# VITAMIN E REQUIREMENTS OF ADULT DOMESTIC CATS (*FELIS CATUS*) FED DIETS CONTAINING HIGH LEVELS OF FISH OIL

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**Yuben Wu**

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| $\alpha$-TO$^{\bullet}$ | $\alpha$-Tocopherol radical |
| $\alpha$-TOH | $\alpha$-Tocopherol |
| $^{1}O_2$ | Active oxygen |
| $^{3}C^{\bullet}$ | Triplet excited carotenoid |
| $^{3}$H-TdR | Tritiated thymidine |
| $^{3}O_2$ | State oxygen |
| AAFCO | Association of America Feed Control Officials |
| Ascorbate$^{\bullet}$ | Ascorbate radical |
| Brdu | 5-Bromo-2'- deoxyuridine |
| Caro | Carotenoids |
| CAT | Catalase |
| Con A | Concanavalin A |
| CPM | Counts per minute |
| ELISA | Enzyme-linked imunosorbent assay |
| FRAP | The ferric reducing ability of plasma |
| GSH | Reduced glutathione |
| GSHPx | Glutathione peroxidase |
| $GSH_{red}$ | Glutathione reductase |
| GSSG | Oxidised glutathione |
| $H_2O_2$ | Hydrogen peroxide |
| HO$^{\bullet}$ | Hydroxyl radical |
| $LO_2^{\bullet}$ | Lipid hydroperoxide radicals |
| LOOH | Lipid hydroperoxides |
| LPO | Lipid peroxides |
| MCDP | 10-N-Methylcarbamoyl-3,7-dimethylamino-10 H-phenothiazine |
| NADP | Triphosphopyridine nucleotide |
| $NO_2^{\bullet}$ | Nitrogen dioxide |
| NRC | National Research Council |
| $O_2^{\bullet-}$ | Superoxide anion |
| OH$^{\bullet}$ | Hydroxyl radical |
| $PA_2$ | Phospholipase $A_2$ |
| PHA | Phytohemagglutinin |
| PHGSHPx | Phospholipid hydroperoxides glutathione peroxidase |
| PUFA | Polyunsaturated fatty acids |
| PWM | Pokeweed mitogen |
| R$^{\bullet}$ | Carbon-centered radical |
| RBC | Red blood cell |
| ROO$^{\bullet}$ | Fatty acid hydroperoxide radicals |
| ROOH | Fatty acid hydroperoxides |
| Se | Selenium |
| SI | Stimulation index |
| SOD | Superoxide dismutase |
| TBA | Thiobarbituric acid |

# ABSTRACT

The vitamin E (α-tocopherol) requirement of adult cats fed diets containing high levels of fish oil was investigated. Thirty-two (16 male, 16 female) adult domestic cats (*Felis catus*) were randomly allocated to four groups according to sex and fed one of four experimental diets (A, B, C, and D) for 126 days. The cats were housed in large outdoor pens in groups of 8 cats. Diets A, B, C and D contained approximately 300 g of fish oil per kg diet dry matter and were supplemented to contain 0, 5, 10, and 15 IU DL-α-tocopheryl acetate per g added fish oil per kg diet, respectively. The diets were provided *ad libitum* with water being available at all times. Food intake was measured daily and body weights were measured at weekly intervals. Blood samples were taken from the jugular vein of each cat at bi-weekly intervals during the study. Blood samples were analysed for plasma α-tocopherol, red blood cell $H_2O_2$ (4 and 2 %) haemolysis, the ferric reducing ability of plasma, plasma lipid peroxides, plasma triglycerides, alkaline phosphatase and whole blood lymphocyte proliferation.

All cats remained healthy throughout the study except one female cat who was removed after 3 weeks due to poor food intake. The four diets were analysed and found to be free of peroxides. The average daily metabolisable energy intake of the cats on diet A, B, C and D at the end of study were similar and were 289, 261, 256, and 267 $kJ \cdot kg^{-1}$ body weight, respectively. No clinical signs of vitamin E deficiency were observed in any of the cats. The plasma α-tocopherol concentrations of the cats in the four groups at the start of the study were not significantly different between the four groups (mean ± SEM, 3.4 ± 0.2 $\mu g \cdot ml^{-1}$). When the cats were fed diet A (unsupplemented), the mean plasma α-tocopherol concentration remained relatively low and the RBC 4 % $H_2O_2$ haemolysis remained high, while the RBC 2 % $H_2O_2$ haemolysis decreased consistently. Plasma lipid peroxides remained relatively low throughout the study. The ferric reducing ability of plasma status was compromised in the cats on the unsupplemented diet. There was no significant ($P < 0.05$) difference in any of the response parameters measured amongst the cats fed diets B, C and D except for the RBC 4 % $H_2O_2$ haemolysis of the cats on diet B which was significantly higher than those on diet C and D at week 4 and week 8, and the LPO value of the cats on diet D which was significantly higher than those of the cats on diet B and C at week 4.

The vitamin E requirement of adult cats fed a high level of fish oil, using the response parameters measured, was estimated to be between 0 and 5 IU of vitamin E per g added fish oil per kg diet. The current recommendation of the Association of American Feed Control Officials (10 IU vitamin E/g fish oil/kg diet) appears to be well in excess. The results from the present study also showed that there was no beneficial effect of dietary vitamin E on whole blood cell proliferation when vitamin E levels were 150 % of the recommendations of the Association of American Feed Control Officials. The vitamin E requirement of adult cats to optimise immune response warrants further investigation.

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# TABLE OF CONTENTS

# LIST OF TABLES

## CHAPTER 1



## CHAPTER 2



## CHAPTER 3

# LIST OF FIGURES

## CHAPTER 1



## CHAPTER 3

# GENERAL INTRODUCTION

The domestic cat (*Felis catus*) is a member of the Felidae family of the order Carnivore and one of the most popular companion animals. Part of their attraction lays in their playful behaviour (Houpt *et al.*, 1988). In recent years researchers have discovered that the relationship between humans and their pets provides numerous physiological and psychological benefits to the owner (Case *et al.*, 1995).

Besides proper health care and medical attention, nutrition is considered to be an important component of the care of cats. Nutritional balance and preferences of diets must be considered when a diet is formulated for cats by an animal nutritionist (Case *et al.*, 1995). It is well known that many cats prefer fish and consequently, numerous cat foods are composed of fish or flavoured with fish (Houpt *et al.*, 1988). However, there have been several reports of vitamin E deficiency in cats as a result of the exclusive feeding of fish and fish based diets (Cordy and Stillinger, 1953; Coffin and Holzworth, 1954; Munson *et al.*, 1958; Griffiths *et al.*, 1960; Watson *et al.*, 1973; Gaskell *et al.*, 1975; Flecknell and Gruffydd-Jones, 1978; Summers *et al.*, 1982; Koutinas *et al.*, 1993; Tidholm, 1996) over the last 50 years.

The vitamin E requirements of other animal species such as humans, rats, pigs, dogs, and guinea pigs have been extensively studied (Van Vleet, 1975; Farrell *et al.*, 1985; Hakkarainen *et al.*, 1986; Jensen *et al.*, 1988a; Mahan, 1991; Meydani *et al.*, 1991; Cho and Choi, 1994; Barja *et al.*, 1996; Wang *et al.*, 1996; Kubo *et al.*, 1997). These studies have demonstrated that the *in vivo* vitamin E requirements are markedly influenced by dietary composition. A high dietary level of polyunsaturated fatty acids increases the requirement for vitamin E as a result of the increased susceptibility of tissues to peroxidation (Duthie, 1993). The dietary vitamin E requirement of cats has been set at 30 IU·kg$^{-1}$dry matter: a figure mostly extrapolated from other animal species (NRC, 1986). In order to prevent vitamin E deficiency in cats fed commercially sold, fish based diets, the Association of America Feed Control Officials (AAFCO, 1997) recommends that diets containing fish oil should be supplemented with 10 IU of vitamin E for every g of fish oil per kg diet. AAFCO (1997) failed to provide evidence to substantiate this value and, therefore, the exact vitamin E requirements of cats fed high levels of polyunsaturated fatty acids are still largely unknown.

The main objective of this study was to determine the vitamin E requirement of cats fed high dietary levels of polyunsaturated fatty acids from fish oil. This study was also undertaken to obtain baseline data on α-tocopherol levels in blood plasma of adult cats, which can be used in the diagnosis of vitamin E deficiency.

# CHAPTER 1

# REVIEW OF LITERATURE

## 1.1 INTRODUCTION

Vitamin E is the term used for eight naturally occurring fat-soluble nutrients with the most potent biological and antioxidant nutrient of the eight being α-tocopherol (Bjørneboe *et al.*, 1990). Vitamin E has a major biological role in protecting polyunsaturated fatty acids and other components of the membranes of all cells from oxidation by free radials. Lipid peroxidation is a chain reaction leading to disturbances in membrane structure and function, which can be stopped by antioxidants. The *in vivo* requirement for vitamin E is markedly influenced by dietary composition. A high dietary intake of polyunsaturated fatty acids increases the requirement for vitamin E as a result of an increased susceptibility of tissue to peroxidation (Duthie, 1993). Clinical manifestations of vitamin E deficiency vary considerably between species. However, in general affected tissues are the neuromuscular, vascular and reproductive systems (Combs, 1992).

The domestic cat is a true carnivore, and is frequently fed commercial diets that contain a high level of polyunsaturated fatty acids. In the past 50 years there have been many reports of vitamin E deficiency in cats. Many of these cases were attributed to the feeding of fish or fish-based diets. Other cases have been attributed to the feeding of commercial cat foods and the feeding of liver. There are no estimates of the vitamin E requirement of adult cats.

This literature review first describes free radicals, lipid peroxidation and the role of major antioxidants within the mammalian body with emphasis on biochemical mechanisms and interactions. Also, dietary factors affecting vitamin E requirement are described as well as targeted tissues and signs of vitamin E deficiency in animals, and the estimated vitamin E requirement of several mammalian species. Methods for the assessment of vitamin E status are presented. Finally, methods of determining vitamin E requirements in animals are discussed.

## 1.2. FREE RADICALS, LIPID PEROXIDATION AND VARIOUS ANTI-OXIDANTS WITHIN THE MAMMALIAN BODY

### 1.2.1 Free radicals and lipid peroxidation

A free radical is any molecular species capable of independent existence (hence the term "free") that contains one or more unpaired electrons (Halliwell, 1994; Halliwell *et al.*, 1995). The most common free radicals within the mammalian body are peroxyl radicals, the superoxide anion ($O_2^{\bullet -}$), the hydroxyl radical ($HO^{\bullet}$)(Machlin and Bendich, 1987; Burton and Traber, 1990). The peroxyl radical has special significance because of its involvement in lipid peroxidation (Burton and Traber, 1990).

The primary target for free radicals are the unsaturated bonds in membrane lipids of cells as well as the protein lipids, e.g. low-density lipoprotein (LDL), within the mammalian body. Lipid peroxidation results in a loss of membrane fluidity, receptor alignment, inactivation of enzymes and potentially in cellular lysis (Niki, 1996). The three-stage process of lipid peroxidation and degradation is outlined in Figure 1.

In the initiation phase (reaction 1), carbon-centred free radicals, $R^{\bullet}$ are produced from a precursor molecule. This reaction may occur *in vivo*, for example, by a metal ion catalysed decomposition of a lipid hydroperoxides (Burton and Ingold, 1989; Burton and Traber, 1990; Burton, 1994).

Initiation: production of $R^{\bullet}$ (carbon-centred radicals). (1)

In the propagation phase, a fraction of the carbon-centred free radicals are converted to peroxyl radicals in a rapid reaction with dissolved molecular oxygen (reaction 2). The chain-carrying species from a relatively unreactive carbon-centred radical are then transformed to a very reactive peroxyl radical. The peroxyl radical can attack any available peroxidisable material, with abstraction of a hydrogen atom (reaction 3a), or addition a double bond (reaction 3b) (Burton and Ingold, 1989; Bjørneboe *et al.*, 1990). The new carbon-centred free radicals proceed by way of reaction 3a and b to yield another peroxyl radical. So a chain reaction is set up and proceeds through the propagation reactions 3a and b for a large number of cycles.

Propagation: $R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$ (fast). (2)

$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$ (H atom abstraction). (3a)

$ROO^{\bullet} + RH \longrightarrow ROORH^{\bullet}$ (Double bond addition). (3b)

In the termination phase, two peroxyl radicals react and result inactive products

(reaction 4) or the peroxyl radicals is inhibited by a chain-breaking antioxidant (Burton and Ingold, 1989). Within the mammalian body, vitamin E (α-tocopherol) is the major and most effective lipid-soluble chain-breaking antioxidant (Burton, 1989; Bjørneboe *et al.*, 1990; Burton and Traber, 1990; Burton, 1994).

Termination: $ROO^{\bullet} + ROO^{\bullet} \longrightarrow$ inactive products. (4)

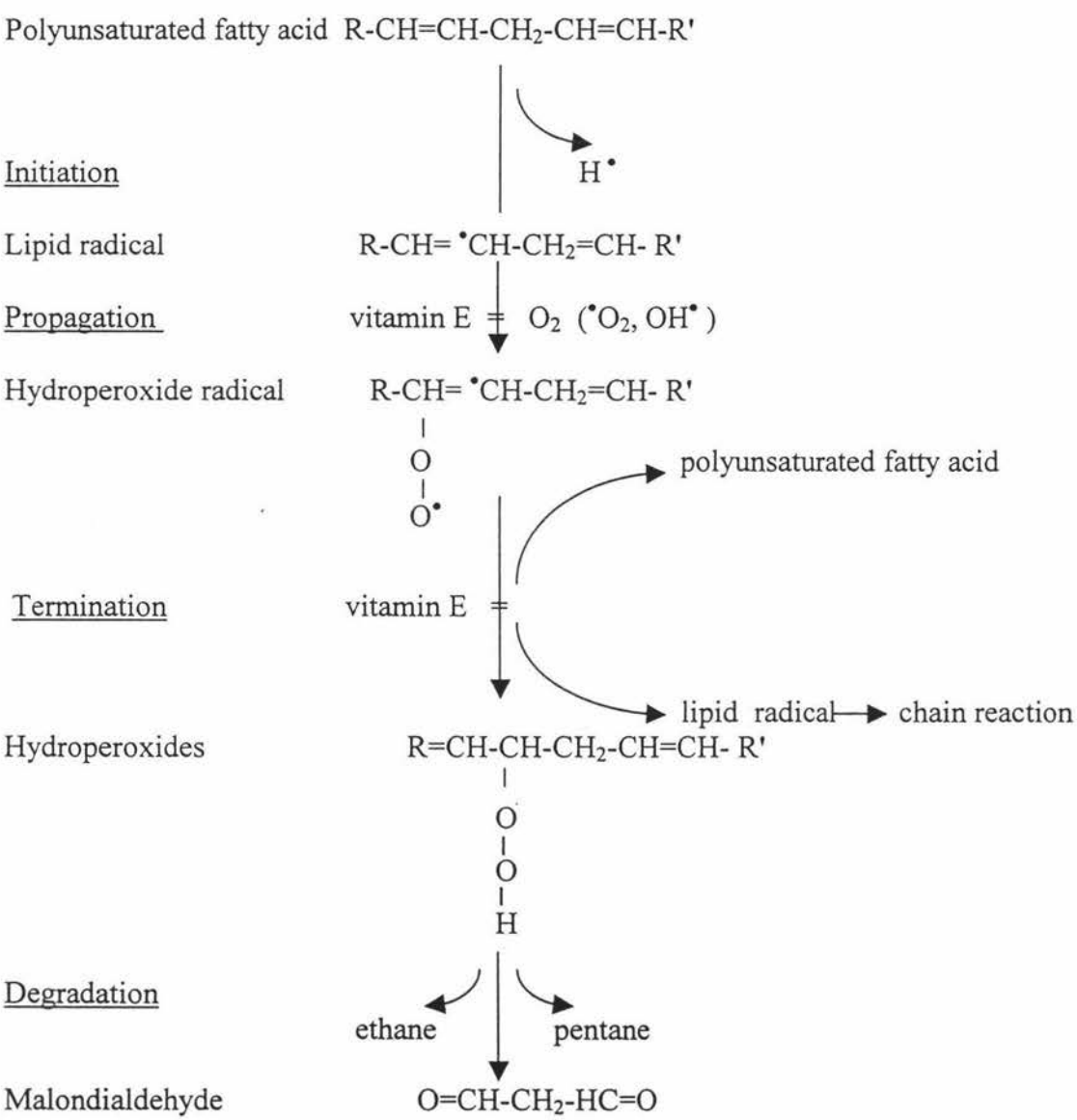


**Figure 1** Schematic presentation of lipid peroxidation (Bjørneboe *et al.*, 1990)

### 1.2.2 Antioxidant nutrients and enzymes, and their interaction within the mammalian body

All mammals have evolved antioxidant defence systems against oxidative damage induced by active oxygen and free radicals (Niki, 1996). The primary defence consists of various antioxidant compounds with different functions such as vitamin E (α-tocopherol), vitamin C (ascorbic acid), β-carotene and uric acid along with various antioxidant enzymes. The notable antioxidant enzymes are glutathione peroxidase (GSHPx), superoxide dismutase (SOD), and catalase (CAT)(Cadenas, 1995). The major antioxidant nutrients, selenium, and enzymes to prevent membranes from lipid peroxidation are described here with emphasis on their biochemical mechanisms and their interactions.

#### 1.2.2.1 Vitamin E (α-tocopherol)

Tocopherols (the four homologues) and tocotrienols are collectively referred to as vitamin E. Of the eight fat-soluble derivatives, α-tocopherol predominates in many species and has the highest biological activity (Bjørneboe *et al.*, 1990). The ability of vitamin E (α-tocopherol) to quench free radicals and act as a terminator of lipid peroxidation is shown in Figure 1. α-Tocopherol inhibits lipid peroxidation by scavenging lipid hydroperoxide radicals, which are intermediates in the chain reactions:

$$TOH + LO_2^{\bullet} \longrightarrow TO^{\bullet} + LOOH$$

Where TOH, $LO_2^{\bullet}$, $TO^{\bullet}$ and LOOH are α-tocopherol, lipid hydroperoxide radicals and α-tocopheroxyl radical and lipid hydroperoxides, respectively (Bjørneboe *et al.*, 1990; Niki, 1996). The α-tocopheroxyl radical may be returned back to α-tocopherol by ascorbate and glutathione (Bjørneboe *et al.*, 1990).

#### 1.2.2.2 Vitamin C (ascorbic acid) and glutathione

Vitamin C is considered to be the most important antioxidant in extracellular fluids (Sies *et al.*, 1992). Glutathione (GSH) can also scavenge various free radicals as well as being a substrate of glutathione peroxidase (Halliwell, 1994). Vitamin C can efficiently scavenge superoxide, hydrogen peroxide, hypochlorite, the hydroxyl radical, peroxyl radicals, and singlet oxygen by donating one electron. However, vitamin C has been shown to be less effective in inhibiting lipid peroxidation initiated by a peroxyl radical initiator than other plasma components, such as α-tocopherol, protein, thiol, urate, and bilirubin (Sies *et al.*,

1992). In addition to direct inactivation of free radicals, vitamin C can also protect membranes against peroxidation by enhancing the activity of tocopherol in which vitamin C regenerates α-TOH from α-TO$^{\bullet}$ (reaction 5)(Moser and Bendich, 1991; Stocker and Bowry, 1996). Moreover, GSH synergistically enhances tocopherol activity in membranes and lipoproteins indirectly as it regenerates ascorbate$^{\bullet}$ back to ascorbate (reaction 6)(Packer and Kagan, 1993). The reaction of α-TO$^{\bullet}$ with ascorbic acid is located at the surface of membranes and lipoproteins (Machlin and Bendich, 1987; Halliwell, 1994; Niki, 1996).

α-TO$^{\bullet}$ + ascorbate $\longrightarrow$ α-TOH + ascorbate$^{\bullet}$ (5)

ascorbate$^{\bullet}$ + GSH $\longrightarrow$ ascorbate + GSSG (6)

1.2.2.3 Uric acid

Uric acid is regarded as one of the most important water-soluble biological antioxidants within the mammalian body (Sevanian *et al.*, 1991). It can scavenge hydroxyl radicals (OH•) as well as hypochlorous acid (CHOCL/$OCL^-$), singlet $O_2$, ozone ($O_3$), nitrogen dioxide ($NO_2$•) and peroxynitrite (Halliwell, 1996). OH• is a powerful initiator of lipid peroxidation (Halliwell, 1996). Uric acid is believed to inhibit lipid peroxidation more effectively than vitamin C. Uric acid inhibits lipid peroxidation by chelating catalytic iron or copper ions. As a result, uric acid reduces the metal ions ability to react with $H_2O_2$ to make the free radical, OH• (Halliwell, 1996). Moreover, an important aspect of uric acid is that it stabilises vitamin C in biological fluids as a result of the inhibition of iron-catalysed oxidation of ascorbate (Sevanian *et al.*, 1991). The normal range of blood uric acid in cats is 0.059 - 0.113 mM, which is lower than in humans (0.2 - 0.04 mM) (Bloom, 1960; Halliwell, 1996). It is unknown how important uric acid is as an antioxidant defence systems within the body of the cat.

1.2.2.4 β-Carotene and other related carotenoids

Independent of its role as a precursor of vitamin A, β-carotene is considered to be an unusual type of lipid antioxidant (Burton and Ingold, 1984; Sies *et al.*, 1992). β-Carotene acts as a lipid-soluble chain-breaking antioxidant at low partial pressures of oxygen. It complements the role of vitamin E which is effective at high oxygen pressure (Burton, 1989).

Carotenoids notably β-carotene and lycopene as well as oxycarotenoids (e.g. zeaxanthin and lutein) exert antioxidant functions in the lipid phase by quenching free radical or $^1O_2$ lycopene, which is the open-chain analogue of β-carotene is the most efficient $^1O_2$ quencher among the biologically occurring carotenoids. The mechanism by which carotenoids protect biological systems against $^1O_2$ mediated damage appears to depend largely on physical quenching (Sies *et al.*, 1992). The deactivation of $^1O_2$ by carotenoids involves transfer of excitation energy from $^1O_2$ to the carotenoid and resulting in the formation of ground state oxygen ($^3O_2$) and triplet-excited carotenoid ($^3Caro^•$)(reaction 7). Then, the energy is dissipated through rotational and vibrational interactions between $^3Caro^•$ and the solvent to recover ground state carotenoid (reaction 8)(Stahl *et al.*, 1994).

$$^1O_2 + Caro \longrightarrow {}^3O_2 + {}^3Caro^• \quad (7)$$

$$^3Caro^• \longrightarrow Caro + \text{thermal energy} \quad (8)$$

Antioxidant properties and the potential biological role of β-carotene and other carotenoids and their implication in human health is an area of current research (Stahl *et al.*, 1994). Cats differ from other mammals in that their natural diet is higher in protein, fat and almost free of carbohydrates (Morris, 1994). Carotenoid pigments are not normally found in plasma or other tissues of cats (NRC, 1986). Because of this, the low intake of β-carotene of cats throughout evolution could have resulted in this antioxidant in defence system not being as important as in other animals.

GLUTATHIONE PEROXIDASE

$$2GSH + H_2O_2 \xrightarrow{GSHPX} GSSG + H_2O$$

$$2GSH + \text{FATTY ACID-OOH} \xrightarrow{GSHPX} GSSG + \text{FATTY ACID-OH} + H_2O$$

GLUTATHIONE REDUCTASE

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

**Figure 2** The glutathione system (Halliwell, 1994).

1.2.2.5 Selenium, glutathione peroxidase, superoxide dismutase and catalase

One of the major physiological roles of selenium (Se) is to function as a general antioxidant as an integral part of glutathione peroxidase (GSHPx) and phospholipid hydroperoxide glutathione peroxidase (PLGSHPx) (Rotruck *et al.*, 1973; Ursini *et al.*, 1985; Bettger, 1993). Se exerts its effect by being required for the synthesis of these enzymes (Bettger, 1993). GSHPx and PLGSHPx can suppress the formation of free radicals by destruction of hydrogen peroxide ($H_2O_2$) and phospholipid hydroperoxides which are the two major free radical precursors within the mammalian body. GSHPx and PLGSHPx are believed to have a different target site in cells. GSHPx mainly acts in the cytoplasm and removes the two free precursors through the glutathione system (Figure 2). It can be seen from Figure 2 that GSHPx remove $H_2O_2$ as well as fatty acid hydroperoxides by using its oxidised reduced glutathione (GSH) to oxidised glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power (Duthie, 1993; Halliwell, 1994; Halliwell *et al.*, 1995). Grossman and Wendel (1983) found that GSHPx has no activity against membrane-bound lipid peroxides. It is likely that the phospholipase $A_2$ enzyme in the membrane of cells cleave fatty acid hydroperoxides from phospholipid and that fatty acid hydroperoxides are further removed by GSHPx (Figure 3)(Ursini and Bindoli, 1987; Duthie, 1993). PLGSHPx specifically reduces membrane-bound lipid hydroperoxides esterified in intact phospholipids (Ursini *et al.*, 1985).

Within the mammalian body, GSHPx works together with several preventive antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) to remove active oxygen ($O_2^{\bullet-}$) and the free radical precursors, $H_2O_2$ and fatty acid hydroperoxides (ROOH) and, therefore, suppress the formation of free radicals. SOD removes $O_2^{\bullet-}$ by accelerating its conversion to $H_2O_2$. The reaction is:

$$2\ O_2^{\bullet-} + H^{\bullet} \longrightarrow H_2O_2 + O_2$$

Catalases in the peroxisomes convert $H_2O_2$ to $H_2O$ and $O_2$ and help to dispose of $H_2O_2$ generated by the action of oxidase enzymes (Halliwell, 1994; Halliwell *et al.*, 1995).

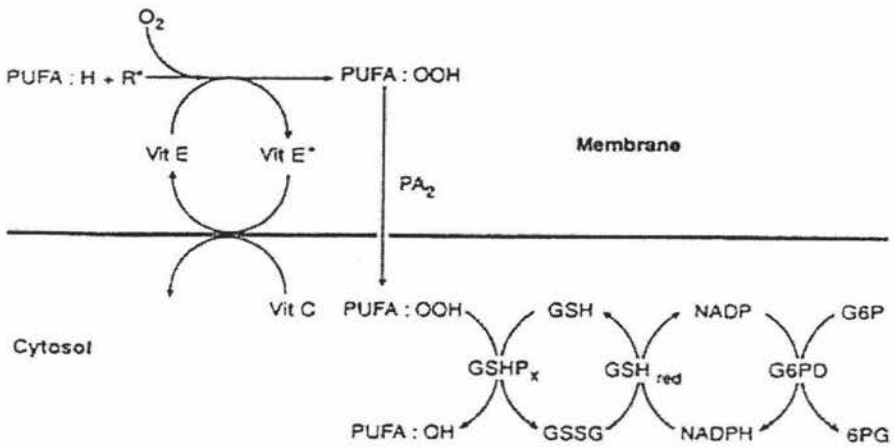


**Figure 3** Interaction of vitamin E and selenium (Duthie, 1993).

R·, free radical; PUFA:H, polyunsaturated fatty acid; PUFA:OOH, polyunsaturated fatty acid hydroperoxides; GSH, reduced glutathione; GSSG, oxidised glutathione; GSHPx, glutathione peroxidase; $PA_2$, phospholipase $A_2$; $GSH_{red}$, glutathione reductase; G6PD, glucose-6-phosphate dehydrogenase; 6PG, 6-phosphogluconate.

1.2.2.6 Interactions of vitamin E with selenium

The antioxidant activities of vitamin E and selenium are closely related and interactive (Duthie, 1993). Vitamin E can protect against many of the symptoms of selenium deficiency and vice versa (McDowell, 1989; Machlin, 1991). Se complementing the antioxidant function of vitamin E is thought to result from reduction of the hydroperoxide production by action of the enzymes, GSH peroxidase and phospholipid hydroperoxide GSH peroxidase. The action by these two Se-containing enzymes allows the vitamin E to exert its chain-breaking action by interacting with lipid peroxyl radicals and therefore spares the requirement for vitamin E (Chow, 1991; Levander, 1992; Duthie, 1993). Combs and Scott (1974) showed *in vitro* lipid peroxidation values in hepatic microsomes from vitamin E-adequate chickens is decreased with the increased dietary supplementation of selenium. Depletion of selenium was found to result in more serious deficiency symptoms including muscular degeneration, and liver necrosis in vitamin E deficient rats (Chow and Chen, 1980; Chow, 1990). However, these symptoms are prevented by selenium included in the diet of rats (Chow, 1991). Synergistic actions of vitamin E and selenium to reduce lipid peroxidation products were demonstrated by Noguchi *et al* (1973). Levels of lipid peroxidation product ($\mu mol \cdot mg^{-1}$ protein) measured

by the thiobarbituric acid (TBA) assay in hepatic microsomes from chickens fed a basal diet was 0.753. The level was decreased to 0.223 and 0.227 in hepatic microsomes from chicken fed basal diet supplemented Se or vitamin E alone, respectively. The level was further decreased to 0.054 when chickens were fed basal diets supplemented with Se and vitamin E.

## 1.3 DIETARY FACTORS AFFECTING VITAMIN E REQUIREMENT OF MAMMALS

There are a number of dietary factors, which affect the vitamin E requirement of animals including humans. These factors may influence the severity of vitamin E deficiency or even alter the syndrome completely (Diplock, 1985). The principal dietary factor affecting vitamin E requirement is polyunsaturated fatty acids (PUFA). To a smaller extent, vitamin E requirements are also influenced by dietary levels of selenium, and sulphur amino acids (SAA). Other dietary factors that influence the vitamin E requirement include synthetic antioxidants, vitamin C, vitamin A, and zinc (Harris and Embree, 1963; Hashim and Asfour, 1968; Diplock, 1985; McDowell, 1989; Machlin, 1991). The following section provides an overview of the above-mentioned dietary factors that affect vitamin E requirement of animals.

### 1.3.1 Polyunsaturated fatty acids

The primary determinant of the vitamin E requirement of animals including humans appears to be the concentration and composition of PUFA in the tissues (Draber, 1993). PUFA are particularly susceptible to oxidation as they contain one or more methylene-interrupted double bonds in their structure (Duthie, 1993). The PUFA content of tissue lipids approaches equilibrium with that of the diet. It has been reported that the dietary vitamin E requirement increases in animals and humans when intake of PUFA increases (Draper, 1980; Machlin, 1991; Hidiroglou *et al.*, 1992). For example, Hidiroglou *et al.* (1992) reported that diets high in linoleate increase the vitamin E requirement to prevent encephalomalacia in the chicken. In humans, Horwitt (1962) demonstrated that doubling the PUFA intake with a fixed amount of dietary vitamin E resulted in a gradual decrease in plasma α-tocopherol.

The levels of PUFA found in unsaturated oil such as cod liver, corn oil, soybean

oil, sunflower seed oil, and linseed oil all increase the vitamin E requirements when incorporated in diets for animals. When diets contain rancid oils and animals consume these, it results in the destruction of body stores of vitamin E (McDowell, 1989). The amount of vitamin E required per g of PUFA is dependent on experimental conditions, species, levels and type of PUFA, and test used which assess the vitamin E status (Draper, 1980). Diplock *et al.* (1967) proposed, after analysis of published results on the vitamin E requirement of experimental animals and human subjects fed diets varying in vitamin E and PUFA content, that the dietary vitamin E requirement per g of PUFA is 0.6 mg. In humans it has been difficult to define the adequacy of diets in term of vitamin E/PUFA ratio, as the composition of human diets is highly variable from time to time and from one to another individual. Plasma vitamin E levels are often used as the criterion to assess dietary vitamin E requirement and a ratio of 0.4 (dietary vitamin E/PUFA) has been suggested to be sufficient in children (Draper, 1980). For a number of species, 0.6 mg of vitamin E per g of PUFA is inadequate and McDowell (1989) noted that 1 mg has been suggested as the minimum. The vitamin E requirement of cats can be expected to higher than other animals, as feline diets often included fish or fish oil that contains a high level of polyunsaturated fatty acids.

### 1.3.2 Selenium

The interrelation between selenium and vitamin E has long been known (Combs *et al.*, 1975; Hill, 1975; Draper, 1980; Levander, 1992). Selenium as well as vitamin E has been shown to be effective in the prevention of nutritional necrotic liver degeneration in rats, as well as in prevention of several field diseases of farm animals, which previously were attributed to vitamin E deficiency. These diseases included nutritional muscular dystrophy in ruminants, exudative diathesis in chickens, necrotic liver degeneration in swine and rat, myopathy of the heart and gizzard in poultry and kidney degeneration in the rat, and mink (Friedrich, 1988).

It is now well accepted that vitamin E and selenium are closely related. Selenium reduces the requirement for vitamin E and delays the onset of vitamin E deficiency syndromes (McDowell, 1989; Machlin, 1991). For instance, vitamin E does not prevent or cure exudative diathesis. However, addition of as little as 0.05 ppm selenium in the diet has been shown to prevent this disease in chickens (Thompson and Scott, 1969). Selenium is known to reduce the requirement for vitamin E at least three ways. It is

required to preserve the integrity of the pancreas, which allows normal fat digestion and thus normal vitamin E absorption. It reduces the amount of vitamin E required to maintain the integrity of lipid membranes via glutathione peroxidase and finally, it aids in an unknown way in retention of vitamin E in blood plasma (McDowell, 1989).

### 1.3.3 Sulphur amino acids

The vitamin E levels required for the prevention of several symptoms of vitamin E deficiency are modified by the intake of sulphur amino acids (Draper, 1980). This effect is most evident in the case of necrotic liver degeneration, which is prevented by vitamin E, cysteine, or selenium (Schwarz and Folta, 1957). Induction of muscular dystrophy in the chicken requires a vitamin E deficient diet containing a low level of sulphur amino acids (Draper, 1980; Machlin, 1991). Although the mechanism of sulphur amino acids in vitamin E deficiency is still not completely understood, it is supposed to be derived from the requirement of SAA in the synthesis of glutathione needed for glutathione peroxidase reaction (Draper, 1993). Leedle and Aust (1990) demonstrated that glutathione can reduce the tocopheroxyl radical. Most of the glutathione in tissues is maintained in the reduced form by glutathione reductase that catalyses the recycling of oxidised glutathione by NADPH (Draper, 1993).

### 1.3.4 Synthetic antioxidants

It is well known that synthetic antioxidants may be used as a substitute for vitamin E (Draper, 1980). N, N'-diphenyl-p-phenylenediamine (DPPD) has been found to be the most effective in the prevention and treatment of deficiency diseases in experimental animals. DPDD as well as ethoxyquine have been shown to replace vitamin E to prevent encephalomalacia, muscular dystrophy and exudative diathesis in chickens (Singsen *et al.*, 1955; Combs and Scott, 1974). The synthetic compound butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are widely used as food and feed antioxidants, have lower biological activity than vitamin E. Other synthetic antioxidants include ethoxyquine (6-ethoxy-2, 2, 4-trimethyl-1, 2-dihydro-quinoline) and methylene blue. The activity of synthetic antioxidants to prevent vitamin E deficiency symptoms has been demonstrated in rats (Draper *et al.*, 1964), chickens (Singsen *et al.*, 1955; Combs and Scott, 1974) and rabbits (Csallany and Draper, 1961).

### 1.3.5 Vitamin C

The functional interrelation between vitamin E and vitamin C has long been recognised. The interaction between these two vitamins is very complex (Chow, 1991). Vitamin C can regenerate or restore the antioxidant properties of vitamin E *in vitro* (Sies *et al.*, 1992). Because of this, the vitamin E requirement should be related to vitamin C status. However, evidence from *in vivo* studies in animals is less consistent.

It was observed that supplementation of diets with ascorbic acid increases the plasma vitamin E level of infants (Arad *et al.*, 1985), vitamin E-deficient guinea pigs (Chen and Chang, 1978) and rats (Chen *et al.*, 1980; Igarashi *et al.*, 1991). For instance, Igarashi *et al.* (1991) studied the effect of vitamin C on vitamin E levels in blood and tissues in rats, and observed that there was a statistically significant increase in the vitamin E levels in plasma, red blood cells, heart, liver, kidneys, spleen and lungs of rats maintained on 50 mg vitamin E·kg$^{-1}$ diet and fed 600 mg vitamin C per kg diet for 6 weeks compared to rats fed 300 mg vitamin C per kg. However, ascorbic acid does not reduce the requirement for vitamin E in pigs *in vivo* (Yen *et al.*, 1985). Burton *et al.* (1990) also found no effect of dietary intake of vitamin C upon vitamin E requirements in the guinea pigs. Burton *et al.* (1993) found that some tissues such as the adrenal gland, lungs, and heart show a significant decrease in vitamin E concentration, when guinea pigs are fed high levels of vitamin C.

### 1.3.6 Vitamin A

The nutritional relationship between vitamin E and vitamin A has been discussed by Green and Bunyan (1969), Draper (1980), Machlin (1991), and Livrea *et al.*, (1996). Vitamin A and E are both very susceptible to peroxidation. As an effective *in vivo* antioxidant, vitamin E can protect vitamin A. High dietary levels of vitamin A have been noted to increase vitamin E requirement of animals as vitamin A depresses utilisation of vitamin E (Schelling *et al.*, 1995). Pudelkiewicz *et al.* (1964) showed that vitamin A acetate in the diet at a level of 50 mg·kg$^{-1}$ or more resulted in lowered liver and plasma tocopherol values in chicken. Blakely *et al.* (1990) reported that high intakes of either retinyl palmitate (42 μmol·kg$^{-1}$ diet) or β-carotene (89 μmol·kg$^{-1}$ diet) depress plasma and liver concentrations of α-tocopherol to about one-half of the normal concentrations in rats. However, Ames (1974) reported that dietary vitamin A supplementation (80, 4,000 and 20,000 IU·100 g$^{-1}$ diet) had no significant effect on the vitamin E requirement of

female rats for reproduction.

### 1.3.7 Zinc

In addition to its role as a component of many enzymes, it is believed that zinc can function as a site-specific antioxidant (Machlin, 1991; Bettger, 1993). Red blood cells from vitamin E- and zinc-deficient animals are very susceptible to peroxidative haemolysis. Supplementation of these diets with zinc or vitamin E results in greater stability of the red blood cells (Machlin and Gabriel, 1980). Bettger *et al.* (1980) showed that skin and joint abnormalities developed in chicken, when zinc-deficient diets containing 30 IU vitamin E per kg were fed. These abnormalities were completely alleviated by the addition of 500 $IU \cdot kg^{-1}$ of vitamin E to the diet. Zinc deficiency in rats also resulted in lowered plasma tocopherol levels (Bunk *et al.*, 1987). These studies suggest that poor zinc status could result in reduced absorption of vitamin E and, therefore, increased vitamin E requirement (Machlin, 1991).

### 1.3.8 Summary

Several dietary factors affect the need of vitamin E in animals including humans. The most important are PUFA and selenium. Vitamin E requirements are increased with increased dietary levels of PUFA. Selenium has a sparing effect on the need for vitamin E. Other main factors that increase vitamin E requirements are a high dietary intake of vitamin A, deficiencies of sulphur amino acids, and deficiencies of zinc. Vitamin E requirements may be decreased with the dietary inclusion of vitamin C and several lipid-soluble synthetic antioxidants (e.g. butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), N,N'-diphenyl-p-p-phenylenediamine (DPPD)).

## 1.4 TARGETED TISSUES AND SIGNS OF VITAMIN E DEFICIENCY

A large number of tissues are affected by vitamin E deficiency in animals (Table 1). In specific, the ones affected appear to depend on the particular species and the dietary levels of other nutrients such as polyunsaturated fatty acids, selenium, and sulphur amino acids (Machlin, 1991). However, in general, the neuromuscular, vascular and reproductive systems are affected by vitamin E deficiency (McDowell, 1989; Combs, 1992). Detailed descriptions of signs of vitamin E deficiency in various animals have

been documented (Friedrich, 1988; McDowell, 1989; Machlin, 1991). Descriptions of the pathology of vitamin E deficiency are also available (Nelson, 1980; Diplock, 1985). The affected tissues and signs of vitamin E deficiency of selected monogastric animal species on pigs, chickens, dogs, rats and cats will be described briefly in the following sections.

### 1.4.1 Affected tissues and signs of vitamin E deficiency in animals

The affected tissues in vitamin E deficiency animals (pig, chicken, dog, rat, cat, mouse, mink, guinea pig, and rabbit) are shown in Table 1. In general, affected tissues include muscle (skeletal, heart and gizzard), nervous system, vascular system, reproductive systems, and adipose tissue as well as the intestines and the liver (Machlin, 1991).

**Table 1 Targeted tissues and vitamin E deficiency symptoms in selected animal species**

| Tissue | Deficiency symptom | Species |
|---|---|---|
| Muscle | | |
| skeletal | Muscular dystrophy | Pig, rat, dog, mink, chicken |
| heart | Muscular dystrophy | Pig, rat, dog, rabbit, guinea pig |
| gizzard | Muscular dystrophy | Turkey |
| Nervous system | | |
| brain (cerebellum) | Encephalomalacia | Chicken |
| nerves | Axonal dystrophy | Rat, dog, mouse, and guinea pig |
| | Lipofuscin accumulation | Rat and mouse |
| Vascular | | |
| blood vessels | Exudative diathesis | Chicken and turkey |
| erythrocytes | Anemia | Pig, rat |
| | *In vitro* haemolysis | Rat, chicken |
| Reproductive | | |
| placental blood vessels | | Pig, rat, mouse, guinea pig, chicken |
| uterus | Fetal death and resorption | Rat |
| testis | Degeneration of epithelium | Pig, rat, rabbit, guinea pig, dog and chicken |
| Adipose tissue | Steatitis (ceroid) | Cat, pig, rat, mouse, and mink |
| Gastrointestine | | |
| intestine | Lipofuscin accumulation | Dog |
| stomach | Gastric ulceration | Pig |
| Liver | Necrosis | Pig, rat and mouse |

Modified from Machlin (1991)

The most common clinical sign of vitamin E deficiency is muscular dystrophy. This occurs in almost all species in the skeletal muscles but is also observed in the heart and some smooth muscles. Some species such as the guinea pig and rabbit develop a

severe debilitating myopathy, when fed diets low in vitamin E. Rats manifest a relatively mild form of myopathy on diets that are free of vitamin E. In contrast, chickens do not develop myopathy unless the diet is simultaneously deficient in sulphur amino acids (Machlin, 1991; McDowell, 1989).

In pigs, vitamin E deficiency causes "Mulberry heart disease". The affected tissues in vitamin E deficiency of pigs include the skeletal and heart muscle, vascular systems, reproductive systems, adipose, stomach and liver. Moreover, clinical signs of vitamin E deficiency in pigs include peripheral cyanosis, dyspnea (abdominal respiration), and a weak pulse (McDowell, 1989). In most cases the clinical signs of vitamin E deficiency in pigs were not observed prior to sudden death. Occasionally it was observed with icterus, difficult locomotion, reluctance to move and weakness (McDowell, 1989).

Three types of diseases are observed as a result of vitamin E deficiency in chickens: exudative diathesis, encephalomalacia and muscular dystrophy (Scott, 1980; McDowell, 1989). The affected tissues in chicken include skeletal muscle, brain, vascular system and reproductive system. Clinical signs of exudative diathesis in chickens include subcutaneous edema, blackening of the affected parts, apathy, and inappetence. Clinical signs of the encephalomalacia which is called "crazy chick disease" is characterised by ataxia, head retraction, and cycling with legs (Scott *et al.*, 1982).

The affected tissues in vitamin E deficiency of dogs include skeletal and heart muscle, central nervous systems, reproductive system, and the intestine. The clinical signs include muscular weakness, subcutaneous edema, anorexia, depression, dyspnea, and eventually coma (Hayes *et al.*, 1970; Van Vleet, 1975).

The signs of vitamin E deficiency in rats include increased red blood cell haemolysis, degeneration of skeletal and heart muscle, lipofuscin accumulation in smooth muscle, fetal death and reabsorption in female rats, degeneration of epithelium of the testes in the male, and liver necrosis (Diplock, 1985; Machlin, 1991).

There are several reasons that account for the wide species difference, the variety of symptoms, and the observation that selenium and /or PUFA may, or may not, affect the manifestation of vitamin E deficiency (Machlin, 1991). First of all, there are species differences in the efficiency with which dietary vitamin E is deposited in tissues. Selenium functions as a constituent of glutathione peroxidase and this enzyme is present in different concentrations in the various tissues of mammalian species (Tappel *et al.*,

1982). Tappel *et al.* (1982) showed that the GSHPx activities in several tissues (liver, kidney, heart, lung, intestine, stomach, muscle, and plasma) of guinea pigs is virtually absent. This may explain why guinea pigs display a serious muscular dystrophy, when fed diets that are a lower in vitamin E. Among tissues investigated in other animals (rat, hamster, mouse, and rabbit), liver and kidney have a relative high activity of GSHPx compared to other tissues e.g. plasma and muscle. For instance, the relative order of activities of GSHPx in the liver of various animal species is: hamster > rabbit = mouse > rat. Finally, the level of labile PUFA, such as cis-11,14-eicosadienoic acid (20:2), cis-5,8,11-eicosatrienoic acid (20:3), arachidonic acid (20:4), cis-5,8,11,14,17-eicosapentaenoic acid (20:5), cis-7,10,13,16,19-docosapentaenoicacid (22:5), and cis-4,7,10,13,16,19-docosahexaenoic acid (22:6), can vary considerably from tissue to tissue and from one species to the other (Machlin, 1991).

### 1.4.2 Affected tissues and signs of vitamin E deficiency in cats

Cordy and Stillinger (1953) reported the first case of vitamin E deficiency in kittens. Cordy (1954) experimentally produced steatitis ("yellow fat disease") in kittens. In this study nine kittens aged 6 to 8 weeks were fed a commercial cat food high in fish content for 8 weeks. Three out of the nine kittens showed severe "steatitis" with pigmentation observed in the adipose tissue of all vitamin E deficient kittens. The study also demonstrated that cats provided with a daily supplementation of 20 or 40 mg of DL-α-tocopherol did not develop steatitis. Gershoff and Norkin (1962) also reported vitamin E deficiency in cats. In their experiment, six kittens aged three to four months were fed a purified diet containing 5 % tuna oil without supplementation of vitamin E. Four kittens showed severe steatitis, myocarditis, and focal myositis of the skeletal muscle (Gershoff and Norkin, 1962). Furthermore, Stephan and Hayes (1978) also reported that signs of the vitamin E deficiency including weight and hair loss, dry and scaly skin, and moderate anaemia were observed when seven cats were fed a semi-purified diets containing 15 % stripped safflower seed oil without vitamin E supplementation.

Since the first report of vitamin E deficiency in kittens by Cordy and Stillinger (1953), there have been many case reports of vitamin E deficiency in both kittens and adult cats (Coffin and Holzworth, 1954; Munson *et al.*, 1958; Griffiths *et al.*, 1960; Kolata, 1971; Watson *et al.*, 1973; Gaskell *et al.*, 1975; Flecknell and Gruffydd-Jones, 1978; Cropper, 1980; Summers *et al.*, 1982; Koutinas *et al.*, 1993; Tidholm, 1996;

Vercelli and Kramer, 1998). Many of the latter cases were attributed to the feeding of fish or fish-based diets and presumably the result of the high intake of PUFA from fish oil. Other cases were attributed to the feeding of commercial cat foods (Cropper, 1980; Vercelli and Kramer, 1998) and the feeding of liver (Kolata, 1971; Watson *et al.*, 1973). All studies and case reports published showed that the adipose tissue was the major affected tissue in vitamin E deficient cats. Selenium deficiency has not been observed in cats (NRC, 1986). Muscle dystrophy has also been observed in the presence of adequate selenium as a result of vitamin E deficiency, when cats were fed diets based on fish (Draper, 1980). Reviewing all of the case reports and experimental studies on vitamin E deficiency in cats over the last five decades, there appears to be no effect of age or sex on the occurrence of the vitamin E deficiency in cats.

The clinical signs of vitamin E deficiency in cats, according to its increasing occurrence, include reduced appetite, body weight and hair loss, depression, dry and scaly skin, tense and sensitive in the abdomen, reluctance to move, and pain which are most evident when the cat is touched on the back lateral and ventral abdomen and base of the tail (Cordy and Stillinger, 1953; Cody, 1954; Coffin and Holzworth, 1954; Munson *et al.*, 1958; Griffiths *et al.*, 1960; Kolata, 1971; Watson *et al.*, 1973; Gaskell *et al.*, 1975; Flecknell and Gruffydd-Jones, 1978; Stephan and Hays, 1978; Cropper, 1980; Summers *et al.*, 1982; Koutinas *et al.*, 1993; Tidholm, 1996; Vercelli and Kramer, 1998).

## 1.5 VITAMIN E REQUIREMENTS OF SEVERAL MAMMALIAN SPECIES

The minimum vitamin E requirement for growing and adult animals of several mammal species is presented in Table 2. It can be seen that the vitamin E requirements, expressed as IU per kg of diets dry matter, for adult pigs, dogs, and rats are comparable at about 30 IU per kg of diet. The vitamin E requirement for the growing mink, rat and dog are also similar (24 to 30 IU per kg of diet), while growing pigs have a lower vitamin E requirement. The vitamin E requirement for growing and adult cats recommended by either NRC (1986) or AAFCO (1997) was 30 IU per kg diet. This value is equal to the vitamin E requirement of the adult rat, dog and pig recommended by the NRC. The vitamin E requirements for cats however were extrapolated from these animals species (Scott, 1980). The vitamin E requirements of both children and adult humans are much lower than that of other monogastric mammals (rat, dog and pig). The vitamin E

requirement presented in Table 2 have been determined based on animals fed diets containing a lower level of PUFA (less than 10 % of diet). It has also been noted that the minimum requirement for vitamin E in species should be increased four to five times when animals are fed diets containing high levels of PUFA. The vitamin E requirement of cats fed diets containing high levels of PUFA from fish oil is at present unknown, although AAFCO (1997) recommended that 10 IU of vitamin E should be added per g of added fish oil per kg of diet. No research has been undertaken to substantiate the accuracy of the latter value.

**Table 2 The minimum vitamin E requirements for growing and adult animals of several mammalian species[a]**

| Species | Growing | Adult | Reference |
|---|---|---|---|
| | ($IU \cdot kg^{-1}$) | | |
| Pig | 16 | 27 | NRC (1988a) |
| Dog | 24 | 27 | NRC (1985) |
| Rat | 30 | 30 | NRC (1995) |
| Human | 5-7 | 8-12 | NRC (1989) |
| Mink | 25 | ND | NRC (1981) |
| Cat | 30 | 30 | NRC (1986) |

ND, not determine.

[a]All diets contain 16.7 MJ $ME \cdot kg^{-1}$ dry matter.

## 1.6 METHODS OF ASSESSING VITAMIN E STATUS

Vitamin E status is best assessed by tissue analysis of tocopherol and in experimental circumstances by evaluation of one or more tests for vitamin E function. Information is more useful if an assessment of selenium status is also made either by tissue analysis of selenium, or by determination of Se-dependent glutathione peroxidase activity (GSHPx) (Combs, 1981). Blood plasma or serum is the most useful tissue to assess the current vitamin E status of an animal. Tests for vitamin E status can include the measurement of red blood cell (RBC) resistance to haemolysis in the presence of dialuric acid or $H_2O_2$, measurement of protection against specific deficiency diseases e.g. encephalomalacia in chicks or exudative diathesis in Se-deficient chicks, and measurement of lipid peroxides (Combs, 1981). Plasma α-tocopherol, RBC haemolysis, and lipid peroxides assays as well as lymphocyte proliferation assays that are most often used in the assessment of vitamin E status are discussed here.

### 1.6.1 Plasma α-tocopherol

The nutritional status of an animal with respect to vitamin E is commonly determined from the plasma (or serum) concentration of α-tocopherol. There is a relatively high correlation between dietary vitamin E level and plasma (serum) α-tocopherol as well as α -tocopherol levels in other tissues e.g. liver. This has been observed in rats (Bieri and Poukka, 1970; Bendich *et al.*, 1986; Cho and Choi, 1994; Farwer *et al.*, 1994), pigs (Wang *et al.*, 1996), rabbits (Verschuren *et al.*, 1990), and mice (Meydani *et al.*, 1987) within a rather wide range of vitamin E intake. Plasma α-tocopherol concentration is not only dependent on dietary intake of vitamin E but also varies with plasma lipoprotein content, as there is a high correlation between plasma concentration of α-tocopherol and total plasma lipids or cholesterol (Traber *et al.*, 1993).

**Table 3 Plasma α-tocopherol status in healthy animals**

| Species | n | α-Tocopherol | | Cholesterol | Ratio[a] |
|---|---|---|---|---|---|
| | | Range | Mean | | |
| | | ($\mu g \cdot ml^{-1}$) | | ($mg \cdot ml^{-1}$) | |
| Cattle | 114 | NA | 2.3 | 1.43 | 1.6 |
| Sheep | 22 | 1.3-2.5 | 1.9 | 0.50 | 3.8 |
| Mute Deer | 14 | 0.8-4.2 | NA | 0.87 | 2.2 |
| Pigs | 10 | 2.0-4.0 | 3.0 | 0.92 | 3.3 |
| Dogs | 25 | 2.7-9.3 | 6.0 | 1.35 | 4.4 |
| Cats | 29 | 3.0-11.0 | 7.0 | 1.67 | 4.2 |
| Mink | 30 | 11.0-22.0 | 14.8 | NA | NA |
| Wolves/foxes | 70 | NA | 5.8 | 2.22 | 2.6 |
| Lions/tigers | 187 | NA | 8.1 | 1.72 | 4.7 |

Adapted from Baker (1986) and Dierenfeld and Traber (1993).
NA, data not available.
[a]Ratio of α-tocopherol to cholesterol.

Plasma levels of α-tocopherol in healthy animals are shown in Table 3. It can be seen that the concentration varies widely among species, 1.9 $\mu g \cdot ml^{-1}$ in sheep to 13.8 $\mu g \cdot ml^{-1}$ in mink (Dierenfeld and Traber, 1993). Plasma cholesterol has been suggested as a more practical component than total lipids for comparative assessment of vitamin E status between species, as vitamin E is transported in plasma lipoproteins and the distribution of various plasma lipoprotein fractions differs considerably among animal species (Dierenfeld and Traber, 1993). Because of this, the ratio of vitamin E to cholesterol is also shown in Table 3. Baker *et al.* (1986) also reported that the plasma α-

tocopherol content in healthy cats ranges from 3 to 11 $\mu g \cdot ml^{-1}$, with a mean plasma α-tocopherol level of 7 $\mu g \cdot ml^{-1}$. Feline (cats, lions and tigers) seem to have the highest vitamin E to cholesterol ratio (4.2-4.7) compared to those of carnivores (pigs, wolves/foxes) (2.6-3.3) except dogs. The herbivores seem to have a lower vitamin E to cholesterol ratio (1.6-3.8).

**Table 4 Plasma α-tocopherol status in selected vitamin E deficient animal species**

| Species | Diet vitamin E | Plasma α-tocopherol | Time | Reference |
|---|---|---|---|---|
| | ($mg \cdot kg^{-1}$ DM) | ($\mu g \cdot ml^{-1}$) | (d) | |
| Pig | 0 | < 0.1 | NS | Wang *et al.* (1996) |
| Rat | 0 | < 0.5 | 63 | Hakkarainen *et al.* (1986) |
| Dog | NS | 0.6 | NS | Hayes *et al.* (1970) |
| | 0.05 | 1.5 | NS | Pillai *et al.* (1991) |
| | 0.08 | 0.07-0.11 | 90 | Pillai *et al.* (1992) |
| Cat | 0 | 0.3[a] | 240 | Gershoff and Norkin (1962) |
| | 0 | 1.2-3.5 | 270-360 | Stephan and Hayes (1978) |
| Cattle | NS | < 1.5 | NS | Adams (1982) |

NS, not specified; DM, dry matter.
[a]Blood serum.

The plasma α-tocopherol status in vitamin E deficient pigs, rats, dogs, cats, and cattle, is shown in Table 4. In general, a plasma α-tocopherol level of less than 1.5 $\mu g \cdot ml^{-1}$ seemed to result in vitamin E deficiency in most monogastric animals (McDowell, 1989). In the case of the cat, plasma α-tocopherol levels of 1.2 to 3.5 $\mu g \cdot ml^{-1}$ were also obtained in the deficient cats (Stephan and Hayes, 1978). It is interesting to note that the first sign of vitamin E deficiency in cats were noted after at least 240 days after the start of feeding a vitamin E free diet. In other animals vitamin E deficiency seem to occur before 60-90 days after the start of feeding a vitamin E free diet.

The quantitative analysis of vitamin E in biological samples e.g. plasma, and in foods and feeds is commonly measured by high performance liquid chromatography (HPLC), as this method is sensitive, accurate, reproducible and allows the identification of different tocopherols (Hashim and Schuttringer, 1966; McMurray and Blanchflower, 1979). Vitamin E is extracted from plasma or feed samples and separated by reverse phase chromatography from other compounds.

### 1.6.2 Red blood cell hydrogen peroxide haemolysis assay

Several functional assays based on RBC cell membrane fragility have been developed to complement plasma or serum α-tocopherol measurements. These include detergent sensitivity, osmotic fragility, spontaneous haemolysis, dialuric acid haemolysis and hydrogen peroxide haemolysis assays. Studies in pigs (Fontaine and Valli, 1977), chickens (Fischer *et al.*, 1970), humans (Gordon and Nitowsky, 1956; Horwitt *et al.*, 1956, Hasim and Asfour, 1968), rats (Bieri and Poukka, 1970), and dogs (Pillai *et al.*, 1992) have demonstrated that the rate of RBC haemolysis correlates inversely with serum or plasma tocopherol levels and increases in vitamin E deficiency. Mehorn *et al.* (1971) indicated, however, that this type of assay lacks specificity and that the values are variable. Changes in the status of other nutrients can influence the rate of RBC haemolysis. For example, in children with cholestasis, the haemolysis test is reported to underestimate the degree of vitamin E deficiency, as indicated by plasma vitamin E, vitamin E to lipid ratio, and malondialdehyde (Cynamon and Isenberg, 1987).

The sensitivity of *in vitro* RBC haemolysis assays as an indicator of vitamin E status varies according to species. Detergent sensitivity of RBC correlates with plasma vitamin E status in goats (Hodate and Hamada, 1984), and sheep (Stevenson and Jones, 1989). In a comparative study of four *in vitro* RBC haemolysis assays in adult dogs, Pillai *et al.* (1992) concluded that the RBC dialuric acid haemolysis assay is the most sensitive *in vitro* assay for diagnosing vitamin E deficiency in adult dogs. However, Bei *et al.* (1995) showed that spontaneous RBC haemolysis is not affected by either vitamin E depletion or supplementation in mice. In humans, the RBC hydrogen peroxide haemolysis assay has been used to assess vitamin E status, but standardisation of the assay has proven to be difficult (Farrell, 1980). Gershoff and Norkin (1962) used the RBC hydrogen peroxide haemolysis assay to assess the vitamin E status of cats. These authors concluded that the hydrogen peroxide haemolysis assay is unlikely to be of much practical value in determining vitamin E deficiency. However, no studies have been undertaken to evaluate this assay in assessing vitamin E status of cats as Gershoff and Norkin (1962) used a standard assay originally developed for humans and rats.

### 1.6.3 Lipid peroxides assays

Vitamin E functions as an antioxidant *in vivo*, protecting membranes from lipid peroxidation. Several assays based on the measurement of products of lipid peroxidation

e.g. lipid hydroperoxides, malondialdehyde, ethane and pentane have been used as a functional test for vitamin E status in animals including humans. These include the thiobarbituric acid assay (Cho *et al.*, 1995; Allard *et al.*, 1997; Vannucchi *et al.*, 1997), breath pentane analysis (Tappel and Dillard, 1981), erythrocyte malondialdehyde release *in vitro* assay (Cynamon *et al.*, 1985) and lipid peroxides assays (LPO) (Allard *et al.*, 1997). The TBA assay and LPO assay are most frequently used. The TBA assay measures the acid breakdown of lipid hydroperoxides into malondialdehyde (MDA), which then reacts with thiobarbituric acid to produce substances that can be measured colorimetrically (Slater, 1984). The LPO assay directly and specifically measures lipid hydroperoxide levels in biological samples e.g. blood plasma or serum. In the presence of haemoglobin, lipid hydroperoxides are reduced to hydroxyl derivatives (lipid alcohols) which oxidatively cleave colorless 10-N-methylcarbamoyl-3,7-dimethylamino-10 H-phenothiazine (MCDP) into methylene blue (Ohishi *et al.*, 1985). A commercial kit is also available. The TBA assay has the advantage of being simple and sensitive (Slater, 1984), but there are also disadvantages. The TBA assay uses thiobarbituric acid that does not react efficiently with some products of the peroxidation such as 4-hydroxyalkenals. Consequently, the TBA reaction may not adequately reflect the biological consequence of lipid peroxidation (Slater, 1984). A second disadvantage is that other degradation products may also react with the reagent. For example, DNA has been shown to be degraded to malondialdehyde (Satoh, 1978). Sialic acid as well as bilirubin also has a great influence on lipid peroxides determination in serum (Satoh, 1978; Slater, 1984; Yagi, 1984). An improved colorimetric TBA assay reported by Satoh (1978) seems to be more suitable for determination of lipid peroxides in blood serum, as the method is free from the interference by sialic acid. A fluorometric TBA assay for blood plasma or serum has been described by Yagi (1976). However, this method is less satisfactory because of lack of reproducibility as indicated by Satoh (1978).

The LPO assay is often used to determine the vitamin E requirement of animals. No sample extraction is required for blood serum or plasma with this method and it quantitates lipid hydroperoxides directly. Furthermore, the LPO assay is applicable to an automated analyser.

### 1.6.4 Whole blood lymphocyte proliferation assay

*In vitro* response of lymphocytes to mitogens has been a widely used laboratory method

to assess the cell-mediated immune response in experimental and clinical immunology (Tham *et al*, 1982; Beharka *et al.*, 1997). Traditionally, lymphocyte proliferation *in vitro* is determined by counting cells directly. These assays are labour-intensive and, therefore, not practical for evaluation of a large number of samples. Methods for measuring the incorporation of [$^3$H]-thymidine into proliferating cells and an improved lymphocyte proliferation ELISA assay have been developed.

The ELISA method is based on the measurement of 5-bromo-2'-deoxyuridine (Brdu) incorporation during DNA synthesis in proliferative cells and has been showed to be accurate, fast, and convenient (Porstmann *et al.*, 1985). A commercial kit for the latter assay is also available. However, the ELISA assay requires separation of lymphocytes from whole blood, and therefore, requires a large volume of whole blood. Furthermore, this assay has been noted to still be laborious (Tham *et al.*, 1982; Jensen *et al.*, 1988b). The latter has led to the development of whole blood lymphocytes proliferation assays. The advantages using whole blood instead of separated lymphocytes includes reduction in size of blood volume, time needed for separation of lymphocytes and, reduction of cost (Fasanmade and Jusko, 1995). *In vitro* blastogenesis of lymphocytes to mitogens using whole blood cultures have been reported in rats (Fasanmade and Jusko, 1995), pigs (Jensen *et al.*, 1988b; Nemec *et al.*, 1994), dogs (Shifrine *et al.*, 1978) as well as cats (Tham *et al.*, 1982). Tham *et al.*, (1982) showed that optimal response for feline lymphocytes to concanavalin A (Con A) was obtained in whole blood cultures incubated for three days and labelled with 1 μCi tritiated thymidine ($^3$H-TdR) for the last 16 hours. The range in stimulation index (SI) of feline lymphocyte to Con A using whole blood cultures was 105 to 186. Tham *et al.*, (1982) also showed that feline lymphocytes responded poorly to phytohemagglutinin (PHA).

The level of *in vitro* proliferation by lymphocytes in response to mitogens has been suggested to be influenced by vitamin E level (Langweiler *et al.*, 1981; Eskew *et al.*, 1985; Bendich *et al.*, 1986; Moriguchi *et al.*, 1990; Meydani *et al.*, 1990; Kowdley *et al.*, 1992). Lymphocytes derived from animals (dogs and rats) including humans maintained on vitamin E-deficient diets show depressed mitogenic response to B-cell or T-cell mitogens. Langweiler *et al.* (1981) reported that vitamin E-deficient dogs decreased lymphocytes response to Con A. Eskew *et al.* (1985) also found that either vitamin E or selenium deficiency suppressed rat lymphocyte proliferation and low levels of dietary vitamin E were insufficient to enhance lymphocyte proliferative responses, even though

the diet contained all other nutrients at recommended levels. Kowdley *et al.* (1992) treated a patient with 100 $IU \cdot d^{-1}$ of oral vitamin E for 3 months. T cells responses to Con A, PHA and delayed-type hypersensitivity were measured before and after vitamin E repletion. The results showed that the patient displayed depressed delayed-type hypersensitivity, decreasing mitogenic response to Con A and PHA which were corrected by vitamin E supplementation.

It has been shown that addition of vitamin E above the recommended levels enhances blastogenesis to mitogen of lymphocyte in various animal species (rats, mice, pigs and calves) as well as humans. Corwin and Shloss (1980a, b) reported that lymphocytes from mice supplemented with varying levels of vitamin E showed enhanced Con A proliferation when dietary levels were in excess of the vitamin E content of the normal diet. Moriguchi *et al.* (1990) also showed that supplementation of rats with 100-2500 ppm vitamin E for 10 days increased mitogenic response of splenocytes to Con A and lipopolysaccharide from *Escherichia Coli*. Bendich *et al.* (1986) demonstrated that a dietary vitamin E concentration of 15 $mg \cdot kg^{-1}$ was adequate to prevent myopathy in rats, while optimal lymphocyte proliferation to Con A and PHA was only obtained at much higher levels of vitamin E (50-200 $mg \cdot kg^{-1}$). Also, lymphocytes from healthy elderly humans consuming a diet supplemented with 800 $IU \cdot d^{-1}$ of vitamin E showed increased mitogenic responses to Con A compared to the cells from elderly subjects consuming a diet with normal level of vitamin E (Meydani *et al.*, 1990). It was suggested by Beharka *et al.* (1997) that immune parameters should be selected as indicator of vitamin E status.

### 1.6.5 The ferric reducing ability of plasma assay

The ferric reducing ability of plasma (FRAP) has been presented as a novel method for assessing water-soluble antioxidant capacity of blood plasma. Those water soluble antioxidants include uric acid, ascorbic acid, bilirubin, trolox (a water-soluble analogy of vitamin E), albumin and the fat soluble antioxidant: α-tocopherol (Benzie and Strain, 1996). Benzie and Strain (1996) reported that α-tocopherol, ascorbic acid, protein, and bilirubin account for 5, 15, 10 and 5 % of the FRAP value of fresh plasma, while uric acid is estimated to contribute around 60 % to the FRAP value. In vitamin E deficient animals FRAP can be reduced and this assay can potentially be used to as a response variable to determine the vitamin E requirement of animals. There are currently no data available on FRAP values in animals including cats.

## 1.7 DETERMINATION OF VITAMIN E REQUIREMENTS OF ANIMALS

The conventional approach taken for determining vitamin E requirements in animals relating to a particular physiological stage is to determine the level of vitamin E required to maintain a specific growth rate, to prevent clinical symptoms of vitamin E deficiency, to prevent lipid peroxidation or achieve a certain RBC haemolysis. Concurrently, plasma or serum tocopherol levels are often determined (Beharka *et al.*, 1997). This approach, however, may not adequately predict the optimal level of vitamin E needed to maintain immunological health. The vitamin E requirement of animals is highly dependent on the response criteria used, as is apparent from the following example. The vitamin E required to prevent spontaneous haemolysis of red blood cells in rats has been suggested to be 14.5 to 20 IU per kg diet when the dietary levels of linoleic acid are less than 10 % (Jager and Houtsmuller, 1970). The vitamin E requirement increases to 22 IU when dietary linoleic acid is increased to 13 % (Jager and Houtsmuller, 1970; Bieri, 1972). The vitamin E required to prevent production of lipid peroxides measured by the TBA method was 30 IU per kg diet, when rats were fed diets containing a high levels of lipid (20 %) (Buckingham, 1985). This value is equal to the vitamin E requirement of rats recommended by the NRC (1995).

**Table 5 Vitamin E requirements of selected animal species using different response criteria[a]**

| Animals | Response criteria | | | Reference |
|---|---|---|---|---|
| | Growth | Deficiency | Immune response | |
| | | ($IU \cdot kg^{-1}$ DM) | | |
| Cat | 30 | 30 | NA | Gershoff and Norkin (1962) |
| Rat | 7.5 | 15 | 50-200 | Bendich *et al* (1986) |
| Dog | 24 | 30 | NA | Van Vleet (1975)<br>NRC (1985) |
| Pig | 10-15 | 10-15 | 60 | Meyer *et al* (1981)<br>Wuryastuti *et al* (1993) |
| Calves | 40 | 15-60 | 200 | Morrill and Reddy (1988)<br>NRC (1988b) |

NA, data not available; DM, dry matter.
[a]All diets contain low levels of PUFA.

Reddy *et al.* (1986) and Beharka *et al.* (1997) noted that vitamin E requirements should also be determined using immune response parameters. The vitamin E

requirements determined by the measurement of growth rate and prevention of signs of vitamin E deficiency using more conventional approaches and by immune response parameters for selected monogastric animal species is listed in Table 5. It can be seen that the vitamin E requirements determined by immune response parameters are 4 to 10 times higher than those obtained by more conventional approaches.

## 1.8 DETERMINATION OF VITAMIN E REQUIREMENT IN CATS

There is little information on the vitamin E requirements of cats. As discussed in section 1.5 of this review, the vitamin E requirements recommended by either NRC (1986) or AAFCO (1997) for growing and adult cats is 30 IU per kg of diet. This value was extrapolated from research on other animal species including pigs, rats and dogs. Vitamin E deficiency in cats was studied by Gershoff and Norkin (1962). These authors demonstrated that diets supplemented with 30 $IU \cdot kg^{-1}$ are sufficient to prevent " steatitis" in kittens. The diets used by Gershoff and Norkin (1962) contained a low level of PUFA (5 % tuna oil). However, the dietary level of vitamin E required to obtain a lower level of the RBC hydrogen peroxide haemolysis in the study of Gershoff and Norkin (1962) was 132 $IU \cdot kg^{-1}$. Based on the RBC hydrogen peroxide haemolysis response, the vitamin E requirement of cats should be higher than then the current recommendations by NRC (1986) and AAFCO (1997). Since the study of Gershoff and Norkin (1962), no further studies have been conducted to determine the vitamin E requirements of cats and at present the vitamin E requirements of cats fed diets containing high levels of polyunsaturated fatty acids are unknown. In order to prevent vitamin E deficiency in cats fed commercially sold, fish oil containing diets, the Association of America feed control officials (AAFCO, 1997) recommended the supplementation of diets containing fish oil with 10 IU of vitamin E for every g of fish oil per kg diet. However, no research has been conducted to substantiate this value and at present it is unknown whether this recommendation is inadequate or provides an excess of vitamin E.

## 1.9 INFERENCES FROM REVIEW OF LITERATURE

A large number of tissues can be affected by vitamin E deficiency in animal species. The specific tissues affected appear to depend on the species but in general affect the

neuromuscular, vascular and the reproductive systems. Two main approaches are often used determining the vitamin E requirement of animals. The conventional approach taken for determining vitamin E requirements in animals is to determine the level of vitamin E required to maintain a specific growth rate, to prevent clinical symptoms of vitamin E deficiency, to prevent lipid peroxidation or achieve a certain RBC haemolysis. The other is to optimise immunological health. There is still a paucity of information on the vitamin E requirement of the adult cat, especially cats that are fed diets containing a high level of fish oil/polyunsaturated fatty acids. "Steatitis" has been reported in cats fed commercial diets and has also been experimentally induced. Based on this review, valuable study objectives would be: (1) determining the requirement for vitamin E (α-tocopherol) of adult cats fed a high level of fish oil/polyunsaturated fatty acids, and (2) provide baseline data on α-tocopherol levels in blood plasma of adult cats fed diets containing high levels of polyunsaturated fatty acids and different levels of vitamin E that may be used in the early diagnosis of vitamin E deficiency.

# CHAPTER 2

# MATERIALS AND METHODS

The study reported here was approved and followed the procedures set out by the Massey University Animal Ethics Committee (Anonymous, 1992).

**Table 1 Ingredient composition of the experimental basal diet**

| Ingredient | Amount ($g \cdot kg^{-1}$ dry matter) |
|---|---|
| Frozen salmon | 159.3 |
| Salmon fish oil | 228.0 |
| Salmon meal protein | 239.3 |
| Soy protein concentrate | 136.3 |
| Rice | 178.8 |
| Sodium chloride | 8.3 |
| Potassium chloride | 8.3 |
| Tomato pomace | 7.6 |
| Pyrophosphate | 7.8 |
| Choline chloride | 5.0 |
| Lecithin | 6.6 |
| Vitamin-mineral premix[a] | 4.8 |
| DL-methionine | 4.0 |
| Citric acid | 3.3 |
| Taurine | 2.7 |

[a]Provided the following ($g \cdot kg^{-1}$ diet): $CaHPO_4 \cdot H_2O$ 31.8, $CaCO_3$ 1.2, $MgSO_4 \cdot 7H_2O$ 4.2, $FeSO_4 \cdot H_2O$ 0.3, $ZnSO_4 \cdot H_2O$ 0.14 and ($IU \cdot kg^{-1}$ diet) vitamin A 14287.5, vitamin $D_3$ 1905, and ($mg \cdot kg^{-1}$ diet) nicotinic acid 57.2, pantothenate 9.5, thiamin hydrochloride 57.2, riboflavin 7.6, pyridoxine hydrochloride 6.7, vitamin $B_{12}$ 0.03, Folic acid 2.9, menadione 1.9, inositol 47.6, β-carotene 5.7, $MnSO_4 \cdot H_2O$ 16.8, $CuSO_4 \cdot 5H_2O$ 21.0, KI 0.18, cyanocobalamin 2.4

## 2.1 ANIMALS AND DIETS

Thirty-two (16 male, 16 female) 5-8 year old domestic short-haired cats (*Felis catus*) from the Heinz Wattie's Companion Animal Nutrition Research Unit (Massey University, Palmerston North, New Zealand) with an initial body weight range of 2292 to 5505 g (mean ± SEM, 3587 ± 146 g) were used. All cats had been fed a moist canned cat food that passed a minimum feeding protocol for proving an adult maintenance claim for a cat

food (AAFCO, 1997) for 6 months. Four male and four female cats were randomly allocated to one of four large outdoor pens and fed one of four experimental diets varying in vitamin E content. The ingredient composition of the experimental basal diet is shown in Table 1. The chemical composition and fatty acid profile of the four experimental diets is shown in Table 2-4.

**Table 2 Chemical composition of the four experimental diets**

| Ingredient | Diet | | | |
|---|---|---|---|---|
| | A | B | C | D |
| | | ($IU \cdot kg^{-1}$ dry matter) | | |
| Vitamin E | 24 | 1197 | 2830 | 4314 |
| | | ($g \cdot kg^{-1}$ dry matter) | | |
| Crude protein | 381 | 393 | 389 | 394 |
| Lipid | 299 | 289 | 276 | 292 |
| Ash | 78.3 | 79.3 | 75.8 | 72.3 |
| Cysteine | 2.1 | 2.5 | 2.4 | 2.1 |
| Methionine | 11.9 | 13.2 | 12.1 | 12.0 |
| Taurine | 3.6 | 3.9 | 3.7 | 3.3 |
| Aspartic acid | 35.1 | 36.7 | 34.8 | 33.8 |
| Threonine | 14.3 | 15.4 | 14.2 | 13.5 |
| Serine | 16.4 | 16.6 | 16.0 | 15.1 |
| Glutamic acid | 53.3 | 54.8 | 52.0 | 52.0 |
| Proline | 24.1 | 22.3 | 23.0 | 22.6 |
| Glycine | 33.4 | 31.2 | 30.6 | 32.2 |
| Alanine | 21.9 | 22.1 | 20.6 | 21.2 |
| Valine | 14.3 | 15.0 | 14.7 | 14.0 |
| Isoleucine | 13.3 | 14.4 | 13.4 | 13.3 |
| Leucine | 23.9 | 25.4 | 24.3 | 23.9 |
| Tyrosine | 10.7 | 10.8 | 10.3 | 10.0 |
| Phenylalanine | 13.7 | 14.4 | 13.8 | 13.6 |
| Histidine | 7.1 | 7.9 | 7.7 | 8.5 |
| Lysine | 21.3 | 23.0 | 21.3 | 21.0 |
| Arginine | 25.9 | 25.9 | 25.0 | 25.3 |

The four experimental diets were formulated from the basal diet and supplemented with 0, 1500, 3000 and 4500 IU DL-α-tocopheryl acetate per kg dry matter, respectively. The fish oil used in the basal diets was a non-stabilised oil and was stored under nitrogen before being included in the diets. The experimental diets were produced in four batches, heat-sterilised and canned by Heinz Pet Products (Terminal Island, Los Angeles, California, USA). All four experimental diets were analysed for nitrogen, lipid, ash,

amino acids, vitamin E (α-tocopherol), and fatty acid content. Diet A, B, C and D contained a calculated metabolisable energy (ME) content of 19.7, 19.5, 19.3 and 19.7 $MJ \cdot kg^{-1}$ dry matter, respectively. The diets were randomly allocated to one of the four pens. The cats were fed to appetite and fresh water was available at all times. The animals were weighed weekly until the end of the study and feed intake of each pen was recorded daily over the 126 d study.

**Table 3 The fatty acid profile of the four experimental diets**

| Name | Fatty acids | Diet | | | |
|---|---|---|---|---|---|
| | | A | B | C | D |
| | | ($mg \cdot g^{-1}$ dry matter) | | | |
| Lauric acid | 12:0 | 1.7 | 1.5 | 1.1 | 2.2 |
| Myristic acid | 14:0 | 14.6 | 10.7 | 14.8 | 15.3 |
| Palmitic acid | 16:0 | 42.6 | 46.0 | 41.5 | 44.7 |
| Palmitoleic acid | 16:1 | 15.5 | 17.0 | 18.3 | 19.7 |
| Stearic acid | 18:0 | 2.0 | 2.0 | 1.8 | 2.1 |
| Oleic acid | 18:1 (n-9) | 11.4 | 10.6 | 10.2 | 10.3 |
| Linoleic acid | 18:2 (n-6) | 55.0 | 55.3 | 59.5 | 63.1 |
| Linolenic acid | 18:3 (n-3) | 17.4 | 17.8 | 18.3 | 20.0 |
| Eicosenoic acid | 20:1 | 24.4 | 22.4 | 23.6 | 22.2 |
| Arachidonic acid | 20:4 (n-6) | 23.0 | 21.4 | 22.3 | 23.9 |
| Eicosapentaenoic acid | 20:5 (n-3) | 17.7 | 17.7 | 18.8 | 19.1 |
| Docosapentaenoic acid | 22:5 (n-3) | 6.2 | 6.4 | 7.1 | 7.1 |
| Docosahexaenoic acid | 22:6 (n-3) | 24.0 | 23.1 | 24.3 | 26.2 |

## 2.2 BLOOD SAMPLE COLLECTION AND PROCESSING

A blood samples (± 2.5 ml) was collected from each cat by jugular venipuncture using a 5 ml springe and size 25 needle at biweekly intervals throughout the experimental period. Each blood sample was quantitatively transferred into a heparinised tube and gently shaken. A subsample (0.25 ml) of the heparinised blood was pipetted into a 1.5 ml Eppendorf tube in a airflow cabinet for the determination of lymphocyte proliferation. Another subsample (0.4 ml) of heparinised blood was pipetted into an alcohol rinsed glass tube (10 x 75 mm) for the determination of red blood cell hydrogen peroxide haemolysis. The remaining heparinised blood (± 1.8 ml) was centrifuged (1200 x g ) for 20 min and 0.5 ml of plasma of each sample was pipetted into a 2.0 ml Eppendorf tube to which 10 µl butylated hydroxytoluene (BHT) solution (0.9 mM in ethanol solvent) had been added for

the analysis of α-tocopherol. Another 0.2 ml of blood plasma was pipetted into a 0.7 ml Eppendorf tube for immediate analysis of lipid peroxides, the ferric reducing ability of plasma (FRAP), alkaline phosphatase and triglycerides using a Cobas Fara II autoanalyser (Hoffmann-La Roche, Basel, Switzerland).

**Table 4 Calculated fatty acid profile of the four experimental diets**

| | Diet | | | |
|---|---|---|---|---|
| | A | B | C | D |
| | | $mg \cdot g^{-1}$ dry matter | | |
| Σ SFA | 60.9 | 60.2 | 59.2 | 64.3 |
| Σ MUFA | 51.3 | 49.9 | 52.0 | 52.2 |
| Σ PUFA | 143.2 | 141.7 | 150.3 | 159.4 |
| Σ (n-6) | 78.0 | 76.7 | 81.8 | 87.0 |
| Σ (n-3) | 65.2 | 65.0 | 68.5 | 72.3 |
| Σ fatty acids | 255.4 | 251.8 | 261.6 | 275.8 |
| Σ n-6/Σ PUFA | 48.4 | 48.7 | 49.0 | 49.9 |
| Σ n-3/Σ PUFA | 37.5 | 38.2 | 37.9 | 38.3 |
| n-6/n-3 | 1.2 | 1.2 | 1.2 | 1.2 |
| Unsaturation index[a] | 222.6 | 221.9 | 225.7 | 225.5 |
| Lipid peroxides | ND | ND | ND | ND |

ND, not detected.
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[a]Unsaturation index equals the sum of the number of double bonds in each fatty acid multiplied by fatty acid percentage of the total fatty acids (Wander *et al.*, 1997).

## 2.3 WHOLE BLOOD AND BLOOD PLASMA ASSAYS

### 2.3.1 Chemicals used

*Plasma vitamin E (α-tocopherol) analysis*: Analar grade ethanol (May and Baker, Dagenham, England), n-hexane, pyrogallol and hydroquinone (BDH, Poole, Great Britain), HPLC grade acetonitrile (AJAX Chemicals, Australia). Vitamin E standard (DL-α-tocopherol) was obtained from Aldrich, Milwaukee, WI, USA.

*Red blood cell hydrogen peroxide haemolysis assay*: Thirty percent hydrogen peroxide (BDH, Poole, Great Britain), aqueous saline and anhydrous dibasic sodium phosphate ($Na_2HPO_4$)(BDH, Poole, Great Britain).

*Plasma lipid peroxides assay:* Reagent 1A: lyophilised enzyme reagent: 1 bottle of ascorbic oxidase and lipoprotein lipase; Reagent 1B: 55 ml of buffer solution. Reagent

2A: lyophilised chromogen reagent, 1 bottle of 10-N-Methylcarbamoyl-3,7-dimethylamino-10 H-phenothiazine (MCDP); Reagent 2B: 110 ml of buffer and haemoglobin. Standard solution: 10 ml of 50 nmol·ml$^{-1}$cumene hydroperoxides.

*Whole blood lymphocyte proliferation assay:* Tritiated thymidine (1 mCi·ml$^{-1}$) (ICN Biochemical, Cleveland, OH), concanavalin (Con A) (Sigma, St Louis, MA, USA), phytohemagglutinin (PHA)(Gibco, Grand Island, NY, USA), pokeweed mitogen (PWM) (Gibco, Grand Island, NY, USA), and tissue culture medium: RPMI 1640 (Gibco, Grand Island, NY, USA), supplemented with 5 % fetal calf serum, 1 mM glutamine, 20 mM HEPES solution, 100 U·ml$^{-1}$ penicillin, 50 μg·ml$^{-1}$, streptomycin, 2.25 mM 2-mercaptoethanol.

*FRAP assay:* Sodium acetate ($C_2H_3NaO_2 \cdot 3H_2O$)(May and Baker, Dagenham, England), glacial acetic acid (BDH, Poole, Great Britain), 2,4,6-tripyridyl-s-triazine (TPTZ)(Sigma, St. Louis, MO, USA), $FeCl_3 \cdot 6H_2O$ (May and Baker, Dagenham, England).

### 2.3.2 Plasma vitamin E (α-tocopherol) analysis

The method for the determination of α-tocopherol in blood plasma was similar to that described by McMurray and Blanchflower (1979) with minor modifications to the extraction procedure of α-tocopherol. A 50 μl plasma sample was pipetted into a 1 ml Eppendorf tube, and 100 μl of ethanol (containing 0.5 g hydroquinone per 100 ml ethanol) was added to precipitate the protein. Hexane (0.5 ml) was added to the Eppendorf tube and vigorously shaken for 1 min. Two liquid phases were separated by centrifugation (2500 x g, 1 min) and 450 μl of the hexane layer (top layer) was then collected for analysis of α-tocopherol. The hexane extract was evaporated to dryness under a stream of $N_2$ and the residue was redissolved in 100 μl ethanol contain 0.5 % (w/v) hydroquinone. α-Tocopherol level in the aliquot was determined by reverse phase HPLC using a Waters Alliance HPLC (Milford, MA) with a Luna 5 μl $C_{18}$ (2) 150 x 4.60 mm column (Phenomenex, Torrance, CA, USA). α-Tocopherol was eluted using acetonitrile (flow rate, 1.5 ml·min$^{-1}$) and detected using a Waters 474 scanning fluorescence detector (excitation wavelength of 296 nm and emission wavelength of 330 nm). DL-α-Tocopherol was quantitated using a vitamin E standard solution (0.25 mg·ml$^{-1}$ in ethanol solvent).

### 2.3.3 Red blood cell hydrogen peroxide haemolysis

RBC hydrogen peroxide haemolysis was determined according to the procedure described by Fischer *et al.* (1970) with minor modifications. Heparinised blood (0.4 ml) was suspended in 2.0 ml phosphate buffered saline (PBS)[1 part 0.9 % saline solution and 1 part phosphate buffered solution (pH = 7.4)] and centrifuged at 1000 x g for 10 min at 4° C. The supernatant was carefully discarded and the RBC's were resuspended in 2.0 ml of 0.9 % (w/v) aqueous saline.

RBC haemolysis was measured by pipetting 0.25 ml of the RBC suspension into each of 6 test tubes. Freshly prepared 4.0 % $H_2O_2$ solution (0.25 ml) was added to each of two tubes (1 & 2) and 2.0 % $H_2O_2$ (0.25 ml) solution was added to each of the other two tubes (3 & 4). Distilled water (1.75 ml) and 0.25 ml phosphate buffered saline were added to each of the two remaining tubes (5 & 6), respectively. All tubes were gently shaken and incubated at 37° C for 2.5 h with gentle shaking every 30 min.

After incubation, 1.5 ml of phosphate buffered saline was added to tube 1, 2, 3, 4, and 6. The tubes were gently shaken and then centrifuged (1000 x g, 10 min) at 4° C. The supernatant was decanted into a 1 ml spectrophotometer cuvettes and the absorption was measured at 540 nm on a spectrophotometer (Pharmacia Biotech, Cambridge, England). The reagent blank was a solution of 0.25 ml saline, 0.25 ml distilled water and 1.5 ml PBS. The extent of haemolysis of the RBC was calculated according to the following equation:

$$\text{Haemolysis (\%)} = \frac{\text{Tube (1\&2 or 3\&4) - Tube 6}}{\text{Tube 5 - Tube 6}} \times 100$$

### 2.3.4 Plasma lipid peroxides

Plasma lipid peroxides were quantitated colorimetrically (675 nm) by measuring methylene blue formation. A commercial LPO-CC kit (Cat. No. CC-004, Kamiya Biomedical Co., Seattle, WA, USA) and a Cobas Fara II autoanalyser (Hoffmann-La Roche, Basel, Switzerland) were used. The results were expressed in $nmol \cdot ml^{-1}$ of plasma.

### 2.3.5 Whole blood lymphocyte proliferation

Whole blood lymphocyte proliferation was carried out by measuring the incorporation of $^3$H-thymidine into proliferating cells from whole blood using a modified method

described by Fasanmade and Jusko (1997). A heparinised blood sample was diluted with supplemented RPMI 1640 to obtain a 1/4 diluted solution. The complete RPMI culture medium (100 µl) was aliquoted into each well of a 96-well flat bottom polystyrene microtiter plate (Nunc, GIBCO BRL, Life Technology Ltd., NZ) and 50 µl culture medium was pipetted into control wells of each sample. Fifty microliter of mitogen working solution (Con A, 20 $\mu g \cdot ml^{-1}$, 1/50 dilution of PHA and PWM solution) was added to the test wells. The optimal dose of Con A, and dilution of PHA and PWM were previously determined. Diluted blood (50 µl) was pipetted into each well and the microtiter plate was incubated at 37° C in a humidified 5 % $CO_2$ incubator for 72 hours. After incubation, 25 µl of diluted tritiated thymidine working solution (1/20 dilution from stocking solution) was added to each well (1.25 µCi/well), and incubation was continued for a further 16 h. The cultures were harvested onto glass-fibre filter papers, using a LKB 1295-001 cell harvester (LKB Wallac, Wallac Oy, Turku, Finland). The filter discs were dried for 2 min in a microwave, and were placed into a plastic bag containing 10 ml scintillation fluid and sealed. The radioactivity in each well was determined using a Wallac 1205 betaplate liquid-scintillation spectrophotometer (LKB Wallac, Wallac Oy, Turku, Finland) and recorded as counts per minute (CPM). The results for each sample were expressed as either CPM or stimulation index (SI) with the latter calculated as the mean of the CPM of the test wells divided by the mean of the CPM of the control wells.

### 2.3.6 The ferric reducing ability of plasma

The ferric reducing ability of plasma was determined by the method described by Benzie and Strain (1996). In this method a Cobas Fara II autoanalyser (Hoffmann-La Roche, Basel, Switzerland) was used to perform the FRAP assay. FRAP reagent was freshly prepared by mixing 10 parts 0.3 $mol \cdot L^{-1}$ acetate buffer (pH = 3.6), 1 part TPTZ solution (10 $mmol \cdot L^{-1}$ TPTZ in 40 $mmol \cdot L^{-1}$ HCL) and 1 part $FeCl_3 \cdot 6H_2O$ solution (20 $mmol \cdot L^{-1}$). Three hundred µl of freshly prepared FRAP reagent was warmed to 37° C in the analyser and a blank reading of absorbance was taken at 593 nm. Ten µl of prepared plasma was added, along with 30 µl $H_2O$. The final dilution of the sample in the reaction mixture was 1/34. Absorbance (A) readings were taken after 0.5 s and every 15 s thereafter during the monitoring period. The change in absorbance ($\Delta A_{593nm}$) between the

final reading selected and the blank reading was calculated for each sample and related to $\Delta A_{593nm}$ of a Fe (II) standard solution tested in parallel.

## 2.4 CHEMICAL ANALYSES

α-Tocopherol in the diets was extracted by the procedure described by Bayfield and Romalis (1979). Analysis of α-tocopherol was identical to the previous described procedure for determination of α-tocopherol in blood plasma. Dry matter was determined in duplicate by drying samples at 70° C to constant weight, while ash was determined by burning samples at 550° C for 16 hours. Lipid was determined by petroleum ether extraction (60-80° C boiling fraction) of duplicate freeze-dried samples (AOAC, 1995). Total nitrogen was determined in duplicate on 0.10-0.15 g samples using a Leco FP-2000 CNS analyser (LECO Corporation, St. Joseph, MI, USA). Crude protein was calculated as total nitrogen multiplied by 6.25. Amino acids were determined on lipid extracted freeze-dried samples using the method described by Hendriks *et al.* (1996). Selenium was analysed in triplicate using inductively coupled argon atomic emission spectrometry (Lee, 1983).

Analysis of lipid peroxides in the diets was carried out by the method of Ueda *et al.* (1986). Fatty acids profiles were obtained using the method described by Sukhija and Palmquist (1988) with the following modifications: toluene was replaced by benzene and Florisil was used instead of activated charcoal to remove pigments. Fatty acid analysis was conducted by gas chromatography (model GC $A_8$, Shiuadzu, Kyoto, Japan) using a stainless-steel column (1.8 m x 3.2 mm) and a flame ionisation detector packed with 10 % EG'SS-X on chromosorb W (80-120 mesh) (Shimadzu, Kyoto, Japan), and oxygen free nitrogen as the carrier gas. The analysis by gas chromatography was isothermally performed at 175° C. Fatty acid standard was purchased from Sigma Chemical Co. (St. Louis, USA). Plasma alkaline phosphatase (EC 3.1.3.1) and triglycerides were determined using commercially available kit (Cat. No. 073634 1 and 073679 1, respectively)(Roche Products NZ Ltd., Auckland, New Zealand).

## 2.5 DATA ANALYSIS

The data were tested for homogeneity of variances using Barlett's test (Snedecor and Cochran, 1980). Where the variance was heterogeneous, the data were either $\log_{10}$ or square root transformed depending the data. All the data were then subjected to analysis of variance using the general linear model (GLM) procedure (SAS, 1990), with diet, gender, time and all the possible interactions between diet, gender and time as variables. When a significant difference ($P < 0.05$) of diet was detected, differences between dietary groups at each time point were identified using Duncan's multiple range test. All statistical analyses were performed using the SAS statistical package (SAS version 6.12, SAS Institute, Gary, NC).

# CHAPTER 3

# RESULTS

## 3.1 BODY WEIGHT AND METABOLISABLE ENERGY INTAKE

All cats remained healthy throughout the study. No signs of vitamin E deficiency were observed in any of the cats. A female cat on diet A was excluded from the study after 3 weeks due to severe loss of body weight (27 %). The food intake of this cat was subsequently measured and found to be 8 g per day.

The average body weight of the cats on the four diets declined during the first week and thereafter increased steadily through the study. The average body weight increase of the cats on diets A, B, C, and D were 1.0, 10.6, 8.7, and 2.6 %, respectively. There was no effect (P = 0.77) of diet on the body weight of the cats on the four diets. However, there was a significant effect of gender, time, and the interaction between diet and gender on the average body weight of the cats (Table 1).

**Table 1 Statistical significance of selected variables on measured response parameters**

| | Response parameter | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Variables | BW | Ptoc | RRCH4 | RBCH2 | FRAP | LPO | PTG | Ratio[a] | PAP |
| Diet | NS | ** | *** | *** | * | * | NS | NS | NS |
| Gender | *** | NS | NS | NS | NS | NS | NS | NS | NS |
| Diet*gender | *** | NS | NS | NS | NS | *** | * | * | NS |
| Time | *** | *** | *** | *** | *** | *** | *** | *** | NS |
| Diet*time | NS | *** | *** | *** | ** | *** | *** | NS | NS |
| Gender*time | NS | NS | NS | NS | NS | NS | NS | NS | NS |
| Diet*gender*time | NS | NS | NS | NS | NS | NS | NS | NS | NS |

NS, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

BW, body weight; Ptoc, plasma α-tocopherol; PTG, plasma triglycerides; RBCH4, Red blood cell 4 % $H_2O_2$ haemolysis; RBCH2, Red blood cell 2 % $H_2O_2$ haemolysis; LPO, lipid peroxides; FRAP, ferric reducing ability of plasma; PAP, plasma alkaline phosphatase.

[a]Ratio of plasma lipid peroxides to triglycerides.

During the first 9 weeks of the study the average daily metabolisable energy (ME) intake of the cats on the four diets expressed per unit body weight varied from 130 to 330

kJ. The average daily ME intake of the cats on diet A, B, C and D during the first week of the study were 143, 212, 226, 138 kJ·kg$^{-1}$ body weight, respectively. The average ME intake during the first 9 weeks of the study was the highest for group B with group D having the lowest ME intake per unit body weight (Figure 1). The average daily ME intake of the cats on diet A, B, C and D during the final week of the study were 289, 261, 256, and 267 kJ· kg$^{-1}$ body weight, respectively.

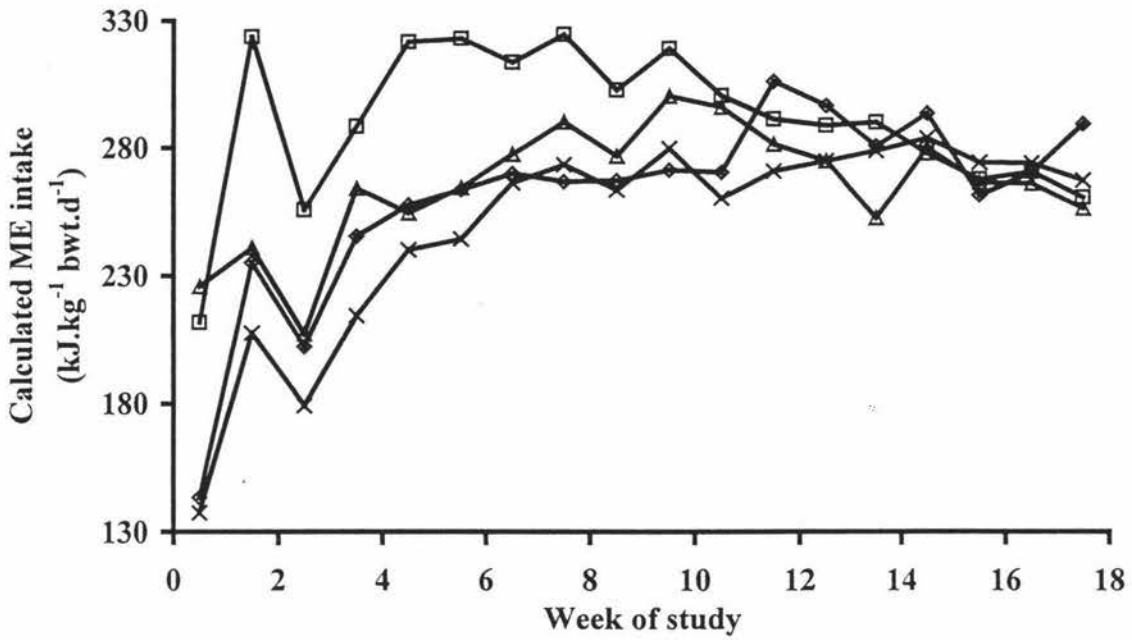


**Figure 1** Average daily ME intake of the adult cats on the four diets (◊, A; □, B; Δ, C; ×, D).

### 3.2 PLASMA α-TOCOPHEROL

There was no significant difference in plasma α-tocopherol concentration of the four groups of cats at the beginning of the study. The mean (± SEM) plasma α-tocopherol concentration of the cats (n = 31) was 3.4 ± 0.2 μg·ml$^{-1}$. The plasma α-tocopherol concentration of the cats on diet A remained low after the diet was fed to the cats (Figure 2). The plasma α-tocopherol level of the cats on diet B, C and D were significantly ($P < 0.05$) increased after 2 weeks on the vitamin E supplemented diets to 17.7 ± 1.4, 27.5 ± 3.2 and 31.1 ± 2.2 μg·ml$^{-1}$, respectively. The plasma α-tocopherol concentration remained relatively constant from 2 to 12 weeks for the four groups of cats and thereafter

increased (Figure 2). The mean (± SEM) plasma α-tocopherol concentration at the end of study were 13.6 ± 1.4, 27.1 ± 2.3, 39.8 ± 4.7, and 46.1 ± 4.3 $\mu g \cdot ml^{-1}$ for the cats on diet A, B, C and D, respectively. There was a significant ($P < 0.01$) effect of diet, time and the interaction between diet and time on plasma α-tocopherol level (Table 1).

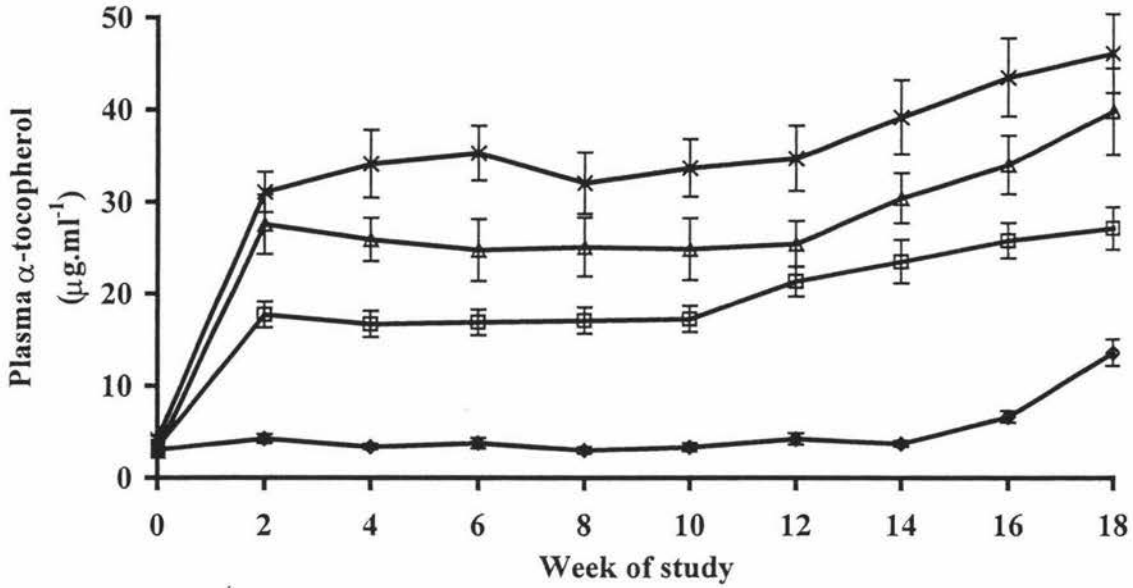


**Figure 2** Mean (± SEM) of plasma α-tocopherol concentration of the adult cats on the four diets (◊, A; □, B; Δ, C; ×, D).

### 3.3 RED BLOOD CELL HYDROGEN PEROXIDE (4 and 2 %) HAEMOLYSIS

There was no difference in RBC 4 % $H_2O_2$ haemolysis among the cats on the four diets at the beginning of the study. The mean RBC 2 % $H_2O_2$ haemolysis of the cats on diet B (83 %) and C (72 %) but not diet D (67 %), were higher than those of the cats on diet C (52 %). The mean (± SEM) RBC haemolysis in the presence of 4 and 2 % $H_2O_2$ of the adult cats at the start of the study were 76 ± 2.3, and 68 ± 4.6, respectively. The RBC 4 % $H_2O_2$ haemolysis of the cats on diet A remained high until 16 weeks and decreased abruptly at week 18 (Figure 3). RBC 2 % $H_2O_2$ haemolysis for the latter group continuously decreased throughout the entire study (Figure 4). When the cats were fed on the vitamin E supplemented diets (B, C and D), RBC $H_2O_2$ (4 and 2 %) haemolysis was decreased and remained relatively constant until the end of the study. There was a significant ($P < 0.01$) effect of diet, time and the interaction between diet and time on RBC 4 % and 2 % $H_2O_2$ haemolysis (Table 1). The RBC 4 % and 2 % $H_2O_2$ haemolysis of the cats on diet A were

significantly higher ($P < 0.001$) than those of the cats on the supplemented vitamin E diets (B, C, and D) throughout the study. There were no significant differences in RBC 4 % and 2 % $H_2O_2$ haemolysis of the cats on the supplemented vitamin E diets (B, C and D) except for the RBC 4 % $H_2O_2$ haemolysis of the cats on diet B which was significantly higher than those on diet C and D at week 4 and week 8.

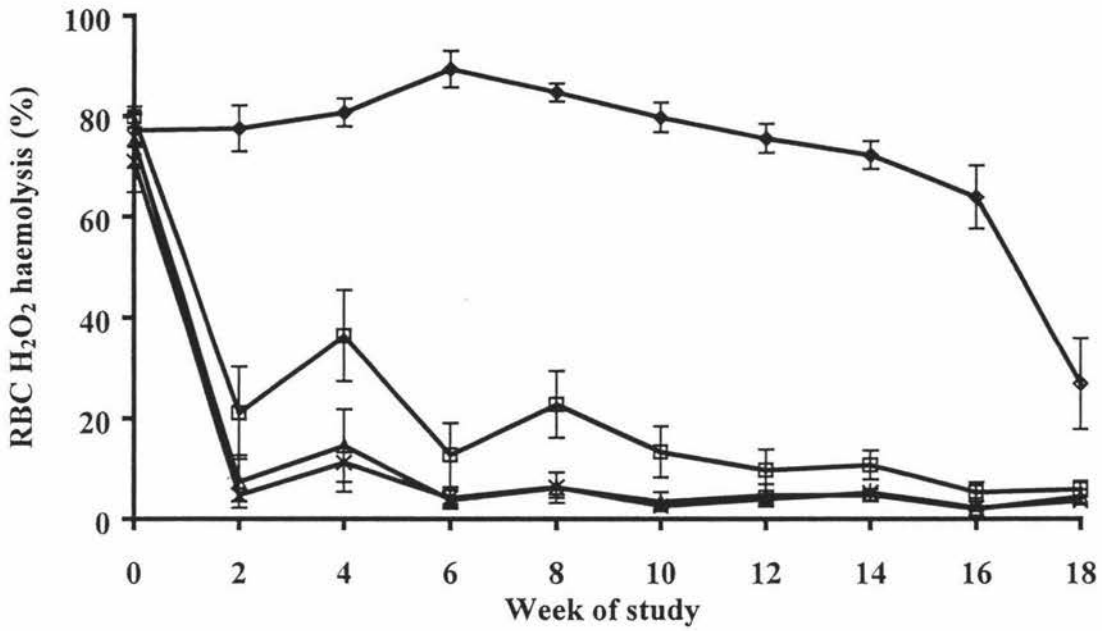


**Figure 3** Mean (± SEM) red blood cell 4 % $H_2O_2$ haemolysis of the adult cats on the four diets (◊, A; □, B; Δ, C; ×, D).

### 3.4 FERRIC REDUCING ABILITY OF PLASMA

There was no significant difference in the average FRAP value of the cats on the four different diets at the beginning of the study. The mean (± SEM) FRAP value for the adult cats (n = 31) at the beginning of the study was 0.66 ± 0.02 $mmol \cdot L^{-1}$. When a new diet was introduced, the FRAP value of the cats on diet A was significantly decreased, and thereafter increased constantly to reach a plateau at week 10. The FRAP value of the cats on diet A increased abruptly before the end of study. The FRAP value of the cats on the vitamin E supplemented diets (B, C and D) increased constantly throughout the entire period except for week 16 when a decrease in FRAP value was observed (Figure 5).

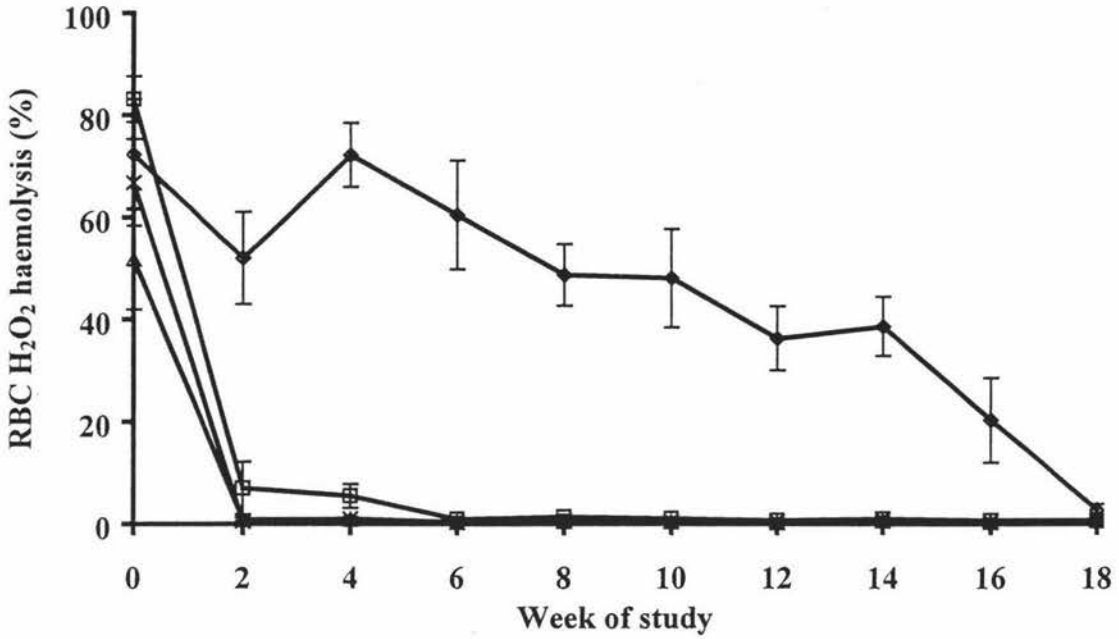


**Figure 4** Mean (± SEM) red blood cell 2 % $H_2O_2$ haemolysis of the adult cats on the four diets (◊, A; □, B; Δ, C; ×, D).

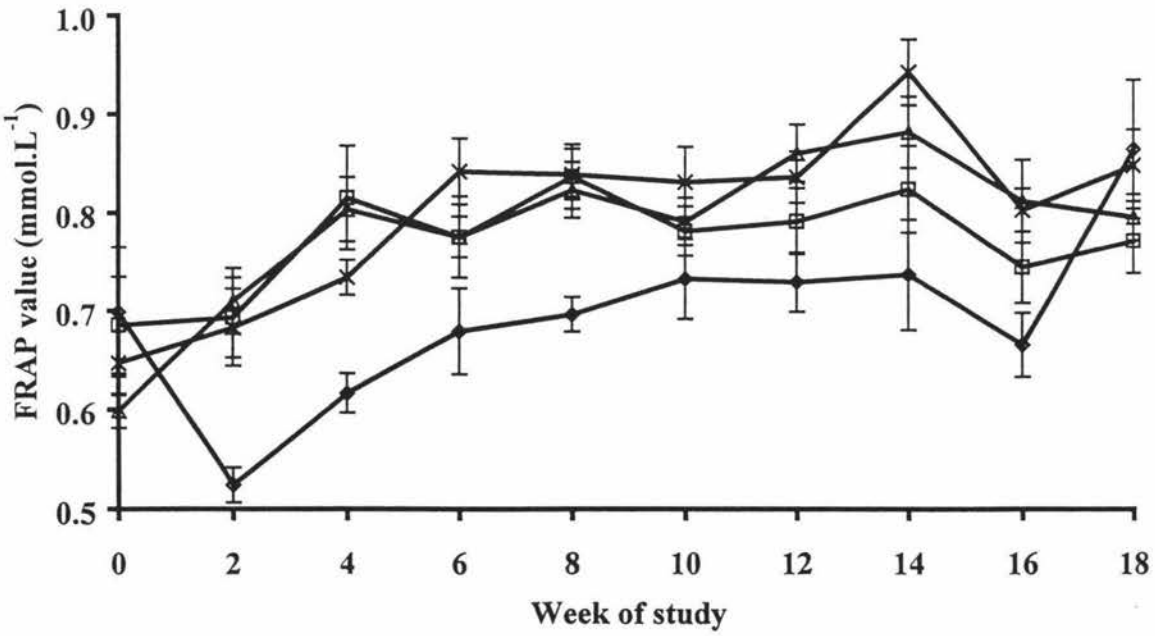


**Figure 5** Mean (± SEM) ferric reducing ability of plasma of the adult cats on the four diets (◊, A; □, B; Δ, C; ×, D).

There was a significant effect of diet, time and the interaction between diet and time on the FRAP status of the cats (Table 1). Cats on diet A had a significantly lower water soluble antioxidant status as measured by the FRAP assay than those on the vitamin E supplemented diets (diet B, C, and D) throughout the entire study except at week 10, 12 and 18. There was no significant difference in FRAP value of the cats on diet B, C and D during the study.

### 3.5 PLASMA LIPID PEROXIDES

The plasma lipid peroxide level of the cats on diet D (22 $nmol \cdot ml^{-1}$) at the beginning of study was significantly ($P < 0.05$) higher than those of the cats on diet A (13), B (11) and C (11 $nmol \cdot ml^{-1}$). The mean (± SEM) of LPO value of the adult cats ($n = 31$) at the start of study was 14 ± 1.5 $nmol \cdot ml^{-1}$. The LPO level of the cats on diet A, B, C and D were significantly ($P < 0.05$) increased after 2 weeks to 28 ± 6, 58 ± 6, 75 ± 11, 51 ± 5 $nmol \cdot ml^{-1}$, respectively and thereafter varied greatly (Figure 6). The mean (± SEM) LPO values of the cats on diet A, B, C, and D at the end of the study were 40 ± 5, 35 ± 3, 36 ± 6, 35 ± 3 $nmol \cdot ml^{-1}$, respectively.

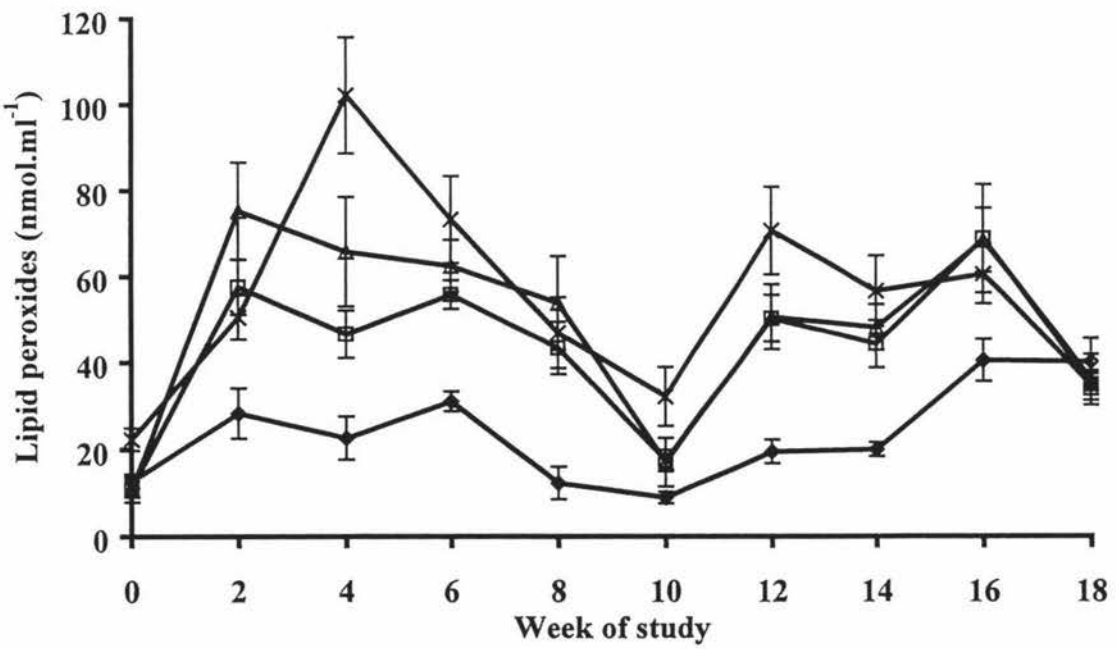


**Figure 6** Mean (± SEM) plasma lipid peroxide levels of the adult cats on the four diets (◊, A; □, B; Δ, C; ×, D).

There was a significant ($P < 0.05$) effect of diet, time and the interaction between diet and time, and diet and gender on the LPO status of the cats (Table 1). Plasma LPO values of the cats on diet A were significantly ($P < 0.05$) lower than those of the cats on the vitamin E supplemented diets (B, C, and D) except at week 10 and 18. There was no significant difference in LPO value of the cats on the vitamin E supplemented diets (B, C and D) except the LPO value of cats on diet D which was higher than those of the cats on diet B and C at week 4. However, when expressing the LPO levels per unit plasma triglycerides, there was no significant effect of diet or interaction between diet and time. There was a significant ($P < 0.05$) effect of time, and the interaction between diet and gender on the ratio of LPO to triglycerides (Table 1).

## 3.6 *IN VITRO* WHOLE BLOOD LYMPHOCYTE PROLIFERATION WITH MITOGEN (CON A, PHA AND PWM) AND WITHOUT MITOGEN

The mean (± SEM) stimulation index of whole blood lymphocytes by mitogens concanavalin A, phytohemagglutinin and pokeweed mitogen of the adult cats ($n = 31$) at the start of the study were 174 ± 15, 57 ± 5, and 97 ± 9, respectively. The mean (± SEM) CPM of unstimulated lymphocytes for the adult cats was 201 ± 9. The pattern of whole blood cell proliferation to Con A, PHA and PWM over time for the cats on diet A, B, C and D were similar (Figure 7, 8 and 9). The response to Con A and PWM was significantly increased at week 2, and thereafter became suppressed throughout the entire period except for a slight increase at week 18. The immune response measured by PHA-stimulated lymphocyte proliferation was suppressed throughout the entire period of the study except for a slight increase at week 18. The incorporation of [$^3$H]-thymidine into DNA of lymphocytes without mitogen (background culture) expressed as CPM increased constantly to reach a plateau at week 8. It remained relatively constant until the end of the study (Figure 10).

There was no effect of selected variables, including diet on immune response parameters measured by i*n vitro* cat whole blood lymphocyte proliferation with mitogen Con A, PHA, and PWM expressed as SI, and without mitogen (background culture) expressed as CPM. However, there was a significant ($P < 0.001$) effect of time on the all immune response parameters measured.

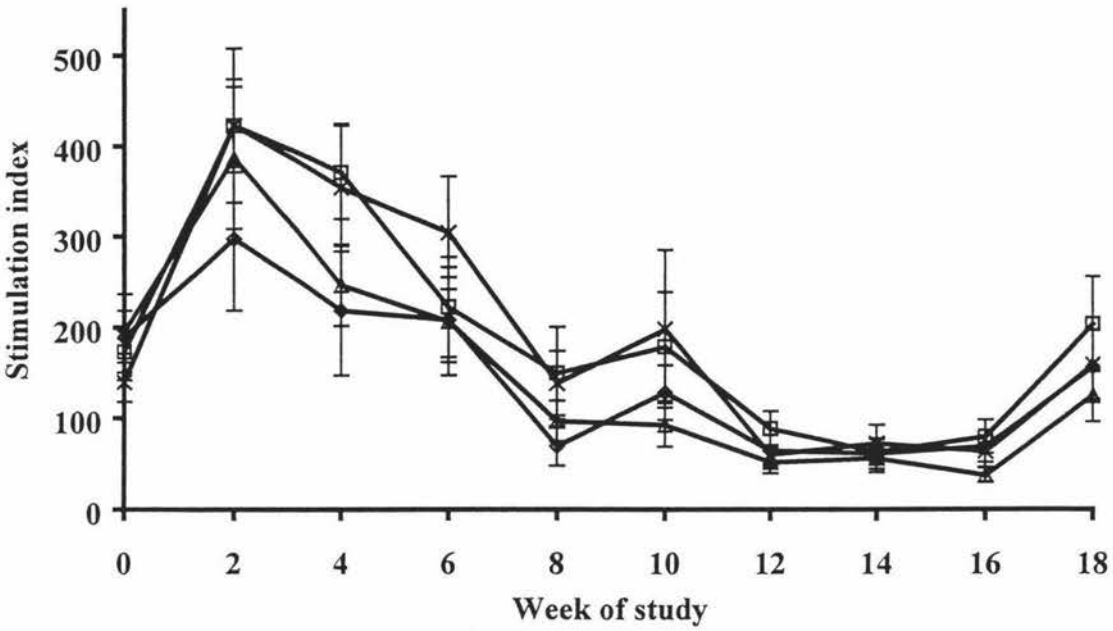


**Figure 7** Mean (± SEM) *in vitro* stimulation index of cat whole blood lymphocytes to concanavalin A (◊, A; □, B; Δ, C; ×, D).

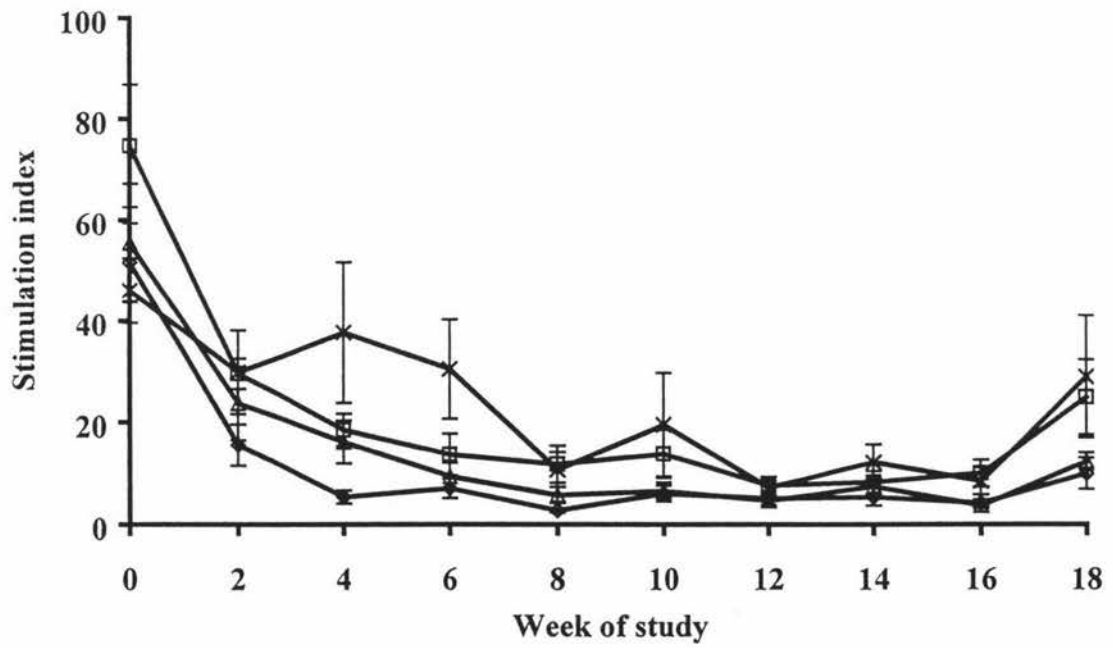


**Figure 8** Mean (± SEM) *in vitro* stimulation index of cat whole blood lymphocytes to phytohemagglutinin (◊, A; □, B; Δ, C; ×, D).

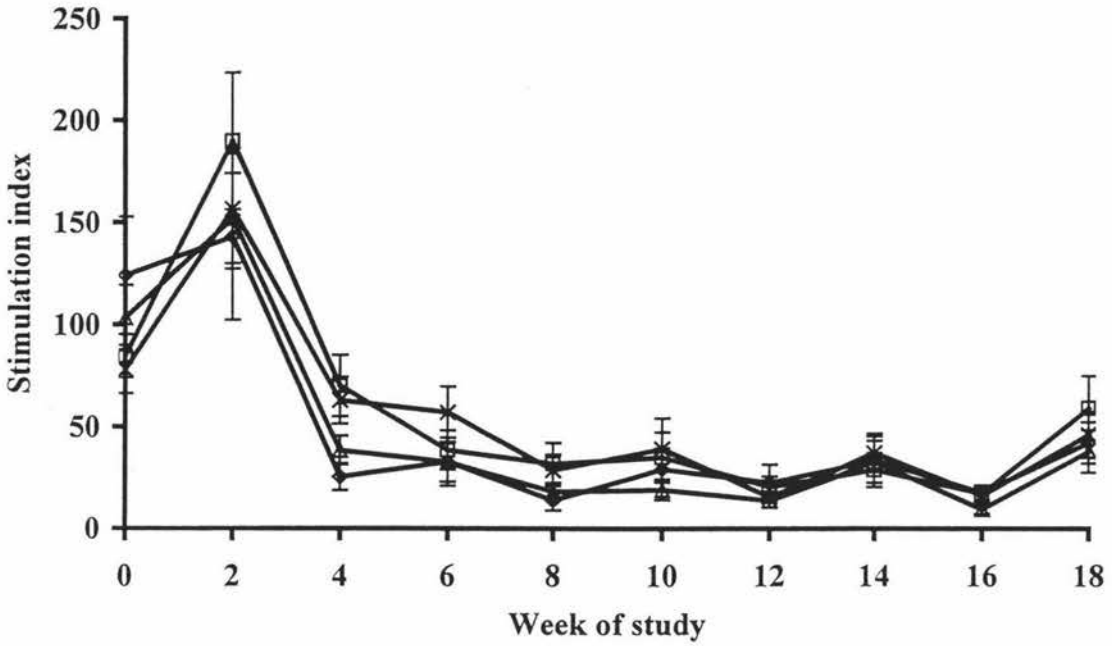


**Figure 9** Mean (± SEM) *in vitro* stimulation index of cat whole blood lymphocytes to pokeweed mitogen (◊, A; □, B; Δ, C; ×, D).

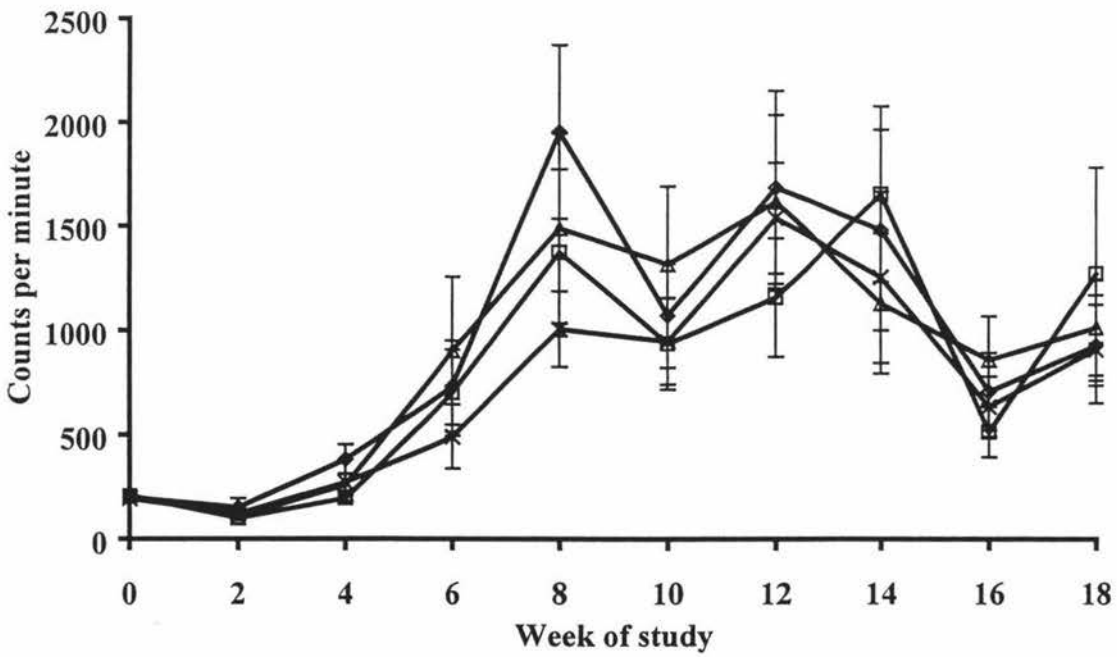


**Figure 10** Mean (± SEM) *in vitro* counts per minute of unstimulated cat whole blood lymphocytes (◊, A; □, B; Δ, C; ×, D).

### 3.7 PLASMA TRIGLYCERIDES AND ALKALINE PHOSPHATASE

There was no significant difference in plasma triglyceride concentration amongst the cats on the four different diets (A, B, C, and D) at the beginning of study. The mean (± SEM) plasma triglyceride concentration of the adult cats (n = 31) at the start of study was 70 ± 24 $mg \cdot dl^{-1}$. The average plasma triglyceride concentration of the cats over time on the four diets (A, B, C and D) was significantly ($P < 0.05$) decreased after 2 weeks and thereafter varied. At the end of study the plasma triglyceride concentration of the adult cats on diet A (57) was significantly ($P < 0.05$) higher than those of the cats on vitamin E supplemented diets (B, 36; C, 32; and D, 27). The average plasma triglyceride level as measured over all the groups was reduced by 47 % at the end of study (Figure 11).

There was no significant effect diet ($P=0.40$) on the plasma triglyceride concentration of the cats. However, there was a significant ($P < 0.05$) effect of time, and the interaction between diet and gender on the plasma triglycerides concentration.

There was no significant effect of any of the variables on plasma alkaline phosphatase (Table 1). The mean (± SEM) plasma alkaline phosphatase levels of the adult cats (n = 31) was 55 ± 1.6 $U \cdot L^{-1}$.

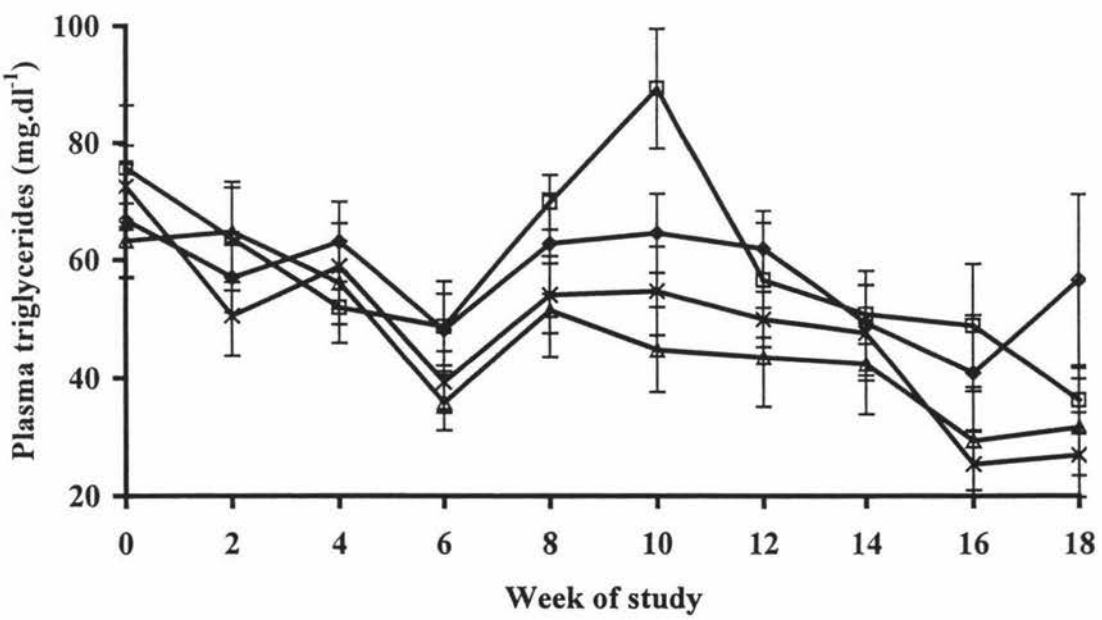


**Figure 11** Mean (± SEM) plasma triglyceride concentration of the adult cats on the four diets (◊, A; □, B; Δ, C; ×, D).

# CHAPTER 4

# DISCUSSION

No clinical signs of vitamin E deficiency were observed in any of the adult cats fed the four different diets. The mean (± SEM) plasma α-tocopherol concentration of the adult cats on the vitamin E unsupplemented diet (A) at the start of the study was 3.4 ± 0.2 $\mu g \cdot ml^{-1}$ and remained relatively constant from week 2 to 12 and thereafter increased until 13.6 $\mu g \cdot ml^{-1}$ at the end of the study. All cats were found to have plasma triglyceride and alkaline phosphatase levels within normal ranges (Fox *et al.*, 1993; AAFCO, 1997). Studies in other animals such as pigs (Wang *et al.*, 1996), rats (Hakkarainen *et al.*, 1986), dogs (Hayes *et al.*, 1970; Pillai *et al.*, 1991, 1992), and cattle (Adams, 1982) have demonstrated that a plasma α-tocopherol concentration of less than 1.5 $\mu g \cdot ml^{-1}$ seems to result in vitamin E deficiency. The mean plasma α-tocopherol concentration of the cats fed diet A was close to the upper limit of that observed for vitamin E deficient cats by Stephan and Hayes (1978). Gershoff and Norkin (1962) studied vitamin E deficiency in growing cats and reported serum α-tocopherol concentrations in vitamin E deficient cats of 0.3 to 5.0 $\mu g \cdot g^{-1}$. Baker *et al.* (1986) reported a plasma α-tocopherol range of 3 to 11 $\mu g \cdot ml^{-1}$ in healthy domestic cats aged 7 months to 8 years old. From the above, it can be seen that the cats fed the unsupplemented diet in the present study maintained an adequate plasma α-tocopherol concentration to prevent vitamin E deficiency symptoms. The gradual increase in plasma α-tocopherol level after week 12 until the end of the study is difficult to explain but may be related to the food intake.

When the new diet was introduced, feed intake of the cats in the four groups was low and the average body weight of the cats decreased. This is as expected as the cats had to adjust to the new diet, which was different from the previous diet fed to the cats. The average ME intake of the cats on the four diets varied (208-258 $kJ \cdot kg^{-1}bwt \cdot d^{-1}$) during the first 9 weeks of the study and thereafter plateaued at approximately 290 $kJ \cdot kg^{-1}bwt \cdot d^{-1}$. The average daily ME intake of the adult cats on the four diets was within the normal range of 210 to 335 $kJ \cdot kg^{-1}bwt \cdot d^{-1}$ (NRC, 1986; Burger, 1993).

NRC (1986) recommends that diets for growing cats should contain 30 IU of α-tocopherol per kg diet with a 4 to 5 fold increase in dietary vitamin E if the diet contains a high level of polyunsaturated fatty acids. AAFCO (1997) recommended that 10 IU of vitamin E should be added per g of added fish oil per kg diet. Based on the data in the present study, the value of 10 IU per g added fish oil per kg diet is well in excess to prevent vitamin E deficiency. RBC membrane fragility has been used as a measure of vitamin E status in many animals including pigs (Fontaine and Valli, 1977), chickens (Fischer *et al.*, 1970), humans (Gordon and Nitowsky, 1956; Horwitt *et al.*, 1956; Hashim and Asfour, 1968), rats (Bieri and Poukka, 1970), dogs (Pillai *et al.*, 1992), and cats (Gershoff and Norkin, 1962; Stephan and Hayes, 1978). Gershoff and Norkin (1962) concluded that the RBC hydrogen peroxide haemolysis test, as performed by these authors, was not a useful assay to measure vitamin E status in growing cats. In the present study, however, differences were observed in the RBC haemolysis measured over time between the four groups. The RBC $H_2O_2$ (4 and 2 %) haemolysis remained relatively high, when the cats were fed the unsupplemented vitamin E diet (A) while RBC $H_2O_2$ (4 and 2 %) haemolysis was significantly decreased when the cats were fed on the vitamin E supplemented diets (B, C and D). There was no significant difference in RBC 4 % and 2 % $H_2O_2$ haemolysis of the cats on the supplemented vitamin E diets (B, C and D) except for the RBC 4 % $H_2O_2$ haemolysis of the cats on diet B which was significantly higher than those on diet C and D at week 4 and week 8. The RBC haemolysis assay as performed in the present study was sensitive to dietary vitamin E levels. To maintain a low RBC haemolysis value, the vitamin E requirement of adult cats fed high dietary levels of fish oil is between 0 and 5 IU per g fish oil per kg of diet.

FRAP has been presented as a new method to assess water-soluble antioxidant status in blood plasma of animals. These plasma water-soluble antioxidants include uric acid, bilirubin, trolox, albumin and α-tocopherol (Benzie and Strain, 1996). There is no data available on baseline FRAP values in cats. In the present study differences were observed in the FRAP value measured over time between the four groups. Cats on diet A had significantly lower plasma FRAP values than the cats on the vitamin E supplemented diets (diet B, C, and D) throughout the entire study except at week 10, 12 and 18. There was no significant difference in FRAP value between the cats on diet B, C and D during the study. These results demonstrate that the water soluble antioxidant status measured by the ferric reducing ability of plasma can be compromised when cats are fed diets low in

vitamin E and that the FRAP assay shows promise as a measure of vitamin E status in cats. Based on the FRAP data, the vitamin E requirement of cats fed high dietary levels of fish oil, in the present study, was between 0 and 5 IU per g fish oil per kg of diet.

Vitamin E is one of the most important fat-soluble antioxidants to protect polyunsaturated fatty acids in membranes against lipid peroxidation (Vannucchi, *et al.*, 1997). Lipid hydroperoxides and malondialdehyde are major products of lipid peroxidation. To the author's knowledge there are no data available on baseline plasma LPO values in cats. The mean (± SEM) LPO value of the adult cats (n = 31) at the start of the study when the AAFCO (1997) tested diet was fed was 14 ± 1 $nmol \cdot ml^{-1}$. Vitamin E has been shown to protect PUFA from lipid peroxidation in several animal species including rats (Cho and Choi, 1994; Cho *et al.*, 1995; Vannucchi, *et al.*, 1997), pigs (Wang *et al.*, 1996) and guinea pigs (Barja *et al.*, 1996). In the present study, there was a significant effect of diet on the plasma LPO value of the cats fed the four different diets. The cats fed the unsupplemented diet had the lowest LPO value while the cats fed the highest dietary level of vitamin E had the highest LPO value. However, when the LPO values were expressed per unit plasma triglycerides, no significant (P = 0.51) effect of diet was observed. These results indicate that the plasma LPO levels of the cats in the present study were related to the level of plasma triglycerides. Kubo *et al.* (1997) showed that vitamin E was unable to prevent an increase in lipid peroxidation products in various tissues (serum, liver and kidney) of rats, as measured by the MDA assay when fed diets containing a high level of docosahexaenoic acid (DHA). Allard *et al.* (1997) demonstrated that human diets when supplemented with n-3 fatty acids result in an increase in lipid peroxidation products measured by either plasma MDA release or LPO, which was not suppressed by vitamin E supplementation. Based on the ratio of lipid peroxidation to plasma triglycerides in the present study, there does not seem to be a need to supplement diets containing a high level of fish oil for adult cats with vitamin E.

Studies in other animals such as dogs (Langweiler *et al.*, 1981), rats (Eskew *et al.*, 1985; Bendich *et al.*, 1986), pigs (Wuryastuti *et al.*, 1993), calves (Reddy *et al.*, 1986) and humans (Meydani *et al.*, 1990; Lowdley *et al.*, 1992) have demonstrated that lymphocyte proliferation to B-cell and T-cell mitogens are influenced by the dietary level of vitamin E and, therefore, are often used as a measure of vitamin E status. Based on studies in other animals (Bendich *et al.*, 1986; Reddy *et al.*, 1986; Wuryastuti *et al.*, 1993), the dietary vitamin E requirement to obtain optimal immunological health of an

animal is 4-10 times higher than the dietary vitamin E level to prevent vitamin E deficiency. Bendich *et al.* (1986) demonstrated that a dietary vitamin E level of 15 IU·$kg^{-1}$ was adequate to prevent myopathy in rats. The vitamin E requirement to optimise T- and B-lymphocyte response to Con A and PHA was much higher (50 to 200 IU·$kg^{-1}$). The data in the present study showed no beneficial effect of dietary vitamin E on whole blood lymphocyte proliferation when levels were 150 % of AAFCO (1997) recommendations. However, the highest dietary vitamin E level in the present study (15 IU·$kg^{-1}$) was approximately three times higher than the recommended minimum dietary vitamin E level for cats (NRC, 1986; AAFCO, 1997). It is unknown if a higher level of vitamin E would result in an increase in lymphocyte proliferation in adult cats.

The present study showed that cat lymphocytes respond poorly to lipopolysaccharides from *Escherichia coli* and that cat lymphocytes respond well to Con A and PWM with PHA showing an intermediate response. The latter was also found by Schultz (1978) and Schultz and Adams (1978). In the present study the response to Con A and PWM when expressed as a stimulation index was found to be significantly increased at week 2, and thereafter became suppressed throughout the entire period except for a slight increase at week 18. The immune response measured by PHA-stimulated lymphocyte proliferation was suppressed throughout the entire period of the study except for a slight increase at week 18. The pattern observed in the present study was mainly due to an increased incorporation of [$^3$H]-thymidine into DNA of lymphocytes without mitogen (background culture). There was no effect of diet on immune response parameters measured by *in vitro* cat whole blood lymphocyte proliferation without mitogen (background culture) expressed as CPM.

There may be several reasons for the low vitamin E requirement estimates of adult cats fed a high level of PUFA from fish oil in the present study. The experimental diets contained a high level of selenium: the analysed Se level in the four diets (A, B, C and D) ranged from 0.95 to 1.03 mg·$kg^{-1}$. These dietary values are within the normal range found in commercial cat foods (Mumma *et al.*, 1986), but approximately 10 times higher than the minimum requirements recommend by the NRC (1986) for growing cats. Combs and Scott (1974) demonstrated that *in vitro* lipid peroxidation values in hepatic microsomes from vitamin E-adequate chickens were decreased with increased dietary supplementation of Se. The reducing effect of high Se levels on the vitamin E requirement is thought to result from the reduction of hydroperoxides through the action of the Se-dependent

enzyme GSHPx. A second possible reason for the low dietary vitamin E requirement estimate may have been the high quality fish oil used in the present study, as evident by the failure to detect lipid peroxides in the diets. In several animal species including rats (Liu and Huang, 1995), chickens (Sheehy *et al.*, 1993) and catfish (Baker and Davies, 1997), it has been demonstrated that tissue α-tocopherol can be depleted when these animals consume diets containing rancid oil. Baker and Davies (1997) showed that plasma α-tocopherol concentrations of catfish fed diets containing oxidised oil were 2.7 times lower than fish fed diets containing fresh oil. Finally, the time required to develop vitamin E deficiency symptoms when fed diets containing a high level of fish oil may be longer than the experimental period in the present study. Gershoff and Norkin (1962) noted the first signs of vitamin E deficiency in growing cats at approximately 240 days after the start of feeding a vitamin E free diet. Stephan and Hayes (1978) noted vitamin E deficiency symptoms after 9–12 months of feeding cats an unsupplemented vitamin E diet. It is, therefore, possible that the experimental period in the present study was too short to show symptoms of vitamin E deficiency. The average plasma α-tocopherol level of the cats fed the vitamin E unsupplemented diet at the end of the present study, however, was 13.6 $\mu g \cdot ml^{-1}$. Gershoff and Norkin (1962) found levels of serum α-tocopherol in vitamin E deficient kittens of 0.3 to 5.0 $\mu g \cdot g^{-1}$. Stephan and Hayes (1978) found levels of plasma tocopherol in the vitamin E deficient cats of 1.2-3.5 $\mu g \cdot ml^{-1}$. It is, therefore, unlikely that the cats in the present study would have developed vitamin E deficiency symptoms when fed the diet for a more extended period of time as the plasma tocopherol levels of the cats in the unsupplemented group were relatively high.

# CHAPTER 5

# CONCLUSION

There were no clinical signs of vitamin E deficiency observed in any of the adult cats in the present study. Various response parameters were used to measure the vitamin E status of the cats including plasma α-tocopherol, red blood cell $H_2O_2$ (4 and 2 %) haemolysis, plasma lipid peroxides, the ferric reducing ability of plasma and whole blood *in vitro* lymphocyte proliferation. Based on the data obtained from these assays, the vitamin E requirement (α-tocopherol) of adult cats fed diets containing a high level of good quality fish oil is between 0 and 5 IU of vitamin E per g added fish oil per kg diet. The vitamin E level recommended by AAFCO (1997) of 10 IU per g added fish oil per kg diet appears to be well in excess. The high level of selenium present in the diets and/or the high quality of the fish oil (without peroxidation) used are believed to be responsible for the lower requirement estimates in the present study. The vitamin E requirement of adult cats fed diets containing a high level of fish oil to optimise immune response parameters warrants further investigation.

# CHAPTER 6

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