

**Immune Response to Vaccination and Selenium and Vitamin E Status
in Horses of Varying Ages**

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Abstract

Immunosenescence refers to alternations in the immune system associated with aging that may subsequently lead to an increased susceptibility to infectious, autoimmune and neoplastic diseases. There are limited studies investigating the effects of immunosenescence on the geriatric equine immune system. The primary and secondary (anamnestic) immune responses may be different in aged horses in comparison to their younger counterparts. Therefore, this raises the question whether or not aged horses would have a protective immune response post vaccine or exposure to a pathogen.

The focus of this study was to investigate the effects of aging on the equine immune system. The first goal was to evaluate the effects of age on specific systemic antibody response to vaccination against rabies and equine influenza. Since horses selected for the test population were naïve to rabies, rabies vaccination was used to evaluate the horse's primary immune response. Healthy aged horses (≥ 20 years) mounted a primary immune response similar to that of younger adult horses (4- 12 years). Since the test population had previously most likely been exposed to equine influenza, vaccination was used to evaluate the secondary (anamnestic) immune response. Healthy aged horses had a significantly reduced anamnestic response in comparison to the younger adult horses even though the aged horses' pre-vaccination titers were higher.

Prior to assessing the immune response of horses in this study a detailed physical examination was performed and results of a CBC and biochemistry profile were analyzed to rule out underlying disease that may impact on their immune response. Serum concentrations of Se, vitamin E, and thyroid hormones were measured to examine the effect of confounding factors on the immune response. Preliminary results showed that significant numbers of horses in PEI had sub-optimal serum Se concentrations. The second focus of this study was to assess the Se and vitamin E status in representative groups of horses (aged pleasure horses, young adult pleasure horses, racehorses, broodmares and their foals) on Prince Edward Island. Resting T4 and T3 serum concentrations were measured and the relationship between the Se status and thyroid hormone concentrations was determined.

The results of this study showed that 79% of the horses tested had inadequate serum Se concentrations and a strong correlation existed between mare's and their foal's serum Se concentration. In contrast, 87% of the horses tested in PEI had adequate concentrations of vitamin E. Selenium concentration affected T4 concentration but there was no effect on T3 concentrations.

These studies demonstrated that the equine geriatric immune system differs from that of younger adult horses and that inadequate Se intake is widespread in the horse population in PEI.

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List of Abbreviations

α -MSH	Alpha-melanocyte Stimulating Hormone
APC	Antigen Presenting Cell
CD3 ⁺	T cells
CD4 ⁺	Helper T cells
CD8 ⁺	Cytotoxic T cells
CAMs	Cell Adhesion Molecules
COX	Cyclooxygenase
DAG	Diacylglycerol
ELISA	Enzyme Linked Immunoassay
EMND	Equine Motor Neuron Disease
Fc	Fragment, crystallizable
FDC	Follicular Dendritic Cells
GPx	Glutathione Peroxidases
HETE	Hydroxyeicosatetraenoic Acid
HI	Hemagglutination-inhibition
HPLC	High Performance Liquid Chromatography
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-6	Interleukin 6
IFN- γ	Interferon-gamma
LCMV	Lymphocytic Choriomeningitis Virus Infection
LFA-1	Leukocyte-function-associated Antigen
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
NK cells	Natural Killer cells
NSAID	Non-steroidal Anti-inflammatory
PEI	Prince Edward Island

PMN	Polymorphonuclear Leukocyte (neutrophil)
PPID	Pituitary Pars Intermedia Dysfunction
PRRs	Pattern Recognition Receptors
RNSA	Rabies Neutralization Serum Assay
Se	Selenium
Se-GPx	Selenium-dependent glutathione peroxidases
SRH	Single Radial Hemolysis
T4	Thyroxine
T3	Triiodothyronine
TCGF	T cell growth factor
TCRs	T cell receptors
TH cells	Helper T cells
TH1	T1 helper cells
TH2	T2 helper cells
TNF- α	Tumor Necrosis Factor-alpha
VEGF	Vascular Endothelial Growth Factor
WMD	White Muscle Disease

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**CHAPTER 1. IMMUNOSENESCENCE AND THE EFFECTS OF
SELENIUM AND VITAMIN E ON THE EQUINE IMMUNE SYSTEM.**

Tammy Lynn Muirhead, J McClure, Jeff Wichtel

1.1. Immunosenescence: changes in innate and adaptive immunity associated with aging

1.1.1. Introduction

Over the last half decade the mean human life span has increased from 50 years to close to 80 years in developed countries. Concomitant with this dramatic increase in life span, there has been an increase in morbidity and mortality in the elderly due to infectious diseases such as pneumonia, urinary tract infections, gastroenteritis and endocarditis (1). This suggests that the immune response of an aged individual has a reduced ability to cope with everyday infectious challenges and poses the question: is increased susceptibility to infectious disease in aged individuals the result of concurrent age-related disease, or can it be attributed to immunosenescence?

Many believe that increasing age leads to a natural decline in immune function (1-3). The term immunosenescence has been used to describe the state of dysregulated immune function that is thought to contribute to increased susceptibility of the elderly to infection, autoimmune disease and cancer (1;4). With advancing age, the immune system has been shown to undergo alterations rather than experiencing an overall decline in function (1;2). Alterations in cell populations, as well as function correlated with normal thymic involution, have been observed in aged individuals. The innate, humoral, and cell-mediated components of the immune system all undergo modifications during the aging process. Unfortunately, some of these age-related changes manifest as an increased susceptibility to disease. The

influence aging has on each of the aforementioned components of the immune system will be discussed in detail in the sections that follow.

Early studies of geriatric immune function generated conflicting results. This was likely due to inclusion of confounding factors through poor study design. Many early studies lacked rigorous inclusion criteria, possibly allowing factors other than age (i.e. concurrent disease, immunosuppressive medications, etc.) to confound the effect of aging alone on immune function. To isolate the effect of aging on immune function the Senieur Protocol was proposed by Lighart *et al.*. By using strict inclusion/exclusion criteria based on clinical and laboratory findings, this protocol ensured that only healthy aged individuals were included in studies of immunosenescence (5).

1.1.2. Innate immune system

The innate immune system functions as the first line of defense against invading pathogens. Macrophages, polymorphonuclear neutrophils (PMN's), and natural killer (NK) cells contribute to innate immunity through processes such as chemotaxis, phagocytosis, natural cytotoxicity, cell interactions and release of soluble mediators (6). The innate immune system attacks invading pathogens until such time as adaptive immunity takes over. The adaptive and innate immune mechanisms are closely linked to provide an effective defense. Consequently, age-related changes in either one of these components will be compounded by compromised efficiency of the other.

Studies investigating the effect of aging on the innate immune system have produced contradictory results. Some studies have documented immunosenescent changes in the adaptive immune system, but report minimal alterations in innate immunity (7-9), while several other studies have shown that there are significant alterations in cells involved in

innate immunity as humans and animals age (10-12). Chronic inflammatory processes in the elderly, however, are consistently documented to be associated with the aging innate immune system (7;8;11;13-15). Claudio Franceschi coined the term inflamm-aging which refers to the chronic, low grade, systemic inflammatory state in the elderly (16). It is characterized by age-related changes in macrophages, neutrophils, and NK cells in terms of alterations in the expression and function of adhesion molecules, and to changes in cytokine production (7;8;11;13-15). The principle focus, however, is on an increased production of pro-inflammatory cytokines from these cells that are believed to be linked to the development of cardiovascular diseases, Alzheimer's disease, insulin resistance and diabetes and osteoarthritis (12;17).

1.1.2.a. Macrophages

Once a pathogen enters the body, the innate immune system serves as the first line of defense. Infectious agents initially encounter tissue macrophages that function directly by phagocytosis, and indirectly through release of mediators or cytokines. Macrophages express several receptors known as pattern recognition receptors (PRRs). These PRRs recognize surface structures that are common to invading pathogens, i.e., lipopolysaccharide (LPS) and mannose receptors. Pattern recognition receptors promote binding of macrophages to a variety of infectious agents, allowing for phagocytosis as well as initiating release of cytokines and inflammatory mediators (6).

Bone marrow hypocellularity, which is commonly observed in the elderly, has been attributed to a finite functional lifespan of hematopoietic stem cells which evolve into monocytes/macrophages (18). In reference to marrow monocytes/macrophages, there is an

age-related decline in CD68⁺ cells (Table 1-1), which are the bone marrow precursors of monocytes/macrophages (11;18). In contrast, a significant increase in marrow macrophage numbers was detected in aged mice using flow cytometry. It was postulated that this may represent a compensatory mechanism for the decreased function in macrophages associated with aging (19).

In contrast to the decreased numbers of marrow monocytes documented in elderly people, circulating monocyte numbers have been shown to be equivalent to those of younger individuals. While the absolute numbers of circulating monocytes appear to be adequate in aged individuals, there is evidence that a greater number of these cells are required for an optimal immune response because of an age-related decrease in the monocyte subpopulation bearing CD11a/CD18 leukocyte-function-associated antigen (LFA-1) (20;21). The LFA-1 is a cell adhesion molecule found on T cells, macrophages and neutrophils. It is involved in recruitment of cells to a site of infection. The LFA-1 binds to other cells, such as antigen presenting cells, and allows T cell proliferation (6). The decrease in the number of LFA-1-bearing monocytes thus results in a decrease in T cell proliferation (20).

Several murine studies have shown that with advancing age macrophage function is altered. Diminished macrophage phagocytic activity has been documented in specific tissues, such as the lungs and skin, in aged mice. One mouse study demonstrated lower clonal growth of alveolar macrophages in aged mice, which are essential for the production of antigen-specific cells so they can effectively combat pathogens. It was postulated that similar alterations in immune function in elderly people may be responsible for the increased incidence of pneumonia amongst geriatric patients (21). Another study in mice investigated the effect of aging on the inflammatory response to dermal injuries. This particular study

showed that there was also impairment in phagocytic activity of the macrophages of aged mice that resulted in delayed wound healing. Diminished signaling capacity of surface receptors on the macrophages in the elderly is the possible cause of their poor phagocytic activity (10).

In response to a wound, macrophages normally migrate to the site of injury and eliminate potential pathogens to aid in wound healing. Macrophages play two distinct roles in wound healing: phagocytosis of wound debris and production of angiogenic and fibrogenic growth factors that promote wound repair (6;10). Studies in aged humans and mice have illustrated that there is delayed macrophage response to the chemotactic stimulus generated by a wound that may contribute to delayed wound healing. These studies also documented that even though there is a delay in their appearance at a site of injury, there is a tendency for significantly higher numbers of macrophages to eventually appear (10;22;23). The reason for the delay is not known, however, higher numbers at the site is thought to compensate for the poor phagocytic activity macrophages present in the elderly (10). Another factor that may contribute to delayed wound healing is that macrophages are a rich source of an angiogenic factor called vascular endothelial growth factor (VEGF) and macrophages from the elderly produce significantly lesser VEGF than the young (22). Therefore, normal angiogenesis cannot occur as quickly in the aged as compared to the young, resulting in delayed wound healing.

Following phagocytosis by macrophages, pathogens are destroyed by enzymes, reactive oxygen species, and nitrogen intermediates. Gomez *et al.* reported an age-related decrease in the amount of reactive oxygen and nitrogen intermediates macrophages could produce (8). This can result in an age-related decline in the intrinsic innate defense

mechanisms. Other studies revealed similar results showing that, in old mice, alveolar macrophages had diminished production of reactive oxygen species and nitric oxide when stimulated by LPS (24;25).

As previously stated, cytokine production from macrophages may be altered with advancing age. Variable changes have been documented in cytokine production in association with aging. These varied results could be due to different experimental conditions, different methods of cytokine detection, or the presence of confounding factors (14). For example, some studies have focused on centenarians. These individuals represent a unique subset of the elderly population with superior immune function. Therefore, the conclusions from these studies may not be a reflection of the normal elderly individual. The most common change seen in the elderly is a greater production of pro-inflammatory cytokines that is often associated with inflamm-aging (16). The high production of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF- α , are of particular interest (26). A review article by Cossarizza *et al.*, stated that the increased production of these cytokines from mononuclear cells in aged individuals, as well as centenarians, was associated with pathological events such as atherosclerosis, osteoporosis, fibrosis, and dementia. The authors state, however, that there are similar levels of these pro-inflammatory cytokines documented in healthy elderly individuals and centenarians without these pathologic outcomes, indicating that increased production of these cytokines may have a different biological significance in different individuals (26). The increase of these cytokines in normal and diseased elderly questions their role in initiating lesions. The consequence of a higher production of pro-inflammatory cytokines from macrophages in the elderly may predispose the aged to inflammatory conditions.

1.1.2.b. Polymorphonuclear neutrophils

Polymorphonuclear neutrophils (PMNs) are another type of phagocyte that is important in the innate immune system. Polymorphonuclear neutrophils are likely the first phagocyte a pathogen encounters. They are normally found in the blood stream; however, upon activation via cytokine signals generated during inflammation, they marginate and undergo selectin-dependent capture. They penetrate the endothelial layer of the vessels and migrate to a site of inflammation (chemotaxis) where they adhere to the extra cellular matrix via integrins. PMNs are capable of ingesting microorganisms or pathogens and eliminating them via respiratory burst which is the rapid release of reactive oxygen species (superoxide radical and hydrogen peroxide) (6).

Quaglino *et al.* state that generally there is a decrease in the function of neutrophils with aging (27). Human and rodent studies have found that the phagocytic capacity, the synthesis of reactive oxygen intermediates and the intracellular killing capacity of neutrophils are impaired in the elderly (7;28-30). Nagel *et al.* found an age-related increase in defects in phagocytic ability which was not influenced by illness (31). Another study by Butcher *et al.* agreed that aging does decrease phagocytic activity that may be due to a decreased expression of CD16 on aged neutrophils (32). Seres *et al.* discovered that a granulocyte-macrophage colony stimulating factor, an important *in vivo* regulator of granulopoiesis and neutrophil function, was incapable of priming neutrophils harvested from elderly patients whereas neutrophils taken from younger subjects responded appropriately. This failure in PMN priming will inhibit superoxide production, intracellular calcium reflux, antibody-dependent cellular cytotoxicity and intracellular killing mechanisms. All of these

components are critical during the earliest phase of infection (21;33). Therefore the elderly may be more susceptible to infectious pathogens due to impairment in their neutrophil killing (20). In another study of geriatric human subjects utilizing chemiluminescence, Aoki demonstrated that there was an impairment in the generation of reactive oxygen species in neutrophils and a decreased reaction to LPS and TNF- α that activate neutrophils at the site of infection in elderly patients (34). This also suggests a sub-optimal immune response in the elderly.

As previously stated, PMNs function through interaction with other cells via cell adhesion molecules (CAMs) such as selectins and integrins. Cell adhesion molecules are cell-surface proteins that are involved in the binding with other cells or with the extra cellular matrix (6). Age-related alterations in the expression of various CAMs has been documented (26). Neutrophil adherence in the elderly has been found to be normal or even enhanced when compared to younger subjects. Elderly subjects have higher number of CD15 and CD11b surface markers when compared to younger subjects, and lack CD11a and CD11c. The CD15 surface marker mediates an increase in neutrophil adhesion with endothelial cells and platelets whereas the CD 11a, b, and c (β 2 integrin family) are involved in cell-substrate adhesion, transduce signals from the extracellular matrix, and regulate growth. These differences in CAMs may account for the increased endothelial adherence of the aged neutrophils observed in the elderly (20;35).

1.1.2.c. Natural killer cells

In addition to macrophages and PMN's, natural killer cells (NK cells) contribute to the innate immune system. NK cells are large, granular, non-T and non-B lymphocytes capable of killing some tumor cells as well as cells infected with intracellular pathogens such as viruses (6). These cells have two alternative mechanisms of lysis. They are capable of direct spontaneous killing (MHC unrestricted cytotoxicity) of neoplastic and virus-infected cells or indirect Fc-receptor-mediated cytotoxicity of antibody coated cells (36).

Studies involving aged human and aged murine subjects demonstrated a consistent increase in cells bearing the NK phenotype (8;15;36-41). Increases in absolute and relative numbers of these cells was observed in the peripheral blood of healthy elderly humans (36;40;41). The age-related increase of cells bearing NK markers, and of non-MHC-restricted T lymphocytes, can be interpreted as a compensatory mechanism for the decrease in T cell numbers typically observed in the elderly (26;37). This will be discussed later.

Studies have also indicated a change in NK cell cytotoxicity as a result of aging. Borrego *et al.* found that, in aged humans, an index of absolute NK cell cytotoxicity remains stable as subjects age, but the relative NK cell cytotoxicity, on a per cell basis, is impaired due to a reduced cellular response to IL-2. Thus, the NK cell expansion may be a compensatory mechanism for decreased NK cell function (42). Natural killer cells have a major role in inhibiting tumor growth and metastasis. The influence of NK activity on the incidence of cancer was investigated in an 11-year study in Japan involving over 3500 young, middle-aged and elderly people. This study demonstrated a higher incidence of cancer in individuals with lower NK cytotoxicity. It also documented higher NK cell activity in the aged subjects as compared to young and middle-aged subjects (43). Plackett *et al.* showed that NK cells

from the elderly are less capable of destroying tumor cells. However, there are increased cell numbers in the elderly, and production of IL-4, which is responsible for B cell activation, is also increased (15).

Research studies on the activity of NK cells in the elderly, in terms of spontaneous killing, have yielded conflicting results (36;39;44). This may be attributed to the fact that a lack of rigorous inclusion and exclusion criteria resulted in the inclusion of individuals with concurrent disease conditions or medications which may have altered immune function (5;37). An article by Cossarizza *et al.*, involving only healthy elderly people and centenarians, investigated how NK cell populations change with aging (26). This study demonstrated an age-related increase in cells from the elderly with high NK activity ($CD16^+CD57^-$) with little alteration in the NK cell numbers with low ($CD16^-CD57^+$) and intermediate ($CD16^+CD57^+$) activity. Sansoni *et al.* agreed with these findings stating that NK activity was preserved in elderly subjects; they could migrate normally in response to chemotactic stimuli and kill target cells efficiently (39). An interesting study by Levy *et al.* investigating 'low NK cell syndrome' illustrated that healthy individuals who had persistently low NK cytotoxicity would be at risk of developing infectious sequelae. They stated that chronological age had both a direct and indirect (via NK activity) association with illness. This study showed that persistently low NK activity is a predictor of impending morbidity and that subjects with high NK activity have greater longevity (45).

1.1.3. Adaptive immune response

The adaptive immune system relies on the actions of three types of lymphocytes: cytotoxic T cells ($CD8^+$), helper T cells ($CD4^+$), and B cells. Each assumes a distinct role in the humoral or cell-mediated immune response. Cytotoxic T cells identify and mediate killing of host cells infected with pathogens. B cells manufacture antibodies that bind to specific epitopes on the surface of the invading pathogens, ultimately causing their destruction. Helper T cells direct both cell-mediated cytotoxic T cells and humoral B cells by means of direct cell interactions, and with the aid of antigen-presenting cells through the release of cytokines. These cytokines attract and stimulate proliferation of lymphoid cells. The generation of memory B and T cells, along with the production of specific antibodies, allow for a faster immune response when the same pathogen is encountered again. In summary, upon infection, adaptive immunity provides protection via recognition of the pathogen, activation of a pathogen-specific immune response, and the development of memory cells for rapid reactivation of the immune system if the pathogen is re-introduced (6;7).

1.1.3.a. T lymphocytes

Alterations in each population of T lymphocytes, whether they are helper, cytotoxic, naïve, memory or suppressor (regulatory) cells, contribute to the modification of the humoral and cell-mediated immune responses typically associated with the aging process. The following T-lymphocyte alterations have been associated with aging: a decline in the number of naïve cells due to diminished thymopoiesis, an increase in the number of memory cells resulting in increased cytokine production, and an accumulation of terminally differentiated effector cells with limited T cell receptors (4).

1.1.3.b.i. T lymphocyte numbers and subpopulations

Reduced immunoresponsiveness in the elderly has been attributed primarily to changes in T cell numbers associated with normal thymic involution. As the thymus involutes, cellularity decreases leading to a decrease in the size of the thymus. The thymic structure changes and becomes disorganized. There is also a reduced rate of T cell migration from the thymus, and alterations in the relative proportions of thymocyte subpopulations. These age-related changes are believed to be the result of either an intrinsic defect within the thymocytes or inability of the stromal cells to differentiate (46). In humans, even when the proportions of the major T cell subsets remain constant, the absolute numbers of the CD3⁺ (T-cells), including both CD4⁺ and CD8⁺ cell populations, decrease with age (26;39). In centenarians, naïve T cells persist, although in reduced numbers, after thymic involution has taken place. Ongoing production of T cells despite thymic involution suggests that peripheral lymphoid organs take over this role (37;47). Interestingly, Bender *et al.* investigated the absolute count of peripheral lymphocytes in elderly men, to determine its relationship with mortality. They found that in healthy elderly people the number of total lymphocytes in circulation does not seem to be affected by age. However, about three years prior to death, a decrease in peripheral lymphocyte count occurs in 92% of the population (48). In a study by Wikby *et al.*, there was a correlation between high CD8⁺ to CD4⁺ ratios and poor T cell proliferation, as well as increased mortality (49).

1.1.3.b.ii. Alteration in T lymphocyte phenotypes

The most consistent feature of T cell immunosenescence is the alteration of T cell phenotypes or subtypes. Helper ($CD4^+$) and cytotoxic ($CD8^+$) peripheral T lymphocytes show mutually exclusive expression of $CD45RA^+$ or $CD45RO^+$, distinguishing them as naïve or memory cells, respectively (37). With advancing age there is the decrease in the proportion of naïve or $CD45RA^+$ cells, with a concomitant increase in the proportion of cells with memory or the $CD45RO^+$ phenotype. This shift from predominantly naïve cells to memory cells may reflect the gradual and cumulative exposure to foreign antigen over time (26;47;50). This, coupled with the decline in the production of newly differentiated naïve T cells, leads to an immune compartment that has remodeled toward memory-like responses, with the capacity to respond to previously encountered antigens, while being less effective when presented with novel antigens (46).

One factor that might contribute to this shift to memory T cells is the effect of the peripheral environment on naïve T cells. Thoman concluded that the aged peripheral microenvironment can cause newly-produced T cells to shift towards the memory phenotype (51). Another factor that can promote the shift to a memory phenotype is increased clonal expansion. A key feature of thymopoiesis is the generation of a T cell repertoire with diverse T cell receptors (TCRs) (6). A diversified population of T cells and TCRs for antigen recognition is essential for protection against the large number of environmental pathogens an individual will typically encounter (46). An age-related loss in the diversity of the T cell repertoire leaves the aged individual more susceptible to new pathogens. Clonal expansion is the normal process of proliferation of antigen-specific lymphocytes in response to antigenic stimulation. It precedes lymphocyte differentiation into effector cells. This allows rare

antigen-specific cells to increase in number for effective response to that particular pathogen (6). Interestingly, the majority of T cells undergoing clonal expansion express CD45R0⁺, known to be the memory phenotype (52). Although the exact mechanism for clonal cell selection is not known, it is hypothesized that it may be derived from latent or subsequent infections. The persistence of these clones in the aged may be the result of defective mechanisms that normally regulate clonal homeostasis, such as apoptosis (2;53). Therefore, the combination of reduced numbers of newly produced naïve T cells, thymic involution, accelerated maturation of newly produced T cells to the mature phenotype, and persistence of antigenic-driven clonal T cell expansion, all act to compromise the diversity of the T cell inventory. Ultimately these factors lead to a shift towards a greater proportion of memory T cell phenotype, as observed in the elderly.

1.1.3.b.iii. Alteration in T lymphocyte function

T cell populations from aged individuals have functional alterations when compared to those from the general population. Alterations in cell signaling transduction, decreased responsiveness to T cell receptor stimulation, and impaired T cell proliferative capacity have been noted. A decline in the frequency of CD4⁺ T cells producing IL-2 and T cells with altered expression of IL-2 receptors has also been reported. These changes have been cited as potential causes of decreased immune response in aged individuals.

Signal transduction in T cells has been thoroughly investigated in elderly subjects. It is the mechanism whereby cells transmit signals from extracellular stimuli to the intracellular compartment, triggering biochemical events at various sites in the cell. This conversion of signals occur through the cell surface receptors that activate intracellular pathways (6). Alterations in these pathways can result in a diminished immune response. Several

researchers have shown that T cells from older donor mice had diminished intracellular calcium mobilization (54-56) and protein phosphorylation (54;57). Patel and Millar concluded that aging results in a global impairment of several distinct protein kinase pathways in both the naïve and memory T cell sets (57). Shi and Miller more specifically demonstrated that declines in mitogen-stimulated phosphorylation are attributable to both tyrosine-specific and serine threonine-specific kinases (58). These kinases are known to undergo qualitative changes with aging resulting in specific differences in patterns of protein phosphorylation of a wide range of substrates that act at intracellular sites (57-60). Specifically tyrosine phosphorylation of the CD3 ζ chain declines with age, both in resting T cells and shortly after activation (61).

Immunologic studies have consistently shown a significant decline in the cytokine interleukin 2 (IL-2) in elderly subjects (7;62;63). IL-2, also known as T cell growth factor (TCGF), is needed for the development of the adaptive immune response (6). It stimulates T lymphocyte division and consequently cell-mediated immunity (6;64). IL-2 is an important T cell derived cytokine that promotes the proliferation and differentiation of lymphocytes into primarily T1 helper cells (TH1) and regulatory T cells.

The decline in IL-2 concentrations with age has ranged from 20% to 90% compared to younger people (20;27;65). This decrease could be the result of the decline in the total number of T cells or, more likely, the shift from mostly naïve cells to predominantly memory cells. Naïve T cells produce mostly IL-2, whereas memory T cells produce other cytokines (66;67). Another reason for the decrease in IL-2 production has been attributed to a decline in IL-2 transcription (67). The induction of IL-2 transcription by mitogens decreases with age. It parallels the age-related decline in IL-2 mRNA levels, supporting this hypothesis (67).

Changes in the number of IL-2 receptors may also pose a problem in the elderly. Memory T cells in the elderly tend to express higher numbers of cell surface IL-2 receptors. The combined decrease of IL-2 secretion and increased number of memory cells with mostly IL-2 receptors manifests as an imbalance. There are greater numbers of this receptor with a lack of the required cytokine that leads to an imbalance, potentially impairing T cell memory quality in aged subjects. (68;69).

One study using transgenic mice to investigate the role of antigenic stimulation in CD4⁺ cells illustrated that there is a shift in this cell population towards a higher proportion of memory T cells in aged mice. The response of naïve aged T cells was diminished, when compared to young mice. There was also deficient production of IL-2 and IL-3, together with decreased lymphocyte proliferation in the absence of the exogenous cytokines (70). Together, these changes result in a hyporesponsiveness of T-cells.

Homann *et al.* conducted a study to compare the function of CD8⁺ and CD4⁺ memory cells in young and aged mice using a lymphocytic choriomeningitis virus infection (LCMV) model. They found that the primary CD8⁺ response was more vigorous in mice less than 5 months of age, but no significant changes were seen in older age groups. Interestingly, re-stimulation with viral proteins showed that the secondary response of the CD8⁺ and CD4⁺ memory cells in older subjects was reduced when compared to younger mice. This illustrated that there were minimal age-related issues in the generation and maintenance of these memory T cells. There were, however, significant defects in their function in recall responses (71).

1.1.3.b.iv. Helper T lymphocytes and cytokines

Helper T cells (TH), also known as CD4⁺ cells, play an important role in both humoral and cell-mediated immunity. TH cells are subdivided into TH1 and TH2 cells. In the humoral response, the generation of memory B cells and production of protective antibodies is dependent of the appropriate function of TH2 cells (6). It is these cells that drive the formation of germinal centers, which is required for B cell expansion and generation of B cell memory (72). Haynes *et al.* carried out several experiments looking at the effects that age has on CD4⁺ function in both primary and memory responses in mice. They found that, in the primary response, there was an age-related decline in naïve CD4⁺ cell helper activity in terms of a reduced germinal center formation, reduced antigen-specific B cell expansion and differentiation, as well as reduced IgG production (63;73-75). The memory response also significantly declined when compared to the younger CD4⁺ memory cells (63;73;76).

TH1 are essential in the response to intracellular pathogens and for the development of functional cytotoxic (CD8⁺) T cells involved in cell-mediated immunity. Cytotoxic T cells play a pivotal role in immunity against viruses (3;6). The CD8⁺ memory cells rapidly generate highly functional effector cells that can destroy cells upon a second exposure to a specific pathogen (77). Several studies have showed that CD8⁺ T cells that were generated without the assistance TH1 were defective in their recall response and that they experience active cell death upon re-stimulation (76;78-80). A shift from the TH1 cell-mediated response to a TH2 humoral response is consistently seen in the elderly (3;76). Interestingly, for a short time after birth, the immune system has an impaired cell-mediated immunity with a strong humoral immunity, or a predominant type 2 response. Type 1 responses, however, predominates shortly after birth (3;50;76).

Alterations in cytokine populations are often seen with aging; thus alterations in the type of immune response also occur. The cytokines associated with a TH1 response include IL-2, IFN- γ , IL-12 and IL-15. The cytokines associated with a TH2 response are IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (3). Some cytokines may up-regulate one immune component and down-regulate another (3;6;20). For example, a major finding in the elderly is a shift from predominately TH1 to TH2 cytokine production. A significant decrease in IFN- γ production and an increase in IL-4 is consistently seen in the elderly (21;81). As previously discussed, there is a significant decline in IL-2 in the elderly and in centenarians associated with significant increase in IL-1, IL-6 and TNF- α . There is also a reduction in certain cytokine receptors on T-cells. Also increased cell death of TH1 compared to TH2 effector cells may play a role in the predominance of a TH2 response in later years of life (66). The increase in tumor incidence, infectious disease and the reoccurrence of latent viral disease seen in the aged is suggestive of a dramatic fall in cellular immune surveillance resulting from the reduced type 1 response.

1.1.3.c. B lymphocytes

B lymphocytes are thymus-independent lymphocytes that develop in the bone marrow and play an important role in the production of antibodies for the humoral immune response. Once a mature B cell encounters and binds with an antigen via cell-surface receptors, proliferation of the cell occurs resulting in an expanded clone of cells for that specific antigen. These activated B cells migrate into secondary lymphoid follicles and form germinal centers around follicular dendritic cell networks. With the aid of TH cells, the cloned cells can differentiate and undergo somatic hypermutation with a propensity for high-affinity

mutants in the germinal centers. This ultimately results in high-affinity long-living memory B cells or antibody-secreting B cells called plasma cells. Plasma cells can no longer interact with TH cells due to very low levels of surface immunoglobulins, and a lack of MHC class II molecules. They also lose their ability to change isotypes or undergo further somatic mutation (6).

Some studies suggest B cells may have reduced ability to become activated and secrete antibodies as aging progresses. Dysfunction in B lymphocyte memory is reported in all phases of the anamnestic response in aged individuals generating initial memory, maintaining viable memory cells, and memory reactivation (2;21). Although the humoral immune response may be delayed or misdirected, it is not entirely absent (1). Age-related decrease in the number of peripheral B cells produced (4;26;37;82-85), changes in the B cell repertoire in both primary and memory cells (83;86-88), and changes in germinal centers have been documented (1;26;88-90).

Several authors state that with increasing age, humoral immune responsiveness and antibody-mediated defense mechanisms are significantly altered (1;4;50;54;82;83;91). Initially, it was assumed that the decrease in response was primarily due to the decline in the TH cells rather than a primary B cell problem. It has been confirmed that age-related changes in T cells do affect B cell activation and differentiation, resulting in an altered humoral response (63;73;76). However, primary B cell alterations have also been documented in the elderly.

The absolute number of B lymphocytes in the body remain relatively stable as people age (2;50;82). There is, however, a decline in peripheral B cells in the aged, especially those cells expressing CD19⁺ (4;37;82;83). CD19 serves as a B cell surface co-receptor that

establishes signaling thresholds critical for B lymphocyte activation and increases B cell responsiveness 100 fold (6). Sato *et al.* showed that CD19⁺-deficient mice had B lymphocytes that were hyporesponsive to transmembrane signals (92). Sansoni and his colleagues also demonstrated that, in healthy elderly people and centenarians, there is a marked decrease in the number of CD19⁺ B lymphocytes (39).

In addition, age-related changes in B lymphopoiesis results in decreased production of B cells (93). Normal development starts with a stem cell which progresses to an early Pro-B cell, to a late Pro-B cell and then to a Pre-B cell. Finally the Pre-B cell develops into an immature B cell (6). A study conducted in 1995 showed that there were markedly decreased numbers of Pre-B cells in aged mice. The Pre-B cell attrition was shown to be associated with a sub-optimal response of the Pro-B cells to IL-7 (94). Interleukin-7 is essential for the maturation of Pro-B cells into Pre-B and Pre-T cells (6). It was also discovered that there was less production of IL-7 by the bone marrow of aged mice (94-96). Both factors contribute to the reduced B cell production with age.

A change in germinal center reactions resulting in alterations in antibody and memory cell formation has been detected in aged humans and animals. During normal germinal center reaction, B cells receive signals to produce high affinity, isotype-switched antibodies which differentiate into memory cells or antibody-forming cells (1). Several studies have revealed that the elderly have smaller and fewer germinal centers that produce antibodies that are characterized by less somatic mutation and lower affinity for binding to an epitope (1;89). There is evidence supporting an impairment in affinity maturation amongst the elderly (97). Also in aged T cells, these cells generate signaling disequilibrium leading to growth of predominantly low affinity B cells. This contributes to a sub-optimal humoral response (89).

These age-related alterations were attributed to a decrease in follicular dendritic cell and TH cell function. A reduced germinal center response in the elderly can hamper the humoral response to an active infection as well as impede the response to a future infection (1). Decreased generation of memory cells in the germinal centers, and poor maintenance of the memory cells, have been documented as age-related changes in B cells (1;88;98).

It has been reported that the quality of serum antibodies, produced in response to specific novel antigens, decline with age. This may manifest as a poor vaccine response (83;99). Schwab *et al.* reported that the specific antibody responses in humans to vaccines for viral (influenza and encephalitis) and bacterial (tetanus toxin, salmonella and pneumococcus) pathogens decreased with age (99). The humoral response itself does not decline with age; instead it is altered due to the diminished antigen-specific production of antibodies.

The antigen-specific response in the elderly is impaired, but this is not reflected in total serum immunoglobulin concentration, which actually increases with age; yet, the number of peripheral B cells produced decline (26;37;39;85). The classes and subclasses of immunoglobulins produced by elderly human beings also undergo significant changes. Both IgG and IgA serum concentrations are shown to be significantly increased, whereas IgM concentrations were unchanged. The IgG subclasses, IgG1, IgG2, IgG3, showed significant increase, whereas IgG4 did not (85). The significance of the increased antibody concentration in healthy old people and centenarians may be a compensatory mechanism, to some degree, for a reduction in cell-mediated immunity as described earlier. There is also evidence of a decrease in antibody diversity in aged humans and mice. This is a direct result of a combination of diminished bone marrow production of new B cells, increased numbers of plasma cells, and expanded B cell clonal expansion (2).

Increased concentrations of autoantibodies have been frequently noted in studies of geriatric subjects. It was originally thought that the frequency of subjects with detectable serum levels of organ-specific and non-organ specific autoantibodies would increase with age. However, plasma collected from healthy elderly subjects and centenarians was practically devoid of organ-specific auto-antibodies (2;26;84), but there was an increase in non-organ specific auto-antibodies, such as rheumatoid factor. This may be due to either genetic factors or aberrant self- recognition (26). Several hypotheses have been proposed to explain age-related changes in the level of autoantibodies. Firstly, an increased number of B cells and plasma cells in organs other than peripheral blood may stimulate autoantibody production. Secondly, an increased lifespan of B cells and plasma cells in germinal centers may be a contributing factor (2).

1.1.3.d. Antigen-presenting cells

Reduced adaptive immunity in the elderly may also be directly linked to antigen presenting cell (APC) dysfunction. Antigen-presenting cells are highly specialized cells that process antigens and display their peptide fragments on the cell surface, together with molecules required for T cell activation. Dendritic cells, macrophages, and B cells are the main APC for T cells (6). Even though APC numbers remain stable with age, their function is altered. Aged macrophages exposed to interferon showed a marked reduction in their ability to up-regulate MHC class II molecules (1;100). Aged murine macrophages stimulated by lipopolysaccharide had significantly higher levels of the inducible cyclooxygenase 2 enzyme (101). This resulted in an increased production of prostaglandin E₂ that is known to inhibit T cell function.

Specialized dendritic cells called follicular dendritic cells (FDC), play an intricate role in antigen presentation via binding of immune complexes. Aged FDCs express an abundance of surface Fc and complement receptors that increase the affinity phagocytic cells have for microbes. These cells produce fewer iicosomes (small fragments coated with immune complexes that separate from the cells early in a secondary antibody response) even though serum concentrations of complement are intact. This results in lower concentrations of CD21L on FDC surfaces, which in turn results in decreased B cell activation. The end result is a functional deficiency of aged FDCs and reducing trapping of immune complexes, leading to immune dysfunction (1;102).

1.1.4. Immunosenescence in horses

It is estimated that geriatric horses (i.e., those being 20 years of age or older) account for approximately 15 % of the equine population in North America (103). There is an ever-increasing demand in private practices and referral centers to provide high quality care for these horses. These older horses experience many changes that may be related to their level of nutrition, their environment, and/or an underlying sub-clinical disease. Thus, it may be difficult to separate normal age-related changes from changes related to a disease. Many aged horses experience declining body condition, muscle tone, and general well-being. It is not known whether these changes contribute to decreased immune function, or is the result of declining immune function (104). A better understanding of immunosenescence in aging horses is needed. There is evidence for an age-related alteration in the ability of horses to respond to antigens, as has been previously described in humans and mice.

To date, few studies have examined the effects of age on the innate, cell-mediated and humoral components of the equine immune system. The innate immune system of the horse

appears to remain intact with age. Horohov and his colleagues, investigating the effects of exercise on the immune response of young and old horses, showed that aged horses (mean age of 25 years) demonstrated lymphokine-activated killer cell activity equivalent to that of younger horses (mean age of 7.5 years) (104). This suggests that these killer cells are able to provide an adequate first line of defense when encountering an antigen. Circulating monocyte and granulocyte counts in young and aged horses were also similar (105). The above findings are in concordance with many of the findings in human studies.

Horses, like other species, experience thymic involution, and have similar modifications in circulating T cell subpopulations (106). T cells of elderly horses show decreased proliferation upon exposure to a mitogen when compared to younger horses (104;107). In these studies T cell proliferation remains decreased after supplementation with recombinant IL-2, suggesting that decreased proliferation cannot be solely attributed to decreased expression of IL-2. This suggests that an age-associated alteration in the T cell IL-2 signaling pathway occurs, probably involving the IL-2 receptor (107). McFarlane *et al.* investigated the age-related quantitative alterations in lymphocyte subsets and found that healthy geriatric horses had decreased absolute total peripheral lymphocyte cell counts, including decreased CD4⁺ and CD8⁺ T cells as compared to young horses. There was also a significant decline in the percentage of CD8⁺ cells resulting in an increased CD4⁺ to CD8⁺ cell ratio in the aged horses. Increased CD4⁺ to CD8⁺ cell ratios has been associated with nonspecific inflammation or immunodeficiency in other species (108). Another study showed an increase in the expression of MHC II on T lymphocytes in adult horses as compared to neonatal foals suggesting a shift toward a larger memory T-cell population with advancing age (109).

Studies on aged horses have also documented changes in the humoral immune response. McFarlane *et al.* demonstrated that aged horses have a significant decline in numbers of peripheral B lymphocytes as compared to younger horses. Measurements of serum immunoglobulins IgG, IgG (T), IgM, and IgA, however, were not significantly different between the age groups (108). Two studies investigating the specific antibody response of aged horses to vaccines and pathogen challenges have shown a blunted response when compared to younger horses (104;110).

1.1.5. Immune response to equine influenza and rabies vaccination in horses.

Horses are routinely vaccinated against equine influenza and rabies in endemic regions. The field efficacy of many commercially available equine vaccines, including influenza and rabies vaccines, is not well-established in young healthy horses, and remains untested in aged horses (111). It is unknown whether these vaccines are capable of preventing disease or infection in aged horses.

The adaptive immune response initiated by vaccination tends to induce either a predominantly cellular or humoral response depending on the type of vaccine administered. In most cases, the best protection is provided by an immune response that mimics the response induced by the naturally occurring disease. A memory response also develops, which provides a more rapid secondary response once the same antigen is encountered. This provides long-term protection following vaccination or natural infection (6;112).

1.1.5.a. Equine influenza vaccination

Equine influenza is a highly infectious disease that is widespread in equine populations. Influenza A virus (H3N8) represents the most common primary respiratory

pathogen of horses in North America (113;114). Equine influenza manifests as pyrexia, depression, anorexia, coughing, nasal discharge and is frequently complicated by secondary bacterial infections (115). Vaccination against equine influenza is commonly employed, however, the degree of protection provided by many of the commercially available vaccines is questionable. Incomplete protection ranging from one month to 15 months in duration has been reported, depending on the influenza vaccine tested (114;116-118). Natural influenza infection, on the other hand, consistently provides strong protection for at least 1 year (114;119). Influenza outbreaks in previously vaccinated horses are common. Authors have attributed this 'vaccine breakdown' to one or more of the following factors: lack of vaccine potency, poor host response to vaccination, and antigenic drift of the virus (114;120;121). A recent study investigating the immune responses to commercial equine influenza vaccines showed that one vaccine yielded significantly higher single radial hemolysis (SRH) values as compared to its competitors (122).

Even though more effective vaccines have recently been developed, there is evidence that, in aged horses, influenza vaccine causes a sub-optimal immune response when compared to younger horses (104;110). Goto *et al.*, studying antibody response to influenza vaccination, showed that the proportion of horses producing hemagglutination-inhibition (HI) antibodies post-vaccination was decreased with advancing age (110). Horohov *et al.* illustrated a significant age-related decline in equine influenza virus-specific antibody responses measured by an ELISA when horses greater than 20 years of age were compared to younger horses (104).

1.1.5.b. Equine rabies vaccination

Rabies is a highly fatal viral disease of the nervous system. Vaccination for this condition is frequently practiced, especially in rabies endemic regions. To date, few studies have investigated the efficacy of rabies vaccination in the horse (123;124). In one study a test vaccine consisting of a minimal effective dose required by federal agencies was administered to nine horses. All vaccinated horses seroconverted, however, when subsequently challenged with rabies virus, all horses developed the disease. Incubation time in vaccinated horses was prolonged compared to the control animals (123). These findings cast doubt on the efficacy of the current commercial rabies vaccines in the face of exposure to the virus.

A novel rabies DNA vaccine has been developed for horses (124). Fischer *et al.* conducted a study to investigate if this vaccine would enhance antibody response. They concluded that the DNA vaccine containing DMRIE-DOPE [N-(1-(2,3-dimyristyloxypropyl)-N,N-dimethyl-(2-hydroxyethyl)ammoniumbromide/dioleoyl phosphatidylethanolamine], stimulated rapid seroconversion after a single immunization. There was a strong anamnestic response, and titers in these horse were generally very high, suggesting that they would be well-protected against a rabies field challenge, however no experimental challenge was conducted (124).

Due to the general lack of studies involving the efficacy of rabies vaccines, there is essentially no information on the ability of aged horses to respond to administration of rabies vaccines.

1.1.6. Summary

Age-related changes in the immune system have been attributed to changes in cell populations and function. The innate component of the immune system appears to be intact in the elderly and likely plays a key role in combating infectious disease. The adaptive component in the immune system, however, undergoes several important age-related changes including alterations in the relative concentrations of different immunoglobulin classes, and development of autoantibodies. The concentration of immunoglobulins increases with age; however, the production of peripheral B cells declines. Signaling, transduction, and IL-2 production/receptors of the T cells are altered. These alterations in the elderly lead to an overall hyporesponsiveness of the immune system, especially in cell-mediated immunity. The most consistent finding in aged subjects is a decrease in the proportion of naïve T cells, with a concomitant increase in memory T cells. Future research will focus on how we can enhance the aged immune system, by reversing these changes or encouraging compensatory mechanisms.

1.2. Selenium and vitamin E

Selenium (Se) and vitamin E are essential dietary components for domestic animals and humans. Both of these nutrients participate in cellular antioxidant systems and have important roles in the immune response and reproductive function. Deficiencies of these nutrients have been associated with a wide range of disorders in domestic livestock (Table 1-2). These nutrients have a complex relationship with each other. The two exert antioxidant effects in tissues, although through independent biochemical pathways and in different locations. Selenium, as a component of the glutathione peroxidases (GPx) family of enzymes, is involved in cellular antioxidant defense by reducing semi-stable hydroperoxides

to less reactive alcohols in a variety of locations. Vitamin E, restricted to the cell membrane, functions to scavenge the free radicals that promote peroxidative chain reactions (125). Therefore, while one may have a sparing influence on a deficiency of the other, neither can fully compensate (126). However, Se and vitamin E are just two of many antioxidants such as, vitamin A, vitamin C and vitamin D that are available for protection of mammalian cells. Deficiency in any one of these may not necessarily result in clinical disease; clinical manifestation, however, may depend on the adequacy of other antioxidants.

In this chapter, the literature on Se and vitamin E will be reviewed both separately and together, since many studies have involved administration of these nutrients in combination.

1.2.1. Selenium

Selenium deficiency is typically seen in geographical areas where the soils are deficient in Se, such as the Great Lakes region, North Eastern and Pacific North Western North America, Northern Europe, China and Australia/New Zealand. Animals that graze on, or are fed, forages grown in these regions are often deficient in Se, unless supplemented. The availability of Se is also dependent on the pH of the soil, climate factors and various farm management practices (127). It is well established that Se uptake by plants is more efficient in alkaline soils which are often found in areas of low rainfall (128). In addition, the frequent application of fertilizers induces rapid plant growth, which has a diluting effect on the Se concentration in the plant. High levels of sulfur or sulfate can compete with Se for absorption sites (129). In humans, clinical Se deficiency has been limited to specific geographic areas with profound soil Se deficiency (i.e. Keshan district of West China). Viral cardiomyopathy is the primary feature of Se deficiency in humans (130). In humans, Se deficiency has sub-

clinical manifestations such as increased susceptibility to physiological stresses and increased susceptibility to an array of diseases such as cancer (131). Selenium is essential for the proper function of various components of the immune system in both humans and animals (132). It is controversial whether Se status in humans can be related to altered incidence and/or increased susceptibility to disease. However, Se deficiencies in farm animals can result in a wide range of diseases and have been reproduced experimentally (Table 2) (132;133).

The biological function of Se is attributed to a variety of selenoproteins, such as glutathione peroxidase (GPx). These selenoproteins, which have a known function, act in three broad areas of cell function: antioxidant activities, thyroid hormone function, and regulation of the activity of redox-active proteins. Selenium is best characterized in its role as an antioxidant. All of these general effects on metabolism can be associated with more specific processes that affect the immune system. Therefore, we can say that the influence of Se on the immune system is multifactorial (133). Selenium-dependent glutathione peroxidases (Se-GPx) are important antioxidant enzymes involved in the reduction of hydrogen peroxide, as well as organic hydroperoxides to less reactive water and alcohols (134). Peroxides are high-energy oxygen containing molecules that are produced during the metabolism of fat. These peroxides can be very destructive to cells. There are four known members of the Se-GPx family of enzymes: cytosolic, phospholipid, extracellular and gastrointestinal enzymes. Thus, Se can act as an antioxidant in the extra cellular space, the cell cytosol, and cell membranes (135;136).

1.2.1.a. Effects of Se on arachidonic acid metabolism

Selenium-GPx has the capacity to regulate the cellular concentrations of lipid hydroperoxides, which can influence the activity of enzymes involved in the arachidonic acid cascade (134). Arachidonic acid is a polyunsaturated fatty acid found in the phospholipids of mammals. It is a biosynthetic precursor of several families of compounds that exert diverse biological effects. The cleaving of arachidonic acid from phospholipids starts the cascade that produces many of these compounds. Arachidonic acid is metabolized by one of two pathways: the cyclooxygenase pathway (COX) or the lipoxygenase pathway (LOX). Ultimately, either of these pathways results in the production of the principle mediators of inflammation (137). Both pathways can be altered by the presence of Se and/or vitamin E (Figure 1-1).

Selenium deficiency in an experimental study involving rats resulted in a decrease of lymphocyte proliferation, which was directly correlated to an increased production of the pro-inflammatory mediators such as, HETE and leukotrienes (138). The likely mechanism hypothesized for this reduction of lymphocyte proliferation is an increase in cellular hydroperoxide tone due to loss of Se-GPX activity. One study in Se-deficient cows showed that concanavalin A-stimulated lymphocyte proliferation was significantly lower when compared to cows with adequate Se concentrations. This may in part be responsible for the decrease in resistance to infectious diseases observed in deficient animals (139). Another study on lymphocytes from Se-deficient cattle illustrated that the LOX pathway can be altered, and subsequent immune function can be impaired. Maddox *et al.* (1991) measured 12-HETE concentration in Se-deficient lymphocytes and found that there was a significant increase in its concentration; 12-HETE has roles in promoting endothelial cell growth, cGMP

production, tumor cell attachment to endothelial cells and regulating prostaglandin and leukotriene production. Interestingly, this study simultaneously revealed that, when bovine lymphocytes from Se-deficient cows were stimulated with concanavalin A, there was a significant decrease in proliferation when compared to lymphocytes from cows with adequate Se levels. It is known that Se-GPx regulates intracellular hydroperoxide levels; in a Se-deficient state, excessive intracellular hydroxides may enhance the production of 12-HETE, potentially diminishing lymphocyte proliferation and affecting the immunologic status of these animals (140).

Selenium also affects the COX pathway. Eicosanoids, namely prostaglandins, thromboxanes and prostacyclines, are the enzymatic oxidation products of arachidonic acid metabolized via the COX pathway (137). They have been linked to the activation of phospholipase D. Phospholipase D has been reported to be important in signal transduction pathways in a number of cell types (141-143). This enzyme catalyzes the hydrolysis of many phospholipids including phosphatidylcholine, to yield phosphatic acid that in turn is hydrolyzed by phosphatic acid phosphatase to form diacylglycerol (DAG). Diacylglycerol is known to activate protein kinase C by increasing its sensitivity to calcium (138). It has been proposed that this signaling pathway is involved in the regulation of many cellular functions, including cell proliferation (144). It was also illustrated that in lymphocytes from Se-deficient rats there was a significant decrease in phospholipase D activation as compared to the rats supplemented with Se. Thus, it was hypothesized that the decrease in Se would ultimately result in dysfunction of immune cells. The addition of prostaglandins to the Se-deficient cells reversed the decreased phospholipase D activity. It was concluded that

decreased phospholipase D activity reduced production of COX pathway metabolites in lymphocytes derived from Se-deficient rats and results in lymphocyte dysfunction (138).

1.2.1.b. Effects of Se in neutrophil function

The role of Se in neutrophil function has been investigated extensively. Neutrophil function is impaired with deficiency of Se, but neutrophil numbers remain constant (133). Neutrophils produce superoxide-derived radicals that kill microbes, however, the production of these highly oxidative substances can have negative effects on the neutrophils themselves, and the surrounding tissues. Selenium deficiency interrupts the balance of pro- and antioxidants (145). Neutrophils from Se-deficient animals have normal phagocytic ability; however there is decreased ability to kill infectious pathogens when compared to neutrophils from animals with adequate serum Se concentrations. This decreased killing ability is correlated to a decrease in cytosolic GPx activity in the neutrophils making the neutrophils susceptible to damage from the reactive oxygen species produced during the respiratory burst (132;145;146). An *in vitro* study measuring the rate of free radical production in stimulated mice neutrophils found that the initial rate of production was the same in the Se-deficient and replenished groups. Only neutrophils from the Se-replenished group, however, were able to continue producing the free radicals over time (146).

The effects of Se and Cu deficiency on neutrophil function has been studied in cattle (145). This study showed that the ability of bovine neutrophils to ingest an organism, *C. albicans*, was not effected by Se or Cu status. There was however, decreased killing of the ingested organism by neutrophils from the Se-, Cu-, and Se-Cu-deficient groups. Again it was postulated that cytoplasmic GPx activity could protect the neutrophil by scavenging

peroxides; thus, the loss of neutrophil GPx activity and subsequent damage to the cells by peroxides could cause the observed decreases in microbicidal activity when an animal was deficient in Se (133;145).

1.2.1.c. Effects of Se in humoral immunity

Studies investigating the effects of Se on antibody production have shown some minimal effects on the magnitude of the total or specific antibody responses in a variety of domestic animals (133;147-149). Selenium supplementation alone has little effect on the antibody response in pigs or ruminants, however, in other species inadequate levels have had adverse effects.

In rats there is decreased IgM, IgG, and IgA titers, and in humans IgG and IgM titers were decreased (132;133). One study evaluated the effect of supplemental Se on humoral antibody production in the equine. This study consisted of 15 ponies that were divided into two groups: diets consisting of low Se concentration (0.02 ppm) and high Se concentration (0.22 ppm). Prior to starting the trial, the ponies were subjected to Se-depleted diets. Each pony was antigenically challenged with ovine packed red blood cells upon receiving its respective diet and again two weeks later. Results showed that the ponies receiving the Se supplemented diet had higher GPx activities and blood Se concentrations. The ponies receiving the Se supplemented diet had significantly increased hemagglutination titers as well as IgG concentrations (147).

In one equine study carried out by Baalsrud and Overnes, the effects on antibody production of Se and vitamin E were investigated. Horses were allocated to four groups: no supplementation, Se supplementation, vitamin E supplementation, and combined Se and

vitamin E supplementation. The horses that received vitamin E and Se had significantly better antibody response to equine influenza virus when compared to the other groups (148). In pigs and ruminants, there was also a non-significant response to dietary or injectible Se supplementation when administered alone; the addition of vitamin E to the diet potentiated the antibody response to both vaccine and antigen exposure (149).

1.2.1.d. Effects Se in thyroid hormone metabolism and function

Selenium deficiency may also impair thyroid function (150). Selenium is required for the conversion of metabolically inactive thyroxine (T4) into the active triiodothyronine (T3) via the enzyme type 1 deiodinase (150). A deficiency in Se results in higher plasma concentrations of T4 and concurrent lower T3 concentrations. This was illustrated in studies investigating calves and ewes grazing on pastures deficient in Se (151;152).

The correlation between thyroid hormones and immune function has been investigated in several studies (153-158). Adequate development and function of antibody and cell-mediated immune responses require normal thyroid hormone concentrations (156;157;159).

One study in mice showed that hypothyroidism could have a negative effect on the development, maintenance, and function of the immune system. In this study, hypothyroid mice had a significant decrease in splenic weight, CD4⁺ splenocyte numbers, and the ratio of CD4⁺:CD8⁺ splenocytes (157). Another murine study demonstrated a bi-directional communication between the thyroid axis and the immune system. Administration of thyroid hormones resulted in increased alloantibodies and lymphocyte proliferation, whereas

lowering thyroid hormones via propylthiouracil therapy resulted in decreased humoral and cell-mediated responses (156).

A study involving humans with differentiated thyroid carcinoma investigated the affects of thyroid hormones on the cell-mediated response. This experiment showed that thyroid hormones do modulate the cell-mediated immune response in humans. Shortly following thyroxine withdrawal, serum levels of IL-18, soluble IL-2 receptors and the percentage NK cells progressively declined (160). Lastly, a recent murine study explored the relationship between thyroid hormones and lymphocyte activity via protein kinase C signaling pathway. This investigation illustrated that there is a thyroid hormone-mediated regulation of protein kinase C content and cytokine production in lymphocytes. They also noted that lymphocytes from hypothyroid mice had lower T and B cell mitogen-induced proliferation when compare to euthyroid mice. Supplementation with T3 restored the lymphocyte proliferation response (155). Therefore, based on the afore-mentioned investigations, we can speculate that the immune function of domestic animals can be affected by Se status, and this maybe mediated in part by changes in thyroid metabolism.

1.2.2. Vitamin E

Vitamin E is widely recognized as a major lipid-soluble anti-oxidant in biological membranes. Vitamin E contributes to membrane stability, regulates membrane fluidity, and protects cellular structures against oxidative stress damage (161;162). This nutrient complements the role of Se. Vitamin E likely enhances immune response and phagocytosis by prevention of lipid peroxidation of cell membranes. Its antioxidant effects also modulate the biosynthesis and activity of important cell regulators, prostaglandins, thromboxane and leukotrienes (163).

Plants synthesize vitamin E, and it is found in adequate amounts for livestock in fresh alfalfa, timothy, meadow fescue and Kentucky bluegrass. Unfortunately, it deteriorates over time when these grasses are stored as hay. Thus, the intake of vitamin E is seasonal in temperate and northern climates and is dependent on several factors: species of plant, plant maturity at harvest and conditions during storage or processing (128;164).

Vitamin E deficiency is rare in humans, but there are three specific situations when it is likely to occur. Vitamin E deficiency is seen in people who cannot absorb dietary fat, in premature or low birth weight infants, and in individuals with rare disorders of fat metabolism. As with Se, vitamin E deficiency is more often associated with sub-clinical manifestations, such as immuno-suppression (165).

1.2.2.a. The role of vitamin E in the arachidonic acid cascade

Similarly to Se, vitamin E can alter the activity of enzymes involved in the arachidonic acid cascade (Figure 1-1). The LOX pathway is dependant on the enzyme 5-lipoxygenase to produce leukotrienes, lipoxins and HETE. Vitamin E inhibits the action of this enzyme (125). Free radicals are essential in lipoxygenase-catalyzed reactions and leukotriene biosynthesis. Vitamin E is hypothesized to interfere with these required free radicals. This hypothesis was supported by the results of a study which revealed elevated levels of leukotriene and 5-HETE in macrophages during vitamin E deficiency (125). T lymphocytes and macrophages are recruited by these leukotrienes. As a result of vitamin E deficiency, there can be increased risk for inflammatory conditions such as atherosclerosis. Thus, maintaining appropriate tissue vitamin E concentrations may lessen the incidence of conditions linked to increased levels of leukotrienes.

Vitamin E can have effects on the COX pathway. Inadequate vitamin E causes immune cell membranes to become unstable and enhances production of immunosuppressants, such as PGE₂, via the COX pathway (162). The excessive production of these eicosanoids leads to an inhibition of T cell proliferation, decreased production of cytokines leading to a decreased TH2 response, and suppression of APCs (166-168). The inhibition of T cell proliferation by PGE₂ has been proven to be due to decreased IL-2 production (169-171).

Supplementation with vitamin E has resulted in a substantial decrease in PGE₂ synthesis and a concurrent increase in T cell proliferation. In one study, administration of vitamin E decreased nitric oxide (NO) production directly and decreased PGE₂ production via the COX pathway. One hypothesis concerning the relationship between NO and PGE₂ postulates that the effect of NO is mediated by peroxynitrite (ONOO), a potent oxidant, which stimulates COX activity. Vitamin E supplementation may reduce this oxidant resulting in decreased PGE₂ synthesis (162;172).

1.2.2.b. The role of vitamin E in cell-mediated immunity

Vitamin E supplementation is associated with significantly enhanced lymphocyte proliferation, increased IL-2 production and enhanced delayed-type hypersensitivity skin responses (173). Supplementation of vitamin E at doses 2-10 times higher than the recommended daily allowance led to significantly increased humoral and cell-mediated immune responses, as well as enhanced phagocytic function in a variety of animal models and in humans (163;173;174). One study in men and women over 60 years of age, given either 800 mg of vitamin E or a placebo for 30 days, found there was an enhanced delayed

hypersensitivity response. There was also a significant increase in both IL-2 production, and mitogen response in the vitamin E supplemented group. It was hypothesized that this change was likely due to the reduction of PGE₂ and plasma lipid peroxide in response to supplementation, since the vitamin E-supplemented group did have a significantly lower concentrations of both metabolites (173). Meydani *et al.* conducted a study to identify the most effective supplemental dose of vitamin E in humans with respect to the cell-mediated immune response. They demonstrated that there was an enhanced delayed hypersensitivity and a better antibody response to vaccine with vitamin E supplementation. This response was found to be optimal at a vitamin E dose of 200 mg/day for an average adult (175).

1.2.2.c. Domestic farm animal studies

The use of vitamin E supplementation has proven to be so beneficial to the immune system that it has been used directly in vaccines. Vitamin E adjuvant vaccines have been developed and tested for practical use in vaccinating farm animals against major diseases. A vitamin E adjuvant used in formulating an *Escherichia coli* (0111:B4) J5 vaccine resulted in a persistently higher IgG antibody titer when compared to the comparable commercial vaccine containing no vitamin E (176). A vitamin E adjuvant vaccine containing heat-killed *Brucella ovis* cells also gave higher antibody titers and better protection when compared to a commercial vaccine with no vitamin E adjuvant. A large field trial in Peru was completed wherein the antibody titers 2 years after *Brucella ovis* vaccination were still higher in the vitamin E adjuvant vaccinated rams than in the rams vaccinated with two popular commercial vaccines. The higher titers probably indicate that the rams vaccinated with the

vitamin E adjuvant vaccine responded better to recurrent infections in the endemic area of the trial, and thus were better protected (174).

Hogan *et al.* investigated the response of bovine neutrophils to parenteral vitamin E. The cows were divided into four groups that received supplemental vitamin E by dietary supplementation, by injection, or by both methods of supplementation. The fourth group did not receive any supplementation. Neutrophils from cows injected with vitamin E had greater intracellular killing ability of bacteria at calving than did neutrophils from placebo-injected cows. Interestingly, the dietary vitamin E during the dry period had no effect on neutrophil function at calving (177).

1.2.3. The role of selenium and vitamin E together.

Treatment with combinations of Se and vitamin E tends to result in a greater immune response when compared to treatment with either alone: these two nutrients appear to have an additive, and perhaps in some cases, synergistic effect on immune function. For example, one study looked at the effect Se and vitamin E on antibody production in sheep after vaccination with a *Chlamydia psittaci* vaccine. There were four groups of sheep given treatments as follows: injectable Se, oral vitamin E, injectable Se/oral vitamin E combination and a control group given saline. The groups supplemented with Se and the vitamin E/Se combination had a significant increase in antibody titer after the first vaccination when compared to the other groups. Sheep that received only vitamin E showed an increase in antibody titer after the second vaccination, however it was moderate in comparison to the Se and Se/vitamin E groups. Supplementation with Se and vitamin E resulted in the highest antibody titer production after the first vaccination. The researchers concluded that the administration of Se led to a superior immune response with higher antibody production in response to the

vaccine. A combination of Se with vitamin E also gave a good immune response but inferior to Se alone. The beneficial effects of vitamin E alone were minimal (178).

Another comparison study was conducted on ovine lymphocytes to illustrate if supplementation with vitamin E and Se could enhance lymphocyte response. Ovine lymphocytes responded to mitogenic stimulation with even a low level of vitamin E and Se when compared to lymphocytes from the control group. The vitamin E, added to lymphocytes in vitro, produced responses that were consistently higher than Se alone, and combination of the two did not lead to a response that was markedly greater than that with vitamin E alone (179).

The cellular immune response in pigs fed a vitamin E and Se-deficient diet was investigated (180). This study investigated lymphocyte proliferation, natural killer cell activity, antibody-dependent cell-mediated cytotoxicity, and respiratory burst of stimulated granulocytes. There was suppression of mitogen-induced lymphocyte proliferation in the pigs fed the deficient diets (180). There were no other significant changes with the other parameters investigated.

Recent research was carried out to explore how vitamin E and Se deficiency leads to immuno-suppression, looking specifically at lymphocyte proliferation. The results suggest that transferrin receptor internalization, which is important for lymphocyte proliferation, is decreased in lymphocytes from vitamin E and Se-deficient animals (181).

The effect of vitamin E and Se status on the humoral immune system was investigated in horses. This study involved 15 horses that were maintained on low Se and vitamin E diets prior to treatment. The horses were divided into groups who were fed diets supplemented with Se, vitamin E, and a combination of the two, or no supplement at all.

After antigen stimulation, the antibody titers were compared. The results showed that there was a significantly higher immunoglobulin response in the horses fed either the vitamin E alone or vitamin E in combination with Se. Selenium supplementation by itself did not generate significantly different response compared to group that did not receive any supplement (148). This reiterates the additive effect of vitamin E and Se on enhancing immune response.

There have been several field studies that have looked at the effects of selenium and vitamin E has on mastitis (182;183). Erskine *et al.* carried out a study on Holstein heifers that received Se-deficient or Se-supplemented diets and were intracisternally challenged with *E. coli*. All heifers established an infection in the inoculated quarter. However, in the Se-deficient group there was greater gland quarter atrophy, agalactia, and a reduction in overall milk yield, when compared to the Se-supplemented group. The Se-deficient heifers had significantly higher bacterial concentrations and duration of infection (183). Another field challenge study by Erskine *et al.* compared mastitis in Se-deficient and Se-supplemented heifers challenged with *Staphylococcus aureus* administered intracisternally. This study yielded similar results with the Se-deficient heifers having higher bacterial concentrations and much faster increase in milk somatic cell counts (SCC) when compared to the Se-supplemented heifers.

There are also some experimental studies that investigated the incidence of mastitis in dairy cattle in cows supplemented with Se and vitamin E. Smith *et al.* carried out a large study where cows were allocated into one of four groups: control, vitamin E- supplemented and Se injected, Se injected and vitamin E-supplemented. The vitamin E supplemented group had a significant 37% decrease in clinical mastitis when compared to all other groups. The

duration of clinical symptoms was reduced by 67% in the cows receiving both Se and vitamin E when compared to the control group (184). Erskine *et al.* investigated the prevalence of mastitis in two dairy herds and mean blood concentrations of Se and GPx activity in these herds. One herd had a high SCC of 700,000 cells/ml while the second had a count of 150,000 cells/ml. Interestingly, the herd with the lower SCC had significantly higher GPX activity and Se concentrations when compared to the other herd (185)

1.2.4. Selenium and vitamin E summary

In summary, Se and vitamin E can influence the immune system through a variety of mechanisms. There is strong evidence that the innate, cell-mediated and humoral components of the immune system can benefit from adequate concentrations of vitamin E and Se.

The initial focus of this thesis is to determine if aging has an effect on the systemic immune response of horses. To achieve this, both healthy young adult horses and aged horses from Prince Edward Island were vaccinated. Serum specific immunoglobulins were measured over time to determine their systemic response to the administered commercial vaccines. Serum Se and vitamin E levels were also determined to help rule out these possible confounding variables.

A concurrent focus of this thesis is to establish the Se and Vitamin E status of the general horse population of Prince Edward Island. To achieve this serum samples from horses in the aforementioned geriatric study as well as racehorses, broodmares and their foals had their serum Se and vitamin E, T3 and T4 levels measured.

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Table 1-1. Expression of CD* markers on various cell types (Adapted from Janeway *et al.*).

CD Antigen	Cellular Expression
CD3	T cells; thymocytes
CD4	TH1 and TH2 helper T cells
CD5	thymocytes; T cells; subset of B cells
CD8	cytotoxic T cells
CD11a	lymphocytes; granulocytes; monocytes; macrophages
CD11b	myeloid cells; NK cells
CD11c	myeloid cells
CD15	neutrophils; eosinophils; monocytes
CD16	neutrophils; NK cells; macrophages
CD18	Leukocytes
CD19	B cells
CD21	Mature B cells; follicular dendritic cells
CD45RO	T cells subsets (memory); B cells; monocytes; macrophages
CD45RA	T cells subsets (naive); B cells; monocytes
CD57	NK cells; subsets of T cells; B cells
CD68	monocytes; macrophages; neutrophils; basophils; large lymphocytes

**: CD stands for cluster of differentiation, which is the set of cell surface molecules that are recognized by a given set of monoclonal antibodies. CD designation is associated with one or more functions. The numbers after the CD represent the order in which the surface molecule was discovered (6).*

Table 1-2. Species-specific diseases caused by selenium and vitamin E deficiency in livestock.

Clinical Disease	Species
White muscle disease (muscular degeneration)	Bovine Caprine Porcine Ovine Equine Avian (turkeys) Avian
Exudative diathesis (generalized edema)	Avian Porcine
Pancreatic fibrosis	Avian
Hepatositis dietetica (enlarged, necrotic liver disease)	Porcine
Mulberry heart disease	Porcine
Blood disorders Heinz body anemia	Bovine Ovine Porcine
Impaired reproductive performance (infertility, early embryonic death, retained placenta, sperm mortality)	Avian Porcine Ovine Bovine
Mastitis	Bovine
Neurologic diseases Equine degenerative myeloencephalopathy Equine motor neuron disease	Equine Equine

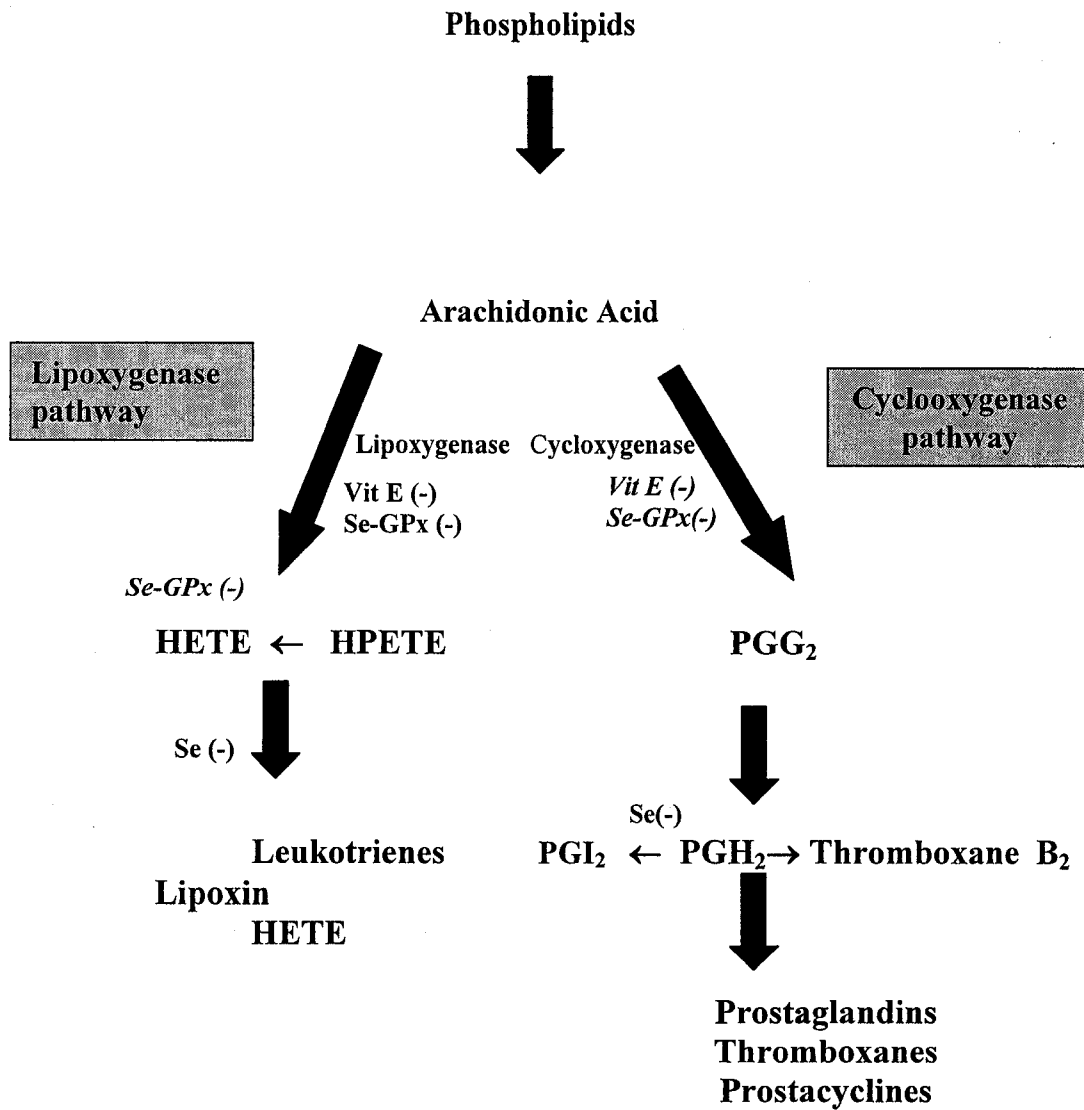


Figure 1-1. The effects of Se and vitamin E on the arachidonic acid cascade (Adapted from Reddanna *et al.*). (Vit E, Vitamin E; Se, selenium; Se-GPx, selenium dependent glutathione peeroxidase; (-)= inhibition)

**CHAPTER 2. THE EFFECT OF AGE ON SYSTEMIC ANTIBODY
RESPONSE FOLLOWING RABIES AND INFLUENZA VACCINATION
IN HEALTHY HORSES.**

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2.1. Abstract

The purpose of this study was to evaluate the effect of age on specific systemic antibody response to vaccination. Thirty-four aged healthy horses (≥ 20 years) and 29 younger adult horses (4-12 years) of various breeds were vaccinated with killed rabies and influenza vaccines. Horses in each age group were allocated to receive either rabies or influenza booster vaccine 4 weeks after the initial vaccination. Serum samples were taken at 0, 4, 8 and 24 weeks. Rabies serum neutralization titers and equine influenza virus specific antibody sub-isotypes (IgGa, IgGb, IgG(T), IgA) as well as single radial hemolysis (SRH) titers were determined.

Anti-rabies titers were similar in the two age groups at all sampling times. Aged horses had higher pre-vaccination anti-influenza titers compared to younger horses but similar post vaccination titers. Younger horses, however, exhibited a significantly greater increase in titer from initial titer compared to aged horses at all sampling times for IgGa and for 8 and 24 weeks for IgGb. The SRH analysis had a similar trend as the IgGa and IgGb ELISA results. There was no detectable serum IgG(T) at any time point. A significant booster vaccine effect was seen for both anti-rabies and anti-influenza titers. Pre-vaccination anti-influenza titer also had a significant effect on subsequent antibody response. In conclusion, we found that healthy aged horses mounted a primary immune response to a killed rabies vaccine similar to that of a younger adult horse; however, aged horses had a significantly reduced anamnestic response to a killed influenza vaccine.

2.2. Introduction

The percentage of geriatric horses within the equine population has increased dramatically in the past decade(1), stimulating an interest in understanding how age affects overall health status. To date, there are limited studies investigating the effect of age on the equine immune system. The innate immune system of older horses, including neutrophils, macrophages and natural killer cells appears to remain intact (2;3); while there is evidence of decline in adaptive immune response including thymic involution and modifications to the circulating T cell populations. Aged horses have a decline in T cell proliferation and shift from a TH1 cell-mediated response to a TH2 humoral response.(2;4) Decreased peripheral B lymphocyte and T cell counts as well as an increase in $CD4^+ : CD8^+$ ratio are found in aged horses (5). There is also some limited evidence that older horses have a reduced specific response to vaccination and pathogen challenges when compared to younger horses (2;6).

The purpose of this study was to evaluate the effect of age on the specific systemic antibody response following rabies and influenza vaccination in healthy horses. Horses involved in this study were from Prince Edward Island, Canada which is free of rabies and thus rabies vaccination is not routinely performed. Enrolled horses had no history of previous rabies vaccination, thus this vaccine was a means of assessing a primary immune response. Since influenza is endemic in the horse population, as reflected by antibody titers before vaccination, influenza vaccination was a means of assessing an anamnestic immune response. Specific aims for this study were to: 1) compare influenza-specific serum IgA, IgGa, IgGb and IgG(T) ELISA titers, as well as anti-influenza single radial hemolysis (SRH) titers in aged and younger adult horses before and after administration of an intramuscular

inactivated influenza vaccine, 2) compare rabies specific serum IgG response in aged and non-aged adult horses after administration of an intramuscular inactivated vaccine, 3) compare the effect of administering a booster vaccination on the specific influenza (IgA, IgGa, IgGb, and IgG(T)) and rabies serum IgG response in aged and younger adult horses, 4) to determine if plasma α -MSH, season, serum Se, T3, T4, breed and sex had an affect on the specific rabies and influenza titers.

2.3. Materials and Methods

2.3.1. Experimental Animals

Sixty-three privately owned healthy horses from various farms across Prince Edward Island, Canada, ranging from 4 to 38 years of age, were included in this study (over 2 years; from 2004 to 2006). Horses were recruited through advertisements and communications with equine clients of the Atlantic Veterinary College. There were 49 light breed horses (23 Quarter horses, 5 Standardbreds, 2 Thoroughbreds, and 19 crossbred horses), 10 ponies, and 4 draft horses. There were 33 females (all non-pregnant and 1 lactating mare) and 30 castrated males enrolled in the study. The horses enrolled in this study historically had not had an influenza vaccination for one year prior to entering the study and had never had a rabies vaccination. For the duration of the study, owners were restricted from administering any further vaccines and were obligated to report if their horse became ill (i.e. respiratory signs). All horses were considered to be healthy based on the results of a general physical exam, complete blood cell count, and biochemical profile. Blood samples were collected into EDTA and serum evacuated tubes. Serum selenium (Se), vitamin E, T3 and T4

concentrations, as well as plasma alpha-melanocyte stimulating hormone (α -MSH) were measured in all horses. The horses were divided into 2 groups: an aged group consisting of horses ≥ 20 years of age ($n = 34$) and a younger group consisting of horses between 4 – 12 years of age ($n = 29$).

2.3.2. Vaccination and Serum Sampling Protocol

Serum samples were collected via jugular venipuncture prior to the initial vaccination and then at 4, 8, and 24 weeks post initial vaccination. All blood samples were allowed to clot, centrifuged, and serum collected for storage at -80°C . After initial blood collection, all horses were administered three intramuscular vaccinations: rabies^a, tetanus^b and a combination equine influenza and equine herpes virus 1-4^c. Based on vaccination history obtained from the owners, the majority of the horses had not received a tetanus vaccination within the previous year; thus all horses were administered tetanus toxoid at the initial vaccination time point. The influenza vaccine was chosen based on its superior ability to produce antibodies post vaccination in comparison to other commercial influenza vaccines (7). Horses in both age groups received either an equine influenza or rabies intramuscular booster vaccine 4 weeks following the initial vaccine. Selection of booster vaccine was based on an initial random coin toss followed by alternating allocation of the two vaccines in each group. Owners were informed of the initial vaccines administered; however, they were blinded as to which booster vaccine their horse received.

2.3.3. Laboratory Analysis

2.3.3.a. Enzyme Linked Immunoassay (ELISA) for Anti-influenza Antibodies

Serum samples were analyzed for Eq/Ky/81 virus specific antibody using a previously described ELISA technique (8). Highly purified Eq/Ky/81 viral antigen obtained from the influenza virus repository at Colorado State University was coated onto 96-well polystyrene plates (Immunlon^d) at 3 hemagglutinin (HA) units per well for IgGa, IgGb and IgA ELISA plates, and 10HA units for IgG(T) ELISA. The plates were then incubated for 2 hours at 37⁰C, washed with PBS, 0.5% Tween (PBST), and blocked overnight at 4⁰C with 1% teleostean gelatin^e-PBST. All plates were stored at -20⁰C with the blocking agent.

Serum samples were diluted in 1% teleostean gelatin-PBST. Dilution of the serum samples varied for the isotypes: IgGa and IgGb were diluted at 1:800, IgA at 1:4 and IgG(T) was undiluted. Samples were incubated on plates in triplicate except for IgA that was in duplicate. Each plate included control wells consisting of a serum sample negative for influenza-specific antibodies, a predetermined series of dilutions of a standard positive equine anti-Eq/Ky/81 virus serum known titer (determined by comparison with a negative serum control in this ELISA system), and a blank. All subsequent incubations were 90 minutes at 37⁰C. Plates were subsequently washed with PBST and incubated with equine immunoglobulin isotype-specific monoclonal antibody (anti-IgGa, IgGb, IgG(T), or IgA) at a dilution of 1:2000. Plates were washed prior to the application of peroxidase-conjugated goat anti-mouse IgG and IgM (H+L) (Affinipure^f). Colour was developed by addition of 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate^g. The optical density of each well was determined with an ELISA reader (Spectra Max 340)^h. Titers were

calculated by the comparison with a standard curve constructed from the dilutions of the positive control serum.

2.3.3.b. SRH for Anti-influenza Titers

A modified SRH assay was carried out by measuring the zone of hemolysis of an agar gel containing viral antigen, ovine erythrocytes and guinea pig complement for each equine sample in the Animal Health Testing Laboratory at the University of Guelph, Guelph, ON. The measurement was in accordance to standard operating procedures established from the Animal Health Trust in Newmarket, England. This was converted to zone areas (mm²) based on tables used for human influenza virus laboratory technique (9;10).

2.3.3.c. Rabies Serum Neutralizing Assay (RSNA)

The measurement of rabies neutralizing antibodies in the equine serum was performed at the Rabies Center of Expertise, Ottawa Lab Fallowfield, Canadian Food and Inspection Agency in Ottawa, ON. The method for this fluorescent antibody virus neutralizing test is an adaptation of a tissue culture neutralization assay according to Smith *et al.* (11). Antibody titers were calculated by the Spearman-Kärber method and converted to International Units (I.U.) by comparison to a positive control serum with a known I.U. (12).

2.3.3.d. Serum Se, Vitamin E, Total T4, Total T3, and Plasma α -MSH Analysis

Serum vitamin E was assayed using a modified high performance liquid chromatography with fluorescence detection (13-15). Serum Se analysis was performed

using a modified method of addition calibrations using a palladium-magnesium nitrate matrix modifier on an atomic absorption spectrometer (16). Both analyses were carried out following standard operation procedures at the Toxicology and Analytical Services Laboratory, Atlantic Veterinary College. Measurement of plasma α -MSH concentration was performed in duplicate using a commercially available radioimmunoassayⁱ validated for use in the horse (17). The intra- and interassay variation for this assay was 5%. Total serum T3 and T4 chemiluminescence enzyme immunoassays were performed at the Diagnostic Laboratory, Atlantic Veterinary College^j.

2.3.4. Statistical Analysis

The vaccine response was measured as the increase in natural log titer values for IgA, IgG_a, IgG_b and anti-rabies titers at weeks 4, 8, and 24 compared to baseline. The vaccine response for SRH was measured as the increase in the actual titer values. Vaccine responses across the three time intervals were calculated for each isotype and analyzed separately by general linear models to evaluate the effects of the following predictors: age (younger vs aged horses), booster vaccine (influenza vs rabies), season (fall- August, September, October, winter- November, December, January, spring- February, March, April, and summer- May, June, and July), adjusted initial titer, breed (pony, light horse, vs draft), sex (female vs castrated male), initial serum Se and vitamin E status (deficient/marginal vs adequate levels) (18;19), serum T3, serum T4, and initial plasma α - MSH. Natural log transformation allowed for modeling based on the normal distribution and for multiplicative effects of the predictors. The initial titer adjustment consisted of subtracting the mean titer within each age group, in order to avoid changing the effect of age. All statistically significant ($p \leq 0.05$) predictors

remained in the model as well as any significant interactions of those predictors. Two-sample tests were used to determine if there was a significant difference in initial α -MSH, Se, vitamin E, T3, and T4 concentrations between the two age groups; a natural log transformation was used for Se and T3 data, and a non-parametric Mann-Whitney test was used for α -MSH. A chi-square analysis was performed on the rabies data to determine if there was an association between age and whether the horses maintained protective titers. All data analysis was performed using statistical software^k.

2.4. Results

2.4.1. Experimental Horses

There were 63 horses enrolled in the study period 0-4 weeks (34 aged horses; 29 younger horses). Three horses were removed from the study between 4 and 8 weeks due to owner non-compliance, leaving 60 horses for the analysis (32 aged horses, mean age of 26.1 years \pm 0.4; 28 younger horses, mean age of 8.3 years \pm 0.9). Between 8 and 24 weeks, three horses died due to unrelated causes, leaving 57 horses for analysis (29 aged horses; 28 younger horses). There were 77% light horses (mean age = 16.5 years), 5% draft breeds (mean age = 23 years), and 18% ponies (mean age = 20 years).

2.4.2. Anti- influenza Antibody Serum Titer Response and the Effects of Age and Pre vaccination Titers

The change in serum anti-influenza titers over time for both the aged and younger horse groups are presented for IgGa, IgGb, and IgA (Table 2-1; Appendix 1). No measurable

IgG(T) was detected throughout the trial (assay sensitivity $\geq 1:155$). Both age groups had measurable IgGa, IgGb and IgA anti-influenza titers prior to vaccination.

Initial IgGa and IgGb titers were significantly higher in aged horses compared to the younger horses ($p= 0.004$ and $p= 0.0027$, respectively). At week 4, there was significant anti-influenza antibody response to the initial vaccination in both age groups for IgGa and IgGb ($p\leq 0.001$). At week 8, the aged and younger horse groups that received an influenza booster on week 4 generated a higher median IgGa and IgGb anti-influenza titer ($p\leq 0.001$) compared to the non-influenza booster groups' whose titers declined by week 8 (Table 2-1). At week 24, all groups had a declining median titers but a booster effect remained for both IgGa and IgGb antibodies ($p= 0.051$ and $p= 0.003$, respectively).

The initial titer had a significant negative effect on the magnitude of change in anti-influenza IgGa and IgGb titers at all subsequent sampling times ($p\leq 0.001$). The younger horses had a significantly greater increase in anti-influenza IgGa titers for the entire sampling period ($p\leq 0.001$) compared to the aged horses (Table 2-1). There was also a significant interaction between age and initial titer for the change in anti-influenza IgGa titers at 4 ($p= 0.032$), 8 ($p= 0.044$), and 24 weeks ($p\leq 0.001$). The impact of initial titer was less pronounced for older horses compared to the younger horses. Similarly, the increase in younger horses' anti-influenza IgGb titers post vaccination was greater compared to the aged horses at 4 ($p= 0.079$), 8 ($p= 0.041$) and 24 weeks ($p= 0.010$) post initial vaccine (Table 2-1). There was no significant interaction between age and initial IgGb titers.

There was a significantly greater increase in anti-influenza IgA titers in aged horses compared to younger horses at 4 weeks ($p\leq 0.001$), 8 weeks ($p= 0.035$), and 24 weeks ($p=$

0.01) (Table 2-1). There was no effect of the influenza booster vaccine on IgA titers (Table 2-1). The increase in SRH titer trends were similar to IgGa and IgGb between the two age groups but were not significant (Figure 2-2; Table 2-2). The influenza SRH analysis revealed a significant booster effect at 8 and 24 weeks ($p \leq 0.001$ and $p = 0.017$, respectively; Table 2-2).

2.4.3. Anti-Rabies Serum Titer Response and the Effect of Age

Anti-rabies titers were detectable (≥ 0.1 I.U.) in 5 horses at the initial sampling time indicating that these horses had likely been previously vaccinated against rabies virus; therefore, they were removed from the analysis leaving 31 aged horses and 24 younger horses. All but 2 horses (aged group) responded to the initial rabies vaccine by 4 weeks (Table 2-3; Appendix 1). One of these aged horses received a rabies booster vaccination but still had minimal serologic conversion (0.57 I.U.).

Younger horses tended to have higher median anti-rabies titers at all time points compared to their aged counterparts but this was not significant (Figure 2-5; Table 2-3). A significant booster effect was observed in the anti-rabies titers at both 8 and 24 weeks ($p \leq 0.001$) (Table 2-3). The median anti-rabies titers at week 8 in the rabies booster groups rose significantly, whereas the non-rabies boosted horses' titers declined. By 24 weeks, all of the horses' titers had declined, although the rabies boosted horses maintained significantly higher anti-rabies titers.

Horses receiving a single dose of rabies vaccine were assessed for serum antibody titers >0.5 I.U. At 8 weeks post-vaccination, only 18% of aged, non-rabies boosted horses

had a serum titer >0.5 I.U., compared to 50% of younger horses but this was not statistically significant ($p=0.08$). By 24 weeks, 89% of non-rabies boosted horses had serum titers <0.5 I.U. All horses, with the exception of one aged horse, receiving rabies booster vaccines had serum titers >0.5 I.U. at 8 weeks, although 28% of these horses had titers below 0.5 I.U. at 24 weeks.

2.4.4. The Effect of Plasma α -MSH and Season on Anti-influenza and Anti-rabies Titers.

Plasma α -MSH concentration in younger horses (mean = 24.4 pmol/L, median = 15.9 pmol/L) was significantly lower than in aged horses (mean = 85.6 pmol/L, median = 47.08 pmol/L, $p < 0.005$) (Table 2-4). There was a significant positive effect of plasma α -MSH on anti-influenza IgA titers at 4 weeks ($p=0.052$) and at 24 weeks ($p=0.007$; Table 2-1). No other significant associations were noted between α -MSH and anti-influenza or anti-rabies titers. Since plasma α -MSH concentrations can fluctuate with season, the effect of season was included in the model for the change in antibody titers, but no significant seasonal effect was found. Most initial samples were collected in the summer (aged- 40%; younger adults- 25%) and fall (aged- 56%; younger adults- 75%) months.

2.4.5. The Effect of Serum Se, Vitamin E, Total T3, and Total T4 Concentrations on Anti-influenza and Rabies Titers

Selenium, vitamin E, total T3 and T4 concentrations were measured in all initial serum samples (Table 2-4). Ninety percent of all the horses in this study had either deficient or marginal serum Se concentration (<0.140 ppm). The horses with adequate Se

concentrations were all in the aged horse group. The aged horses had significantly higher vitamin E concentrations compared to the younger horses ($p= 0.001$). Twenty-three percent of the horses had deficient serum vitamin E concentrations ($<150 \mu\text{g/dL}$). However, neither Se nor vitamin E status had a significant impact on antibody production following vaccination.

None of the horses in this study had a plasma T3 concentration below the reference range ($<0.4 \text{ nmol/L}$) and only 9% of the horses (4 aged, 2 young) had a low T4 concentration ($<15 \text{ nmol/L}$).¹ The younger horses had a significantly higher serum T4 concentration as compared to the aged horses ($p= 0.026$). There were no effects of T3 or T4 concentrations on antibody production following vaccination.

2.4.6. The Effect of Breed and Sex on Anti-influenza and Rabies Titers

Light horse breeds had a significant increase in anti-influenza IgGb titers at 24 weeks ($p= 0.013$) compared to the pony and draft horse breeds (Table 2-1). Breed also had significant effects on the change in the mean SRH influenza titers at all sampling time periods ($p= 0.017$, $p\leq 0.001$, and $p\leq 0.001$, respectively) with the light horses having a greater increase in SRH levels compared to the pony and draft breeds.

Females had a significantly greater change in anti-rabies titers at the 8-week sampling time ($p= 0.049$) (Table 2-1). There were no other significant associations between gender and change in antibody titer.

2.5. Discussion

2.5.1. Primary Immune Response

A primary immune response occurs when an animal is exposed to an antigen for the first time. This response involves recruitment of naïve lymphocytes which become activated, proliferate and finally create memory T-cells and B-cells which have a role in providing protection from subsequent challenges by the same antigen (20). Studies in aged people and mice reveal a shift in lymphocyte populations, with a greater number of memory cells and fewer naïve cells in the aged subjects. This potentially could result in a reduced capacity of the elderly to respond to a novel pathogen compared to younger subjects (21-24). Impaired primary immune response has also been reported in aged horses (2;4). In aged horses, it has been shown that there is a shift in lymphocyte subsets and a decrease in peripheral lymphocytes including a decrease in CD4 and CD8 cells that could alter immune function (5). Assessment of primary immune response has traditionally been performed by measuring antibody production following vaccination using a novel antigen such as sheep red blood cells or bacteriophage λ (25;26).

In the study reported here, antibody production following exposure to rabies antigen was used to measure primary immune response. This was possible because the study was performed on Prince Edward Island, which is rabies free, thus most horses are not rabies vaccinated. Therefore, after excluding horses with a history of rabies vaccination or a detectable baseline rabies serum titer, response to rabies vaccination was used to assess a primary immune response. There was no significant difference in the anti-rabies neutralizing antibody titer response between the aged and the younger horses. However the anti-rabies

titers generated by both the younger and aged horses that received a single dose of rabies vaccine were generally low. At 8 weeks post-vaccination, 82% of aged horses and 50% of younger horses had anti-rabies titers of <0.5 I.U. A titer of <0.5 I.U. in rabies vaccinated humans is an indication for a booster vaccine (27). By 24 weeks, 89% of horses receiving a single vaccine dose had anti-rabies titers below 0.5 I.U.. In comparison, all but one horse receiving a booster rabies vaccine had titers >0.5 I.U. at 8 weeks post initial vaccination although 28% of these horses had serum titers below this level at 24 weeks. Currently there is no recommended protective titer level established for horses. The current labeled protocol for the commercial rabies vaccine used in this study is a single initial vaccination followed with annual boosters^a. This recommendation was established based on experimental challenge in horses; unfortunately serum titers were not monitored during this challenge trial (personal communication; Dr. Normand Plourde, Merial Canada Inc., QC). These findings indicate that: 1) aged horses may have greater difficulty maintaining an adequate antibody titer after a single rabies dose compared to younger horses, 2) the current rabies vaccination label recommendation of a single dose being administered on primary vaccination may need to be reconsidered and 3.) vaccination protocol for horses against rabies may need to be re-evaluated for horses that have inadequate Se concentrations.

2.5.2. Secondary Immune Response

Since influenza is endemic in the equine population, response to influenza vaccination was used to evaluate a secondary or anamnestic immune response. All horses had evidence of previous exposure to the influenza antigen as evidenced by detectable serum

IgGa, IgGb, IgA, and SRH anti-influenza titers prior to vaccination. The younger horses had a significantly greater magnitude of increase in IgGa and IgGb post-vaccination compared to aged horses. The SRH titers post-vaccination followed a similar trend but did not reach statistical significance.

Pre-vaccination anti-influenza IgGa and IgGb titers were significantly higher in aged horses but antibody titers were similar among both age groups that received the same booster treatment at all post-vaccination time points. These findings were consistent with another study investigating the efficacy of commercial influenza vaccines in horses which also demonstrated that younger horses had lower antibody titers in comparison to older horses which suggests that the older horses were better protected against infection (28). One explanation for the higher magnitude change in influenza titers in younger horses is that they started at lower pre-vaccination titers, which allowed for a greater antibody response post-vaccination. However for IgGa and IgGb, both age and initial titer remained significant predictors of change in magnitude of influenza titer response, even with the pre-vaccination age group interaction included in the model. This indicated these 2 factors are each significant predictors of antibody response.

The serum anti-influenza IgA titers in the aged horses had a small but significant increase post initial vaccination while none was seen in the younger horses. There was no further increase in IgA titers post booster vaccine. The actual serum IgA titers in both age groups were very small in comparison to the IgGa and IgGb titers making the biological significance of this finding questionable. The majority of the IgA immunoglobulin is found in mucosal surfaces where its activity is most important for protection against respiratory

viral infections (20). Thus, the mucosal IgA titers would be more relevant but were not measured in this study.

High initial anti-influenza titers in the older horses compared to the younger horses may be the result of a greater number of exposures to influenza virus. Alternatively it may be the consequence of an aged-related switch to a predominantly TH2 response. As people age, the TH1 cell-mediated immune response declines and the TH2 humoral response predominates (29-31). While advanced age favors a humoral response, humoral function does decline, resulting in a dampened antibody response when challenged (29-31). Similarly, Horohov *et al.* found that aged horses had a 10-fold lower anti-influenza antibody response than younger horses (2). This is consistent with the blunted anti-influenza antibody response observed in the aged horses in this study.

Anti-influenza IgG(T) was not detected at any time point throughout this study. In a previous study, influenza naïve feral ponies serum IgG sub-isotype response was evaluated after challenged with influenza virus (8). These ponies mounted a strong IgGa and IgGb response but no IgG(T) response. In our study, both young and aged horses demonstrated a post-vaccination IgG sub-isotype response typical of natural infection.

SRH titers are known to correlate well with antibody concentrations specific for equine influenza virus neutralization and protection (32-35). The SRH titer corresponding to protection varies among studies. Mumford *et al.* found that a pre-viral challenge SRH level of $\geq 74 \text{ mm}^2$ correlated with protection against disease and $\geq 100 \text{ mm}^2$ significantly reduced the time of virus shedding (35). In a similar study, all the ponies that generated SRH titers $\geq 154 \text{ mm}^2$ following vaccination were protected against subsequent infection (no virus

cultured) while ponies that generated titers $\geq 85 \text{ mm}^2$ were protected against clinical disease (34). Another study carried out by Mumford *et al.*, showed a 90% protection against influenza infection at a SRH titer of $\geq 165 \text{ mm}^2$ (36). In our study, the younger horses had a pre-vaccination mean SRH titer of 31 mm^2 while the aged horses had a mean SRH titer of 89 mm^2 . All horses had a strong SRH titer that persisted above the pre-vaccination titers for the 24-week duration of this study period. At 24 weeks post-vaccination, 82% of the horses in this current study maintained SRH titers that would be considered protective of clinical disease ($>85 \text{ mm}^2$). Thus, the difference between age groups in magnitude of antibody response following influenza vaccination observed in the current study would unlikely be clinically significant.

2.5.3. Plasma α -MSH Concentrations and Season

α -MSH is a hormone known to increase in the plasma of equids with pituitary pars intermedia dysfunction (PPID), a prevalent disease of aged horses. PPID is associated with development of secondary infections, such as bacterial sinusitis, endoparasitism and subsolar abscesses. The loss of immunocompetency observed in horses with PPID is presumed to result from increased plasma concentrations of immunosuppressive hormones including α -MSH and cortisol. The effect of α -MSH on vaccination response has not been previously examined. In our study, we did not find an association between plasma α -MSH concentration and IgGa or IgGb immunoglobulins. High plasma α -MSH concentration production was associated with increased production of anti-influenza IgA titers. The biological significance of this finding is unknown.

Plasma α -MSH concentration fluctuates with season (17). McFarlane *et al.* found that there was a significantly greater concentration of plasma α -MSH in horses and ponies tested in late summer and fall compared to the spring. The analysis showed no significant seasonal effect, and no confounding effect of season on α -MSH. Interestingly, even though there were a high percentage of horses from both groups sampled in the fall, there was still a significantly higher α -MSH concentration in the aged horse group. There were 6 aged horses that had very high concentrations.

2.5.4. Serum Se and Vitamin E Concentrations

Selenium and vitamin E exert similar antioxidant effects in cells via independent pathways at different sites (37). Selenium and vitamin E supplementation in Se-deficient ponies enhanced primary immune response with significantly higher serum immunoglobulin IgG titers against tetanus and influenza A compared to the non-supplemented group (38). In addition, the study documented that supplementation consisting of Se and vitamin E or vitamin E alone yielded higher IgG titers against the same antigens as compared to non-supplemented or Se only supplemented groups (38). In our study, Se and vitamin E status had no significant effect on the immune response in either group of horses. However, approximately 89% of the horses in this study had Se concentrations below adequate levels, while 39% of the horses had inadequate vitamin E concentrations. This high proportion of horses with an inadequate Se status in both age groups may have reduced our ability to identify an effect of Se deficiency on the immune response. It is also possible that the

inadequate selenium levels contributed to the poor primary immune response to a single dose of rabies vaccine.

2.5.5. Breed and Sex

All horses in our study were categorized as a light horse, pony or draft horse. Breed had a significant effect on the change in anti-influenza IgGb titers from 0 to 24 weeks with the light horse breeds having approximately 2 times higher antibody titers compared to the pony and draft horse breeds. Analysis of SRH influenza titers also showed a significant breed effect at each follow-up time of this study with light horses having significantly higher titer changes. These differences may be partly the result of the uneven distribution of the breeds and the differences in the average age of the 3 breed groups. A breed/age interaction was not detected; however the small numbers of draft horses and ponies in the study may have limited our ability to find such an interaction.

Sex was not a significant factor except for a single, weakly significant finding for anti-rabies titers at 8-weeks with females having a greater increase in anti-rabies titers compared to geldings. In human studies, females mount a stronger immune response to vaccines when compared to males due to their increased levels of estrogen (39;40). This may be the explanation for the gender effect in this study, however, it was only significant at the 8-week sampling period. Further studies would be necessary to explore this theory in horses.

2.6. Conclusion

In summary, aged horses mounted a primary immune response to a rabies vaccine equivalent to younger adult horses. However, many horses receiving a single rabies vaccine failed to maintain likely protective titers by 24 weeks post-vaccination. Aged horses had a significantly higher initial anti-influenza titer compared to the younger horses. The younger adult horses exhibited a significantly greater magnitude of change in the anti-influenza titers throughout the entire study in comparison to the older horses. This reduced response in the aged horses is likely the consequence of aging as well as preexisting higher pre-vaccination titers. Further studies are needed to better define the influence of age and initial titer concentrations have on antibody production.

Other factors such as Se and vitamin E status were determined in this study to evaluate their effects on the equine immune response. Even though there was no effect on the horse's antibody response, the study did illustrate that there was a significant number of horses in PEI that had sub-optimal serum Se concentrations. Chapter 3 of this thesis focuses on the Se and Vitamin E status of the general horse population of Prince Edward Island. Serum samples from horses in the aforementioned geriatric study as well as racehorses, broodmares and their foals had serum Se, Vitamin E, T3 and T4 levels measured.

2.7. Footnotes

- a.** Imrab®, Meril Incorporated, Baie d'Urfé, Quebec.
- b.** Super-Tet® with Havlogen®, Intervet Canada Ltd., Whitby, Ontario
- c.** Calvenza EIV/EHV®, Boehringer Ingelheim, Ingelheim, Germany.
- d.** Immunlon 2HB®, VWR International, Mississauga, Ontario.
- e.** Teleostean gelatin, Sigma, Ottawa, Ontario.
- f.** Affinipure®, Jackson ImmunoResearch Laboratories, INC, West Grove, PA.
- g.** ABTS substrate, Roche Diagnostics Corporation, Laval, Quebec.
- h.** Spectra Max 340 using SOFT Max® Pro Windows version 1.1, Molecular Devices, Sunnyvale, CA.
- i.** Euria- α -MSH RIA, American Laboratory Products Co., Windham, NH.
- j.** Immulite® Total T4 and T3, Diagnostic Products Corporation, Los Angeles CA.
- k.** Minitab 14®, Minitab Inc., State College PA.
- l.** Atlantic Veterinary College internal reference intervals for equine T3 and T4.

2.8. Acknowledgments

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Table 2-1. Magnitude of change in ln (titer) from pre-vaccination titer for anti-influenza IgA, IgGa and IgGb.

Time (wks)	Model [®]	Group	Magnitude of titer change*					
			IgA	p-value	IgGa	p-value	IgGb	p-value
0- 4	Age	Young	0.580 (0.375,0.901)	0.001	21.80 (13.44,35.38)	0.001	8.03 (5.12,12.6)	0.079
		Old	2.149 (1.436,3.216)		5.70 (3.62,8.96)		4.64 (3.17,7.07)	
	Initial titer				0.001		0.001	
	Initial titer/ age interaction				0.032			
	α- MSH			0.052				
0- 8	Age	Young	0.615 (0.417,0.908)	0.035	22.42 (15.30,32.87)	0.001	12.3 (7.82,19.3)	0.041
		Old	1.092 (0.758,1.572)		4.37 (3.06,6.29)		6.50 (4.22,9.83)	
	Booster	Influenza	0.468 (0.319,0.687)	0.355	21.91 (15.03,31.94)	0.001	16.0 (10.26,24.98)	0.001
		Rabies	0.724 (0.500,1.602)		4.48 (3.11,6.44)		4.94 (3.22,7.60)	
	Initial titer					0.001		0.001
	Initial titer/ age interaction					0.044		
0- 24	Age	Young	0.383 (0.233,0.630)	0.010	6.62(4.36,10.08)	0.001	3.43 (2.14,5.50)	0.010
		Old	1.093 (0.680,1.758)		1.87 (1.24,2.83)		1.78 (1.11,2.89)	
	Booster	Influenza	0.682 (0.454,1.023)	0.719	4.73 (3.11,7.20)	0.051	3.67 (2.28,5.92)	0.003
		Rabies	0.615 (0.410,0.922)		2.62 (1.74,3.96)		1.67 (1.03,2.71)	
	Initial titer					0.001		0.001
	Initial titer/ age interaction					0.001		
	Breed	Pony					1.75 (0.767,3.17)	
		Light					4.37 (3.32,5.74)	0.013
		Draft					1.98 (0.679,5.79)	
α- MSH			0.007					

* : Titer at each time period was X times greater than the value at 0 weeks with a confidence interval of 95% in ().

® : Each model initially included age, booster effect, initial titers, α-MSH, serum Se, serum vitamin E, serum T3, serum T4, breed, season, and sex. Only significant predictors and significant interactions between predictors were kept in the models and shown in this table.

Table 2-2. Change in anti-influenza titer for SRH analysis

Time	Model [®]	Group	LSM	Standard Error	P-value
0- 4 weeks	Age	Young	153.6	8.112	0.096
		Old	168.1	7.768	
	Breed	Pony	156.4	10.42	0.017
		Light horse	182.1	4.895	
		Draft	144.0	16.42	
0- 8 weeks	Age	Young	153.3	7.376	0.078
		Old	167.3	7.034	
	Booster	Influenza	176.0	7.476	0.001
		Rabies	144.6	6.980	
	Breed	Pony	140.6	9.567	0.001
		Light horse	182.5	4.438	
		Draft	157.7	14.86	
0- 24 weeks	Age	Young	98.64	9.585	0.191
		Old	111.9	9.854	
	Booster	Influenza	117.8	9.738	0.017
		Rabies	92.75	9.769	
	Breed	Pony	73.48	11.93	0.001
		Light horse	145.9	5.654	
		Draft	96.37	21.50	

[®] :Each model initially included age, booster effect, initial titers, α -MSH, serum Se, serum vitamin E, serum T3, serum T4, breed, season, and sex. Only significant predictors and significant interactions between predictors were kept in the models and shown in this table

Table 2-3. Magnitude of change in ln (titer) anti-rabies titers.

Time	Model [®]	Group	Magnitude of titer change*	P-value
0- 4 weeks	Age	Young	22.2 (12.3,40.3)	0.225
		Old	13.8 (8.17,23.1)	
0- 8 weeks	Age	Young	69.2 (37.7,126)	0.301
		Old	45.4 (26.8,77.5)	
	Booster	Influenza	10.6 (5.98, 18.7)	0.001
		Rabies	295 (164, 528)	
	Sex	Male	36.8 (20.0, 67.4)	0.049
		Female	85.1 (48.9, 148)	
0- 24 weeks	Age	Young	17.5 (10.1,30.3)	0.302
		Old	11.9 (7.13,19.7)	
	Booster	Influenza	6.15 (3.49,10.5)	0.001
		Rabies	33.7 (19.7,57.7)	

[®] :Each model initially included age, booster effect, initial titers, α - MSH, serum Se, serum vitamin E, serum T3, serum T4, breed, season, and sex. Only significant predictors and significant interactions between predictors were kept in the models and shown in this table

* :Titer at each time period was X times greater than the value at 0 weeks with a confidence interval of 95% in ().

Table 2-4. Mean serum variables and standard deviations in aged and younger horses.

	Aged horses	Young horses
α- MSH (pmol/L)	85.6 \pm 77.3	24.4 \pm 32.3 [⊗]
T4 concentration (nmol/L)	25.4 \pm 7.09	28.5 \pm 9.75 [*]
T3 concentration (nmol/L)	1.16 \pm 0.0761	1.19 \pm 0.0568
Selenium (ppm)	0.0832 \pm 0.0509	0.0563 \pm 0.0265
Vitamin E (μg/dL)	312 \pm 149	195 \pm 104 [*]

^{*}: *p-value of < 0.05 by two-sample t-test.*

[⊗]: *p-value of < 0.05 by Mann-Whitney test.*

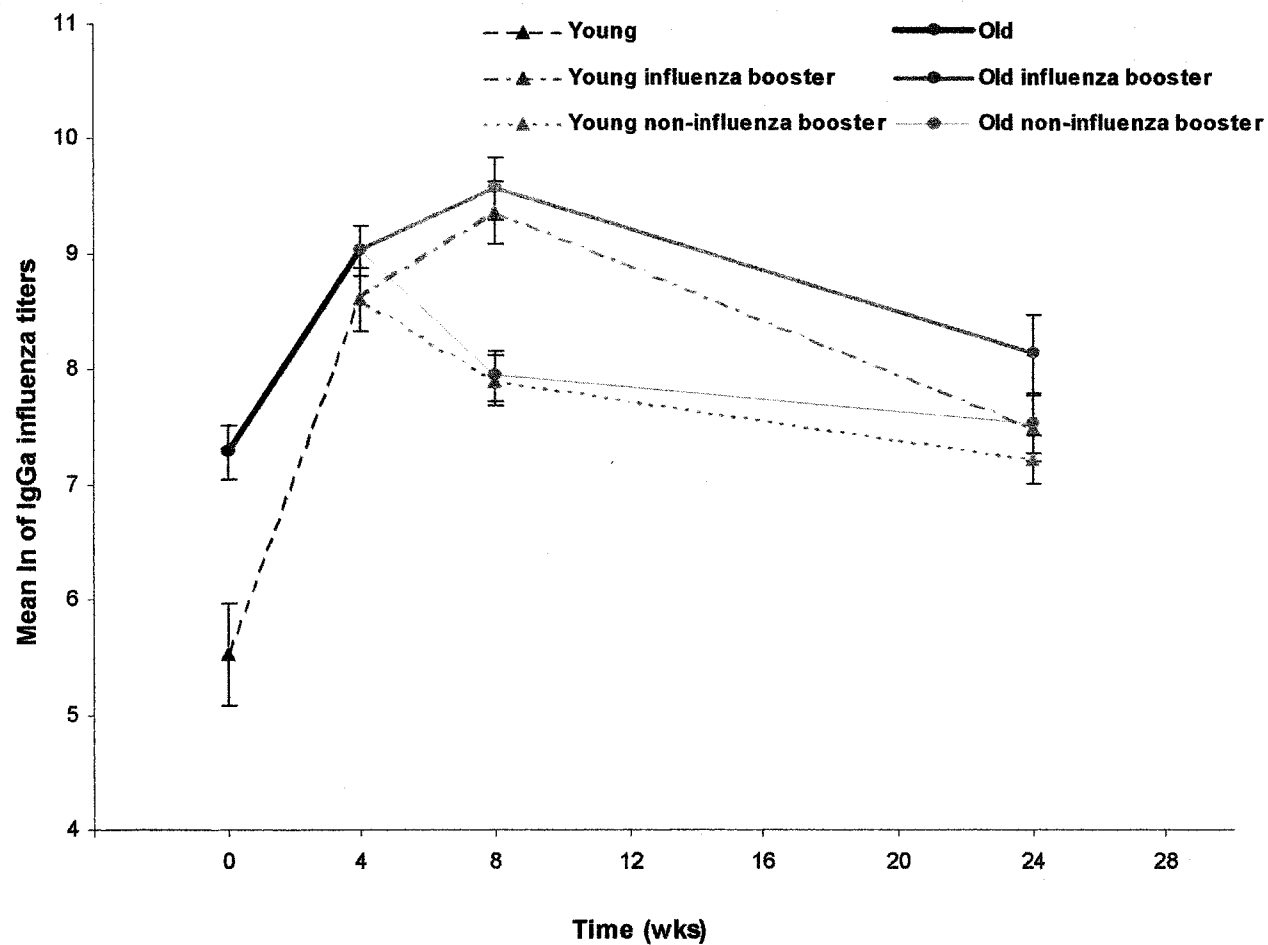


Figure 2-1. Mean \pm SEM ln of serum IgGa titers for influenza in aged (20 years plus) and young adult horses (4-12 years) pre-and post-vaccination with commercial killed influenza vaccine.

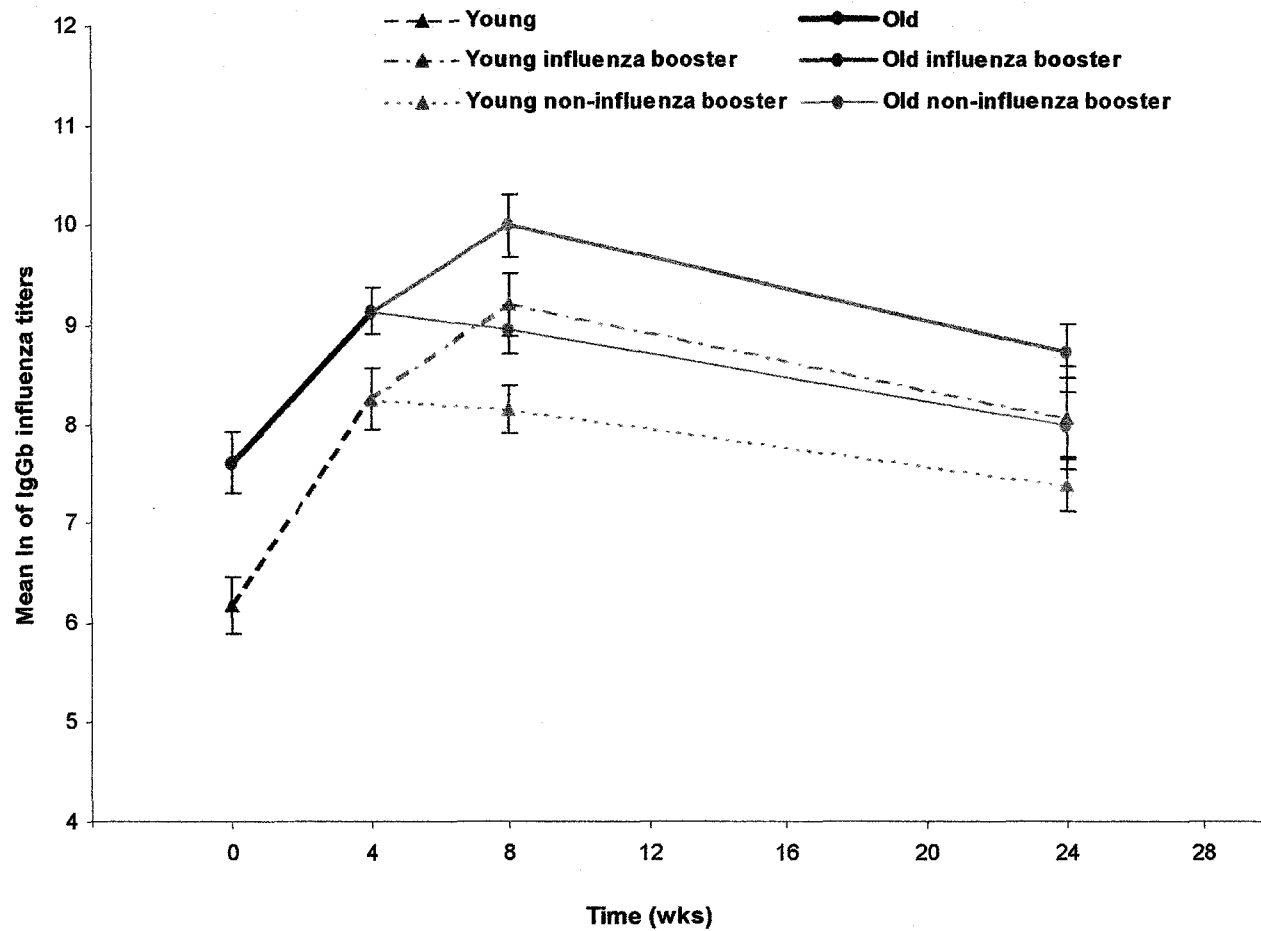


Figure 2-2. Mean \pm SEM ln of serum IgGb titers for influenza in aged (20 years plus) and young adult horses (4-12 years) pre- and post-vaccination with commercial killed influenza vaccine.

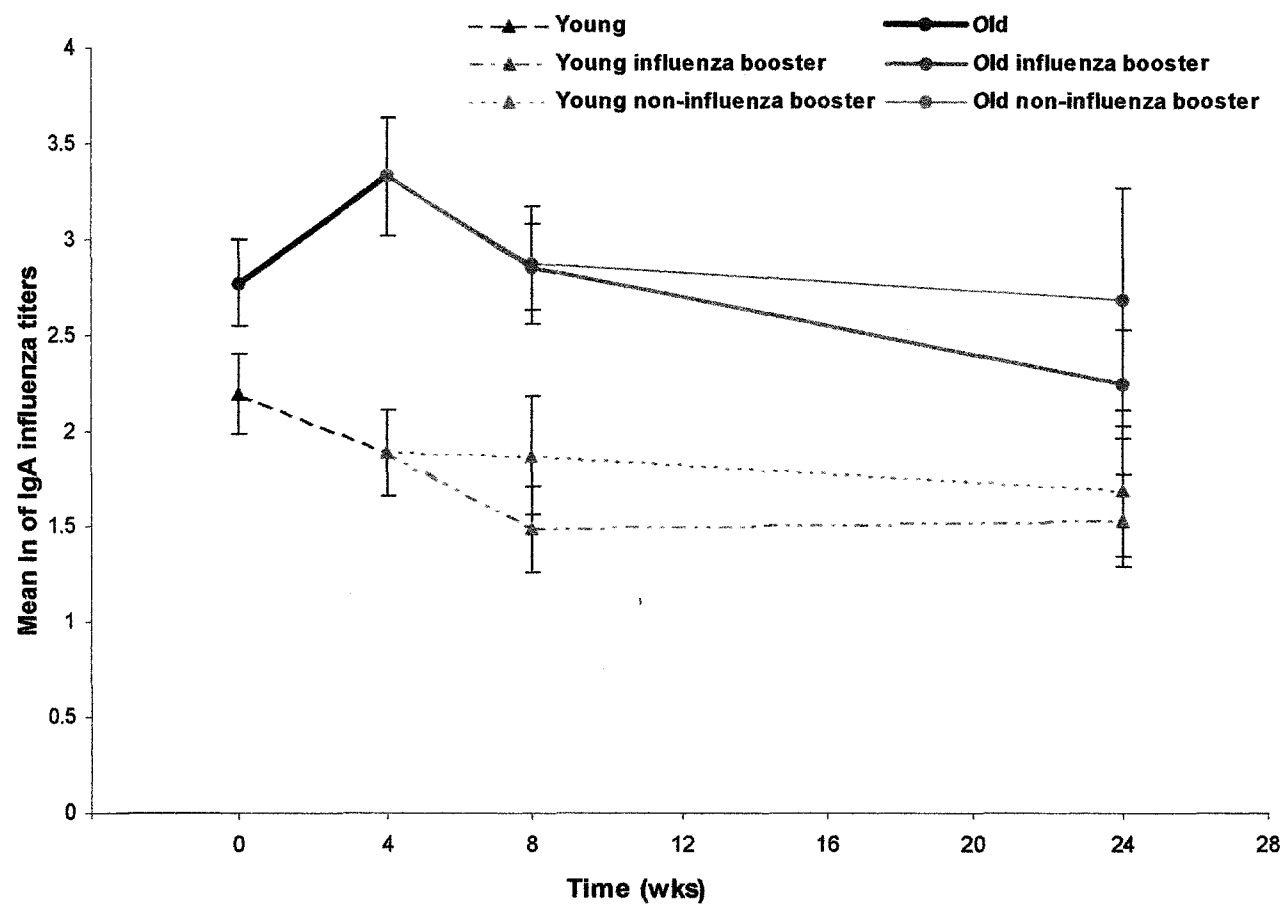


Figure 2-3. Mean \pm SEM ln of serum IgA titers for influenza in aged (20 years plus) and young adult horses (4-12 years) pre- and post-vaccination with commercial killed influenza vaccine.

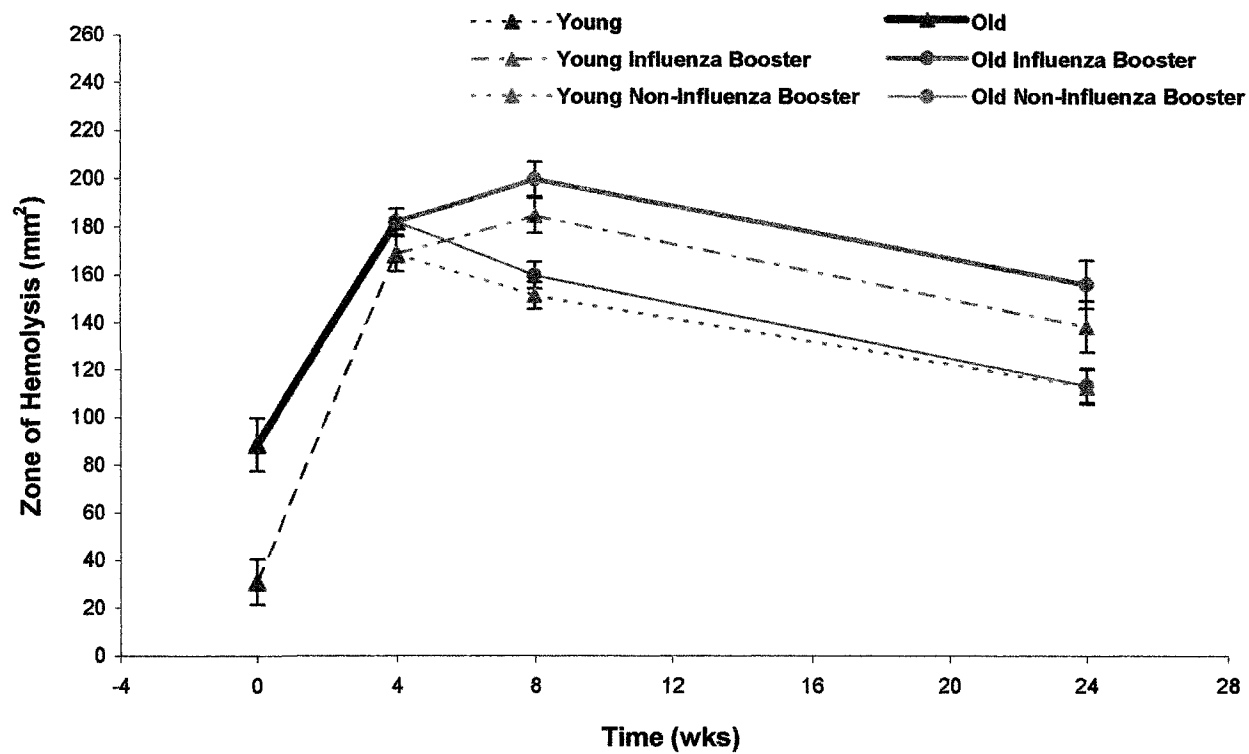


Figure 2-4. Mean \pm SEM of SRH zones of hemolysis for influenza in aged (20 years plus) and young adult horses (4-12 years) pre- and post-vaccination with commercial killed influenza vaccine.

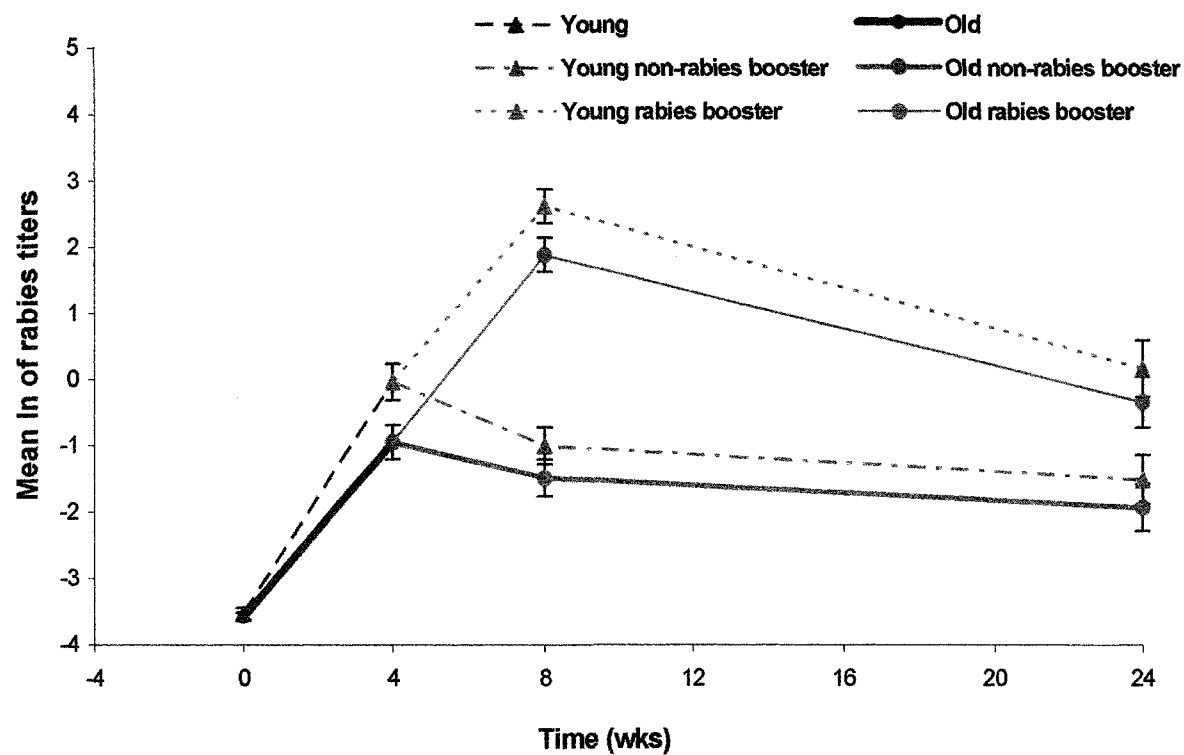


Figure 2-5. Mean \pm SEM ln of serum rabies titers in aged (20 years plus) and young adult horses (4-12 years) pre- and post-vaccination with commercial killed rabies vaccine.

**CHAPTER 3. THE SELENIUM AND VITAMIN E STATUS OF HORSES
IN PRINCE EDWARD ISLAND.**

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To be submitted to CVJ

3.1. Abstract

Serum selenium (Se) and vitamin E concentrations were measured in 201 horses from Prince Edward Island (PEI). Horses were selected from 5 demographic groups: aged pleasure horses, young adult pleasure horses, racehorses, broodmares and their foals. Approximately 79% of all horses had inadequate Se concentrations based on currently accepted reference ranges. The aged and young adult pleasure horses had the highest prevalence of inadequate serum Se concentrations (82% and 97%, respectively). Racehorses and broodmares had significantly higher serum Se concentrations compared to the pleasure horse groups ($p < 0.001$, respectively). There was an effect of season on serum Se concentrations; horses tested in the winter months had significantly higher concentrations compared to those tested in the spring. Eighty-seven percent of the horses in this study had adequate serum vitamin E concentrations. The aged pleasure horses, broodmares, and racehorses all had significantly higher serum vitamin E concentrations compared to the young adult pleasure horses ($p < 0.001$). Selenium status was positively associated with vitamin E status in racehorses. Selenium concentration affected T4 concentration such that with every 0.1 unit increase in Se concentration there was a 3.8 unit increase in T4 concentration ($p = 0.012$). T3 concentrations were adequate in all horse groups. There was a strong correlation between mares and their foals for Se serum concentration (0.805; $p < 0.001$); however, there was minimal correlation between mare and foal concentrations of vitamin E and T4.

3.2. Introduction

Selenium (Se) and vitamin E are essential dietary nutrients for livestock. The Maritime Provinces, including PEI, are known to have soils that have insufficient Se concentrations to meet the requirements of livestock. Animals consuming locally grown feeds are at risk of becoming deficient unless supplemented. Vitamin E, however, is found in adequate amounts in pasture at early stages of growth; however, this vitamin deteriorates over time when these grasses mature or are stored as hay or silage. Thus vitamin E intake in forage-fed livestock is likely to fluctuate throughout the year, being lowest during the winter months when livestock are fed conserved forages only (1;2). Despite this, the prevalence of Se and vitamin E deficiency in the equine population of PEI has not been previously investigated. A recent study investigating the Se status of dairy herds in PEI showed that despite supplementation, 59% of the herds surveyed had either a marginal or deficient Se status, suggesting that Se deficiency may remain more widespread on PEI than previously recognized (3).

Selenium and vitamin E are essential components of several major metabolic pathways and have complementary roles as antioxidants. A deficiency of Se or vitamin E diminishes protection against oxidative stress, making the cell membrane more susceptible to disruption (4). The most common clinical manifestations of decreased anti-oxidant activity associated with vitamin E and/or Se deficiency are muscular and neuromuscular diseases, such as white muscle disease (WMD), equine motor neuron disease (EMND), and equine degenerative myeloencephalopathy (EDM) (2;5-8). Deficiencies in Se and vitamin E have

also been associated with significant decrease in immune response in horses when compared to horses that received supplementation (9-11).

Thyroid hormone metabolism is dependent on Se status: 3'5' tri iodothyronine deiodinase, a Se-containing enzyme, is responsible for converting thyroxine (T4) to the more active 3',5',3'-triiodo-L-thyronine (T3) (12). Calves and lambs grazing on Se-deficient soils have increased circulating T4 concentrations and decreased circulating T3 concentrations compared to animals supplemented with Se (13;14). Alterations in thyroid hormone concentrations may be responsible for the poor growth, ill thrift and lowered performance commonly noted in Se-deficient herds. To our knowledge, the relationship between Se status and resting thyroid hormone concentrations in horses has not been previously investigated.

The objectives of this study were as follows: 1.) to estimate the prevalence of Se and vitamin E deficiencies in representative groups of horses on Prince Edward Island, 2.) to measure resting T4 and T3 concentrations in serum and examine the relationship between the Se status and thyroid hormone concentrations, and 3.) to determine if feeding and supplementation practices are associated with Se and vitamin E status.

3.3. Materials and Methods

3.3.1. Experimental Animals

The experimental animals were selected from the general horse population in PEI as samples of convenience. The horses represented specific demographic groups: group 1 consisted of 34 aged pleasure horses (20 years and older), group 2 consisted of 31 younger adult pleasure horses (4-12 years of age), group 3 consisted of 36 Standardbred racehorses,

and groups 4 and 5 consisted of 50 Standardbred broodmares and their foals (<72 hours of age), respectively. Both groups of pleasure horses were initially recruited for the immunological study in chapter 2. The racehorses were from the Charlottetown Driving Park and were incorporated in the study on a volunteer basis after horse owners/trainers were informed of the purpose of the study. All samples were collected in one day (2006) and the owners/trainers were informed of the results of the blood work. Groups 4 and 5 were previously recruited for another immunological study in 2001 and serum samples had been stored in cryovials at -80°C (15).

To be eligible for inclusion, all horses must have been living on PEI, under the same ownership, and under the care of the same individual for at least the 3 months prior to enrolment in the study. Demographic groups 1 and 2 consisted primarily of light horses ($n = 51$), ponies ($n = 10$) and draft breeds ($n = 4$). These two groups had an additional serum Se and vitamin E concentration assay performed 6 months after the initial sample collection.

3.3.2. Laboratory Analysis

Blood samples were collected via jugular venipuncture into evacuated tubes. Samples were allowed to clot, centrifuged, and serum was decanted into cryovials for storage at -80°C until assays were performed. Vitamin E was assayed using modified high performance liquid chromatography (HPLC) with fluorescence detection (16). Serum Se analysis was performed using a method of additions calibration using a palladium-magnesium nitrate matrix modifier on an atomic absorption spectrometer (17-19). Both analyses were carried out following standard operating procedures at the Toxicology and Analytical Services Laboratory at the

Atlantic Veterinary College, Charlottetown, Prince Edward Island. Serum T4 and T3 chemiluminescence enzyme immunoassays were performed at Diagnostic Services, Atlantic Veterinary College, Charlottetown, Prince Edward Island^a.

3.3.3. Statistical Analysis

Descriptive statistics are presented in tabular form classified according to accepted clinical reference ranges for serum Se (20), vitamin E (21), T4, and T3 concentrations^c (22). General linear models were used to evaluate factors influencing serum Se, vitamin E, T4, and T3 concentrations; the serum Se, vitamin E, and T4 concentrations were square root transformed to meet the model assumptions. The following categorical predictors were included in the initial model: demographic group (young, old, racehorses, broodmares), breed (pony, light horse, draft), sex, and season of sampling (fall- August, September, October, winter- November, December, January, spring- February, March, April, and summer- May, June, and July). Selenium and vitamin E values were included in the initial models for vitamin E and Se, respectively. Selenium, vitamin E, and the respective thyroid hormone were included in the initial models for the thyroid hormones (T4 and T3). To avoid any intermediate effects of these predictors on the comparison of horse groups, the predictors were centered by subtracting horse group means. Many of these predictors were correlated to demographic group. Without transformation, there would likely be confounding between the predictor and group. By using this transformation, it is specifically testing whether there is an association between the predictor and the variable of interest. All two-factor interactions were assessed. Predictors significantly associated with the dependent variable ($p \leq 0.05$) were

retained in the model. Effects of significant categorical predictors were presented as least square means with pairwise comparisons adjusted by the Bonferroni method. Foals were excluded from the linear models as the distribution and range of values obtained for the foals made comparisons with other groups inappropriate. Also, Se values from foals were highly correlated to those of their respective mares; thus, their respective mares may have confounded their results. Pearson's correlations were used to quantify the relationship between mares' and their foals' serum Se, vitamin E, T4, and T3. Samples of serum Se and vitamin E taken initially and after 6-month for the aged and young adult pleasure horses were compared by paired t-tests. The effects of feed type and Se and vitamin E supplementation were assessed in linear models as described above with demographic groups included to control for confounding.

3.4. Results

3.4.1. Se Status

Two hundred-one horses (151 adults and 50 foals) from PEI were included in this study. Seventy-nine percent of these horses had serum Se concentrations that fell below the range considered adequate (Table 3-1). The younger adult pleasure horses and foals had the highest prevalence of inadequate serum Se concentrations (97% and 96%, respectively). Seventy-two percent of the broodmares were also below recommended serum Se concentrations. The lowest prevalence of inadequate serum Se concentration was in the racehorses (45% marginal and 0% deficient; Table 3-1).

Serum Se concentrations were affected by demographic group ($p \leq 0.001$; Table 3-2). Racehorses and broodmares had significantly higher serum Se concentrations when compared to the young and aged pleasure horses (Table 3-2; Figure 3-1). There was no significant difference between the racehorses and the broodmares or between young and aged pleasure horses (Table 3-2; Figure 3-1). There was also a significant seasonal effect on the Se concentrations ($p = 0.041$; Table 3-2). The horses tested in the winter months had significantly higher Se concentrations compared to the Se concentrations in horses tested during the spring months.

There was a moderate correlation between the broodmare and foal Se concentrations ($r = 0.546$; $p \leq 0.001$; Table 3-3). Five mares were considered outliers because their serum Se concentrations were very much higher than the other mares (range: 0.203-0.232 ppm) indicating they had likely been recently supplemented; when these mare/foal pairs were removed from the analysis, there was a high correlation between Se concentrations of mares and their foals (0.805 ; $p \leq 0.001$; Figure 3-2).

The aged and younger adult pleasure horses had serum samples taken 6 months following the initial sampling (data not shown); there was no significant change in the concentrations between sampling times for either of the demographic groups.

3.4.2. Vitamin E Status

Adequate serum vitamin E concentrations were found in 86% of all adult horses and foals (Table 3-1). The group with the highest prevalence of inadequate vitamin E status was the young adult pleasure horses (55%) followed by the aged pleasure horses (24%). Vitamin

E concentrations in the racehorse and broodmare groups were mainly within the normal range; the few inadequate horses had only marginal levels (3% and 2%, respectively).

The aged pleasure horses, racehorses, and broodmares had significantly higher serum vitamin E concentrations compared to the young adult pleasure horses ($p \leq 0.001$; Table 3-2; Figure 3-3). There was no significant difference between the aged pleasure horses, racehorses, and broodmares. There also was a significant group by selenium interaction ($p=0.010$; Table 3-2). The effect of Se concentration on vitamin E concentration was evident only in the racehorses where every 0.1 unit of increase in serum Se concentration was associated with a 5.8 unit increase in serum vitamin E concentration (Table 3-2). There was no significant change in the prevalence of vitamin E deficiency at the 6-month sampling time period for aged and young adult pleasure horses.

There was no significant correlation between mares' and their foals' vitamin E concentrations ($r= 0.232$; $p\text{-value}= 0.104$; Table 3-3).

3.4.3. Thyroid Hormones

None of the horses in this study had hypothyroidism, defined as concentrations of both thyroid hormones which fall below the range considered adequate, based on normal reference ranges for T4 and T3. All horses had adequate T3 values. The highest prevalence of inadequate serum T4 concentrations was found in the racehorse group (78% inadequate) followed by the broodmares (58% inadequate). The other groups had low prevalence of inadequate serum T4 (Table 3-1).

Three horses were removed from the dataset used in the general linear model based on unusually high T4 concentrations (range: 92.8-291 nmol/L; normal adult range: 15-51 nmol/L). The results presented in Table 2-2 are based on square-root transformed T4 values; the ordering and significance between horse groups was unchanged for analysis of the full dataset based on the optimal transformation of T4 values determined by the Box-Cox procedure. Serum T4 concentrations were significantly different between demographic groups ($p \leq 0.001$; Table 3-2; Figure 3-4). The highest T4 concentrations were seen in the young adult and aged pleasure horses followed by broodmares, and racehorses (Table 3-2). There was also an effect of Se on the T4 concentration such that every 0.1 unit of increase in serum Se concentration was associated with 3.8 units increase in T4 concentration.

All horses had adequate T3 concentrations and there were no significant group differences in the T3 concentration. There was a moderately significant negative mare-foal correlation for T3 concentrations ($r = -0.442$; $p = 0.001$).

3.4.4. Feeding and Supplementation Data

All horses were fed locally grown hay. All horses, with the exception of the racehorses, were allowed to graze on pasture during the grazing months. All racehorses were given a commercially prepared feed (complete pelleted feed or sweet feed which contained ~50-55 ppm of Se), while only 52% of the young adult pleasure horses and 56% of the aged pleasure horses were fed a pelleted feed or sweet feed. Locally grown grains, such as corn and oats, were given to some racehorses (53%), young adult pleasure horses (7%), and the aged pleasure horses (9%). The diet of the broodmares was not known (Table 3-4).

Selenium supplementation in the form of either a fortified salt block or free choice mineral was made available to 45% of young adult pleasure horses, 50% of aged pleasure horses, and 22% of the racehorses. Vitamin E supplementation in the form of a powder top dress was given to a limited number of horses: 3% in the young adult and aged pleasure horses and 14% to the racehorses (Table 3-4).

When controlling for confounding by demographic group, there was no effect of feeding or supplementation found on Se or vitamin E concentration.

3.5. Discussion

3.5.1. Se Status

Forages and grains grown throughout the Atlantic provinces are generally considered to be deficient in Se. Livestock being fed solely on these locally grown feedstuffs are at high risk of Se deficiency (23;24). In this study, a significant proportion of horses on PEI had serum Se concentrations considered inadequate. Seventy-nine percent of all the horses tested had either deficient or marginal serum Se concentrations. These results support previous studies carried out on livestock in PEI investigating Se status (3;23).

We confirmed that horses grazing on forages grown in PEI are unlikely to receive sufficient Se. This is based on the high prevalence of young adult pleasure horses that had deficient or marginal Se status (97%) combined with the fact that the majority of these horses were being fed solely locally grown hay or were on pasture. Only 52% of the young adult pleasure horses were fed feeds with known Se concentrations. In comparison, all of the

racehorses (routinely fed a set amount of commercially prepared ration) had a much lower prevalence of inadequate Se concentrations (45%). Additional Se supplementation in the form of a loose mineral or a communal block was offered to 45% of the young pleasure horses. None of the horses that received Se supplementation other than a commercially prepared feed had an adequate Se status. It appears that the mode of supplementation (i.e. communal fortified salt block or powder, frequency of supplementation, etc.), the amount of commercial feed given and the consistency of feeding commercial feeds all played an important role in the efficacy of the supplementation. Interestingly horses given the additional supplementation in the form of loose or block mineral had higher Se concentrations based on raw means; however, the supplemented horses still yielded inadequate Se concentrations based on established reference values. Again this questions the effectiveness of certain modes of supplementation.

The horses that received additional Se supplementation were supplemented with inorganic Se sources which have been shown to be effective (25;26). The current dietary Se requirement for horses, in all physiological states, is 0.1 mg/kg of DM (NRC, 1989). This questions the effectiveness of how supplementation was administered to the horses in this study. A Se block offered to a horse does not ensure that they will consume the essential nutrients they are offered and often there are multiple horses in one paddock with one available block. To ensure that a powder form is ingested it should be offered within a palatable feed (i.e. mixed with a grain or wet mash). In many cases, the powder may fall on the ground or filter down in the mixed feed to be left in a feeding dish uneaten while the more desirable feedstuffs are consumed.

The aged pleasure horse group also had a significant number of Se deficient or marginal horses at 82%. The majority of these horses were fed in the same manner as the younger pleasure horses; however, some of these horses received specially formulated feeds for geriatric horses. Eighteen of the 34 horses received feeds with established Se concentrations of 50-55 ppm. In 17 of the 34 aged pleasure horses Se was supplemented as some form of inorganic Se and only 4 of these horses had adequate serum Se concentrations. The previously discussed problems with Se supplementation methods might be also a factor with this group. As horses become geriatric (20 plus years), their owners are more apt to pay attention to their diets to help maintain an adequate weight such as providing good quality forage and geriatric formulated diets. This may account for the fewer numbers of Se deficient or marginal horses in this group compared to the younger pleasure horses.

The racehorses had the lowest prevalence of deficient or marginal Se levels (45%). All of the horses in this group were offered feeds that contained added Se at known concentrations. Interestingly, only 8 racehorses received additional oral Se supplementation beyond that already present in the feed; however, all racehorses were in training and received rations consisting of grains, pelleted feeds and good quality hay. The consistent feeding and the fact that racehorses are typically fed larger quantities of commercially prepared feedstuffs compared to pleasure horses likely contributed to the significantly lower number of Se deficient or marginal horses in this group.

The incidence of broodmares with inadequate Se status was 72%. This was not significantly different from the racehorses. Unfortunately, the exact feedstuffs and supplementation with Se in these horses was not known. The majority of broodmares would

likely be maintained on higher concentrate diets to allow for their increased energy needs during the 3rd trimester of pregnancy and lactation. It is also common practice by some broodmare owners to administer an injectable form of Se/vitamin E approximately 1 month prior to foaling, even though this may only give a transient rise in concentrations. These management practices might account for the lower number of broodmares with inadequate Se concentrations.

Approximately two-thirds of the foals in this study had inadequate Se concentrations. This places these foals at higher risk for WMD. Mares with deficient serum Se concentrations in late gestation are at significantly higher risk of giving birth to a foal with WMD (8). As with the broodmares, the Se supplementation history was not known at the time of serum collection. Oral Se supplementation to the mares during the entire pregnancy is the best preventative measure for avoiding Se-deficient newborn foals. The majority of the Se-deficient foals were born to Se-deficient or Se-marginal broodmares while only three Se-deficient foals were born to Se-adequate mares. The Se concentration of the foal was strongly correlated to that of the mare. These findings are consistent with previous research (2;8;27).

Selenium concentrations appeared to be affected by season. Horses tested during the winter months had significantly higher serum Se concentrations compared to horses tested in the spring. The reason for this is not known. However, many horse owners may offer commercial feeds or simply increase the amount of feed they are administering over the winter months to provide the extra energy that would be required during the colder temperatures to maintain their body condition. The significance of this relationship is suspect

because the broodmares were sampled in the spring and all the racehorses were sampled in the summer; therefore, there was some confounding between group and season in the data.

Subsequent serum samples were collected on the adult and aged pleasure horses 6 months after the initial serum sampling time. The owners were informed of their horses' serum Se status approximately 1-2 days post initial serum sampling and were consulted about how to manage supplementation if needed. Unfortunately, few horse owners chose to supplement their deficient or marginal horses with an additional Se product; thus there was no significant difference in the Se concentrations from the two sampling times. Only 3 horses were given oral powdered form of Se after consultation and only 1 horse achieved adequate Se concentrations after 6 months of supplementation.

3.5.2. Vitamin E Status

Vitamin E is readily available in fresh pastures such as alfalfa, timothy, meadow fescue and Kentucky bluegrass; however, vitamin E will deteriorate over time when these grasses are stored as hay. Thus in the Atlantic region, one would expect a seasonal affect for the amount of vitamin E intake (1;28). Approximately 87% of the horses that were tested in this study had adequate serum vitamin E concentrations. Only 10 of these horses (1 adult pleasure horse, 1 aged pleasure horse and 8 racehorses) were supplemented with oral vitamin E. The supplementation history of the mares and foals was not known. Feeding of fresh pasture, good quality hay, and complete pelleted feeds may explain adequate vitamin E concentrations (1). The young adult pleasure horses had the highest prevalence of inadequate vitamin E concentration (39%). This again may be attributed to feeding and management

practices such as feeding minimal and inconsistent amounts of concentrates. Interestingly there was no effect of season on vitamin E concentrations; too few horses tested in the winter months may be the reason a seasonal effect was not detected.

There was a significant interaction between group and Se concentrations on vitamin E concentration (Table 3-2). It showed that as the Se concentration increased so did the vitamin E concentration; however, this relationship was only seen among the racehorses. The effect of Se on vitamin E makes biological sense based on the fact that these essential nutrients are supplemented together in the majority of commercial feeds and supplements. Another factor to consider is the regimented feeding schedule of the racehorses in comparison to the other groups where these horses are fed consistent and usually large amounts of feed.

None of the adult and aged pleasure horses were administered vitamin E supplementation 6 months after the initial serum collection time and there was no significant difference in the vitamin E concentrations at the two sampling times.

3.5.3. Thyroid Hormones

As previously stated, all horses in this study had adequate T3 concentrations. The racehorses had a significantly higher incidence of low T4 concentrations with 78% of those horses having a low T4. One reason for this could be the use of exogenous compounds such as phenylbutazone. Phenylbutazone, a frequently used non-steroidal anti-inflammatory (NSAID), inhibits iodide oxidation and coupling of iodotyrosines resulting in reduced thyroid concentrations for up to 10 days once administration has ceased. Phenylbutazone also competes with T4 for binding sites on plasma proteins, and it may decrease production of

thyroid stimulating hormone (29;30). Fifty eight percent of the broodmares had low T4 concentrations (Table 3-1). The high prevalence of broodmares with low T4 concentrations may also be the result of postpartum effects. Various studies have shown that in late-term pregnancy, mares have either normal or decreased serum thyroid hormone concentrations (31;32). The foals had very high T3 and T4 concentrations compared to the other groups. It is well established that foals have much higher levels of these hormones at birth which decrease with age (33). Interestingly there was no correlation between the mares and their foals' T4 concentrations but there was a negative correlation between their T3 concentrations. The normally high thyroid hormone concentrations foals have at birth may have contributed to these findings.

It is well established in several species that as serum Se concentrations decrease to inadequate levels, serum T4 levels increase. A Se-containing enzyme, 3'5' triiodothyryne deiodenase, is responsible for converting thyroxine (T4) to the more active form 3,5,3-triiodo-L-thyronine (T3). A lack of Se prevents this conversion and leads to an increase in T4 and a decrease in T3 (13;14). All the horses in this study had normal T3 concentrations even with inadequate Se concentrations. This study showed that there was a positive correlation between Se and T4 concentration. This finding is contrary to published data in other livestock species (13), and may be related to the iodine content of typical Se supplements fed to horses (data not available). However, it may be that in horses the activity of 3'5' triiodothyryne deiodenase is not as closely related to Se status as in other species.

3.6. Conclusion

In summary, our study confirmed that Se deficiency in the equine population of PEI is a major concern. An appreciable number of the horses in each demographic group investigated had inadequate serum Se concentrations. The small proportion of horses receiving Se supplementation in this survey (26%) may also explain in part these low serum Se concentrations. The significant positive correlation shown between the Se concentrations of mares and their foals emphasizes the importance of routine Se supplementation to broodmares throughout the gestation.

Our study also illustrated that the prevalence of inadequate serum vitamin E concentrations in horses on PEI is less than that of Se. The majority of the horses tested in this study had adequate levels of vitamin E. We also found that serum Se concentrations were positively associated with serum T4 concentrations, contrary to published findings in other livestock species.

3.6. Footnotes

- a. Immulite[®] Total T4 and T3, Diagnostic Products Corporation, Los Angeles, CA, USA.
- b. Minitab 14[®], Minitab Inc., State College PA, USA.
- c. Atlantic Veterinary College internal reference intervals for adult equine T3 and T4.

3.7. Acknowledgements

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Table 3-1. Mean \pm SD and prevalence (% of demographic group) of selenium and vitamin E deficiencies in the horse population on PEI, broken down by sub-population, and the proportions of these sub-populations having inadequate resting thyroid hormone serum concentrations.

	Young adult pleasure horses (n = 31)	Aged pleasure horses (n = 34)	Racehorses (n = 36)	Broodmares (n = 50)	Foals (n = 50)	Total horses (n=201)
Se concentrations						
Mean \pm SD	0.061 \pm 0.031	0.080 \pm 0.051	0.14 \pm 0.032	0.11 \pm 0.031	0.059 \pm 0.053	
% Deficient (0.008-0.0053 ppm)	36	41	0	6	60 *	29
% Marginal (0.0053-0.120 ppm)	61	41	45	66	36	50
% Adequate (0.140-0.250 ppm)	3	18	55	28	4	21
Vitamin E concentrations						
Mean \pm SD	192.8 \pm 100.3	321.3 \pm 153.6	374.8 \pm 119.8	343.7 \pm 93.4	408.0 \pm 156.8	
% Deficient (< 150 μ g/dL)	39	9	0	0	0	8
% Marginal (150-200 μ g/dL)	16	15	3	2	0	6
% Adequate (200-1000 μ g/dL)	45	76	97	98	100	86
T4 concentrations						
Mean \pm SD	29.34 \pm 9.730	24.46 \pm 119.8	10.18 \pm 5.039	26.11 \pm 47.27	281.9 \pm 78.6	
% Inadequate (< 15 nmol/L)	6	12	78	58	2	32
% Adequate (15-51 nmol/L) (Foal: 279-464 nmol/L)	94	88	22	42	98	68
T3 concentrations						
Mean \pm SD	1.26 \pm 0.420	1.29 \pm 5.04	1.29 \pm 0.673	1.76 \pm 2.47	12.89 \pm 3.714	
% Inadequate (< 0.4 nmol/L)	0	0	0	0	0	0
% Adequate (0.4-1.7nmol/L) (foal: 10-19 nmol/L)	100	100	100	100	100	100

*:Se-deficient foals were born to 27 of the 36 Se-deficient / marginal broodmares, while Se-deficient foals were born to only 3 of the 14 Se-adequate mares.

Table 3-2. Results of final linear models for Se, Vitamin E, and T4 data collected from horses in the PEI population.

Outcome	Effect	Category	Estimate [⊗]	SE	P-value
Se	Group	Young pleasure	0.227	0.019	0.001
		Aged pleasure	0.286	0.018	
		Racehorses	0.392	0.020	
		Broodmares	0.369	0.019	
	Season	Winter (Nov, Dec, Jan.)	0.421	0.051	0.041
		Spring (Feb, March, April)	0.272	0.020	
		Summer (May, June, July)	0.308	0.010	
		Fall (Aug., Sept., Oct.)	0.303	0.014	
Vitamin E	Group	Young pleasure	13.39	0.58	0.001
		Aged pleasure	17.42	0.56	
		Racehorses	19.13	0.54	
		Broodmares	18.37	0.46	
	Se(Group)	Young pleasure	-1.95	19.45	0.010
		Aged pleasure	13.44	11.05	
		Racehorses	58.38	17.08	
		Broodmares	-7.075	8.713	
T4	Group	Young pleasure	5.333	0.171	0.001
		Aged pleasure	4.887	0.163	
		Racehorses	3.101	0.158	
		Broodmares	3.784	0.139	
	Se	n/a	3.830	1.781	0.033
T3	Group	Young pleasure	1.258	0.095	0.784
		Aged pleasure	1.171	0.090	
		Racehorses	1.287	0.088	
		Broodmares	1.201	0.077	

[⊗]:Regression coefficients for continuous predictors and least squares means for categorical predictors, computed at the mean Se in the group, when Se is present in the model. The estimates for Se, vitamin E and T4 refer to a square-root transformed scale.

Table 3-3. Correlation of Se, vitamin E, T4, and T3 between mares and their foals.

	Pearson Correlation (r)	P-value
Se concentrations	0.805 [⊗]	0.001
Vitamin E concentrations	0.232	0.104
T4 concentrations	-0.210	0.143
T3 concentrations	-0.442	0.001

[⊗]: Correlation based on 45 pairs of mares and foals. Five mares and their foals were determined to be outliers, therefore were removed from the analysis. With all 50 pairs, the $r = 0.546$ and $p \leq 0.001$.

Table 3-4. Percent (%) of horses in each demographic group offered feeds and mineral supplements.

	Aged pleasure horses (n = 34)	Young adult pleasure horses (n = 31)	Racehorses (n = 36)
Commercially prepared feed*	56 (19)	52 (16)	100 (36)
Grain	9 (3)	7 (2)	53 (19)
Mineral supplements			
Vitamin E (powder)	3 (1)	3 (1)	22 (8)
Se (fortified salt block or powder)	21 (17)	45 (14)	22 (8)

*: Analysis of feed on commercial label indicates that they contain both Se (~50-55ppm) and vitamin E (~70-85 IU/lb).

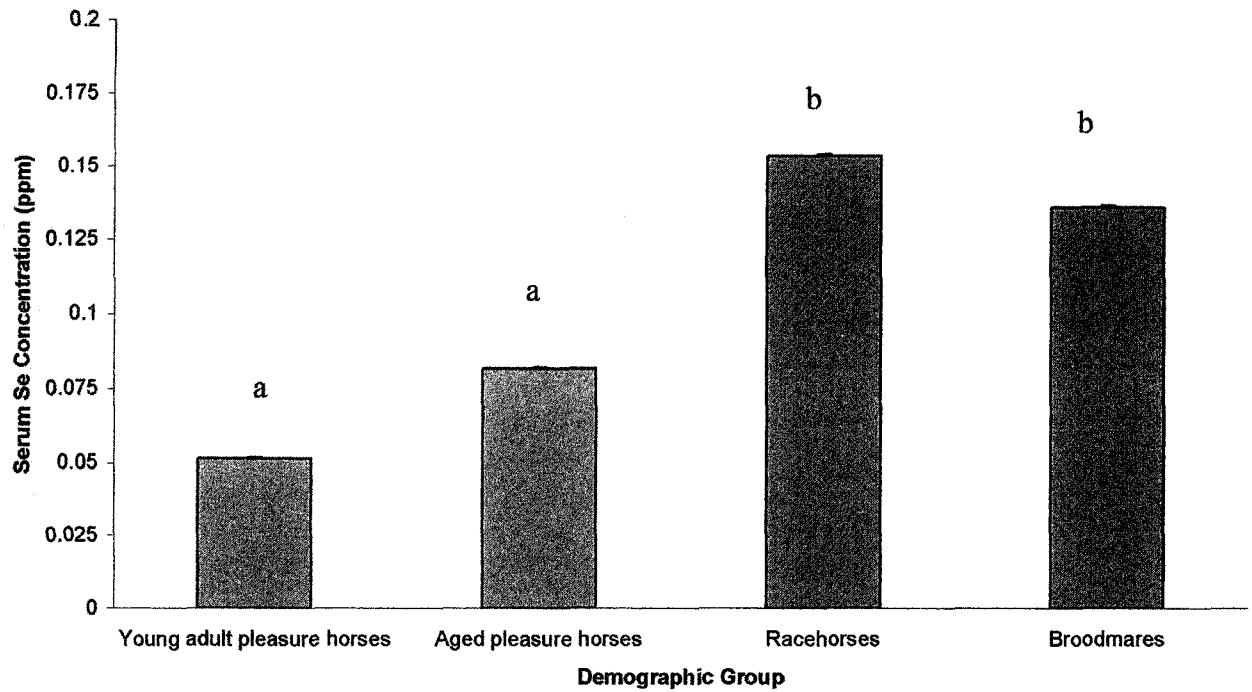


Figure 3-1. Comparison of the LSM \pm SEM for serum Se concentrations between demographic horse groups on PEI. Means with differing superscripts are significantly different ($p < 0.05$).

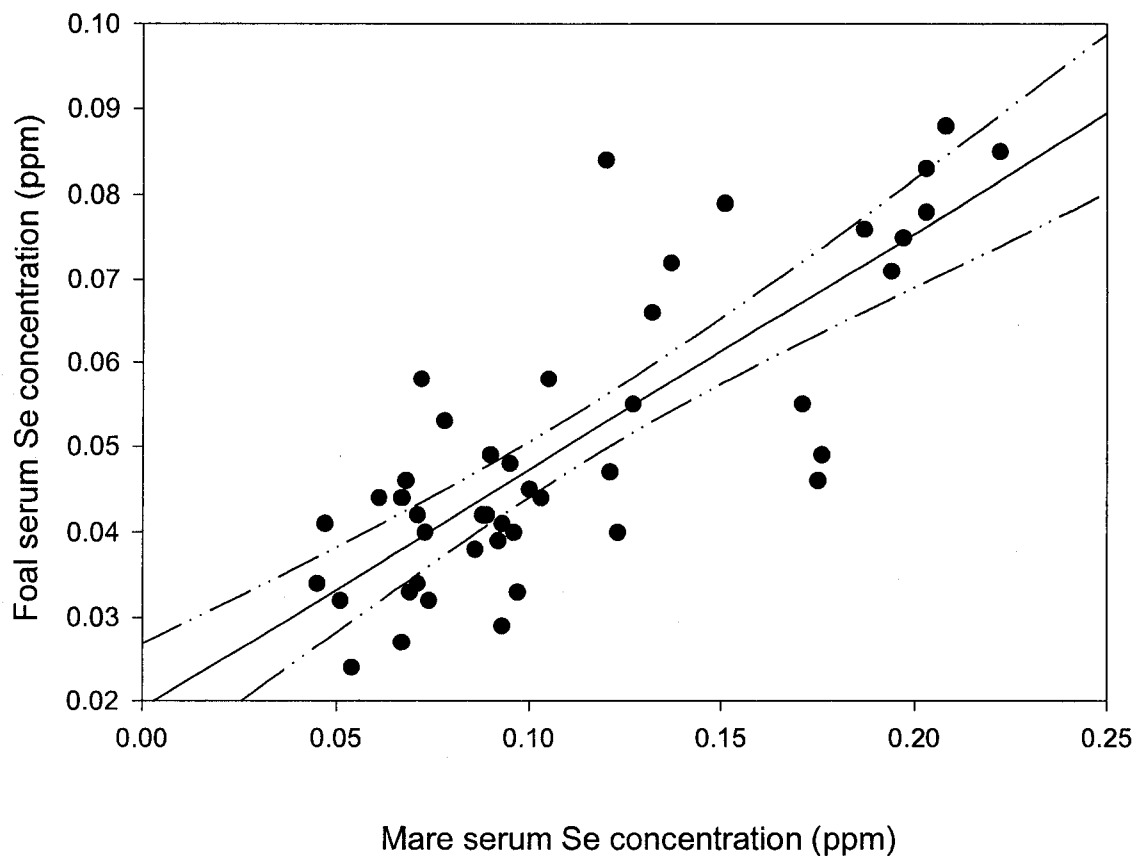


Figure 3-2. The relationship between broodmare serum Se concentration (ppm) and their foals' serum Se concentration. The correlation line is shown with upper and lower 95% confidence limits. Analysis has omitted 5 mares and their foals that were determined to be outliers ($n=45$; Pearson's correlation = 0.805; $p < 0.001$).

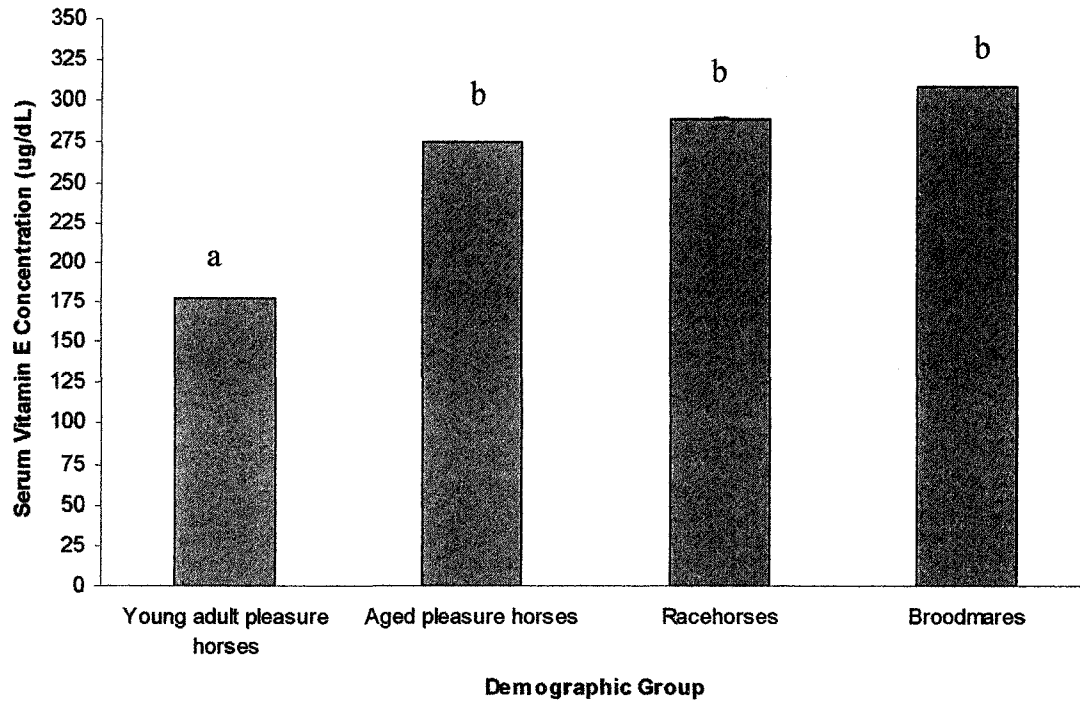


Figure 3-3. Comparison of the LSM \pm SEM for serum vitamin E concentrations between demographic horse groups on PEI. Means with differing superscripts are significantly different ($p < 0.05$).

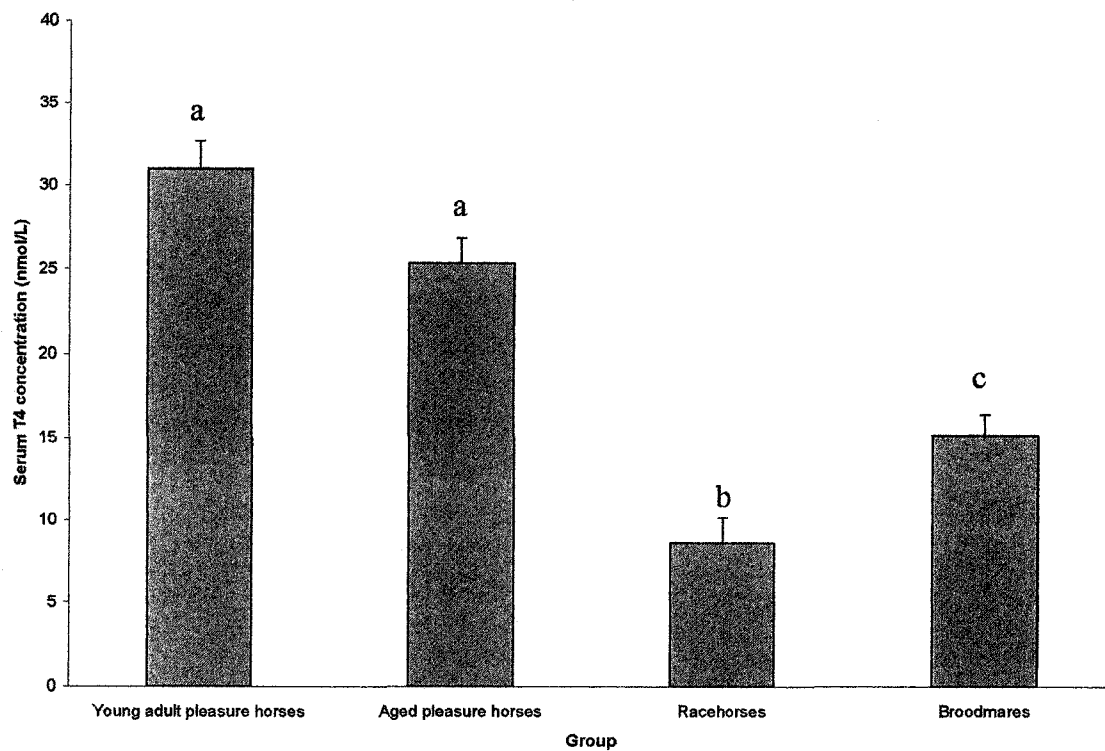


Figure 3-4. Comparison of the LSM \pm SEM for serum T4 concentrations between demographic horse groups on PEI. Means with differing superscripts are significantly different ($p < 0.05$).

CHAPTER 4. SUMMARY AND GENERAL DISCUSSION

4.1. Introduction

The initial objective of this research was to investigate the effect of age on the specific systemic antibody response in the horse following vaccination against rabies and influenza. Prior to this study, there was minimal research carried out on aged horses to determine if their immune system was capable of responding to vaccination that was similar to younger horses. Ultimately this study helped establish whether vaccination protocols in the aged horse need modification.

As previously stated, enrolled horses had no history of previous rabies vaccination, thus this vaccine was a means of assessing a primary immune response. Since influenza is endemic in the horse population, as reflected by antibody titers before vaccination, influenza vaccination was a means of assessing an anamnestic immune response.

In the initial stages of the study, serum Se and vitamin E concentrations were measured on the pre-vaccination samples of all horses. We were interested in assessing Se and vitamin E because deficiency of either could be related to a sub-optimal immune response. Preliminary data indicated that 93% of the first 42 horses tested were found to have inadequate serum Se concentrations based on the accepted reference ranges (1). Based on these preliminary results, a second research objective was developed: to examine the prevalence of Se and vitamin E deficiency in representative groups of horses on Prince Edward Island. Research in other livestock species has established relationships between Se status and circulating thyroid hormone concentrations so we elected to examine this relationship because we could find no published data on this topic in the horse. Lastly, we wished to determine the degree of correlation between maternal and neonatal serum Se,

vitamin E, T4 and T3 concentrations because there was little published information on these relationships (Chapter 3).

4.2. Age and Immune Response

Serum samples were analyzed for influenza (ELISA) and rabies (RSNA) specific antibodies prior to and following vaccination. The RSNA results indicated that the aged horses could have a primary immune response equivalent to that of younger adult horses. Unexpectedly, the rabies titers in all horses declined dramatically 6 months following the initial vaccination. This decline in titers below human protective titers of <0.5 I.U., was not foreseen and was the result of receiving no booster vaccination (as per vaccine label instructions) (2). To our knowledge this is a finding that has not been previously published and we suggest further research should be done to determine if a booster vaccination is necessary following an initial rabies vaccination (Chapter 2). Also, there was a significant percentage of the horse population tested (89%) that had inadequate Se concentrations in each age group; thus, a more controlled investigation into how Se deficiency could effect the response to the rabies vaccination over time should also be considered.

The previously described ELISA technique used in this study on consecutive serum samples from aged and young adult horses performed well in detecting specific antibodies for equine influenza (3). The pre-vaccination samples showed that aged horses had significantly higher titers in comparison to that of the younger adult horses. Repeated exposure to infection or a shift to a predominantly TH2 response with age may be the cause

of these high titers (4-6). Titers in both age groups remained similar in both age groups post vaccination. These results were consistent with a previous study (7).

Even though the older horses had a dampened increase in magnitude of titers compared to the younger adult horses, their serum titer levels were similar post vaccination. These results indicated that while the aged horses did have a secondary immune response to influenza vaccination, it was reduced compared to the younger adult horses or possibly horses have a maximum titer they reach and aged horses are already closer to the maximum through their higher lifetime exposure.

One limitation to this study was the number of horses recruited. The inclusion criteria set for this study, such as vaccination history, ownership and recent history of illness, limited the number of horses able to participate. This was especially noted in the young adult horse group. Accompanying this was the reduction of horse numbers by half when the different vaccines were allotted to each age group. If there were more horses involved there would have been more potential to detect effects of the various parameters we measured.

Another limitation may have been the use of multiple vaccines at the initial stages of the study. The initial proposal for this study called for 25 horses per group but the initial proposal did not have a booster vaccination in its protocol. This initial sample size was based on a power calculation of 85% probability of detecting a difference of 50% between each age group. The addition of a booster vaccination to the protocol resulted in the number of horses in each age group reduced by half for the 8 week and 6 month sampling period. Also, potentially the use of more than one vaccine per horse may have affected the immune response and altered the results.

The influenza vaccine chosen for this study may have also been a limitation. The vaccine for this study was chosen based on published data that showed this particular influenza vaccine yielded significantly higher single radial hemolysis (SRH) values as compared to another vaccine on the market (8). If this vaccine truly does stimulate such a profound antibody response, it may have limited our ability to detect a difference between the two age groups. A less antigenic vaccine may have better demonstrated a sub-optimal immune response in aged horses. A study comparing two vaccines with known differing capabilities of stimulating an immune response in both age groups would investigate this potential limitation.

4.3. Se and Vitamin E Status

One of the most dramatic findings was that 79% of all horses tested in our study had inadequate serum Se concentrations, indicating that a deficiency in dietary Se is very prevalent among horses in PEI. While this could be explained by the low Se concentrations typical of forages grown in PEI, it could also be a reflection of inappropriate reference ranges. The reference ranges currently in use have been based on little experimental data compared to other livestock species. For instance, in cattle and sheep, reference ranges have been developed based on the results of many supplementation response trials measuring, for instance, growth in young animals (9). Further work is indicated to better define the Se requirements and reference ranges for the horse population.

Despite the lack of effect of Se and vitamin E status on immune response in both age groups, it is well established that these two nutrients play an important role in immune

function (10-12). The fact that most study horses had inadequate Se concentrations (79%) may have made comparison difficult and thus not allowed for a Se effect to be detected. A randomized clinical trial with control and supplemented horses would help in assessing whether the measures of immune response utilized in this study are affected by Se status.

As previously mentioned, supplementation with Se is generally highly correlated with Se status (13). However in our study, a history of Se supplementation (in various forms) did not result in significantly higher serum Se concentrations in the supplemented horses with the exception of racehorses and broodmares where commercially prepared feeds would be fed regularly. This suggests that typical modes of supplementation may be ineffective, or that there were underlying effects that were not detected in our study. Horse groups with the highest feed requirement (racehorses and broodmares) had the highest serum Se concentrations, suggesting that feed amount offered and consistency may be more important than feed type; however, amount offered and consistency was not recorded in this study. Studies are in progress that compare various methods of supplementation, such as inorganic Se salts, organic Se (yeast) and Se-treated forage, which were sparked from the findings of this research (personal communication; Dr. Julia Krause, Atlantic Veterinary College, Charlottetown, PE).

There was also a positive association with serum Se and T4 concentrations (coefficient= 3.830), a finding which is not consistent with previous research (14). This may be related to the iodine content of typical mineral supplements fed to horses. Another explanation may be that in horses the activity of 3'5' triiodothyronine deiodenase is not as closely related to Se status as in other species.

An additional finding was a highly positive correlation between mares and their foal's serum Se ($r = 0.805$) illustrating the importance of Se supplementation in the gestational period of the mare. This high correlation was expected based on published literature (15-17). The vitamin E, T4, and T3 concentrations between the mares and their foals were not strongly correlated in this study. Studies in various species, other than horses, have shown that there is limited placental transfer of vitamin E from the dam to the fetus (18-20). Colostrum, however, is a good source of vitamin E for the neonate (19;21). These two facts may account for the lack of strong correlation between the mares and their foals. The thyroid hormones may not be correlated based on the vastly different normal reference ranges between adult horses and neonatal foals (22). Baseline thyroid hormone concentrations should be a good indicator of thyroid function; however, measuring concentrations in horses following a thyroid releasing hormone challenge might provide more information (23).

Vitamin E deficiency was not prevalent in PEI horses, even though all of the horses' diets consisted of hay, some of which was stored for long periods of time. Evaluation of tissue samples of liver or muscle for vitamin E status may be more sensitive compared to serum and might have resulted in different results. Also, a re-evaluation of the reference ranges for vitamin E may be warranted as was suggested previously for Se reference values. Seasonal effect on vitamin E serum concentrations was analyzed; however, there was no seasonal effect detected in this study as would have been expected based on published literature (24-26).

One limitation to this study is the lack of consistent dietary information collected on each horse group. Unfortunately, when this study was initiated a complete feeding history of

the aged and young adult pleasure horses was not recorded while on the study. The information was collected retrospectively, leaving much room for error and inconsistencies. Also, the serum samples used for the broodmares and their foals were taken from another study; no feeding or supplementation history (i.e. dietary or injectable Se supplementation) was available. Even though attempts were made to collect this data, the information was not consistent and unreliable due to passing to time and changing of management practices. Overall this research did provide important information on the Se status of horses in PEI and has set the stage for further research that is currently in progress.

4.4. Future Directions

Although the research within this thesis does provide insight into the immune response and Se and vitamin E status of horses in varying ages in PEI, there is still a need for additional research. One aspect that was not addressed directly in this thesis was the specific alterations in T cell, B cell, and cytokine function in horses as they age. The ability of T cells to respond to antigens can be investigated through various assays that determine function via detection of cytokines (ie. IL-2, IFN- γ), determine structure of the T cells via specific MHC molecules, and determine what precursor T cells proliferate post vaccination (27).

Research has shown that aged horses, along with other species, switch from a primarily TH1 (cell mediated) response to a TH2 (humoral) response (28;29). Further research focusing on the lymphoproliferation of mononuclear cells and antibody production in aged horses post vaccination would compliment the results of this thesis.

More extensive research should be done involving a greater numbers of horses in comparison to this research. Ideally a trial whereby horses are given one of the two vaccines used in this study with having subsequent serum samples collected over a longer period of time should be carried out. Once the horse has completed this phase of the research it can be vaccinated with the second vaccine and undergo the same sampling regime. This will eliminate the potential of there being a vaccine interaction resulting in a altered immune response.

The ELISA performed in this research detected minimal anti-influenza serum IgA in comparison to the other immunoglobulins. The majority of IgA is produced in the mucosal surfaces, where its activity is most important against respiratory viral infections (30). Performing nasal swabs concurrently with the fore mentioned research to detect mucosal IgA could potentially help better define the immune response of horses.

Interestingly, the RSNA analysis indicated that anti-rabies titers declined rapidly to questionable protective levels within 6 months following the initial vaccine. There is limited research to date on measuring anti-rabies antibodies in horses post vaccination (31;32). The current labeled protocol for the commercial rabies vaccine used in this study is a single initial vaccination followed with annual boosters, which was based on experimental challenge in horses (personal communication; Dr. Normand Plourde, Merial Canada Inc., QC). Further investigations are needed to establish if a 2 or 3 dose protocol for horses receiving their initial rabies vaccination is more appropriate to ensure adequate antibody production and protection. A controlled study looking at horses' rabies specific titers post initial and booster vaccination over a period of 1-2 years would be desirable. A challenge study would

ultimately be best for establishing equine protective levels but is questionable from an animal welfare standpoint.

More extensive research should be done on the effects of Se and vitamin E on the equine immune response. Even though the results from this study did not indicate that Se and vitamin E status did have a significant effect on the immune response, 89% of the horses incorporated in the study had inadequate Se concentrations, possibly reducing the ability to detect an effect (Chapter 2). Currently there are studies in progress that are extensions of this study. One study is a controlled immunologic study involving equal numbers of horses with both adequate and inadequate serum Se concentrations with organic, inorganic and no supplementation may better illustrate the effect Se status could have on the immune response (personal communication; Dr. Julia Krause, Atlantic Veterinary College, Charlottetown, PE). This investigation should help establish a clearer picture of the effects Se and vitamin E on the equine immune response.

In addition, further investigation to examine the correlation between mares and their foals' serum Se concentrations and the benefit to the overall health status of the equine neonate is warranted. Based on the results from our research, another study is in progress focusing on Se source on Se status and immune function in horses. This study is looking at dietary Se in gestational mares and how it may affect foals Se status and immune function (personal communication; Dr. Julia Krause, Atlantic Veterinary College, Charlottetown, PE).

Lastly, to help identify the cause of the positive relationship between Se and T4 seen in this thesis, further research should be carried out. A first step might be to investigate Se and thyroid function using a thyroid-releasing hormone challenge. This test could be carried

out on the two groups of horses differing in Se status to determine if thyroid function is truly affected by Se status in the horse. An investigation specifically focusing on the relationship between Se and 3'5' triiodothyronine deiodinase activity in horses specifically would be warranted. A study measuring the relationship of 3'5' triiodothyronine deiodinase activity to Se status in supplemented and non-supplemented horses would be useful; however, there are several Se-dependent mammalian deiodinases (33;34) and it may be necessary to measure the activity of each of these to determine if any or all deiodinase activity is Se-dependent in the horse, as has been found in other species.

4.5. Conclusion

In summary, this thesis has contributed to our knowledge of immune function and micronutrient nutrition the horse. It has established that the aged geriatric immune system does indeed differ from the young adult horse. It has led the way to help understand changes in the aged equine immune system and can be used to initiate further research in this area. In the process we have identified that the current rabies vaccination recommendations may be sub-optimal. This thesis also provides insight into the Se status and vitamin E in horses in PEI and likely Atlantic Canada. It illustrates the importance of dietary supplementation in many demographic groups of horses and points to a general failure to ensure adequate trace element intake in horses in this region.

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APPENDIX

Appendix 1.

Anti-influenza and rabies titers for aged and younger adult horses. Data presented as median titers except SRH data presented as means.

Sampling Time		0		4 Weeks		8 Weeks		24 Weeks	
Horse Groups	Aged	Control	Aged	Control	Aged	Control	Aged	Control	
Anti-influenza Titers									
IgG(a)	1:3,777	1:602	1:16,077	1:16,073					
Influenza Booster					1:16,328	1:8,691	1:3,408	1:2,261	
Rabies Booster					1:3,337	1:2,483	1:2,398	1:1,010	
IgG(b)	1:2,783	1:316	1:10,077	1:3,954					
Influenza Booster					1:23,645	1:12,154	1:6,276	1:5,240	
Rabies Booster					1:9,772	1:4,446	1:4,757	1:1,710	
IgA	1:13	1:10	1:21	1:7					
Influenza Booster					1:14	1:4	1:6	1:4	
Rabies Booster					1:11	1:8	1:9	1:6	
SRH (mm ²)	88.6	30.79	181.90	168.43					
Influenza Booster					199.69	184.5	155.93	138.1	
Rabies Booster					159.60	151.2	113.4	112.8	
Rabies Titers (I.U.)	0.03	0.02	0.400	0.705					
Influenza Booster					0.160	0.500	0.110	0.285	
Rabies Booster					9.120	18.08	0.810	1.710	