The Effects of Supplemental Vitamin E and Selenium on Feline Immunity

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Abstract

Both vitamin E and selenium are essential for optimal immune function and their supplementation in the diet is known to enhance various immune parameters in many species. Immune function may be enhanced further by their combined supplementation (Kubena & McMurray, 1996). There have been very few studies on the effects of vitamin E supplementation on immune function in the cat and it appears that vitamin E does not produce the same enhancement of immune parameters that has been found in other species, although older cats may benefit from supplementation (Hayek *et al.,* 2000). No studies have investigated the effects of selenium supplementation or of combined vitamin E and selenium supplementation on immune function in the cat. The aim of this study was to examine the effects of both single and combined supplementation of vitamin E and selenium on immune function in the cat.

The 4 week study followed a 3x3 factorial design with 9 diets, including a control or basal diet containing 68.2 IU/kg DM Vitamin E and 0.38 mg/kg DM selenium, and 8 diets supplemented with moderate or high levels of Vitamin E (250 or 500IU/kg DM diet) and/ or Selenium (2 or 10mg/kg DM diet). Blood samples were analysed for immune cell phenotype expression, lymphocyte proliferation to concanavalin A and phytohaemagglutinin, phagocytosis, immunoglobulin G concentration and prostaglandin E_2 concentration. Results were analysed in SAS by mixed procedure repeated measures analysis.

Vitamin E supplementation at both a moderate and high level were found to significantly increase lymphocyte proliferative responses to concanavalin A and phytohaemagglutinin, whether or not selenium was supplemented in the diet. Phagocytic activity was significantly in increased by vitamin E and combined vitamin E and selenium supplementation. Selenium supplementation alone had no significant effect on any of the immune parameters measured. None of the supplemental diets were found to have a significant effect on the expression of immune cell phenotypes, immunoglobulin G concentration or prostaglandin E2 concentration. Overall, a moderate level (250 IU/kg DM) of vitamin E supplementation may benefit feline immune health when supplemented in the diet. A higher level of vitamin E

supplementation is unlikely to offer any added benefit to immune health and would add unnecessary cost to the manufacture of the diet. Selenium supplementation appears to offer no benefit to immune health in cats.

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List of Abbreviations

APC	Antigen Presenting Cell
CAT	Catalase
CoQH	Coenzyme Q
Con A	Concanavalin A
DM	Dry Matter
DNA	Deoxyribonucleic Acid
GPx	Glutathione Peroxidase
H	High
H_2O_2	Hydrogen Peroxide
IgG	Immunoglobulin G
IU	International Units
M	Moderate
MHC	Major Histocompatability Complex
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
O_2	Oxygen
PGE2	Prostaglandin E2
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PHA	Phytohaemagglutinin
Se	Selenium
SOD	Superoxide Dismutase
Vit E	Vitamin E

1. Literature Review

1.1 Introduction

The diet can provide not only the nutrients required for survival, but may also have added benefits that can maintain and improve health (Hayek *et al.,* 2004; Bontempo, 2005). Over recent times, functional foods and nutraceuticals have played a major role in human nutrition due to the realisation that the diet can influence health and the occurrence of disease, and a large industry has been developed to provide the consumer with products designed to address various health issues (Rutherfurd-Markwick & Hendriks, 2003; Hayek *et al.,* 2004). This phenomenon has now spread to the pet food industry; many owners consider their pets to be important members of the family and seek ways to maintain the health of their pets. Feeding premium diets and supplements designed to enhance pet health is becoming a popular means to do this, particularly as these diets are becoming more available (Rutherfurd-Markwick & Hendriks, 2003; Bontempo, 2005).

Free radicals are produced by the cells of the immune system and are important for defence of the host against infection (Bendich, 1990; Knight, 2000). However, excess free radical activity is known to have a negative impact on immune function and has been implicated in the development of many diseases (Chow 1991; Halliwell *et al.,* 1995). The knowledge that antioxidants can protect the body from free radical damage has led to the accumulation of a large body of research into the effects of antioxidant supplementation on immune function. Research has found a positive effect of supplementation on a number of immune cell activities and disease resistance in many species, such as chickens, pigs and cattle (Chew, 1996; Finch & Turner, 1996). The supplementation of vitamin E or selenium in the diet is known to modulate immune function and their combined supplementation has been found to produce even greater benefits (Chew, 1996; Finch & Turner, 1996). This is thought to be due to the synergistic antioxidant effects of vitamin E and selenium in the cell membrane, and their control of arachidonic acid metabolism (Chew, 1996; Kubena & McMurray, 1996). However, there has been very little published on the effects of vitamin E on feline immune function and there is no available information on the effects of dietary

supplementation of selenium, or a combination of vitamin E and selenium on immune function in cats.

The cat has evolved as an obligate carnivore and has developed several metabolic adaptations that result in it having differing nutritional requirements to other species (MacDonald *et al.,* 1984; Morris, 2002; Rutherfurd-Markwick & Hendriks, 2003). Due to the unique metabolism of the cat, it is possible that vitamin E and selenium may not have the same effects on immune function that have been reported in other species.

The aim of this study was to determine whether the addition of supplemental vitamin E and selenium to the diet, either individually or in combination, had any effect on various immune parameters in the cat. The hypothesis of this study was that while vitamin E supplementation was unlikely to enhance immune function parameters in the cat, combined supplementation of vitamin E and selenium may have a positive effect on these parameters.

1.2 The Immune System

The role of the hosts' immune system is to prevent and fight infection by pathogens and other foreign agents (Felsburg, 1994; Grimble, 1997; Calder & Kew, 2002). The immune system is made up of two parts; the innate immune system, which is non-specific, and the acquired immune system, which can identify specific antigens (Felsburg, 1994; Knight, 2000; Parkin & Cohen, 2001; Calder & Kew, 2002). The innate and acquired immune systems interact, which is an essential process for complete protection of the body against infection (Felsburg, 1994; Chew, 1996; Parkin & Cohen, 2001; Calder & Kew, 2002). Communication between the cells of the immune system is achieved by the use of chemical signals, which allows interaction between the two systems (Parkin & Cohen, 2001; Calder & Kew, 2002; Hayek *et al.,* 2004), and also allows cells to accurately locate and become activated at the site of infection (Parkin & Cohen, 2001; Hayek *et al.,* 2004).

1.2.1 Innate Immunity:

Innate immune activity utilises physical barriers, chemicals and microbial compounds, along with some non-specific immune cell activity (Felsburg, 1994; Erickson *et al.,*

2000; Parkin & Cohen, 2001; Hayek *et al.,* 2004). These physical barriers and compounds include the skin and mucous membranes; the acidic and enzymatic secretions of the sebaceous glands; the actions of mucous and cilia of the respiratory tract to trap and expel antigens from the body; the enzyme and acid secretions of the digestive system; the colonisation of the gut with bacteria; the motility of the gastrointestinal tract; and the acidity and flushing of the urogenital tract (Felsburg, 1994; Erickson *et al.,* 2000).

If an antigen passes the hosts' physical barriers, the cells of the innate immune system become the next line of defence, performing a number of functions such as the destruction of parasites by eosinophils (Felsburg, 1994; Parkin & Cohen, 2001); inflammatory reactions produced by mast cells and basophils (Matés *et al,* 2000; Parkin & Cohen, 2001); the destruction of tumour and virus infected cells by natural killer cells (Felsburg, 1994; Erickson *et al.,* 2000; Hayek *et al.,* 2004); antigen presentation to cells of the acquired immune system (Chew, 1996; Parkin & Cohen, 2001) and phagocytosis and killing by neutrophils, macrophages and monocytes (Felsburg, 1994; Chew, 1996; Kubena & McMurray, 1996; Knight, 2000).

Phagocytosis is a process where an antigen is ingested by an immune cell, and destroyed by the release of compounds within the cell (Felsburg, 1994; Erickson *et al.,* 2000; Knight, 2000). Destruction of antigens can occur by two methods. The first method, termed the respiratory burst, is an oxygen (O_2) dependent reaction where oxygen molecules are reduced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme that catalyses the conversion of oxygen to the superoxide radical and hydrogen peroxide. This results in the release of toxic free radicals such as hydrogen peroxide, the hydroxyl radical and singlet oxygen (Chew, 1996; Parkin & Cohen, 2001). The second method is oxygen independent, and involves the release of various toxic cationic proteins and enzymes present within the cytoplasmic granules of the phagocyte (Chew, 1996; Knight, 2000; Parkin & Cohen, 2001). In addition to this, phagocytic cells also release pro-inflammatory mediators such as cytokines (interleukin-1 and -6), tumour necrosis factor, prostaglandins and leukotrienes, which play an important role in the immune inflammatory response and the stimulation of lymphocyte activity (Grimble, 1997).

The cells of the innate immune system are able to differentiate between self and non-self and undertake actions to destroy antigens, but do not develop a specific response to each antigen. They serve to contain the infection, until an antigen specific response can be developed by the cells of the acquired immune system (Hayek *et al.,* 2004).

1.2.2 Acquired Immunity:

The acquired immune response is based around the recognition of an antigen by specific memory T and B cells and subsequent actions to destroy the antigen (Felsburg, 1994; Parkin & Cohen, 2001; Calder & Kew, 2002). Antigen is presented to the T or B cell in association with an antigen presenting cell (APC) (Chew, 1996; Parkin & Cohen, 2001). The APC's include macrophages, monocytes, Langerhans cells and Kupffer cells (Kubena & McMurray, 1996). Antigen presenting cells ingest antigens as they come into contact with them in the body and, after internal processing of the antigens, present them on the cell surface in conjunction with a surface protein called the major histocompatability complex (MHC). Endogenous antigen, such as that produced by viral infection or tumour proteins, is presented in association with the MHC class I, while exogenous antigen is complexed to MHC class II on the cell surface (Parkin & Cohen, 2001; Calder & Kew, 2002). T cells activated by antigen presentation flow to the site of infection to destroy the antigen, while activated B cells release specific antibodies into the blood and body fluids (Felsburg, 1994; Parkin & Cohen, 2001; Calder & Kew, 2002). Once an antigen has been encountered by the lymphocytes of the acquired immune system memory cells are developed so that any subsequent infection with the same antigen can be defended more quickly and efficiently (Felsburg, 1994; Chew, 1996; Kubena & McMurray, 1996; Parkin & Cohen, 2001).

The two main T cell types are T helper cells and cytotoxic T cells (Felsburg, 1994; Chew, 1996; Bendich, 1999; Parkin & Cohen, 2001). A third type of T cell is the T suppressor cell (Chew, 1996; Kubena & McMurray, 1996; Bendich, 1999). Cytotoxic T cells and T helper cells target intracellular antigens that cannot be removed by the innate immune system. The cytotoxic T cells combat viral and tumour infection (Kubena & McMurray, 1996; Parkin & Cohen, 2001), and T helper cells aid in the killing of other intracellular pathogens (Parkin & Cohen, 2001; Hayek *et al.,* 2004). T suppressor cells are thought to down-regulate T and B cell functions to maintain normal immune activity and prevent autoimmunity, hypersensitivity and tissue damage (Sheffy & Schultz, 1979;

Kubena & McMurray, 1996). The receptors on the surface of T cells recognise antigen and the MHC cell markers (Chew, 1996). T helper cells ($CD4^+$) associate with the antigen-MHC class II complex, and cytotoxic T cells ($CD8^+$) with the antigen-MHC I complex, on the cell surface of antigen presenting cells (Parkin & Cohen, 2001; Calder & Kew, 2002; Hayek *et al.,* 2004).

There are two forms of the T helper cell: T helper 1 cells and T helper 2 cells (Bendich, 1999). T helper 1 cells control the cell mediated immune response (Sheffy & Schultz, 1979; Felsburg, 1994; Parkin & Cohen, 2001) and release interleukin 2, which promotes proliferation of other T cells and also stimulates cytotoxic T cells to divide and release cytotoxic molecules (Felsburg, 1994; Parkin & Cohen, 2001). T helper 1 cells also release interferon γ, which increases the cytotoxicity of natural killer cells and activates macrophages to attack intracellular pathogens including fungi, mycobacteria and protozoa (Parkin & Cohen, 2001). T helper 2 cells promote a humoral response (Sheffy & Schultz, 1979; Felsburg, 1994; Chew, 1996; Parkin & Cohen, 2001), that both regulates antibody production by B cells and increases inflammation by the release of interleukins 4, 5, 6 and 10 (Felsburg, 1994; Bendich, 1999; Parkin & Cohen, 2001). Cytotoxic T cells bind to cells infected with specific antigens and release granules containing enzymes into the cell cytoplasm. The actions of these enzymes lead to DNA fragmentation, apoptosis and killing of the infected cell. Cytotoxic T cells also induce cell apoptosis by binding to molecules on the surface of the infected cell (Parkin & Cohen, 2001).

B cells produce antibodies to fight infection (Sheffy & Schultz, 1979; Felsburg, 1994; Chew, 1996). The actions of antibodies released by B cells complement those of the innate immune system and include functions such as adherence to mucosal membranes, opsonisation of bacteria for phagocytosis and the sensitisation of infected cells and tumour cells for antibody-dependent killing by cytotoxic cells (Felsburg, 1994; Parkin & Cohen, 2001). Antibody dependant cellular cytotoxicity is a process where antigen coated with antibody can be recognized and destroyed by lymphocytes and phagocytes (Kubena & McMurray, 1996).

1.3 Free Radical Production and Activity

A free radical is any molecule that contains one or more unpaired electrons (Bendich, 1990; Halliwell & Chirico, 1993; Halliwell *et al.,* 1995). When a free radical comes into contact with a non-radical molecule they can react to create a new radical product in several ways: by joining together to produce a new radical compound; by the removal an electron from the non-radical; and by donation of an electron to the non-radical (Halliwell *et al.,* 1995). In each case, the resulting molecule contains an unpaired electron, making it a new radical. This process of electron donation and removal can continue with other non-radical molecules to begin a damaging chain reaction. If two free radicals interact, they can join to form a covalent bond and become a stable non-radical molecule (Halliwell & Chirico, 1993; Halliwell *et al.,* 1995).

There are many free radical species produced in the body, the most notable being hydrogen peroxide, the superoxide anion, the hydroxyl radical and singlet oxygen (Chow 1991; Halliwell & Chirico, 1993; Halliwell *et al.,* 1995). Hydrogen peroxide and superoxide are generally low level free radicals, however in excess they can cause cellular damage. They may also generate other, more damaging, radicals such as the hydroxyl radical (Halliwell & Chirico, 1993; Halliwell *et al.,* 1995). The hydroxyl radical is highly reactive and often responsible for damaging chain reaction initiation and lipid peroxidation (Halliwell & Chirico, 1993; Halliwell *et al.,* 1995). Singlet oxygen is a highly reactive molecule that can react directly with fatty acids to cause lipid peroxidation (Halliwell & Chirico, 1993; Halliwell *et al.,* 1995)

1.3.1 Lipid Peroxidation:

The initiation of lipid peroxidation occurs when a free radical species ($R^{\cdot}$) comes into contact with, and removes a hydrogen atom from a fatty acid or fatty acyl chain (-CH). The removal of the hydrogen atom leaves an unpaired electron attached to the carbon atom of the fatty acid, thus creating a lipid radical ($C^{\cdot}$) (Figure 1.1 a) (Halliwell & Chirico, 1993). This lipid radical can undergo rearrangement and reaction with O_2 to form a peroxyl radical ($-CO_2^{\cdot}$) (Figure 1.1 b) (Halliwell & Chirico, 1993; Wang & Quinn, 2000). The peroxyl radical may then react with nearby fatty acids and begin a chain of damaging lipid peroxidation reactions (Figure 1.1 c) (Bendich, 1990; Halliwell & Chirico, 1993). Peroxyl radicals may also cause cell damage by reacting with

proteins in the cell membrane or they may join with other peroxyl radicals (Halliwell & Chirico, 1993).

$$-CH + R^{\cdot} \rightarrow C^{\cdot} + RH \qquad (a)$$

$$-C^{\cdot} + O_2 \rightarrow -CO_2^{\cdot} \qquad (b)$$

$$-CO_2^{\cdot} + -CH \rightarrow -CO_2H + -C^{\cdot} \qquad (c)$$

Figure 1.1: Mechanisms of lipid peroxidation by free radical species. (a) lipid peroxidation; (b) formation of peroxyl radical; (c) continuation of lipid peroxidation chain reaction (Halliwell *et al,* 1995)

The composition of fatty acids in the cell affects the length of the lipid peroxidation chain reaction, as the greater the number of double bonds in the fatty acid molecule, the greater its susceptibility to free radical attack (Halliwell & Chirico, 1993). Polyunsaturated fatty acids contain many double bonds, which is the reason for their high susceptibility to free radical attack (Chow 1991; Halliwell & Chirico, 1993). The level of protein in the cell membrane also affects lipid peroxidation, due to the susceptibility of protein to attack by peroxyl radicals (Halliwell & Chirico, 1993). The concentration of oxygen and antioxidants in the cell can also influence lipid peroxidation (Halliwell & Chirico, 1993). Immune cell membranes contain high levels of polyunsaturated fatty acids and are therefore highly susceptible to lipid peroxidation. Maintaining adequate antioxidant concentrations in these cells is important for immune health (Knight, 2000).

1.3.2 Activation of Free Radicals:

Free radical production occurs naturally in the body (Halliwell & Chirico, 1993; Halliwell *et al.,* 1995; McKenzie *et al.,* 1998). In fact, free radical production is a necessary event for many normal metabolic processes (Bendich, 1990; Chew, 1996; Matés *et al.*, 1999). Natural free radical production can occur due to the respiratory burst of phagocytes (McKenzie *et al.,* 1998), production by immune cells such as lymphocytes and fibroblasts (Halliwell & Chirico, 1993; Halliwell *et al.,* 1995), the release of transition metal ions (Chow 1991; Halliwell *et al.,* 1995), reduced antioxidant levels (Chow; 1991) and leakage from the electron transport chain (Bendich, 1990;

Halliwell *et al.,* 1995; Matés *et al.*, 1999; 2000). Free radicals are also produced in the body due to reactions with external factors such as toxins, pollutants or certain drugs (Chow 1991; Halliwell *et al.,* 1995; Chew, 1996) and radiation (Chow 1991; Halliwell *et al.,* 1995). Any source of free radical production can lead to tissue damage (Halliwell & Chirico, 1993; Halliwell *et al.,* 1995; Chew, 1996) and many disease conditions can be caused by, or result in the formation of, free radicals (Halliwell *et al.,* 1995). Free radical activities have been implicated in autoimmune diseases, cardiovascular disease and cancer (Chow 1991).

1.4 Antioxidant Activity

The body contains antioxidant enzymes and nutrients along with other mechanisms to prevent free radical damage (Chow 1991; Halliwell & Chirico, 1993; Matés *et al.,* 2000). The antioxidant enzymes and nutrients are responsible for neutralising free radicals and maintaining the reduction-oxidation balance in cells (Chow 1991; Halliwell *et al.,* 1995; Chew, 1996). Deoxyribonucleic acid (DNA) repair mechanisms that remove and re-synthesise damaged DNA and the binding of transition metals, such as iron and copper, to transport proteins, are non-antioxidant mechanisms that protect the body from free radical damage (Halliwell *et al.,* 1995).

1.4.1 Antioxidant Enzymes:

The antioxidant enzymes prevent free radical production by interacting with precursor molecules at the initiation phase to prevent radicals from interacting with other molecules in the body (Bendich, 1990; Matés *et al.*, 1999). Superoxide dismutase (SOD) functions by converting the superoxide anion ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) (Figure 1.2 a). Catalase (CAT) then converts the H_2O_2 to water (H_2O) and O_2 (Figure 1.2 b) (Babior, 1984; Bendich, 1990).

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow[SOD]{} H_2O_2 + O_2 \quad \text{(a)}$$

$$H_2O_2 + H_2O_2 \xrightarrow[\text{Fe-CAT}]{} 2H_2O + O_2 \quad \text{(b)}$$

Figure 1.2: Antioxidant mechanisms of superoxide dismutase (a) and catalase (b) (Bendich, 1990)

Glutathione peroxidase (GPx) performs the same function as catalase, reducing hydrogen peroxide to water and oxygen in the presence of reduced glutathione (GSH), and producing oxidised glutathione (GSSG) in the process (Figure 1.3 a) (Babior, 1984; Kiremidjian-Schumacher & Stotzky, 1987; Bendich, 1990; Halliwell *et al.,* 1995). Glutathione peroxidase is also capable of inactivating lipid peroxides (LOOH), to produce a stable lipid aldehyde (LOH) (Figure 1.3 b) (Bendich, 1990; Matés *et al.*, 1999; 2000; Rayman, 2000). Glutathione reductase reduces oxidised glutathione back to reduced glutathione using NADPH as an energy source (Figure 1.3 c) (Halliwell *et al.,* 1995; Matés *et al.*, 1999; Knight, 2000). This activity of glutathione peroxidase is considered to be one of the most important antioxidant defences in the body (Halliwell *et al.,* 1995; Matés *et al.*, 1999).

$$H_2O_2 + 2GSH \xrightarrow[GPx]{} 2H_2O + GSSG \quad \text{(a)}$$

$$LOOH + 2GSH \xrightarrow[GPx]{} LOH + GSSG + H_2O \quad \text{(b)}$$

$$GSSG + NADPH + H^+ \xrightarrow[\text{Glutathione Reductase}]{} 2GSH + NADP^+ \quad \text{(c)}$$

Figure 1.3: Antioxidant mechanisms of glutathione peroxidase (a & b) and glutathione reductase (c) (Bendich, 1990)

Various nutrients are required for the synthesis of antioxidant enzymes including selenium, zinc, copper, riboflavin, vitamin B6 and the sulphur amino acids (Grimble, 1997). A deficiency in a nutrient required for the production of antioxidant enzymes

can result in reduced antioxidant activity and increased oxidative stress (Halliwell *et al,* 1995).

1.4.2 Antioxidant Nutrients:

Any radicals that are not neutralised by antioxidant enzymes can be neutralised by interaction with antioxidant nutrients (Matés *et al.*, 1999). Antioxidant nutrients prevent free radical production by donating an electron to the radical, resulting in a stable product (Bendich, 1990; Chew, 1996). Antioxidant nutrients are not radicals, therefore when they react with a free radical they become a weak radical themselves that must be recycled by other antioxidants (Halliwell *et al.,* 1995). Vitamin C, vitamin E and β-carotene are the major chain breaking antioxidants. Vitamin C is water soluble (Bendich, 1990; Chow 1991; Halliwell *et al.,* 1995; Knight, 2000), while vitamin E and β-carotene are lipid soluble antioxidants (Chew, 1996). Some other important antioxidant molecules include the flavonoids, uric acid, glutathione and ubiquinol (Halliwell *et al.,* 1995).

1.5 Effects of Free Radicals and Antioxidants on the Immune System

The controlled production of free radicals is known to be important in the regulation of immune function (Bendich, 1990; Bendich, 1999; Knight, 2000). However, they may also cause cell and tissue damage, which may be detrimental to the immune system (Bendich, 1990; Bendich, 1999; Knight, 2000). Damage may be direct, due to free radicals attacking molecules, or indirect due to the activation of other compounds after the initial attack (Halliwell & Chirico, 1993). Oxidative stress can have a great effect on immune function since the cells of the immune system contain many polyunsaturated fatty acids in their cell membranes (Meydani & Beharka, 1996; Hayek *et al.,* 2000; Knight, 2000; Matés *et al.,* 2000) and produce and release high amounts of free radicals as part of their normal activity (Knight, 2000).

Free radical attack causing lipid peroxidation, as described in Section 1.3.1, alters the structure of the cell membrane and leads to reduced membrane fluidity and a loss of function (Chow 1991; Halliwell & Chirico, 1993; Bendich, 1999). This in turn reduces the ability of immune cells to divide and create new cell membranes, an event that is essential for immune defence (Bendich, 1990). The cell membranes of immune cells

also contain many receptors essential for cellular communication, such as lymphocyte subset receptors; receptors of the MHC; and those required to process cytokines, antibodies, and hormones (Bendich, 1990; Knight, 2000). Any alteration of the cell membranes of immune cells can adversely affect cell signalling and reduce cell responsiveness (Bendich, 1990; Knight, 2000). Other effects of free radical attack on the cell include DNA strand breakage and an increase in intracellular calcium molecules (Halliwell & Chirico, 1993; Halliwell *et al.,* 1995). DNA damage by free radicals can result in mutations, altered cell proliferation activity and reduced ability to produce critical factors, including various enzymes (Bendich, 1999). A rise in calcium levels can result in the activation of proteases and nucleases, which attack the cell cytoskeleton and DNA (Halliwell & Chirico, 1993).

The release of reactive oxygen species and other toxic products by phagocytes assist in the killing of pathogens (Babior, 1984; Bendich, 1990; Matés *et al.,* 2000). However, these radicals can cause damage to cells and tissues, including those of the immune system, when they are released from the cell (Babior, 1984; Bendich, 1990; Turner & Finch, 1991; Chew, 1996). While the phagocyte normally has mechanisms to prevent the release of oxidants into surrounding tissues, leakage can occur and affect nearby cells (Babior, 1984), and the phagocyte itself may be damaged or killed by its own release of oxidant products (Babior, 1984; Kiremidjian-Schumacher & Stotzky, 1987; Bendich, 1990; Rooke *et al.,* 2004).

Antioxidants provide protection against free radical damage, and can also modulate the activity of the immune system (Bendich, 1990; Tengerdy, 1990; Hayek *et al.,* 2000; Knight, 2000). The cells of the immune system contain higher levels of antioxidants than other cells in the body, highlighting their importance in maintaining immune cell structure and function (Knight, 2000). Under normal conditions antioxidant defences can be up-regulated to counter increased free radical production, however, if free radical production is excessive, these defence systems may be unable to cope (Halliwell *et al.,* 1995; Matés *et al.*, 1999). This may lead to reduced immune function and possibly inflammatory and autoimmune conditions (Matés *et al.*, 1999; Arthur *et al.,* 2003).

While antioxidants cannot completely protect the body from oxidation, their defence can be improved with an increased dietary intake of antioxidant micronutrients

(Grimble, 1997; Matés *et al.*, 1999). Antioxidant supplementation has been shown to reverse many of the effects of oxidative stress, however, the magnitude of this response is dependent on the prior nutrient status of the individual (Grimble, 1997). Many activities of the immune system are affected by the intake of antioxidant micronutrients (Grimble, 1997), and their addition to the diet can prevent diseases caused by free radical damage in the body (Bontempo, 2005).

A balance between free radical and antioxidant activity is essential to ensure that free radicals are able to carry out beneficial activities while ensuring they do not reach harmful levels (Bendich, 1990; Halliwell & Chirico, 1993; Halliwell *et al.,* 1995; Matés *et al.*, 1999). This balance is also required for optimum immune function (Bendich, 1990; Knight, 2000; Wang & Quinn, 2000). The oxidant to antioxidant balance is essential to maintain immune cell membrane structure, and functions such as gene expression, signal transduction and the production of nucleic acids and cellular proteins (Knight, 2000; Wang & Quinn, 2000).

1.6 Effects of Nutrient Supplementation on Immune function

Deficiencies of the essential nutrients are a known cause of reduced immune function (Kelleher, 1991; Kubena & McMurray, 1996; Calder & Kew, 2002; Dall'Ara, 2003; Hayek *et al.,* 2004), with reintroduction of these nutrients restoring immune function (Calder & Kew, 2002). Similarly, an excess of some nutrients may also have negative effects on immunity (Calder & Kew, 2002; Dall'Ara, 2003; Hayek *et al.,* 2004). Supplementation with individual nutrients can enhance immune function, however there is generally a greater enhancement when all nutrients are at optimum levels in the diet (Kubena & McMurray, 1996).

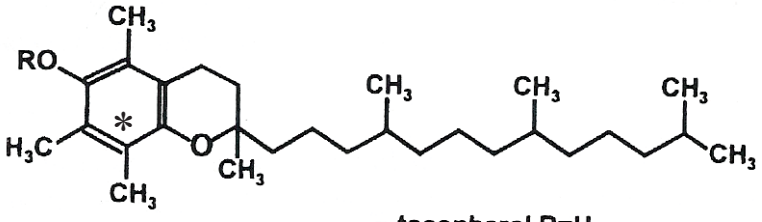
Antioxidants are commonly added to animal diets to maintain nutritional quality, prevent the destruction of nutrients in the feed, and to ensure that the feed remains palatable to the animal (Rutherfurd-Markwick & Hendriks, 2003). The health benefits of this practice have been realised in recent years, and include the prevention of diseases such as cancer and heart disease and a reduction in the rate of ageing (Rutherfurd-Markwick & Hendriks, 2003). Antioxidant supplementation studies have been carried out in several production animal species, and the results have shown that antioxidants,

either individually or in combination, have the ability to improve host resistance to disease (Chew, 1996). There is also a growing body of evidence suggesting that antioxidant nutrients may benefit health when added to companion animal diets. So far evidence has shown similar benefits of antioxidant supplementation in companion animals to those found in other species, for example β-carotene supplementation was found to enhance humoral and cell-mediated immune function in cats, and reverse the decline in immune function associated with aging in elderly dogs (Massimino *et al*, 2003). Other antioxidants that have been studied in the diet of cats and dogs include vitamin E, vitamin C, β-carotene, lutein and isoflavonoids (Allison *et al,* 2000; Baskin *et al.,* 2000; Scott *et al,* 2002). Diets including a combination of antioxidants may have greater health benefits than the addition of only one antioxidant to a diet (Rutherfurd-Markwick & Hendriks, 2003). Studies using various antioxidant mixtures in cats and dogs have shown improved serum vitamin levels, a reduction in lipid peroxidation and reduced deleterious effects of exercise on immune function (Rutherfurd-Markwick & Hendriks, 2003; Hayek *et al.,* 2004; Bontempo, 2005).

1.7 Vitamin E and Immune Function

1.7.1 Vitamin E:

Vitamin E is a term used to describe a group of tocopherol and tocotrienol compounds, with the most active form being α-tocopherol (See Figure 1.4) (Bendich & Machlin, 1988; Chow 1991; Wang & Quinn, 2000; NRC, 2006).



(Indicates chromanol ring)*

Figure 1.4: Chemical structure of α-tocopherol (Hayek *et al.,* 2000)

Vitamin E is able to donate an electron to a free radical to neutralise it, preventing the free radical mediated chain reaction that results in cell damage (Bendich, 1990; Wang & Quinn, 2000). The ability of vitamin E to quench free radical activity is related to its structure. A stable structure is maintained by delocalisation of the unpaired electron around the chromanol ring, which prevents the molecule becoming highly reactive (Wang & Quinn, 2000).

Vitamin E protects the lipids of the cell membrane from free radical damage and lipid peroxidation, a function it performs synergistically with glutathione peroxidase (Bendich, 1990). Vitamin E can quench peroxyl radicals (LOO˙) produced during the oxidation of polyunsaturated fatty acids in the cell membrane by reacting with them to produce non-reactive lipid hydroperoxides (Figure 1.5) (Halliwell *et al.,* 1995; Chew, 1996; Wang & Quinn, 2000).

LOO˙ + α-tocopherol → tocopherol radical˙ + LOOH

Figure 1.5: Quenching of the peroxyl radical by vitamin E (Halliwell *et al.,* 1995)

Vitamin E becomes a weak radical itself as a result of its interaction with free radicals, however it can be regenerated by ascorbate (vitamin C) (Figure 1.6 a), glutathione (Figure 1.6 b) and coenzyme Q (CoQH) (Figure 1.6 c) (Halliwell *et al.,* 1995).

tocopherol radical$^{\cdot}$ + ascorbate → α-tocopherol + semidehydroascorbate$^{\cdot}$ (a)

tocopherol radical$^{\cdot}$ + glutathione → α-tocopherol + glutathione$^{\cdot}$ (b)

tocopherol radical$^{\cdot}$ + $CoQH_2$ → α-tocopherol + $CoQH^{\cdot}$ (c)

Figure 1.6: Regeneration of vitamin E by vitamin C (a), glutathione (b) and coenzyme Q (c) (Halliwell *et al.,* 1995)

Vitamin E activity maintains membrane fluidity by preventing lipid peroxidation (Bendich, 1999), and can form complexes with other compounds in the lipid bi-layer that may stabilise the cell membrane (Wang & Quinn, 2000).

Vitamin E is an essential nutrient for immune function (Beharka *et al.,* 1997; Wang & Quinn, 2000). The importance of this vitamin is highlighted by the fact that the cells of the immune system contain higher levels of vitamin E than other cells in the body (Bendich, 1990; Beharka *et al.,* 1997; Hayek *et al.,* 2000). The antioxidant activity of vitamin E explains a great deal of its protective effects on immune function (Sheffy & Schultz, 1979; Meydani *et al.,* 1986; Bendich, 1990). Free radicals and lipid peroxidation have a suppressive effect on the immune system and the antioxidant activity of vitamin E is thought to reduce these effects and enhance the immune response (Chew, 1996; Calder & Kew, 2002). Vitamin E may also regulate immune function by reducing the activity of immunosuppressive compounds, such as prostaglandin E_2 (Tengerdy, 1990; Beharka *et al.,* 1997; Hayek *et al.,* 2000), and the cell signalling molecule, nuclear factor κB (Rooke *et al.,* 2004).

1.7.2 Prostaglandin Production:

Prostaglandin E_2 is produced during the metabolism of arachidonic acid by the cyclo-oxygenase pathway (Hayek *et al.,* 2000; Wu *et al.,* 2001; Miles *et al.,* 2003). While low levels of prostaglandin E_2 are necessary for normal immune cell function, high levels

are immunosuppressive (Meydani & Beharka, 1996). A vitamin E deficiency results in the increased production of immunosuppressive eicosanoids, including prostaglandin E_2 (Erickson *et al.,* 2000; Knight, 2000). In contrast, vitamin E supplementation is known to inhibit the production of prostaglandin E_2 (Sheffy & Schultz, 1979; Bendich *et al.,* 1986; Meydani *et al.,* 1986). This is thought to be due to the free radical scavenging effect of vitamin E, as it can neutralise the hydroperoxides required for the activation of the cyclo-oxygenase enzyme (See Figure 1.7, Section 1.8.2) (Wu *et al.,* 2001).

The effect of vitamin E on prostaglandin E_2 production may be more effective in older individuals, as vitamin E may reverse the increase in cyclo-oxygenase activity that occurs with age (Meydani & Beharka, 1996; Hayek *et al.,* 2000; Wu *et al.,* 2001), most likely by preventing free radical production (Wu *et al.,* 2001). Increased prostaglandin E_2 production and reduced immune function have been observed in aged rats and mice (Meydani *et al.,* 1986), and several studies have reported a reduction in prostaglandin E_2 and improvement in immune cell production and activity when vitamin E has been administered in aged mice (Meydani & Beharka, 1996; Wu *et al.,* 2001). Therefore, vitamin E may be an important supplement for preventing age related immune suppression and inflammation caused by prostaglandin E_2 (Meydani *et al.,* 1986; Wu *et al.,* 2001).

1.7.3 Cytokine Production:

Some immune enhancing effects of vitamin E may be related to its stimulation of interleukin-2 production. Vitamin E supplementation enhances the production of interleukin-2 by T helper 1 cells, and is an important promoter of T and B cell proliferation and differentiation. Vitamin E is therefore an important regulator of the length and potency of the immune response. Interleukin-2 also induces cytokine release by activated T cells (Beharka *et al.,* 1997). The release of tumour necrosis factor-α and interleukin-6 are also known to be affected by vitamin E status (Beharka *et al.,* 1997).

1.7.4 Polyunsaturated Fatty Acids:

The level of vitamin E required by a cell increases as the level of polyunsaturated fatty acids in the cell membrane increases (Sheffy & Schultz, 1979; Tengerdy, 1990; Chow 1991; Dall'Ara, 2003). Polyunsaturated fatty acids must be present in the correct ratio to promote immunity, while excess amounts reduce immune function (Dall'Ara, 2003). Polyunsaturated fatty acids are known to modulate humoral and cell-mediated immunity (Dall'Ara, 2003). They can alter macrophage function and reduce lymphocyte proliferative activity, possibly by changing the composition, fluidity and function of the cell membrane. The addition of vitamin E to the cell can regulate these effects (Kubena & McMurray, 1996). For this reason it is recommended that vitamin E addition to pet foods be increased when there is a high ratio of polyunsaturated fatty acids in the food (Hayek *et al.,* 2000; Bontempo, 2005; NRC, 2006). The level and composition of fat intake can alter arachidonic acid production, and subsequently prostaglandin production, which can greatly alter immune function (Sheffy & Schultz, 1979; Kubena & McMurray, 1996).

1.7.5 Effects of Vitamin E Supplementation on Immune Function:

While a deficiency of any vitamin can lead to reduced immune function, studies with rats show that vitamin E deficiency suppresses immune function even when all other nutrients are at adequate levels in the diet, demonstrating the importance of vitamin E to immune function (Bendich *et al.,* 1986). A loss of immune function is often the earliest sign of vitamin E deficiency in many species (Bendich *et al.,* 1986; Kubena & McMurray, 1996), while the restoration of vitamin E status restores immune function (Meydani & Beharka, 1996; Grimble, 1997; Hayek *et al.,* 2000), and supplementation can lead to further enhancement of the immune system (Meydani & Beharka, 1996). It is suggested that the level of vitamin E supplementation required to enhance the immune system is around 4-10 fold greater than the maintenance requirement (Hendriks *et al.,* 2002; Rutherfurd-Markwick & Hendriks, 2003). Evidence from studies in various species has suggested that vitamin E supplementation may also be beneficial to the immune system of companion animals (Rutherfurd-Markwick & Hendriks, 2003).

The use of supplemental vitamin E may be especially beneficial in the aged animal (Tengerdy, 1990; Calder & Kew, 2002), as immune function is reduced in the elderly, partly due to increased free radical stress. Fewer infections are reported in elderly

humans taking vitamin E supplements and other parameters of immune function, such as the mitogenic response and antibody production in response to vaccination, have also been improved (Meydani & Beharka, 1996).

The effects of vitamin E supplementation on immune cell function and resistance to pathogens has been studied in various production and laboratory animals and in humans (Beharka *et al.,* 1997; Calder & Kew, 2002). These studies have focused on various immune parameters and have shown that vitamin E supplementation can influence both the innate and acquired immune systems. For example, it has been suggested that phagocytic activity may be enhanced by vitamin E supplementation, which is likely to be due to the effects of vitamin E on the production of immunosuppressive compounds such as free radicals and prostaglandin E2 (Beharka *et al.,* 1997). Vitamin E supplementation may also influence the lymphocyte proliferative response, possibly by its effects on interleukin-2 production (Beharka *et al.,* 1997). Some examples of the effects of vitamin E on immune function are shown in Table 1.1.

Table 1.1: Effects of vitamin E supplementation on different immune parameters

↑ proliferation in response to concanavalin A, phytohaemagglutinin and lipopolysaccharide (Mouse: Meydani & Beharka, 1996), (Rat: Grimble, 1997)
↑ response to concanavalin A (Mouse: Meydani *et al.,* 1986; Moriguchi *et al.,* 1990; Meydani & Beharka, 1996), (Elderly Human: Moriguchi *et al.,* 1990; Knight, 2000)
↑ response to phytohaemagglutinin and concanavalin A (Piglet: Finch & Turner, 1996)
↑ response to phytohaemagglutinin, lipopolysaccharide, pokeweed mitogen and concanavalin A (Calf: Finch & Turner, 1996)
↑ T cell numbers (Rat: Grimble, 1997)
↑ delayed type hypersensitivity response (Aged Mouse: Meydani *et al.,* 1986; Calder & Kew, 2002), (Elderly Human: Bendich, 1990; Kelleher, 1991; Meydani & Beharka, 1996; Bendich, 1999; Knight, 2000), (Mouse: Meydani & Beharka, 1996)
↑ natural killer cell activity (Rat: Moriguchi *et al,* 1990; Meydani & Beharka, 1996)
↑ phagocytosis by neutrophils (Premature human infant: Calder & Kew, 2002)
↑ phagocytic activity of macrophages (Rat: Moriguchi *et al,* 1990)
↑ viral resistance (Aged Mouse: Calder & Kew, 2002)
↑ protection against infectious disease (Rabbit: Meydani & Beharka, 1996)
↑ response to *E. coli* (Chicken: Chew, 1996; Meydani & Beharka, 1996; Grimble, 1997), (Pig: Meydani & Beharka, 1996), (Turkey: Grimble, 1997)
↑ response to *Chlamydia* (Lamb: Chew, 1996)
↑ antibody response to Hepatitis B, tetanus toxoid and pneumococci (Elderly Human: Meydani & Beharka, 1996; Bendich, 1999; Calder & Kew, 2002)
↑ antibody production (Pig, Sheep, Cow: Finch & Turner, 1996), (Sheep: Rooke *et al.,* 2004)
↓ prostaglandin E_2 Production (Mouse: Bendich, 1990; Meydani & Beharka, 1996), (Elderly Human: Bendich, 1990; Kelleher, 1991; Bendich, 1999; Knight, 2000)
↑ interleukin-2 production (Elderly Human: Kelleher, 1991; Bendich, 1999; Knight, 2000), (Aged Mouse: Meydani & Beharka, 1996; Calder & Kew, 2002)
↑ interferon-γ release by spleen lymphocytes (Aged Mouse: Calder & Kew, 2002)
↑ superoxide production by neutrophils (Cow: Finch & Turner, 1996)

Key: ↑ Increased; ↓ Decreased

While many positive effects of vitamin E supplementation on immune function have been reported, as outlined in Table 1.1 above, other studies have reported mixed results, with either no significant improvements to immune function found, or with some immune parameters enhanced and others showing little or no improvement (Kelleher, 1991; Calder & Kew, 2002). A reduction in free radical production may be beneficial in some individuals where oxidation of host tissues can exacerbate a disease state, however in healthy individuals or those with impaired immunity this effect may inhibit removal of infection (Erickson *et al.,* 2000). For example, excessive vitamin E supplementation may reduce the ability of phagocytic cells to kill pathogens, as it can neutralise the free radicals produced by the cell to fight infection (Erickson *et al.,* 2000; Calder & Kew, 2002). Several studies of the effects of vitamin E supplementation on

phagocytic activity in humans have reported a reduction in free radical production and killing of pathogens (Kelleher, 1991; Meydani & Beharka, 1996; Calder & Kew, 2002).

1.7.6 Vitamin E Studies in the Cat:

The minimum vitamin E requirement for cats is 30 IU/kg DM (Hayek *et al.,* 2000; Hendriks *et al.,* 2002; NRC, 2006; Yu & Paetau-Robinson, 2006), well below the amount thought to be required for optimal immune response in this species (Hayek *et al.,* 2000). The optimal vitamin E requirement for immune response has been suggested to be around 200 IU/kg DM in humans (Calder & Kew, 2002). A study of the levels of various vitamins in pet foods found that vitamin E concentrations ranged between 15-440 IU/kg DM. It has been suggested that a vitamin E concentration of over 550 IU/kg diet would benefit the antioxidant status of pets (Bontempo, 2005). Serum vitamin E levels have been found to increase in cats consuming a diet containing supplemental vitamin E (Hendriks *et al.,* 2002; Yu & Paetau-Robinson, 2006).

Several studies have reported the benefits of vitamin E supplementation in preventing free radical related damage in the cat. In a study of antioxidant protection in cats presented with an oxidative challenge, a diet calculated to give each animal 165-220 IU vitamin E per day was found to protect against oxidation, although this diet also contained other supplements that could also prevent oxidation (Hill *et al,* 2005). Another study reported that vitamin E and ascorbic acid were shown to have only a small effect on Heinz body anaemia produced in cats by feeding onion powder or propylene glycol, but may exert a positive effect by conserving other antioxidant compounds within the body (Rutherfurd-Markwick & Hendriks, 2003). In a study of the effects of nutrient supplements on oxidative stress in cats with renal insufficiency, animals fed a diet supplemented with vitamin E (815 IU/kg DM), β-carotene and vitamin C showed reduced levels of DNA damage due to oxidants (Yu & Paetau-Robinson, 2006). As these antioxidant nutrients were tested together, it is unknown if any one nutrient had a greater effect than the others (Yu & Paetau-Robinson, 2006).

While these studies show that vitamin E supplementation can protect the cat from the adverse effects of oxidants, there is very little data available regarding the effect of vitamin E on immune function in the cat (Hayek *et al.,* 2000; 2004). A recent study of the vitamin E requirements of cats found no enhancement in lymphocyte proliferation to

concanavalin A when a vitamin E supplemented diet of 1,100 IU/kg DM, 2,800 IU/kg DM or 4,300 IU/kg DM was fed for 26 weeks (Hendriks *et al.,* 2002). In another study, 38 young and old cats were fed a diet containing 60 IU/kg DM vitamin E for a 60 day period before half were put on a diet supplemented with 250 IU/kg DM or 500 IU/kg DM vitamin E for a further 60 days (Hayek *et al.,* 2000). As would be expected, serum vitamin E levels in both young and old cats consuming the supplemented diet were significantly higher than those of controls. The older cats had a greatly reduced T cell proliferative response to concanavalin A and phytohaemagglutinin compared to young cats, but no differences were seen in the B cell response to pokeweed mitogen between the age groups. This suggests than in cats, the loss of immune function with age affects T cells to a greater degree than other immune cells, as has been noted in other species (Hayek *et al.,* 2000). Supplementation of vitamin E at 250 IU/kg DM had no effect on T or B cell proliferation in young cats, however older cats on this diet showed a significant improvement in response to concanavalin A compared with age matched controls (Hayek *et al.,* 2000). The lymphocyte proliferative response to concanavalin A was also significantly higher in aged cats consuming 500 IU/kg DM compared to age matched controls. There was a significant increase in the response of young cats to pokeweed mitogen consuming the 500 IU/kg diet in comparison to age-matched controls (Hayek *et al.,* 2000). Supplementation of vitamin E at either level did not significantly increase lymphocyte proliferation to phytohaemagglutinin in either age group. Prostaglandin E_2 production was reduced in cats consuming the 500 IU/kg DM compared to controls in both age groups (Hayek *et al.,* 2000).

Cats are known to experience the same decline in immune function with age that has been noted in other species (Hayek *et al.,* 2000; Campbell *et al.,* 2004). A study by Campbell *et al*. (2004) reported a reduction in total white blood cell counts, lymphocyte and eosinophil counts and the CD4:CD8 ratio in older cats compared to younger cats. However, neutrophil, basophil and monocyte counts were not found to decline with age (Campbell *et al.,* 2004). The study by Hayek *et al* (2000) suggests that the decline in immune function of aged cats can be prevented to some extent by vitamin E supplementation of 250 IU/kg, however a higher level of dietary vitamin E supplementation provided no further enhancement of immune function and did not enhance immune function to the same degree as has been observed in other species (Finch & Turner, 1996: Meydani & Beharka, 1996; Grimble, 1997).

1.7.7 Safety of Vitamin E Supplementation:

There are very few cases of vitamin E toxicity in animals or humans. Studies have shown that cats, dogs, rabbits, frogs and monkeys develop no signs of toxicity at a dose of 220 IU/kg body weight. Side effects of vitamin E supplementation have only been observed in animals at doses above 1,100 IU/kg body weight (Bendich & Machlin, 1988). Vitamin E supplementation may exacerbate defects in blood coagulation occurring due to vitamin K deficiency (Bendich & Machlin, 1988), and dosages of vitamin E above 2000 IU/kg body weight have been associated with blood coagulation dysfunction (Hill *et al.,* 2005). In a human trial spanning seven months, a dosage of 220 IU/day was suggested to be the optimal level of vitamin E supplementation for immune enhancement, while a dose of 880 IU/day was found to inhibit antibody production in response to tetanus toxoid, diphtheria and pneumococci vaccinations (Meydani *et al.,* 1997). Others have reported negative effects when dosages of over 330 IU/day vitamin E were taken by human subjects: Baehner *et al.* (1977) showed reduced bacterial killing by neutrophils in a seven day trial and Devaraj *et al.* (1996) showed reduced respiratory burst of monocytes and release of interleukin-1β over a 14 week trial. A study in which human subjects consumed a 1760 IU vitamin E supplement daily reported the reduced bactericidal killing ability of phagocytes (Kelleher, 1991).

1.8 Selenium and Immune Function

1.8.1 Selenium:

An adequate intake of selenium is required for an optimal immune response (Kiremidjian-Schumacher & Stotzky, 1987; Spallholz *et al.,* 1990; McKenzie *et al.,* 1998). High levels of selenium have been detected in organs associated with the immune system, such as the bone marrow, thymus, intestine, liver, spleen and lymph nodes, and in immune cells such as lymphocytes, granulocytes and macrophages (Spallholz *et al.,* 1990). The level of selenium required to enhance immune system function appears to be much greater than that required for normal antioxidant and enzymatic functions (Rayman, 2000). However, excessive selenium intakes have been shown to have an inhibitory effect on the immune system (Kiremidjian-Schumacher & Stotzky, 1987; Kubena & McMurray, 1996). Aged people have been found to have reduced plasma selenium, along with reduced activity of glutathione peroxidase, suggesting that selenium intake is important for the maintenance of immune function in the elderly (Knight, 2000).

Selenium is thought to modulate immune function and reduce inflammation by three main mechanisms. These include its incorporation into glutathione peroxidase, which performs antioxidant removal of radical peroxides and its control of the lipoxygenase and cyclo-oxygenase pathways of the arachidonic acid cascade, which controls the synthesis of leukotrienes, thromboxanes, prostaglandins and lipoxins. Selenium can also modulate the products of the respiratory burst of phagocytes (Spallholz *et al.,* 1990; Rayman, 2000; Rooke *et al.,* 2004). In addition to the removal of immunosuppressive free radicals and the control of arachidonic acid metabolism, selenium increases expression of the high-affinity interleukin-2 receptor on T cells, thereby increasing T cell activity (McKenzie *et al.,* 1998). Selenium containing enzymes also control the production of pro-inflammatory cytokines such as interleukins-1 and 6 and tumour necrosis factor-α, and prevent the activation of the pro-inflammatory nuclear factor κB cascade by the removal of free radicals from the cell (Rooke *et al.,* 2004).

1.8.2 Arachidonic Acid Metabolism:

The production and release of arachidonic acid from cell membranes has been linked to free radical reactions and lipid peroxidation. The products of the arachidonic acid cascade, such as prostaglandins and leukotrienes, also require free radical intermediates for their formation (Bendich, 1990). Low levels of hydroperoxides activate the enzymes lipoxygenase and cyclo-oxygenase, which have some control over prostaglandin and leukotriene synthesis, while high hydroperoxide levels can inactivate these enzymes (Halliwell & Chirico, 1993; Rooke *et al.,* 2004).

Figure 1.7 outlines the main aspects of arachidonic acid metabolism by the lipoxygenase and cyclo-oxygenase pathways and includes the points of control by hydroperoxides, vitamin E and glutathione peroxidase. Briefly, arachidonic acid is metabolised by the cyclo-oxygenase pathway to produce prostaglandin G_2 (PGG_2), which is then reduced to prostaglandin H_2 (PGH_2). The activity of cyclo-oxygenase is the rate limiting step in this pathway, and is controlled by hydroperoxide levels. Several enzymes then convert PGH_2 to prostaglandins (PG) and thromboxane (TXA_2). The lipoxygenase pathway metabolises arachidonic acid to produce hydroxyeicosatetraenoic acids (HETE) and leukotrienes (LT) (Wu *et al,* 2001). Both pathways require hydroperoxide and glutathione peroxidase for activation. Glutathione peroxidase and vitamin E prevent excessive hydroperoxide production and regulate arachidonic acid metabolism (Spallholz *et al,* 1990; Wu *et al.,* 2001).

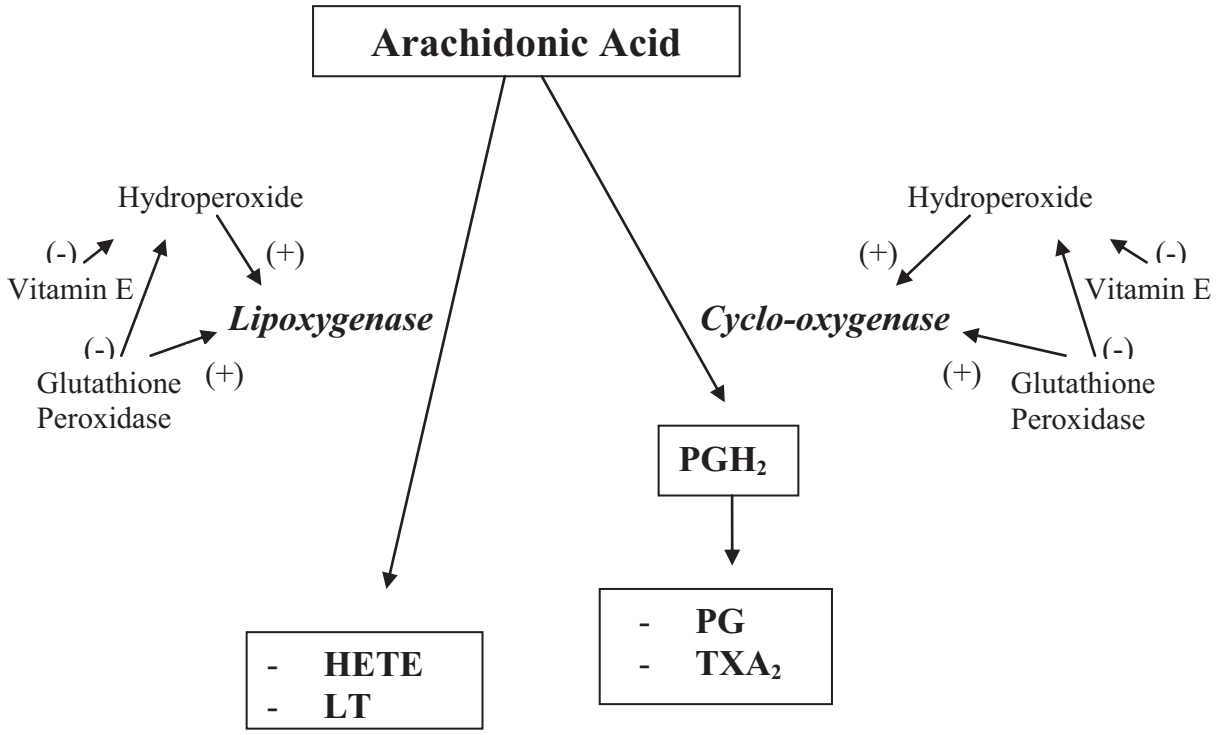


Figure 1.7: Metabolism of arachidonic acid

Glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase reduce PGG_2, the precursor of PGH_2, in the cyclo-oxygenase pathway of the arachidonic acid cascade. These enzymes are also responsible for the reduction of the hydroxyeicosatetraenoic acids in the lipoxygenase pathway, leading to the synthesis of leukotrienes and lipoxins (Spallholz *et al,* 1990). Studies have shown reduced eicosanoid production when both selenium and glutathione peroxidase are deficient, outlining their importance in the metabolism of arachidonic acid (Spallholz *et al.,* 1990; McKenzie *et al.,* 1998). Selenium and its associated enzymes also control the level of hydroperoxides produced within the lipoxygenase and cyclo-oxygenase pathways, and in this way can regulate the production of pro-inflammatory prostaglandins and leukotrienes (Rayman, 2000; Arthur *et al.,* 2003).

1.8.3 Cancer Prevention:

Selenium appears to have anti-carcinogenic activities, and increased intake of this mineral has been linked to the reduced incidence of some forms of cancer (Kiremidjian-Schumacher & Stotzky 1987; Spallholz *et al.,* 1990; McKenzie *et al.,* 1998; Rayman, 2000; Simcock *et al.,* 2004). These preventative effects are most likely due to the antioxidant, anti-inflammatory and immuno-stimulatory effects of selenium (McKenzie *et al.,* 1998; Simcock *et al.,* 2004), and the production of anti-tumourigenic metabolites, such as methyl selenol, that inhibit the metabolism of tumour cells, inhibit angiogenesis and cause apoptosis of cancer cells (Rayman, 2000). Selenium supplementation has also been shown to increase the ability of lymphocytes to develop into cytotoxic T cells and kill tumour cells and also to increase natural killer cell activity (McKenzie *et al.,* 1998; Rayman, 2000). Selenium appears to modulate these functions by its ability to up-regulate the expression of receptors for interleukin-2 on the surface of activated lymphocytes and natural killer cells (Turner & Finch, 1991; Rayman, 2000; Rooke *et al.,* 2004). This allows increased interaction with interleukin-2, which regulates cell growth and expansion and differentiation into cytotoxic T cells (Rayman, 2000).

1.8.4 Selenium Deficiency and Supplementation:

Various studies in animals and humans have shown that both selenium deficiency and toxicity suppress immune function, while adequate and supplementary selenium can improve immune function (Kiremidjian-Schumacher & Stotzky 1987; Spallholz *et al.,* 1990; Rooke *et al.,* 2004). Selenium supplementation leads to the decreased production of hydrogen peroxide and superoxide (Spallholz *et al.,* 1990), and reduced lipid peroxidation (Kiremidjian-Schumacher & Stotzky, 1987). Table 1.2 outlines some of the effects of selenium on immune function found in studies in various species.

Table 1.2: Effects of selenium supplementation on different immune parameters

↑ T cell response to pokeweed mitogen (Cow: Finch & Turner, 1996; McKenzie *et al.,* 1998)
↑ lymphocyte proliferation to phytohaemagglutinin and pokeweed mitogen (Pig, Lamb: Kiremidjian-Schumacher & Stotzky, 1987)
↑ lymphocyte response to phytohaemagglutinin *in vitro* (Mouse: Turner & Finch, 1991), (Human: McKenzie *et al.,* 1998)
↑ lymphocyte response to phytohaemagglutinin, concanavalin A and pokeweed mitogen (Lamb: Finch & Turner, 1996)
↑ development of cytotoxic T cells (Mouse: Spallholz *et al.,* 1990), (Human: Rayman, 2000)
↑ development of T suppressor cells (Mouse: Spallholz *et al.,* 1990)
↑ delayed type hypersensitivity response (Mouse: McKenzie *et al.,* 1998)
↑ natural killer cell activity (Human: McKenzie *et al,* 1998; Rayman, 2000), (Mouse: McKenzie *et al.,* 1998)
↑ natural killer cell induced tumour cytotoxicity (Rat: Kiremidjian-Schumacher & Stotzky, 1987)
↑ neutrophil migration and superoxide activity (Cow: Kiremidjian-Schumacher & Stotzky, 1987; McKenzie *et al.,* 1998)
↑ neutrophil migration and phagocytic activity (Goat: Finch & Turner, 1996)
↑ killing by macrophages *in vitro* (Guinea Pig: Kiremidjian- Schumacher & Stotzky, 1987), (Human: McKenzie *et al,* 1998)
↑ resistance to infection (Calf: Finch & Turner, 1996), (Sheep: Rooke *et al.,* 2004)
↑ antibody response to virus *in vitro* (Cow: McKenzie *et al.,* 1998)
↑ antibody production (Mouse, Rat, Rabbit, Cow: Kiremidjian-Schumacher & Stotzky, 1987), (Cow, Sheep: Finch & Turner, 1996), (Sheep: Rooke *et al.,* 2004)
↓ B cell lipoxygenase activity *in vitro* (Human: McKenzie *et al.,* 1998)
↑ expression of the high affinity interleukin-2 receptor (Mouse: McKenzie *et al.,* 1998)
↑ interferon production (Human: Kiremidjian-Schumacher & Stotzky, 1987)
↓ lipid peroxidation (Guinea Pig: Kiremidjian-Schumacher & Stotzky, 1987)
↑ tumour killing ability (Human: Rayman, 2000)
↑ tumour cytotoxicity of macrophages (Mouse: Erickson *et al.,* 2000)
↓ tumour growth (Rat, Mouse: Kiremidjian-Schumacher & Stotzky, 1987)

Key: ↑ Increased; ↓ Decreased

1.8.5 Studies of Selenium Activity in the Cat:

There have been very few studies of the selenium requirements of the cat (Simcock *et al.,* 2004). The minimum selenium requirement for the cat is 0.3 mg/kg DM (NRC, 2006), which is based on studies in other animal species (Wedekind *et al.,* 2000; NRC, 2006). Studies to determine the requirement for selenium in kittens suggest that diets for growing kittens must contain a minimum of 0.4 mg/kg DM (Wedekind *et al.,* 2000) or 0.5 mg/kg DM to account for low bioavailability (Wedekind *et al.,* 2003b). There is no data available describing selenium deficiency or toxicity in the cat (Simcock *et al.,* 2004), however deficiency appears to be uncommon and has not been observed in cats (NRC, 2006). The symptoms of selenium deficiency in dogs include muscle weakness, oedema, anorexia, depression, dyspnoea and progression into a coma (Simcock *et al.,* 2004). A study of the selenium requirements of kittens found that a selenium deficiency led to a reduction in the rate of hair growth, while high doses of selenium appeared to have no deleterious effects (Wedekind *et al.,* 2003a).

While the cat appears to require the same essential minerals as other animals, there may be differences in transport, metabolism and storage specific to the cat (MacDonald *et al.,* 1984). Therefore, the cat may have differences in its metabolism of selenium that require a different dietary intake of this mineral (Simcock *et al.,* 2004). Several studies have noted that there are differences in selenium uptake and concentration in the blood of cats compared to other species. For example, one study reported that while serum selenium concentrations in dogs and chicks reach a plateau at a level above the animal's requirements, the serum concentration in the cat continues to rise linearly (Wedekind *et al.,* 2003a). Similarly, in a study of the selenium requirement of kittens, no plateau was reached for plasma selenium levels and plasma levels continued to increase as selenium intake was increased (Wedekind *et al.,* 2003b). Serum selenium concentrations are 50-70% higher in cats than dogs consuming diets containing similar amounts of selenium, and the cat has been observed to have around a five-fold higher serum selenium concentration than that of other species (Wedekind *et al.,* 2003b). A large variation in serum selenium concentration between cats has also been noted, which is related to the amount of selenium in the diet (Simcock *et al.,* 2004).

The differences noted between cats and other species, in terms of the regulation of selenium levels in the blood, suggest that there may also be other differences in

selenium metabolism in the cat compared to other species, possibly related to differences between carnivores and omnivores (Wedekind *et al.,* 2003b). There is no information available on the effects of selenium on the feline immune system.

The addition of selenium to some pet food diets may be advisable as bioavailability appears to be low in pet foods at around 30% in canned diets (Wedekind *et al.,* 1998; 1999; 2000; 2002; 2003b) and 50% in extruded diets (Wedekind *et al.,* 1998; 1999; 2003b). In a study of the concentrations of selenium in various commercial cat and dog foods, selenium concentrations were found to be much higher in the cat foods, especially in those containing sea food (Simcock *et al.,* 2004). All pet foods analysed met the minimum requirements set by AAFCO of 0.10 and 0.11 mg/kg diet for dogs and cats, respectively (Simcock *et al.,* 2004). It is not known if these levels provide for optimum health in these species, or what an optimal level would be. It has been suggested that due to the low bioavailability of selenium in pet foods, some pets may not be consuming enough selenium to maintain good health (Simcock *et al.,* 2004).

1.8.6 Safety of Selenium Supplementation:

In most species, selenium intake must be contained within a narrow range to prevent signs of deficiency or toxicity (Turner & Finch, 1991; Rayman, 2000; Simcock *et al.,* 2004). In humans, a selenium intake of up to 0.45 mg/day is considered safe, while symptoms of toxicity occur at intakes above 0.9 mg/day (McKenzie *et al.,* 1998). Excessive selenium intake is reached at 5 mg/kg diet in dogs (Wedekind *et al.,* 2002; Simcock *et al.,* 2004), so a maximum intake of 2 mg/kg diet is recommended (Wedekind *et al.,* 2003a). Signs of toxicity noted in the dog include nausea, vomiting, diarrhoea, lack of appetite leading to anorexia and reduced growth, hair loss and a coarse coat, increased respiration and cardiovascular changes (Simcock *et al.,* 2004).

There is no recommended maximum selenium intake for cats (Wedekind *et al.,* 2003a). Some wet cat foods have been shown to have levels which are potentially toxic for other species (5 mg/kg diet), however low bioavailability in seafood products may prevent toxicity, and cats may be able to tolerate a much higher level of selenium than other species (Simcock *et al.,* 2004). As cat foods can contain very high levels of selenium, with one study finding that commercial moist diets contained between 0.16 and 6.12

mg/kg DM (Simcock *et al,* 2004), it is important to determine a safe upper limit for this species (Wedekind *et al.,* 2003a).

1.9 Combined Vitamin E and Selenium Supplementation and Immune Function

As mentioned in sections 1.7 and 1.8, both selenium and vitamin E are required in adequate amounts for optimum immune function, however these nutrients demonstrate sparing effects in order to maintain immune function if either nutrient is not present in adequate amounts (Turner & Finch, 1991; Chew, 1996; Kubena & McMurray, 1996; Knight, 2000). If both are deficient, immune function will be reduced to a greater degree than if only one is deficient (Chow 1991; Turner & Finch, 1991; Kubena & McMurray, 1996). Combined supplementation of these nutrients may have a greater positive effect on immune function than supplementation of either nutrient individually (Kubena & McMurray, 1996).

1.9.1 Vitamin E and Selenium Deficiency and Supplementation:

The majority of studies investigating the effects of interactions between selenium and vitamin E on the immune system have been based on deficiencies of these nutrients. Deficiencies in both selenium and vitamin E in laboratory and production animals have been reported to decrease natural killer cell activity (Knight, 2000); T cell cytotoxicity (Knight, 2000); phagocytic function and killing ability (Chew, 1996; Finch & Turner, 1996; Kubena & McMurray, 1996); antibody production (Sheffy & Schultz, 1979; Turner & Finch, 1991; Finch & Turner, 1996); and lymphocyte response to mitogens such as concanavalin A, phytohaemagglutinin and pokeweed (Sheffy & Schultz, 1979; Turner & Finch, 1991; Chew, 1996; Finch & Turner, 1996). Various microbiological infections, such as *Candida albicans* and *Staphylococcus aureus*, may be linked to a deficiency in both vitamin E and selenium and these conditions have been reproduced in laboratory animals in experiments where the animals were injected with these pathogens (Turner & Finch, 1991).

Dietary supplementation with vitamin E or selenium has been shown to produce a number of immuno-stimulatory effects in many species (Sheffy & Schultz, 1979; Rooke *et al.,* 2004). While there have been many studies on the effect that supplementation of

these nutrients has on immune function individually, fewer studies have been carried out on the effect of a combination of these nutrients on immune function. These studies have reported the enhancement of some aspects of immunity, above that produced by individual supplementation of either nutrient, when selenium and vitamin E intakes are increased. However, the majority of these studies were in animals that were deficient in one or both nutrients at the beginning of the trial (Kubena & McMurray, 1996). Studies in which animals with adequate vitamin E and selenium status were fed a diet supplemented with vitamin E and selenium show a significant enhancement of immune function, although this response is higher in deficient animals (Finch & Turner, 1996). Therefore, further investigation is required to determine the benefit of combined selenium and vitamin E supplementation on immune function. Table 1.3 outlines some effects of combined selenium and vitamin E supplementation on immune function found in studies in different species.

Table 1.3: Effects of vitamin E and selenium supplementation on different immune parameters

↑ lymphocyte proliferation to phytohaemagglutinin (Pig: Kiremidjian-Schumacher & Stotzky, 1987; Finch & Turner, 1996)
↑ response to phytohaemagglutinin and pokeweed mitogen (Sheep: Finch & Turner, 1996)
↑ lymphocyte response (Dog, Lamb, Cow: Finch & Turner, 1996)
↑ B cell count (Cattle: Kubena & McMurray, 1996)
↑ phagocytosis and bactericidal activity of neutrophils *in vitro* (Cattle: Chew, 1996)
↑ superoxide production by neutrophils (Pig: Finch & Turner, 1996)
↑ microbicidal activity of neutrophils (Cow: Finch & Turner, 1996)
↑ resistance to experimental infection (Chicken, Duck, Pig: Finch & Turner, 1996)
↑ antibody production in response to typhoid vaccination (Rabbit: Kiremidjian-Schumacher & Stotzky, 1987)
↑ antibody production in response to sheep red blood cells or tetanus toxoid (Mouse: Kiremidjian-Schumacher & Stotzky, 1987)
↑ antibody production (Chicken, Horse, Pig, Rat: Finch & Turner, 1996) (Sheep: Rooke *et al.,* 2004)
↑ immunoglobulin production (Rat: Kubena & McMurray, 1996), (Pig: Chew, 1996; Finch & Turner, 1996), (Lamb: Finch & Turner, 1996)
↑ humoral response (Horse, Lamb: Finch & Turner, 1996), (Sheep: Rooke *et al.,* 2004)
↑ overall immune function (Chicken: Kubena & McMurray, 1996)

Key: ↑ Increased; ↓ Decreased

While benefits of selenium and vitamin E supplementation have been found in some species, in others there appears to be little effect. For example, while there was a

positive synergistic effect of selenium and vitamin E supplementation on antibody production in horses (Baalsrud & Overnes, 1986) and pigs (Peplowski *et al,* 1980), no benefit was seen in studies of ruminant species (Larsen *et al,* 1988a; Droke & Loerch, 1989). It is suggested that differences in vitamin E and selenium metabolism by ruminant and monogastric species could account for the differing effects of these nutrients on immune enhancement (Finch & Turner, 1996). It is important to note, however, that the design of an experiment can affect the results obtained and make comparisons of studies both within and between species very difficult. Various factors such as species, age, sex, initial nutrient status, the immune parameter being studied and the chemical form of the supplement, can all affect the response to vitamin E and selenium supplementation and the dosage required. This is highlighted by the often conflicting results found in various scientific studies (Finch & Turner, 1996; Rooke *et al.,* 2004). The health of the species being tested must also be taken into account when evaluating the benefits of supplementation. For example, while immuno-compromised individuals, such as the aged, may benefit from supplementation, a healthy population may show no improvement (Calder & Kew, 2002). The safety of combined supplementation of these nutrients must also be considered. *In vitro* addition of high levels of vitamin E and selenium to lymphocytes taken from mice and production animals were toxic to these cells, suggesting that excessive supplementation may be toxic *in vivo* (Finch & Turner, 1996).

No studies have been carried out looking at the effects of a combination of vitamin E and selenium on immune function in the cat or dog. These studies must be undertaken to determine if vitamin E and selenium supplementation can enhance immune function in both of these species.

1.10 Safety and Efficacy of Nutrient Supplementation

Due to pet owners becoming more concerned about, both their own, and their pet's, health, the demand for nutraceuticals and functional foods for companion animals is expected to continue to increase (Rutherfurd-Markwick & Hendriks, 2003). To ensure that these demands can be met and the safety of animals ensured, scientific testing needs to be carried out to determine whether particular nutrients have beneficial effects in companion animals (Calder & Kew, 2002; Rutherfurd-Markwick & Hendriks, 2003;

Bontempo, 2005). Little research has been conducted to determine the bioavailability, nutrient interactions and the requirements for individual nutrients in companion animal diets (Bontempo, 2005). Species differences in metabolism and physiology make it important that a supplement is tested in the species in question, since a compound that is beneficial in one species could be harmful or toxic to another (Calder & Kew, 2002; Rutherfurd-Markwick & Hendriks, 2003).

An excess of one nutrient may have an inhibitory effect on the actions of other nutrients essential for immune function (Kubena & McMurray, 1996; Calder & Kew, 2002), while others may complement each other. A particular nutrient may also improve the function of one immune cell type, but may at the same time inhibit the actions of another at the same dosage (Calder & Kew, 2002). It is also important to remember that an increase in immune cell numbers or particular cellular activities following dietary supplementation does not necessarily mean that the host will be less susceptible to disease (Calder & Kew, 2002; Rutherfurd-Markwick & Hendriks, 2003). However, the additional studies required to determine this are often difficult to perform, require a large amount of time, money and subjects (Rutherfurd-Markwick & Hendriks, 2003), and often have a high ethical cost, hence the reliance on assessing the impact on specific immune assays (K. Rutherfurd-Markwick, Personal Communication, May 11, 2010).

1.11 Summary

Vitamin E and selenium supplementation have been reported to enhance immune function in various animal species and in humans. The antioxidant effects of vitamin E and selenium in the cell membrane and control of the production of prostaglandin E_2 are thought to be the major mechanisms by which vitamin E and selenium enhance immune function (Chew, 1996; Kubena & McMurray, 1996). There have been few studies on the effects of supplemental vitamin E on immune function in the cat. While vitamin E supplementation enhanced some immune function parameters in older cats in one study, little benefit has been seen in young, healthy cats (Hayek *et al.,* 2000). No studies have looked at the effects of selenium or the combined supplementation of vitamin E and selenium on immune function in the cat. Studies are required to determine if vitamin E and selenium supplementation can provide the same positive effects on immune function in healthy adult cats as has been reported in other species.

Vitamin E supplementation at a level of around 4-10 fold greater than the minimum dietary requirement has been found to be the appropriate level of supplementation to enhance immune function in many species (Hendriks *et al.,* 2002; Rutherfurd-Markwick & Hendriks, 2003). Aged cats were reported to have an enhanced response to T and B cell mitogens when consuming a diet containing a 250 IU/kg DM vitamin E supplement, around eight times the minimum dietary requirement of 30 IU/kg DM. The consumption of a diet containing 500 IU/kg DM (over 16 times the minimum requirement) did not further enhance immune function in aged cats. In young animals, little benefit was seen at either level of supplementation (Hayek *et al.,* 2000).

Studies of the effects of selenium on immune function have found enhanced immune activity over a range of levels from 0.5 - 5 mg/kg diet (Kiremidjian-Schumacher & Stotzky, 1987). High levels of selenium in the diet have been found to be toxic in many species, however the cat does not seem to be as susceptible (Simcock *et al.,* 2004). Combined vitamin E and selenium supplementation studies have found a possible synergistic effect of these nutrients on immune function in some species, although many studies using combined supplements have used animals deficient in one or both nutrients, which can influence the outcome of these studies (Kubena & McMurray, 1996). Studies of combined vitamin E and selenium supplementation in healthy animals with an adequate nutrient status prior to the study have found some enhancement of immune function, although not as great as that seen in deficient animals (Finch & Turner, 1996). When comparing results between studies it is also important take into account confounding factors, such as the age, sex, health and species of subjects, which can affect the outcome of a study (Finch & Turner, 1996).

1.12 Aims of Research

The aim of this study is to determine what, if any, effect there is of vitamin E and selenium supplementation on immune parameter function in the cat. It is hypothesised that vitamin E supplementation may have little effect on immune parameter function; however combined supplementation of vitamin E and selenium could result in enhancement of the immune parameters being tested. The results of this study will add to the current literature on the effects of vitamin E supplementation on immune parameters and provide information on some parameters that have not yet been tested.

Since selenium and combined supplementation of vitamin E and selenium have not been studied in the cat previously, the results of this study will provide new information that may form the basis for further study on this topic.

In the present study, two supplemental levels of vitamin E of 250 IU/kg DM and 500 IU/ kg DM will be added to a diet containing 68.2 IU/kg DM vitamin E, to give final concentrations of 318.2 IU/kg DM and 568.2 IU/kg DM. This allows direct comparison with the study undertaken by Hayek *et al.* (2000), which is the only similar feline study available. Due to the low toxicity of vitamin E (Bendich & Machlin, 1988), the levels of supplementation used in this study are considered to be safe. Selenium will be supplemented to a diet containing 0.38 mg/kg DM selenium, at either 2 mg/kg DM or 10 mg/kg DM, to give final concentrations of 2.38 mg/kg DM and 10.38 mg/kg DM. The ability of cats to tolerate high levels of selenium in the diet (Simcock *et al.,* 2004) allows the higher level of supplementation to be tested in this species, while the lower level is similar to that used in other studies, so will allow comparisons to be made to them. In addition to the four test diets containing a supplement of vitamin E or selenium, four diets will assess the effect of combined supplementation of vitamin E and selenium using all possible combinations of the levels stated above.

2. Materials and Methods

2.1 Animals

All procedures involving animals in this study were approved by the Massey University Animal Ethics Committee (Ethics number 06/74). Seventy-two healthy male and female short haired, domestic cats, ranging in age from 1.6 – 12.8 years, were divided into nine groups of eight cats and housed in colony cages at the Centre for Feline Nutrition, Massey University, Palmerston North, New Zealand. There were 40 neutered and 32 unneutered animals in the study. Food and water was provided *ad libitum.* The cats weighed between 2.36 to 6.86 kg at the beginning of the trial and weight was monitored weekly for the duration of the four week trial. Whole blood and saliva samples were taken from each cat at the beginning of the trial and at two and four weeks. Blood samples (2-3 ml) were taken by jugular venipuncture and collected in sterile tubes coated with lithium heparin (Becton Dickinson Instruments, Cambridge, MA, USA). Figure 2.1 outlines the design of the trial. During the one week acclimatisation period all animals were fed the basal diet, after which they were fed one of nine test diets containing supplemental vitamin E and/ or selenium for four weeks, excepting the control group, which remained on the basal diet for the duration of the trial.

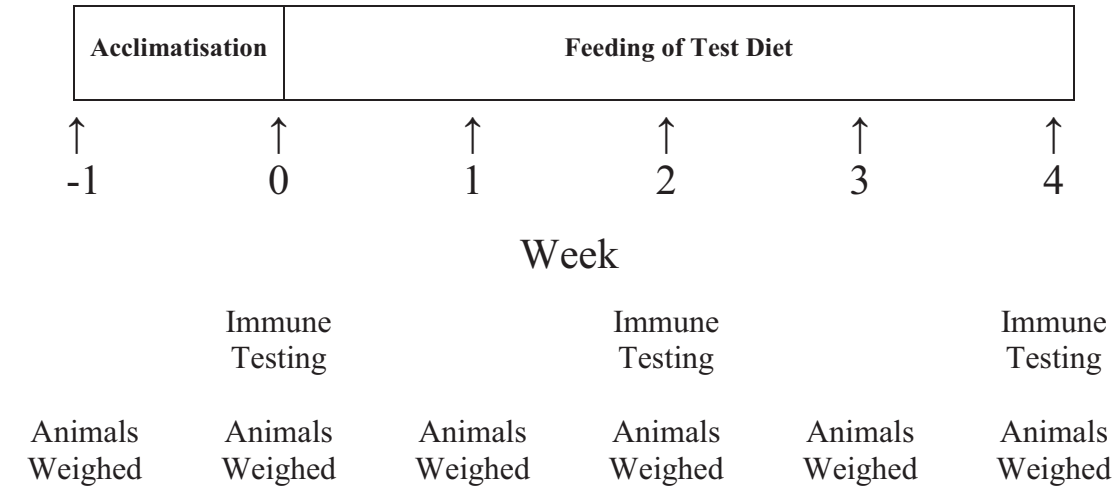


Figure 2.1: Experimental trial design

The test diets include 2 vitamin E supplemented diets at moderate (250 IU/kg DM) and high (500 IU/kg DM) levels and 2 selenium supplemented diets at moderate (2 mg/kg DM) and high (10 mg/kg DM) levels. Four mixed vitamin E and selenium supplement diets will also be tested, containing all possible combinations of the doses above. This follows a 3x3 factorial statistical model with the fixed effects of vitamin E, selenium and week. The factorial design of the trial is shown in Figure 2.2.

		Selenium	
	0	M	H
Vitamin E	M	MM	MH
	H	HM	HH

Key: 0= Control; M= Moderate; H= High. Vitamin E supplement; Selenium supplement

Figure 2.2: Factorial design of trial

2.2 Limitations of Study

The study was restricted to a period of 4 weeks, which limits the ability to determine the long term effects of supplementation. The animals were grouped according to their normal housing arrangements in the colony, resulting in variations in age and sex between groups. Due to the complexity of the factorial design, it was not possible to extend the trial to perform a cross over experiment where the animals were assigned to a different diet half way through the trial to account for individual group variations in response to supplementation.

2.3 Diets

Vitamin E (Lutavit E50, lot number 00504409TO) and selenium (Sel-plex, batch number: 225333) were obtained from BASF, Manukau City, Auckland, NZ and Alltech Inc, Nicholasville, KY, USA, respectively. Nine test diets were used, four containing two levels of vitamin E or selenium, four containing different combinations of vitamin E and selenium, and a control diet. The base diet contained 68.2 IU/kg DM vitamin E and 0.38 mg/kg DM selenium. The amount of supplemental vitamin E and/ or selenium added to each diet is outlined in Table 2.1. Each test diet was prepared daily by mixing

the powder supplements with a canned commercial jellimeat cat food in an electric mixer for around 10 seconds and then fed to each group of cats. Sufficient test diet was prepared daily to ensure that the diets were fed *ad libitum*, and food trays were weighed daily to monitor the amount of food left each day. The amount of food offered was altered as required.

Table 2.1: Supplemental levels (wt/kg DM) of vitamin E and/ or selenium added to diets

Group	Supplemental Vitamin E	Supplemental Selenium	Group symbols
C	-	-	C
MVitE	250 IU	-	↑E
HVitE	500 IU	-	↑↑E
MSe	-	2 mg	↑Se
HSe	-	10 mg	↑↑Se
MVitE-MSe	250 IU	2 mg	↑E, ↑Se
MVitE-HSe	250 IU	10 mg	↑E, ↑↑Se
HVitE-MSe	500 IU	2 mg	↑↑E, ↑Se
HVitE-HSe	500 IU	10 mg	↑↑E, ↑↑Se

Key: C= Control; HVItE= High vitamin E; HSe= High selenium; MVitE= Moderate vitamin E; MSe= Moderate selenium.

2.4 Immunophenotyping

The level of expression of $CD4^+$ (T helper cell), $CD8^+$ (cytotoxic T cell), B cell and $CD14^+$ (monocyte) antigens on peripheral blood leucocytes and granulocytes was analysed by flow cytometry. Antibodies (Serotec, Raleigh, NC, USA) were either feline-specific (CD4, CD8) or canine-specific (B cells, CD11b) (canine-specific antibodies had been shown to cross react with the relevant feline antigen by the supplier). 5µl of appropriate antibody was added to 100 µl of whole heparinised blood in a 2 ml eppendorf tube. The tube was mixed and then incubated in the dark at room temperature for 20 minutes. 1 ml of PBS was then added and the tube was mixed and spun in a centrifuge at 358 x g for 10 minutes. The supernatant was discarded and the cells fixed by adding 100 µl of 8% formaldehyde, incubating for 1 minute and adding 1 ml ACK lysis buffer. This was incubated for 10 minutes then centrifuged at 358 x g for 10 minutes. The pellet was washed with 1 ml PBS then underwent centrifugation at 358

x g for 10 minutes. The pellet was re-suspended in 0.5 ml PBS, and the samples transferred to FACS tubes and analysed by flow cytometry using a FACS-Calibur flow cytometer (Becton Dickinson Instruments, Cambridge, MA, USA). Data from each sample was collected for 10 000 gated events.

2.5 Lymphocyte Proliferation

A whole blood cell proliferation assay was modified from previously published methods (Saker *et al*., 2001). Lithium heparin-treated peripheral whole blood was diluted 1:4 in complete RPMI-1640 medium (supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin sulphate and 50 μM 2-mercaptoethanol (all reagents from Gibco, Poole, UK). 100 μl of the diluted blood was added in quadruplicate to the wells of a 96-well, flat-bottomed tissue culture plate (Greiner, Frickenhausen, Germany), and cultured in the presence of either 5 μg/ml concanavalin A (Sigma, St Louis, USA), 1:49 diluted phytohaemagglutinin (Gibco, Poole, UK), or complete RPMI-1640 substituted for the mitogen in the control wells. The cells were cultured for 48 hours at 37°C in a 5% humidity, CO_2-air atmosphere, before being pulsed for 18 hours with 0.5 μCi methyl-^{3}H-thymidine (Amersham Biosciences, Little Chalfont, UK) per well. Each plate was harvested onto a 96-well glass fibre mat using a cell harvester (Tomtek, Orange, CT, USA) and counted using a Wallac-Trilux 1450 Microbeta liquid scintillation and luminescence counter (Boston, MA, USA). Stimulation index was calculated as counts per minute (CPM) in wells with mitogen divided by CPM in wells without mitogen.

2.6 Phagocytosis

The phagocytic capacity of peripheral blood leucocytes was analysed by flow cytometry. 5 μl of FITC-labelled *Escherichia coli* bacteria (1 x 10^9/ml) (Molecular Probes Inc, Eugene, OR, USA) was mixed with 100 μl of whole blood and incubated for 30 minutes at 37ºC. Several controls were also prepared: one containing blood and no *E. coli* was processed according to the remainder of the protocol, and for the others blood was incubated in the absence of *E. coli*, fixed with 100 μl of 8% para-formaldehyde, H_2O was added , and then 5 μl of *E. coli* was added. Immediately following incubation, the cells were fixed with 100 μl of 8% para-formaldehyde, and the erythrocytes lysed by the addition of 1ml of ice-cold water. Samples were left for

10 minutes, centrifuged (2700 x g) and the pellet was re-suspended in 500 µl of phosphate buffered saline, and 50 µl of 4% Trypan blue added to quench extraneous fluorescence. The phagocytic activity was determined using a FACS-Calibur flow cytometer (Becton Dickinson Instruments, Cambridge, MA, USA).

2.7 Immunoglobulin G ELISA

An enzyme linked immunosorbent assay (ELISA) was used to measure the IgG concentration in heparinised cat plasma samples. A 96-well plate was coated overnight at 4°C with 100 µl per well of mouse anti-feline IgG (MCA 624; Serotec, Raleigh, NC, USA) (1/500 dilution in carbonate coating buffer). Unbound sites were blocked by adding 200 µl of blocking buffer per well (1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)), and incubating for 60 minutes at 37°C. The plate was washed three times with buffer (PBS + 0.05% Tween 20). Diluted sample or standard aliquots of 100 µl per well were added in triplicate to the plate and incubated for 90 minutes at 37°C. The plate was then incubated for 60 minutes at 37°C with 100 µl/well of secondary antibody peroxidase-conjugated affinity purified goat anti-feline IgG (H+L) (Jackson ImmunoResearch laboratories, West Grove, PA, USA). The plate was washed three times with buffer (PBS + 0.05% Tween 20) after each incubation. 100 µl TMB (3, 3', 5, 5;-TetraMethylBenzidine) (Zymed Laboratories Inc, South San Francisco, CA, USA) was then added to each well, and the colour allowed to develop for 20 minutes. The reaction was then stopped by adding 50 µl per well of 1M H_2SO_4, and the absorbance measured at 450 nm (Wallac 1420 Multilabel Counter, Turku, Finland).

2.8 Prostaglandin E_2 ELISA

200 µl blood samples (diluted as for lymphocyte proliferation) were prepared in duplicate, either stimulated or un-stimulated with concanavalin A (100 µl) and incubated for 48 hours at 37 ºC. After being centrifuged at 358 x g for 10 minutes the supernatants were transferred to a 96 well plate and stored at -20ºC before analysis. The prostaglandin E_2 concentration of feline plasma was measured using a commercially available competitive ELISA kit (R & D Systems, Minneapolis, MN, USA). Samples and reagents were prepared and added to 96 well microplates that were pre-coated with goat anti-mouse polyclonal antibody, as directed. 50 µl of primary antibody solution

was added to each well, followed by 50 µl of prostaglandin E_2 conjugate. The plates were covered and incubated for 16-20 hours at 2-8˚C. The plates were then washed four times in an automatic plate washer with wash buffer (PBS concentrate provided in kit). 200 µl of substrate solution was added to each well and the plates were covered and incubated at room temperature for 20 minutes. 50 µl of stop solution was then added to each well and the plates were read at 450 nm using a Wallac 1420 Multilabel Counter (Turku, Finland).

2.9 Data Analysis

The results were analysed by a mixed procedure repeated measures analysis in the SAS program (SAS, 1999). The model included the fixed effects of selenium, vitamin E and week and their interaction and cat nested within selenium x vitamin E combination as a random effect. Significance was set at $p < 0.05$. Results are presented as mean ± SEM, unless otherwise stated.

3. Results

3.1 Animal Age and Body Weight

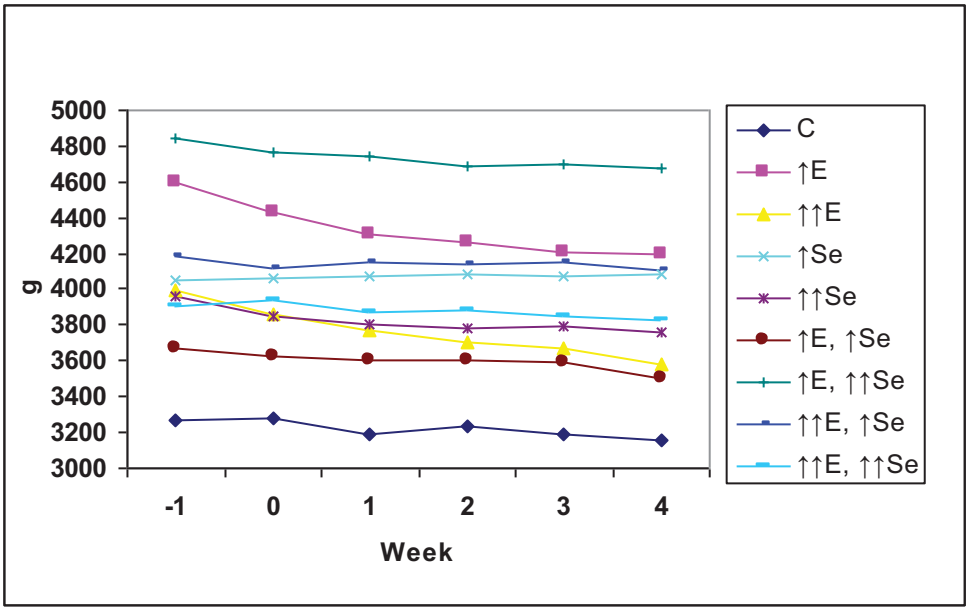
There were 72 cats in this trial; 39 males and 33 females. The average age of the cats was 4.71 (± 0.14) years. Table 3.1 shows the average age and sex distribution of the cats in each group.

Table 3.1: Average age (years) (± SEM) and number of male and female cats in each dietary group

	Diet								
	C	**↑E**	**↑↑E**	**↑Se**	**↑↑Se**	**↑E, ↑Se**	**↑E, ↑↑Se**	**↑↑E, ↑Se**	**↑↑E, ↑↑Se**
Average	8.48 ± 0.83	3.31 ± 0.52	2.83 ± 0.43	2.94 ± 0.51	6.33 ± 1.72	3.31 ± 0.67	4.90 ± 0.80	5.10 ± 0.91	5.22 ± 1.24
Male	1	4	3	6	7	4	8	5	1
Female	7	4	5	2	1	4	0	3	7

Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) (n=8)

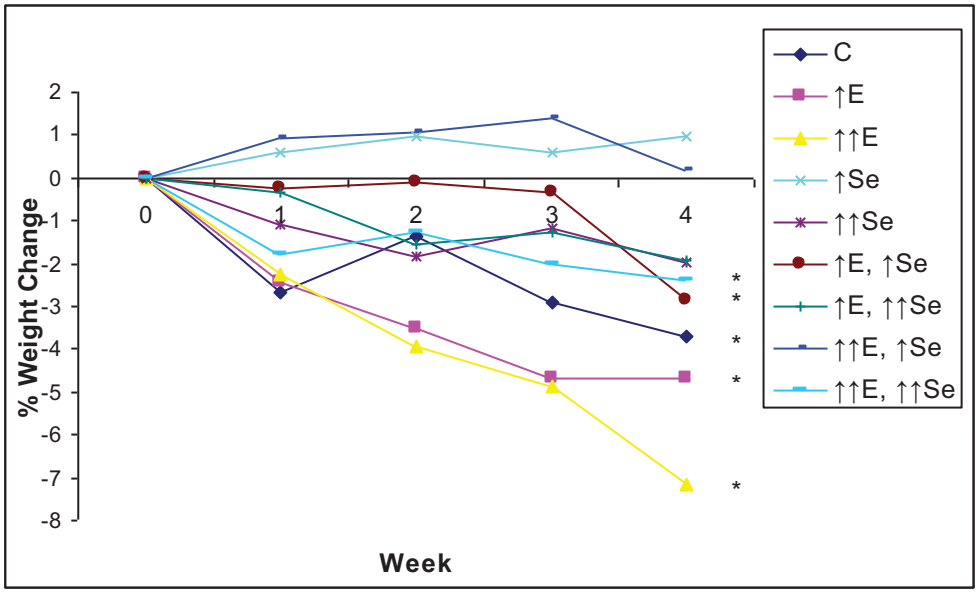
The average age of cats in the control group was significantly higher (p=0.001) than that of the MVitE, HVitE, MSe and MVitE+MSe groups. There were no other significant differences in age between groups. There was a large variation in the average body weight of each group, partially due to the number of male and female animals in each group, with males being on average 1264 g heavier than females (females 3267 g ± 20.4; males 4531 g ± 29.5). There was also a greater variation in the body weights of males, as compared to females. The group with the highest average body weight (4737 g ± 25.8) in the trial was the MVitE+HSe group, in which all the cats were males, while the group with the lowest average body weight (3218 g ± 19.6) during the trial was the control group, with seven females and one male. The average total body weight of each group prior to, and during the trial is shown in Figure 3.1.



Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) (n=8)

Figure 3.1 Average body weight (g) of cats fed one of nine diets containing supplemental levels of vitamin E and/ or selenium before and during trial

To adjust for the different sex ratios between groups, the changes in body weight are expressed as percentage change from week 0 to give a clearer view of the changes that occurred over time. Figure 3.2 shows the average percentage body weight change of cats consuming each of the nine dietary regimes. There was a reduction in body weight in all groups apart from the MSe and HVitE+MSe groups during the trial. Overall, there was a very strong effect of week ($p<0.0001$) on the weight of cats. There was also a strong effect of the interaction of selenium x week ($p=0.0004$) on body weight. The statistics summary table for body weight, and tables of all results, are given in Appendix 1. There was a significant reduction in body weight in five groups during the trial; the control group between weeks 0 and 4 ($p=0.0165$); the MVitE group between weeks 0 and 2 ($p=0.0007$) and weeks 0 and 4 ($p<0.0001$); the HVitE group between weeks 0 and 2 ($p=0.0018$), weeks 2 and 4 ($p=0.0157$) and weeks 0 and 4 ($p<0.0001$); the MVitE+MSe group between weeks 0 and 4 ($p=0.0191$); and the HVitE+HSe group between weeks 0 and 4 ($p=0.0369$).



*Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) *= Significant change from week 0 ($p<0.05$) (n=8 per group)*

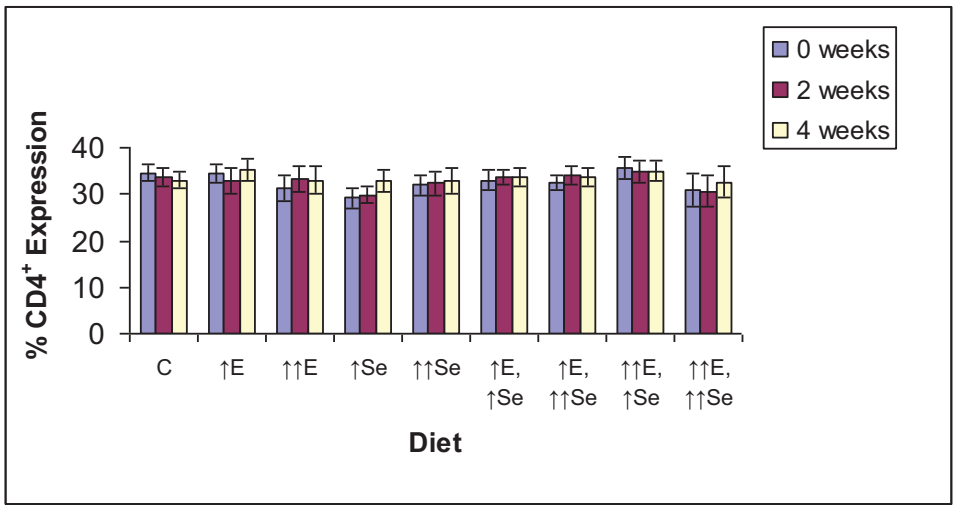
Figure 3.2: Average change in body weight (%) over time of cats fed one of nine diets containing supplemental levels of vitamin E and/ or selenium before and during trial

In the HVitE+HSe group and the MVitE+MSe group, the significant reduction in group weight (-2.37% and -2.85% respectively), during the trial is primarily due to a large reduction in the weight of male animals (HVitE+HSe: -5.08%; MVitE+MSe: -3.88%). There was less change in the body weight of females in these groups (HVitE+HSe: -1.99%; MVitE+MSe: -1.83%). There was a large reduction in the weights of both male and female cats in the HVitE (-7.15%) and MVitE (-4.67%) groups. These two groups experienced the greatest decline in body weight during the trial and were also noted to have the lowest food intakes overall.

3.2 Immune Cell Phenotypes

3.2.1 $CD4^+$ Cell Marker:

The levels of expression of the T helper cell marker $CD4^+$ in the blood of cats fed a diet containing the nine different dietary regimes are shown in Figure 3.3. Statistical analysis indicated a significant effect of week ($p=0.0283$) and the interaction of selenium x vitamin E x week ($p=0.0161$) on $CD4^+$ cell expression. Overall, there was little change in the expression of the $CD4^+$ cell marker in response to vitamin E or selenium supplementation over the duration of the trial and no differences between groups ($p>0.05$).

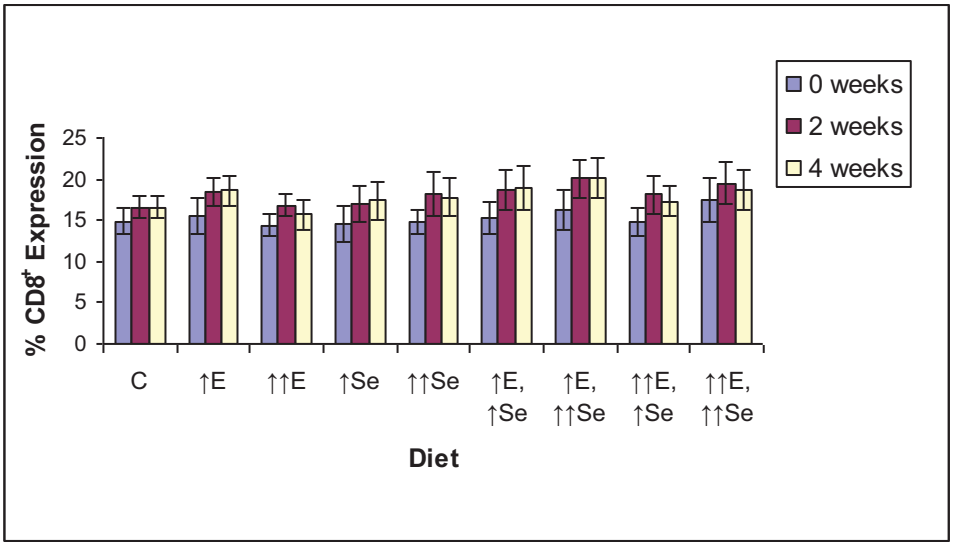


Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) (n=8)

Figure 3.3: $CD4^+$ cell expression (%) in blood (± SEM) collected from cats fed one of nine diets containing supplemental levels of vitamin E and/ or selenium over a four week period

3.2.2 $CD8^+$ Cell Marker:

The levels of expression of the cytotoxic T cell marker $CD8^+$ in the blood of cats fed a diet containing the nine dietary regimes are shown in Figure 3.4. There was a significant effect of week ($p<0.0001$) on the expression of the $CD8^+$ cell marker, with the level of expression of the $CD8^+$ cell marker significantly greater after weeks 2 and 4 of the trial, compared to week 0, in all groups. However, there were no significant differences in the level of expression of the $CD8^+$ marker between groups at any time during the trial ($p>0.05$).



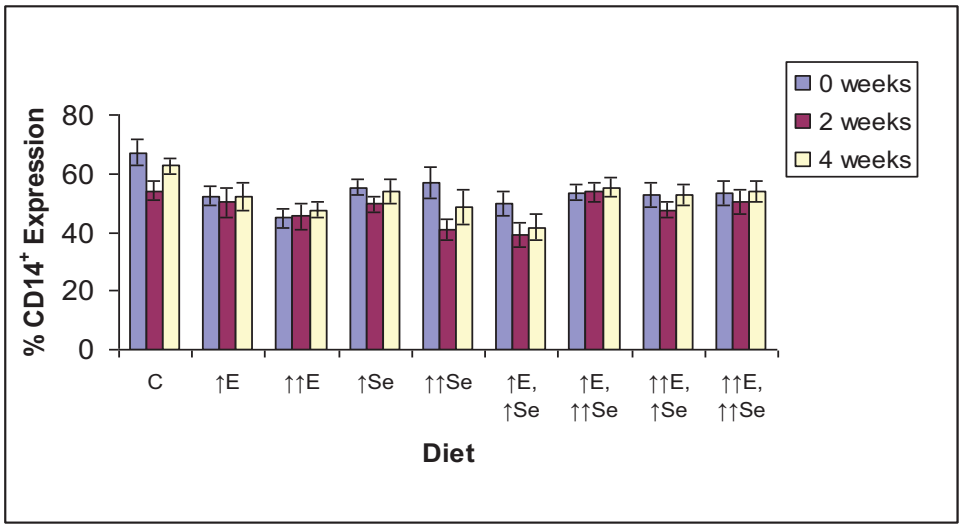
Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) (n=8)

Figure 3.4: $CD8^+$ cell expression (%) in blood (± SEM) collected from cats fed one of nine diets containing supplemental levels of vitamin E and/ or selenium over a four week period

3.2.3 $CD14^+$ Cell Marker:

The levels of expression of the monocyte cell marker $CD14^+$ in the blood of cats consuming a diet containing the nine dietary regimes are shown in Figure 3.5. In the control group there was a reduction in the expression of the $CD14^+$ cell marker between weeks 0 and 2 ($p=0.0013$), however expression increased between weeks 2 and 4 ($p=0.0338$). The level of $CD14^+$ cell marker expression declined in the MVitE+MSe group between weeks 0 and 2 ($p=0.0102$), and although expression increased after 4 weeks supplementation (week 2 and week 4 $p=0.5616$), it was still significantly lower than in week 0 ($p=0.0447$). $CD14^+$ cell marker expression was numerically higher in the control group compared to all other groups during the trial. At time 0, $CD14^+$ cell

marker expression was significantly greater in the control group than in the MVitE ($p=0.0081$); HVitE ($p<0.0001$); MSe ($p=0.0319$); MVitE+MSe ($p=0.0018$); MVitE+HSe ($p=0.0149$); HVitE+MSe ($p=0.0093$); and HVitE+HSe groups ($p=0.0135$). Expression of the $CD14^+$ cell marker was highly variable within most of the groups, including in the control group. There was a significant effect of week ($p<0.0001$) on the expression of the $CD14^+$ cell marker, indicating that the expression of the $CD14^+$ cell marker was affected by time. This was most clearly seen in the reduction in expression of the cell marker at week 2 of the trial in the majority of groups. There was also a significant interaction of selenium x vitamin E ($p=0.0089$), however the effect of supplementation on this parameter does not appear to be great, and is probably not biologically significant.

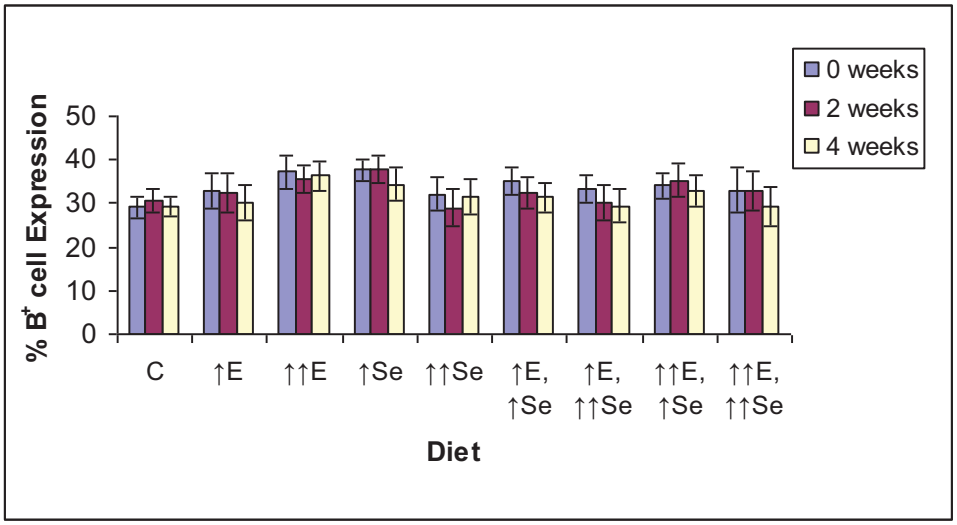


Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) (n=8)

Figure 3.5: $CD14^+$ cell expression (%) in blood (± SEM) collected from cats fed one of nine diets containing supplemental levels of vitamin E and/ or selenium over a four week period

3.2.4 B^+ Cell Marker:

Figure 3.6 shows B^+ cell expression in the blood of cats fed each of the nine dietary regimes. There was a general downward trend during the period of supplementation in the level of expression of B^+ cells in the majority of groups in the trial, reflected in the significant effect of week ($p=0.0011$) on these results. Overall, there was no significant effect of vitamin E or selenium on B^+ cell expression ($p>0.05$).



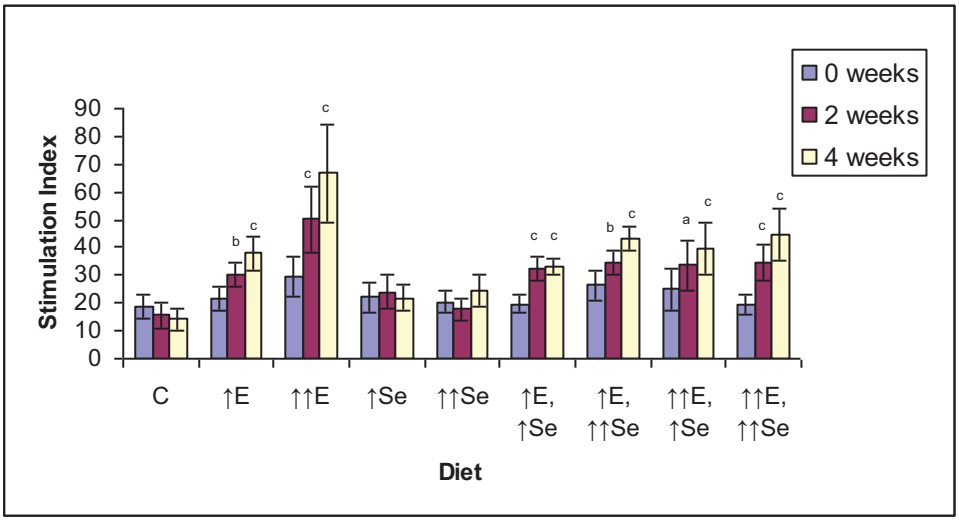
Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) (n=8)

Figure 3.6: B^+ cell expression (%) in blood (± SEM) collected from cats fed one of nine diets containing supplemental levels of vitamin E and/ or selenium over a four week period

3.3 Lymphocyte Proliferation

3.3.1 Concanavalin A:

The proliferative responses of lymphocytes to the T cell mitogen Concanavalin A (Con A) in cats fed diets containing the nine different dietary regimes are shown in Figure 3.7. There was a significant effect of week ($p<0.0001$). There was also a significant effect of vitamin E ($p=0.0017$) and the interaction of vitamin E x week ($p<0.0001$) on the lymphocyte proliferative response to Con A, indicating a strong effect of vitamin E supplementation over time. The initial response of lymphocytes to Con A (week 0) were similar across all nine groups.



Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$ *(n=8)*

Figure 3.7: Lymphocyte proliferative responses to Concanavalin A (Con A) in blood (± SEM) collected from cats fed one of nine diets containing supplemental levels of vitamin E and/ or selenium over a four week period

Table 3.2 summarises the results from the groups where there was a significant increase in the proliferative response of lymphocytes to Con A observed during the trial. In the MVitE group, there was an increase in lymphocyte proliferation between weeks 0 and 2 ($p=0.0034$) and weeks 0 and 4 ($p<0.0001$). Proliferation also rose numerically between weeks 2 and 4, however this was not statistically significant ($p=0.1041$). Similarly, in the HVitE group, the lymphocyte proliferative response increased over time, with significant differences between weeks 0 and 2 ($p=0.0003$) and weeks 0 and 4 ($p<0.0001$), but again not between weeks 2 and 4 ($p=0.0966$). While there were large numerical differences between these groups, there were also high degrees of variability

and thus no significant differences were found between the MVitE and HVitE groups in this trial. There were no significant changes in lymphocyte proliferative responses to Con A in the MSe or HSe groups during the trial.

Table 3.2: Vitamin E supplemented groups in which the lymphocyte proliferative response to Concanavalin A (Con A) (± SEM) increased significantly during a 4 week trial

	Diet					
Week of Trial	**↑E**	**↑↑E**	**↑E, ↑Se**	**↑E, ↑↑Se**	**↑↑E, ↑Se**	**↑↑E, ↑↑Se**
0	21.29 ± 4.36	29.45 ± 7.35	19.67 ± 3.42	26.56 ± 5.40	24.87 ± 7.65	19.58 ± 3.60
2	30.25[b] ± 4.67	50.05[c] ± 11.67	32.54[c] ± 4.29	34.75[b] ± 4.33	33.49[a] ± 9.02	34.56[c] ± 6.44
4	38.06[c] ± 6.14	66.68[c] ± 17.54	33.08[c] ± 3.10	43.14[c] ± 4.30	39.56[c] ± 9.08	44.50[c] ± 9.54

Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) [a]*p<0.05;* [b]*p<0.01;* [c]*p<0.001 (n=8)*

In the MVitE+MSe group, lymphocyte proliferation rose significantly in response to Con A between weeks 0 and 2 ($p<0.0001$). This level was maintained to the end of the trial (weeks 0 and 4 $p<0.0001$) with no significant increase occurring between weeks 2 and 4. The proliferative response also increased between weeks 0 and 2 ($p=0.0034$) and weeks 0 and 4 ($p<0.0001$) in the MVitE+HSe group, again with no significant change between weeks 2 and 4 ($p=0.1003$). In the HVitE+MSe group there was a significant rise in proliferation noted between weeks 0 and 2 ($p=0.0479$), weeks 2 and 4 ($p=0.0373$), and weeks 0 and 4 ($p<0.0001$). The lymphocyte proliferative response to Con A increased over the trial period in the HVitE+HSe group, with highly significant differences between weeks 0 and 2 ($p=0.0003$) and weeks 0 and 4 ($p<0.0001$), but no significant increase between weeks 2 and 4 ($p=0.1287$). Lymphocyte proliferation to Con A did not vary significantly between the four combined supplement groups during the trial.

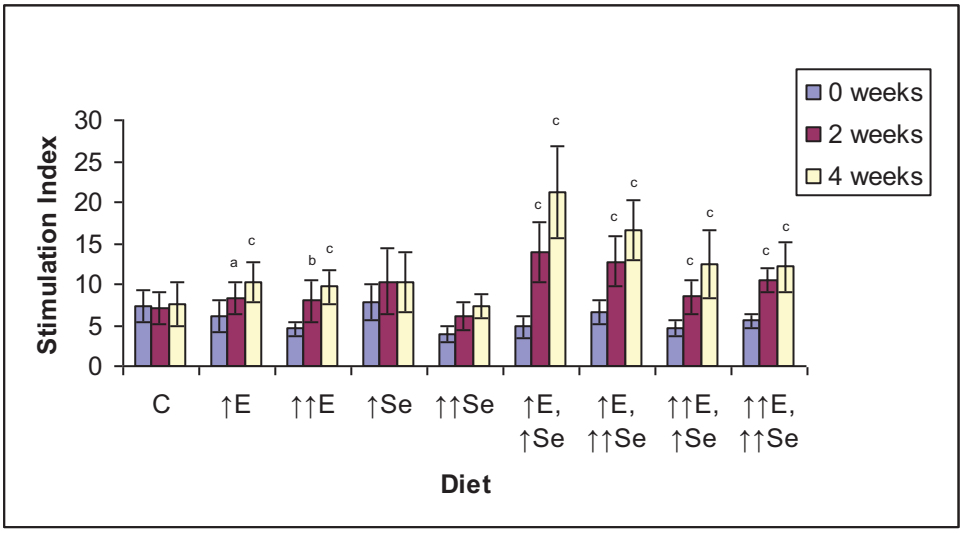
In terms of vitamin E dosage, there appeared to be little difference in response between a high or moderate level of supplementation, as there were no significant differences between these groups during the trial. The rate of response to supplementation was similar in the majority of groups, with increases occurring between weeks 0 and 2 but

little further change between weeks 2 and 4. Only the HVitE+MSe group experienced a significant increase in response to supplementation between weeks 2 and 4 of the trial.

There was a small but significant reduction in the proliferative response of lymphocytes to Con A in the control group between weeks 0 and 4 (p=0.0256). This was the only group in which there was a significant reduction in lymphocyte proliferation during the trial.

3.3.2. Phytohaemagglutinin:

The results of the analysis of lymphocyte proliferative responses to the T cell mitogen phytohaemagglutinin (PHA) in cats fed the nine dietary regimes are shown in Figure 3.8. The effect of time on proliferation is evidenced by the significant effect of week ($p<0.0001$) on these results, with increased proliferation in many groups after weeks 2 and 4 of the trial. There was also a significant effect of vitamin E x week (p=0.0001), which indicates a strong effect of supplementation of this nutrient over time in this trial. The initial responses of lymphocytes to PHA (week 0) were similar across all nine groups.



Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$ *(n=8)*

Figure 3.8: Lymphocyte proliferative responses to phytohaemagglutinin (PHA) in blood (± SEM) collected from cats fed one of nine diets containing supplemental levels of vitamin E and/ or selenium over a four week period

The proliferation of lymphocytes in response to PHA increased between weeks 0 and 2 (p=0.0144) and weeks 0 and 4 (p=0.0005) in the MVitE group, with no significant

increase observed between weeks 2 and 4 (p=0.2798). Similarly, in the HVitE group, the proliferative response increased between weeks 0 and 2 (p=0.0060) and weeks 0 and 4 (p<0.0001), but not between weeks 2 and 4 (p=0.1695). There were no significant differences in the values obtained at weeks, 0, 2 and 4 between the two groups. While the proliferative responses of lymphocytes to PHA increased in the MSe and HSe groups during the trial, this was not significant in either group.

In the MVitE+MSe group, the lymphocyte proliferative response increased in each consecutive sampling period with significant differences between weeks 0 and 2 (p<0.0001), weeks 2 and 4 (p=0.0461) and weeks 0 and 4 (p<0.0001). This group also had the greatest increase in the proliferative response of lymphocytes to PHA in the study. The lymphocyte proliferative response to PHA also increased in the MVitE+HSe group between weeks 0 and 2 (p=0.0003) and weeks 0 and 4 (p<0.0001). However, although the level of proliferation increased between weeks 2 and 4, this was not significant (p=0.1055). The response to PHA also increased in the HVitE+MSe group between weeks 0 and 2 (p=0.0005) and weeks 0 and 4 (p<0.0001), with a non-significant rise occurring between weeks 2 and 4 (p=0.2386). In the HVitE+HSe group, there was an increase in proliferation between weeks 0 and 2 (p=0.0003) and weeks 0 and 4 (p<0.0001), but again the increase between weeks 2 and 4 was not significant (p=0.6442). When the performance of the four combined supplement groups were compared, no significant differences in response to PHA were observed between them during the trial. The results from groups in which there was an increase in the lymphocyte proliferative response to PHA are shown in Table 3.3.

Table 3.3: Vitamin E supplemented groups in which the lymphocyte proliferative response to phytohaemagglutinin (PHA) (± SEM) increased significantly during a 4 week trial

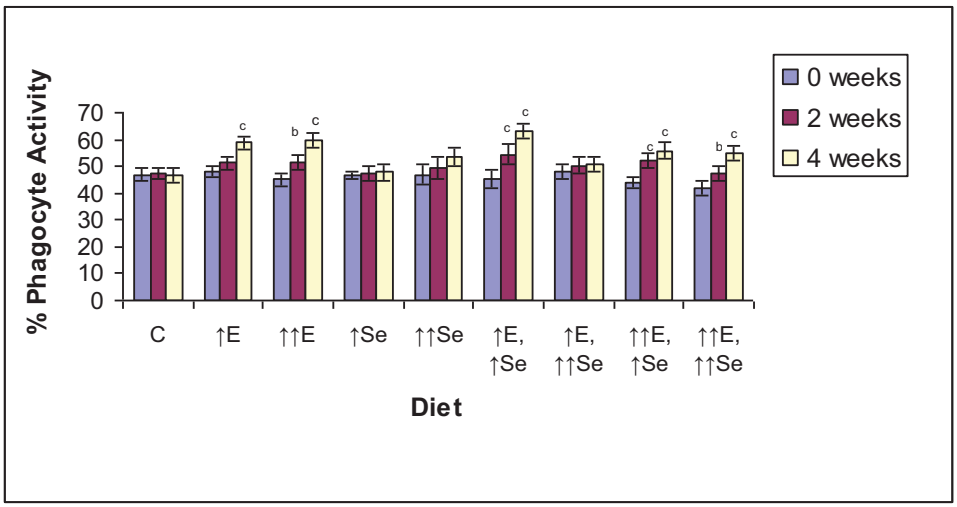
	Diet					
Week of Trial	**↑E**	**↑↑E**	**↑E, ↑Se**	**↑E, ↑↑Se**	**↑↑E, ↑Se**	**↑↑E, ↑↑Se**
0	6.20 ± 2.00	4.60 ± 0.90	4.78 ± 1.33	6.61 ± 1.40	4.70 ± 1.00	5.50 ± 0.90
2	8.24[a] ± 2.00	7.91[b] ± 2.60	13.91[c] ± 3.60	12.80[c] ± 3.10	8.51[c] ± 2.10	10.42[c] ± 1.42
4	10.25[c] ± 2.50	9.70[c] ± 2.10	21.20[c] ± 5.54	16.51[c] ± 3.62	12.45[c] ± 4.80	12.12[c] ± 3.01

Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) [a]p<0.05, [b]p<0.01, [c]p<0.001 (n=8)

The rates of response to dietary supplementation were similar to those seen for Con A, with a significant increase in proliferation to PHA occurring between weeks 0 and 2 of the trial, and little change between weeks 2 and 4 in all but the MVitE+MSe group, where proliferation was seen to increase significantly between each time point.

3.4 Phagocytic Activity

The results of the analysis of peripheral blood leukocyte (PBL) phagocytic activity in cats fed the nine dietary regimes are shown in Figure 3.9. There was a significant effect of week ($p<0.0001$) on these results suggesting that the level of PBL phagocytic activity changed over time, which is reflected in the increased values seen in most groups at weeks 2 and 4 of the trial. The interaction of vitamin E x week ($p<0.0001$) and selenium x vitamin E x week ($p=0.0002$) indicates that there was an effect of dietary supplementation over time in this trial. The initial levels (week 0) of PBL phagocytic activity were similar across the nine groups.



Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) [a] *p<0.05;* [b] *p<0.01;* [c] *p<0.001 (n=8)*

Figure 3.9: Phagocytic Activity (%) of peripheral blood leukocytes in blood (± SEM) collected from cats fed one of nine diets containing supplemental levels of vitamin E and/ or selenium over a four week period

In the MVitE group, there was little change in PBL phagocytic activity between weeks 0 and 2 ($p=0.1034$), however activity did increase significantly between weeks 2 and 4 ($p=0.0005$) and weeks 0 and 4 ($p<0.0001$). In the HVitE group, there was a consistent increase in PBL phagocytic activity over the time of the trial, with significant differences noted between weeks 0 and 2 ($p=0.0017$), weeks 2 and 4 ($p=0.0001$) and weeks 0 and 4 ($p<0.0001$). PBL phagocytic activity did not differ significantly between the two vitamin E treatment groups during the trial. There was no change in PBL phagocytic activity in the MSE group during the trial ($p=0.6265$), and while phagocytic

activity increased at each time point in the HSe group, this was not significant (p=0.0966).

There was a significant increase in PBL phagocytic activity in the MVitE+MSe group between weeks 0 and 2 (p<0.0001), weeks 2 and 4 (p<0.0001) and weeks 0 and 4 (p<0.0001). In the HVitE+MSe group there was an increase in PBL phagocytic activity between weeks 0 and 2 (p=0.0002), which was maintained at week 4 of the trial (week 0 vs week 4 p<0.0001), however there was little change between weeks 2 and 4 (p=0.0710). In the HVitE+HSe group, PBL phagocytic activity increased between each sample point with significant differences between weeks 0 and 2 (p=0.0087), weeks 2 and 4 (p=0.0007) and weeks 0 and 4 (p<0.0001). There was not a significant increase in phagocytic activity in the MVitE+HSe group at any time point during the trial. The MVitE+MSe group showed the greatest increase in phagocytic activity among the mixed supplement groups and activity was significantly higher in this group after week 4 in comparison to the HVitE+HSe (p=0.0374), and MVitE+HSe groups (p=0.0026). Phagocytic activity was significantly greater in the HVitE (p=0.0245) and MVitE (p=0.0498) groups compared to the MVitE+HSe group after week 4 of the trial. Table 3.4 summarises the results for those groups in which PBL phagocytic activity increased during the trial.

Table 3.4: Vitamin E and / or selenium supplemented groups in which peripheral blood leukocyte phagocytic activity (± SEM) increased significantly during a 4 week trial

	Diet				
Week of Trial	**↑E**	**↑↑E**	**↑E, ↑Se**	**↑↑E, ↑Se**	**↑↑E, ↑↑Se**
0	48.00 ± 2.12	45.10 ± 2.40	45.23 ± 3.43	44.08 ± 2.05	41.94 ± 2.59
2	51.31 ± 2.50	51.70[b] ± 2.63	54.44[c] ± 3.73	52.00[c] ± 2.61	47.45[b] ± 2.59
4	58.70[c] ± 2.31	59.90[c] ± 2.90	63.02[c] ± 2.71	55.80[c] ± 3.15	54.70[c] ± 2.83

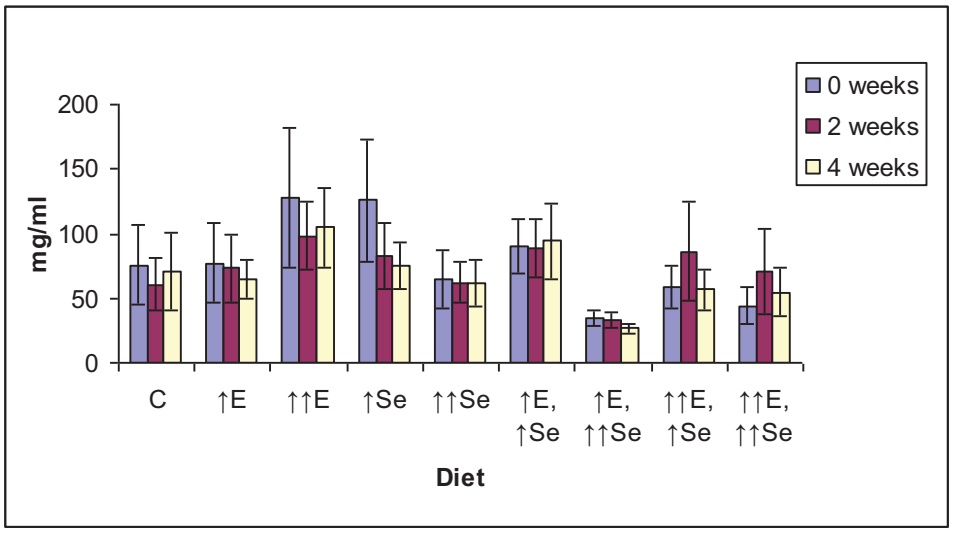
Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) [a]p<0.05; [b]p<0.01; [c]p<0.001 (n=8)

The pattern of response to supplementation was similar in the HVitE, MVitE+MSe and HVitE+HSe groups with the level of PBL phagocytic activity continuing to rise between each time point in the trial. In contrast, there was an initial sharp increase in

activity between weeks 0 and 2 in the HVitE+MSe group, but no further increase after this time. In the MVitE, the rate of response was slower, with PBL phagocytic activity not increasing significantly until week 4 of the trial.

3.5 Immunoglobulin G ELISA

Immunoglobulin G (IgG) levels in the blood of cats fed a diet containing the nine dietary regimes are shown in Figure 3.10. There was a large amount of variability in these results indicated by the large standard errors in all groups. There were no significant changes in the level of IgG in the blood within groups during the trial, nor was there any significant effect of either vitamin E or selenium, or their interaction, on these results ($p>0.05$).

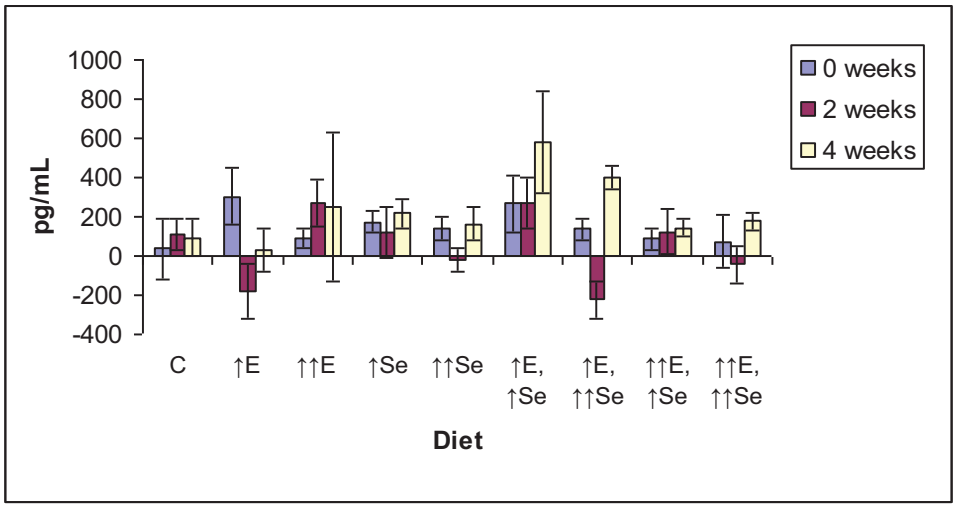


Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) (n=8)

Figure 3.10: Immunoglobulin G concentration in blood (± SEM) collected from cats fed one of nine diets containing supplemental levels of vitamin E and/ or selenium over a four week period

3.6 Prostaglandin E2 ELISA

The results of the competitive ELISA of prostaglandin E_2 concentration in the blood of cats fed a diet containing the nine dietary regimes are shown in Figure 3.11. The results are reported as the difference between un-stimulated samples and samples stimulated with Con A. Statistical analysis of the results found a significant effect of week ($p=0.0139$) on the prostaglandin E_2 concentration, suggesting an effect of time on these results. There was no apparent effect of dietary supplementation of vitamin E and/ or selenium on prostaglandin E_2 production ($p>0.05$).



Diet Key: C= Control; ↑↑E= HVitE (500 IU/kg diet); ↑↑Se= HSe (10 mg/kg diet); ↑E= MVitE (250 IU/kg diet); ↑Se= MSe (2 mg/kg diet) (n=8: ↑↑E ,↑↑Se; ↑Se; ↑↑E; ↑E) (n=6: ↑↑E, ↑Se; ↑E, ↑Se) (n=5: ↑↑Se) (n=4: C; ↑E, ↑↑Se)

Figure 3.11: Prostaglandin E_2 concentration (pg/mL) (difference between un-stimulated and stimulated samples) in blood (± SEM) collected from cats fed one of nine diets containing supplemental levels of vitamin E and/ or selenium over a four week period

4. Discussion

Dietary supplementation of vitamin E and selenium, both individually and in combination, have been reported to enhance immune cell function in many species and may help to prevent disease. However, there has been very little data published on the effects of these supplements on the feline immune system (Hayek *et al.,* 2000; 2004). The cat has evolved as an obligate carnivore, requiring a diet of animal tissue to provide several nutrients that this species can no longer metabolise itself (MacDonald *et al.,* 1984; Morris, 2002). Due to this unique metabolism it is possible that dietary supplements may not have the same effects on immune function in the cat that have been reported in other species. In fact, the cat is known to differ from other species in its metabolism of selenium, as the concentration of selenium in serum does not appear to reach a plateau with increased dietary intake of this mineral (Wedekind *et al.,* 2003a; 2003b), and cats appear to be able to tolerate a much higher level of dietary selenium compared to other species (Simcock *et al.,* 2004). For this reason, the effects of selenium on immune function may also differ in the cat when compared to other species.

The few available studies on the effects of vitamin E on immune function in the cat have not found the same enhancement of immune parameters as in other species, with the exception of elderly cats (Hayek *et al.,* 2000; Hendriks *et al.,* 2002), which may also suggest contrasting metabolism of this nutrient in the cat. However, before supplemental concentrations of both of these nutrients are added to cat diets, it is important to determine their safety and efficacy in this species. Determining the safety of supplemental dosages of selenium in the diet is especially important, as high intakes of this mineral are toxic to other species (Turner & Finch, 1991), although the low bioavailability of selenium in companion animal diets, and apparent increased tolerance to selenium, appear to reduce the risk of toxicity in the cat (Simcock *et al.,* 2004).

The aim of this study was to determine the effects of supplemental vitamin E and selenium, both individually and in combination, on selected immune function parameters in the cat. Eight supplemented diets were tested including a moderate vitamin E diet (250 IU/kg DM diet), a high vitamin E diet (500 IU/kg DM diet), a

moderate selenium diet (2 mg/kg DM diet), a high selenium diet (10 mg/kg DM diet), and four diets containing supplements of both vitamin E and selenium using combinations of the dosages mentioned above (MVitE+MSe; MVitE+HSe; HVitE+MSe; and HVitE+HSe). The supplements were added to a nutritionally balanced base diet already containing adequate levels of vitamin E (68.2 IU/kg DM) and selenium (0.38 mg/kg DM), as recommended by the American Association of Feed Control Officials (AAFCO, 2009), and were fed to cats *ad libitum* over a four week period.

All cats remained healthy during the trial, although there was a decline in body weight in all groups, except for the MSe group and the HVitE+MSe group, during both the one week acclimatisation period and the subsequent four week trial period. This decline was significant in five groups, including the control group. One factor that may have contributed to loss of body weight in the animals in this trial is the normal seasonal loss of weight seen in de-sexed males coming out of winter in the colony (D. Thomas, Personal Communication, May 11, 2010). This appears to have been a major factor in the HVitE+HSe group and the MVitE+MSe groups, as there were more males than females in these groups and the males lost more weight than the females. The animals were also likely to have had a reduced feed intake and therefore, body weight at this time of year due to an increase in the environmental temperature and day length (D. Thomas, Personal Communication, May 11, 2010). The two groups with the greatest loss in weight, the HVitE group and the MVitE group, had lower food intakes compared to the other groups, which explains the loss of weight seen in these groups. As there was a general decline in the body weight of the cats during the one week acclimatisation period of the trial, prior to the period of supplementation, it is likely that the loss of weight seen during the trial was partly due to the environmental factors mentioned above, rather than the experimental diets alone. Whether the reduced feed intake of the animals had an effect on the results of the trial is not known, although it is likely that this resulted in reduced amounts of the vitamin E and selenium supplements in the diets being consumed. This may have masked some of the positive, or negative, effects of supplementation on immune parameter function. There is no comparable data available from other feline studies on the maintenance of weight during feed trials.

Supplementation had little effect on the level of expression of the different immune cell phenotypes measured in this study. Vitamin E and selenium supplementation had no effect on the expression of the $CD4^+$ T cell marker. There was an increase in $CD8^+$ cell marker expression in all groups, including the control group, after weeks 2 and 4 of the trial when compared to baseline values. Since this increase occurred in all groups it can be assumed that the increase in $CD8^+$ cell marker expression during the trial was unrelated to the inclusion of the dietary supplements in the diet and therefore, these nutrients did not increase the expression of the $CD8^+$ cell marker. There were changes in the level of expression of the $CD14^+$ cell marker in all supplemented groups during this trial, however the $CD14^+$ population also fluctuated significantly in the control group over the same period so it is assumed that these changes were due to normal variations in $CD14^+$ marker expression or due to difficulties in the measurement of this marker, rather than an effect of the supplements in the diet. There was a general decline in the B^+ cell population in the majority of groups as the trial progressed, showing again that diet had no significant effect on B^+ cell expression.

While there was no effect of supplementation on cell phenotypes in this trial, studies in other species have reported changes in T and B cell populations when vitamin E and/ or selenium were supplemented in the diet (Spallholz *et al.,* 1990; Kubena & McMurray, 1996; Grimble, 1997). The greatest effects of vitamin E supplementation on T cell expression have been shown in elderly humans. A study in elderly humans noted a 50% increase in the percentage of T cells when vitamin E was supplemented at 110 IU/day for 28 days. This was due to an increase in the number of $CD4^+$ T cells, with no change seen in the number of $CD8^+$ T cells (Purkins *et al.,* 1990). In an earlier 28 day study using a mixed supplement of 100 mg/day vitamin C, 2.4 mg/day vitamin A and 55 IU/day vitamin E, a similar change in the T cell population was found in elderly patients ranging in age from 77 – 96 years (Penn *et al.,* 1989). As the T cell population is known to decline with age (Campbell *et al,* 2004), the increases seen in the human trials in response to vitamin E supplementation could reflect a return to a normal level seen in younger adults, whereas in the current trial the cats in the treatment groups, with an average age of 4.24 years, would be more likely to have a healthy population of T cells and therefore, vitamin E supplementation may be less likely to have an effect in these animals. Therefore, the contrasting results obtained in the current study in cats and the

two trials in humans may be due to the difference in age between subjects and/or species differences.

In female mice fed a diet containing 0, 0.2 or 2 mg/kg diet selenium per day for five months, the ratio of $CD4^+$ to $CD8^+$ cell levels in the thymus was reduced as the selenium level increased in the diet, suggesting that selenium supplementation can alter the expression of $CD4^+$ and $CD8^+$ T cell markers (Spallholz *et al.,* 1990). This change in ratio was caused by a large reduction in $CD4^+$ cell expression and a small increase in $CD8^+$ expression. Selenium supplementation had no effect on the B^+ cell population of spleen cells (Spallholz *et al.,* 1990). While the current trial in cats did not find the same effect of selenium on the T cell population as that seen in mice, this may be related to the much shorter four week trial period used, in comparison to the five month study in mice. The apparent tolerance of cats to selenium (Wedekind *et al.,* 2003a; 2003b; Simcock *et al.,* 2004) may also explain the differing outcomes of these trials.

From the results of the current trial, it appears that supplemental vitamin E and selenium do not alter T and B cell populations in healthy adult cats, however a study of longer duration would be required to determine the long term effects of these nutrients, especially that of selenium. Part of the rationale behind determining proportions of cell phenotypes is to determine the safety of the supplements, as alteration in the percentages of T and B cell populations could lead to animals being more susceptible to certain types of disease or infection (K. Rutherfurd-Markwick, Personal Communication, November 16, 2010), therefore from a safety perspective it is beneficial that the cell phenotypes did not change significantly.

In the current trial, analysis of the lymphocyte proliferative response of cats to the T cell mitogen Con A showed significant increases in proliferation in both the MVitE and HVitE groups. Proliferation to Con A also increased significantly in all the mixed supplement groups, (MVitE+MSe; MVitE+HSe; HVitE+MSe; and HVitE+HSe) although statistical analysis showed that only the vitamin E in the diet was having a significant effect in these groups. There were no significant differences between any groups receiving supplemental vitamin E, which suggests that a higher dosage of vitamin E does not have any further positive effect on lymphocyte proliferation to Con A than a moderate dose. A similar result occurred in the analysis of lymphocyte

proliferative responses to PHA, where there was a significant increase in proliferation in all the vitamin E and mixed supplement groups but, once again, no significant differences between groups. Again, only the vitamin E in the diet had a significant effect in these groups. Similar results in response to vitamin E supplementation have been reported in rats (Bendich *et al.,* 1983). Rats fed a diet supplemented with 220 IU/kg DM diet vitamin E for 20 weeks had an increased mitogenic response to Con A and PHA (Bendich *et al.,* 1983). A further study in rats by the same authors found that the optimum lymphocyte proliferative response to both Con A and PHA was reached with a diet containing 55 IU/kg DM vitamin E, while the 220 IU/kg DM diet or 1100 IU/kg DM diet did not lead to any further significant enhancement in a 10 week trial, in fact the level of stimulation appeared to decrease at the 1100 IU/kg DM diet level (Bendich *et al.,* 1986). These results support the findings of current trial to some extent, as vitamin E supplementation of 500 IU/kg DM diet (568.2 IU/kg DM diet total dose) did not further enhance the lymphocyte proliferative response compared with the diet supplemented with 250 IU/kg DM vitamin E (318.2 IU/kg diet total dose). The lower optimum level of vitamin E supplementation of 55 IU/kg DM diet in the study in rats (Bendich *et al.,* 1986) may reflect a species difference. In this study, a diet containing vitamin E at a level of 68.2 IU/kg DM (control diet) produced a sub-optimal level of stimulation to Con A or PHA in cats, as these parameters both increased with supplementation, however further study at supplementary levels between 68.2 IU/kg DM and 318.2 IU/kg DM may identify an optimum dose at a lower level than that used in the current study.

In contrast to the present study, previous studies in cats have not found a positive effect of vitamin E on the lymphocyte proliferative response to mitogens. A recent study of the vitamin E requirements of adult cats found no enhancement in lymphocyte proliferative responses to Con A when a diet containing a 1,100 IU/kg DM, 2,800 IU/kg DM or 4,300 IU/kg DM vitamin E supplement was fed for 26 weeks (Hendriks *et al.,* 2002). It is possible that the high doses in the Hendriks study had an inhibitory effect on the proliferative response to Con A, as was found in the rat study by Bendich *et al.* (1986). In the current study, the enhancement of proliferation to Con A appeared to taper off above 250 IU/kg DM, with little difference seen between the 250 IU/kg DM and 500 IU/kg DM vitamin E groups. Further study is required to establish whether

doses higher than 500 IU/kg DM do lead to inhibition of the proliferative response of lymphocytes to Con A.

In the study by Hayek *et al.* (2000), supplementation of vitamin E for two months at 250 IU/kg DM diet had no effect on the lymphocyte proliferative response to Con A or PHA in young cats, however older cats on this diet showed a significantly increased response compared to age matched controls (Hayek *et al.,* 2000). At higher levels of vitamin E supplementation (500 IU/kg DM diet), there was a non-significant increase in the lymphocyte proliferative response of young cats to PHA. Little extra benefit was seen at the same level of supplementation in older cats (Hayek *et al.,* 2000). While these results appear to contradict those of the current study, the age of the cats may need to be taken into account when comparing the outcomes of both studies. The average age of the young cats in the study by Hayek *et al.* (2000) was 2.65 years and the older cats 9.92 years, whereas the average age of animals in the treatment groups in this study was 4.24 years. The generally higher age of the animals in this study may explain at least to some extent the greater response to the mitogens seen when vitamin E is supplemented in the diet compared to that seen by Hayek *et al.* (2000).

The literature describing the effects of selenium supplementation on lymphocyte proliferative responses in other species shows mixed outcomes. Selenium supplementation of 0.1 mg/kg DM diet increased the mitogenic response of lamb lymphocytes to PHA and pokeweed mitogen, however 0.5 mg/kg DM selenium supplementation led to a reduced mitogenic response (Larsen *et al,* 1988b), while pigs fed a selenium supplement of 0.05-0.1 mg/kg DM for 12 weeks showed increased lymphocyte proliferative responses to PHA (Larsen & Tollersrud, 1981).

There are few reports of the effects of combined supplementation of vitamin E and selenium on the lymphocyte proliferative response to T cell mitogens. However, a positive effect of these nutrients on proliferation to PHA was found in pigs consuming a combined supplement of vitamin E (44 IU/kg DM) and selenium (0.05-0.1 mg/kg DM) for 12 weeks. This response exceeded that of the single vitamin E or selenium supplementation (Larsen & Tollersrud, 1981). The results of the current trial suggest that additional selenium, either alone or in combination with vitamin E, had no effect on lymphocyte proliferation to the T cell mitogens Con A and PHA in the cat. Selenium

was also not found to have a negative effect on lymphocyte proliferative responses to Con A and PHA. The contrasting results between this study and those in domestic farmed species above are likely to be related to species differences. As cats are known to be tolerant to high concentrations of selenium in the diet that are toxic in many other species (Simcock *et al.,* 2004), the sensitivity of immune cells to this nutrient may be lower in the cat than in other species. The differences between studies may also exist because farmed species are more prone to nutrient deficiency (Finch & Turner, 1996), and therefore may not have been in optimum condition prior to the trials. In addition, the longer trial periods used in the farmed species studies may also account for the different outcomes of these trials when compared to the current trial. While only vitamin E was found to have a significant effect on proliferation to Con A and PHA in the current trial in cats, selenium is likely to have had a sparing effect on vitamin E in the cell membrane, which could contribute to the positive effects on lymphocyte proliferation seen in this study (Turner & Finch, 1991; Chew, 1996; Kubena & McMurray, 1996; Knight, 2000).

In this study, phagocytic activity was significantly enhanced in the MVitE, HVitE, MVitE+MSe, HVitE+MSe and HVitE+HSe groups. Interestingly, the MVitE+HSe group did not display an increase in activity, unlike the other combined supplement groups and it is possible that the high level of supplementation of selenium in comparison to vitamin E supplementation had a negative effect on phagocytic activity. Human studies have reported an increased phagocytic response when vitamin E was supplemented in the diet (Kelleher, 1991; Meydani & Beharka, 1996). Elderly humans taking a vitamin E supplement of 400 IU vitamin E for several days displayed enhanced phagocytic activity, although the supplement also decreased bactericidal activity, which was thought to be due to reduced hydrogen peroxide production caused by the supplement (Meydani & Beharka, 1996). Similarly, humans taking a supplement of 1936 IU/day vitamin E had greater neutrophil phagocytic activity but reduced bactericidal activity (Kelleher, 1991), and another study in 13 adult males and five boys also reported reduced bactericidal activity when a supplement of 330 IU/day vitamin E was taken by subjects for three weeks (Prasad, 1980).

Excessive vitamin E supplementation may neutralise free radicals produced during the respiratory burst to fight infection, reducing the killing ability of the phagocytic cell

(Erickson *et al.,* 2000; Calder & Kew, 2002). While reduced free radical production may benefit some individuals with free radical related disease conditions, such as ischemia or chronic inflammatory diseases, in healthy individuals or individuals with impaired immune function reduced free radical production may inhibit their defence against infection (Erickson *et al.,* 2000). While phagocytic activity was increased by vitamin E and selenium supplementation in the current study, bactericidal action was not measured, so it is not known if the ability of these cells to destroy antigens with free radicals was reduced by the antioxidant activities. In future studies, the ability of phagocytes to destroy antigens should also be analysed to determine if there is any effect on the production of free radicals by phagocytic cells when vitamin E and selenium are supplemented in the diet of cats.

There are few available studies on the effects of combined supplementation of vitamin E and selenium on phagocyte activity. However, a study by Hogan *et al.* (1990) reported that supplementation of vitamin E (90 IU/kg DM diet) and/ or selenium (0.45 mg/kg DM diet) for 30 days had little effect on the phagocytic activity of neutrophils in dairy cows, while microbicidal activity was increased. In contrast, ageing mice fed a diet containing supplemental vitamin E (211 IU/kg diet), selenium (0.73 mg/kg diet), vitamin C (693 mg/kg diet) and β- carotene (34 mg/kg diet) for 15 weeks had a significantly higher level of phagocytic activity (Alvarado *et al.,* 2006). In the current trial, there was a significant increase in phagocytic activity in the combined supplement groups, although this was at a similar level to that seen in the single vitamin E supplement groups. If selenium supplementation is to have any benefit above that of vitamin E supplementation alone, it may be in increasing microbicidal activity, such as was seen in the dairy cow study by Hogan *et al.* (1990), mentioned above. Further studies would be required to establish if this occurs in the cat.

Vitamin E and selenium supplementation had no effect on immunoglobulin G production in the current trial, which may be due to the lack of a vaccine or infection challenge being used to elicit a response. Studies in other species that have reported changes in IgG production in response to supplementation with vitamin E and selenium have used experimental infection or vaccination with antigens. For example, vitamin E supplementation has been reported to increase IgG production to a bovine herpes virus vaccine in calves supplemented with 125, 250 or 500 IU/ day for 24 weeks from birth

(Reddy *et al.,* 1987). Vitamin E supplementation has also been found to increase antibody production in response to experimental infection and vaccination in many domestic species (Finch & Turner, 1996). Apart from the lack of a vaccine challenge, the differences in results obtained between the current trial and the calf trial (Reddy *et al.,* 1987) may be related to the nutritional status of the animals prior to the study, where, as previously stated, there is a higher likelihood of nutrient deficiency in farmed species kept under stressful environmental conditions (Finch & Turner, 1996), compared to healthy adult cats fed a balanced diet. In addition, the immune systems of the calves may still have been developing, which may have also altered the production of IgG in response to supplementation.

There are conflicting reports on the effect of selenium supplementation on IgG production. Mice consuming a selenium supplement of 1-3 mg/kg DM diet for six weeks showed increased production of IgG and immunoglobulin M (IgM) in response to infection with the SRBC antigen (Spallholz *et al.,* 1973). Selenium supplementation had no effect on IgG production in the current cat trial, which is in agreement with the study by Blodgett *et al* (1986), in which weaned piglets consuming diets containing 0.3-1.5 mg/kg DM selenium for 39 days showed no significant increase in IgG in response to lysozyme and ribonuclease. Similar studies in pigs and ruminant animals have also reported little or no effect of this nutrient on IgG production (Finch & Turner, 1996).

While selenium supplementation does not appear to have led to enhanced IgG production in the current trial, there also do not seem to have been any adverse effects arising from supplementation with this nutrient at doses of 2 and 5 mg/kg DM diet. This contrasts with results from a study in rats consuming a high level of selenium (5 mg/kg) in drinking water for 10 weeks, where reduced antibody production in response to infection with keyhole limpet haemocyanin was observed (Koller *et al.,* 1986). This highlights the potentially damaging effects of excessive selenium supplementation in some species. A longer trial period, such as that used in the rat study, may determine if longer term supplementation has a detrimental effect on this parameter in cats. Although, given the apparent high tolerance of cats to selenium (Wedekind *et al.,* 2003a; 2003b; Simcock *et al.,* 2004), the same negative effects on IgG production may not occur in this species.

Vitamin E and selenium regulate prostaglandin E_2 production and prevent it reaching concentrations in the cell that are immunosuppressive (Meydani & Beharka, 1996; Rayman, 2000; Arthur *et al.,* 2003). Vitamin E supplementation resulted in reduced prostaglandin E_2 production when young and old mice consumed 605 IU/kg DM diet for six weeks (Meydani *et al.,* 1986). In a study in young and old mice fed a diet supplemented with 33 IU/kg DM or 550 IU/kg DM vitamin E for 30 days, there was no change in the production of prostaglandin E_2 in un-stimulated macrophages in response to supplementation and overall, there were very low levels of prostaglandin E_2 produced in these cells. In macrophages stimulated with lipopolysaccharide, prostaglandin E_2 production was significantly higher than in un-stimulated cells for all groups. Aged mice consuming a 550 IU/kg DM vitamin E supplement also experienced a reduction in prostaglandin E_2 levels (Wu *et al.,* 2001). In rats consuming supplemental selenium in drinking water at 0.5, 2 or 5 mg/kg for 10 weeks, there was a significant reduction in prostaglandin E_2 production in the 5 mg/kg group in comparison to the control group (Koller *et al.,* 1986).

These studies all produced results which contrast with the results of the current trial in cats, where the majority of groups had similar levels of prostaglandin E_2 production in stimulated or un-stimulated samples and there was no reduction in prostaglandin E_2 levels caused by supplementation. The current study also produced results which conflicted with those of Hayek *et al.* (2000), where both young and old cats consuming a diet containing 500 IU/kg DM vitamin E for 2 months had reduced prostaglandin E_2 levels compared to age matched controls. However, due to the evaporation of a large amount of samples, the results of this analysis are not conclusive and it is uncertain what effect vitamin E and selenium had on prostaglandin E_2 concentrations in this trial. Therefore, further study is required to provide more information on the effects of vitamin E and selenium supplementation on prostaglandin E_2 production in the cat.

Lymphocyte proliferation and phagocytic activity were increased in the current study, possibly due to the antioxidant effects of vitamin E and selenium. . Vitamin E acts as a free radical scavenger in the cell membrane and is important in protecting fatty acids in the cell membrane from lipid peroxidation (Bendich, 1990; Halliwell *et al.,* 1995; Chew, 1996; Wang & Quinn, 2000). This activity maintains the stability and functions of the cell membrane (Bendich, 1999; Wang & Quinn, 2000). Selenium, as a

component of the enzyme glutathione peroxidase, also plays an important role in the protection of the cell from the effects of free radicals (Spallholz *et al.,* 1990; Rayman, 2000; Rooke *et al.,* 2004). Vitamin E and selenium are also important regulators of the arachidonic acid cascade and the production of prostaglandin E_2 by their control of the level of hydroperoxides required for the activity of cyclo-oxygenase enzymes (Wu *et al.,* 2001). Reduced prostaglandin E_2 production is known to result in increased lymphocyte proliferation in response to mitogens and increased antibody production (Hayek *et al.,* 2000). Both of these nutrients also affect the activity of T cells by their effects on interleukin-2 production. Vitamin E supplementation can enhance interleukin-2 production (Beharka *et al.,* 1997), while selenium increases the expression of high-affinity interleukin-2 receptors on T cells (McKenzie *et al.,* 1998). The increases in lymphocyte proliferation in this study may be attributed in part to the effects of vitamin E on interleukin-2 production and activity, as interleukin-2 promotes T cell proliferation and activity (Beharka *et al.,* 1997). Future studies on the use of vitamin E and selenium supplementation in the cat could include a measure of the concentration of interleukin-2 in the blood to determine their effects on this cytokine, and hence provide more information on the mechanisms by which these nutrients affect lymphocyte proliferation and activity. As already mentioned, it is not known if the increases in phagocytic activity and lymphocyte proliferation in this study were related to a reduction in the production of prostaglandin E_2, due to the unreliable results for this parameter. It is possible that this may have occurred as vitamin E supplementation was found to reduce prostaglandin E_2 production in both young and aged cats in the study by Hayek *et al.* (2000).

There is a large body of information outlining the positive effects of vitamin E and selenium on various parameters of immune function (Kelleher, 1991; Finch & Turner, 1996; Rayman, 2000; Rooke *et al,* 2004). However, the results from different studies are often conflicting due to differences between species, the age and health of subjects, or differences in experimental design, such as the chemical form of the supplement and the route of administration. The nutrient status of subjects can also greatly affect the outcome of a study. As can be expected, nutrient deficient subjects generally show a greater response to supplementation with vitamin E and selenium than do those with adequate levels of these nutrients in the diet prior to a study. It can be difficult to ascertain the initial nutrient status of subjects in a study as this is sometimes not

reported, which also complicates direct comparison between studies (Finch & Turner, 1996). The results of studies in animals with an adequate nutrient status prior to a study are more likely to be representative of the normal population, especially in companion animal studies. In this discussion an attempt has been made to use examples of dietary supplementation studies where subjects had an adequate initial vitamin E and selenium status prior to the study to allow better comparison with the current research in cats. The plasma concentrations vitamin E and selenium of the cats were not measured in this study. However, as the cats were consuming a complete and balanced diet prior to the study, it is assumed that the nutrient status of the animals was adequate.

There does not appear to have been any effect of the dosage of vitamin E and selenium in this study as lymphocyte proliferation to Con A and PHA, and phagocytic activity increased by a similar amount at both moderate and high levels of supplementation. There was a uniquely elevated response of lymphocytes to Con A in the HVitE group, however the cause of this is unknown. The lack of a significant effect of dosage on these results is in agreement with other studies where supplementation with high levels of vitamin E did not lead to greater increases in immune function than at a moderate level in both rats and cats (Bendich *et al.,* 1986; Hayek *et al,* 2000). While a higher dosage did not appear to lead to further enhancement of immune function in the parameters mentioned above, this may not be true for other immune parameters. For example, in the study by Hayek *et al,* (2000), prostaglandin E_2 production in cats was only reduced by the 500 IU/kg vitamin E supplement.

In the current study, there does not seem to have been a synergistic effect of combined supplementation of vitamin E and selenium compared to the supplementation of vitamin E or selenium alone on lymphocyte proliferation and, while there was a significant effect of vitamin E x selenium on phagocytic activity, the mixed supplement groups did not have a greater level of activity compared to the single vitamin E supplemented groups. This is in contrast to the reports of others that suggest there is a synergistic effect of these two supplements leading to enhancement of immune function to levels above that of single supplementation (Kubena & McMurray, 1996). For example, a synergistic effect of vitamin E and selenium was found in the study by Larsen & Tollersrud (1981), where there was an increase in the mitogenic responses to PHA in pigs consuming a low level supplement of 44 IU/kg DM vitamin E and 0.05-0.1 mg/kg

DM selenium for 12 weeks versus that of a single supplement. However, many studies that have noted a synergistic effect of these nutrients have been in animals with a nutrient deficiency in vitamin E and selenium (Finch & Turner, 1996). The cats used in the current trial had adequate intakes of vitamin E and selenium prior to the trial, and this may explain why these nutrients were not found to have a synergistic effect. Selenium supplementation alone did not lead to increased phagocyte or lymphocyte function, but also did not appear to have a negative effect on these functions. It is interesting to note, however, that phagocytic activity did not increase significantly in the MVitE+HSe group, as occurred in the three other combined supplement groups. This may suggest that a high level of selenium had a suppressing effect on phagocytic activity in the absence of a concurrent high level of vitamin E supplementation.

The vitamin E and selenium content of commercial cat diets available at supermarkets range from basic diets adhering to the minimum standards of 30 IU/kg DM vitamin E and 0.10 mg/kg selenium, as required by the American Association of Feed Control Officials (AAFCO, 2009), to more expensive diets containing 70 – 100 IU/kg vitamin E and 0.30 mg/kg selenium. Diets claiming to promote optimum health and condition were found to contain between 475 – 500 IU/kg vitamin E and 0.30 mg/kg selenium. The results of this study suggest that the addition of supplemental vitamin E and selenium to some of the basic supermarket diets may enhance immune function in cats, however the vitamin E content need not be as high as that found in the optimal health promoting products mentioned above, as the diet containing 568 IU/kg DM did not lead to any greater enhancement of immune function than did the 318 IU/kg DM diet in this trial.

While there was a positive effect of vitamin E and selenium on phagocytic activity and of vitamin E on lymphocyte proliferation in response to Con A and PHA in this study, it may not necessarily lead to greater resistance to disease in cats (Calder & Kew, 2002; Rutherfurd-Markwick & Hendriks, 2003). Studies involving experimental infection with antigens could be used to determine if there is a benefit of supplementation with these nutrients in disease prevention and recovery, however there are animal welfare concerns with this type of experimentation. Alternatively, the health and incidence of disease in animals consuming a diet containing supplemental vitamin E and selenium could be monitored long term. However, it is very difficult to determine that a dietary

treatment is responsible for a lack of disease incidence, as this process requires large numbers of animals to be studied for an extended period, and is therefore very costly as well as time consuming (K. Rutherfurd-Markwick, Personal Communication, May 11, 2010).

Dietary supplementation with vitamin E and selenium may enhance some immune parameters while having no effect, or a possible negative effect on other parameters (Calder & Kew, 2002). For this reason, it is important to conduct further testing to increase the body of information in this area and to confirm that the immune enhancing effects of vitamin E, and to a lesser extent selenium, found in this study are beneficial to overall health. To do this, a wider range of immune parameter tests could be studied over a longer period of time. Future studies in this area should also focus on the mechanisms that led to these effects, such as antioxidant activity, interleukin-2 production and prostaglandin E_2 production. The respiratory burst of phagocytic cells should also be assessed to determine if high levels of antioxidants reduce beneficial free radical production in these cells as has been found in other species. There should also be a focus on the effects of vitamin E and selenium supplementation on various other factors of immune function in the cat, such as antibody production, as there is a lack of knowledge in this area.

The results of this study are interesting and are in contrast to the stated hypothesis, which predicted that vitamin E supplementation was unlikely to have a positive effect on the function of immune parameters, while a combined supplement of vitamin E and selenium was hypothesised to lead to enhancement of the immune parameters tested. This hypothesis was based on the results of the studies by Hayek *et al.* (2000) and Hendriks *et al.* (2002), who found no effect of vitamin E supplementation on the lymphocyte proliferative responses of healthy adult cats to Con A and PHA. In the current study, it was somewhat surprising to find that vitamin E supplementation did elicit an increase in lymphocyte proliferative responses to Con A and PHA in the cats, as well as an increase in phagocytic activity. Meanwhile, the synergistic effect of combined vitamin E and selenium supplementation that has been reported by other authors in a study in pigs (Larsen & Tollersrud, 1981) was not found to occur with the immune function parameters tested in this study. As stated earlier, it is likely that the unique metabolism of selenium in the cat (Simcock *et al.,* 2004) may be responsible for

the lack of immune enhancement by selenium in this study. While these differ from the predictions made prior to the study, they provide useful new information that can be added to the body of research on the effects of dietary vitamin E and selenium supplementation on immune function in the cat.

5. Conclusion

There was a positive effect of vitamin E, or a combination of vitamin E and selenium, on phagocytic activity, while only vitamin E supplementation enhanced lymphocyte proliferative responses to Con A and PHA in this study. High levels of supplementation did not lead to any greater enhancement of immune function than moderate levels of supplementation in this study and combined vitamin E and selenium supplementation did not lead to significantly greater immune function than did vitamin E supplementation alone. There was no effect of supplementation on immune cell phenotype expression, IgG production or the prostaglandin E_2 concentration in the blood. Overall, from the results of this study, a supplemental level of 250 IU/kg DM vitamin E appears to be adequate to enhance some parameters of immune function in the cat. A higher level of vitamin E supplementation is unlikely to offer any added benefit and would add unnecessary cost to the manufacturing of the diet. As single selenium supplementation did not have an effect on immune function and combined supplementation of vitamin E and selenium did not enhance immune function above the level of single vitamin E supplementation, there seems to be no benefit to adding a selenium supplement to the diet.

Topics for further research:

- Effects of vitamin E and selenium supplementation on the production of free radicals by phagocytic cells
- Establishing whether selenium supplementation can increase microbicidal activity in cats
- Effects of vitamin E and selenium supplementation on prostaglandin E_2 production in the cat
- Measurement of the concentration of interleukin-2 in the blood of cats in response to vitamin E and selenium supplementation to determine their effects on this cytokine, and to provide more information on the mechanisms by which these nutrients affect lymphocyte proliferation and activity
- Studies of a longer trial period and testing of a wider range of immune parameters, such as antibody production, to build on the body of knowledge on the role of vitamin E and selenium in immune health in the cat

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Appendix 1

Statistics Summary Tables

Weight:

Effect	Nominator DF	Denominator DF	F Value	Pr > F
Selenium	2	63	1.07	0.3476
Vitamin E	2	63	1.80	0.1743
Selenium x Vitamin E	4	63	2.17	0.0821
Week	2	126	24.17	<.0001
Selenium x Week	4	126	5.47	0.0004
Vitamin E x Week	4	126	1.49	0.2097
Selenium x Vitamin E x Week	8	126	0.74	0.6584

Immune Cell Phenotype Expression:

$CD4^+$:

Effect	Nominator DF	Denominator DF	F Value	Pr > F
Selenium	2	62	0.17	0.8458
Vitamin E	2	62	0.27	0.7669
Selenium x Vitamin E	4	62	0.47	0.7573
Week	2	124	3.67	0.0283
Selenium x Week	4	124	0.40	0.8080
Vitamin E x Week	4	124	0.05	0.9949
Selenium x Vitamin E x Week	8	124	2.47	0.0161

$CD8^+$:

Effect	Nominator DF	Denominator DF	F Value	Pr > F
Selenium	2	63	0.56	0.5722
Vitamin E	2	63	0.47	0.6274
Selenium x Vitamin E	4	63	0.07	0.9904
Week	2	126	60.65	<.0001
Selenium x Week	4	126	0.53	0.7163
Vitamin E x Week	4	126	2.00	0.0982
Selenium x Vitamin E x Week	8	126	0.37	0.9365

$CD14^+$:

Effect	Nominator DF	Denominator DF	F Value	Pr > F
Selenium	2	63	1.13	0.3295
Vitamin E	2	63	2.14	0.1257
Selenium x Vitamin E	4	63	3.71	0.0089
Week	2	125	11.05	<.0001
Selenium x Week	4	125	0.16	0.9558
Vitamin E x Week	4	125	2.38	0.0552
Selenium x Vitamin E x Week	8	125	1.12	0.3548

B^+ cells:

Effect	Nominator DF	Denominator DF	F Value	Pr > F
Selenium	2	63	0.69	0.5043
Vitamin E	2	63	0.29	0.7495
Selenium x Vitamin E	4	63	0.48	0.7539
Week	2	125	7.21	0.0011
Selenium x Week	4	125	0.92	0.4574
Vitamin E x Week	4	125	0.62	0.6505
Selenium x Vitamin E x Week	8	125	1.23	0.2876

Lymphocyte Proliferation:

Concanavalin A:

Effect	Nominator DF	Denominator DF	F Value	Pr > F
Selenium	2	62	0.01	0.9948
Vitamin E	2	62	7.07	0.0017
Selenium x Vitamin E	4	62	0.79	0.5383
Week	2	117	47.62	<.0001
Selenium x Week	4	117	0.78	0.5424
Vitamin E x Week	4	117	12.27	<.0001
Selenium x Vitamin E x Week	8	117	1.33	0.2337

Phytohaemagglutinin:

Effect	Nominator DF	Denominator DF	F Value	Pr > F
Selenium	2	62	0.46	0.6363
Vitamin E	2	62	2.44	0.0956
Selenium x Vitamin E	4	62	0.77	0.5475
Week	2	119	73.96	<.0001
Selenium x Week	4	119	2.40	0.0539
Vitamin E x Week	4	119	6.35	0.0001
Selenium x Vitamin E x Week	8	119	1.98	0.0540

Phagocytic Activity:

Effect	Nominator DF	Denominator DF	F Value	Pr > F
Selenium	2	63	0.32	0.7296
Vitamin E	2	63	1.98	0.1461
Selenium x Vitamin E	4	63	0.78	0.5436
Week	2	126	80.04	<.0001
Selenium x Week	4	126	1.11	0.3538
Vitamin E x Week	4	126	10.25	<.0001
Selenium x Vitamin E x Week	8	126	4.15	0.0002

Immunoglobulin G:

Effect	Nominator DF	Denominator DF	F Value	Pr > F
Selenium	2	63	2.34	0.1050
Vitamin E	2	63	0.16	0.8548
Selenium x Vitamin E	4	63	0.94	0.4474
Week	2	125	0.87	0.4214
Selenium x Week	4	125	0.65	0.6259
Vitamin E x Week	4	125	0.90	0.4654
Selenium x Vitamin E x Week	8	125	0.55	0.8161

Prostaglandin E_2:

Effect	Nominator DF	Denominator DF	F Value	Pr > F
Selenium	2	48	2.85	0.0674
Vitamin E	2	48	0.49	0.6163
Selenium x Vitamin E	4	48	2.07	0.0988
Week	2	79	4.52	0.0139
Selenium x Week	4	79	1.21	0.3145
Vitamin E x Week	4	79	1.72	0.1542
Selenium x Vitamin E x Week	8	79	0.80	0.6028