Rabies antibody production in response to vaccination
The Canadian Food Inspection Agency’s Rabies Laboratory measured rabies antibody titers in serum using a competitive ELISA (34). In this test, both serum and a labeled monoclonal antibody (Mab) were applied to virus coated plates at the same time, and antibodies in the serum competed with the labeled Mab for antigen binding sites. The results were reported as percent (%) inhibition of binding of a rabies virus glycoprotein-specific peroxidase-labeled Mab to microtiter plates coated with ERA (Evelyn-Rokitnicki-Abelseth) variant rabies virus. An inhibition value of 20% or greater was considered positive for detection of glycoprotein-specific antibodies in the horse serum, indicative of seroconversion.
Relative cytokine gene expression measured by Real Time (RT)-PCR

Relative cytokine gene expression was measured in stimulated lymphocytes and neutrophils. Lymphocytes were suspended in 1 ml of RPMI+ at a concentration of 0.5x10^6/ml. An unstimulated sample was analyzed at baseline before the beginning of the feeding trial. A stimulated and an unstimulated sample for each animal were analyzed at one and three months after beginning the feeding trial. Following analysis, a stimulation index (SI) was calculated for each cytokine using the following formula:

\[ SI = \frac{\text{relative quantity (RQ) stimulated}}{\text{RQ unstimulated}}. \]

Lymphocytes were stimulated with 2 μg/ml of ConA in RPMI+ and an equal volume of RPMI+ was added to the unstimulated cells. All cells were incubated at 37°C for 24 hours in a 5% CO\textsubscript{2} air humidified atmosphere. Following incubation, the sample was centrifuged at 16,000g in a microfuge for three minutes, after which time the cell pellet was resuspended in RNA later (Sigma, Oakville, ON, Canada). The samples were frozen at -20°C and stored for further analysis.

Neutrophils from each animal were also suspended in 1 ml of RPMI+ at a concentration of 0.5x10^6/ml. The sampling time points and sample processing was identical to the procedure used for the lymphocytes except that the neutrophils were stimulated with lipopolysaccharide (LPS) at 50 ng/ml for four hours.

Gene expression assays were performed at the Gluck Equine Research Center at the University of Kentucky. All samples from an individual horse were analyzed in one run. During the first step of the analyses, the samples were thawed and the RNA was purified using the RNeasy® Micro Kit (Qiagen®, Valencia, CA, USA). This commercial kit is specially designed for the purification of RNA from small samples. A carrier RNA, supplied with the kit, was also used to increase the total yield of RNA.
Following RNA purification, reverse transcription reactions were performed as previously described (35) using 12μl of each RNA sample and reverse transcription master mix (Promega, Madison, WI, USA). The reactions were incubated at 42°C for 15 minutes and 95°C for 5 minutes. The cDNA was stored at -20°C until analyzed by RT-PCR.

Cytokine gene expression was measured by RT-PCR as described previously (36, 37), using equine specific intron-spanning primer/probe sets for IFNγ, IL-2, IL-5, IL-10, and tumor necrosis factor alpha (TNFα) for the lymphocyte samples and IL-1, IL-6, IL-8, IL-12, and TNFα for the neutrophil samples. The cytokines were chosen based on their importance in immunological processes involving the cell types of interest as well as availability; their major functions are summarized in Table 2.1.

Each reaction contained 4.5μl of cDNA and 5 μl TaqMan Gene Expression Master Mix (ABI) and 0.5 μl primer and probes. All reactions were incubated in duplicate wells at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds in an Applied Biosystems 7900 sequence detection system. Beta-glucuronidase (β-GUS) was used as the housekeeping gene for the lymphocyte samples and beta-2-microglobulin (B2M) was the housekeeping gene for the neutrophil samples (38). After recording the cycle threshold (C_T) for each lymphocyte cytokine, the ΔΔC_T method was used to calculate relative changes in cytokine gene expression (39), where ΔΔC_T = [(Avg. gene of interest C_T - Avg. β-GUS C_T)horse - (Avg. gene of interest C_T - Avg. β-GUS C_T)calibrator]. The same formula was applied to the neutrophil cytokine results, using the results for B2M instead of β-GUS. The relative changes in gene expression before (baseline sample, used for calibrator calculation) and after initiation of treatment were
measured for each gene of interest. The calibrator was calculated from the mean $\Delta C_T$ obtained from the baseline samples for each individual gene. Through this method, the baseline (pre-treatment) value was included in the calculation of the relative quantification (RQ) value for the one and three month samples. The use of a calibrator helps to reduce the effect of between horse variations. Results are expressed as RQ of each cytokine after stimulation calculated as $2^{-\Delta C_T}$, at one and three months after initiation of dietary treatment. Results are also expressed as the stimulation index (SI), calculated as RQ stimulated/RQ unstimulated for the same two time periods.

**Statistical analysis**
Statistical analysis was performed with commercial software (Intercooled Stata 9.2, StataCorp, College Station, TX), using repeated measures analysis of variance (ANOVA). Comparisons between means were made using the following pre-planned contrasts: supplemented versus unsupplemented, and organic versus inorganic. Values for neutrophil phagocytosis, lymphocyte blastogenesis, relative cytokine gene expression, and the stimulation indices for gene expression were log10- transformed before statistical analysis to normalize their distribution; where the time × treatment interaction was not significant, values are presented for the treatment main effect (i.e. the effect of dietary treatment at one and three months combined). Lymphocyte blastogenesis and gene expression medium controls were analyzed using ANOVA. Results are presented as means with standard error (SE), unless otherwise indicated.
Results
The mean (± SE) age (in years) of horses from each experimental group was as follows: control group, 9.4 (4.93); inorganic group, 12.4 (4.28); and organic group, 12.0 (5.24), with no significant differences in age between groups. The individual ages (in years) of the horses in the study were as follows: group 1: 6, 7, 7, 9, 18; group 2: 8, 8, 13, 16, 17; group 3: 6, 7, 14, 15, 18. No systemic illness was observed in any of the horses during the study period, except for a mild impaction colic in a horse in the organic Se group during the last month of the study. This horse was treated medically and the problem resolved within two days, and data for this horse were retained.

Blood selenium parameters and glutathione peroxidase activity
At the end of the experimental period, all measures of blood Se were significantly higher in horses receiving supplementary Se (inorganic and organic groups) when compared to horses in the control group (Figures 2.1 and 2.2). Further, horses in the organic Se group had significantly higher plasma and red blood cell Se concentrations when compared to horses in the inorganic Se group, with the greatest difference in red blood cell Se concentrations. The effects of time and time × group interaction were significant for each measure of Se status (p < 0.01). Mean (± SE) Se concentrations at the end of the study for the control, inorganic and organic groups were 0.030 (0.001), 0.144 (0.004) and 0.166 (0.008) ppm Se in plasma, and 0.085 (0.011), 0.299 (0.008) and 0.442 (0.020) ppm Se in red blood cells, respectively (all pairwise group comparisons significantly different, p < 0.05).

Mean glutathione peroxidase activity (± SE) at the end of the trial was higher in the horses in the two Se groups when combined and compared to horses in the control group.
(p <0.001), however there was no significant difference between the two Se-supplemented groups (Figure 2.3).

Lymphocyte blastogenesis
Lymphocyte blastogenesis in response to two concentrations of the mitogen Concanavalin A (ConA) was not significantly affected by Se supplementation or time. Stimulation indices (± SE) at study end, using 5 μg/ml of Con A were 42 (7.48), 38.2 (8.94), and 47.4 (21) for the control, inorganic and organic groups, respectively; stimulation indices at study end using 2 μg/ml of Con A, were 70.6 (13), 91.2 (26.6), and 79 (13.9) for the control, inorganic and organic groups, respectively. There was no significant difference between the groups for the results of the medium control (0 ConA). The complete data for lymphocyte blastogenesis is presented in Appendix E.

Neutrophil phagocytosis
Phagocytosis of fluorescent beads, measured using flow cytometry, was also not significantly affected by Se treatment or time. The complete data for phagocytosis is presented in Appendix F.

Rabies antibody production in response to vaccination
Rabies antibody production, measured by percent inhibition using a competitive ELISA, was not affected by Se supplementation. All but one horse had baseline values below 20% inhibition, indicative of immunologic naivety to rabies virus. One horse had a baseline value of 45% inhibition, indicative of previous immunization against rabies.
This horse was removed from the statistical analysis of rabies antibody production data.

Seroconversion (increase from < 20% to > 20% inhibition) occurred in all previously naïve animals after initial vaccination, and percent inhibition increased in all animals after the booster vaccination (Figure 2.4). Percent inhibition (SE) after initial vaccination was 61.2 (13.6), 35.25 (7.7), and 40.6 (5.6) for the control, inorganic and organic groups, respectively. Percent inhibition after booster vaccination was 86 (4), 87 (2.7), and 90.4 (2.7) for the control, inorganic and organic groups, respectively.

Cytokine gene expression measured by RT-PCR

Horses receiving organic Se had a higher mean SI for relative expression of IL-5 by lymphocytes when compared to horses receiving inorganic Se or no supplementary Se (p<0.05); there was no significant time by treatment interaction so the Se treatment main effect, based on the relative gene expression at one and three months, is presented (Figure 2.5). There was no significant difference between the groups when comparing the results of the media control in regards to IL-5 gene expression. Gene expression of the remaining lymphocyte cytokines (IFNγ, IL-2, IL-10, TNFα) was not significantly altered by Se supplementation. The complete set of means (± SE) of the relative cytokine gene expression of stimulated cells and the stimulation indices are reported in Appendix G.

Relative gene expression of the neutrophil cytokines was affected by Se supplementation, but the effect of Se source depended on the cytokine measured. Horses receiving Se, either organic or inorganic, had higher mean relative expression of IL-1 in neutrophils when compared to horses receiving no supplementary Se but the effect of source of Se was not significant (Figure 2.6). Horses receiving inorganic Se had
significantly higher mean relative expression of IL-8 in stimulated neutrophils when compared to horses receiving no supplementary Se, but mean expression for horses receiving organic Se was not significantly different from horses receiving either inorganic or no Se (Figure 2.7). For the remaining cytokines (IL-6, IL-12, TNFα) measured in the neutrophil samples a usable signal could also not be detected with RT-RCR.

Discussion
The addition of Se to the ration of experimental horses resulted in higher concentrations of Se in plasma and red blood cells, and higher activity of blood glutathione peroxidase. Further, adding Se yeast, an organic form, resulted in superior Se concentrations in plasma and red blood cells when compared to inorganic Se. These results are in general agreement with the findings of Calamari et al. (26), who found that Se yeast fed to horses was more effective than sodium selenite at increasing total Se concentration in the blood of horses, but was not more effective at increasing glutathione peroxidase-1 activity over a 16-week experimental period. Glutathione peroxidase did not reach a plateau in activity by the end of the present study (Figure 2.3). To compare the effect of Se source at steady-state, glutathione peroxidase activity in blood may need to be followed for more than four months following a change in Se intake, due to the time required to completely replace the circulating erythrocyte population.

While the availability of inorganic versus organic Se has not been extensively studied in horses, this area of research has received a great deal of attention in other domestic animal species. Mahan, et al. (22, 40, 41) demonstrated that supplementation of pigs with
either inorganic or organic Se, resulted in significant differences in not only Se retention, tissue Se concentration, and serum Se concentration, but in colostrum and milk Se concentrations as well, with organic Se supplementation being more effective overall. Bovine studies also have shown that organic Se is more effective than inorganic Se at raising the concentration of Se in colostrum, milk, whole blood and serum (23, 24, 42).

Selenoproteins are involved in many aspects of cell biochemistry and function. Through its incorporation into selenoproteins, Se is involved in antioxidant defense, redox regulation, and other cellular processes in many cell types, including cells of both the innate and adaptive immune systems (1, 11). Dietary Se appears to be important for an adequate immune response; however, the mechanisms by which Se influences the immune system are not fully understood. Selenium deficiency has been shown to affect both innate and adaptive immunity, including neutrophil and macrophage function, lymphocyte function, and antibody production (10, 43-45). The role of Se deficiency in inflammation, infection, allergy, cancer, and ageing is an active research area, and immunological processes are an important aspect of the pathophysiology of these conditions (46).

To date, there are only two published studies in adult horses and ponies investigating the relationship between Se status of the animal and immune response, both examining humoral responses. One study showed an increase in antibody response after vaccination against influenza in horses after supplementation with vitamin E and Se when compared to no supplementation or supplementation with Se alone (8). Another study in ponies found a higher concentration of immunoglobulins after antigen challenge with sheep red blood cells in ponies supplemented with Se compared to unsupplemented ponies (9).
Both studies compared the feeding of an inorganic Se supplement to unsupplemented controls.

In the present study, we examined the effects of Se supplementation and Se source on parameters of both innate and adaptive immunity in adult horses. Neutrophil phagocytic activity was not significantly different between the groups. Both the phagocytic and microbicidal ability of neutrophils have been previously studied in relation to Se status in other animal species (10, 11). Based on studies with neutrophils from goats Se deficiency can lead to a decrease in phagocytic activity as well as decreased production of and response to chemotactic factors (47, 48), which play an important role in recruiting immune cells to the site of infection.

The effect of Se status on lymphocyte function has mainly been studied by investigating in vitro blastogenesis of lymphocytes after mitogen stimulation. Studies in different species indicate that Se and vitamin E deficiency lead to a decrease in lymphocyte proliferation in response to some mitogens (phytohemagglutinin, ConA, pokeweed) (44, 49-51). In the present study, no effect of Se supplementation on lymphocyte proliferation could be observed. This may be due, in part, to the relatively small number of animals in each group, as this biological assay can be quite variable, both between and within animals, on different testing days. Other possibilities include the potential for a synergism of Se with vitamin E, as previous studies in pigs and dogs used a combination of Se and vitamin E (44, 49). Additionally, the incubation time of the lymphocytes with the mitogen or the choice of mitogen may play a role. Roy et al. (50) only found a significant difference in human lymphocyte blastogenesis after 72 hours of
incubation with phytohemagglutinin when comparing Se-deficient and Se-supplemented individuals, but not after 48 hours.

The effect of dietary Se on immunoglobulin production has also been investigated in people, horses, cattle, sheep, and pigs (8, 9, 51-53); generally, Se-supplemented individuals appear to have an increased production of specific immunoglobulins following antigenic stimulation compared to Se-deficient individuals. Results from a recent study in cattle indicated, however, that the effect of Se supplementation on antibody titers may be influenced by the amount of Se supplemented, and that excessive doses of Se may lead to immune suppression (54). In the present study, no difference was seen in the humoral immune response to rabies vaccination between the control and Se-supplemented groups. These findings are similar to findings in cattle by Reis et al. (54), which found that supplementation of Se-deficient 12 months old calves with different doses of Se, or no Se, did not affect their rabies antibody titers post vaccination (determined by rapid fluorescent focus inhibition test and fluorescent inhibition microtest). However, the amount of Se supplemented did affect the persistence of titers. Only calves receiving 3-6 mg Se daily had antibody titers considered to be protective against rabies for the entire study period of 120 days. As the horses in the present study received a booster vaccine, the duration of protective titers after only one vaccination could not be evaluated for the three groups, but could be subject to future research as it has been shown that rabies antibody titers declined to low titers within six months in horses that only receive one vaccination (55). Most of the horses in the study by Muirhead et al. were also Se-deficient.
In the study reported here, relative cytokine gene expression in lymphocytes and neutrophils was measured using RT-PCR. The goal was to investigate the differences in relative change in gene expression of cytokines produced by these cells after stimulation that might be related to Se content or source. The cytokines measured were IFNγ, IL-2, IL-5, IL-10, and TNFα for the lymphocyte samples. For neutrophil samples IL-1, IL-6, IL-8, IL-12, and TNFα were tested but only IL-1 and IL-8 yielded results for analysis, which may be due to only a small amount of RNA present in the original sample, resulting in failure to detect amplification during RT-PCR. Alternatively, there may have been insufficient expression of these genes.

Cells of the innate and adaptive immune system mediate their effects through the production and release of different cytokines. The effects of Se status on cytokine gene expression in immune cells have not been well investigated in domestic animal species, but deserve consideration as a mechanism through which Se could influence the immune response and thus was considered to require further study. Recent research has shown that Se can attenuate pro-inflammatory gene expression in macrophages of mice, and therefore has an important role as an anti-inflammatory agent in those cells (12). The study by Vunta et al. (12) demonstrated a significant decrease in LPS-induced expression of the pro-inflammatory genes cyclooxygenase-2 (COX-2) and TNFα via the inhibition of mitogen-activated protein (MAP) kinase pathways, which are involved in activation of COX-2 and other pro-inflammatory genes. The expression of COX-2 is regulated by the transcription factor nuclear factor kappa B (NF-κB), which is involved in the induction of the inflammatory response. This transcription factor can be induced by pro-inflammatory stimuli such as LPS, TNFα, and ovalbumin. Research has shown that Se status can
modulate the activity of NF-κB in murine monocytes and macrophages (56-58), which may be an explanation as to how differences in Se status could also alter cytokine gene expression.

A study which investigated the effect of Se supplementation on human immune cell function demonstrated an increased expression of the IL-2 receptor in T-lymphocytes following Se supplementation (50). A more recent study in mice investigated the difference in cytokine profile of lymphocytes from mesenteric lymph nodes in Se-deficient mice compared to mice of adequate Se status after infection with Cryptosporidium parvum (13). Results from that study showed that lymphocytes from mice with an adequate Se status produced higher levels of IFNγ, IL-2, and IL-4 throughout the course of infection compared to Se-deficient mice.

In the present study, horses receiving organic Se had a higher mean stimulation index for lymphocyte expression of IL-5 when compared to horses receiving inorganic Se or no supplementary Se (p<0.05; Figure 2.5). Lymphocytes from horses receiving organic Se increased expression of IL-5 about 6-fold when stimulated with 2 μg/ml of ConA compared to unstimulated lymphocytes from the same horses. However, in horses receiving inorganic or no Se supplementation, IL-5 expression was no different in stimulated lymphocytes when compared to unstimulated lymphocytes. Interleukin-5 is secreted by T-helper 2 lymphocytes and is involved in the later stages of B-lymphocyte activation during which it contributes, together with IL-6, to the differentiation of B-lymphocytes into antibody-secreting plasma cells (59, 60). Interleukin-5 has been shown, in mice, to promote IgA secretion in plasma cells that have already undergone isotype switching (60). However, clonal expansion of B-lymphocytes, which precedes
differentiation, requires the presence of IL-4, another T-helper 2 derived cytokine (60). Interleukin-4 expression was not measured in this study, but should be considered in future studies. Exposure to IL-5, among other cytokines (IL-4, IL-9, IL-10, IL-13), also favors the development of T-helper 2 lymphocytes from naïve CD4 T-lymphocytes (61). Further, IL-5 stimulates proliferation, cell activation, and differentiation of eosinophils (62).

There was no difference between the groups in the relative gene expression of the remaining lymphocyte cytokines included in the analysis. The finding that there was no difference in IL-2 gene expression was consistent with the finding that there was no effect of Se supplementation or source on lymphocyte blastogenesis, as IL-2 functions as a T-lymphocyte growth factor.

The relative cytokine gene expression of neutrophils was significantly different between the dietary treatment groups, both for IL-1 and IL-8. Horses receiving either organic or inorganic Se had higher mean relative expression of IL-1 when compared to horses receiving no supplementary Se. In this case, source of Se was not important. In contrast, horses receiving inorganic Se had the highest mean relative expression of IL-8 by stimulated neutrophils. Interleukin-8, also known as CXCL8, is a chemotactic factor, which plays an important role in the recruitment of leukocytes to the site of infection as well as the activation of neutrophils, including respiratory burst and the release of lysosomal content (63). Based on these results, there is evidence that the Se status of the host influences neutrophil function in adult horses.

The effect of Se status on neutrophil function has been extensively studied in cattle, particularly dairy cows. One study demonstrated that dietary supplementation of cattle
with Se led to a more rapid neutrophil influx into milk following intramammary bacterial challenge as well as increased intracellular killing of ingested bacteria by neutrophils when compared to cattle not receiving supplementary Se (64). The difference in neutrophil influx is indicative of a difference in chemotactic response similar to the findings described in goats (47, 48). Another study in postparturient Holstein cows showed that higher blood Se concentrations resulted in enhanced neutrophil function, measured as increased neutrophil adhesion to nylon fibers and superoxide production (65). In the study by Cebra et al. (65) all cows had blood Se concentrations within the reference range determined to prevent deficiency, but the cows with Se concentrations above the reference range had increased neutrophil function compared to cows within the reference range. This finding indicates that the Se requirements for prevention of classical deficiency syndromes and an optimal immune function may differ, at least in regards to neutrophil function.

Based on previous research findings, which have demonstrated that Se can alter the activity of the transcription factor NF-κB (56-58), one may speculate that cytokine gene expression can be influenced by Se through a similar mechanism. Activation of NF-κB by the Toll pathway results in the production of immune mediators, including cytokines and chemokines (66). Specific toll-like receptors, which are the first component of this pathway, can be activated through binding of pathogens (67). If differences in Se status can affect the activity of NF-κB one may speculate further that inorganic and organic Se supplementation can have different effects on cytokine gene expression because different Se sources result in a difference in Se status. According to results from a study in humans, Se supplementation (100 μg of Se as sodium selenite daily) can result in
increased expression of genes involved in protein biosynthesis and gene translation in lymphocytes of healthy individuals (68). The authors hypothesized that this upregulation may result in increased selenoprotein synthesis leading to an increase in lymphocyte activity. There is evidence that Se can affect gene expression on a transcriptional and translational level, and these mechanisms may be involved in the changes seen in relative cytokine gene expression after Se supplementation of horses.

In summary, Se supplementation of adult horses with inorganic or organic Se led to an increase in measures of Se status in blood, with horses receiving organic Se having the highest concentrations of Se in plasma and red blood cells. Dietary Se content and source appear to influence immune function in horses, elaborated as increased lymphocyte expression of IL-5 and neutrophil expression of IL-1 or the organic Se group and IL-1 and IL-8 in the inorganic Se group. These results suggest potential mechanisms by which Se supplementation might influence immune function in horses.
Table 2.1  Summary of relevant functions of cytokines measured during lymphocyte and neutrophil gene expression (69)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Selected functions of cytokine</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>T-cell growth &amp; functional differentiation to T-helper 1 lymphocytes; macrophage activation</td>
</tr>
<tr>
<td>IL-1</td>
<td>Important mediator of inflammatory reactions; endogenous pyrogen; stimulation of T-helper lymphocytes; promotion of B-lymphocyte proliferation and immunoglobulin synthesis</td>
</tr>
<tr>
<td>IL-2</td>
<td>Growth factor for all subpopulations of T-lymphocytes; proliferation of activated B-lymphocytes</td>
</tr>
<tr>
<td>IL-5</td>
<td>B-lymphocyte differentiation into plasma cells; development of T-helper 2 lymphocytes; proliferation, activation, differentiation of eosinophils; augmented IgA secretion</td>
</tr>
<tr>
<td>IL-8</td>
<td>Neutrophil activation; enhancement of metabolism of reactive oxygen species; chemotaxis</td>
</tr>
<tr>
<td>IL-10</td>
<td>Important anti-inflammatory cytokine - inhibits synthesis of several cytokines, including IL-1, IL-2, IL-6, IFNγ, TNFα</td>
</tr>
<tr>
<td>TNFα</td>
<td>Phagocytosis; initiation of adaptive immune response; over expression associated with effects leading to inflammatory response syndrome during gram-negative septicemia</td>
</tr>
</tbody>
</table>
Figure 2.1  Mean (± SE) plasma Se concentration in adult horses (n=5 per group) receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se; * the mean of horses receiving supplementary Se is significantly different from the mean of the control group (p < 0.05); ** all pairwise mean comparisons are significant (p < 0.05)
Figure 2.2  Mean (± SE) red blood cell Se concentration in adult horses (n=5 per group) receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se; * the mean of horses receiving organic Se is significantly different from the mean of the horses receiving inorganic or no Se (p < 0.05); ** all pairwise mean comparisons are significant (p < 0.05)
Figure 2.3  Mean (± SE) blood glutathione peroxidase activity in adult horses (n=5 per group) receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se; *the mean of horses receiving supplementary Se is significantly different from the mean of the control group (p < 0.05)
Figure 2.4 Percent inhibition of serum anti-rabies antibodies prior to vaccination, prior to booster vaccination (eight weeks later), and at the end of the study (four weeks after booster) in adult horses (n=5 per group) receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se.
Figure 2.5  Mean stimulation index (SI) for expression of IL-5 in lymphocytes from adult horses (n=5 per group) receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se; a,b means with different superscripts are significantly different (p<0.05).
Figure 2.6  Mean relative quantification of IL-1 expression in stimulated neutrophils from adult horses (n=5 per group) receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se; a,b means with different superscripts are significantly different (p<0.05)
Figure 2.7 Mean relative quantification of IL-8 expression in stimulated neutrophils from adult horses (n=5 per group) receiving supplementary inorganic Se, organic Se, or receiving no supplementary Se; a,b means with different superscripts are significantly different (p<0.05)
References


CHAPTER 3: THE EFFICACY OF SELENIUM TREATMENT OF FORAGE FOR THE CORRECTION OF SELENIUM DEFICIENCY IN HORSES

Abstract
Fortification of plants with Se has been used to increase the Se intake of humans and farm animals residing in Se-deficient regions but its use has not previously been investigated for Se supplementation of horses in North America. The aims of this study were to evaluate the effect of Se-enriched fertilizer application on the Se concentration of fresh forage and hay, and to test the effect of feeding Se-enriched hay on the Se status of horses fed a basal diet containing inadequate Se (< 0.1 ppm dry matter (DM)). This method of Se supplementation was further compared to a more conventional method using Se-containing trace mineral blocks. Application of Se-enriched fertilizer resulted in fresh forage and hay with significantly higher Se content when compared to untreated fresh forage and hay from the fields that were studied. The concentration of Se in fresh forage and hay was increased from concentrations considered inadequate to meet the dietary requirements for horses to those considered adequate. Both Se-enriched hay and the Se-containing trace mineral block significantly increased the plasma and red blood cell Se concentrations of adult horses. However, horses fed Se-enriched hay had significantly higher plasma Se concentrations when compared to horses with access to the Se-containing trace mineral block, with mean plasma Se concentrations falling in the reference range considered adequate (> 0.14 ppm) only for the horses fed Se-enriched hay. In summary, application of Se-enriched fertilizer increased the Se concentration of hay to a degree sufficient to meet the published dietary requirement for horses (> 0.1 ppm...
of diet dry matter), and feeding of Se-enriched hay was an effective method of Se supplementation of horses.
Introduction
Selenium, a trace element which is essential to all animal species, is a component of several important selenoproteins such as the antioxidant enzymes glutathione peroxidase and thioredoxin reductase (1, 2). Herbivores obtain Se from plants which in turn take it up from the soil. Selenium is taken up by plants more readily in geographical areas with arid climate and alkaline soil; such conditions are typical of the mid-western regions of North America. The coastal regions of North America and the area around the Great Lakes have lower soil Se concentrations and these regions tend to have relatively acidic soils and higher rainfall. Under acidic soil conditions Se more readily forms complexes with iron (iron hydroxides) which have low solubility, making it less available to plants; furthermore, as a consequence of higher rainfall, Se tends to leach from the soil (3-5).

Most plants take up Se in direct relationship to the soil Se concentration but a few plants accumulate Se (Se-accumulating plants) (6-8). Selenium-accumulating plants can be found in the arid mid-west of North America and include two-grooved milkvetch, prince’s plume and woody asters. Selenium metabolism differs between Se-accumulators and non-accumulators with Se-accumulators mainly synthesizing non-protein amino acids such as methylselenocysteine and selenocystathionine while the predominant form of Se in non-accumulators is selenomethionine (8, 9). Out of the different forms of Se, selenate is most bioavailable to the plant and transported more rapidly from the roots to the leaves than selenite or selenomethionine. Selenite is quickly converted to organic forms of Se, such as selenomethionine, which are mainly retained in the roots. Due to the chemical resemblance of Se and sulfur, selenate uptake is mediated by sulfate transporters in the roots of the plants, using an active transport mechanism within the
plasma membrane of root epidermal cells. Organic forms of Se are also taken up actively whereas selenite accumulates in plant roots through passive diffusion (9, 10). Once it reaches the leaves, selenate gets taken up into the chloroplasts and is metabolized by enzymes utilized in sulfate assimilation. During this process selenate is reduced to selenide which is then used for synthesis of selenocysteine. Selenocysteine can further be metabolized to selenomethionine and both are incorporated into proteins through nonspecific substitution of selenocysteine and selenomethionine with cysteine and methionine, respectively (10). Since selenate and sulfate utilize the same metabolic pathway in plants, sulfate can interfere with Se assimilation of the plant. In non-accumulating plants the presence of sulfate inhibits selenate uptake whereas Se-accumulators take up selenate preferentially over sulfate (9, 10).

A recent study (11) has shown that the majority of horses on Prince Edward Island (PEI), Canada, are deficient or marginally deficient based on common measures of Se adequacy using accepted references ranges of plasma Se for adult horses (deficient 0.008-0.053, marginal 0.053-0.120, adequate 0.140-0.250 ppm (12)). Pleasure horses appear to have the highest prevalence of inadequate blood Se concentrations when compared to broodmares and racehorses (11). Ensuring effective Se supplementation of horses living on PEI poses a challenge, especially for pleasure horses, many of which do not receive well-balanced concentrate feeds or mineral supplements on a regular basis. Therefore, a goal of this research was to evaluate methods of Se supplementation that may be more efficacious in situations where delivery of supplementary Se through a concentrate ration is not practical.
For Se supplementation of equine feeds, no specific regulations currently exist. The
Canadian Food Inspection Agency recommends Se to be incorporated into complete
feeds for non-food producing livestock at “nutritional levels” but does not specify what
these might be (13). Nutritional guidelines for horses are also provided by the National
Research Council (NRC) (14): the Se requirement for mature idle horses was estimated
by the NRC to be 0.1 ppm of diet dry matter.

Supplementary Se can be added to concentrate feed or included in a trace mineral
block in order to make it available to horses living in Se-deficient areas. Commonly used
inorganic forms of Se include sodium selenite and sodium selenate. A study which
compared the bioavailability of selenite and selenate in cattle, sheep, and horses found
that these compounds were equally effective at increasing serum Se concentration and
 glutathione peroxidase activity (15). Organic Se is commonly supplemented in the diet as
Se-fortified yeast. The predominant form of Se in Se yeast is selenomethionine (1, 16,
17). Fortification of food crops with Se is another option for Se supplementation and is
common practice in some regions of the world with naturally low soil Se concentration;
in these locations, Se-containing fertilizer is used to increase the Se content of the plants
used for human consumption (7, 18). Selenium-enriched fertilizer has been used
successfully in increasing the Se concentration of forage offered to cattle and sheep (19-21);
however, this is not currently common practice in North American farming regions,
and its use has only recently been investigated in horses in Europe (22).

Based on the location of PEI in a Se-deficient region and the apparent prevalence of
Se-deficiency among the PEI horse population, the aims of the study reported here were
to evaluate the effect of applying Se-enriched fertilizer to fields on Se concentration of
fresh forage and hay, and to test the effect of feeding Se-enriched hay on the Se status of horses. This method of Se supplementation was further compared to a more conventional method using inorganic Se-containing trace mineral blocks. The specific hypotheses to be tested were that the application of Se-enriched fertilizer increases the Se concentration of forage grown on the fertilized soil, and that the Se status of Se-deficient adult horses fed Se-treated forage is enhanced when compared to horses with access to a Se-containing trace mineral block, or horses receiving no supplementary Se.

**Materials and methods**
In the first part of the study, a Se-containing fertilizer additive was used to produce Se-fortified hay which, together with unfortified hay, was used in the second part of the study. Two similar fields on a cooperating farm were divided into four plots each to avoid bias due to topography or soil type, using stakes to mark each plot (Figure 3.1). Forages growing on these two fields were permanent-sward timothy grass (field A) and a timothy-alfalfa-mix (field B), respectively. The fields were located adjacent to each other and had similar soil type. The farm had been determined to produce Se deficient hay (0.05 ppm) during the previous season. The plots on each field were assigned a treatment using systematic random assignment within each field and then received nitrogen phosphate potassium (NPK) fertilizer treatment based on their assignment, either with or without sodium/barium selenate (Selcote Ultra®, CropCare Holdings Ltd, Nelson, New Zealand) added at the recommended rate of 1 kg per hectare (ha). Selcote Ultra® contains 10 g Se per kg in the form of sodium and barium selenate. This formulation is intended to result
in immediate (sodium selenate) and slow (barium selenate) release of Se into the soil (20).

Prior to fertilizer application, core soil samples were taken from both fields to determine the ideal choice of fertilizer. In each field, the soil core samples were taken at a depth of ten centimeters from ten locations and then thoroughly mixed to achieve a representative sample. Based on the results of the soil analysis (Appendix H), a low phosphate NPK fertilizer (15.5.15) was chosen and applied at a rate of 225 kg/ha. Selcote Ultra® was blended with approximately half of the nitrogenous fertilizer according to the recommendations of the manufacturer at a rate intended to deliver 10 g Se per ha (1 kg Selcote Ultra®/ha).

Fresh forage samples (whole plant) were obtained from each field before treatment and before the fields were divided into plots by walking diagonally across the field and taking grab samples every ten steps. These samples were combined, dried, ground, and submitted for Se analysis by fluorimetric determination of Se (23). Furthermore, samples were taken from each plot one and two months after fertilizer application by walking diagonally across the plot and combined within plot and then processed and analyzed as described above.

Hay was harvested two months after the fertilizer was applied. Hay bales from each treatment were identified with different color baling twine, and core samples from the permanently identified hay bales were collected approximately one month after harvest and analyzed in an identical fashion to the fresh forage samples (see Appendix A for detailed description of forage Se analysis).
In the second part of the study, fifteen adult, non-pregnant, non-lactating Standardbred horses were subjected to a feeding trial. The use of animals in this study was approved by Animal Care Committee of the University of Prince Edward Island, Canada. Horses were randomly assigned to one of three experimental groups, with five horses per group. The horses were housed in individual stalls to ensure that the assigned diets were provided accurately. All horses were fed hay and locally grown whole oats according to NRC maintenance requirements (24). Dietary treatments were assigned as follows: Group 1. Low-Se hay with no supplementary Se, Group 2. Low-Se hay, supplemented with free choice access to a sodium selenite-containing trace mineral block containing 14 mg/kg of Se (Sifto® Canadian Stockman, Mississauga, ON), Group 3. High-Se hay, with no supplementary Se. Horses in groups 1 and 3 had constant free choice access to a non-Se containing trace mineral block (Sifto® Canadian Stockman, Mississauga, ON). Researchers and animal care staff working with the horses were blinded regarding the experimental treatments. A composite sample of the oats was analyzed for Se using the same method as for the forage Se analysis.

Blood samples were obtained from the jugular vein using EDTA and heparinized vacuum tubes prior to the onset of treatments, and one, two, and three months after beginning treatment. Plasma and whole blood Se concentrations were measured using atomic absorption spectrophotometry (25; Appendix A). Red blood cell Se concentration was calculated based on the following formula:

Red blood cell Se = whole blood Se − [(1 − hematocrit) x plasma Se] / hematocrit,
as previously described (26).
Whole blood glutathione peroxidase activity was measured for each animal at the beginning and end of the trial. Blood was collected into heparinized vacuum tubes and was transferred into several aliquots of 2ml Eppendorf® microfuge tubes and frozen at -20°C until analysis, using a commercial assay (Ransel® by RANDOX laboratories) based on the method by Paglia and Valentine (27). Specifically, this is a UV method which measures the decrease in absorbance of light at 340 nm when glutathione is oxidized by cumene hydroperoxide catalyzed by glutathione peroxidase.

Statistical analysis was performed using analysis of variance (ANOVA) and repeated measures ANOVA (Intercooled Stata 9.2, StataCorp, College Station, TX). Comparisons of means were performed by student’s t-test, simple ANOVA, or ANOVA contrasts for the repeated measures model. The forage data was tested for plot and field effect using stepwise univariate ANOVA.

Results

Feed analysis
The Se concentration of the oats used in the feeding trial was 0.05 ppm DM. By the time of harvest, the botanical composition of the timothy hay and the timothy-alfalfa-mix were visually very similar; further, proximate analysis (Appendix I) for crude protein and calcium yielded similar results for the two fields (crude protein 9.54 % and 10.13% DM basis, respectively; calcium 0.34 % and 0.29 % DM basis, respectively). Univariate analysis of the forage Se concentration showed no evidence of a plot or field effect. Based on these results, data was combined over plot and field for statistical analysis, and
the hays derived from the two fields were not considered separately for the purposes of
the feeding trial.

The mean (± SE) forage Se concentrations as well as the differences between the
treatment groups are shown in Figure 3.2. Application of Se-enriched fertilizer resulted in
fresh forage and hay with significantly higher Se content when compared to fresh forage
and hay derived from untreated plots (Figure 3.2). At one month after fertilizer
application the Se concentration in one of the untreated plots in field B was high (0.43
ppm) which was considered to be an outlier most likely due to labeling or laboratory
error and was removed from further analysis. The concentration of Se in fresh forage and
hay was increased from concentrations considered inadequate to meet the dietary
requirements of horses to those considered adequate (> 0.1 ppm DM). The concentration
of Se in treated fresh forage was three times higher at one month after application when
compared to two months after application (p < 0.05). There was no significant difference
in Se concentration between samples of fresh forage obtained immediately before
harvest, and the hay that was baled (Figure 3.2).

**Analysis of blood Se parameters**
Both Se-enriched hay and the Se-containing trace mineral block significantly increased
the plasma and red blood cell Se concentrations of the experimental horses (Figures 3.3
and 3.4). Further, horses fed high-Se hay had significantly higher plasma Se
concentrations when compared to horses with access to the Se-containing trace mineral
blocks. Mean (± SE) plasma Se concentrations at the end of the study for groups 1, 2, and
3 were 0.062 (0.0043), 0.128 (0.0057) and 0.164 (0.0158) ppm, respectively (p<0.0001
for group, n = 5 per group). Thus, by the end of the feeding trial, mean plasma Se concentrations fell in the reference range considered adequate (> 0.14 ppm) (12) only for the horses fed Se-enriched hay. Mean red blood cell Se concentrations at the end of the study for group 1, 2, and 3 were 0.089 (0.0174), 0.206 (0.0221) and 0.252 (0.0227) ppm, respectively. Both treatments groups were significantly different from the controls, but the difference between the two treatments did not reach significance for group. The effect of time and group by time interaction was statistically significant both for plasma and red blood cell Se (p < 0.01).

Whole blood glutathione peroxidase activity
After three months on the experimental diets there were no significant differences in mean glutathione peroxidase activity between the experimental groups (results for group as well as group by time interaction); however, if the groups receiving Se supplementation were combined together, horses in these groups had significantly higher glutathione peroxidase activity when compared to the unsupplemented controls (p = 0.04) (Figure 3.5).

Discussion
Effect of Se-enriched fertilizer on forage Se concentration
After application of the nitrogenous fertilizer, fresh forage Se concentrations increased significantly in the plots receiving Secote Ultra®. Small variations over time in the Se content of the areas receiving no added Se were not significant and likely occurred due to trends in plant maturity. No plot or field effect could be observed in regards to Se
concentration even though different forages were grown on the two fields. This finding was surprising because forages containing legumes, such as alfalfa, generally have a higher protein concentration than grass forages. As a result a higher Se concentration of legume-containing forage could be expected as Se in non-accumulators is mainly stored in plant proteins by incorporation of selenocysteine and selenomethionine. In this case, the botanical composition of the swards became more similar over time, perhaps because harvest was delayed due to inclement weather and competition from the timothy resulted in a negligible final alfalfa content in Field B. Reflecting this, the crude protein and calcium concentrations of the hays from each field were very similar which may explain the absence of plot and field effect.

The Se concentrations from forage samples obtained at time of harvest (two months after treatment) were similar to those found in the hay on a DM basis (Figure 3.2). This suggests that there was no significant loss of Se during the preservation process. The high forage Se concentration one month after fertilizer application may have been a result of leaf contamination with the fertilizer. However, this is unlikely due to the high rainfall experienced over the growing period. More likely, the high Se concentration at one month was due to the release of the sodium selenate component of the fertilizer prills which are designed for quick dissolution and absorption by the plant (20). Furthermore, plant maturation may also have influenced the Se concentration as the protein concentration decreases during plant maturation. The pattern in pasture Se concentrations found in the study reported here were in part comparable to those determined by a study in Oregon which evaluated the effect of Selcote Ultra® on alfalfa and orchard grass Se concentration (28). In the Oregon study the alfalfa (two different varieties: Rampage and
Rebel) Se concentration was 0.02 ppm DM for both varieties before Selcote Ultra® application, 0.7 and 0.47 ppm DM one month after Se application, and 0.23 and 0.25 ppm DM two months after Se application. The Se concentration of orchard grass was 0.01 ppm DM before Se application and 0.20 and 0.17 ppm DM one and two months after Se application, respectively. Therefore, the alfalfa results are comparable both at one and two months after Selcote Ultra® and the results for orchard grass are comparable at two months after Se application. Taking both the findings from the Oregon and the PEI studies into consideration, harvesting forage one month after Selcote Ultra® application would likely have resulted in hay with much higher concentration of Se than the hay used in this study which was obtained by harvesting two months after application. Research findings have suggested that Selcote Ultra® may not have to be re-applied every year (20, 28) as plant Se levels remain high, most likely due to the slow release formulation of the fertilizer. Whelan et al. suggested that Selcote Ultra® application results in adequate forage Se concentration for up to four years when applied at a rate of 10g Se/ha (20). Recommendations on the application interval cannot be made based on the results from the study reported here as the Se concentration of the fresh forage was not measured in the following year.

PEI is located in a Se-deficient region. Several previous studies have investigated the relationship between the Se status of herbivores, the geographical area where the animals reside, and the occurrence of Se-responsive diseases. Kubota et al. (3) analyzed the Se concentration of alfalfa and other forage crops in different geographical areas of the United States and defined four areas based on plant Se concentration: IV: plants with more than 0.1 ppm of Se (mid-west), III: plants with variable concentrations of Se
(western states, Kentucky, Tennessee, Mid-Atlantic states), II: plants with less than 0.1 ppm of Se (north-east and Great Lakes region), and I: plants with less than 0.05 ppm of Se (south-eastern and northwestern coastal regions). A study performed in southern Ontario, located in the Great Lakes region, found that the majority of forage plants sampled had low Se concentrations (0.03 to 0.089 ppm) (4). Results from another study evaluating the Se status of horses in Virginia and Maryland indicated that the source of hay fed to horses was one factor that influenced their blood Se status (29). A more recent study investigated the Se concentration in beef from different geographical areas of the United States. The authors found that geographic origin was an important predictor of Se content of skeletal muscle, as well as Se concentration of whole blood, diaphragm muscle, liver, and hair. Further, soil and forage Se concentrations were correlated with skeletal muscle Se concentration (30).

Fortification of food crops for human consumption is already common practice in some areas with Se-deficient soils, especially in Europe (7, 18). This method has further been investigated in cattle and sheep (19, 21, 31); however, it is not currently common practice as a method of Se supplementation in North America. One study demonstrated a slight increase in blood Se and a significant increase in milk Se in dairy cattle after offering hay from selenized soil (19). The same study also found that the hay from selenized soils was superior to sodium selenite (mixed into barley) in raising the Se levels in whole blood and organs of fattening bulls and sheep. A study performed on PEI in 1989 and 1990 investigated the use of Se fertilizer on beef cow/calf farms and its effects on calves from birth to weaning (31). Results indicated that Se fertilizer was a successful method of Se supplementation, leading to higher pasture and animal blood Se levels on
treated farms when compared to control farms; however, no significant differences could be observed in average daily gain or adjusted weaning weights on treated compared to untreated farms. The treatments were not conducted within one farm but on different farms which may have been a confounding factor. In a more recent study, Se enriched fertilizer was spread on pasture and fields in which barley and maize were grown (21). Cows grazing the pasture and feeding on silage from the grass and maize, as well as winter barley, had increased blood Se concentrations when compared to a no-Se control group. Recently, a European study has investigated the use of Se-enriched fertilizer on feedstuffs in horses (22) and found it to be an effective method of increasing the Se content of barley, straw, and hay, even though a lower concentration of fertilizer had been applied (4g Se/ha for barley and straw; 3g Se/ha for hay).

The results of the study reported here show that the changes observed in the concentration of Se in treated fresh forage, and the hay derived from that forage, were sufficient to correct the low Se content of the forages. Initially the fields contained Se at concentrations considered inadequate to meet the dietary requirements of horses (< 0.1 ppm DM) (14); however, following treatment, they increased to concentrations considered adequate (> 0.1 ppm DM). Therefore, based on the results from this study application of Se-enriched fertilizer can increase the Se concentrations of fresh forage and hay to a degree sufficient to meet the published dietary requirement for horses.

Effect of feeding Se-treated forage on the Se status of horses

Based on the results reported here, both Se-enriched hay and the Se-containing trace mineral blocks significantly increased measures of Se status in horses. Reference ranges
are available for plasma Se concentration (12) and based on these reference ranges, mean plasma Se concentrations of horses at the beginning of the study fell in the lower end of the range considered marginal (0.053-0.120 ppm). By the end of the study, mean plasma Se concentrations were in the range considered adequate for horses fed Se-enriched hay, but not for those having access to the fortified block or for controls. Feeding of hay derived from Se-treated forage was thus an effective method for increasing measures of Se adequacy in horses raised in this Se-deficient region.

Both plasma and red blood cell Se concentrations were measured in this study as they reflect the amount of Se contained in two distinct Se pools (32): red blood cell Se is almost entirely composed of Se in the form of selenocysteine, as part of the enzyme glutathione peroxidase. As such, changes in red blood cell Se in response to supplementation can be taken as a reflection of availability of Se for incorporation into selenoproteins. Red blood cell Se takes longer to respond to Se supplementation because the selenocysteine is incorporated into red blood cell glutathione peroxidase only at the time of erythropoesis (33). In contrast, Se in plasma reflects short term changes in Se intake (26). Results from the study reported here show a significant difference in plasma Se concentration between the horses in group 2 (Se-containing trace mineral block) and group 3 (Se-enriched hay), with higher plasma Se in the horses receiving the Se-enriched hay. There was also a trend of greater red blood cell Se in forage-fed horses; however, it did not reach statistical significance. If the study had continued for another month the difference in red blood cell Se concentration would most likely have been more prominent as this measure of Se status is considered a long term indicator of Se status. No significant difference in glutathione peroxidase activity could be observed between
the two groups receiving supplementary Se. This finding is consistent with results from previous studies which could not identify a difference in the influence of different Se sources on glutathione peroxidase activity. Calamari et al. (34) found that Se yeast or sodium selenite did not differ in their effect on glutathione peroxidase-1 activity. Similarly, the study reported in Chapter 2 confirmed that glutathione peroxidase activity will increase after Se supplementation of deficient animals and can be used to assess the overall Se status of an animal, but no difference in the enzyme activity could be observed when comparing the two dietary sources of Se. The study by Hornick et al. (22), similarly found an increase in plasma Se in the group receiving Se-enriched feedstuffs when compared to the control group receiving no supplementary Se and no significant difference in glutathione peroxidase activity between the two groups. However, all horses had plasma Se concentrations in the upper range of marginal and adequate glutathione peroxidase concentrations at the beginning of the feeding trial and it is not clear from the study design if the horses were depleted of Se prior to the study.

The increased Se concentrations in horses provided by the treated hay may be a result of the form of Se provided by the plant. Non-Se-accumulating plants predominantly take up Se as selenate from the soil which is then metabolized by the plant to selenocysteine and selenomethionine which are inserted into plant proteins thereby resulting in an organic form of Se (6, 9). The differences in bioavailability of inorganic and organic Se result from differences in absorption and metabolism of the Se compounds. Selenomethionine is absorbed and metabolized in a similar way as the amino acid methionine, utilizing active transport mechanisms (sodium-dependent neutral amino acid transport system) across intestinal membranes (35). After absorption, selenomethionine
can be further metabolized to selenocysteine and inserted into selenoproteins or stored in organs with a high rate of protein synthesis, such as red blood cells, liver, kidneys, pancreas, gastrointestinal mucosa, and skeletal muscle (1, 35). In contrast, inorganic Se compounds, like other minerals, are absorbed through passive diffusion and a relatively small proportion is incorporated in proteins and retained, whereas the remainder is excreted through the urine and feces (36).

In mammals, selenomethionine and selenocysteine are metabolized in different ways (1, 7). Selenomethionine is randomly substituted for methionine during protein synthesis through addition to the growing peptide chain by methionine transfer RNA. Therefore, in mammalian species Se is stored in proteins mostly as selenomethionine. Selenocysteine on the other hand, is required at the active site of selenoproteins, such as glutathione peroxidase and thioredoxin reductase, where selenocysteine insertion into the primary protein structure takes place during translation (37) and is therefore considered to be the 21st proteinogenic amino acid. Both selenomethionine and selenocysteine are valuable Se sources for mammals and were likely both available to the horses receiving Se-enriched forage in this study. However, the forage was not specifically analyzed for Se compounds. Determining the various Se compounds found in the forage Se would have been useful and should be considered in future studies.

**Conclusion**

Application of Se to pasture grown on Se-deficient soils increased the Se concentrations of hay to a degree sufficient to meet the published dietary requirement for horses. Feeding of Se-enriched hay was an effective method for Se supplementation of horses,
and should be considered as an alternative Se source for horses where daily supplementation via the concentrate ration is not practical. Depending on other components of the diet and the time from treatment of the fields to harvest, it appears that feeding Se-fortified hay to horses may obviate the need to supply supplementary Se by other means.
Figure 3.1  Schematic showing the two experimental fields in relation to each other as well as the division into untreated and selenium-treated plots within each field.
Figure 3.2  Forage selenium concentration (means ± SE, DM basis) prior to (entire field) application of nitrogenous fertilizer either with or without added selenium, 1 and 2 months after fertilizer application, and in hay one month after harvest; means with different superscripts differ significantly (p < 0.05). Pre-application results represent results for entire field.
Figure 3.3  Mean (± SE) plasma selenium concentration in horses provided with a selenium-containing trace mineral block, fed selenium-fortified hay, or left unsupplemented (p<0.0001 for group, n = 5 horses per group); * the mean of horses receiving supplementary selenium is significantly different from the mean of the control group (p < 0.05); ** all pairwise mean comparisons are significant (p < 0.05)
Figure 3.4 Mean (± SE) red blood cell selenium concentration in horses provided with a selenium-containing trace mineral block, fed selenium-fortified hay, or left unsupplemented (n = 5 horses per group); * the mean of horses receiving supplementary selenium is significantly different from the mean of the controls (p < 0.05)
Figure 3.5  Mean (± SE) blood glutathione peroxidase activity in horses provided with a selenium-containing trace mineral block, fed selenium-fortified hay, or left unsupplemented (n = 5 horses per group); * the mean of horses in the two groups receiving supplementary selenium combined is significantly different from the mean of the controls (p < 0.05)


CHAPTER 4: THE EFFECTS OF SELENIUM SOURCE ON MEASURES OF SELENIUM STATUS OF MARES AND SELENIUM STATUS AND IMMUNE FUNCTION OF THEIR FOALS

Abstract
Among horses, foals are the age group most at risk of clinical disease associated with selenium (Se) deficiency, yet there is little information available on the effects of maternal Se supplementation on the Se status and immune function of neonatal foals. This study examined the effects of source of supplementary Se (inorganic versus organic) on measures of Se status in the blood of mares and their foals, together with colostrum and milk Se concentrations. The effect of source of Se on immune function of foals was also examined, including lymphocyte proliferation in response to the mitogen concanavalin A, neutrophil phagocytic activity, and relative cytokine gene expression of stimulated lymphocytes (IFNγ, IL-2, IL-5, IL-10, TNFα) and neutrophils (IL-1, IL-6, IL-8, IL-12, TNFα). Twenty Standardbred mares were randomly assigned to one of two treatment groups (10 animals per group). Mares in each group received the same balanced ration except for the source of supplementary Se: one group received organic Se (as Se yeast) and the other group received inorganic Se (as sodium selenite), each incorporated into the ration to deliver 0.3 ppm supplementary Se. Each mare was maintained on the experimental diet from two months before her estimated due date until their foal was one month old. There were no significant differences in maternal plasma or red blood cell Se concentrations between the organic and inorganic groups at any time during the study. The same was true in the foals at birth. At one month of age, there was a significant between-group difference in red blood cell Se concentration of foals (p < 0.05), with higher levels in the organic group. Selenium source did not affect colostrum
or milk Se concentration; however, colostrum and milk Se concentrations were closely correlated with the plasma and red blood cell Se concentrations in the mare. This finding may be of future use to predict Se in colostrum and milk based on the Se status of the mare during late pregnancy. Selenium source did appear to influence relative gene expression of IL-2, TNFα, and IFNγ in lymphocytes; however, no Se source consistently led to higher expression. To conclude, the source of dietary Se did not influence blood or milk Se parameters of mares, but it did affect red blood cell Se of one month old foals. The source of dietary Se may influence the immune function of foals through changes in relative gene expression of some lymphocyte cytokines.
Introduction
The effect of the maternal Se intake on the Se status of offspring has received considerable attention in humans and domestic animal species (1), however, in horses, little information is currently available. In domestic livestock, both trans-placental Se transfer, as well as transfer through colostrum and milk, can influence the Se status of the fetus and neonate (2, 3). Colostrum and milk Se concentrations depend on both the amount and source of Se (inorganic versus organic) available in the maternal diet; organic Se supplementation in cows has led to higher Se concentration in milk compared to inorganic Se (2-6). Similarly, colostrum and milk Se concentrations after foaling were higher in mares receiving organic Se compared to those receiving inorganic Se (7) and foals born to mares receiving organic Se had higher serum Se concentrations at birth.

Neonatal and growing foals are most at risk of clinical disease associated with Se deficiency, yet the effect of maternal Se supplementation on the health of foals has not been extensively studied. White muscle disease (WMD) or nutritional muscular dystrophy in foals is the principal Se-responsive disease of horses (8-10). Furthermore, Se deficiency has increasingly been associated with alterations in immune function in various species, leading to increased interest in the relationship between maternal and neonatal Se status and neonatal immune function (7, 11-14).

Previously we have shown that dietary Se, both concentration and source, influenced immune function in adult horses, and that these effects may be mediated in part by alterations in relative cytokine expression of neutrophils and lymphocytes (Chapter 2). These results support and extend the results of previous research showing that Se deficiency affects both innate and adaptive immunity, including neutrophil and
macrophage function, lymphocyte function, and antibody production in humans and animals (15-20).

The equine neonate is at high risk of infectious disease and sepsis, so studies examining the effect of Se supplementation on the immune response of neonates are of particular interest. A study in rats found that thymocytes from neonates receiving low-Se milk showed impaired activation in vitro. Further, the percentages of CD8 cytotoxic T-lymphocytes, CD2 T-lymphocytes, B-lymphocytes, and natural killer cells were decreased in the spleen of neonates nursed by mothers receiving a low-Se diet (14). A study of Se supplementation and immune function in foals, the only of its kind so far published, found greater antibody titers to influenza virus in foals born to mares supplemented with Se at 3mg per day compared to mares receiving 1mg Se per day (7). Further, Se supplementation of mares with either an inorganic or organic source at a rate of 3 mg of Se per day was associated with a higher concentration of serum IgG in foals at two weeks of age compared to foals born to the mares receiving a daily amount of 1 mg of Se (21).

The purpose of this study was to expand our previous work from Chapter 2 in which we demonstrated differences in measures of Se status and immune function in adult horses supplemented with either inorganic or organic Se. In this case the focus is on foals, the group most at risk for developing a Se-responsive disease. Measurements of interest included the effect of Se source on colostrum and milk Se concentrations as well as plasma and red blood cell Se concentrations of mares and foals. Additionally, measures of innate and adaptive immune function of foals were studied, including lymphocyte proliferation in response to the mitogen concanavalin A, neutrophil...
phagocytic activity, and relative cytokine gene expression of lymphocytes and neutrophils.

**Materials and Methods**

**Experimental animals and study design**

Twenty multiparous Standardbred mares from three farms on Prince Edward Island (PEI) were included in the study. The experimental animals were kept at their home herd for the duration of the study. The medical and reproductive history of each mare was recorded using a standardized questionnaire (Appendix J). The mares were due to foal between April and June, 2008.

The mares were placed on experimental feed two months before their estimated due date (based on a gestational period of 11 months) and the feeding trial was continued until the foals were one month of age. During the experimental period the horses were housed, fed locally-grown hay determined to be Se-deficient. Horses had daytime access to pasture where available. The only concentrate fed to the mares was the experimental ration, described below, and no other Se-containing supplements were offered to the experimental animals during the duration of the study.

Prior to the beginning of the study, the animals were randomly assigned to one of two experimental groups, each containing 10 mares: horses received either a complete pelleted feed (16% protein) containing organic Se (Se yeast), or the same feed containing inorganic Se (sodium selenite). Both feeds were formulated by a commercial feed mill (Shur-Gain Atlantic, Moncton, NB, purchased at Phillips Feed Services Ltd.) to deliver 0.3 ppm supplementary Se in the total ration. The primary investigator and owners were
blinded as to group assignment of each mare. In addition to the experimental concentrate feed, the broodmares had free choice access to locally grown, low-Se roughage (hay or haylage).

Samples of each of the two experimental concentrate feeds, as well as all forages being fed on each of the three farms, were analyzed for Se content; proximate analysis was also performed (Appendix K). Forage Se concentrations varied between 0.05 and 0.08 ppm. The experimental concentrate feeds contained 0.47 ppm and 0.53 ppm Se on a dry matter basis for the feeds with added organic and inorganic Se, respectively. Selenium analyses of feed samples were performed using fluorimetric determination of Se (22) (Appendix A). The experimental concentrate feeds were analyzed prior to the beginning of the study.

The amount of experimental feed offered daily was adjusted for each individual mare based on live weight measured using a weight tape. Concentrate offered was calculated to meet NRC requirements for each mare, taking into consideration requirements for gestation and lactation (23) (Appendix L).

The owners of the broodmares signed a consent form for each animal included in the study (Appendix M) and the use of animals in this study was approved by the Animal Care Committee of the University of Prince Edward Island.

**Blood selenium parameters**

Blood samples were obtained from the jugular vein of the mares (using EDTA vacuum tubes) prior to onset of treatments, one month after the onset of treatment, at foaling, and one month after foaling. Blood samples were collected from the jugular vein of foals
within the first 12 hours of life, and again at one month of age. Plasma and whole blood Se concentrations were measured using atomic absorption spectrophotometry (24; Appendix A) and red blood cell Se concentration was estimated using the following formula after sub-sampling for estimation of hematocrit:

\[
\text{Red blood cell Se} = \text{whole blood Se} - \left[\left(1 - \text{hematocrit}\right) \times \text{plasma Se}\right] / \text{hematocrit},
\]
as previously described (25). Both plasma and whole blood samples were frozen at -20°C until Se analysis was performed. All measurements were performed at the Atlantic Veterinary College.

**Colostrum and milk selenium concentrations**

A colostrum sample was obtained from each mare within the first 12 hours following parturition. Milk samples were obtained one and four weeks after foaling. Colostrum and milk samples were centrifuged at 16,000 g and the fat layer was removed. The remaining sample was frozen at -20°C until analysis. All samples were analyzed using fluorimetric determination of Se (22) (Appendix A).

**Complete blood count and immunoglobulin G (IgG) analyses**

Blood was obtained from each foal from the jugular vein 18 to 24 hours after birth, using an EDTA vacuum tube and a serum tube with no anti-coagulant. A complete blood count (CBC) was performed on blood from the EDTA tube for each foal, to identify evidence of inflammation. Foals were excluded from the study if they had an abnormal CBC reflective of inflammation together with collaborating clinical signs, as assessed during a physical examination performed by the primary investigator.
Serum concentration of IgG was estimated at one day of age to assess passive transfer of immunity using the glutaraldehyde coagulation screening test (26, 27). If the IgG concentration was classified as <800 mg/dl (suggestive of inadequate passive transfer of immunity) based on the glutaraldehyde coagulation test, a confirmatory SNAP® ELISA (IDEXX laboratories Inc., Portland, ME, USA) was also performed because the glutaraldehyde coagulation test has been shown to have a poor specificity for IgG <800 mg/dl (28). If the IgG concentration of a foal was classified as <800 mg/dl using both the glutaraldehyde coagulation test and the SNAP® test, the owner was informed and the option of a plasma transfusion was discussed. If the IgG concentration was classified as <400 mg/dl, according to the SNAP® test, a plasma transfusion was highly recommended, regardless of the apparent health status of the foal. The decision to perform a plasma transfusion was made by the owner, and any foal that received a plasma transfusion was excluded from the study.

Serum from foals collected at 18-24 hours of age was frozen at -80°C for quantitative serum IgG concentration at a later date using radial immunodiffusion (RID; VMRD, Pullman, WA, USA) following the manufacturers instructions.

Lymphocyte blastogenesis in response to the mitogen Concanavalin A (ConA)
Heparinized blood samples were collected from each foal one day after birth (7 ml) and again at one month of age (14 ml). During the first step, the blood was mixed with an equal volume of RPMI-1640 (Sigma, Oakville, ON, Canada). The blood-RPMI mixture was layered onto Ficoll-Paque Plus (Amersham Biosciences, Baie d’Urfé, QC, Canada) and centrifuged at 400g for 30 minutes at 4°C to separate the lymphocyte fraction from
the whole blood. After separation, the lymphocyte layer was harvested and the cells were washed twice with sterile saline (700g for 5 minutes) and the cell pellet was resuspended in 1ml of RPMI+ (RPMI-1640 with 100 Units/ml of Penicillin and 100 μg/ml of Streptomycin, 2 mM glutamine, and 10% heat inactivated fetal calf serum). The cells were counted and cell viability was evaluated using the trypan blue exclusion method (29).

The cell number was adjusted to 2x10^6/ml and the cells were dispensed at 100 μl/well in a 96 well plate (BD Falcon, VWR, Mississauga, ON, Canada) to which an equal volume of ConA (Sigma, Oakville, ON, Canada) diluted in RPMI+ was added to give a final concentration of 5, 2, and 0 μg/ml of ConA. All samples were run in quadruplicate. Cellular proliferation in response to mitogen stimulation was determined using [3H]thymidine incorporation into cellular DNA. The cells were incubated at 37°C for 48 hours in a 5% CO₂ air humidified atmosphere, after which 1 μCi of [3H]thymidine (GE Healthcare, Baie d’Urfé, QC, Canada) was added to each well. The cells were incubated for 18 hours with the [3H]thymidine and were then harvested onto glass fiber filtermats (Skatron Instruments Inc., Sterling, VA, USA) using a Skatron semi-automatic cell harvester (Sterling, VA, USA). Finally, the amount of radioactivity (counts per minute, CPM) incorporated into cellular DNA was detected with a Wallac Microbeta Trilux 11450 (Perkin Elmer, Woodbridge, ON, Canada) liquid scintillation counter. Stimulation indices (SI) were calculated using the following formula: the CPM of the stimulated cells divided by the CPM of the unstimulated cells.
Neutrophil phagocytosis of fluorescent beads measured by flow cytometry

Neutrophils were isolated from the red blood cell fraction after Ficoll-Paque separation of foal lymphocytes on day 30. First, the red blood cells were washed twice with saline at 700g for five minutes. This was followed by red blood cell lysis using a method modified from Raidal et al. (30). To lyse the red blood cells, 19ml of lysis solution (0.8% ammonium chloride, 0.08% sodium EDTA, 0.08% sodium carbonate) were added to 1ml of blood at room temperature. After ten minutes, 19ml of saline were added and the mixture was pipetted through a 50 micron nylon mesh to eliminate remaining cellular debris. This was then centrifuged at 700g for five minutes. The cell pellet was resuspended in 10ml of lysis solution, after which 19ml of saline were added. This mixture was again centrifuged at 700g for five minutes and the cell pellet was then washed twice with saline. Finally, the cell pellet was resuspended in 1ml of saline. After isolating the neutrophils, the cells were counted and their viability was assessed using the trypan blue exclusion method (29).

To evaluate phagocytosis, neutrophils were incubated with 2μm polystyrene fluorescein (FITC) labeled beads (Polysciences, Warrington, PA, USA). Incubation continued for 30 minutes at 37°C in the dark on a shaker. To differentiate beads that were attached to the outside of the cell from engulfed beads (evidence of phagocytosis activity), a negative control was included for each sample. This control was prepared on ice and 0.2% PBS-EDTA (0.2g EDTA in 100ml PBS) was added to inhibit attachment and internalization of the beads; previously shown by Raidal et al. (30).

Fluorescence of each sample was measured using flow cytometry (FACSCalibur, Becton-Dickinson, San Jose, CA, USA) and the phagocytic activity was reported as the difference between the sample and the control (% gated cells).
Relative cytokine gene expression measured by (real-time) RT-PCR

Relative cytokine gene expression was measured in foal lymphocytes on days 1 and 30 and in neutrophils on day 30. Two methods were used for preparing lymphocytes from day 30: a standard lymphocyte preparation and a cryopreserved lymphocyte preparation.

**Standard lymphocyte preparation**

Lymphocytes were suspended in 1 ml of RPMI+ at a concentration of 0.5x10^6/ml. Lymphocytes were stimulated with 2 μg/ml of ConA in RPMI+ and an equal volume of RPMI+ was added to the unstimulated cells. All cells were incubated at 37°C for 24 h in a 5% CO₂ air humidified atmosphere. Following incubation, the sample was centrifuged at 16,000g in a microfuge for three minutes, after which time the cell pellet was resuspended in RNAlater (Sigma, Oakville, ON, Canada). The samples were frozen at -20°C and stored for further analysis.

**Cryopreserved lymphocyte preparation**

For the cryopreserved preparation, cytokine gene expression was measured in cryopreserved lymphocytes. The assay was performed only on lymphocytes from foals at one month of age because the samples from the one day old foals did not yield enough lymphocytes. First, the remaining lymphocytes not used for the blastogenesis assay were added to 1ml of freezing media (10% DMSO and 90% fetal calf serum) and frozen at -80°C over night after which the samples were stored in liquid nitrogen until further analysis.

At the Gluck Equine Research Center, the cells were removed from the liquid nitrogen, thawed in a water bath (37°C) and immediately transferred into media (90%
RPMI and 10% fetal equine serum). The cells were centrifuged at 300g for 10 minutes, after which RPMI was added to the cell pellet. The lymphocytes were then counted and their viability assessed with trypan blue.

The lymphocytes of each animal (2x10^6/ml) were stimulated with phorbol myristate acetate (PMA) for four hours. An unstimulated control was included for all except two foals (not enough cells available in those cases). After four hours RNA STAT™ (Tel-Test, Inc.) was added to the samples, which were then frozen at -80°C until further processing.

In the next step, RNA was isolated from the RNA STAT™ using the following steps: homogenization, RNA extraction with chloroform, RNA precipitation with isopropanol, and RNA wash with 75% ethanol. After RNA isolation the samples were again frozen at -80°C. Reverse transcription, PCR, and calculation of RQ values were performed as described below.

*Neutrophil preparation*

Neutrophils from each one month old foal were also suspended in 1 ml of RPMI+ at a concentration of 0.5x10^6/ml. The sample processing was identical to the procedure used for the lymphocytes except that the neutrophils were stimulated with lipopolysaccharide (LPS) at 50 ng/ml for four hours.

*RNA isolation and measurement of relative cytokine gene expression*

Gene expression assays were performed at the Gluck Equine Research Center at the University of Kentucky. All samples from an individual foal were analyzed in one run.
During the first step of the analyses, the samples were thawed and the RNA was purified using the RNeasy® Micro Kit (Qiagen®, Valencia, CA, USA). This commercial kit is specially designed for the purification of RNA from small samples. A carrier RNA, supplied with the kit, was also used to increase the total yield of RNA.

Following RNA purification, reverse transcription reactions were performed as previously described (31) using 12μl of each RNA sample and reverse transcription master mix (Promega, Madison, WI, USA). The reactions were incubated at 42°C for 15 minutes and 95°C for 5 minutes. The cDNA was stored at -20°C until analyzed by RT-PCR.

Cytokine gene expression was measured by RT-PCR as described previously (32, 33), using equine specific intron-spanning primer/probe sets for IFNγ, IL-2, IL-5, IL-10, and tumor necrosis factor alpha (TNFα) for the lymphocyte samples and IL-1, IL-6, IL-8, IL-12, and TNFα for the neutrophil samples. The cytokines were chosen based on their importance in immunological processes involving the cell types of interest as well as availability; their major functions are summarized in Table 4.2.

Each reaction contained 4.5μl of cDNA and 5 μl TaqMan Gene Expression Master Mix (ABI) and 0.5 μl primer and probes. All reactions were incubated in duplicate wells at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds in an Applied Biosystems 7900 sequence detection system. Beta-glucuronidase (β-GUS) was used as the housekeeping gene for the lymphocyte samples and beta-2-microglobulin (B2M) was the housekeeping gene for the neutrophil samples (34). After recording the cycle threshold (C_T) for each lymphocyte cytokine, the ΔΔC_T method was used to calculate relative changes in cytokine gene expression (35), where ΔΔC_T = [Δ(Avg.
gene of interest $C_T - \text{Avg. } \beta\text{-GUS } C_T)_{\text{foal}} - (\text{Avg. gene of interest } C_T - \text{Avg. } \beta\text{-GUS } C_T)_{\text{calibrator}}$. The same formula was applied to the neutrophil cytokine results, using the results for B2M instead of $\beta$-GUS. The calibrator was calculated from the mean $\Delta C_T$ of all one day old foals for the lymphocyte samples and 30 day old foals for the neutrophil samples. The use of a calibrator helps to reduce the effect of between foal variations.

Results are expressed as RQ of a specific cytokine produced by the cell (lymphocyte or neutrophil) after stimulation calculated as $2^{-\Delta C_T}$.

**Statistical analysis**

Statistical analysis was performed with commercial software (Intercooled Stata 9.2, StataCorp, College Station, TX). Analysis of variance (ANOVA) and repeated measures ANOVA was used for testing the effect of experimental treatment on continuous outcomes. Values for relative cytokine gene expression, and the stimulation indices for gene expression, were log$_{10}$-transformed before statistical analysis as their distribution was not normal. Following log$_{10}$-transformation the data did have a normal distribution in those cases where there was sufficient data. Categorical data from the immunoglobulin screening tests were analyzed using the chi-square test. Pairwise correlation coefficients for colostrum / milk Se and mare blood Se measurements (plasma and red blood cells) were calculated. Results are presented as means with SE, unless otherwise indicated.
Results

Experimental animals
Nutritional management prior to the beginning of the feeding trial was reflective of the practices of each of the co-operating farms. Nineteen of the 20 mares were considered deficient for Se status (<0.053 ppm), whereas one mare had an adequate plasma Se concentration (> 0.14 ppm), based on accepted reference ranges (36).

Of the 20 foals born, data from 18 of these foals were retained for analysis at one day of age (nine from each group), and 16 foals were available for follow up at 30 days of age (eight from each group). Foals were lost to follow up for the following reasons: one foal died shortly after birth (presumed septicemia; organic Se group), one foal was sick at birth and was consequently hospitalized (presumed septicemia; Se inorganic group), one foal was diagnosed with failure of passive transfer of immunity and received a plasma transfusion (inorganic Se group), and one foal was euthanized (presumed septicemia; organic Se group). Thirteen foals were male (nine from mares in the organic Se group and four from mares in the inorganic Se group) and five were female (all from mares in the inorganic Se group). None of the foals in the study were considered premature (born at < 320 days gestation). None of the foals remaining in the study were born after a dystocia. No farm effect could be observed. One horse occasionally stood out in the analysis, but removing this horse from the statistical analysis did not alter the results.

Measures of selenium status
Mean (± SE) plasma Se concentrations for mares prior to the onset of treatment were 0.009 (0.003) ppm and 0.011 (0.003) ppm for the inorganic and organic groups, respectively. These values are reflective of low Se intake, falling in the range considered
inadequate (< 0.14 ppm), with all but one mare falling into the deficient range (< 0.053 ppm), based using currently accepted reference ranges (36).

Mare plasma and red blood cell Se concentrations increased in both groups following the onset of the experimental treatment (p < 0.001; Figures 4.1 and 4.2). However, there was no significant effect of Se source on plasma or red blood cell Se concentration in mares during the 3 months of the study. The results were not significantly altered by removing the mare with adequate Se status from the analysis.

Maximal plasma Se concentrations were observed in mares at the time of foaling (Figure 4.1). The mean (± SE) plasma Se concentrations at this time were 0.084 (0.015) ppm and 0.099 (0.011) ppm for the organic and inorganic groups, respectively. Despite the steep increase in concentration following the onset of the experimental treatments, plasma Se at foaling did not increase sufficiently to meet the criterion for adequacy (> 0.14 ppm).

One month after foaling, Se concentration in plasma was decreased when compared to the values at foaling (p < 0.001) being 0.057 (0.010) ppm and 0.054 (0.007) ppm for the organic and inorganic groups, respectively. In contrast, no significant change was observed in mare red blood cell Se concentration between foaling and one month after foaling (p = 0.277; Figure 4.22).

In foals, mean (± SE) plasma Se concentration at birth was 0.037 (0.005) ppm for foals of mares in both groups (Figure 4.3). These values fell below the published reference range for plasma Se concentrations in neonatal foals (0.06 to 0.1) (36, 37)).

By one month of age, the plasma Se concentrations did not significantly change when compared to the concentrations soon after birth (Figure 4.3). However, red blood cell Se
concentration increased between birth and 1 month of age ($p = 0.001$; Figure 4.4) and there was a tendency to a significant time $\times$ treatment interaction ($p = 0.07$) such that at one month of age, the red blood cell Se concentration tended to be higher in foals born to mares in the organic group ($p = 0.096$). When either foal red blood cell Se concentration at birth or mare’s milk Se concentration at one month was used as a covariate in the model, red blood cell Se concentration was significantly higher in the foals born to mares in the organic group when compared to those from the inorganic group ($p < 0.05$).

Colostrum and milk selenium concentrations
The effect of Se source on colostrum or milk Se concentration, measured at foaling, and at 1 and 4 weeks of lactation, was not significant (Figure 4.5). Selenium concentration declined over time ($p < 0.05$) with the highest concentrations found in colostrum. Reference ranges for milk Se have been published (36), however, the effect of stage of lactation is not specified. Based on these reference ranges, milk Se concentrations of 0.015-0.04 ppm are considered adequate. Sixteen mares in week 1 (six from the organic Se group, ten from the inorganic Se group) and seven mares in week 4 (three and four from organic and inorganic Se groups, respectively) fell in the range considered adequate. In week 1, the remaining mares had marginally deficient milk Se (0.008-0.015 ppm). In week 4, two mares had deficient milk Se (0.005-0.008 ppm) and seven were marginally deficient.

Significant correlations were present between colostrum/milk Se concentrations and measures of Se in the blood of mares (Table 4.1). The strongest relationships were observed between values in red blood cell and colostrum; relationships between plasma
and milk at one week of lactation were moderate, while those at one month were weak and not significant. Colostrum Se concentrations were moderately to highly correlated (r > 0.6) to measures of red blood cell Se concentration obtained from as early as 2 months before foaling, to the day of foaling. This was not affected by dietary Se treatment.

Immunoglobulin G (IgG) analysis
Categorization by the two IgG screening tests did not differ between experimental treatments. The results of the RID assay did not differ between treatments, and there was no significant relationship between RID results and the colostral Se concentration. The results of the three tests for each foal are presented in Appendix N.

Lymphocyte blastogenesis in response to the mitogen Concanavalin A (ConA)
Lymphocyte blastogenesis in response to ConA was not affected by the experimental treatment. The mean (± SE) stimulation indices for lymphocytes stimulated with ConA at a concentration of 5 μg/ml from foals at one day of age were 168.5 (39.63) and 230.63 (46.87), and at one month of age were 225.75 (29.78) and 214.88 (54.18), for foals from mares fed inorganic and organic Se, respectively. For lymphocytes stimulated with ConA at a concentration of 2 μg/ml, the values for foals at one day of age were 199.38 (46.35) and 273.88 (61.86), and at one month of age were 294 (39.90) and 318.5 (58.63), for foals from mares fed inorganic and organic Se, respectively. There was no significant difference between the groups for the results of the medium control (0 ConA).
Neutrophil phagocytosis of fluorescent beads measured by flow cytometry
Measurement of neutrophil phagocytic activity in foals at one month of age did not yield any usable results because neutrophils showed no phagocytic activity in most of the samples.

Relative cytokine gene expression measured by (real-time) RT-PCR
The mean (± SE) relative cytokine gene expression of lymphocytes and neutrophils are reported in Appendix O. Some samples did not yield enough RNA to generate valid amplification data and therefore the numbers of foals used to generate each mean are different among cytokines and are reported in the relevant tables.

For the standard lymphocyte preparation, one month old foals in the organic group had higher relative gene expression for IL-2 when compared to foals in the inorganic group (p = 0.05; Figure 4.6). At one day of age, foals in the organic group tended to have higher relative gene expression for IFNγ (p = 0.072) while at one month of age, foals in the inorganic group tended to have higher relative gene expression for this cytokine (p = 0.059) (Figure 4.7). For the cryopreserved lymphocyte preparation from foals at one month of age, TNFα gene expression was higher in foals from the inorganic group (p = 0.04) (Figure 4.8). Mean (±SE) viability of the cryopreserved lymphocytes was 82.56 (1.31) percent.

There was no significant difference in relative gene expression of the neutrophil cytokines IL-1 and IL-8. For the remaining cytokines measured in the neutrophil samples no usable signal could be detected with RT-RCR, which may have been due to the small amount of RNA present in the original sample or lack of expression of these genes.
Discussion
Results from the present study show that the form of Se fed to mares during late pregnancy and early lactation can affect measures of Se status in their foals. Significantly higher red blood cell Se concentration was observed in one month old foals when organic Se was fed, when compared to inorganic Se. However, colostrum and milk Se concentrations were not affected by Se source in this study. The form of Se fed to mares influenced measures of immune response in their foals, specifically the relative gene expression of IL-2, TNFα, and IFNγ in lymphocytes.

Although the importance of Se for animal health has been well established, to date only one comparable study has been published which investigated the effect of Se source on the Se status of mares and their foals (7). In that study, fifteen mares (five per group) were supplemented with 3 mg Se/day (either organic or inorganic), or 1 mg inorganic Se/day, from 55 days prior to foaling until 56 days post-foaling. These authors showed that serum Se concentrations were greater in mares receiving organic Se when compared to mares receiving inorganic Se. Foals born to mares receiving organic Se also had higher serum Se concentrations (7). In the present study Se source resulted in differences in red blood cell Se concentration in one month old foals, but not in other measures of Se status. In both studies, mares were supplemented for a similar duration at the end of pregnancy with a similar amount of Se; however the basal Se content of the diet and the initial Se status of the mares in the study by Janicki et al. (7) was not specified, which may in part account for the differences observed.

A significant difference in foal red blood cell Se could be observed at one month of age when foal red blood cell Se concentration at birth or mare’s milk Se at one month was used as a covariate. Interestingly, this difference between the groups was not
reflected in the plasma Se concentration. One possible explanation for this finding could be that the difference in red blood cell Se reflects a difference in transplacental transfer of Se with the usual delay in red blood cell Se response. This delay is due to the fact that Se is incorporated into red blood cells only at time of erythropoiesis. If the difference observed is a result of transplacental transfer, this would also explain why foal red blood cell Se increased even though mare milk Se decreased between one and four weeks post partum.

The observed difference in Se source on foal red blood cell Se in the present study may have been more prominent if supplementation of mares would have commenced earlier during pregnancy which could be investigated in a future feeding trial. Furthermore, since plasma and red blood cell Se of mares did not reach levels considered adequate for adult horses, higher concentrations of dietary Se may be required for broodmares in this region. The decision to supplement 0.3 ppm Se in the concentrate feed and begin Se supplementation of the mares two months prior to their anticipated due date had been based on results from our study in adult horses (Chapter 2), where adequate Se concentrations were achieved in all horses and significant differences in measures of blood Se could be observed between the inorganic and organic group two months into the feeding trial. The reason for this discrepancy is unclear; one explanation may be that trans-placental transfer of Se from the dam to the fetus could have occurred, decreasing the amount of Se available to the mare. To determine the degree of trans-placental transfer of Se, Se would have to be measured in the blood of the foal prior to colostrum intake. This was originally intended, however, for logistical reasons, a pre-suckle sample could not be obtained from most of the foals. The degree to which Se is transferred across
the placenta and whether there is a difference in trans-placental transfer between inorganic and organic Se has not been studied in horses. The occurrence of trans-placental transfer of Se has been demonstrated in cattle (2, 3), but transport mechanisms have not been studied in domestic animal species. Additionally, the mares could have "lost" Se into the colostrum. Other factors that may have influenced the results include variations in amount of feed offered, feed uptake, and feeding mistakes during the experiment.

Janicki et al. (7) found that colostrum and milk Se concentrations were higher in mares receiving the organic Se supplement, which was not observed in the present study. This discrepancy may have been due in part to sampling technique; the present study was conducted in the field and obtaining milk samples of consistent volume, at a consistent time post-suckle, was difficult which may have led to excessive variation in the protein composition of the sample. In future studies it may be helpful to measure Se in a certain fraction of the milk or corrected for variation in milk components such as proteins. A recent bovine study found that most of the Se increase in milk occurs in the β-lactoglobulin-α-lactalbumin fraction in whey (38). Whey proteins make up around twenty percent of the protein in milk of cows, by weight, and lactoglobulin is the most common whey protein. As mentioned previously, differences in the initial blood Se status of the mares may also have explained some of the discrepancy in the results for colostrum and milk Se, if the mares in the study by Janicki et al. (7) had adequate blood Se when they entered the study.

There was a moderate to high degree of correlation between measures of Se concentration in colostrum and plasma/red blood cell Se of the mares. Plasma and red
blood cell Se concentrations of mares during late pregnancy appear to be useful in predicting their colostrum Se concentration at foaling. Furthermore, mare plasma and red blood cell Se at birth was highly correlated with colostrum Se concentration. This finding is in agreement with results from bovine and porcine studies where colostrum and milk Se concentrations were found to be influenced by the maternal Se status (2-6). The low maternal blood Se may partly also have resulted from loss of Se into the colostrum and milk, which may explain why mare plasma Se decreased after foaling.

There is little published research on the influence of Se status and dietary form of Se on immunity of foals. In their study, Janicki et al. (7) found greater antibody titers to influenza virus in foals from mares supplemented with Se at a rate of 3 mg daily when compared to mares receiving 1 mg Se per day; however, the form of Se did not influence titers. The same researchers found that Se source did not affect colostral IgG concentration or foal serum IgG at 12 hours of age. Selenium supplementation of 3 mg Se daily was associated with a higher concentration of serum IgG in foals at two weeks of age (21) when compared to supplementation of 1 mg Se per day. These findings suggest that the influence of Se on foal immunity should be investigated further.

The influence of maternal Se status and Se supplementation on the immunity of offspring has been studied more extensively in other domestic animal species. Selenium supplementation of pregnant cows and ewes led to higher colostral immunoglobulin concentrations compared to unsupplemented animals (12, 13) and Se fortification of colostrum increased serum IgG of newborn calves in one study (39); however, higher concentrations of Se in colostrum (5 ppm) appeared to inhibit absorption in the same study. Similarly, research in lambs demonstrated an increase in lymphocyte proliferation
in response to mitogens (phytohaemagglutinin, pokeweed mitogen, Con A) at lower
doses of Se (0.1 ppm) as sodium selenite or selenomethionine; however, high doses of Se
(0.5 ppm) led to a decrease in mitogen response (40). While the aforementioned studies
demonstrate that Se supplementation can affect immunity or measures of immune
function in young animals, none of these studies specifically compared organic and
inorganic forms of supplementary Se.

Results from the present study suggest that the form of Se fed to the mare may
influence immune function of their offspring through alterations in relative cytokine gene
expression of the neonate’s lymphocytes. Specifically, relative gene expression of IL-2 in
ConA simulated lymphocytes and TNFα in PMA stimulated lymphocytes were affected
by the form of Se fed. For IFNγ expression of ConA stimulated lymphocytes there was a
tendency towards a difference between the groups for both one day and one month post
partum, but it was not statistically significant. Interestingly, the Se source leading to
greater relative gene expression depended on the cytokine measured. Due to this finding,
and the low and variable number of foals that could be included in the analysis, it can
only be concluded from this data that Se source appears to influence relative gene
expression of some lymphocyte cytokines in foals, but the form of Se mostly altering
gene expression might differ depending on the cytokine. However, these results need to
be verified in foals that were supplemented with enough Se to reach blood Se
concentrations within the range considered adequate.

Similarly to the findings by Janicki et al. (21), serum IgG of one day old foals was not
affected by dietary Se source. Serum IgG concentration was not measured at a later time
point during our study.
The results from mares and foals presented in this chapter build upon findings from non-pregnant, non-lactating adult horses reported in Chapter 2. In those adult horses, dietary Se content and source influenced immune function in horses: alterations in lymphocyte expression of IL-5, and neutrophil expression of IL-1 and IL-8 were observed. Together, these two studies provide evidence for an effect of source of dietary Se on cytokine gene expression. The mechanisms involved in the effect of Se on gene expression have not been established. There is evidence that Se can affect gene expression on a transcriptional and translational level, and these mechanisms may be involved in the changes seen in relative cytokine gene expression in this study. Previous research has demonstrated that Se can alter the activity of the transcription factor NF-κB (41-43) and according to results from a study in humans, Se supplementation (100 µg of sodium selenite daily) can result in increased expression of genes involved in protein biosynthesis and gene translation in lymphocytes of healthy people (44).

In the present study, no effect of Se source on lymphocyte proliferation or neutrophil phagocytic activity was observed. In case of the lymphocyte proliferation assay, this may have been due in part to the relatively small number of foals in each group, as this biological assay can be quite variable, both between and within animals, on different testing days.

The neutrophils from the foals at one month of age showed no measurable phagocytic activity in most of the samples, which may have resulted from the assay design or the low numbers of cells available. The same assay design that had been established for a previous study in horses (Chapter 2) was used for the foal neutrophil samples. Variables which may affect the outcome of the phagocytosis assay include cell isolation procedures
and cell concentration, opsonin concentration, type of particle phagocytized, phagocyte to particle ratio, time and temperature at which the samples are kept from collection to testing, shaking speed during incubation, incubation time and method of counting (45). One study looking at the influence of plasma treatment on neutrophil phagocytosis of yeast in foals found that the phagocytic activity of foal neutrophils was lower than that of adult horses when autologous serum was used as an opsonin but it was increased when pooled adult horse serum or anti-yeast IgG was used as an opsonin (46). In another study the addition of serum from adult horses did not significantly increase the phagocytic activity of foal neutrophils (45). A likely cause for the failure of the assay in this case was the low number of neutrophils available which was evident during the analysis with the flow cytometer shown as the number of gated cells. Neutrophil phagocytic activity was only measured in the sample taken after one month as other studies have shown a decreased phagocytic activity in young foals between birth and three weeks of age (45, 47). Furthermore, a smaller amount of sample was available for cell isolation on day one post partum as samples for the CBC also had to be collected.

In summary, the Se source fed to mares during late pregnancy and early lactation can influence the red blood cell Se concentrations of their foals at one month of age with organic Se leading to higher red blood cell Se when compared to inorganic Se. Mare blood and milk Se as well as foal blood Se at birth were not affected by Se source. This difference in Se source may be more prominent if supplementation of mares commenced earlier during pregnancy or if a higher concentration of Se was supplemented to the diet. Furthermore, the blood Se status of mares during late pregnancy is correlated with their colostrum Se and early lactation milk Se. Selenium source influenced relative gene
expression of some lymphocyte cytokines, namely IL-2, TNFα, and IFNγ; however, no
Se source consistently led to increased expression of these cytokines.
Table 3.1 Correlations coefficients, with accompanying p-values, for the relationship between colostrum or milk selenium (Se) concentration and plasma or red blood cell (RBC) selenium concentration in broodmares. Value in blue indicates a significant relationship.

<table>
<thead>
<tr>
<th></th>
<th>Colostrum Se</th>
<th>Milk Se week 1</th>
<th>Milk Se week 4</th>
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<tr>
<td><strong>Plasma Se</strong></td>
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<tr>
<td>2 months prep-</td>
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<td>1 month prep-</td>
<td>0.4652</td>
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<tr>
<td>1 month prep-</td>
<td>0.0599</td>
<td>0.1760</td>
<td>0.3638</td>
</tr>
<tr>
<td><strong>Plasma Se</strong></td>
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<tr>
<td>Foaling</td>
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<td><strong>Plasma Se</strong></td>
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<td>0.3352</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>1 month prep-</td>
<td>0.7948</td>
<td>0.5917</td>
<td>0.1411</td>
</tr>
<tr>
<td>1 month prep-</td>
<td>0.0001</td>
<td>0.0076</td>
<td>0.6022</td>
</tr>
<tr>
<td><strong>RBC Se</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foaling</td>
<td>0.8288</td>
<td>0.5358</td>
<td>0.0476</td>
</tr>
<tr>
<td>0.0000</td>
<td>0.0180</td>
<td>0.8610</td>
<td></td>
</tr>
<tr>
<td><strong>RBC Se</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 month post-</td>
<td>0.4842</td>
<td>0.3748</td>
<td>-0.0112</td>
</tr>
<tr>
<td>1 month post-</td>
<td>0.0794</td>
<td>0.1526</td>
<td>0.9673</td>
</tr>
</tbody>
</table>
Table 4.2  Summary of relevant functions of cytokines measure during lymphocyte and neutrophil gene expression (48)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Selected functions of cytokine</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>T-cell growth &amp; functional differentiation to T-helper 1 lymphocytes; macrophage activation</td>
</tr>
<tr>
<td>IL-1</td>
<td>Important mediator of inflammatory reactions; endogenous pyrogen; stimulation of T-helper lymphocytes; promotion of B-lymphocyte proliferation and immunoglobulin synthesis</td>
</tr>
<tr>
<td>IL-2</td>
<td>Growth factor for all subpopulations of T-lymphocytes; proliferation of activated B-lymphocytes</td>
</tr>
<tr>
<td>IL-5</td>
<td>B-lymphocyte differentiation into plasma cells; development of T-helper 2 lymphocytes; proliferation, activation, differentiation of eosinophils; augmented IgA secretion</td>
</tr>
<tr>
<td>IL-8</td>
<td>Neutrophil activation; enhancement of metabolism of reactive oxygen species; chemotaxis</td>
</tr>
<tr>
<td>IL-10</td>
<td>Important anti-inflammatory cytokine - inhibits synthesis of several cytokines, including IL-1, IL-2, IL-6, IFNγ, TNFα</td>
</tr>
<tr>
<td>TNFα</td>
<td>Phagocytosis; initiation of adaptive immune response; over expression associated with effects leading to inflammatory response syndrome during gram-negative septicemia</td>
</tr>
</tbody>
</table>
Figure 4.1 The effect of dietary selenium source on plasma selenium concentration in broodmares (n=10 per group). Inorganic selenium (sodium selenite) or organic selenium (selenium yeast) was fed from two months before foaling to one month after foaling, to deliver 0.3 ppm supplementary selenium on a ration drymatter basis. Effect of time p<0.001
Figure 4.2 The effect of dietary selenium source on red blood cell selenium concentration in broodmares (n=10 per group). Inorganic selenium (sodium selenite) or organic selenium (selenium yeast) was fed from two months before foaling to one month after foaling, to deliver 0.3 ppm supplementary selenium on a ration drymatter basis. Effect of time p<0.001
Figure 4.3 The effect of dietary selenium source (inorganic vs. organic) on plasma selenium concentration in their foals (n=9 and n=8 per group at birth and 1 month, respectively). Supplementary selenium was fed to broodmares from two months before foaling to one month after foaling.
Figure 4.4 The effect of dietary selenium source (inorganic vs. organic) on red blood cell selenium concentration of foals (n=9 and n=8 foals per group at birth and 1 month, respectively). Supplementary selenium was fed to broodmares from two months before foaling to one month after foaling. Time × treatment interaction, p = 0.07; * effect of selenium source at 1 month p < 0.05 (when either foal red blood cell Se concentration at birth or mare’s milk Se concentration at one month was used as a covariate)
The effect of dietary selenium source on colostrum and milk selenium concentrations in broodmares (n=10 and n=8 mares per group for colostrum and milk, respectively). Inorganic selenium (sodium selenite) or organic selenium (selenium yeast) was fed from two months before foaling to one month after foaling, to deliver 0.3 ppm supplementary selenium on a ration drymatter basis. Effect of time p < 0.05
Figure 4.6  Mean relative quantification of IL-2 expression of ConA stimulated lymphocytes of one month old foals born to mares receiving either inorganic or organic Se (number of foals per group: inorganic n=5, organic n=4). Effect of group p = 0.05
Figure 4.7 Mean relative quantification of IFNγ expression of ConA stimulated foal lymphocytes from foals born to mares receiving either inorganic or organic Se at a) one day and b) 30 days post partum (number of foals per group: a) inorganic n=3, organic n=4; b) inorganic n=5, organic n=6). Effect of group a) p = 0.072; b) p = 0.059
Figure 4.8  Mean relative quantification of TNFα expression of PMA stimulated foal lymphocytes (previously cryopreserved) from one month old foals born to mares receiving either inorganic or organic Se (number of foals per group: inorganic n=5, organic n=4). Effect of group p = 0.04
References


CHAPTER 5: DISCUSSION

Introduction
Selenium (Se) is an essential trace element and Se deficiency can have serious consequences for animal health. Selenium is incorporated into various selenoproteins and participates in many metabolic processes, including (and most notably) antioxidant defense mechanisms. For this reason, Se deficiency increases the risk of oxidative-induced damage to cells or cellular components. Plant-derived foods and feedstuffs are the major dietary source of Se for herbivores, as plants assimilate and metabolize Se, although Se is not essential to plant health and survival.

The Se status of horses not receiving Se supplementation is largely dependent on the geographical area in which they reside (1, 2). Selenium-deficient areas include most coastal regions and the area around the Great Lakes of North America, parts of Europe (Belgium, Sweden, Slovakia, France, Germany, Italy), parts of Asia (south-west China, Siberia, North Korea, Turkey), Egypt, and New Zealand. Many of these regions have significant livestock populations.

The most common clinical manifestation of Se deficiency in large animals is white muscle disease (WMD), mainly affecting neonates and young animals. Even though the roles of oxidants and antioxidants in the pathophysiology of WMD have been recognized for over half a century (3, 4), Se deficiency and WMD are still relatively common problems and diagnoses of Se deficiency and WMD are made each year in Se-deficient regions, including the teaching hospital of the Atlantic Veterinary College. In support of this empirical observation, a recent cross-sectional study of 201 horses living on Prince Edward Island (PEI) indicated that the majority of these horses were either deficient or
marginally deficient based on accepted references ranges for adult horses (5). Mean serum Se concentration of the horses studied by Muirhead et al. (article in press) differed by population group (5): racehorses and broodmares had significantly higher mean serum Se concentrations when compared to pleasure horses. The authors concluded from these results that Se deficiency is widespread in the horse population on PEI with pleasure horses being overrepresented. It was hypothesized that pleasure horses, in comparison to horses in training or broodmares, may receive less balanced concentrate feeds, and may therefore be particularly at risk for low blood Se concentrations.

In a similar survey of the Se status of 346 horses in Virginia and Maryland, researchers found a wide range of blood Se concentrations and the Se status was influenced by management practices, such as feeding of supplements, origin of hay, and time spent on pasture (6). These research results suggested that achieving Se adequacy was problematic in horse populations in many locations in North America, but that there was potential to favorably influence Se status through management.

Preliminary results from the survey of the PEI horse population (201 horses) by Muirhead et al. (5) led us to conclude that the majority of horses on PEI did not receive sufficient supplementary Se to ensure Se adequacy. In addition to WMD, impaired immune function has increasingly been associated with Se deficiency in other species, creating a risk for an increased susceptibility to disease.

The potential health implications of Se deficiency together with the lack of research concerning the relative effectiveness of the available modes of supplementation led to the program of research described in this thesis. We chose initially to examine a range of available Se supplementation methods and their effect of the Se status of horses, with the
final goal of being able to provide recommendations on effective Se supplementation of horses to horse owners/caretakers, veterinarians, and nutritionists.

**Research outcome: selenium supplementation**

In Chapters 2 and 3, the effectiveness of the following methods of Se supplementation was examined in adult horses: sodium selenite or Se yeast added to concentrate feed, provision of sodium selenite-containing trace mineral block, and Se-fortified hay harvested after application of Se-enriched fertilizer. All of these methods of Se supplementation were efficacious for raising measures of blood Se status into the range considered adequate, or close to adequate in the case of the Se-containing trace mineral blocks. In particular, the finding that application of Se-enriched fertilizer can increase the Se concentrations of fresh forage and hay, and that the feeding of Se-fortified hay can raise measures of Se status in blood from the range considered deficient or marginal to the range considered adequate, suggests that fortified hay can be used as an alternative Se source in horses, including pleasure horses not receiving significant Se on a regular basis via their concentrate ration.

Demonstrating the efficacy of selenized fertilizer for increasing the Se content of *in situ* pasture suggests that this might be a novel method of providing supplementary Se to horses spending significant parts of the year at pasture. Further studies on the duration of effect on pasture Se concentration of spring application of Se-enriched fertilizer, at various rates, would need to be performed before definitive recommendations could be made.
As a result of its metabolism, organic Se has greater bioavailability to mammals compared to inorganic Se (7-9) and organic Se products such as selenized yeast have been on the market for some years. Owners, managers and nutritional advisors often question whether such products should be included in equine rations on a regular basis, but there is little information in the literature regarding the relative efficacy of inorganic and organic Se in improving the Se status and health of Se-deficient horses. Our finding in Chapter 2 that organic Se is superior to inorganic Se when it comes to increasing the blood Se concentration of adult horses is in agreement with a study by Calamari et al. (10). To evaluate differences between inorganic and organic Se further, Calamari et al. (10) measured the proportion of total Se as selenomethionine and selenocysteine in blood and plasma of horses. They reported that selenocysteine was the predominant form of Se in blood and it increased after inorganic and organic Se supplementation. Furthermore, Calamari et al. (10) established that the difference in Se concentration between the horses receiving inorganic or organic Se was a result of a greater increase of selenomethionine in the blood and plasma of horses supplemented with organic Se. Therefore it seemed that the higher blood Se concentration after Se yeast supplementation is mainly in the form selenomethionine, the major component in Se yeast.

In our studies in adult horses (Chapters 2 and 3), we did not measure the different components of Se in the blood; however, short and long term effects of Se supplementation were measured by determination of plasma and red blood cell Se, respectively. Additionally, the activity of the selenocysteine-containing antioxidant enzyme glutathione peroxidase in blood was measured. Based on previous findings by Calamari et al. (10) and our findings in adult horses (Chapters 2 and 3), it appears that
blood glutathione peroxidase activity will increase in horses receiving Se supplementation, but its activity is not influenced by dietary Se source. This may partly be due to the fact that the higher concentration of Se in the blood after supplementation with organic Se is in the form of selenomethionine and not selenocysteine.

Broodmares and their foals are a group within the equine population that deserves special attention when it comes to Se status and supplementation. According to the findings by Muirhead et al. (article in press), many broodmares, out of 50 mares studied, on PEI had blood Se concentrations that placed them into the deficient or marginal range (6 % deficient, 66 % marginal, 28 % adequate) (5). Consequently, broodmares and their foals living on PEI and in other Se-deficient areas would greatly benefit from effective Se supplementation. With the study, described in Chapter 4, one of our aims was to determine if an organic Se source should be recommended over an inorganic Se source when supplemented to broodmares during late gestation and early lactation. The results showed that the Se source fed to mares during late pregnancy and early lactation can influence the red blood cell Se concentrations of their foals at one month of age, with organic Se resulting in significantly higher red blood cell Se when compared to inorganic Se; however, other measures of blood Se status did not differ between animals receiving inorganic or organic Se.

Prior to this research, only one study on the effect of dietary Se source on the Se status of broodmares and their offspring had been published (11), in which mares were supplemented with either organic or inorganic Se. With their study, Janicki et al. (11) showed that serum Se concentrations were greater in mares receiving organic Se when
compared to mares receiving inorganic Se. Foals born to mares receiving organic Se also
had higher serum Se concentrations (11). In our study, dietary Se source resulted in
differences in red blood cell Se concentration in one month old foals, but not in other
measures of Se status. In both studies, mares were supplemented for a similar duration at
the end of pregnancy with a similar amount of Se; however the basal Se content of the
diet and the initial Se status of the mares in the study by Janicki et al. (11) was not
specified, which may in part account for the differences observed.

Janicki et al. (11) found in the same study that colostrum and milk Se concentrations
were higher in mares receiving the organic Se supplement, which was not observed in our
study. This discrepancy may have been due in part to sampling technique; our study was
conducted in the field and obtaining milk samples of consistent volume and at a
consistent time post-suckle was difficult, which may have led to excessive variation in
the protein composition of the sample. As mentioned previously, differences between the
studies in the initial Se status of the mares may also have explained some of the
discrepancies in the results for colostrum and milk Se.

The observed difference in Se source on foal red blood cell Se in our study may have
been more prominent if supplementation of mares would have commenced earlier during
pregnancy which could be investigated in a future feeding trial. Furthermore, since
plasma and red blood cell Se of mares did not reach levels considered adequate for adult
horses, higher concentrations of dietary Se may be required for broodmares. The decision
to supplement 0.3 ppm Se and begin Se supplementation of the mares prior to their
anticipated due date had been based on results from the study in adult horses (Chapter 2),
where significant differences in measures of blood Se could be observed between the
inorganic and organic group two months into the feeding trial. However, a significant
difference between inorganic and organic Se supplementation could not found in the
broodmares at any time during the study (Chapter 4). The reason for this discrepancy is
unclear; one explanation may be that trans-placental transfer of Se from the dam to the
fetus could have occurred, decreasing the amount of Se available to the mare. To
determine the degree of trans-placental transfer of Se, Se would have to be measured in
the blood of the foal prior to colostrum intake. This was originally intended in the study
described in Chapter 4, however, for logistical reasons, a pre-suckle sample could not be
obtained from most of the foals. The degree to which Se is transferred across the placenta
and whether there is a difference in trans-placental transfer between inorganic and
organic Se has not been studied in horses, to date. The occurrence of trans-placental
transfer of Se has been demonstrated in cattle (12, 13), but transport mechanisms have
not been studied in domestic animal species. Earlier studies in people have shown that Se
utilizes sulfate transport mechanisms across the human placenta and the amount of Se
transported in this manner is influenced by the amount of sulfate present as the two
competitively inhibit each others uptake (14, 15). A similar Se transport mechanism may
also exist in domestic animal species, however, due to the different types of placentation
between the species one should not simply extrapolate from research in humans.

Although the effect of maternal dietary Se source on the Se status of the newborn has
been studied in cattle, pigs, and horses (11, 16, 17), none of the studies have specifically
addressed trans-placental Se transfer by obtaining pre-suckle samples. In all three
species, previous research has shown that Se yeast supplementation of mothers resulted
in higher blood Se in their offspring at birth (11, 16, 17). If more effective trans-placental
transfer of organic Se is partly responsible for this phenomenon that may be an explanation for the higher foal red blood cell Se in the organic group in our study (Chapter 4). The fact that this difference could not be observed at birth could be explained by the delayed increase seen in red blood cell Se after Se supplementation resulting from incorporation of Se into red blood cells at the time of erythropoiesis.

An additional finding which may have clinical implications, is that the blood Se status of mares during late pregnancy was found to be positively correlated with colostrum and early lactation milk Se concentrations. Measurement of blood Se levels of the dam prior to foaling may aid in identifying foals at risk of developing Se-responsive diseases.

As we learn more about Se nutrition in horses, we become more confident at making recommendations regarding Se supplementation in this species; however as is often the case, new questions arise and many things remain unclear. Still, valuable conclusions could be reached from the research reported in this thesis. We have confirmed earlier findings by Muirhead et al. (5; article in press) that measures of Se in the blood of horses fed locally grown feeds without Se supplementation fall in the range considered inadequate; that supplementation with Se through a range of modes (mineralized block, selenite, Se-yeast, fortified forage) will increase Se intake into the range considered adequate; that, similar to other species, supplementation of non-pregnant adult horses with organic forms of Se tends to lead to higher measures of Se status when compared to inorganic forms of Se. In broodmares, supplementation commencing earlier during pregnancy and using higher doses of Se should be studied to gain more insight, especially since the superiority of organic Se has been previously reported (11). Furthermore, providing horses with
adequate amounts of Se is not necessarily dependent on feeding of Se in a concentrate feed or mineral supplement since Se-fortified hay, and perhaps pasture, are viable and practical alternative sources of Se for adult horses. It would appear that the widespread occurrence of Se inadequacy on PEI is a reflection of management failure: it is likely that many owners do not follow the established recommendations for Se supplementation. However, when recommended methods and rates are followed by horse owners, they are likely to result in Se adequacy.

**Research outcome: selenium and immune function**

In addition to WMD, the most well studied clinical manifestation of Se deficiency in livestock, other Se-deficient states have been associated with a variety of *subclinical* problems in humans and animals, including reduced thyroid function, altered immunity, and increased susceptibility to disease. Since many disorders associated with suboptimal Se status do not present with specific clinical signs, tests related to Se status in the research areas of interest - such as antioxidant defense mechanisms, immune response, or thyroid function - are required.

Selenium deficiency has been shown to alter both innate and adaptive immunity, including neutrophil and macrophage function, lymphocyte function, and antibody production in humans and animals (18-22). Since disease prevention is an important aspect of veterinary medicine, we wished to investigate whether Se affects measures of immune function in horses. Identifying the most effective method of supplementation may help to prevent acute disease and control chronic conditions in horses by improving their immunity. To date, there are only two published studies in adult horses and ponies.
which have looked at the relationship between Se status and the immune response. Both studies investigated humoral immune response to supplementation with inorganic Se (18, 19).

The limited research of the effects of Se status and source of Se on the immune response of horses led us to investigate this relationship further. In Chapter 2 the effect of Se supplementation and Se source on neutrophil phagocytic activity, lymphocyte blastogenesis in response to the mitogen ConA, and relative cytokine gene expression of stimulated lymphocytes and neutrophils in adult horses are described. Results indicated that Se supplementation can increase the relative gene expression of IL-1 and IL-8 in LPS stimulated neutrophils with no effect of Se source in the case of IL-1 and inorganic Se supplementation resulting in higher gene expression when compared to organic Se supplementation in the case of IL-8. Selenium supplementation also appears to increase relative IL-5 gene expression in stimulated horse lymphocytes, with organic Se resulting in higher gene expression compared to inorganic Se. The results of the relative cytokine gene expression of adult horse lymphocytes and neutrophils are the first to indicate that Se source can influence lymphocyte and neutrophil function. The effect of Se supplementation on lymphocyte cytokine gene expression has not been previously reported in horses, but has been studied in mice (23). Results showed that lymphocytes from Se-adequate mice produced higher levels of IFNγ, IL-2, and IL-4 throughout the course of an infection, compared to Se-deficient mice. In our study in adult horses, no difference could be observed in IFNγ or IL-2 expression between the different treatment groups. Not finding a difference in relative IL-2 gene expression was consistent with the finding that there was no effect of Se supplementation or source on lymphocyte
blastogenesis in adult horses (Chapter 2), as IL-2 functions as a T-lymphocyte growth factor. Similarly, like IL-2, the amount of IFNγ expressed increases with lymphocyte proliferation and both the expression of IL-2 and IFNγ did increase in lymphocytes after stimulation, however there were no differences between the three treatment groups. Interleukin-4, the third cytokine with higher gene expression in Se-adequate mice (23), was not measured in the horses studied in Chapter 2. Interleukin-4 is involved in B-lymphocyte activation where it results in clonal expansion. Interleukin-5 has many roles when it comes to influencing immune function, but it is also involved in the later stages of B-lymphocyte activation; it contributes, together with IL-6, to the differentiation of B-lymphocytes into antibody-secreting plasma cells. Based on the findings by Wang et al. (2009) and our finding that IL-5 gene expression was affected by treatment, measurement of IL-4 expression should be considered in future equine studies.

The results obtained in mice and horses suggest that Se supplementation might influence immune function through alteration in cytokine gene expression, however, the exact mechanisms through which Se alters the immune response have not yet been determined. The effect of micronutrients on gene expression is an emerging area of research, and although only a few studies have investigated the effect of Se on gene expression, there is some evidence that Se exerts its influence at the molecular level. Based on previous research findings, which have demonstrated that Se can alter the activity of the transcription factor NF-κB (24-26), one may speculate that cytokine gene expression in our studies was influenced by Se through a similar mechanism. Activation of NF-κB by the Toll pathway results in the production of immune mediators, including
cytokines and chemokines (27). Specific toll-like receptors, which are the first component of this pathway, can be activated through binding of pathogens (28).

Research has shown, that Se, as part of selenoproteins, can both activate and inhibit NF-κB (29). Many questions remain as to how Se influences molecular mechanisms such as changes in NF-κB activity. It appears that both reactive oxygen species as well as thioredoxin-1, as part of the thioredoxin reductase system, can affect NF-κB activity. Contradicting findings of the molecular actions of Se, such as promotion versus inhibition of NF-κB, or cell growth, may result from differences in dose and source of Se provided; cellular redox balance has also been proposed as a determining factor (29).

According to results from a study in humans, Se supplementation (sodium selenite) can result in increased expression of genes involved in protein biosynthesis and gene translation in lymphocytes of healthy individuals (30). Based on these findings, the authors hypothesized that this up-regulation may result in increased protein synthesis in lymphocytes which may contribute to an increase in lymphocyte activity after Se supplementation. To conclude, there is evidence that Se can affect gene expression on a transcriptional and translational level, and these mechanisms may be involved in the changes seen in relative cytokine gene expression after Se supplementation.

If differences in Se status can affect the activity of NF-κB differently, one may speculate further that the dietary source of Se may indirectly affect cytokine gene expression because different dietary Se sources can result in a difference in Se status. Additionally, changes in Se-containing protein concentrations, or selenocysteine-containing enzyme activity, based on the dietary Se source provided, may affect the
transcription factor activity resulting in changes in gene expression. However, these relationships remain to be studied and are at this point purely speculative in horses.

The study presented in Chapter 2 contributed evidence that Se supplementation of Se-deficient adult horses can increase measures of innate and adaptive immune function, namely relative gene expression of neutrophil and lymphocyte cytokines. Based on the results from Chapter 2, no definitive conclusion can be made as to which Se source should be preferred for improving the immunity of adult horses.

New insights could be gained into the effects of Se supplementation and dietary source of Se on the immune function of horses. In the adult horses studied in Chapter 2, no difference was seen in the humoral immune response between the control and Se-supplemented groups. Neither Se supplementation nor the source of Se had an effect on antibody titers. These findings differ from earlier studies in horses and ponies, which did show a difference in humoral immune response based on Se status (18, 19). Our findings in horses are, however, similar to a study looking at antibody response to rabies vaccination in cattle by Reis et al. (31), which found that supplementation of Se-deficient 12 months old calves with different doses of Se, or no Se, did not affect their antibody titers. However, the amount of Se supplemented did affect the persistence of titers. Only animals receiving 3-6 mg Se daily had antibody titers considered to be protective against rabies for the entire study period of 120 days. As the horses in the present study received a booster vaccine, the duration of protective titers after only one vaccination could not be evaluated for the three groups, but could be subject to future research as it has been shown that rabies antibody titers will decline in horses that only receive one vaccination (32).
As a novel approach gene expression of neutrophil and lymphocyte cytokines was evaluated in this thesis and its use to measure immune function shows promise as a method to study the relationship between Se and immunity. Although no definitive conclusions could be drawn from the results reported in this thesis, further studies investigating changes in gene expression in response to Se supplementation are warranted.

In addition to studying the effects of dietary Se on measures of immune function in adult horses, we were interested in the effect of maternal dietary Se source on the immunity of their offspring. Within the horse population, foals are particularly susceptible to impairment of the immune response, especially if failure of passive transfer of immunity occurs. Additionally, the immune response of healthy foals has been shown to differ from that of adults, increasing the risk of certain diseases in young foals (33-37). Because enhancement of immunity may benefit foals during the “vulnerable period”, we were interested in studying the effect of different dietary Se sources on the immune response of young foals.

In the study described in Chapter 4, the aim was to determine if the dietary Se source offered to broodmares during late pregnancy and early lactation would influence measures of immune function in their foals. Results indicated that Se source may influence the relative cytokine gene expression of foal lymphocytes. Specifically, relative gene expression of IL-2 and TNFα appeared to be different between foals born to mares receiving organic Se and foals born to mares receiving inorganic Se. For IFNγ expression of stimulated lymphocytes, there was a trend toward a difference between foals born to
organic or inorganic Se-supplemented mares for both one day and one month post partum, but it was not statistically significant. Interestingly, there was a discrepancy as to which Se source led to higher relative gene expression of the different cytokines, similar to results in adult horses as reported in Chapter 2.

The study reported in Chapter 4 is the first to look at the effect of Se source on relative cytokine gene expression in cells of the immune system of foals. Generally speaking, few studies report on the effect of Se on gene expression.

To date, only one equine study has investigated the effect of Se source on the immune response of foals and found greater antibody titers to influenza virus in foals from mares supplemented with Se at a rate of 3 mg daily (11). Both inorganic and organic Se were used and compared in the study but no difference between the two Se sources could be found. The same researchers found that that Se source did not affect colostrum immunoglobulin (IgG) concentration or foal serum IgG at 12 hours; however, Se supplementation of mares with either source at a rate of 3 mg of Se per day was associated with a higher concentration of serum IgG in foals at two weeks of age (38). Taking this limited information into account, there is evidence to suggest that the Se status of the foal can affect its immune response; more work needs to be done, however, to establish the mechanisms through which this is accomplished.

Future research directions
Many questions remain with regard to Se research in horses. To date, the ideal amount of daily Se supplementation has yet to be established. The dilemma which practicing veterinarians and researchers face is that, even though the problems associated with Se
deficiency are well known, the intake and type of Se required to ensure optimal health are not. Current reference ranges are applied to all adult horses without taking into account their intended exercise level or reproductive status. No well-recognized guidelines for Se supplementation (dose, frequency, type) exist for horses. Since Se is essential for antioxidant defense mechanisms, Se requirements of horses may vary by their "pro-oxidant load". Pagan et al. (39) studied differences between inorganic and organic Se supplementation in exercising horses and found increased urinary excretion of Se in horses after exercise when these horses had previously received sodium selenite. The authors concluded from this finding that Se requirements of exercising horses may depend on the dietary source of Se. Based on their findings, horses that are exercised regularly and are receiving inorganic Se, may require higher a dose of inorganic Se compared to horses receiving organic Se for the same amount of exercise. It is in the interest of equine health that these issues need to be addressed and more specific information made available.

The current program of study was not designed to answer this question, but instead examined a range of supplementation methods, testing whether they result in blood Se measures that we currently consider to reflect adequacy. No regulation exists for Se supplementation of equine feeds for either amount or source of Se and only guidelines are provided by the National Research Council (NRC) (40). The Se requirement for mature idle horses was estimated by the NRC to be 0.1 ppm (0.1 mg/kg) of diet dry matter, however, it has been suggested that 0.3 ppm would be a more appropriate recommendation for exercising horses (39). Calamari et al. (10) did evaluate different doses of Se yeast (0.2, 0.3, and 0.4 mg/kg dry matter) in their study in adult horses and
found a linear dose effect for blood Se concentration, but all three doses resulted in plasma Se within the adequate range. To date, there are no specific guidelines for broodmares or horses with different workloads and similar reference ranges are applied to all adult horses.

In an ideal world, uniform recommendations for the amount of Se supplementation and desired blood Se status should be established for the different groups within the horse population. The challenge we are facing in this case is that Se deficiency does not usually present with clinical signs of WMD, the basis for the current reference ranges, is not the main concern, particularly in adult horses. Other considerations when trying to determine the level of Se supplementation and the desired blood Se status include the role of Se in thyroid function and immunity, amongst others. Additionally, there are several areas of Se research in humans and other animal species, which have not yet been explored in horses, but may also be relevant to equine health, including allergic processes, aging and fetal development.

The host antioxidant status has been studied in other species by researchers looking at the immune response during allergic diseases such as asthma. Results have been contradictory: Se plays a role in reducing oxidative stress in the lower airways during asthma, but also has an enhancing effect on the immune responses, mainly T helper 2 responses, that are part of the pathophysiology of asthma (41, 42). Glutathione peroxidase activity appears to be up-regulated in severe asthma (43), which is a likely explanation for the protective effect of Se against oxidative stress. A positive correlation between antioxidant (vitamin C and Se) levels and allergen responses to house dust mites
was demonstrated in another study (41), raising the question as to whether antioxidant supplementation of asthma patients may increase disease severity.

A study performed in mice found that Se intake and airway inflammation do not appear to be related in a simple dose-dependent manner (44). The same study also found an up-regulation of glutathione peroxidase-1 and selenoprotein P within the lung of the mice after antigen challenge. Since allergic airway inflammation, including inflammatory airway disease and recurrent airway obstruction, is a common problem of horses this may be an interesting research focus for investigating the effect of Se supplementation and Se source. Studies have shown that recurrent airway obstruction in horses is associated with evidence of oxidative stress in the lung (45) as well as lower levels of ascorbic acid (46). Furthermore, one study found that acute organic dust exposure resulted in significant antioxidant depletion in the trachea, which may reflect inflammation and oxidative processes in peripheral airways (47). There is some evidence that antioxidant supplementation of horses with recurrent airway obstruction may be beneficial. In one study antioxidant treatment (consisting of vitamins E and C, and Se) significantly improved exercise tolerance and significantly reduced endoscopic inflammatory score in horses with recurrent airway obstruction (48). In this study, plasma uric acid concentrations were significantly reduced, suggesting down-regulation of the xanthine-dehydrogenase and xanthine-oxidase pathways.

Oxidative stress also plays an important role in the aging process. Aging is accompanied by chronic inflammation and oxidative stress, leading to an impairment of immune function. During the process of aging, an imbalance develops between prooxidants and antioxidant with a shift towards prooxidants. The host antioxidant status
has been studied in relation to immune function during aging in mice and humans.
Research in prematurely aging mice suggests that supplementation with adequate levels
of antioxidants may help to improve leukocyte function and restore redox balance (49).
Leukocytes depend on both oxidant and proinflammatory compounds to carry out
different functions, including activation, proliferation, differentiation, and phagocytosis,
and the leukocytes themselves may be affected by oxidative damage by production of
reactive oxygen species. Supplementation of Se and other antioxidants appears to
improve innate as well as acquired immune function in the elderly (50). Previous research
has proven an altered immune response in old horses (32, 51, 52), including elevated
cytokine production characteristic of “inflamm-aging”, reduced antibody response to
influenza vaccination, and decreased T-lymphocyte proliferation. The effect of Se status
and source on immune function of old horses has not been investigated to date and may
be another relevant area of future research.

Besides its integral role in antioxidant defense mechanisms, Se status also affects
endocrine function. Thyroid hormones are essential for many aspects of normal
metabolism, growth and development. In addition to iodine, which is a central component
of thyroid hormones, Se is essential for thyroid hormone synthesis, activation, and
metabolism (53). The relationship between Se status and thyroid hormone concentrations
has been investigated in domestic animals. One study in cows grazing pasture with a low
Se content revealed low serum T3 concentrations (54). In another study, Se
supplementation increased the plasma concentration of T3 and the ratio of T3:T4 (55). In
lambs with Se deficiency myopathy, serum T3 concentrations and the T3:T4 ratio were
significantly decreased (56, 57). In contrast, a study in adult horses found that plasma thyroid hormone levels were not affected by Se source or dose (10).

Normal term foals have been shown to have markedly increased circulating concentrations of free thyroid hormones at birth whereas premature foals have significantly lower serum concentrations of total and free fractions of thyroid hormones compared to normal foals (58). Thyroid insufficiency may be involved in the development of problems commonly seen in premature foals such as inability to maintain body temperature, decreased surfactant production leading to respiratory distress syndrome and hyaline membrane disease, and musculoskeletal abnormalities. Since Se status affects thyroid hormone metabolism it would be interesting to investigate the effect of Se status on thyroid hormone metabolism in term and premature foals, and to determine if Se could alleviate some of the problems associated with prematurity in foals. In this context, the effect of prenatal Se supplementation should also be considered.

Both prenatal and postnatal Se supplementation appears to be essential for antioxidant function of the offspring. In pigs a low-Se diet fed during gestation led to an increase in fetal oxidative stress, measured as fetal liver hydrogen peroxide and malondialdehyde (MDA), an indicator of lipid peroxidation (59). It appears that Se intake during gestation can affect levels of mRNA encoding for glutathione peroxidase-1 during porcine fetal development (60). After research had shown that selenomethionine can influence gene expression in the intestine of the mouse (61), a study in chickens found that maternal supplementation with vitamins and trace minerals, including Se, can influence intestinal development of the offspring through alteration of gene expression in the intestine (62). The study in mice indicated that a low-Se status can up-regulate genes involved in DNA
damage and oxidative stress and down-regulate the expression of genes involved in
detoxification (61). Based on the evidence for the importance of Se during development,
a study similar to the study described in Chapter 4 should be conducted, but Se
supplementation should begin prior to pregnancy.

The effect of nutritional status on DNA damage and repair is the focus of a research
area called nutrigenomics, which investigates the effect of diet on gene expression (63-
65). The risk for developmental and degenerative disease increases with DNA damage
which is dependent on nutritional status including optimal concentrations of
micronutrients. As mentioned previously, a study in mice indicated that a low-Se status
up-regulates genes involved in DNA damage and oxidative stress and down-regulates the
expression of genes involved in detoxification (61). A recent study investigating the
effect of selenomethionine on genome stability measured in human peripheral blood
lymphocytes found that Se as selenomethionine may improve genome stability at
concentrations up to 430 μg Se/liter, but that higher doses may be cytotoxic (66). The
field of nutrigenomics could be another research focus for the important antioxidant
micronutrient Se especially if Se is used at supranutritional levels. Research to date
shows that higher levels of Se may be required for the influence of Se status on the
immune system compared to the antioxidant status of the individual (11, 67, 68);
however, very high levels of Se are cytotoxic and may result in undesired effects.

Concluding remarks
To conclude, Se is an essential micronutrient of horses, including its effect on the
antioxidant status and immune response. Many areas of Se research still need to be
investigated in the horse, including the role of Se during development, the effect of Se on thyroid function of the neonate, the effect of Se on the immune response of old horses, the effect of Se on chronic inflammatory processes such as recurrent airway obstruction, and the effect of different Se levels on genome health.

We have learned from the work summarized in this thesis that different methods of Se supplementation, including Se-fortified forage, can be used to improve the Se status of horses in Se-deficient regions. In adult, non-pregnant horses, organic Se is consistently superior to inorganic Se in raising blood Se; however, this effect of dietary Se source could not be demonstrated as clearly in broodmares and their foals. We have further gained insight that Se may contribute to alterations in immune function of horses through changes of cytokine gene expression, however, the significance of these observations remains to be clarified.
References


64. Fenech M. Genome health nutrigenomics and nutrigenetics--diagnosis and nutritional treatment of genome damage on an individual basis. Food Chem Toxicol 2008;46(4):1365-70.


APPENDIX A: DETERMINATION OF SELENIUM

Fluorimetric determination of Se in biological material (Excerpt from Animal Health Center SOP # TOX-016-02)

Purpose

The fluorometric determination of selenium in biological material.

Principle of Method

Samples are digested with a mixture of perchloric and nitric acids at high temperature and the selenium is converted to the $\text{Se}^{4+}$ form to react with 2,3-diaminonaphthalene. The resulting complex is extracted into cyclohexane and its fluorescence (excitation 375 nm and emission 525 nm) is quantitatively measured.

Quality Control

Control sample with known value of Se.

References


Detection limit

0.005 μg Se/g sample
Determination of plasma and whole blood Se using atomic absorption (AA) spectrophotometry (Excerpt from AVC Toxicology and Analytical Services SOP)

Purpose
To determine the amount of Se in animal plasma/whole blood.

Principle of Method
Plasma/whole blood is analysed by a method of additions calibration using a palladium-magnesium nitrate matrix modifier on a furnace AA. A Se lamp emits light at a wavelength which is absorbed by Se as it is released during atomization. The amount absorbed is detected, then quantitated by method of additions calibration using plasma/whole blood with a known value of Se spiked with 0.05, 0.15, and 0.30 ppm Se standards.

Quality Control
Run one plasma/whole blood sample with known value of Se for each 10 unknowns, at the beginning, middle, and end of runs.

Reference
Determination of Se in human serum by GFAAS with transverse heated graphite atomizer and longitudinal Zeeman effect background correction. Michaela Feuerstein and Gerhard Schlemmer, Perkin Elmer Bodenseewerk, Uberlingen, Germany, 2000.

Detection limit
0.005 ppm
**APPENDIX B: BASAL DIET CALCULATION FOR HORSES IN CHAPTER 2**

<table>
<thead>
<tr>
<th><strong>Dry matter basis</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (%)</td>
<td>90</td>
</tr>
<tr>
<td>Crude Protein (%)</td>
<td>9.65</td>
</tr>
<tr>
<td>ADF (%)</td>
<td>36.52</td>
</tr>
<tr>
<td>TDN (%)</td>
<td>59.93</td>
</tr>
<tr>
<td>NEL (Mcal/kg)</td>
<td>1.28</td>
</tr>
<tr>
<td>EST D.E. (Mcal/kg)</td>
<td>2.53</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>0.49</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>0.28</td>
</tr>
<tr>
<td>Magnesium (%)</td>
<td>0.22</td>
</tr>
<tr>
<td>Potassium (%)</td>
<td>1.33</td>
</tr>
<tr>
<td>Copper (ppm)</td>
<td>12.16</td>
</tr>
<tr>
<td>Zinc (ppm)</td>
<td>21.84</td>
</tr>
<tr>
<td>NEg (Mcal/kg)</td>
<td>0.72</td>
</tr>
<tr>
<td>NEm (Mcal/kg)</td>
<td>1.42</td>
</tr>
</tbody>
</table>

Digestible energy: 2.53 Mcal/kg DM

NRC maintenance requirements for digestible energy: 16.7 Mcal/kg (average, 500kg live weight)

\[
16.7/2.53 = 6.6 \text{ kg hay (DM per head per day)}
\]
APPENDIX C: PILOT TRIAL LYMPHOCYTE BLASTOGENESIS

During the pilot trial, concentrations of 0.5, 1, 2, and $5 \times 10^6$ cells/ml were used and $2 \times 10^6$ cells/ml appeared to produce the best proliferation as determined by SI. The concentrations of ConA used in the trial were 10, 5, 2, 1, and 0.5 µg/ml. The cells were incubated for 48 hours or 72 hours. The results of the pilot study indicated that $2 \times 10^6$ cells/ml, incubated for 48 hours with 5 and 2 µg/ml of ConA yielded the best results in regards to cell proliferation.
APPENDIX D: PILOT TRIAL NEUTROPHIL PHAGOCYTOSIS

Before the study, a pilot trial was performed to assess different methods to inhibit phagocytosis. Other methods used, which were not successful in inhibiting phagocytosis, included cytochalasin B (3μl/0.5x10^6 cells) at room temperature, cytochalasin B (3μl/0.5x10^6 cells) at 37°C, cytochalasin B (30μl/0.5x10^6 cells) at room temperature, and incubation at 4°C. The attempt to quench fluorescence of adhered beads with trypan blue (200μg/ml) was also unsuccessful.
### Table 1

Lymphocytes blastogenesis (mean ± SE) in response to two concentrations of Con A at study start, 1 and 3 months after dietary assignment, in adult horses (n=5 per group) receiving supplementary inorganic selenium, organic selenium, or receiving no supplementary selenium.

<table>
<thead>
<tr>
<th></th>
<th>Study start</th>
<th>1 month</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Con A 5 μg/ml</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Se</td>
<td>28.2 (3.0)</td>
<td>12.2 (1.8)</td>
<td>42.0 (7.5)</td>
</tr>
<tr>
<td>Inorganic Se</td>
<td>35.4 (7.9)</td>
<td>15.6 (3.3)</td>
<td>38.2 (8.9)</td>
</tr>
<tr>
<td>Organic Se</td>
<td>33.4 (8.6)</td>
<td>10.6 (2.6)</td>
<td>47.4 (21.0)</td>
</tr>
<tr>
<td><strong>Con A 2 μg/ml</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Se</td>
<td>59.6 (8.3)</td>
<td>67.2 (12.2)</td>
<td>70.6 (13.0)</td>
</tr>
<tr>
<td>Inorganic Se</td>
<td>78.2 (22.2)</td>
<td>80.4 (17.6)</td>
<td>91.2 (26.6)</td>
</tr>
<tr>
<td>Organic Se</td>
<td>84.2 (20.7)</td>
<td>67.4 (7.0)</td>
<td>79.0 (13.9)</td>
</tr>
</tbody>
</table>
APPENDIX F: NEUTROPHIL PHAGOCYTOSIS DATA FROM CHAPTER 2

**Table 1** Mean (± SE) neutrophil phagocytic activity (%) at study start, 1 and 3 months after dietary assignment, in adult horses (n=5 per group) receiving supplementary inorganic selenium, organic selenium, or receiving no supplementary selenium

<table>
<thead>
<tr>
<th></th>
<th>Study start</th>
<th>1 month</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phagocytosis (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Se</td>
<td>5.8 (2.6)</td>
<td>15.6 (2.2)</td>
<td>20.8 (1.8)</td>
</tr>
<tr>
<td>Inorganic Se</td>
<td>4.2 (1.4)</td>
<td>16.8 (2.8)</td>
<td>19.8 (3.6)</td>
</tr>
<tr>
<td>Organic Se</td>
<td>5.8 (1.7)</td>
<td>14.4 (3.9)</td>
<td>32 (8.3)</td>
</tr>
</tbody>
</table>
APPENDIX G: CYTOKINE GENE EXPRESSION DATA FOR CHAPTER 2

Table 1  Relative cytokine gene expression of stimulated lymphocytes (mean RQ ± SE) 1 and 3 months after dietary assignment in adult horses (n=5 per group) receiving supplementary inorganic selenium, organic selenium, or receiving no supplementary selenium

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>No selenium</th>
<th>Inorganic selenium</th>
<th>Organic selenium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 month</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>161.68 (22.08)</td>
<td>185.24 (68.97)</td>
<td>249.74 (100.74)</td>
</tr>
<tr>
<td>IL-2</td>
<td>5.89 (1.78)</td>
<td>5.46 (1.52)</td>
<td>6.28 (1.78)</td>
</tr>
<tr>
<td>IL-5</td>
<td>85.55 (9.08)</td>
<td>222.32 (81.45)</td>
<td>493.75 (275.81)</td>
</tr>
<tr>
<td>IL-10</td>
<td>98.64 (18.37)</td>
<td>139.98 (37.70)</td>
<td>148.05 (27.52)</td>
</tr>
<tr>
<td>TNFα</td>
<td>17.05 (4.26)</td>
<td>20.66 (7.25)</td>
<td>23.61 (3.87)</td>
</tr>
<tr>
<td></td>
<td>3 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>75.02 (28.80)</td>
<td>87.17 (35.72)</td>
<td>88.02 (41.20)</td>
</tr>
<tr>
<td>IL-2</td>
<td>3.29 (0.82)</td>
<td>5.78 (2.98)</td>
<td>3.29 (1.01)</td>
</tr>
<tr>
<td>IL-5</td>
<td>32.02 (11.69)</td>
<td>64.77 (54.40)</td>
<td>67.48 (22.06)</td>
</tr>
<tr>
<td>IL-10</td>
<td>66.87 (15.25)</td>
<td>145.40 (58.33)</td>
<td>68.75 (19.97)</td>
</tr>
<tr>
<td>TNFα</td>
<td>10.02 (1.77)</td>
<td>13.34 (4.06)</td>
<td>10.58 (3.36)</td>
</tr>
</tbody>
</table>
Table 2  Stimulation indices of relative lymphocyte cytokine gene expression (mean RQ ± SE) 1 and 3 months after dietary assignment in adult horses (n=5 per group) receiving supplementary inorganic selenium, organic selenium, or receiving no supplementary selenium.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>No selenium</th>
<th>Inorganic selenium</th>
<th>Organic selenium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 month</td>
<td>3 months</td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>23.69 (7.77)</td>
<td>58.26 (35.56)</td>
<td>31.00 (9.14)</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.55 (0.40)</td>
<td>1.20 (0.47)</td>
<td>2.09 (1.01)</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.28 (0.42)</td>
<td>2.17 (0.73)</td>
<td>7.69 (3.72)</td>
</tr>
<tr>
<td>IL-10</td>
<td>7.26 (1.32)</td>
<td>14.59 (7.42)</td>
<td>19.08 (10.33)</td>
</tr>
<tr>
<td>TNFα</td>
<td>3.66 (1.26)</td>
<td>3.68 (1.35)</td>
<td>3.68 (0.60)</td>
</tr>
<tr>
<td></td>
<td>3 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>19.99 (9.88)</td>
<td>14.22 (4.22)</td>
<td>31.64 (18.52)</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.28 (0.46)</td>
<td>0.93 (0.38)</td>
<td>1.37 (0.63)</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.23 (0.47)</td>
<td>0.16 (0.07)</td>
<td>3.44 (2.31)</td>
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<tr>
<td>IL-10</td>
<td>6.16 (1.95)</td>
<td>15.47 (8.79)</td>
<td>14.67 (7.00)</td>
</tr>
<tr>
<td>TNFα</td>
<td>3.18 (0.64)</td>
<td>3.63 (0.89)</td>
<td>3.79 (1.15)</td>
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</table>
Table 3  Cytokine gene expression of stimulated neutrophils (mean RQ ± SE) 1 and 3 months after dietary assignment in adult horses (n=5 per group) receiving supplementary inorganic selenium, organic selenium, or receiving no supplementary selenium

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>No selenium</th>
<th>Inorganic selenium</th>
<th>Organic selenium</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1 month</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1</td>
<td>6.025 (0)</td>
<td>87.162 (6.281)</td>
<td>40.288 (3.182)</td>
</tr>
<tr>
<td>IL-8</td>
<td>14.961 (0)</td>
<td>178.782 (13.158)</td>
<td>32.444 (14.749)</td>
</tr>
<tr>
<td></td>
<td>3 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1</td>
<td>14.118 (7.926)</td>
<td>28.065 (8.145)</td>
<td>21.300 (9.808)</td>
</tr>
<tr>
<td>IL-8</td>
<td>10.272 (4.384)</td>
<td>78.434 (9.996)</td>
<td>18.424 (4.628)</td>
</tr>
</tbody>
</table>
Table 4  Stimulation indices of relative neutrophil cytokine gene expression (mean RQ ± SE) 1 and 3 months after dietary assignment in adult horses (n=5 per group) receiving supplementary inorganic selenium, organic selenium, or receiving no supplementary selenium

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>No selenium</th>
<th>Inorganic selenium</th>
<th>Organic selenium</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>0.596 (0)</td>
<td>4.702 (0)</td>
<td>0.639 (0.427)</td>
</tr>
<tr>
<td>IL-8</td>
<td>3.918 (0)</td>
<td>2.307 (2.143)</td>
<td>0.415 (0.282)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>No selenium</th>
<th>Inorganic selenium</th>
<th>Organic selenium</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>1.481 (0.956)</td>
<td>0.768 (0.1258)</td>
<td>1.122 (0.808)</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.563 (0.281)</td>
<td>1.840 (0.737)</td>
<td>1.219 (0.610)</td>
</tr>
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<td></td>
<td>Timothy field</td>
<td>Timothy/Alfalfa field</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------</td>
<td>-----------------------</td>
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</tr>
<tr>
<td>Organic matter (%)</td>
<td>3.1</td>
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</tr>
<tr>
<td>pH</td>
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<td>645</td>
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<td>Potash (ppm)</td>
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<td>Calcium (ppm)</td>
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<td>Magnesium (ppm)</td>
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<tr>
<td>Boron (ppm)</td>
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<tr>
<td>Copper (ppm)</td>
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<td>Zinc (ppm)</td>
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<td>Manganese (ppm)</td>
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<td>Iron (ppm)</td>
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<tr>
<td>Sodium (ppm)</td>
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<td>19</td>
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<tr>
<td>Aluminum (ppm)</td>
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<td>1008</td>
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<tr>
<td>Lime index</td>
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APPENDIX I: HAY ANALYSIS CHAPTER 3

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<th>Timothy</th>
<th>Timothy/Alfalfa</th>
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<tr>
<td>Dry matter (%)</td>
<td>85.9</td>
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<tr>
<td>Crude Protein (%)</td>
<td>9.54</td>
<td>10.13</td>
</tr>
<tr>
<td>ADF (%)</td>
<td>35.81</td>
<td>38.31</td>
</tr>
<tr>
<td>NDF (%)</td>
<td>61.48</td>
<td>66.51</td>
</tr>
<tr>
<td>TDN (%)</td>
<td>60.99</td>
<td>56.43</td>
</tr>
<tr>
<td>NEL (Mcal/kg)</td>
<td>1.30</td>
<td>1.23</td>
</tr>
<tr>
<td>EST D.E. (Mcal/kg)</td>
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<td>Calcium (%)</td>
<td>0.34</td>
<td>0.29</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
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<td>Magnesium (%)</td>
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</tr>
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<td>Potassium (%)</td>
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<td>Copper (ppm)</td>
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<td>4.31</td>
</tr>
<tr>
<td>Zinc (ppm)</td>
<td>21.00</td>
<td>14.77</td>
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<tr>
<td>NEg (Mcal/kg)</td>
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<td>0.57</td>
</tr>
<tr>
<td>NEm (Mcal/kg)</td>
<td>1.45</td>
<td>1.27</td>
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</tbody>
</table>
APPENDIX J: QUESTIONNAIRE FOR OWNERS OF MARES IN CHAPTER 4

Questionnaire

Name of mare:
Age:
Owned since:
Number of foals:
Previous health problems:
Previous reproductive problems:
Problems with previous foals:
Vaccinations status:
De-worming protocol:
Pasture turn out:
Feed (type/amount):
Supplements:
Feed storage:
<table>
<thead>
<tr>
<th>Dry matter basis</th>
<th>Farm 1</th>
<th>Farm 2</th>
<th>Farm 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (%)</td>
<td>90.8</td>
<td>93.9</td>
<td>94.8</td>
</tr>
<tr>
<td>Crude Protein (%)</td>
<td>6.28</td>
<td>8.32</td>
<td>6.75</td>
</tr>
<tr>
<td>ADF (%)</td>
<td>40.25</td>
<td>35.58</td>
<td>46.30</td>
</tr>
<tr>
<td>TDN (%)</td>
<td>54.37</td>
<td>61.33</td>
<td>45.34</td>
</tr>
<tr>
<td>NEL (Mcal/kg)</td>
<td>1.16</td>
<td>1.31</td>
<td>0.97</td>
</tr>
<tr>
<td>EST D.E. (Mcal/kg)</td>
<td>2.31</td>
<td>2.58</td>
<td>1.97</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>0.14</td>
<td>0.36</td>
<td>0.43</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>0.20</td>
<td>0.19</td>
<td>0.17</td>
</tr>
<tr>
<td>Magnesium (%)</td>
<td>0.08</td>
<td>0.20</td>
<td>0.18</td>
</tr>
<tr>
<td>Potassium (%)</td>
<td>1.57</td>
<td>0.82</td>
<td>1.15</td>
</tr>
<tr>
<td>Copper (ppm)</td>
<td>2.65</td>
<td>3.05</td>
<td>3.29</td>
</tr>
<tr>
<td>Zinc (ppm)</td>
<td>19.51</td>
<td>13.43</td>
<td>12.86</td>
</tr>
<tr>
<td>NEg (Mcal/kg)</td>
<td>0.58</td>
<td>0.76</td>
<td>0.35</td>
</tr>
<tr>
<td>NEm (Mcal/kg)</td>
<td>1.28</td>
<td>1.46</td>
<td>1.05</td>
</tr>
</tbody>
</table>
APPENDIX L: RATION COMPOSITION FOR MARES IN CHAPTER 4

<table>
<thead>
<tr>
<th>Mare and foal ration</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>% Dry matter</td>
<td>90</td>
</tr>
<tr>
<td>Energy</td>
<td>3.50 MCal</td>
</tr>
<tr>
<td>Crude protein</td>
<td>160.00 g</td>
</tr>
<tr>
<td>Lysine</td>
<td>8.03 g</td>
</tr>
<tr>
<td>Calcium</td>
<td>9.70 g</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>5.70 g</td>
</tr>
<tr>
<td>Magnesium</td>
<td>2.36 g</td>
</tr>
<tr>
<td>Sodium</td>
<td>4.00 g</td>
</tr>
<tr>
<td>Iron</td>
<td>37.20 mg</td>
</tr>
<tr>
<td>Copper</td>
<td>36.50 mg</td>
</tr>
<tr>
<td>Manganese</td>
<td>60.30 mg</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.15 mg</td>
</tr>
<tr>
<td>Selenium</td>
<td>2.93 mg</td>
</tr>
<tr>
<td>Cobalt</td>
<td>9570.00 IU</td>
</tr>
<tr>
<td>Iodine</td>
<td>2145.00 IU</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>200.00 IU</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.73 mg</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>2.64 mg</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>0.74 mg</td>
</tr>
<tr>
<td>Niacin</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>15.39 mg</td>
</tr>
<tr>
<td>Calcium/Phosphorus-ratio</td>
<td>0.75 mg</td>
</tr>
<tr>
<td>Crude protein</td>
<td>45.7 g/MCal</td>
</tr>
</tbody>
</table>

214
APPENDIX M: CONSENT FORM FOR OWNERS OF MARES IN CHAPTER 4

Consent Form

Hereby I,  , give permission to include my horse,  , in the study entitled The effect of organic and inorganic selenium supplementation on selenium status of mares and selenium status and immune function of their foals performed by J. Wichtel, F. Markham, J McClure, M. McNiven, M. Wichtel, and J. Montgomery.

I understand and agree that the above named animal (and her foal) can receive no other selenium supplementation or selenium and vitamin E injection for the duration of the study.

I understand that I am responsible for initiating treatment and covering any medical cost that might be necessary after the routine health check of my foal, that is part of the study.

____________________  ______________________
Name                  Date
APPENDIX N: FOAL SERUM IMMUNOGLOBULIN CONCENTRATION

Table 1 Foal serum immunoglobulin concentration (mg/dl) from screening tests and radial immunodiffusion (RID); group 1 = organic Se, group 2 = inorganic Se

<table>
<thead>
<tr>
<th>Foal ID</th>
<th>Group</th>
<th>Glutaraldehyde coagulation test</th>
<th>SNAP® test</th>
<th>RID</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>1</td>
<td>&gt;800</td>
<td>N/A</td>
<td>1567</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>&gt;800</td>
<td>N/A</td>
<td>1271</td>
</tr>
<tr>
<td>G</td>
<td>1</td>
<td>400-800</td>
<td>400-800</td>
<td>342</td>
</tr>
<tr>
<td>H</td>
<td>1</td>
<td>&gt;800</td>
<td>N/A</td>
<td>1652</td>
</tr>
<tr>
<td>J</td>
<td>1</td>
<td>400-800</td>
<td>400-800</td>
<td>520</td>
</tr>
<tr>
<td>N</td>
<td>1</td>
<td>&gt;800</td>
<td>N/A</td>
<td>1144</td>
</tr>
<tr>
<td>R</td>
<td>1</td>
<td>&gt;800</td>
<td>N/A</td>
<td>1206</td>
</tr>
<tr>
<td>T</td>
<td>1</td>
<td>&gt;800</td>
<td>N/A</td>
<td>2650</td>
</tr>
<tr>
<td>U</td>
<td>1</td>
<td>&gt;800</td>
<td>N/A</td>
<td>1835</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>&gt;800</td>
<td>N/A</td>
<td>2148</td>
</tr>
<tr>
<td>D</td>
<td>2</td>
<td>&lt;400</td>
<td>&lt;400</td>
<td>92</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>&gt;800</td>
<td>N/A</td>
<td>1271</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>&gt;800</td>
<td>N/A</td>
<td>2650</td>
</tr>
<tr>
<td>K</td>
<td>2</td>
<td>&gt;800</td>
<td>N/A</td>
<td>No sample</td>
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<tr>
<td>M</td>
<td>2</td>
<td>&gt;800</td>
<td>N/A</td>
<td>3826</td>
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<tr>
<td>O</td>
<td>2</td>
<td>&lt;400</td>
<td>&lt;400</td>
<td>308</td>
</tr>
<tr>
<td>Q</td>
<td>2</td>
<td>&lt;400</td>
<td>&lt;400</td>
<td>1339</td>
</tr>
<tr>
<td>S</td>
<td>2</td>
<td>&gt;800</td>
<td>N/A</td>
<td>1741</td>
</tr>
</tbody>
</table>
Table 1 Relative cytokine gene expression of ConA stimulated foal lymphocytes mean (± SE) one day post partum; number of foals per group specified for each cytokine

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Organic Se</th>
<th>Inorganic Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>19.098 (10.998)</td>
<td>4.017 (2.232)</td>
</tr>
<tr>
<td></td>
<td>n=4</td>
<td>n=3</td>
</tr>
<tr>
<td>IL-2</td>
<td>21.223 (10.820)</td>
<td>3.573 (1.017)</td>
</tr>
<tr>
<td></td>
<td>n=3</td>
<td>n=3</td>
</tr>
<tr>
<td>IL-5</td>
<td>not enough observations</td>
<td>not enough observations</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.917 (1.329)</td>
<td>0.750 (0.249)</td>
</tr>
<tr>
<td></td>
<td>n=3</td>
<td>n=3</td>
</tr>
<tr>
<td>TNFα</td>
<td>1.849 (0.716)</td>
<td>1.400 (0.480)</td>
</tr>
<tr>
<td></td>
<td>n=4</td>
<td>n=4</td>
</tr>
</tbody>
</table>
Table 2  Relative cytokine gene expression of ConA stimulated foal lymphocytes mean (± SE) one month post partum, number of foals per group specified for each cytokine

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Organic Se</th>
<th>Inorganic Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>14.803 (6.566)</td>
<td>43.769 (16.087)</td>
</tr>
<tr>
<td></td>
<td>n=6</td>
<td>n=5</td>
</tr>
<tr>
<td>IL-2</td>
<td>3.012 (1.221)</td>
<td>1.016 (0.277)</td>
</tr>
<tr>
<td></td>
<td>n=4</td>
<td>n=5</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.533 (0.276)</td>
<td>0.492 (0.224)</td>
</tr>
<tr>
<td></td>
<td>n=3</td>
<td>n=4</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.310 (0.071)</td>
<td>0.685 (0.230)</td>
</tr>
<tr>
<td></td>
<td>n=5</td>
<td>n=5</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.421 (0.207)</td>
<td>1.253 (0.387)</td>
</tr>
<tr>
<td></td>
<td>n=4</td>
<td>n=5</td>
</tr>
</tbody>
</table>
Table 3  Relative cytokine gene expression of PMA stimulated foal lymphocytes mean (± SE) one month post partum (cryopreserved cells), number of foals per group specified for each cytokine

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Organic Se</th>
<th>Inorganic Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>16.401 (2.372)</td>
<td>20.794 (6.521)</td>
</tr>
<tr>
<td></td>
<td>n=4</td>
<td>n=5</td>
</tr>
<tr>
<td>IL-2</td>
<td>89.225 (21.961)</td>
<td>120.436 (56.561)</td>
</tr>
<tr>
<td></td>
<td>n=4</td>
<td>n=5</td>
</tr>
<tr>
<td>IL-5</td>
<td>not enough observations</td>
<td>not enough observations</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.395 (0.124)</td>
<td>0.916 (0.314)</td>
</tr>
<tr>
<td></td>
<td>n=4</td>
<td>n=4</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.951 (0.230)</td>
<td>1.409 (0.309)</td>
</tr>
<tr>
<td></td>
<td>n=4</td>
<td>n=5</td>
</tr>
</tbody>
</table>
Table 4  Relative cytokine gene expression of LPS stimulated foal neutrophils mean (± SE) one month post partum, number of foals per group specified for each cytokine

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Organic Se</th>
<th>Inorganic Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>0.9086 (0.171)</td>
<td>1.5341 (0.294)</td>
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<tr>
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<td>n=6</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.9825 (0.958)</td>
<td>1.3023 (0.404)</td>
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<tr>
<td></td>
<td>n=8</td>
<td>n=7</td>
</tr>
</tbody>
</table>