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THE ANTIOXIDANT ROLE OF -TOCOPHEROL IN THE NUTRITION

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THE ANTIOXIDANT ROLE OF α-TOCOPHEROL IN THE NUTRITION OF THE AFRICAN CATFISH, *CLARIAS GARIEPINUS* BURCHELL

by

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A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

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Department of Biological Sciences
Faculty of Science

In collaboration with
Department of Nutrition
University College Cork
Cork, Eire.

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ABSTRACT

Rémi Thomas Merrett Baker 'The antioxidant role of α-tocopherol in the nutrition of the African catfish, *Clarias gariepinus* Burchell'

The role of the lipid-soluble antioxidant α-tocopherol (vitamin E) in the nutrition of African catfish (*Clarias gariepinus* Burchell) has been investigated with respect to effects on growth, certain indices of health and prevention of tissue lipid peroxidation.

Although juvenile *C. gariepinus* were not found to show an immediate dependency on vitamin E for growth, even during rapid growth stages, a minimum requirement of α-tocopherol based on the prevention of hepatic lipid peroxidation was established at 30-40 mg α-tocopherol per kg dry diet. This is in line with published values for numerous cultured fish species. Tissue α-tocopherol accretion was found to be dependent on dietary dose of the vitamin (supplied as all-rac-α-tocopheryl acetate) and, with the exception of blood-plasma, increased tissue tocopherol status resulted in increased stability against lipid peroxidation.

Assessment of iron-ascorbate induced peroxidation (as TBARS) revealed that feeding catfish with diets containing α-tocopherol at several times the currently supplemented level, improved the stability of catfish muscle and this has fillet quality implications. Similarly, heightened fillet vitamin E content resulted in lower moisture exudation from previously frozen fillets, thus demonstrating the role of α-tocopherol in the maintenance of membrane integrity.

Oxidation of dietary oils and elevated ingestion of iron were found to be antagonistic of tissue vitamin E concentration. Consumption of feed containing rancid oil had marked effects on tissue fatty acid composition and these effects were modulated by increased intake of α-tocopheryl acetate.

In catfish fed diets containing similar amounts of ascorbate, a higher α-tocopherol supplement marginally improved ascorbate retention indicating a possible sparing of vitamin C by vitamin E.

The present work will contribute significantly to the understanding of antioxidant nutrition in this poorly researched cultured species. Furthermore, these investigations have improved and strengthened the database of knowledge accumulated within the field of nutrition of finfish.

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Reports submitted to Industrial partners

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-'The effect of α-tocopherol supplementation in practical diets for the African walking catfish, *Clarias gariepinus*'.

-'The effect of dietary supplementation of vitamin C derivatives at two levels of α-tocopherol inclusion in practical diets for African catfish fry, *Clarias gariepinus*'.

-'Modulation of the nutritional stress imposed by oxidised dietary oil, by the supplementation of dl-α-tocopheryl acetate into diets for the African catfish (*Clarias gariepinus* Burchell, 1822).'

-'Histological and haematological examination of African catfish fed diets varying in all-rac-α-tocopheryl acetate inclusion and oil oxidative state.'

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Signed .....R.B.L.....

Date .....1/8/96.....

CHAPTER 1

GENERAL INTRODUCTION

1.1 Discovery and Chemical Structure of the E-Vitamers

Vitamin E, the fat-soluble antioxidant micro-nutrient, was postulated to exist in 1922 when wheat-germ oil was observed to protect rats against the foetal resorption caused by rancid lard. The factor responsible was known not to be a water-soluble nutrient, nor could it have been vitamin A or D since cod-liver oil was unsuccessful in preventing the foetal-resorption process. Subsequently, vitamin E was more precisely named *tocopherol*, a name derived from the Greek 'tocos' meaning 'birth' and 'phera' meaning 'which confers'. The tag '-ol' was added to reflect the alcohol group within its configuration.

Isolation of various vitamers of the vitamin E group revealed the existence of four tocopherol isomers (α-, β-, γ- and δ-tocopherols) and the four related *tocotrienols*. The structures of these compounds, and in addition the structure of α-tocopheryl acetate (a stable form of the vitamin, commonly added to feeds) are presented in figure 1.1.

Biosynthesis of vitamin E analogues is achieved by plants, in the following manner. Homogentisic acid provides the aromatic ring (chromanol head) on to which geranyl-geranyl phosphate is added to form a side chain (phytyl tail). This yields δ-tocotrienol. α-, β- and γ-tocotrienol are formed by successive methylation of the ring. The tocopherols are derived upon reduction of the side-chain of the latter pre-cursor forms (Bender, 1992).

1.2 Intake and Metabolism of Vitamin E

Natural sources of vitamin E are mainly of plant origin since it is plants that synthesize the vitamin *de novo*. Of these, cereal and nut oils represent the richest sources of the various vitamers, although green vegetables also contain significant quantities. Since animals cannot synthesize vitamin E, they are totally dependent on dietary sources of the

vitamin. Therefore, animal tissues rely on dietary intake if they are to consequently represent a vitamin E source in the food chain (Machlin, 1991).

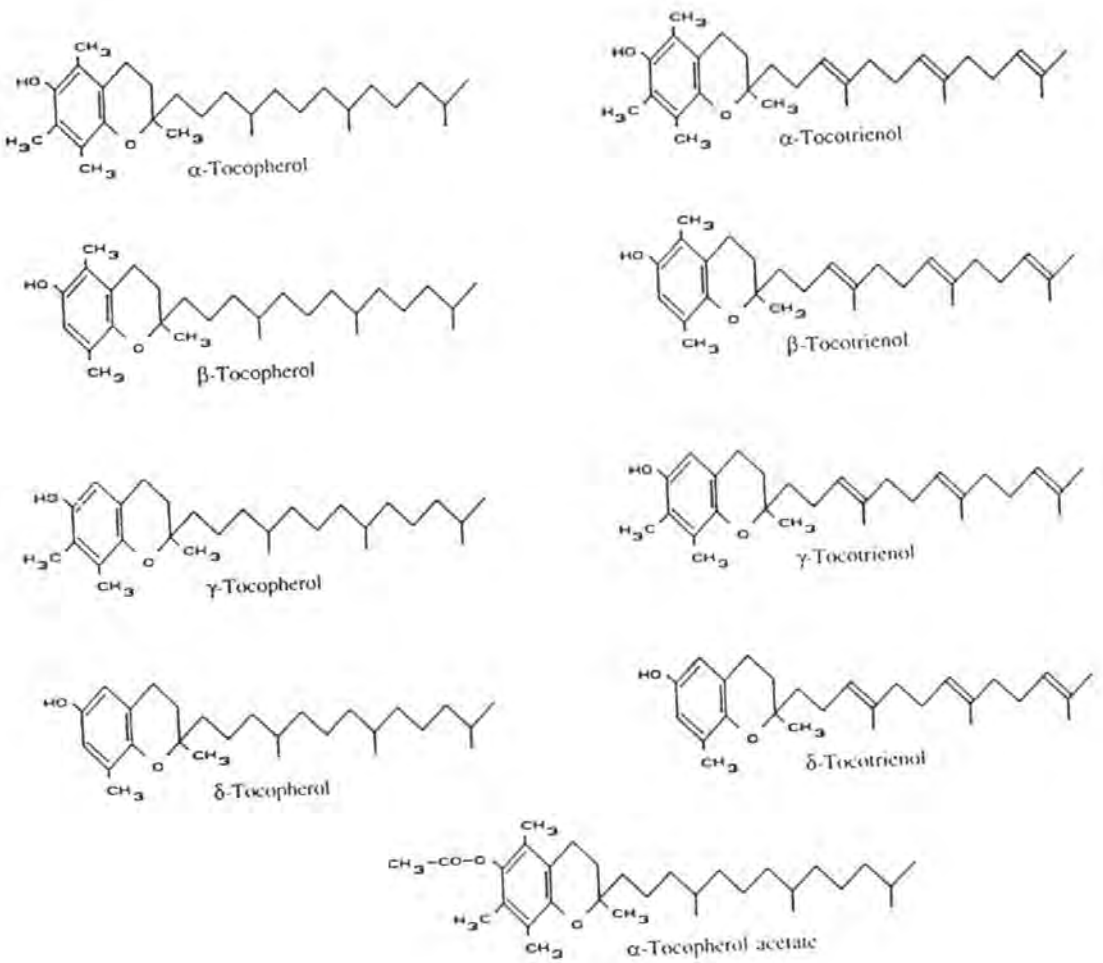


Figure 1.1 Structures of the tocopherols, tocotrienols and α-tocopheryl acetate

In both animals and man, assimilation of dietary vitamin E occurs through the following mechanism (compiled from Bender (1992), Dutta-Roy *et al.* (1994) and Traber and Kayden (1989)).

Firstly, absorption of the vitamin occurs across the intestinal barrier. In the case of ester derivatives of the vitamin (such as the acetate), the free tocopherol must be produced through hydrolysis by pancreatic esterase, in order to allow absorption (Muller *et al.*, 1976). Tocopherol uptake relies on the formation of lipid micelles by the action of pancreatic lipases and bile salts, thereby allowing the passage of lipids, and associated lipid-soluble components, across the intestinal brush-border and into the enterocyte (Gallo-Torres, 1970; MacMahon and Thompson, 1970). The site of maximal absorption in rats is the junction between the upper and middle thirds of the small intestine (Hollander *et al.*, 1975).

Once in the enterocyte (intestinal mucosal cell), the free tocopherols are incorporated into lipoproteins such as the chylomicrons and very low density lipoproteins (VLDLs), and from there into systemic circulation via the mesenteric lymphatic system (Bjorneboe *et al.*, 1986). Whilst in circulation, the chylomicrons and VLDL are available for metabolism and transfer of some α-tocopherol into tissues. This process is mediated by the action of lipoprotein lipase. The chylomicron remnants and accumulations of low density lipoprotein (LDL) and high density lipoprotein (HDL) are then destined for modification in the liver (Cohn *et al.*, 1992).

Uptake and storage of vitamin E by the liver is then followed by the slow release of α-tocopherol (and to a much lesser extent γ-tocopherol) in VLDL. This VLDL is metabolised gradually into LDL (Traber *et al.*, 1988), again by the action of lipoprotein lipase. This enzyme may also be responsible for the tocopherol transfers between LDL and HDL in plasma.

To some extent, erythrocytes may also play a part in tocopherol transport, since there is

a relatively large amount of the vitamin in the membranes of erythrocytes and these are in rapid equilibrium with plasma vitamin E pools.

Because of the fact that LDL preferentially transports α-tocopherol rather than γ-tocopherol (Dutta-Roy *et al.*, 1989;Traber and Kayden, 1989), only α-tocopherol and its acetate shall be discussed (unless otherwise stated). Reference to vitamin E in this text will be reserved as a general descriptor for all tocopherol and tocotrienol derivatives possessing any degree of α-tocopherol biological activity.

Incorporation of α-tocopherol into animal tissues proceeds by one of two mechanisms. The vitamer can be released from lipoproteins by the action of a lipoprotein lipase, hydrolysing the triglycerides in the chylomicrons, and other lipoproteins, thus liberating the vitamin for transfer into tissues. LDL-bound tocopherol can also be taken up via the LDL receptors on cell membrane surfaces (Gotto *et al.*, 1986).

Tissue α-tocopherol retention is dependent on specific cytosolic binding proteins (Kaplowitz *et al.*, 1989). These proteins are believed to be further responsible for the differences in biological activity (potency) of the various tocopherol/tocotrienol isomers. Values for the biological activity of these vitamers are quoted relative to the activity of pure D-α-tocopherol. Activities of the D- forms of vitamin E compounds (expressed as biological activity per mole) are as follows: α-tocopherol, 1.0; β-tocopherol, 0.49; γ-tocopherol, 0.10; δ-tocopherol, 0.03; α-tocotrienol, 0.29; β-tocotrienol, 0.05; γ- and δ-tocotrienol, undefined; α-tocopheryl acetate and succinate, 1.03. It should be noted that the latter of these compounds has a higher biological activity than pure D-α-tocopherol due to the fact that the free tocopherol is susceptible to oxidation prior to intestinal absorption. The all-rac-α-tocopheryl acetate form (racemic mixture), which is typically used as a feed supplement (due to its stability against oxidation) has a biological activity of 0.76.

Within tissues, α-tocopherol is transported from the binding proteins in the cytosol, to the cell membranes where a majority of the vitamin is associated with membranal lipids. The stereochemistry of the phytyl tail (side chain) influences the incorporation of the α-tocopherol molecule into the membrane. This tail bonds to membrane phospholipids allowing the chromanol head to be exposed at the membrane/cytosol interface (see figure 1.2). The orientation of the chromanol head within biomembranes is of considerable significance and will be discussed relative to α-tocopherol's function.

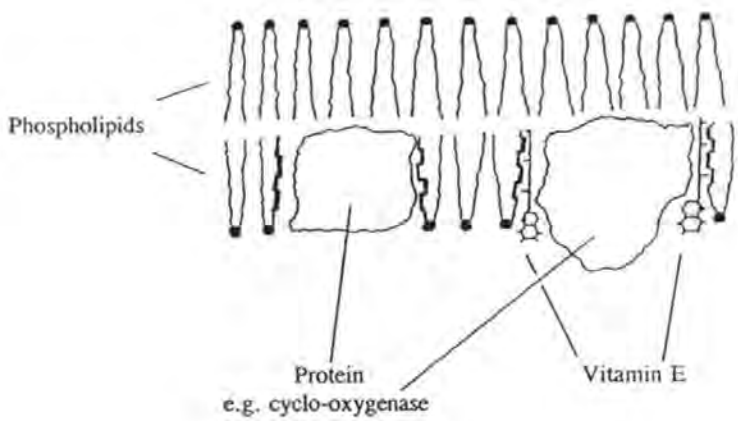


Figure 1.2 Membrane localization of α-tocopherol.

Vitamin E is cleared from tissues by its oxidation (possibly to the tocopherol quinone or hydroquinone (see figure 1.3) and subsequent removal via bile or excretion by the skin, or to a lesser extent in urine. The latter route tends to play a very minor role in the removal of vitamin E from the body. Bender (1992) states that urine tocopherol-metabolites ("Simon's metabolites") accounted for only 1% of tocopherol from a labelled test-dose. Significant amounts of α-tocopherol were seen by Shiratori (1974) on the skin of rats, demonstrating a cutaneous excretory route in the rat model.

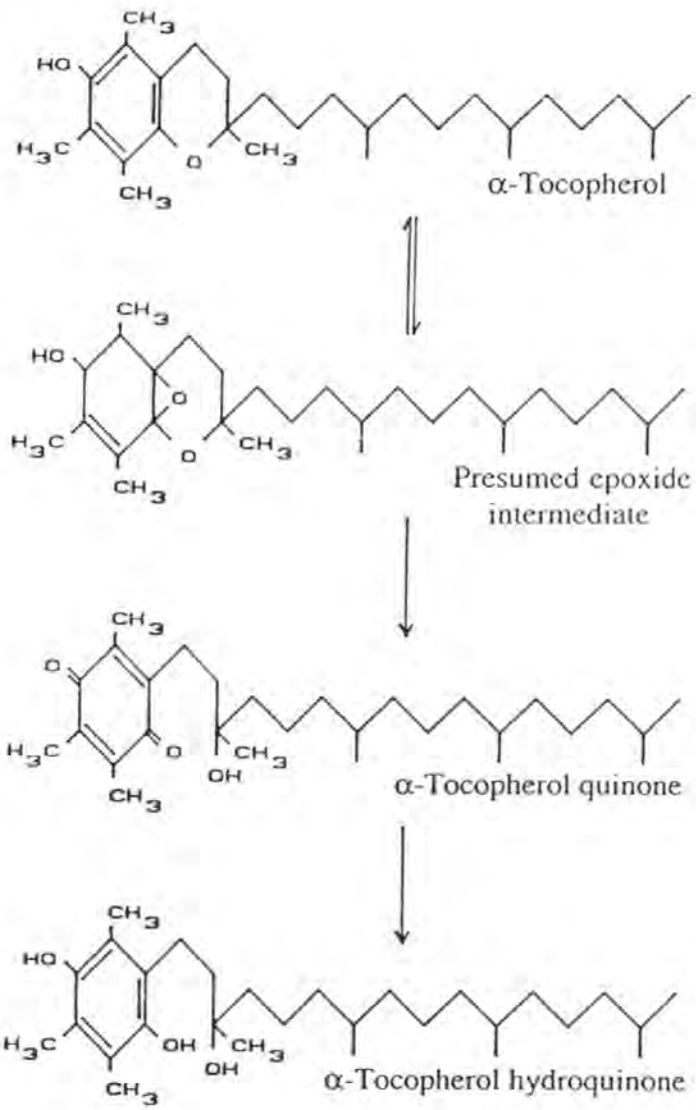


Figure 1.3 Oxidation of α-tocopherol to the quinone and hydroquinone

1.3 Biological Functions

1.3.1 Antioxidant Role of α-tocopherol

In biological systems, vitamin E (chiefly α-tocopherol), functions primarily as a non-specific, membrane-bound antioxidant, protecting tissue-lipids from free-radical mediated peroxidative modification.

Free radicals are any chemical species capable of independent existence that contain one or more unpaired electrons (Halliwell and Gutteridge, 1993). The unpaired electron conveys a considerable degree of reactivity to the radical due to the fact that radicals will attempt to stabilise their configuration by filling their outer electron orbital, by either sharing an electron with another radical (mutual stability), to form a dimer, or by taking an electron from another source, such as a membrane-bound fatty acid molecule. Hydrogen ion abstraction ensures that stability of the initial radical is achieved, although as a result this initiates secondary reactions by creating a new radical and this radical may in turn abstract further hydrogen ions, thus generating a chain reaction event.

In the case of the array of feed and tissue damaging free radicals, oxygen derived free radicals (or 'oxy-radicals') represent the focus of much research since it is mostly these species that behave as radical-chain-reaction initiators, giving rise to highly reactive lipid peroxyl radicals. The peroxidation of fatty acids is represented summarily in figure 1.4. Oxy-radicals are also capable of self-insertion into unsaturated portions of the fatty acid carbon chain (see figure 1.4). Poly-unsaturated fatty acids (PUFAs) or highly unsaturated fatty acids (HUFAs) would obviously be more susceptible to such modification, than the fatty acids containing few sites of unsaturation.

Incorporation of singlet oxygen across carbon to carbon double bonds, leads to the formation of aldehydes and ketones. The fatty acid carbon chain may then fragment to yield short chain (C2-11) products of lipid peroxidation (Grosch, 1987). This phenomenon forms the basis of numerous biochemical assays for oxidative damage in tissues and feeds. The assay for thiobarbituric acid reactive substances (TBARS) is one such test. Thiobarbituric acid forms a coloured complex, mainly with malonaldehyde (MA) or malondialdehyde (MDA) ($CH_2(COH)_2$), which can be measured due to its spectral properties.

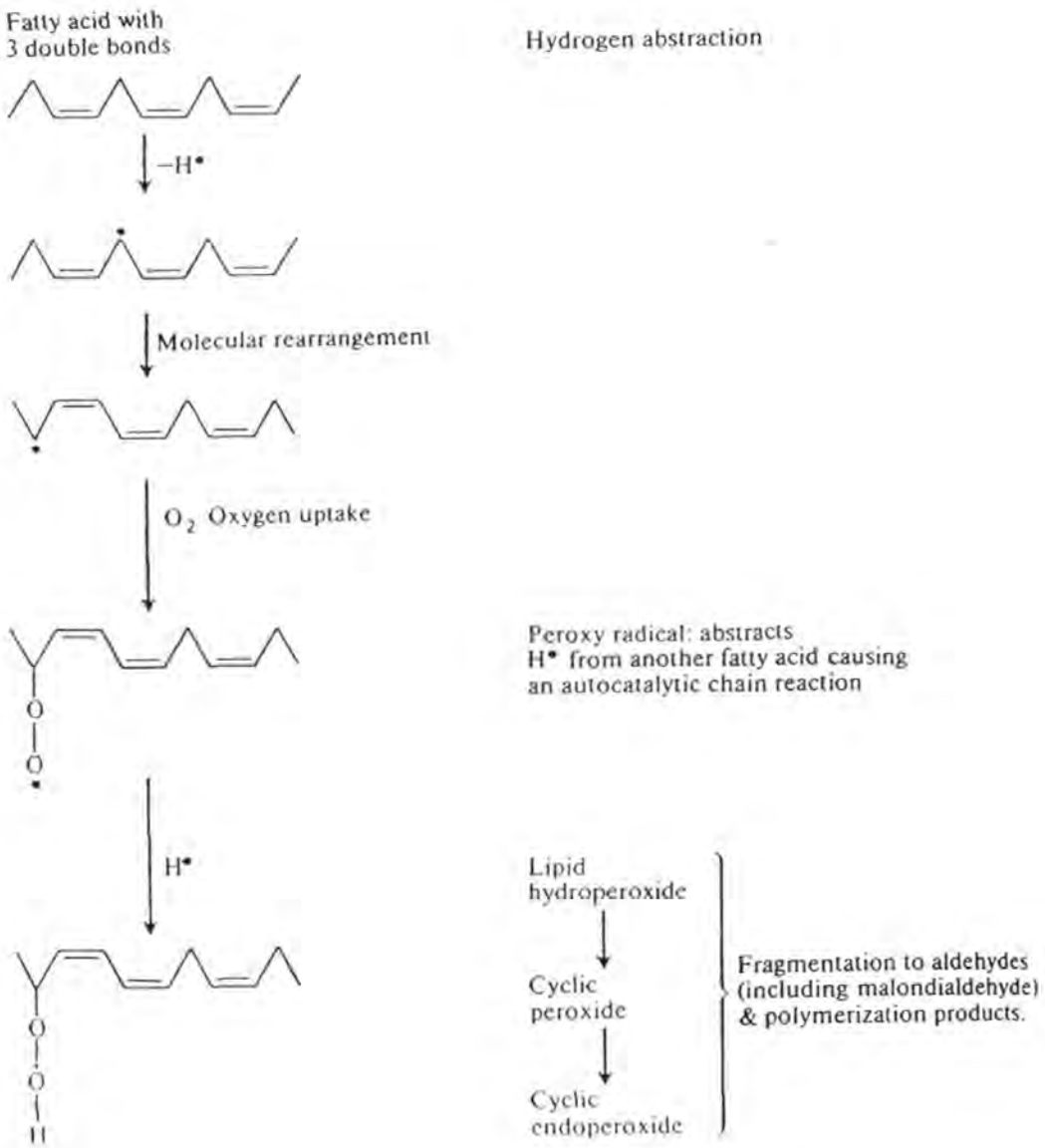


Figure 1.4 Simplified overview of unsaturated fatty acid modification by free-radicals

Where oxy-radicals function *in vivo*, both modes of oxidation occur resulting in changes in the chemical nature of the afflicted tissue. These changes may give rise to structural problems within membranes, due to fatty acid configurational changes. Additionally, in food, organoleptic properties may be altered giving rise to characteristic off-tastes (Grosch, 1987; Mottram, 1987).

Fortunately, biological materials are not defenceless against the free radical attack. Antioxidant defences, of which α-tocopherol is one, function to minimise radical mediated tissue injury. Owing to the location of α-tocopherol, its primary function is to arrest the process of lipid peroxidation in biomembranes. This is achieved at the membrane surface, by the hydroxyl group on the chromanol head portion of the molecule. α-tocopherol functions by donation of its hydrogen ion from the hydroxyl group to the reactive free radical (most commonly a lipid peroxyl radical), thereby blocking the lipid peroxidation cascade (see figure 1.5).

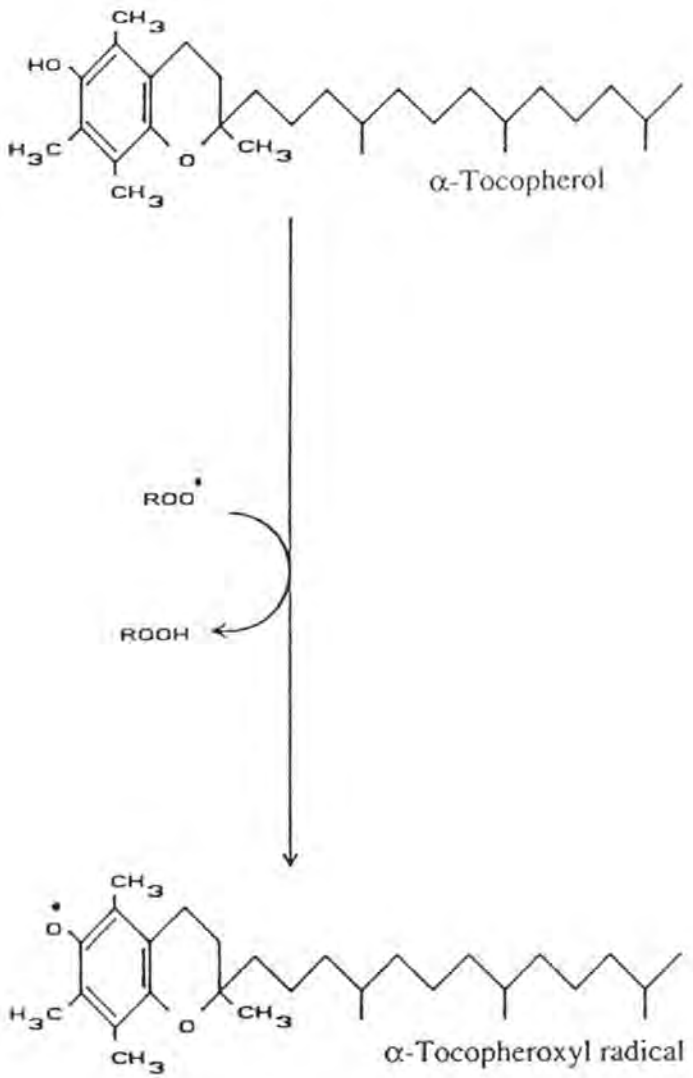


Figure 1.5 Free-radical quenching effect of α-tocopherol.

It must be noted however, that peroxyl radicals are formed deeper in the membrane, away from α-tocopherol's reactive group. The mechanism relies on the fact that on formation of the lipid peroxyl radical, the fatty acid hydrophobicity is changed, thus enabling the damaged fatty acid to diffuse outward to the membrane-cytosol interface. Here the damage induced by the radical is repaired, though the fatty acid molecule would probably remain 'floating' at the interface (Morrissey *et al.*, 1994). In doing so however, α-tocopherol is converted into a free radical itself. This α-tocopheroxyl radical is relatively stable, therefore does not perpetuate the chain-reaction. The α-tocopheroxyl radical can be removed from the system upon dimerization with any peroxyl radical or by reduction back to its α-tocopherol form.

This latter process can be achieved by water-soluble reducing agents such as ascorbate (vitamin C). Obviously this is only possible since the chromanol head lies at the cytosol/membrane interface, allowing reactions between the water-soluble ascorbate and the hydrophobic tocopherol. Elucidation of the α-tocopherol/ascorbate synergy will follow somewhat later in the text.

The implications of α-tocopherol protection of PUFAs are great. Prevention or control of fatty acid modification has far reaching consequences in human and animal health as well as the maintenance of fatty acid composition in biological materials for human and animal consumption.

Recently it has been proposed that α-tocopherol may be capable of pro-oxidative action. Frei and Gaziano (1993) performed a number of *in vitro* studies at different concentrations of metal ions and aqueous peroxyl radicals (the relevance of metal ions to oxidation will be outlined later). It was concluded that α-tocopherol acted as a chain transfer agent and not as a radical trap when in a system containing low levels of peroxyl radicals. Therefore α-tocopherol was judged to promote metal-ion independent peroxidation. It was further postulated that in LDL at near-depleted ascorbate levels, vitamin E's pro-oxidant role may

prevail over the antioxidant mechanisms. Indeed, at low metal ion concentrations, increasing vitamin E may increase peroxidation in LDL. It should be noted that all of these theories regarding pro-oxidation by vitamin E stem from *in vitro* or isolated cell-fraction work. These techniques, though offering interesting results, may be over-simplified and may not represent what happens *in vivo*.

Aside from the above mechanisms causing potential deleterious effects, toxicity of α-tocopherol is very low indeed. Tokuda and Takeuchi (1995) fed rainbow trout a diet containing 10,000 mg (10 g) α-tocopherol per kg diet and noticed no liver dysfunction. However, hepatic lipid levels (excluding α-tocopherol) were significantly elevated in the fish fed this high dose.

1.3.2 The possible role of α-tocopherol in eicosanoid modulation.

Eicosanoids are a group of hormone-like fatty acid derivatives, responsible for, amongst other things, the inflammatory response and vascular permeability (Bergström *et al.*, 1964; Dahlen *et al.*, 1983) Specifically, they are derived from the oxidative conversion of arachidonic acid (20:4 n-6)(and to a lesser extent other PUFAs) via one of two pathways. Through the cycloxygenase pathway, prostaglandins and thromboxanes are produced by a series of reductases and isomerases. These enzymes exploit the unsaturated nature of the carbon chain to produce a five carbon ring, from which two side chains can be modified to give a variety of prostaglandin and thromboxane homologues (Stryer, 1988).

Alternatively, leukotrienes may be derived via the lipoxygenase pathway. Lipoxygenase initiates chain modifications in the twenty carbon chains of arachidonic or eicosapentaenoic acid, to yield a structure unmodified from the parent PUFA, in terms of degree and site of unsaturation (Stryer, 1988).

Essentially, both of the pathways outlined above rely on free radical controlled modification of the PUFAs, after excision of the fatty acids from their membrane matrix.

The excised PUFA is converted (by cycloxygenase or lipoxygenase) into an endoperoxide intermediate which is then further modified to yield the eicosanoids (Rice and Kennedy, 1988). Potentially, therefore, α-tocopherol could exert a modulatory effect, dictating the production of the eicosanoids by trapping the peroxidative free radicals destined for eicosanoid production, or by regulating the availability of arachidonic acid to the phospholipase excisor (Diplock *et al.*, 1989). Possible evidence linking vitamin E status to eicosanoid production has been observed in investigations where there was an increase in prostaglandin synthesis, in selected tissues from vitamin E deficient animals (Koff *et al.*, 1981). Alternatively, Chan and Leith (1981) reported decreased prostacyclin in aortas removed from vitamin E deficient rabbits.

The interference in eicosanoid production could possibly be influenced by dietary means, through supplementation of α-tocopherol and/or selected fatty acids. Modulation of prostaglandin regulated inflammation, organ blood-flow, cross-membrane ion-transport or synaptic transmission, or leukotriene mediated smooth-muscle contraction, bronchoconstriction and vascular permeability may be achieved through the action of α-tocopherol, and this may have significant medical implications.

1.3.3 Regulation of membrane fluidity

Both the specific stereochemistry of the α-tocopherol molecule and the antioxidant capabilities have regulatory effects on the fluidity of biomembranes, especially those rich in arachidonic acid (Bender, 1992).

On the whole, membrane fluidity is dictated by a combination of cholesterol and phospholipid PUFA composition. High levels of PUFAs and low cholesterol concentrations tend to increase membrane fluidity (Rice and Kennedy, 1988). In addition, the presence of α-tocopherol plays a significant part in ordering the fatty acids in the membrane. This is achieved by the phytyl tail of α-tocopherol, which will interact closely with the

methylene-interrupted double-bonds of arachidonate and other long chain PUFAs (Diplock and Lucy, 1975). As well as this, the chromanol head is able to contribute to membranal fluidity by the influence of the hydroxyl group on the lipid/water interface, and its role in weakening van der Waals interactions between the fatty-acyl chains (Gomez-Fernandez *et al.*, 1989). The membrane stability achieved will result in minimising the ability of excision of arachidonic acid by phospholipase, thus demonstrating the modulation of eicosanoid production as described earlier (section 1.3.2).

In its antioxidant role, α-tocopherol exerts a further beneficial effect on membrane integrity. Uncontrolled membrane-PUFA peroxidation would decrease membrane-fluidity by reducing the amount of membrane associated PUFAs and increasing the cross-linking of those peroxidixed fatty acids. Evidently, tocopherol presence would slow the rate of peroxidation and therefore maintain membrane structural integrity.

1.4 Interactions of α-tocopherol

1.4.1 α-tocopherol and lipids

Many of the lipid/vitamin E interactions have already been previously described.

Most of these relationships have shown the antagonism between unsaturated lipids and α-tocopherol, whereby increasing tissue levels of PUFAs necessitates increased α-tocopherol for protection against peroxidation. With respect to dietary intakes of these nutrients, the same is true, although balancing the dietary requirements of each is seldom a problem since foods high in PUFAs are often good sources of vitamin E (Bender, 1993).

Aside from the antagonistic relationship exhibited between vitamin E and lipids, there exists a positive interaction when dietary lipid levels are low. In this instance the transport of α-tocopherol is dependent on the availability of lipid micelles for absorption. Similarly, vitamin E transport within the body is dependent on total plasma lipids (Bender, 1992).

1.4.2 The effect of other antioxidants

The term 'antioxidants' represents rather a large grouping of assorted vitamins, enzymes and synthetic antioxidants. Many, if not all of these act in concert with α-tocopherol, defending biological materials against a host of free radicals.

Firstly, we shall consider the role of vitamins A and C in their synergistic support of α-tocopherol.

Vitamin A (retinol and β-carotene) has a well-defined role in visual systems of animals, and an important role in the control of growth and tissue differentiation. In addition, β-carotene functions as a potent free radical trapping antioxidant, especially in systems where radicals are generated by photo-reactions (Halliwell and Gutteridge, 1993). Possibilities exist therefore, for a synergistic relationship where vitamin A (as β-carotene) and vitamin E may spare each other through mutual protection. Though this has been demonstrated *in vitro*, little evidence has come to light on this *in vivo* synergy in plant or animal systems. Carotenoids such as astaxanthin and canthaxanthin may also exhibit sparing action since in membrane models they have been seen to effectively scavenge free radicals (Palozza and Krinsky, 1992). Also, it has been demonstrated that vitamin E is able to protect these pigments within fish tissues (Pozo *et al.*, 1988).

Ascorbate (vitamin C) has been observed to interact with α-tocopherol *in vitro* by regenerating α-tocopherol from the tocopheroxyl radical (McCay, 1985). Further studies reported by Hilton (1989) have shown a sparing effect by ascorbate when rats were deficient in vitamin E.

In its recycling role, ascorbate donates a hydrogen ion to the tocopheroxyl radical, to become monodehydroascorbate (see figure 1.6). Monodehydroascorbate can also be recycled back to the reducing form (ascorbate) by reducing agents in the aqueous phase. The sparing of α-tocopherol could also be achieved by ascorbate, by directly reducing reactive radical species prior to their damaging bio-membranes.

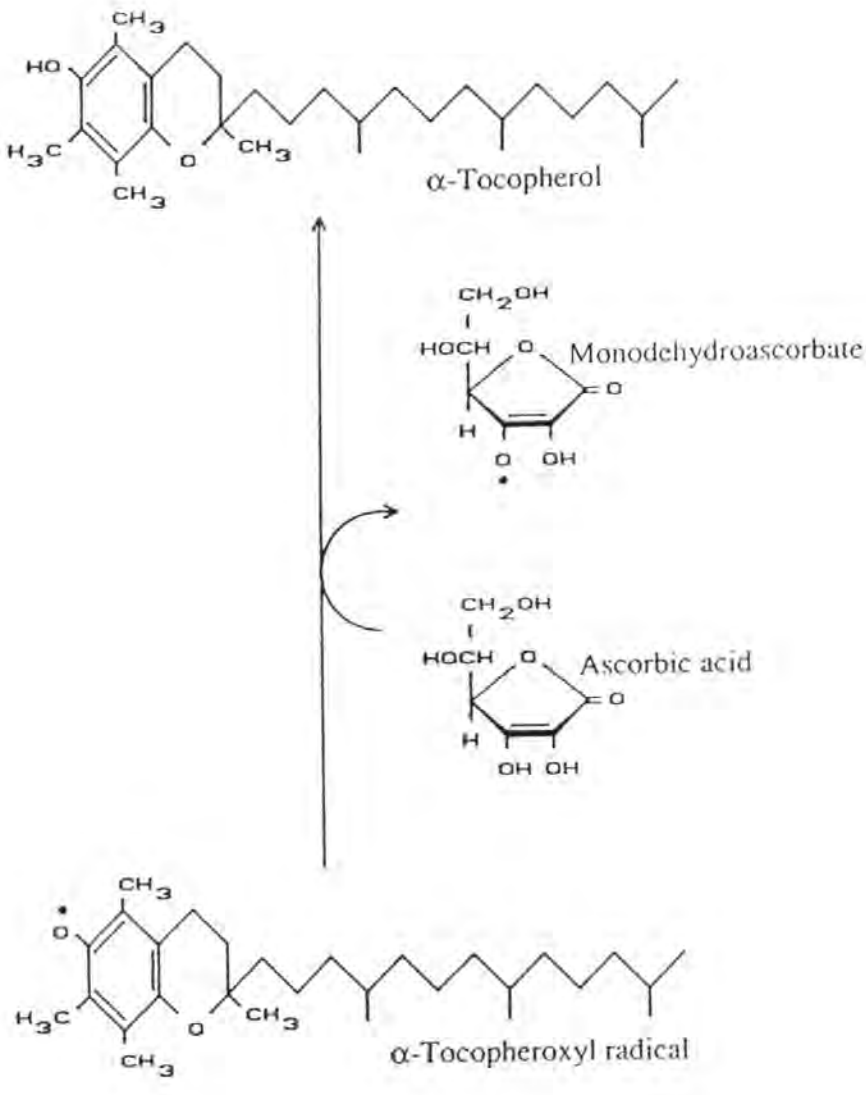


Figure 1.6 Reduction of the tocopheroxyl radical by vitamin C (ascorbate) to α-tocopherol

Vitamin C may accelerate peroxidative damage at high ascorbate concentrations. This pro-oxidant damage is due to the fact that ascorbate is able to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), when free iron is available in solution. Ferrous ions are then capable

of hydroxyl radical generation by way of the Fenton reaction (Halliwell and Gutteridge, 1993). It should be noted that ferric ions are capable of oxidative damage by direct electron scavenging. These points will be mentioned briefly later. Enzymic defences against free radical mediated tissue-damage are also capable of sparing α-tocopherol. A battery of antioxidant enzymes exist in animal tissues whose roles are to 'intercept', or aid in the interception of, specific free radical species in the aqueous phase.

Many of the enzymes, though capable of independent action, function sequentially in the removal of oxy-radicals. Catalase, superoxide dismutase (Zn-, Cu-, and Mn- SOD) and glutathione peroxidase (GSH-Px) are ubiquitous in biological systems exposed to the stresses associated with oxygen-derived radicals.

Catalase is capable of removal of hydrogen peroxide from cells by degradation to water and oxygen. This is of importance when we consider that hydrogen peroxide is the primary source of the highly reactive hydroxyl radical. SOD functions on superoxide radicals to produce hydrogen peroxide. Already it is evident that the enzymes must cooperate in the protection of tissues since SOD is producing hydrogen peroxide as a by-product of superoxide removal, and this is the substrate for catalase.

The seleno-enzyme, glutathione peroxidase can also act on hydrogen peroxide in addition to the hydroxyl radical and lipid-peroxyl radicals. In this role glutathione peroxidase catalyses oxidation of two molecules of substrate (glutathione) to yield a glutathione dimer and reduced product. In many biological systems there also exists selenium-independent glutathione peroxidase activity, and glutathione transferases may also take part in similar reactions. Despite these overlaps in enzyme roles, many researchers have concentrated on glutathione peroxidase in investigations related to antioxidant status. Glutathione peroxidase also represents the link accounting for the selenium / tocopherol synergy and as such has been extensively studied by Walsh *et al.* (1993) in their work on calves. The findings of that research demonstrated the fact that GSH-Px activity increased in response to vitamin

E deficiency. This is evidence of a compensatory action. Above the requirement levels of tocopherol, no sparing of GSH-Px was noted. Sakai and co-workers (1992) also noticed elevated antioxidant enzyme activities in response to the nutritional stress of feeding rancid oils, although other research in the field has not established such a phenomenon.

Synthetic antioxidants such as butylated hydroxy toluene (BHT) or butylated hydroxy anisole (BHA) have been used to great success in the protection of oxidizable components of animal feeds or feed ingredients. Incorporation of these alternative antioxidants into ingredients has significantly reduced lipid-peroxidation damage in feeds, though consumer pressure is being applied to minimize the use of these compounds in order to reduce carry-over into humans (Bailey and Um, 1992). This obviously moderates the use of widespread addition of these compounds into feeds destined for food-animals. The radical sequestering abilities of the synthetic antioxidants has also led to their use in assays where protection of oxidizable components is desired.

1.4.3 Antagonism of vitamin E by transition metals

Transition metals may exist in one of a number of valencies. As such, they have the ability to either accept or donate electrons depending on their state. Ferrous ions (Fe^{2+}) can donate electrons and in doing so become ferric ions (Fe^{3+}). These two iron states are both capable of exerting a pro-oxidant effect, though each by different mechanisms. Ferrous ions are chiefly responsible for pro-oxidation via Fenton reactions, in which non-radicals such as hydrogen peroxide are split to yield hydroxyl radicals (Halliwell and Gutteridge, 1993). These radicals are extremely reactive and may cause lipid peroxidation unless halted by antioxidants. Ferric ions are capable of exerting direct oxidant pressure by electron abstraction from molecules. Additionally, in systems where there exists a large pool of antioxidants such as ascorbate, these may reduce ferric ions to the ferrous state, thus perpetuating free radical mediated damage of tissues. Evidently, the best defence from

metal induced attack is to effectively chelate the metal into protein matrices such as enzymes. In the case of iron, the largest pool is within erythrocytes as haemoglobin, although transferrin and ferritin also contribute to control of tissue iron levels (Halliwell and Gutteridge, 1993).

Metal overloads in which basically there is insufficient protein to envelope the metal, leads to unbound forms of these transition elements being free in solution, and hence free to participate in Fenton type reactions. Pro-oxidation of tissues by transition metals accounts for most of their toxic effects. Whether in iron, copper, manganese *etc.* overload, lipid peroxidation has often been observed and in some cases this has been accompanied by a depletion of tissue vitamin E (Kirchin *et al.*, 1992).

1.5 Effects of tocopherol deficiency

1.5.1 α-tocopherol deficiency in man and animals

This section has been compiled from the excellent reviews of Rice and Kennedy (1988), McDowell (1989), Machlin (1991) and Morrissey *et al.* (1994). Additional material has been incorporated and will be acknowledged where appropriate.

Although vitamin E was not isolated and identified until the late 1930s, effects of its deficiency were observed in 1920 by researchers who demonstrated induced rat sterility by the consumption of certain milk products. Two years later, the factor necessary for normal reproduction in rats was termed vitamin E, although the exact structure of α-tocopherol was not determined until 1938.

Much of the early work into vitamin E deficiency was based on terrestrial animals, since symptoms of deficiency were not recognised in humans. In 1931, Pappenheimer and Goettsch (cited in Machlin, 1991) demonstrated that vitamin E was necessary in preventing muscular wasting in rabbits and guinea pigs, and in preventing nutritional encephalomalacia ('crazy chick disease'). The latter disorder is characterised by locomotor

incoordination. Further work on chicks revealed that muscular dystrophy and exudative diathesis afflicted deficient individuals. To date, the list of identified vitamin E deficiency symptoms in land animals is extensive (see table 1.1).

It has been noted that some of the symptoms characteristic of vitamin E insufficiency may be prevented by increased selenium status or the dietary incorporation of synthetic antioxidants, although in many roles, vitamin E is exclusive.

As described earlier, deficiency can be exarcebated by antagonistic action from increased oxidative stress, whether from elevated oxygen partial pressure, increased pro-oxidation from metals, rancidity of lipid sources or the heightened potential for peroxidation caused by increased dietary polyunsaturated fatty acid intake. Any of these antagonists will also compromise human tocopherol status.

Human studies on vitamin E deficiency were quick to try and focus on the effects of the vitamin on reproductive ability. Vitamin E was sensationally claimed to increase virility in adults, though most research demonstrated that increased vitamin E dose had little or no effect on the measured reproduction parameters. In fact, no human deficiency pathology was identified until it was noticed that the debilitating neuropathy in patients suffering from a severe fat malabsorption disease, was due to tocopherol deficiency. Since then, cases have appeared mainly in infants due to the low tissue levels of vitamin E at birth, coupled to high PUFA intakes from milk formulae. Manufacturers have taken steps to reduce PUFA and increase the vitamin E concentrations in these products. It would appear that effectiveness of vitamin E assimilation in the gut improves throughout childhood, so that after infancy, few cases of deficiency are evident. Nevertheless, these symptoms have come to light: haemolytic anaemia, low haemoglobin, reticulocytosis, hyperbilirubinemia, elevated platelet count, hyperaggregability of platelets, increased risk of intraventricular haemorrhage and increased severity of retinopathy of prematurity in low-birth weight infants; reduced erythrocyte half-life, axonal dystrophy and neuromuscular deficits in

children with severe lipid malabsorption; reduced erythrocyte half-life, ceroid deposition in smooth muscle and neuromuscular deficit in adults with malabsorptive disorders, or familial or experimental deficiency. Increased tissue status of vitamin E, whether by oral or intra-muscular injection, has been shown to abrogate the effects listed above.

Table1.1 Effects of vitamin E deficiency in terrestrial animals

Tissue	Symptom	Animal affected
Muscle		
Skeletal	Necrotizing myopathy	monkey, pig, rat, dog, rabbit, guinea-pig, horse, calf, lamb ¶, kid ¶, mink ¶, chicken, duck §, antelope.
Heart	Necrotizing myopathy	pig, rat, dog, rabbit, guinea pig, calf, cow, sheep, goat, baboon, antelope, elephant, deer,
Gizzard	Necrotizing myopathy	turkey.
Reproductive		
Placental blood vessels	Foetal death and resorption	pig, rat, mouse, guinea pig, cow, ewe ¶, chicken.
Uterus	Lipofuscin accumulation	rat.
Testis	Epithelial degeneration	monkey, pig, rat, rabbit, guinea pig, hamster, dog, chicken.
Gastrointestinal		
Intestine	Lipofuscin accumulation	dog.
Stomach	Gastric ulceration	pig.
Adipose	Lipofuscin accumulation	pig, rat, mouse, hamster, cat, mink.
Vascular		
Blood vessels	Exudative diathesis †	chicken, turkey.
	Fibrinoid degeneration	pig.
Erythrocytes	Anaemia	monkey, pig, rat.
	Haemolysis (*in vitro*)	monkey, rat, chicken.
Platelets	Increased number	rat.
	Incr. aggregation (*in vitro*)	rat.
Eyes	Cataracts	turkey embryo, rabbit.
	Retinal degeneration	dog, monkey, rat.
Nervous system		
Brain (cerebellum)	Encephalomalacia ‡	chicken.
Nerves	Axonal dystrophy	monkey, rat, dog, duck, mouse, guinea pig.
	Lipofuscin accumulation	rat, mouse.
Liver	Necrosis †	pig, rat, mouse.

¶ vitamin E ineffective if diets are severely deficient in selenium; both nutrient required.
§ preventable by sulphur amino acids.
† preventable by selenium.
‡ neural changes may be secondary to vascular pathology.

1.5.2 α-tocopherol deficiency in fish

Fish are predominantly more susceptible to free radical mediated lipid peroxidation since their tissues contain relatively higher amounts of PUFA and HUFA (highly unsaturated fatty acids) when compared to terrestrial animals (humans included) (Sargent *et al.*, 1989). It therefore follows that their requirement for vitamin E will also be greater in order to protect these delicate fats.

In a natural environment, fish of all trophic levels may be expected to always be sufficient in vitamin E because of the synthetic capabilities of algae. α-tocopherol is then transferred up the food chain via consumed tissues.

Under culture situations however, conditions of increased stress from crowding (and therefore disease), environmental factors (mainly temperature) and a reliance upon artificial feeds has led to the appearance of numerous deficiency syndromes in a number of cultures fish species.

Vitamin E deficiency may be caused by either depletion of tissue α-tocopherol by free radical stressors, inadequacy of supplemented vitamin E or by the degradation of tocopherol from the diet. One would not expect the latter to occur when tocopherol esters are employed as the vitamin E form, but in situations where unprotected α-tocopherol is the sole source, this may be rapidly oxidised in the diet. This is of considerable importance where untreated meat by-products, such as poultry offal, are fed directly to fish in polyculture systems. In one reported case involving the culture of catfish (*Clarias Sp.*) in Thailand, poultry offal was being transported by road to the culture ponds (Pearson, 1993). Due to a combination of high temperatures and long transport times (several days), the food was often degraded prior to feeding. The effect was to induce jaundice in the catfish, though this disease may not necessarily be directly linked to antioxidant status.

Conditions that have been firmly associated with vitamin E deficiency mainly include necrosis of a number of organs, although other effects are also documented.

In carp (*Cyprinus carpio*) Watanabe *et al.* (1970a,b) demonstrated that α-tocopherol insufficiency led to muscular dystrophy ("Sekoke disease"), poor growth, exudative diathesis, exopthalmia and lordosis. Satoh and co-workers (1987) report reduced appetite and decreased liver weights in tilapia (*Tilapia nilotica*). Blue tilapia (*Oreochromis aureus*) were afflicted by poor growth, skin and fin haemorrhages, anorexia, impaired erythropoiesis, muscle degeneration, splenic and hepatic ceroid deposition, and lack of skin colour (Roem *et al.*, 1990). Salmonids deficient in vitamin E exhibit dystrophic muscle, impaired erythropoiesis, extreme anaemia, susceptibility to handling-stress, high mortality, serous fluid in the body cavity, and exudative diathesis (NRC, 1993). Many symptoms of deficiency cannot be separated from those associated with the consumption of oxidised oils. Evidently this is due to their similarity in mode of action *i.e.* effects are caused by free radical mediated lipid peroxidation. As such, information from research into effects of autoxidised oil may well illustrate vitamin E deficiency conditions. Stephan *et al.* (1993) demonstrated that seabass (*Dicentrarchus labrax*) suffered from growth depression, anorexia, skeletal myopathy, liver, pancreas and kidney necrosis, hepatic ceroidosis, and haemosiderine in the spleen. Channel catfish (*Ictalurus punctatus*) fed on rancid feeds were predisposed to muscular dystrophy, fatty livers, anaemia, exudative diathesis and depigmentation (NRC, 1993). Cowey and co-workers (1984), on the other hand, could find no pathology in rainbow trout fed moderately oxidised oil. Differences in weight gain, haematocrit, erythrocyte fragility, mortalities and indices of lipid peroxidation were seen between trout from treatments supplemented or deficient in vitamin E.

Through experimentation, minimum requirement levels of vitamin E have been defined for a great deal of fish species, in order to negate the problems of deficiency diseases and to satisfy growth performance criteria. These requirement levels are set out in NRC (1993), although it must be understood that dietary composition and oxidative stresses would obviously alter actual requirements. Nevertheless, those values serve as good guidelines

for the feed formulator. Nowadays, salmonid feeds are supplemented at between 100 and 400 mg α-tocopheryl acetate per kg diet, though the requirement is quoted as 25-100 mg per kg.

1.6 Benefits of supplementation of vitamin E into fish diets

It is evident that supplementation of vitamin E into fish diets would be a worthwhile strategy. Low-level inclusion of α-tocopheryl acetate as a feed ingredient would overcome any potential processing and storage losses of the vitamin, though may not confer any advantage to the fish itself. Augmented addition of vitamin E into compounded fish feeds has been seen to offer considerable advantages in terms of health and stability against lipid peroxidation.

Increased dietary α-tocopherol concentration has been noted to modulate immune response in many fish. A concise review of this topic has been produced by Lall and Olivier (1993). Autotoxicity of lipid peroxides and reactive oxygen species can cause destruction of vital defence components such as neutrophils and macrophages by disruption of phospholipid membranes. Antioxidant protection of these non-specific defences could possibly promote the longevity of such cells, thus promoting effective functioning. Although many studies have examined various parameters associated with the immune response, experimenters have mainly concerned themselves with assessing the response of vitamin E deficient individuals and individuals adequate in the vitamin. Few studies have explored possibilities of heightened supplementation of vitamin E in order to improve immune status above basal levels. Conferring such an advantage would be extremely desirable in high-density culture situations. To date, results are inconsistent with many conflicting observations. Lall *et al.* (1988) and Hardie *et al.* (1990) could find no beneficial effects of increased vitamin E concentration in feeds for Atlantic salmon (*Salmo salar*), although in the latter study the complement system was compromised in the low-tocopherol treatment.

It has also been shown that high levels of vitamin E (2500 mg per kg) will suppress immune function (Blazer, 1991) by blocking the controlled free radical mechanisms harnessed for destruction of pathogens. All in all, it seems unlikely that super-supplementation will prove advantageous in ameliorating immune response of fish culture, though more research is required since advances in this field would be beneficial to the expanding aquaculture industry.

An area of research in which super-supplementation has proved worthwhile is in the prevention of fish product deterioration. For some time it has been known that increased dietary tocopherol leads to increased tissue levels of the vitamin. This in turn means that tissues will have a greater capacity to deal with free radicals, even *post mortem*. Meat products from animals fed increased doses of vitamin E will be more resistant to oxidation in storage or display prior to retail. This parameter has been measured by evaluation of many different products of lipid peroxidation. Determination of selected aldehydes and/or volatiles has yielded important results with respect to kinetics of autoxidation (Shahidi *et al.*, 1987; Ajuyah *et al.*, 1993). One of the most widely applied tests is the assay for thiobarbituric acid reactive substances (TBARS). This method is based on the formation of a coloured complex on reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), though other compounds may also form an adduct with TBA. Sheehy and co-workers (1993a) demonstrated that muscle from poultry fed incremental doses of α-tocopheryl acetate, would be more stable in storage in response to increases of the vitamin. Storage stability in that study was assessed by inducing peroxidation through incubation of tissue homogenates with an iron-ascorbate solution. Of course, this parameter could equally be measured after extended storage, subjection of tissues to increased partial pressures of oxygen, or by alternative pro-oxidation. Previous research in poultry had already revealed that heightened vitamin E status improved taste and odour of chick meat (Laksesvela, 1960).

These sorts of studies have been carried out in a number of fish species. Increased tissue α-tocopherol has been observed to decrease TBARS formation in rainbow trout (*Oncorhynchus mykiss)*(Hung *et al.*, 1980; Hung and Slinger, 1982; Boggio *et al.*, 1985; Frigg *et al.*, 1990), channel catfish (*Ictalurus punctatus*)(O'Keefe and Noble, 1978; Gatlin *et al.*, 1992) and red sea bream (*Pagrus major*)(Murata and Yamauchi, 1989), implying that fish product shelf-life can be lengthened through dietary means.

Storage stability of meat products can also be evaluated visually in some cases. For beef, it is usual for meat to change colour upon oxidation. The desirable 'cherry red' colour is due to levels of oxymyoglobin and this is often associated with freshness. Upon oxidation however, oxymyoglobin is converted to metmyoglobin resulting in browning of the meat (Arnold *et al.*, 1993; Lanari *et al.*, 1993) and lessened appeal to consumers. Vitamin E postponed this effect, thereby extending the shelf life of beef fillets. Another situation where colour is of great importance to the consumer, is in some fish products. Cultured salmon or rainbow trout are fed diets containing astaxanthin or canthaxanthin in order to impart an orange/red colour to the flesh. *In vivo* these pigments may be protected from oxidation (Sigurgisladottir *et al.*, 1994) though stability of pigment colour is not maintained any better in tocopherol supplemented fish products.

Additional to product quality with regards to longevity, it is also important to consider the nutritional ('eating') quality of meats destined for human consumption. So far it is obvious that tissue α-tocopherol concentration is dependent mainly on dietary vitamin E intake. Consumption of meat products containing elevated vitamin E would prove beneficial in boosting man's antioxidant status, with important repercussions in health status. Since tissue lipids are also stabilised *post mortem*, toxicity of oxidised oils is also avoided.

Table fish could represent a major source of dietary vitamin E in countries practising intensive aquaculture, and much scope exists to influence human health through manipulation of fish diets.

With all of the above considerations, it is evident that merely establishing a minimum requirement for a particular species, is not entirely satisfactory. Supplementing fish diets with supra-nutritional levels of vitamin E would be beneficial in terms of maintaining product quality as well as providing a vehicle for boosting the α-tocopherol status of humans. Scope exists for studies in the field of vitamin E nutrition, particularly with respect to tissue 'health'. Although much work has been carried out in antioxidant nutrition in finfish, a species as yet unexplored is a cultured tropical food fish, the African catfish.

1.7 The African catfish (*Clarias gariepinus* Burchell)

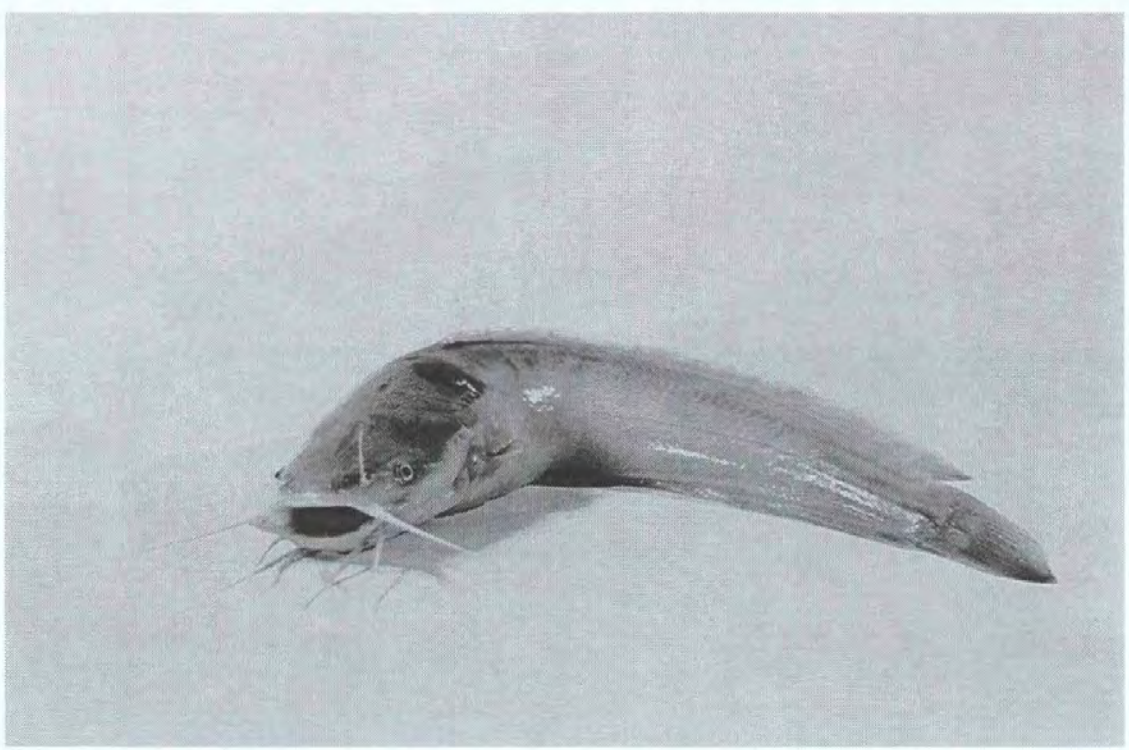
Clarias gariepinus (Burchell 1822) (plate 1.1), is an omnivorous freshwater fish species indigenous to Africa and found over an extremely large latitudinal range (Haylor, 1990). The catfish is capable of air breathing from very early in development and this is beneficial in many culture situations. Extracting oxygen from the air means that *Clarias* are able to tolerate the low water oxygen tensions associated with fairly anoxic ponds and with the low concentration of oxygen encountered in high density culture.

Clarias are known to be efficient feeders (Verreth and Eding, 1993) and this too, adds to the suitability of this species for farming. Due to the aforementioned factors, the African catfish is currently cultured in China, the Philippines, Indonesia, Thailand, Brazil and in several European countries (Verreth and Eding, 1993).

Clarias products include mainly fresh fillets, although great product variability exists even from this one cut. Depending upon size, sex and obviously dietary regime, fillets may be more suitable for retail as fresh or smoked products.

Despite the large increase in interest in this species over the last decade, advances have tended to focus on genetic or macro-nutrient research.

Of the macro-nutrients, the protein nutrition of *Clarias* has been best studied. Henken and co-workers (1986) defined an optimum dietary protein concentration for fry, of 30 % at



a lipid level of 6 %. For larger fish, using 8 % lipid at a 24 ^{0}C culture temperature, 50 % protein supported better growth than a diet containing 30 % crude protein. Evidently, this highlights how the provision of increased lipid (supplying more non-protein energy) allows for heightened protein utilization for growth. Despite the requirement level having been defined under certain culture conditions, workers still rely on commercially available trout feeds with satisfactory results (Degani *et al.*, 1988).

Within the field of vitamin nutrition, little research has been carried out, since on the whole *Clarias* have performed well on trout feeds as discussed above. To date, qualitative requirements for thiamine, riboflavin, pyridoxine, pantothenic acid, folic acid, niacin and ascorbic acid have been established in the Asian catfish (*Clarias batrachus*)(Butthep *et al.*, 1983). Additionally, Mgbenka (1991) has declared the vitamin C requirement of *Clarias gariepinus* (supplied as L-ascorbic acid) as 60 mg per kg diet. Evidently, there is scope for detailed micro-nutrient studies, and with the marketability of catfish products, optimization of aspects of storage quality would appear to be an area meriting attention. With the current interest in natural antioxidant nutrition, it would seem extremely beneficial to examine the role played by vitamin E in preventing lipid peroxidation in tissues of the African catfish.

1.8 Research aims

The current series of investigations aims to establish the efficacy of α-tocopherol supplementation (as all-rac-α-tocopheryl acetate) in diets for the African catfish (*Clarias gariepinus*) in modulating free radical mediated peroxidation of tissue lipids. Additionally, the effects of other dietary parameters (such as dietary pro- and antioxidants) upon α-tocopherol's functioning will be assessed.

It is expected that, through these investigations, the field of *Clarias* nutrition will be strengthened by the elucidation of the role of dietary vitamin E in this poorly researched

cultured species.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 The test-diets

2.1.1 Feed formulation

Semi-practical test-diets were formulated by a feed formulation software package ('Ultramix' V2.12, A.G. Munford, University of Exeter, Exeter, Devon, U.K.) to cater for the known requirements of the African catfish. Where nutrient requirement levels were not known for this species, values from other omnivorous, warm-, fresh- water fish were assumed. In addition, the formulation was weighted in favour of locally available feed-stuffs.

A generalised formulation is presented in table 2.1.

The semi-practical diets were designed to contain approximately 50% protein by dry weight. This was above levels normally associated with *Clarias* feeds (Henken *et al.*, 1986) to ensure that the maximum growth potential was realised. Protein was supplied in the form of brown Chilean fishmeal (Crediton Feedmills, Crediton, Devon, U.K.), meat and bone meal and blood meal (Carne and Sons, Callington, Cornwall, U.K.). The latter also served as a feeding attractant (S.J. Davies (1996), pers. comms.).

The lipid contribution to the diet was supplied partially by the fishmeal, though a bulk of the lipid was provided through a 1:1 cod-liver oil ('Boost', Seven Seas Ltd., Hull, U.K.): corn oil ('Mazola', C.P.C. (U.K.) Ltd., Esher, Surrey, U.K.) mix. This ensured an adequate balance of n-3/ n-6 fatty acids (N.R.C., 1993). Actual levels of dietary lipid varied between investigations, though values were always within the range 10-13%. Note that conventional, commercially available cod-liver oil was replaced by an oil, unsupplemented with antioxidants.

Corn-starch, dextrin (Sigma Chemical Company Ltd., Poole, Dorset, U.K.) and Molasses

Table 2.1 Generalised composition of practical test diets for the African catfish, *Clarias gariepinus*.

Ingredient	%inclusion
Fishmeal (Chilean)	60.00
Meat and bone meal	10.00
Bloodmeal	2.00
Cod-liver oil (BOOST, Seven Seas Ltd.)	2.50-3.00
Corn oil (MAZOLA, CPC U.K. Ltd.)	2.50-3.00
Corn starch:Dextrin (3:2)	15.35-16.35
Molasses	1.00
Mineral premix (see table 2.2)	5.00
B-complex vitamin premix (see table 2.3)	0.10
Fat-soluble vitamin premix (see table 2.4)	0.05
Macro-vitamin premix (see table 2.5)	0.50

Table 2.2 Mineral premix composition

Mineral salt		Inclusion (g kg^{-1}dry diet)
Calcium orthophosphate	$CaHPO_4.2H_2O$	12.000
Magnesium sulphate	$MgSO_4.7H_2O$	4.8450
Sodium chloride	NaCl	2.2800
Potassium chloride	KCl	1.9000
Iron sulphate	$FeSO_4.7H_2O$	0.9500
Zinc sulphate	$ZnSO_4.7H_2O$	0.2090
Manganese sulphate	$MnSO_4.4H_2O$	0.0960
Copper sulphate	$CuSO_4.5H_2O$	0.0298
Cobalt sulphate	$CoSO_4.7H_2O$	0.0181
Calcium iodate	$CaIO_3.6H_2O$	0.0112
Chromic chloride	$CrCl_3.6H_2O$	0.0048
Sodium selenite	Na_2SeO_3	0.0025
(α-cellulose)	(filler)	(27.6536)

50 g total premix in 1 kg dry diet (5% inclusion).

Table 2.3 B-complex vitamin premix composition (except B12-cyanocobalamin †)

vitamin	inclusion (mg kg^{-1} dry diet)
B1-Thiamine hydrochloride	50
B2-Riboflavin (feed grade-96%)	52.1
B6-Pyridoxine hydrochloride	40
Calcium pantothenate	100
Niacin	200
H2-Biotin (2%)	300
Folic acid (90%)	16.7
(α-cellulose - filler)	(241.2)

† B12-cyanocobalamin required = 0.05 mg kg^{-1} dry diet

1 g premix in 1 kg diet by dry weight (0.1%)

Table 2.4 Fat-soluble vitamin premix composition (except vitamin E †)

vitamin	inclusion (mg kg^{-1} dry diet)
A-Vitamin A palmitate (1.7 $X10^6$ I.U. g^{-1})	2
D-Rovimix D3-500 (5 $X10^5$ I.U. g^{-1})	20
K-Menadione sodium bisulphite (51%)	80
(α-cellulose - filler)	(398)

† Vitamin E (all-rac-α-tocopheryl acetate) added as required.

0.5 g premix in 1 kg dry diet (0.05%)

Table 2.5 Macro-vitamin premix composition

vitamin	inclusion (mg kg^{-1} dry diet)
Inositol	200
Choline chloride	2000
Ascorbic acid	500
(α-cellulose- Filler)	(2300)

5 g premix in 1 kg dry diet (0.5%)

(British Sugar plc., Norwich, U.K.) constituted the carbohydrate component of the formulation, useful for possible protein sparing and also due to their pellet-binding properties.

Minerals were added to diets in the form of a pre-mix, designed on the basis of maximum requirements of fish as described in N.R.C. (1993). Inclusions of metal salts in the pre-mix are shown in table 2.2.

Pre-mixes were also used to provide the necessary vitamins in the diets. The fat-soluble vitamin (except vitamin E), B-vitamin and macro-vitamin pre-mixes were formulated to satisfy the requirements of salmonids as set out by Cho and co-workers (1985). Tables 2.3, 2.4 and 2.5 present the compositions of the B-vitamin, fat-soluble vitamin (except vitamin E) and macro-vitamin pre-mixes respectively. These vitamins were supplied by Roche Products Ltd. (Colborn Dawes, Heanor, Derbyshire, U.K.), Lonza U.K. Ltd. (Cheltenham, Gloucestershire, U.K.) and Takeda Chemical Company (Tokyo, Japan). Of the pre-mixes, only the macro-vitamin premix had to be prepared fresh on the day of diet manufacture. The others could be made well in advance and stored at -20^{0}C.

Vitamin E was supplied to the diet as a spray-dried acetate ester of α-tocopherol, available commercially as Rovimix E-50 SD (50 % purity) (Roche Products Ltd., Colborn Dawes, Heanor, Derbyshire, U.K.). Actual inclusion levels of this vitamin varied between each investigation, therefore values are presented in the appropriate chapter.

2.1.2 Diet manufacture and storage

Batches of 2 or 3 kg of food were prepared as follows. Dry powdered ingredients were individually weighed into a suitably sized container, then thoroughly mixed in the bowl of a Hobart A120 food processor (Hobart Manufacturing Company Ltd., London, U.K.). The oil was then added very gradually in a continuous, slow-pouring action. During further mixing, the molasses was added to the mash. Finally, an appropriate volume of tap-water

was then added during continuous mixing to yield a dough considered sufficiently moist for extrusion. Typically 300 cm^3 of water per kg of dry matter was used.

Pellet extrusion was achieved using the extruder assembly of the Hobart processor, equipped with a 3 mm die. The resulting diet-strings were then spread thinly on to trays and air-dried at 40°C in a fan-assisted drying-cabinet. The dried diets were then stored in black polythene bags inside air-tight bins. Samples of diets were withdrawn immediately after manufacture and stored at -20°C prior to analysis of proximate composition, α-tocopherol content, and in some instances, fatty acid determination.

2.2 The nutrition trials

2.2.1 Experimental animals

African walking catfish, *Clarias gariepinus* Burchell (1822), of mixed sexes were spawned in-house (for procedure see section 2.9) and grown up to a suitable starting weight (0.6-60 g) on commercial trout feeds. After careful grading fish were stocked into the experimental system.

2.2.2 Culture facility

The experimental system comprised of eight, 80 litre, circular, poly-ethylene tanks, each supplied with re-circulated fresh-water at a rate of 5 litres per minute, via 2 cm diameter, drilled spray-bars. Water aeration was achieved by means of a single air-stone.

Water-flow within the tanks was unidirectional, with outflow water, and accompanying detritus, being drawn from the bottom of the tank, up through a standpipe and into the filtration system. The standpipe also maintained a fixed water depth.

All outflow water passed through a series of filtration measures. Initially, particulate material was removed in a bottom-draining conical chamber, on top of which was a 5 μm nylon mesh. Solids were able to either settle in the bottom of the cone, to be flushed away

by draining, or removed as water passed through the mesh. This mesh was cleared daily. Water then entered a baffled chamber containing a high surface-area medium. Further removal of suspended solids occurred here, the material forming a sludge which was disposed of during a periodic system over-haul. Finally, prior to being pumped back into the tanks, the water entered a bio-filter chamber. Here, water passed over a trickle-plate and then through plastic bio-spheres on which denitrifying bacteria had established. This ensured efficient degradation of toxic-ammoniacal waste into nitrites and then nitrates. Regular water changes helped to maintain water-quality.

During experimental periods, system temperatures were maintained between 26 and 27^{0}C and the photoperiod controlled to provide 12 hours light, 12 hours dark.

2.2.3 Feeding

Fish were manually fed test diets two or three times daily, at a restricted ration level varying between each experiment. The percent body-weight to be fed per day was calculated on the basis of weekly biomass weighings, adjusted daily to correct for mortalities.

During weighing periods, fish populations were moved, from the tank in which they had spent the preceding week, into the next tank in the system. This tank rotation precluded any possible tank induced (position) effects.

Actual rations supplied were also corrected to allow for dietary moisture content.

By careful observation of the feeding behaviour of the fish, over feeding was minimised.

2.2.4 Tissue sampling

Selection of fish prior to sampling of tissues occurred in one of two ways. Either, the first three or four fish netted per tank were taken and tissues excised, or fish whose weights were close to the tank mean were taken in an attempt to sample representatively. Samples

of fresh fish tissue were excised and immediately placed into pre-labelled, poly-thene, re-sealable bags. These were then stored at -70^{0}C until required. Samples were analysed within 2 months of sample collection, priority being given to specific samples if analyte degradation was likely.

Blood was collected and stored according to the procedure described in section 2.6.1.

2.2.5 Growth performance and nutrient utilisation parameters.

A number of parameters describing growth performance and feed utilisation efficiency were applied to treatments, upon termination of the nutrition trials. These are defined accordingly.

2.2.5.1 Specific Growth Rate

Specific growth rate (SGR) is used to compare growth of fish on a relative daily basis and is expressed as the percent increase from initial live weight over a defined period of time.

$$SGR\ (\%\ d^{-1}) = \frac{\ln w2 - \ln w1}{T} \times 100$$

where:

w2= final weight (g)
w1= initial weight (g)
T= defined time period (days)

2.2.5.2 Feed efficiency

Feed efficiency (FE) relates to the ability of the feed to support weight gain with respect to the amount of feed consumed. In other words, the extent to which feed is utilised for growth.

$$FE = \frac{\text{live weight gain (g)}}{\text{amount dry food fed (g)}}$$

2.2.5.3 Protein utilisation.

The utilisation of protein for growth may be expressed as either the protein efficiency ratio (PER) or the apparent net protein utilisation (ANPU). The protein efficiency ratio simply quantifies the weight gained by the animal with respect to the amount of protein consumed and hence may be calculated thus:

$$PER = \frac{\text{live weight gain (g)}}{\text{protein fed (g)}}$$

Fillet protein deposition (FPD) was developed as a parameter relating the utilisation of dietary protein to its deposition in the skeletal muscle (fillets) of the fish, and hence indicates the efficiency of protein retention. Fillet protein deposition was calculated in a similar manner to apparent net protein utilisation (ANPU) except that allowances were made for the contribution of fillets to the total body weight.

$$FPD\ (\%) = \frac{(F-I)}{P} \times 100$$

Where:

F = weight (g) of protein in fillets at end of experiment
I = weight (g) of protein in fillets at start of experiment
P = weight (g) of protein consumed by the fish

note: F and I derived thus: protein concentration (g g^{-1} tissue) x body-weight x proportional contribution of fillets to body-weight.

2.2.5.4 Hepatosomatic index.

The hepatosomatic index (HSI) is a measure of the contribution of liver weight to the total body-weight of the fish and is calculated thus:

$$HSI\ (\%) = \frac{\text{liver weight (g)}}{\text{body-weight (g)}} \times 100$$

2.3 Proximate chemical composition

2.3.1 Determination of moisture content

The moisture content of feed and fish carcasses was determined according to A.O.A.C. (1990). In summary, samples of feed materials, tissues or entire fish carcasses were weighed and dried to a constant final weight at 105^{0}C in a fan-assisted Pickerstone E 70F oven (R.E. Pickerstone Ltd., Thetford, Norfolk, U.K.). The percentage moisture in the sample was calculated thus:

$$\text{moisture (\%)} = \frac{\text{change in weight (g)}}{\text{initial weight (g)}} \times 100\ \%$$

2.3.2 Determination of crude protein content.

The protein content of feed and fish carcasses was determined by the Kjeldahl method. Typically, 500 mg of dried feed or carcass was weighed into a borosilicate digestion tube containing 20 cm^3 of concentrated H_2SO_4 (Sp.Gr. 1.84) and 2 Kjeldahl catalyst tablets (2 x 3 g K_2SO_4, 105 mg $CuSO_4.5H_2O$ and 105 mg TiO_2. Thompson and Capper Ltd., Runcorn, Cheshire, U.K.). Digestion was carried out in a Gerhardt Kjeldatherm digestion block (C. Gerhardt Laboratory Instruments, Bonn, Germany) for 30 minutes at 250^{0}C followed by a further 2 hours at 380^{0}C with the acid fumes collected and neutralised by 15 % NaOH in a Gerhardt Turbosog unit. After cooling, the Gerhardt Vapodest 3S distillation unit diluted the sample with distilled water and neutralised with 40 % NaOH. The ammonia in the sample was then collected into 50 cm^3 of saturated orthoboric acid (H_3BO_3) by steam distillation. Using BDH '4.5' indicator, the distillate was titrated against 0.25 M HCl and the percentage protein in the dry sample determined thus:

$$\text{\% crude protein} = \frac{\text{(sample titre (ml) - blank titre (ml))} \times 0.25 \times 14 \times 6.25}{\text{sample weight (mg)}} \times 100\ \%$$

Where:

0.25 = [HCl] in moles

14 = Relative atomic mass of nitrogen
6.25 = Constant describing relationship between nitrogen and protein content of sample.

2.3.3 Determination of total lipid.

Total lipid in the samples of feed and carcass was determined by either Soxhlet extraction or a method derived from the preparative procedure described by Barnes and Blackstock (1973).

In order to carry out the Soxhlet extraction, 5.0 g of dried sample was weighed into a cellulose extraction thimble (Whatman) which was fitted to a Gerhardt Soxtherm unit. The sample was refluxed with 130 cm^3 of petroleum ether (40-60 fraction) for 40 minutes in the "circulation" mode. The "recovery" mode was then employed to reduce the solvent volume to below 5 mm from the base of the extraction thimble. This was followed by a further 70 minutes of refluxing with the Soxtherm in the "circulation" mode. After this period the Soxtherm was again set for "recovery" and the remaining solvent removed from the collected lipid residue by evaporation. The change in weight of the collecting vessel was proportional to the lipid content of the sample and hence the percentage of lipid in the dry sample was calculated as follows:

$$\% \text{ lipid} = \frac{\text{mass of lipid residue (g)}}{\text{sample weight (g)}} \times 100\ \%$$

The alternative method of lipid determination was derived from that used for the extraction of lipids by Barnes and Blackstock (1973) and was followed by a gravimetric determination of the lipid content of the solvent extract. In this modification, 500 mg of dry material was weighed into a 50 cm^3 conical flask to which 10 cm^3 of chloroform : methanol (2:1) was added. The flasks were sealed and left overnight at room temperature. At the end of this period the extract was suction-filtered through a Whatman No. 2 filter into a test tube and the residue in the conical quantitatively removed using a further 10 cm^3 of chloroform

: methanol. Duplicate 5 cm^3 aliquots were transferred to pre-weighed test tubes and the solvent evaporated at 60^0C using a water bath. The weight gained by the test-tube was attributable to the lipid slick left behind by solvent evaporation. This was proportional to the lipid content of the sample and hence the percentage of lipid in the dry material was calculated thus:

$$\% \text{ lipid} = \frac{4 \times \text{weight gain of tube (g)}}{\text{sample weight (g)}} \times 100 \%$$

2.3.4 Determination of ash content.

The ash content of the dry material was determined in accordance with A.O.A.C. (1990). 500 mg of dry sample were accurately weighed into a pre-weighed crucible and heated for 8 hours at 525^0C in a Carbolite GLM 11/7 muffle-furnace (Carbolite Furnaces Ltd., Bamford, Sheffield, U.K.). The residue in the crucible was the non-combustible, or ash, component of the sample. Re-weighing of the crucible plus contents, and comparison with the weights of each before combustion, yielded the differential which was then used to derive the value for sample ash content. The calculation of percentage ash in the sample was as follows:

$$\% \text{ ash} = \frac{(\text{weight of crucible + residue}) - \text{weight of crucible (g)}}{\text{sample weight (g)}} \times 100 \%$$

2.4 Determination of micro-nutrients

2.4.1 Determination of α-tocopherol by High Performance Liquid Chromatography

2.4.1.1 Conventions adopted

All solvents used were of HPLC grade or better. Other chemicals were of a similar grade to ANALAR.

Exposure of samples to light and high temperatures was avoided.

Samples were stored on ice during work-up.

All automatic pipettes used for quantitative volume delivery (homogenate, final solvent) were calibrated.

Stock solutions were made fresh for each batch of analyses. *N.B.* No stock solutions were kept for longer than 1 month, even at 4^0C.

2.4.1.2 Extraction Procedure for tissues

Approximately 1 g of tissue (accurately weighed and recorded) was homogenised in 9 cm^3 1.15% potassium chloride (1.15% KCl) for 30 seconds, using a glass homogeniser. To 1 cm^3 of the homogenate in a screw-capped glass tube, 2 cm^3 1% pyrogallol solution (made fresh daily in absolute alcohol) was added and the tube vortexed briefly. The pyrogallol functioned as an aqueous antioxidant, protecting the vitamin E in the assay. 0.3 cm^3 saturated potassium hydroxide (KOH) was pipetted into the tube and the mixture vortexed vigorously for 30 seconds. At this stage the tube contents turned brown due to the KOH/pyrogallol reaction. The tubes were then capped and incubated in a water-bath at 70^0C for 30 minutes. Samples were shaken periodically. After incubation, tubes were cooled on ice and then had 1 cm^3 distilled water followed by 4 cm^3 hexane (containing 0.005% butylated hydroxy toluene) added.

The non-saponifiable lipids were extracted into the hexane layer by vigorous shaking.

Phase separation was assisted by centrifugation at 1500 x g for 10 minutes,

As much of the hexane layer (upper phase) as possible was removed to a cooled, glass-tube, using a pasteur pipette. The remaining mixture was re-extracted twice with 2 cm^3 hexane/BHT, pooling the hexane layers each time.

Pooled hexane layers were evaporated in a water bath at 37^{0}C. under nitrogen gas, supplied via an eight-port manifold system.

Re-dissolution of the residue in 500 μl absolute alcohol (dilution factor applied depending on tissue and dietary tocopherol dose)(table 2.6) and filtration through a 0.2 μm syringe-filter (Whatman 0.2 μm, 13 mm nylon-membrane), into a teflon-capped, 2 cm^3 vial then enabled samples to be stored at -20^{0}C., prior to injection onto the HPLC system.

The extraction procedure was slightly modified for blood-plasma. In this case, 1 cm^3 absolute alcohol was added to 500 μl plasma. This was then vortexed briefly, 1 cm^3 hexane/BHT was added, the tube vortexed vigorously, centrifuged at 1500 x g for 10 minutes, then the hexane layer removed to a cooled tube. Re-extraction (twice) with 1 cm^3 hexane/BHT and pooling of the hexane layers was followed by the evaporation, re-dissolution and storage stages as previously described for other tissues.

2.4.1.3 Extraction procedure for feeds

This was as for other tissues except for that 200 mg ground feed was homogenised in 9.8 cm^3 distilled water.

2.4.1.4 HPLC conditions

α-tocopherol was quantified using a Kontron HPLC system, equipped with a syringe-loading injector, a Hypersil BDS C18 column and a fluorescence detector (excitation 293 nm, emission 326 nm, Sensitivity setting according to table 2.6). The mobile phase used was methanol/water (98:2 v/v) at a flow rate of 2.0 cm^3/min. Under these conditions the

run-time was around 10 minutes.

2.4.1.5 Quantification

Sample α-tocopherol concentrations were calculated relative to an external α-tocopherol standard (Sigma T-3251)(standard concentration set relative to expected sample concentration (table 2.6) on the basis of peak areas. This was performed by an Integrator software package (Kontron PC Integration Pack 3.9, Kontron Instruments). Sample injection was preceded by the duplicate injection of the standard. Re-injection of the standard was carried out after every six sample determinations. All samples were also injected in duplicate.

Tissue α-tocopherol concentrations were expressed as $\mu g\ g^{-1}$ or $\mu g\ cm^{-3}$ blood-plasma.

Feed α-tocopherol concentrations were expressed as mg kg^{-1} air-dried feed *i.e.* values were corrected to account for dietary moisture content.

Typically, the coefficient of variance (C.V.) for the assay was less than 5 %. This was calculated after ten determinations of α-tocopherol from the same homogenate.

Table 2.6 Sample dilution factors, standard concentrations and detection criteria for the determination of α-tocopherol by HPLC with fluorescence detection (ex.293 nm, em.326 nm)

Tissue	Tocopherol dose (mg kg^{-1})‡	Dilution factor †	Tocopherol standard ($\mu g\ cm^{-3}$)	Fluorescence detector sensitivity settings (V)
Muscle	0-200	1	5	500
	200-500	2		
Liver	0-200	1	20	350
	200-500	5		
Heart	0-200	1	5	550
	200-500	2		
Spleen	0-200	1	5	500
	200-500	2		
Blood-plasma	0-200	1	40	400
	200-500	2		
Feeds	0-200	1	10	500
	200-500	2		

‡ For tissues : dietary dose as fed to fish ; For diets: as supplemented into formulation.

† Dilution factor applied to samples prior to determination of tissue α-tocopherol

2.4.2 Determination of Ascorbic acid (Vitamin C) levels in tissues and feeds.

2.4.2.1 Extraction procedure

About 100 mg (accurately weighed and recorded) of tissue was excised and immediately stored on ice. The pre-weighed tissue was homogenized in ice-cold extraction medium (5.4 cm^3 $HClO_4$ + 5% TCA + 0.2 g EDTA made up to 250 cm^3 (Dabrowski and Hinterleitner, 1989) in a glass-homogeniser for 2 minutes. (100 mg tissue per 1 cm^3 extraction medium was used).

Homogenates were centrifuged for 30 minutes at 27000 x g and the supernatant transferred to clean test-tubes and recentrifuged if particulate matter remained.

At this point the homogenates could be stored frozen for later use, though a storage period of over 2 weeks was avoided due to possible deterioration of the samples.

The procedure for feeds differed somewhat from that for tissues.

Samples of 2 g of evenly ground feed were agitated for 15 minutes, with 10 cm^3 of extraction medium, in a conical flask.

Filtration of the mixture through Whatman No. 1 paper removed any suspended feed particles from the filtrate, thus preparing the samples for frozen storage until required.

2.4.2.2 Determination

After having established linearity for the following assay, in the range of 0-40 μg cm^{-3} L-ascorbic acid, a single standard was used in the routine determination of selected vitamin C analogues in feeds and tissues. This method was modified from that of Thomas *et al.* (1982) and Carr *et al.* (1983).

All samples were assayed in duplicate.

250 μl aliquots of the supernatant were pipetted 3 glass test-tubes labelled A, B, and C respectively. The tubes were dealt with as follows, according to their label:

Series A tubes + standards:

To each tube 25 μl of (0.2 %) 2,6-dichlorophenol-indophenol (2-6 Dye) was added and the tubes incubated for 10 minutes.

After incubation, 25 μl of distilled water was added and a further incubation period of 1 hour, in darkness, at room temperature was used.

Series B tubes:

25 μl of (0.2%) 2,6-Dye was added to each tube and the tubes incubated at room temperature for 10 minutes.

25 μl of 1% $KBrO_3$ was pipetted into all tubes and again a 1 hour incubation at room temperature followed.

Series C tubes:

To each tube, 50 μl of distilled water was added and tubes incubated at room temperature for 1 hour.

Upon completion of the 1 hour incubation stage for all tubes, 250 μl (2%) Thiourea and 250 μl of (2%) dinitrophenyl-hydrazine (DNPH) (2 g DNPH made up to 100 cm^3 in 12 M H_2SO_4) were added . All series were then incubated for 3 hours at 60^0C.

After incubation, 0.5 cm^3 ice-cold (18M) H_2SO_4 was added carefully into all tubes, the tubes centrifuged for 10 minutes at 5000 x g to remove turbidity and the samples left to stand at room temperature for 30 minutes. The absorbance of each sample at 524 nm was then read against a blank using a dual-beam spectrophotometer.

Derivation of the absorbances of L-Ascorbate and L-Ascorbyl-2-sulphate from the absorbances obtained from the different tube series proceeded as follows:

Abs. attributed to L-ascorbic acid (AA)= A - C

Abs. attributed to L-ascorbyl-2- sulphate (AS)= B - A.

Determination of the concentrations of each analogue proceeded according to the following formula:

$$[AA]\ (\mu g\ cm^{-3}\ supernatant) = \frac{Abs.AA}{Abs.Std} \times 40$$

[AS] was determined in a similar manner.

These were expressed as $\mu g\ g^{-1}$ tissue by determining the amount of tissue in the homogenate (or feed in the filtrate).

2.4.3 Determination of fatty acids in feeds and tissues.

2.4.3.1 Extraction of fatty acids from tissues.

Fatty acids were extracted from tissues by a slightly modified version of that of Burton *et al.* (1985).

2.5 g of muscle, or 1 g of liver tissue, were homogenised in a glass homogeniser with 7.5 cm^3 distilled water. To 1 cm^3 homogenate in a glass screw-capped tube, 1 cm^3 60 mM sodium dodecyl sulphate (SDS) solution was added and the mixture vortexed briefly. 2 cm^3 ethanol was then added and once again the mixture vortexed. In order to separate the lipid fraction, 2 cm^3 n-hexane (+0.005% butylated hydroxy toluene (BHT)) was pipetted into the tube and the contents vortexed for 30 seconds. After 2 minutes of centrifugation at 1500 X g, 1 cm^3 of organic layer was transferred to a screw-capped (foil-lined) vial and stored at -20°C.

2.4.3.2 Extraction of fatty acids from feeds.

The extraction of fatty acids from feed samples differed from the method outlined above. In this instance, 1 g of finely-ground diet sample was soaked for 12 hours in 9 cm^3 2:1 chloroform methanol (+ 0.005% BHT) in a sealed, 20 cm^3 screw-capped bottle. Samples were shaken periodically to assist the extraction. After this period the suspended material was allowed to settle and 2 cm^3 of the solvent/lipid mixture pipetted into a screw-capped glass tube, and dealt with as directed for tissue supernatants.

2.4.3.3 Methyl-esterification of fatty acids.

Preparation of methyl-esters of the fatty acids proceded as directed by Slover and Lanza (1979).

Having extracted the fatty acids in the manner described in section 2.4.3.1 or 2.4.3.2, the hexane/ lipid mix from the vial was quantitatively transferred into a screw-capped glass tube with minimal additional hexane. This was evaporated at 37 ^{0}C under a stream of nitrogen gas. To the residue in the tube, 1 cm^3 methanolic 0.5M NaOH was added, the tube securely capped and heated at 100^{0}C in a boiling water-bath for 15 minutes. The tube was then cooled before addition of 2 cm^3 14% BF_3/methanol (Sigma Chemical Company Ltd., Poole, Dorset, U.K.). Once more the tube was securely capped and placed in a boiling water-bath for 15 minutes. After boiling, the tube was cooled and 1 cm^3 hexane and 2 cm^3 saturated aqueous NaCl was added to the contents and the tube vortexed for 1 minute. Phase-separation occurred with 2 minutes of leaving the tube to stand.

The resulting upper layer (hexane) was transferred to a 45 x 11 mm vial containing a 1 mm layer of anhydrous sodium sulphate (Na_2SO_4). The vial was capped and shaken and then allowed to stand for 20 minutes. A pasteur-pipette was then used to transfer the hexane (containing the fatty acid methyl-esters (FAMEs)) to a 2 cm^3 crimp-capped glass vial. In this state the FAMEs could be stored for several months at -20^{0}C.

2.4.3.4 Determination of FAMEs by gas chromatography.

Separation was achieved on a Hewlett-Packard 5890 series II gas chromatograph fitted with a wax column (DB-WAX, 30 m x 0.32 mm x 0.25 μm film), using the following conditions:

Injector and detector temperature: 250^{0}C.

Injection criteria: 1 μl volume, 50:1 split.

Carrier gas criteria: H_2 at 50 cm^3 min^{-1}, 70 kPa column head pressure.

Temperature programme: 60-230^{0}C at 2^{0}C min^{-1}, hold 20 min.

Peaks were identified by mass spectroscopy (mass range 35-500) using a Hewlett-Packard 5970 series mass-selective detector. Results were expressed as % contribution of the FAME to the total chromatogram area.

2.4.4 Determination of total iron

Preparation of samples prior to iron (Fe) analysis required ARISTAR quality chemicals or equivalent, and all glassware was acid washed and rinsed in ultra-pure water (HPLC grade) prior to analysis.

Livers, skeletal muscle, plasma and diets were analysed for total iron content using the procedure below.

Dried tissues and diets were finely ground and immediately transferred into sealed, marked vials in a desiccating chamber for storage until required.

Either 100 mg of tissue or diet, or 300-400 μl blood-plasma was accurately dispensed into screw-capped glass tubes and pre-digested overnight in 5 cm^3 of concentrated nitric acid. After this initial stage, tubes were heated in a water bath at 100 ^{0}C for 30 minutes and then allowed to cool back to room temperature. Once sufficiently cool, tubes were carefully uncapped and allowed to stand for a further 5 minutes in a fume cupboard until all acid fumes had been vented. 1 cm^3 of hydrogen peroxide (30% w/v H_2O_2) was added to the sample tubes which were then capped and reheated in the water bath as described above. After cooling and re-venting tubes, resulting solutions were made up to 10 cm^3 using ultra-pure Milli-Q water and transferred into sealed plastic vials before analysis of iron (Fe) by AAS.

The quantification of iron in the prepared samples was performed using a Varian SpectrAA

600 flame atomic absorption spectrometer, equipped with a Varian SPS.5 auto-sampler. An external standard was used in calibration of the instrument (Merck-BDH, Poole, Dorset, U.K.), with sample absorbance determined at 492.3 nm.

2.5 Determinants of extent of oxidation

2.5.1 Spectrophotometric determination of tissue TBARS by iron-ascorbate stimulated lipid peroxidation.

A set of four glass screw-capped tubes, labelled '0', '50', '100' and '200' reflecting respective incubation times, were prepared for each sample.

To each tube, 500 μl tris-malate buffer (80 mM Tris (Sigma T-1387) adjusted to pH 7.8 with 0.1M maleic acid), 200 μl 5mM ferrous sulphate solution and 200 μl 2mM ascorbic acid solution (prepared fresh daily) were added. In addition, 2 cm^3 TBA/TCA/HCl reagent (150 g trichloroacetic acid and 3.73 g thiobarbituric acid dissolved in 1 litre 0.25M hydrochloric acid) was added to each '0' tube.

Using a glass homogeniser, 1 g of sample tissue (weighed accurately and recorded) was homogenised in 9 cm^3 1.15% potassium chloride solution (1.15% KCl) for 30-45 seconds. 100μl of tissue homogenate was pipetted into each tube and vortexed for 10 seconds. Immediately, the tubes labelled '50', '100' and '200' were placed into a 37^{0}C water bath. Samples were shaken periodically during incubation. After incubation for the necessary length of time, the appropriate tube was removed and immediately had 2 cm^3 TBA/TCA/HCl reagent added. This was obviously omitted for tubes labelled '0'. These were handled in the same manner as other tubes after this stage.

Tubes were vortexed for 10 seconds and placed in a boiling water bath for 15 minutes in order to allow TBA/MDA adduct formation. Following boiling, all tubes were centrifuged at 1500 x g for 15 minutes at ambient temperature.

The supernatant was transferred into a 2 cm^3 cuvette using a pasteur pipette and the

sample's absorbance against a blank (500 μl tris-malate buffer, 200 μl ferrous sulphate solution, 200 μl ascorbic acid solution, 100 μl 1.15% KCl and 2 cm^3 TBA/TCA/HCl reagent) determined at 535 nm using a dual-beam spectrophotometer.

Values of TBARS, expressed as nmol MDA equivalents g^{-1} of tissue, were obtained using the molar extinction coefficient of malondialdehyde (MDA)(1.56 X 10^5 M^{-1} cm^{-1}).

$$\text{MDA (nmol MDA equivalents } g^{-1} \text{ tissue)} = \frac{1923.06 \text{ X Abs}}{\text{tissue wt (g)}}$$

2.5.2 Determination of TBARS in dietary oils, or feed-extracted oils.

The assay was applied in a similar manner to that for TBARS determination in tissues. Differences between methods are as follows:

Only the set of tubes labelled '0' in section 2.5.1 was required. In other words, determination of TBARS was carried out without stimulated lipid-peroxidation. In place of the iron and ascorbate solutions, 400 μl of distilled water was pipetted into the tubes. Sample preparation was carried out as outlined below.

-Dietary oils

2 g of fresh oil or 0.5 g of oxidised oil, were dissolved and made up to 100 cm^3 with Chloroform-methanol-antioxidant mixture (2:1 chloroform:methanol + 0.01% BHT). 100 μl of oil solution was then used in the assay as described in section 2.5.1 in place of the homogenate. The reagent blank contained just 100 μl chloroform-methanol-antioxidant mixture instead of 1.15% KCl.

Calculation of TBARS was achieved thus:

$$\text{mmol MDA equivalents } g^{-1} \text{ oil} = \frac{0.192306 \times \text{abs.} \times 100\dagger}{\text{sample weight (g)}}$$

† solvent volume (cm^3) used in sample dissolution

-Oils extracted from feeds

A crude 2:1 chloroform:methanol extraction was performed in order to extract the oil from ground samples of feed. This was carried-out in teflon-sealed bottles, into which about 0.5 g of feed and 5 cm^3 solvent was placed. After soaking for 6 hours, with continuous agitation, the mixtures were filtered through Whatman No. 1 filter-paper and 100 μl of this filtrate was introduced into the TBARS assay as previously described (section 2.5.1).

Calculation of TBARS concentration was according to the following formula:

$$\text{TBARS (mmol MDA equivalents) } g^{-1} \text{ dry food} = \frac{0.192306 \times \text{abs.} \times 5}{\text{sample weight (g)}} \times m$$

where: m= correction for diet moisture content (100/% dry matter)

2.5.3 HPLC determination of Malondialdehyde (MDA)

All solvents used were of HPLC grade or better. Other chemicals were of a similar grade to ANALAR.

Samples were stored on ice during work-up.

All automatic pipettes used for quantitative volume delivery (homogenate, final solvent) were calibrated.

Stock solutions were made fresh for each batch of analyses. *N.B.* No stock solutions were kept for longer than 1 month, even at 4^0C.

2.5.3.1 Sample preparation

Preparative steps necessary for the accurate determination of malondialdehyde in tissues were carried out as follows. To 8 cm^3 of tissue homogenate (10 % tissue in distilled water) 2 cm^3 of precipitate solution (30 mM H_2SO_4, 15 mM Na_2WO_4) was added. The mixture was then centrifuged at 1500 x g for 10 minutes.

For analysis of dietary oils, solutions of oil were prepared. Thus, 0.05-0.4 g of oil to be tested were dissolved in 10 cm^3 2:1 chloroform/methanol. This solution was then diluted fivefold in 40 % ethyl alcohol in distilled water.

2.5.3.2 Formation and subsequent determination of TBA/MDA adduct

Determination of concentration of malondialdehyde (MDA) in tissues and dietary oil was carried out as follows. 500 μl of supernatant, oil solution or external standard (4 μM 1,1,3,3-tetraethoxypropane (Sigma Chemical Company Limited, Poole, Dorset, U.K.)) was pipetted into replicate screw capped glass tubes. To all tubes 50 μl 10 mM butylated hydroxytoluene (BHT) in ethyl alcohol, and 3 cm^3 0.44 M orthophosphoric acid (H_3PO_4) were added, the tubes vortexed for 10 seconds and then left for 10 minutes. After this time, 1 cm^3 40 mM thiobarbituric acid (TBA) was added and all tubes were heated at 90 ^{0}C for 45 minutes. Solutions were cooled on ice before being injected on to a Perkin Elmer series 2 liquid chromatography system fitted with a 5 μm x 4.6 mm x 0.25 m Hypersil BDS C18 column (Shandon HPLC, Runcorn, Cheshire, U.K.). The eluate was passed through a Perkin Elmer LC-75 spectrophotometric detector set at 532 nm and the resulting peaks integrated manually. The mobile phase was 65 % 50 mM KH_2PO_4-KOH pH7 / 35 % methanol pumped at 1 cm^3 min^{-1}. Under these conditions the retention time for the TBA/MDA adduct was about 6 minutes. A coefficient of variance achieved for this assay was 8.2 %.

2.5.4 Determination of the Anisidine value of an oil (BS 684: section 2.24: 1989)

Oils to be used as feed ingredients and oils extracted from biological matrices were reacted with ρ-anisidine in order to determine the Anisidine value. This value is indicative of the amount of aldehydes (principally 2-alkenals) in a given sample.

About 2 g of the oil to be tested (or 0.5 g if the oil was oxidised) were accurately weighed into a 25 cm^3 volumetric flask, labelled 'C'. The oil was dissolved in iso-octane (2,2,4-trimethylpentane) and made up to the mark. A screw-capped glass test-tube, labelled 'A', was set-up per replicate. To tube 'A', 5 cm^3 of the test solution from flask 'C' was added. At this stage, a tube labelled 'B' was designated as a blank. To this tube 5 cm^3 of iso-octane was added. 1 cm^3 of anisidine reagent (125 mg ρ-anisidine dissolved in glacial acetic acid and made up to 50 cm^3 in volumetric flask) was pipetted into all tubes, the tubes capped, vortexed for 10 seconds and then left to incubate at 22-24^{0}C for 8 minutes in darkness. After incubation, the solutions were transferred to clean dry spectrophotometer cuvettes, and the absorbances of solutions 'A' (reacted sample) and 'C' (unreacted sample) measured against 'B' (Blank) at 350 nm in a dual-beam spectrophotometer.

Anisidine values (AV) were calculated in accordance with the following formula:

$$AV = \frac{25((1.2*Abs_A)-Abs_C)}{\text{sample wt (g)}}$$

Anisidine value: 100 times the increase in absorbance, measured at a wavelength or 350 nm in a 10 mm cell, of a test solution (1 g oil in 100 cm^3 solution) when reacted with ρ-anisidine under the conditions of the test.

2.6 Haematological techniques

2.6.1 Collection of blood-samples

Blood samples were taken from fish using the following procedure.

Clarias were removed from their tanks and individually anaesthetised with ethyl ρ-amino benzoate ('benzocaine') dissolved in absolute alcohol. This was dosed into a vessel containing water from the experimental system. Once the catfish was just under anaesthetic (characterised by loss of stability and flaccid body-posture on handling) the fish was placed on an appropriate non-slip surface and the blood sample withdrawn thus:

A 2 cm^3 syringe equipped with a 23G, 25 mm needle, was heparinised and the needle inserted laterally into the catfish, at the fish's midpoint, adjacent to the lateral line. The needle was gently pushed until resistance was felt from the backbone. At this stage, the syringe was inclined so as to deviate the needle ventrally from the backbone. Upward pressure on the syringe-piston then drew up a required blood-sample volume (normally around 1.5 cm^3). This was transferred into pre-labelled micro-centrifuge tubes for future centrifugation.

Having been bled, catfish were then monitored during recovery in vigorously aerated system water.

2.6.2 Haematocrit

Haematocrit was determined immediately after the blood was collected from the fish. Samples of whole blood were drawn by capillarity into heparinised micro-haematocrit tubes (Hawksley, Lancing, Sussex, U.K.) and sealed at one end with Cristaseal (Hawksley). The tubes were then spun for 5 minutes at 14000 x g in a haematocrit centrifuge and the packed cell volume determined thus:

$$\text{haematocrit } \% = \frac{\text{length of packed red cell column (mm)}}{\text{total length of blood column (mm)}} \times 100\ \%$$

2.6.3 Separation of blood-plasma

Blood-plasma separation was achieved by the centrifugation of whole blood at 6000 x g for 5 minutes in a micro-centrifuge, using capped, polypropylene Eppendorf tubes. After centrifugation, the plasma was decanted into clean, labelled Eppendorfs for storage at -70^{0}C.

2.6.4 Total haemoglobin.

Total whole blood and plasma haemoglobin were determined with a minimum of stasis by the cyanmethemoglobin method as described by Sigma procedure No. 525 (Sigma Chemical Company Limited, Poole, Dorset, U.K.). The method procedes as follows: 200 μl of plasma or 20 μl whole blood were added to 5.0 cm^3 of Drabkins reagent, vortexed and incubated at room temperature for 15 minutes. The absorbance of the solution was then measured against a blank (Drabkins solution only) at 540 nm using a dual-beam U.V./ Vis. spectrophotometer. The concentration of total plasma haemoglobin was determined by reference to a calibration curve established using cyanmethemoglobin standard solutions in the range 0-180 mg cm^{-3}.

2.7 Determination of exudative fillet-moisture loss

A portion of lateral muscle from each fish (1 cm thick, 50 cm^2) was excised, weighed and placed on to an absorbent pad enclosed in a sealable polythene bag. All samples were frozen at -20^{0}C for 12 hours and subsequently allowed to thaw at 6^{0}C for either 48 or 96 hours in a domestic refrigerator equipped with fluorescent tube lighting. After the thawing period, muscle samples were reweighed and the percentage moisture lost calculated.

2.8 X-radiography of *Clarias*

Clarias individuals were anaesthetised in accordance with the procedure set out in section 2.6.1. Fish were then placed onto the 24 x 30 cm film cassette (AGFA Blue R4, Curix screens, AGFA-Gevaert N.V., Belgium) and exposed to an appropriate X-ray dose from a Phillips Practix X-ray unit. This was varied according to subject density, by means of either the voltage selector (X-ray penetration power (normally 40-50 kV, fixed 0.2 mA)), exposure time selector (0.1 or 0.2 seconds) or by adjusting the distance between the subject and the X-ray source. Film sheets were developed manually under brown light (Kodak filter No. 67) . Films were immersed in developer (G150, AGFA-Gevaert N.V., Belgium) for 3 minutes, then rinsed in tap-water before a final 3 minute immersion in a fixative (G350, AGFA-Gevaert N.V., Belgium). After being rinsed once more in water, films were air-dried at 40^{0}C. Viewing of the films on a light-table enabled observations to be made.

2.9 Induced spawning of *Clarias*.

Spawning of *Clarias* was achieved following the procedure of DeLeeuw *et al.* (1985). Preparation of the hormone solution was as follows. 1 mg of LH-RH (Lutenizing hormone-Releasing hormone (Sigma L-4513)) was dissolved in 1 cm^3 of distilled water. This was divided into 10 portions (10 x 100 μl) and stored at -20^{0}C.

One portion of hormone solution was dissolved into 2 cm^3 of a carrier solution (5 mg pimozide (Sigma P-1793) in a solution of 0.8 g NaCl + 0.1 g sodium metabisulphite + 0.25 g bovine serum albumin in 100 cm^3 of distilled water).

The hormone/ carrier mix was then injected into a suitable female fish at the base of the pectoral fin. After 16 hours the female was lightly anaesthetised, in accordance with section 2.6.1, and stripped of eggs.

The eggs were dispersed slightly with about 50 cm^3 of buffered saline and then mixed with

the sperm from a sacrificed male. Fertilised eggs were then distributed evenly between several tanks of a suitable re-circulation system.

CHAPTER 3

CHANGES IN TISSUE α-TOCOPHEROL STATUS AND DEGREE OF LIPID PEROXIDATION WITH VARYING α-TOCOPHERYL ACETATE INCLUSION IN DIETS FOR THE AFRICAN CATFISH.

3.1 Introduction

The E-vitamer, α-tocopherol, has been the focus of much attention in both human and animal health studies, due to its free radical quenching, lipid protection role (Packer and Landvik, 1989). In humans, dietary tocopherol supplements are suggested to decrease the incidence of coronary heart disease (Byers, 1993; Stephens *et al.*, 1996), possibly slow down or prevent the growth of some neoplasias (Crawford, 1993), reduce the prevalence of cataracts (Robertson *et al.*, 1989) and lessen the effects of arthritis (Machtey and Ouaknine, 1978). Animals too may benefit from dietary α-tocopherol inclusion, though this benefit would vary from one species to another. Increased dietary levels have been correlated to increased tissue status of the vitamin, resulting in improvements in cellular integrity and functioning (Packer and Landvik, 1989). In addition, stabilizing tissue lipids leads to a post-mortem protection of tissues against free radical mediated peroxidative deterioration and this has a beneficial role in the storage stability of meat products destined for human consumption.

Vitamin E supplementation (commonly as the ester, α-tocopheryl acetate) has been observed to prolong the time that meat products such as beef (Arnold *et al.*, 1993), veal (Engeseth *et al.*, 1993), pork (Whang *et al.*, 1986; Asghar *et al.*, 1991), poultry (Sheehy *et al.*, 1993a) and fish may be stored.

Fish in particular are prone to detrimental oxidative lipid modification, due to the high degree of poly-unsaturation of their lipids. This renders the fish susceptible to radical damage *in vivo*, as well as potentially increasing the risk of human tissue to radical attack

on consumption of the fish product. Studies on salmonids (Hung and Slinger, 1982; Frigg *et al.*, 1990), channel catfish (O'Keefe and Noble, 1978) and red seabream (Murata and Yamauchi, 1989) have demonstrated the protective effects of vitamin E against lipid peroxidation in tissues subjected to differing storage conditions or *in vitro* oxidative challenge.

No such studies however, have been performed on the African catfish (*Clarias gariepinus* Burchell, 1822), a cultured freshwater species currently attracting interest due to its rapid growth rate, potentially high stocking density (Verreth and Eding, 1993) and high filletable proportion (pers. obs.).

The objectives of the investigation are to determine the efficacy of incremental all-rac-α-tocopheryl acetate addition into low-tocopherol practical diets (basal diet), in the prevention of lipid peroxidation in muscle and liver tissues of *C.gariepinus*, and to examine the effects on growth and selected health criteria.

3.2 Materials and Methods

3.2.1 Reagents

All reagents used were ANALAR grade obtained from Sigma Chemical Company Limited, Poole, Dorset, U.K., and Merck Ltd., Poole, Dorset, U.K.. All-rac-α-tocopheryl acetate (Rovimix E-50 SD) was a generous gift from F. Hoffmann- La Roche, Basel, Switzerland. All other vitamins were kindly donated by Colborn-Dawes, Heanor, Derbyshire, U.K.

3.2.2 Fish stock, experimental facilities and diets.

African catfish, *Clarias gariepinus*, juveniles were spawned in-house (according to section 2.9) and grown to approximately 60g, on commercial trout feeds, prior to commencement of the trial. Juveniles of mixed sexes and of mean weight 60g were stocked into the culture facility described in section 2.2.2.

A low-tocopherol, basal practical diet was formulated as described in section 2.1.1, with the following modifications.

Lipid was delivered in the form of a cod-liver oil: corn oil mix (1:1) at a level of 5% providing a balance of n-3 and n-6 fatty-acids.

The constituents and respective dietary inclusion levels for the basal diet are presented in table 3.1. Dietary manufacture was carried out as detailed in section 2.1.2.

Compared to calculated values, proximate analysis of the finished test diets confirmed the values of macronutrients in the feeds. These data are presented in table 3.2.

Levels of dietary vitamin E (supplied as all-rac-α-tocopheryl acetate) were set in relation to levels reported by other workers in a variety of fish species. Inclusions of α-tocopheryl acetate were 0, 80, 200 and 500 mg kg^{-1} dry diet, in order to provide a low tocopherol diet, a diet containing above the supposed requirement levels of other warm-water species (estimated at 80mg kg^{-1} from N.R.C. (1993)), and two diets containing graded high levels of vitamin E respectively. HPLC determination of α-tocopherol content, seen in table 3.3

confirmed the actual values of α-tocopherol in the diets to be very close to those intended.

3.2.3 Feeding regime and nutrition trial protocol

The *Clarias* were uniformly graded and assigned, 16 per tank, into eight tanks (four treatments duplicated). A representative number of surplus fish (n=8) were removed initially for fillet composition analysis and the remaining fish weighed individually at the start of the feeding trial.

A food ration level of up to 2 % body weight per day was employed, with three times daily feeding, this ration being calculated on a dry matter basis. All fish were weighed individually, every second week in order to determine feed totals for the proceeding weeks. On termination of the 70 day feeding trial, individual fish weights were recorded and three fish per tank removed for proximate analysis. In addition, eight fish from each treatment (four per tank) were randomly netted and desanguinated by caudal venipuncture (section 2.6.1) in order to determine haematocrits (% packed cell volume). After centrifugation, plasma samples were frozen at -80°C prior to further analysis. Tissue samples from these fish were removed and also stored at -80°C until analysis for α-tocopherol and thiobarbituric acid reactive substances (see 3.2.5) .

3.2.4 Growth and nutrient utilization parameters

The following parameters were calculated (according to the formulae described in section 2.2.5) from data acquired from the feeding trial; percent change in mean body weight, daily feed intake, specific growth rate (SGR) and feed efficiency (FE). In addition, protein efficiency ratio (PER) and fillet protein deposition (FPD) were calculated after proximate analysis of test diets and fillets.

Additional to the final body weights on termination of the trial, liver weights were recorded in order to allow calculation of the hepatosomatic index (% contribution of liver

weight to the total body weight).

3.2.5 Analytical methods

The proximate composition of fish fillets and the dietary analyses were performed using the standard AOAC (1990) chemical procedures for moisture, crude protein (N X 6.25), and ash. Lipid determination was carried out gravimetrically after using the Folch cold lipid extraction as described by Barnes and Blackstock (1973). These procedures are described in section 2.3.

α-tocopherol was extracted and determined from muscle, liver and plasma samples using the method of Buttriss and Diplock (1984) as outlined in section 2.4.1. Dietary α-tocopherol assays were performed by P.J.A. Sheehy (Department of Nutrition, University College Cork, Republic of Ireland) in accordance with the method of Brubacher and co-workers (1985).

Thiobarbituric acid reactive substances (TBARS) were quantified by determination of equivalents of the lipid peroxidation product, malondialdehyde (MDA). Samples of fillet and liver were frozen at -20^{0}C until analyses were performed using a modification of the method of Kornbrust and Mavis (1980) (see section 2.5). Values were calculated by the method of Beuge and Aust (1978) and expressed as nmol MDA equivalents per mg tissue, using $E_{MDA} = 1.56 \times 10^5$ /M per cm.

Colour photography of fillets under controlled lighting conditions allowed qualitative inter-treatment comparisons to be made. Fillets were stored refrigerated at 4^{0}C for several days, each in individual, resealable polythene bags.

3.2.6 Statistical analysis

ANOVA ($P<0.05$) coupled with Duncan's new multiple range test (Steel and Torrie, 1960) were performed in order to facilitate inter-treatment comparisons while linear regression ($P<0.05$) was used to assess the relationship between dietary α-tocopherol inclusion and tissue levels of the vitamin. Analyses were performed using the statistical software package 'Statgraphics'.

Table 3.1 Composition of practical test diets for the African catfish, *Clarias gariepinus*.

Ingredient	%inclusion
Fishmeal (Chilean)	60.00
Meat and bone meal	10.00
Bloodmeal	2.00
Cod-liver oil	2.50
Corn oil	2.50
Corn starch:Dextrin (3:2)	16.35
Molasses	1.00
Mineral premix*	5.00
B-complex vitamin premix†	0.10
Fat-soluble vitamin premix‡	0.05
Macro-vitamin premix§	0.50

* Mineral salt inclusion (g kg^{-1}dry diet):
Calcium orthophosphate ($CaHPO_4.2H_2O$) 12.000, Magnesium sulphate ($MgSO_4.7H_2O$) 4.8450, Sodium chloride (NaCl) 2.2800, Potassium chloride (KCl) 1.9000, Iron sulphate ($FeSO_4.7H_2O$) 0.9500, Zinc sulphate ($ZnSO_4.7H_2O$) 0.2090, Manganese sulphate ($MnSO_4.4H_2O$) 0.0960, Copper sulphate ($CuSO_4.5H_2O$) 0.0298,Cobalt sulphate ($CoSO_4.7H_2O$) 0.0181, Calcium iodate ($CaIO_3.6H_2O$) 0.0112, Chromic chloride ($CrCl_3.6H_2O$) 0.0048, Sodium selenite (Na_2SeO_3) 0.0025. Filler (α-cellulose) 27.6536.

† B-vitamin inclusion (mg kg^{-1} dry diet):
B1-Thiamin hydrochloride 50, B2-Riboflavin (feed grade-96%) 52.1, B6-Pyridoxine hydrochloride 40, Calcium pantothenate 100, Niacin 200, H2-Biotin (2%) 300, Folic acid (90%) 16.7. Note B12 added separately at 0.05 mg kg^{-1} dry diet. Filler (α-cellulose) 241.2.

‡ Fat-soluble vitamin inclusion (mg kg^{-1} dry diet):
A-Vitamin A palmitate (1.7×10^6 I.U. g^{-1}) 2, D-Rovimix D3-500 (5×10^5 I.U. g^{-1}) 20, K-Menadione sodium bisulphite (51%) 80. Filler (α-cellulose) 398.

§ Macro-vitamin inclusion (mg kg^{-1} dry diet):
Inositol 200, Choline chloride 2000, Ascorbic acid 500. Filler (α-cellulose) 2300.

Table 3.2 Proximate composition of basal practical test diet (bracketed values depict theoretical composition as calculated from NRC (1993))

	Moisture (%)	Protein (% DM)	Lipid (% DM)	Ash (% DM)	Residual
Mean	5.52	49.29 [49.35]	12.18 [11.87]	14.47 [14.14]	18.54
S.E.	0.246	0.275	0.414	0.069	
n ¶	12	10	8	12	

¶ number of determinations from a single diet.

Table 3.3 Assayed α-tocopherol in test diets (mg kg^{-1} dry diet)

α-tocopheryl acetate inclusion (mg kg^{-1} dry diet)		0	80	200	500
	Mean	2.15	78.40	179.16	475.78
	S.E.	0.205	2.410	17.868	35.031
	n ¶	4	4	4	4

¶ number of extractions from each test diet.

3.3 Results

As can be seen in table 3.4, no significant differences (P=0.993) between treatments were detected with respect to mean final body weight. No obvious trends were apparent in any growth related index. Fillet protein deposition (FPD) and protein efficiency ratio (PER) values were slightly elevated in the low tocopherol group, though this could not be tested statistically due to the fact that the indices were calculated on the basis of the feed consumption of all fish in each treatment. Individual fish food intake was therefore not known.

From the values for the fillet composition of catfish fed the different diets (table 3.5), it is evident that carcass contributions from moisture, protein or ash did not vary significantly (P>0.05) between dietary treatments.

Levels of fillet-lipid contribution were observed to fluctuate, sometimes significantly (P=0.057), between treatments.

Comparison of hepatosomatic indices of catfish after the ten week investigation (table 3.6) yielded no significant differences (P=0.198) between treatments.

Figure 3.1 presents values for the haematocrits of catfish on termination of the feeding trial. Significant differences (P=0.025) existed between the highest supplemental tocopherol treatment (500mg kg^{-1}) and the treatments fed the lowest levels of α-tocopherol (0 and 80 mg supplemental α-tocopherol kg^{-1} dry feed). Haematocrits decreased with increase in α-tocopherol administration.

Data from the HPLC determination of α-tocopherol from the non-saponifiable fraction of catfish muscle, liver and blood-plasma are presented in table 3.7. Regression analysis indicates a direct relationship between dietary and muscular α-tocopherol (R-sq= 0.83, n= 24). Similarly, α-tocopherol concentrations in the plasma and liver can be seen to increase concomitant with an increase in the dietary level (R-sq= 0.82 and 0.93

respectively, n= 24). Inter-treatment differences in fillet, plasma or liver tocopherol were statistically significant ($P<0.05$).

The results of studies on the oxidative stability of *Clarias* fillets and liver are presented in table 3.8. Table 3.8 and figure 3.2 clearly show that elevating dietary α-tocopherol inclusion decreased resulting TBARS formation in fillets. At all incubation times, it was apparent that the extent of TBARS generation (in malondialdehyde (MDA) equivalents) was progressively less with increasing dose of all-rac-α-tocopheryl acetate. These differences were statistically significant ($P=0.001$) prior to and following the incubation phase.

Liver TBARS were observed to be influenced in the same way as already described in muscle tissues. Table 3.8 and figure 3.3 demonstrate the significant inverse relationship between dietary α-tocopherol and TBARS formation in the liver tissue of *Clarias*. At this juncture it is worthwhile noting that, although no significant differences were detected between treatments using the range test, suppression of data from the unsupplemented α-tocopherol treatment revealed statistically significant inter-treatment differences ($P=0.037$) between tocopherol supplemented diets. This is depicted in the insert to figure 3.3.

Visual evaluation of fillet colour as an index of the extent of oxidation yielded no trends (plate 3.1); no inter-treatment differences were seen, even after 14 days of refrigerated storage at 4^0C.

Table 3.4 Growth related performance and nutrient utilization of catfish fed experimental diets after 70 days.

α-tocopheryl acetate inclusion (mg kg^{-1})	0	80	200	500	*
Mean initial weight (g)	60.23	60.04	60.21	60.22	±2.466
Mean final weight (g)	191.40	194.86	193.66	193.7	±22.19
% change in mean weight	217.79	224.56	221.66	221.64	
Specific Growth Rate (%d^{-1})	2.75	2.80	2.78	2.78	
Feed Efficiency	1.68	1.69	1.69	1.69	
Protein Efficiency Ratio	3.48	3.42	3.42	3.36	
Fillet Protein Deposition (%)	23.09	22.53	22.46	22.30	

* ± SEM (Standard error of their pooled means)

Table 3.5 Proximate composition of *Clarias* fillets expressed as a percentage of the fresh fillet mass.

mg α-tocopheryl kg^{-1} dry diet		Moisture (%)	Crude Protein(%)	Lipid (%)	Ash (%)
0	Mean	77.27	19.41	3.972[a]	1.07
	S.E.	0.165	0.133	0.320	0.018
	n ¶	5	5	5	5
80	Mean	77.71	19.34	3.072[b]	1.06
	S.E.	0.322	0.257	0.292	0.018
	n	5	5	5	5
200	Mean	77.64	19.28	3.227[ab]	1.12
	S.E.	0.247	0.169	0.212	0.050
	n	5	5	5	5
500	Mean	77.93	19.42	2.973[b]	1.11
	S.E.	0.384	0.128	0.256	0.030
	n	5	5	5	5
Initial	Mean	78.61	19.06	2.274	1.14
	S.E.	0.177	0.147	0.120	0.015
	n	8	8	8	4

[ab] Values carrying common superscripts are not significantly different ($P>0.05$). Initial fillets have been excluded from the ANOVA.

¶ number of fish analysed per treatment. Each value represents the mean of 'n' duplicate determinations.

Table 3.6 Hepatosomatic indices (%) of *Clarias* after 10 weeks of feeding test diets.

mg α-tocopherol kg^{-1} dry diet		0	80	200	500
	Mean	1.374	1.373	1.474	1.559
	S.E.	0.054	0.085	0.052	0.083
	n ¶	10	10	10	10

No significant differences exist between treatments ($P > 0.05$).
¶ number of livers per treatment (i.e. 5 per tank, 10 per treatment).

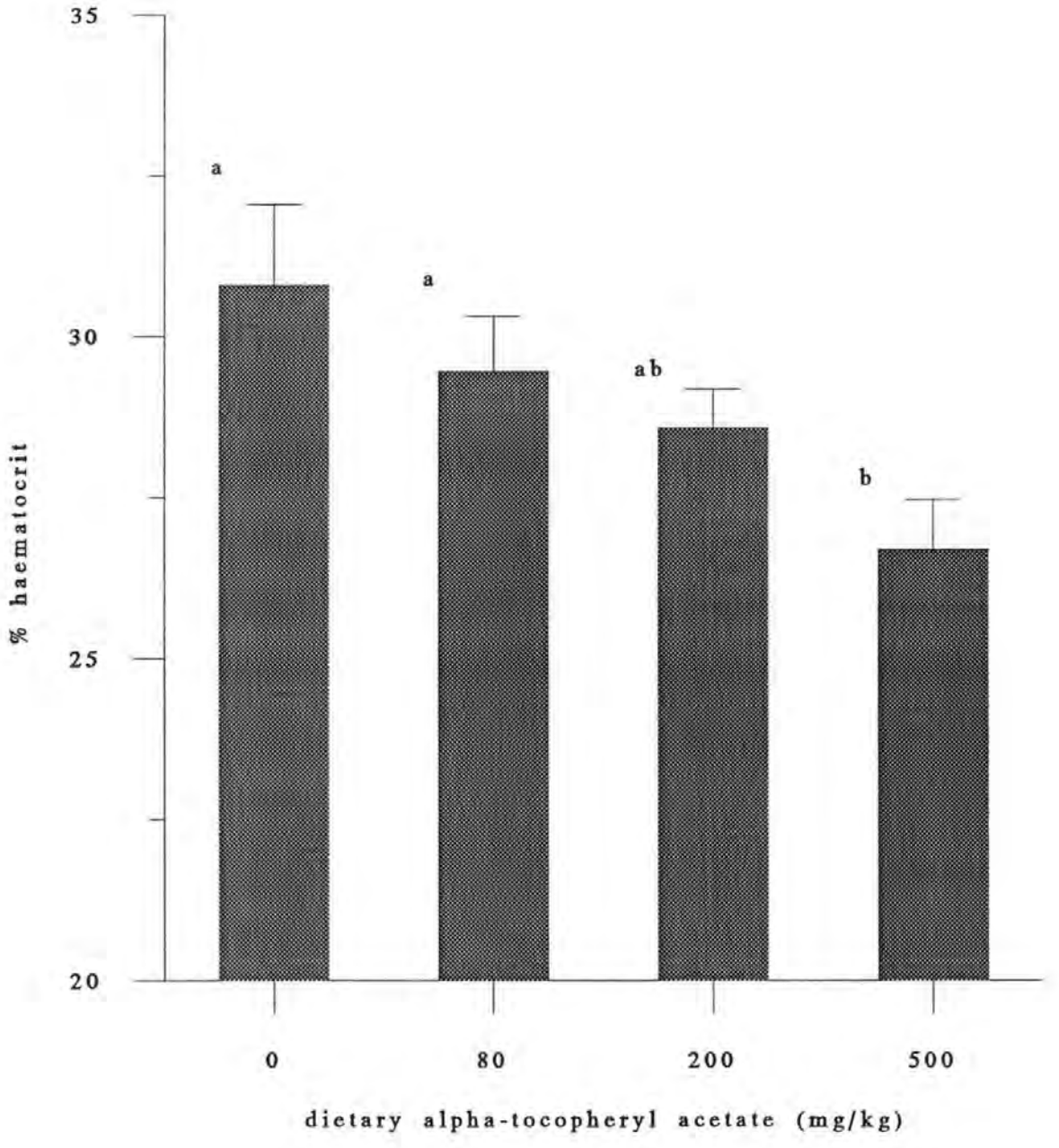


Fig. 3.1. Haematocrits (% Packed Cell Volume) from catfish fed diets supplemented with α-tocopheryl acetate. Points are means of 8 determinations, with standard errors represented by vertical bars. Values sharing common superscripts are not significantly different ($P > 0.05$).

Table 3.7 Assayed α-tocopherol in *Clarias* fillets, livers and blood-plasma after 70 days of feeding test diets.

Dietary α-tocopheryl inclusion (mg kg^{-1})		0	80	200	500
		α-tocopherol content (μg g^{-1}tissue or ml^{-1}plasma)			
Muscle	mean	2.19^{a}	6.16^{b}	10.80^{c}	17.12^{d}
	s.e.	0.82	0.67	0.88	1.63
	n	5	5	5	5
Liver	mean	37.20^{a}	212.20^{b}	724.41^{c}	3160.77^{d}
	s.e.	13.21	8.02	37.07	285.54
	n	5	5	5	5
Plasma	mean	4.39^{a}	14.85^{b}	31.04^{c}	51.25^{d}
	s.e.	0.68	1.01	3.47	6.13
	n	5	5	5	5

^{abcd} Within rows, values sharing common superscripts are not significantly different ($P > 0.05$).

n= number of duplicate extractions per treatment.

Table 3.8 TBARS (nmol MDA equivalents mg^{-1} tissue) in *Clarias* muscle and livers after iron/ascorbate induced lipid peroxidation. Values are expressed as means of ten determinations per treatment ± standard errors .

mg α-tocopherol kg^{-1} dry diet	time (mins)	Muscle TBARS (nmol MDA mg^{-1} tissue)	Liver TBARS (nmol MDA mg^{-1} tissue)
0	0	$0.148^{a}\pm0.020$	$0.180^{a}\pm0.013$
	50	$0.968^{a}\pm0.074$	$2.145^{a}\pm0.202$
	100	$1.175^{a}\pm0.064$	$3.529^{a}\pm0.228$
	200	$1.405^{a}\pm0.085$	$4.682^{a}\pm0.139$
80	0	$0.082^{b}\pm0.005$	$0.088^{b}\pm0.015$
	50	$0.538^{b}\pm0.102$	$0.181^{b}\pm0.016$
	100	$0.864^{b}\pm0.138$	$0.186^{b}\pm0.016$
	200	$1.192^{ab}\pm0.178$	$0.218^{b}\pm0.017$
200	0	$0.075^{b}\pm0.006$	$0.074^{b}\pm0.011$
	50	$0.341^{c}\pm0.038$	$0.139^{b}\pm0.009$
	100	$0.570^{c}\pm0.115$	$0.167^{b}\pm0.015$
	200	$0.958^{b}\pm0.176$	$0.187^{b}\pm0.012$
500	0	$0.058^{b}\pm0.004$	$0.066^{b}\pm0.006$
	50	$0.215^{d}\pm0.014$	$0.115^{b}\pm0.006$
	100	$0.232^{d}\pm0.025$	$0.135^{b}\pm0.009$
	200	$0.524^{c}\pm0.125$	$0.152^{b}\pm0.009$

[abcd] Values within columns carrying common superscripts are not significantly different ($p>0.05$). ANOVA were performed with values from common incubation times, comparing between dietary treatments.

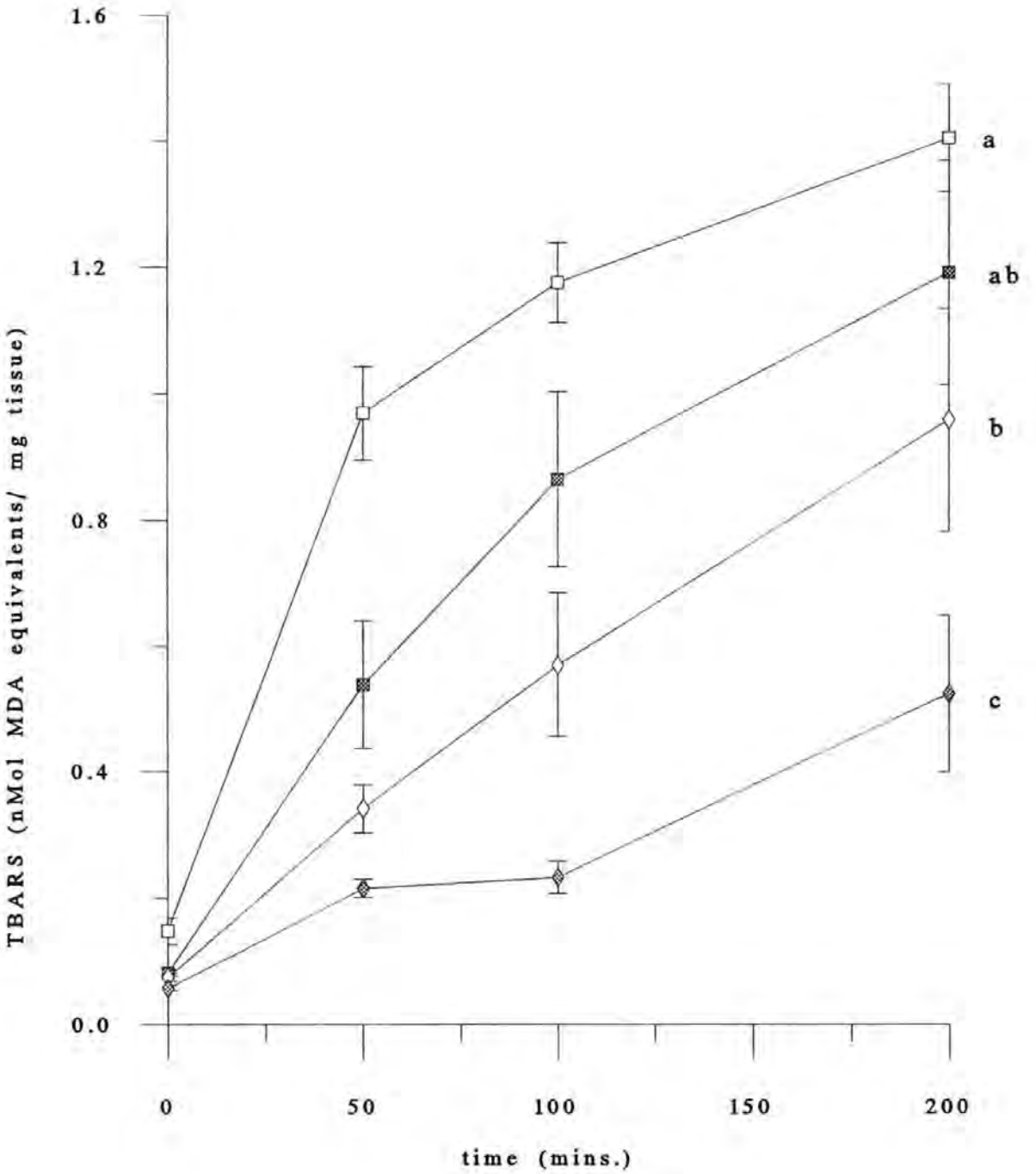


Figure 3.2 TBARS as a measure of iron-ascorbate induced lipid peroxidation in muscle (fillet) from catfish fed diets supplemented with all-rac-α-tocopheryl acetate (0mg kg^{-1}, □; 80mg kg^{-1}, ■; 200mg kg^{-1}, ◇; 500mg kg^{-1}, ◆). Points are means of 10 determinations with standard errors represented by vertical bars. Values possessing common superscripts are not significantly different ($P > 0.05$).

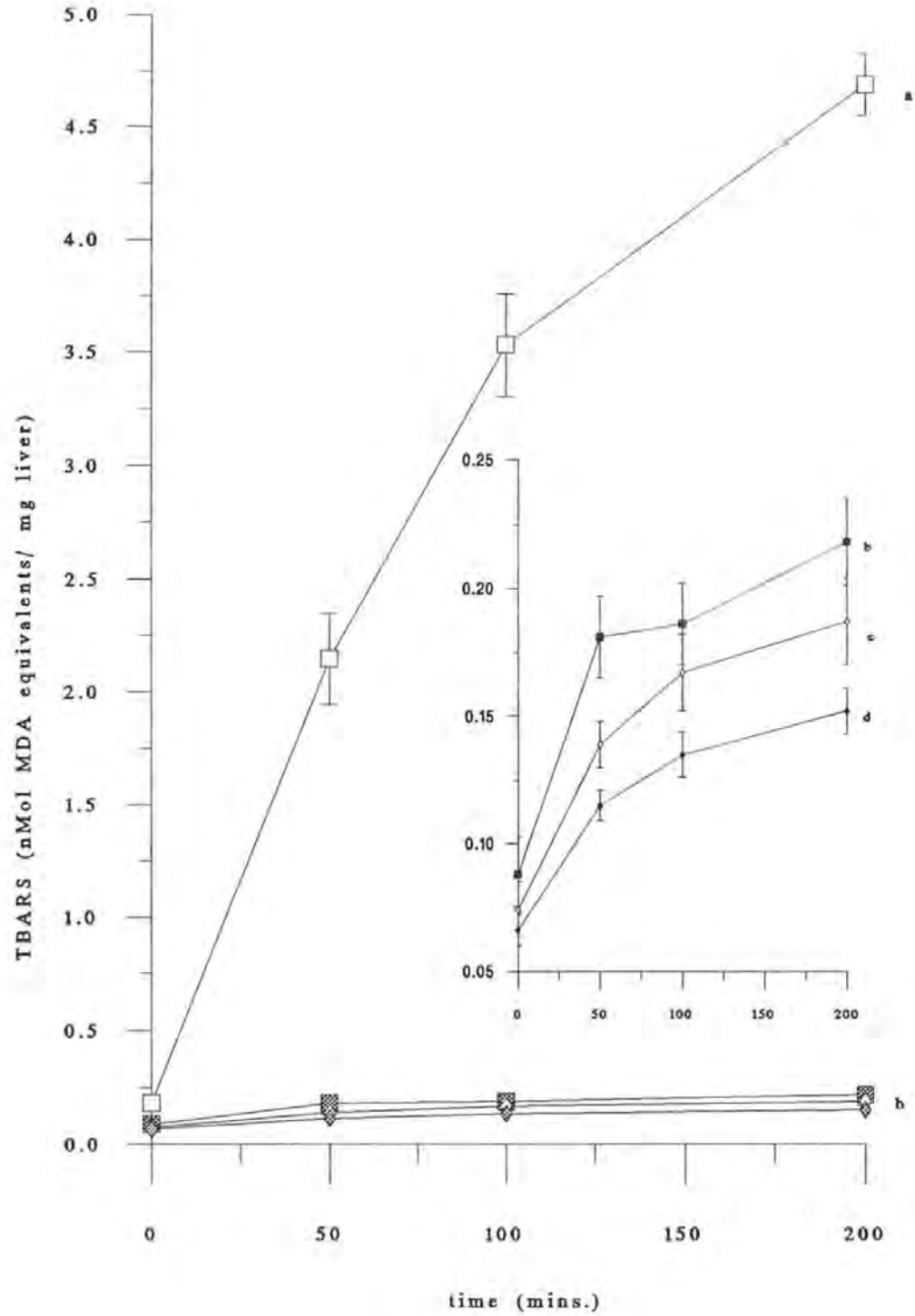
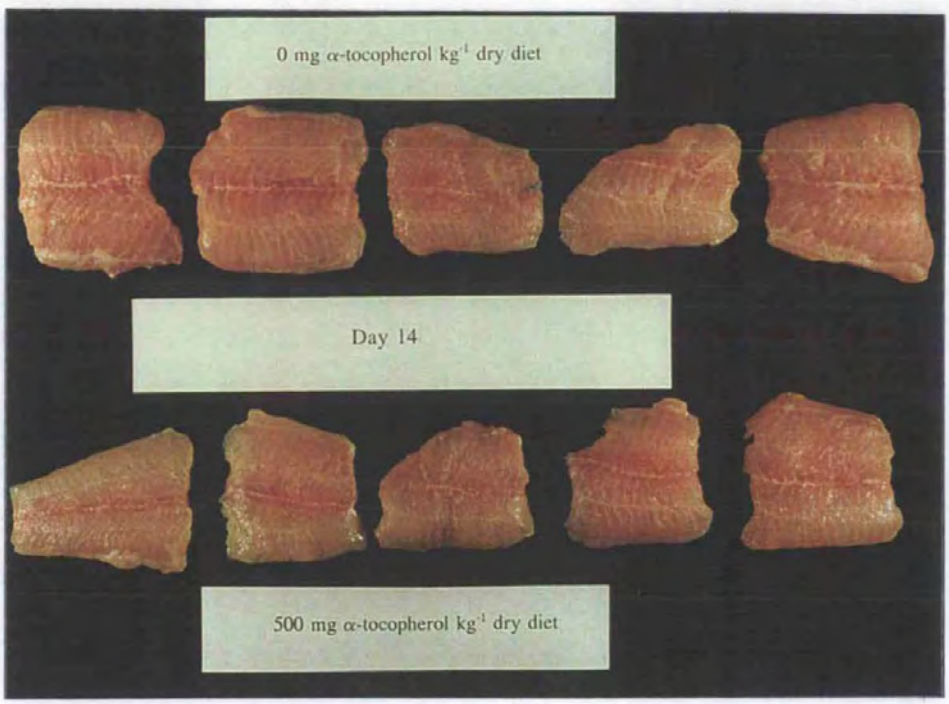


Figure 3.3 TBARS as a measure of iron-ascorbate induced lipid peroxidation in livers from catfish fed diets supplemented with all-rac-α-tocopheryl acetate (0mg kg^{-1}, □; 80mg kg^{-1}, ■; 200mg kg^{-1}, ◇; 500mg kg^{-1}, ◆). Points are means of 10 determinations with standard errors represented by vertical bars. Values possessing common superscripts are not significantly different ($P > 0.05$).


0 mg α-tocopherol kg-1 dry diet
Day 14
500 mg α-tocopherol kg-1 dry diet

3.4 Discussion

Absence of statistically significant growth related trends over the seventy day feeding trial, during which there was a threefold increase in body weights of all fish would imply that no metabolic advantage was conferred to catfish consuming higher α-tocopheryl acetate rations. Inter-treatment differences resulting from increased tocopherol supplementation may have been expected to occur as a result of peroxidation suppression. Peroxidation of inadequately protected erythrocyte membrane lipids manifests as reduced haematocrit values as seen in many vitamin E deficient animals. This phenomenon has already been reported by Lovell *et al.* (1984) in channel catfish (*Ictalurus punctatus*) and Moccia *et al.* (1984) in rainbow trout (*Oncorhynchus mykiss*). In this study however, haematocrits were observed to decrease significantly with increase in plasma α-tocopherol. To the authors knowledge, this has never been reported in the literature for fish or other vertebrate species.

It would seem unlikely that the vitamin E doses under study would cause a decrease the haematocrit through a damaging pro-oxidant action since it is widely recognised that α-tocopherol plays a protective role in erythrocytes, promoting membrane fluidity, regulating permeability and thus decreasing the tendency of red-blood cells to haemolyse (Niki *et al.*, 1989; Bender, 1992). From this we may assume that the erythrocytes from treatments receiving the higher α-tocopheryl acetate doses will have more structural integrity. We are therefore left with the dilemma of explaining the increased haematocrit in the lowest tocopherol treatment. Increased packed-cell volumes may result from increased erythropoiesis, haemoconcentration or an increase in red blood cell internal volumes (Wedemeyer and Yasutake, 1977). No mechanism is apparent to account for the former possibility. Indeed Roem and co-workers (1990) report impaired erythropoiesis in vitamin E deficient tilapia (*Oreochromis aureus*). An erythrocyte volume increase would appear feasible to explain these findings. But since membrane fluidity decreases with decrease in

vitamin E, the erythrocyte would be expected to lyse with expansion of its membrane. Therefore the increased cell volume would rely on an elevated fluid infiltration sufficient to increase volume without compromising membrane integrity. Validation of this hypothesis would depend on the determination of further haematological parameters such as blood-cell counts and erythrocyte dimension analysis. Haemoconcentration through the loss of fluid from blood vessels to other tissues may also account for the increase in haematocrit in fish fed tocopherol depleted diets. Poston *et al.* (1976) and Blazer and Wolke (1984) observed slightly increased plasma protein concentrations in vitamin E deficient Atlantic salmon (*Salmo salar*) and rainbow trout (*O. mykiss*) respectively. This would suggest possible haemoconcentration, despite reduced haematocrits in the former study, since Poston *et al.* (1976) also reported the incidence of exudative diathesis (fluid infiltration into the visceral cavity).

In all treatments, it was seen that tissue TBARS (thiobarbituric acid reactive substances) values were inversely related to the level of α-tocopherol inclusion in the diet. Elevating dietary tocopherol was effective in suppressing the formation of TBARS in the meat fillets, thus conferring stability to the lipid in muscle-tissue membranes against oxidative insult. This is consistent with the findings of studies on poultry (Sheehy *et al.*, 1991, 1993a), beef (Arnold *et al.*, 1993), pork (Whang *et al.*, 1986), veal (Engeseth *et al.*, 1993) and fish species such as the rainbow trout (*Oncorhynchus mykiss)* (Hung and Slinger, 1982; Boggio et al., *1985; Frigg* et al., 1990), red seabream (*Chrysophrys major*) (Murata and Yamauchi, 1989) and channel catfish (*Ictalurus punctatus*) (O'Keefe and Noble, 1978; Gatlin *et al.*, 1992). Storage-induced changes in fillet colour were not observed in this study. The oxidation of *Clarias* fillet myoglobin to metmyoglobin (causing a browning effect) did not become apparent, in contrast to findings in beef (Arnold *et al.*, 1993). This may have been due to the fact that catfish muscle contains relatively lower concentrations

of myoglobin, so that differences may have been masked by the background flesh colour. The relationship between fillet TBARS value and dietary tocopherol appears relatively linear amongst the tocopherol-supplemented treatments and this is obviously due to the fact that muscle tocopherol assimilation from the dietary source was linear as well (R-sq= 0.83), as seen from the HPLC determination of α-tocopherol in *Clarias* muscle and has been already observed in other species by the majority of workers in this field (Hung and Slinger, 1982; Boggio *et al.*, 1985; Frigg *et al.*, 1990; Gatlin *et al.*, 1992).

Assuming that oxidative degradation of lipids whilst in frozen storage (1 week) prior to analysis was minimal, the amount of TBARS in fillets at 'time 0' of the incubation stage of the assay (basal TBARS) may reflect the *in vivo* TBARS concentration. This would demonstrate the 'oxidative state' of the catfish, since it may be assumed that the iron-ascorbate peroxidative action had not had time to damage the tissue lipids. These results show that, assuming that all fillets would be subjected to the same degree of oxidative attack from oxy-radicals *in vivo*, muscle tissues from catfish fed increasing levels of α-tocopheryl acetate were progressively more successful in preventing peroxidative damage. In addition, supplementation of α-tocopheryl acetate above the level considered normally as the vitamin E requirement for this species (80-100 mg kg^{-1} air-dried feed), was proved to further benefit the meat tissues with respect to the oxidative stability and therefore have significant commercial implications when considering product quality. The commercial viability of super-doses of dietary vitamin E inclusion, with respect health and tissue condition, remains to be examined in this species

Determination of the degree of peroxidation in liver tissue did not reveal the dose dependant response to the same extent as observed in muscle. Since the liver tends to accumulate lipids (Sargent *et al.*, 1989), making it a target organ for peroxidation, slight vitamin E insufficiencies may manifest as large increases in the resulting products of lipid peroxidation. Supplementation of α-tocopheryl acetate, satisfying estimated vitamin E

requirement levels, was able to significantly reduce TBARS formation in the liver. Doses of tocopherol above the requirement (200 and 500 mg kg^{-1} feed) were only able to marginally improve the stability against peroxidation. This does not reflect the liver tocopherol content as determined by HPLC. In this case, the assimilation of tocopherol from dietary sources was linear (R-sq= 0.93) with respect to the dose, yet there was no associated linear increase in oxidative stability as measured by TBARS. This may be due to the battery of other antioxidant defenses present in the liver. Suppression of TBARS formation may have been achieved effectively until the vitamin E became limiting. This buffering phenomenon by hepatic antioxidants, other than tocopherol, would preclude the use of liver TBARS as an indicator of vitamin E status in the catfish, certainly at the doses used in this study.

As a secondary indicator of liver condition, the hepatosomatic index was determined. This parameter provided no statistically significant trend. Livers from fish fed the requirement of tocopherol, or higher tocopherol doses, may have been expected to be larger than livers from fish fed diets low in vitamin E, since some degeneration of liver tissues through lipid peroxidation would have been possible. This has been observed in Atlantic salmon, *Salmo salar* (Smith, 1979), through histological examination.

In conclusion, this study has demonstrated that, at the dietary lipid level employed in this investigation, supplementation of vitamin E in the form of all-rac-α-tocopheryl acetate, results in a concomitant increase in tissue α-tocopherol concentration and a progressive protection of tissues of *Clarias gariepinus* against the peroxidative damage of oxy-radicals both *in vivo* and under forced oxidation.

Further research must aim to elucidate the effect of vitamin E supplementation in aquaculture-type diets of varying lipid status. With the current emphasis on the production of high energy diets, dietary lipid inclusion levels have been steadily increasing. This not

only has a direct effect on the lipid content of fish tissues, but also on the susceptibility of these tissues to lipid oxidation. Vitamin E supplementation is likely to be an important dietary strategy in order to maintain fish health and the quality of products destined for human consumption.

CHAPTER 4

THE QUANTITATIVE REQUIREMENT FOR α-TOCOPHEROL BY JUVENILE AFRICAN CATFISH

4.1 Introduction

In previous work (chapter 3; Baker and Davies, 1996) the author has demonstrated uptake of α-tocopherol by *Clarias gariepinus*, from a dietary source, and that the vitamin functions as an antioxidant within catfish tissues. Furthermore, a dose response was observed in muscle and hepatic tissues with respect to the protection of tissues from lipid peroxidation. The study evaluated the efficacy of dietary tocopherol concentrations over a broad range surrounding an estimated requirement (0-500 mg kg^{-1}) for maximal growth in this species, and therefore a more precise estimation of absolute requirement for α-tocopherol is to be undertaken.

It is appropriate to define what is implied as 'requirement', since many response criteria maybe assessed. For example, the minimum requirement of a particular nutrient for maximum growth is not necessarily the same as that level required for optimum functioning of the immune system. Requirements may also be expressed as the nutrient-dose necessary to saturate enzyme systems, maximise hepatic storage or prevent certain pathologies, therefore one must state the basis of requirement. Consequently, declared requirements for α-tocopherol stated within the text of this chapter, will be qualified by associated response criteria.

From NRC (1993) data it was estimated by the author that the dietary requirement for α-tocopherol to satisfy optimum growth in *Clarias gariepinus*, would lie within the range 20-100 mg α-tocopherol per kg air-dried feed, though these values were obtained for unrelated fish species (common carp (Watanabe *et al.*, 1970b), blue tilapia (Roem *et al.*, 1990), channel catfish (Lovell *et al.*, 1984)) and may therefore not reflect what may be

required by African catfish. Additionally, studies have employed varied diet formulations, further complicating the estimation of vitamin E requirements.

It is known that many factors may affect vitamin E requirements in animals, and these have been briefly introduced in chapter 1. Research in the field of fish nutrition has demonstrated that these factors act similarly in many fish species.

Of the nutritional factors influencing vitamin E status in fish, level and degree of polyunsaturation of dietary lipids has been most extensively investigated.

On work in common carp (*Cyprinus carpio*), Watanabe *et al.* (1981) demonstrated increased requirement for α-tocopherol when purified diets were supplemented with methyl esters of fish oils. Boggio and co-workers (1985) noted that the dietary requirement of α-tocopheryl acetate for prevention of vitamin E deficiency signs, was higher in rainbow trout (*Oncorhynchus mykiss*) fed fish-oil diets as compared to diets containing swine fat. In that study, for any given level of supplemental dietary tocopherol, tissue levels of this nutrient were depleted by heightened PUFA (polyunsaturated fatty acid) intake, implying the utilization of vitamin E in preventing tissue damage. An enriched PUFA diet fed to sturgeon (*Acipenser naccarii*) also led to decreased hepatic tocopherol relative to fish fed a control diet, despite the former containing ten times more α-tocopherol (Randall *et al.*,1992).

Dietary lipid composition has been taken into consideration when declaring requirements of α-tocopherol in studies on channel catfish (*Ictalurus punctatus*) (Lovell *et al.*, 1984), common carp (*C. carpio*) (Watanabe *et al.*, 1981), Nile tilapia (*Oreochromis niloticus*) (Satoh *et al.*, 1987), blue tilapia (*Oreochromis aureus*) (Roem *et al.*, 1990) and rainbow trout (*O. mykiss*) (Boggio *et al.*, 1985). Roem and co-workers (1990) expressed the tocopherol requirement relative to dietary lipid and arrived at values of 10 mg kg^{-1} of diet employing a 3 % lipid diet, and 25 mg kg^{-1} diet using a 6 % lipid diet. These values are lower than many reported values for other fish species, though this may be due to the

synthetic antioxidant content of the diets (120 mg BHA kg^{-1} diet). On the basis of test diets employed, Roem's team recommended that 3-4 mg α-tocopheryl acetate should be supplemented into diets, per percent corn oil per kg of diet for blue tilapia fingerlings. As well as oil composition, oil quality will also affect absolute requirements for vitamin E (Hashimoto *et al.*, 1966). Oxidation of dietary lipids is known to heighten the requirement of the vitamin, though this topic will be covered in more detail in chapter 5. Temperature has been recorded to influence membrane structure (Greene and Selivonchick, 1987). In colder waters fish will incorporate more PUFA into their membranes, in order to increase membranal-fluidity and as such more vitamin E is necessary to protect these lipids from oxidative damage. Cowey *et al.* (1984) considered this phenomenon in the culture of rainbow trout (*Oncorhynchus mykiss*) and stated that no exact requirement for dietary α-tocopherol could/should be derived for fish under certain culture situations due to environmental-factor fluctuations.

Other nutritional factors which may influence vitamin E requirements of finfish include the presence and abundance of alterative antioxidants and antagonists (pro-oxidants). In situations where selenium may be limiting, the minimum requirement for vitamin E may increase due to the possibility that selenium-dependent glutathione peroxidase (Se-GSH-px), will not be available in sufficient quantity to remove damaging hydroxyl radicals from biological systems. This being the case, vitamin E will be utilised rapidly in chain termination, upon lipid modification by hydroxyl radicals. Heisinger and Dawson (1983) (cited in Bell *et al.*, 1985) reported selenium-independent GSH-px activity in selenium deficient bullheads (species unspecified), therefore this may compensate somewhat for Se-GSH-px inactivity, although Cowey (1986) states that there is no evidence for such a system in trout. However, a compensatory increase in GSH-S-transferase activity was observed in vitamin E deficient rainbow trout and this enzyme is known to inhibit lipid

peroxidation *in vitro* (Cowey, 1986).

Since vitamin C also has an antioxidant role, it may be expected that low dietary concentrations of this nutrient may allow heightened activity of free radical cascade systems, and this may necessitate increased vitamin E to prevent tissue-lipid damage. This is described further in chapter 6 and therefore, to avoid unnecessary repetition, will not be covered here.

Inclusion of synthetic antioxidants (eg. BHA, BHT, ethoxyquin) in feeds and feed ingredients may modulate α-tocopherol requirement, by partially sparing the need for vitamin E. This may be achieved by several mechanisms. Firstly, it is expected that the synthetic antioxidants will prevent loss of the E-vitamers from feeds and ingredients, by preventing lipid peroxidation in these systems. Additionally, in halting rancidifying processes in lipids, these compounds will reduce the consumption and assimilation of pre-formed hydroperoxides which may degrade to produce free radicals *in vivo*. However, Gatlin and co-workers (1992) did not observe any tocopherol sparing by BHA, BHT, ethoxyquin, or Endox™ (commercial antioxidant product: BHA + disodium ethylenediaminetetraacetate, mono- and di-glycerides) with respect to tissue accretion of tocopherol and prevention of peroxidation.

Transition metals such as iron and copper are known to stimulate lipid peroxidation *in vitro* (Halliwell and Gutteridge, 1993) and this topic is explored in chapter 7. As the investigation in chapter 7 will demonstrate, in nutritional metal-overload, pro-oxidation by orally-administered metals can compromise the antioxidant defence system, thereby increasing the need for dietary tocopherol to combat peroxidative challenge.

As demonstrated above, establishment of a vitamin E minimum requirement level would be confounded by numerous considerations. Studies should therefore define diets and culture conditions, especially when factors depart from those employed under typical

culture situations.

Previous research has established requirements for vitamin E in many fish species. In order to prevent pathologies associated with vitamin E deficiency and allow optimum growth, levels of 10-25 mg α-tocopheryl acetate per kg diet for blue tilapia (Roem *et al.*, 1990), 50 mg α-tocopheryl acetate per kg diet for Nile tilapia (Satoh *et al.*, 1987), 30-50 mg α-tocopheryl acetate per kg diet for channel catfish (Murai and Andrews, 1974; Wilson *et al.*, 1984), 25 mg α-tocopheryl acetate per kg diet for rainbow trout (Hung *et al.*, 1980) and 50-100 mg α-tocopheryl acetate per kg diet for common carp (Watanabe *et al.*, 1970ab, 1981) have been quoted in the literature. It must be noted that the relatively high dietary vitamin E supplementation recommendations in published works on common carp probably result from experimental design, since 50 mg α-tocopheryl acetate per kg diet appears to be the lowest tested dose in most instances (see Watanabe *et al.* (1970ab, 1981), Aoe *et al.* (1972)).

Results from chapter 3 demonstrate that *Clarias* fed a basal diet containing no supplemental vitamin E (assayed at 2.15 mg α-tocopherol kg^{-1} dry diet) performed as well as supplemented treatments with respect to growth and feed utilization over the eight week trial. During the trial, fish grew three-times in body weight. It must be noted however, that in this study, starting weights of catfish were about 60 g and this implies a sizeable body pool of the vitamin due to pre-trial feeding on commercial diets containing 200 mg kg^{-1}.

It is expected that the current investigation will determine the dietary α-tocopherol requirement of juvenile African catfish (*Clarias gariepinus*), based on appropriate response criteria. Catfish will be reared at normal culture temperatures, and fed practical type diets,

defined with respect to selenium and vitamin C inclusion level and amount of PUFA. Doses of vitamin E under test will range from 0 to 100 mg kg^{-1} dry-diet supplied as the stable ester, all-rac-α-tocopheryl acetate. Starting weights of catfish will be lower than those employed in chapter 3, and the trial duration will be extended by 4 weeks, thus allowing for depletion of hepatic-tocopherol stores accumulated prior to trial commencement.

4.2 Materials and Methods

4.2.1 Chemicals

All chemicals used were of ANALAR grade or better, and purchased from Sigma Chemical Company Limited, Poole, Dorset, U.K., and Merck Limited, Poole, Dorset, U.K.. All-rac-α-tocopheryl acetate (Rovimix E-50 SD) was a generous gift from Roche Products, Heanor, Derbyshire, U.K.

4.2.2 Fish stock, Experimental facilities and Diets

African catfish (*Clarias gariepinus*) of mixed sexes were spawned in house according to the procedure described in section 2.9, and grown to 10 g. These juveniles, of mixed sexes, were stocked into 80 litre tanks of a freshwater closed recirculation system. System water temperatures were maintained at 27^{0}C, the photoperiod held at 12 hours light:12 hours dark, and water quality parameters measured and adjusted as appropriate.

A low-tocopherol, basal practical diet (table 4.1) was formulated as described in section 2.1.1, but modified as follows:

Cod-liver/ corn oil (1:1) was included at 6 % of the formulation in order to increase the energy density of the feed, to maximise realised growth potential.

Levels of dietary vitamin E (supplied as all-rac-α-tocopheryl acetate) were set in relation to levels reported by other workers in a variety of fish species. Incremental levels from 0 to 100 mg α-tocopheryl acetate per kg of air-dried feed were selected, thereby offering a broad range of doses either side of an estimated requirement based on salmonids (NRC, 1993). Inclusion levels of Rovimix E-50 SD (donated by Roche Products Ltd., Heanor, Derbyshire, U.K.) were selected to fulfil these criteria, though 10 % additional was incorporated to allow for potential processing losses of the vitamin. In all, eight diets were made as detailed in section 2.1.2. These diets were designed to contain 0, 5, 15, 30, 45,

60, 75 or 100 mg α-tocopheryl acetate per kg of air-dried feed.

4.2.3 Dietary analyses

Proximate analysis (AOAC, 1990; described in section 2.3) of the completed test diets confirmed values to be close to calculated values (see table 4.2).

HPLC determination of α-tocopherol content of the pelleted feeds (according to the method in section 2.4.1) established values to be close to intended. These data are presented in table 4.3 and are expressed as mg α-tocopherol kg^{-1} dry diets.

Since dietary polyunsaturated fatty acid (PUFA) content has been seen to affect vitamin E requirement in a number of species (Watanabe *et al.*, 1977; Boggio *et al.*, 1985), dietary oils were characterised by GC-MS as described in section 2.4.3 and values reported in table 4.4.

Selenium content of the basal diet was calculated from NRC (1993) values for raw materials and the selenium supplement in the premix. From these data the dietary selenium concentration was established at 2.34 mg kg^{-1} dry diet

4.2.4 Feeding regime and Nutrition trial protocol

The fish were uniformly graded and assigned, twenty per tank, into the eight tanks of the experimental system.

Clarias were fed three times daily for twelve weeks, at a ration level of up to 3 % body weight per day (calculated on a dry matter basis). Rations dispensed were recalculated weekly to allow for body-weight changes.

On completion of the feeding period, individual fish weights were recorded and selected tissues were excised for analysis. Additionally, eight fish were desanguinated by caudal venipuncture (section 2.6.1), haematocrits (section 2.6.2) and total haemoglobin (section 2.6.4) determined and the bloods centrifuged to enable plasma storage at -80^{0}C for future

analysis.

4.2.5 Growth and nutrient utilization parameters

The following parameters were calculated, according to the formulae in section 2.2.5, from data acquired from the feeding trial; percent change in mean body weight, specific growth rate and feed conversion efficiency.

4.2.6 Hepato- and Haematological techniques

Additional to the final body weights on termination of the trial, the liver weights of 8-12 fish per treatment were recorded in order to allow calculation of the hepatosomatic index (% contribution of liver weight to the total body weight).

Plasma samples (stored at -80^{0}C) were subjected to the cyanmethemoglobin method for analysis of haemoglobin (possible crude index of spontaneous haemolysis)(section 2.6.4), determination of plasma α-tocopherol (section 2.4.1), and determination of plasma malondialdehyde (MDA) concentration (according to section 2.5.3). Coupling plasma haemoglobin and whole-blood haemoglobin (% whole-blood haemoglobin contained within plasma) was performed in order to render the plasma haemoglobin interpretation of spontaneous haemolysis more sensitive and negate differences caused by varied haematocrit.

4.2.7 Analytical methods

α-tocopherol was extracted from muscle, liver and blood-plasma samples, and quantified using the method outlined in section 2.4.1.

Tissue TBARS concentrations were determined as detailed in section 2.5.1 with the exception that no iron-ascorbate forced peroxidation was carried-out. The volume occupied

by the iron sulphate and ascorbic acid solutions were replaced by 400 μl of distilled water. Values were expressed as nmoles MDA equivalents per g tissue, or per ml plasma. Samples of muscle, liver and plasma were frozen at -80^{0}C until analyses were performed.

4.2.8 Statistical analysis

After variance checks and log(10) or square-root data transformations where appropriate, measured parameters from individual groups were compared using one-way ANOVA coupled with Duncan's multiple range test (Steel and Torrie, 1960). Differences between treatments were considered significant when $P<0.05$. Regression analysis ($P<0.05$) was used in the determination of relationship between tissue and dietary α-tocopherol concentrations. Break-point analysis was possible on hepatic TBARS concentrations, upon performing linear regressions on data sub-sets. Analyses were performed using the statistical software package *Statgraphics* 6.1 (Manugistics Incorporated, Rockville, MD, U.S.A.).

Table 4.1 Composition of practical test diets for the African catfish, *Clarias gariepinus*.

Ingredient	%inclusion
Fishmeal (Chilean)	60.00
Meat and bone meal	10.00
Bloodmeal	2.00
Cod-liver oil	3.00
Corn oil	3.00
Corn starch:Dextrin (3:2)	15.35
Molasses	1.00
Mineral premix[1]	5.00
B-complex vitamin premix[2]	0.10
Fat-soluble vitamin premix[3]	0.05
Macro-vitamin premix[4]	0.50

[1] Mineral salt inclusion (g kg^{-1}dry diet):
Calcium orthophosphate ($CaHPO_4.2H_2O$) 12.000, Magnesium sulphate ($MgSO_4.7H_2O$) 4.8450, Sodium chloride (NaCl) 2.2800, Potassium chloride (KCl) 1.9000, Iron sulphate ($FeSO_4.7H_2O$) 0.9500, Zinc sulphate ($ZnSO_4.7H_2O$) 0.2090, Manganese sulphate ($MnSO_4.4H_2O$) 0.0960, Copper sulphate ($CuSO_4.5H_2O$) 0.0298,Cobalt sulphate ($CoSO_4.7H_2O$) 0.0181, Calcium iodate ($CaIO_3.6H_2O$) 0.0112, Chromic chloride ($CrCl_3.6H_2O$) 0.0048, Sodium selenite (Na_2SeO_3) 0.0025, Filler (α-cellulose) 27.6536.

[2] B-vitamin inclusion (mg kg^{-1} dry diet):
B1-Thiamine hydrochloride 50, B2-Riboflavin (feed grade-96%) 52.1, B6-Pyridoxine hydrochloride 40, Calcium pantothenate 100, Niacin 200, H2-Biotin (2%) 300, Folic acid (90%) 16.7. **Note** B12 added separately at 0.05 mg kg^{-1} dry diet.Filler (α-cellulose) 241.2.

[3] Fat-soluble vitamin inclusion (mg kg^{-1} dry diet):
A-Vitamin A palmitate (1.7×10^6 I.U. g^{-1}) 2, D-Rovimix D3-500 (5×10^5 I.U. g^{-1}) 20, K-Menadione sodium bisulphite (51%) 80.Filler (α-cellulose) 398.

[4] Macro-vitamin inclusion (mg kg^{-1} dry diet):
Inositol 200, Choline chloride 2000, Ascorbic acid 500, Filler (α-cellulose) 2300.

Table 4.2 Proximate composition of basal practical test diet (bracketed values depict theoretical composition as calculated from NRC (1993)).

	Moisture (%)	Protein (% DM)	Lipid (% DM)	Ash (% DM)	Residual
Mean	5.96	47.01 [49.35]	11.64 [11.87]	16.04 [14.14]	19.35
S.E.	0.139	0.238	0.063	0.118	
n	12	8	12	12	

n=number of determinations from a single diet.

Table 4.3 Assayed α-tocopherol in test diets (mg kg^{-1} dry diet). Values were obtained from 3 extractions of each diet.

Inclusion (mg all-rac-α-tocopheryl acetate kg^{-1} dry diet	Assayed α-tocopherol in test diets (mg kg^{-1} dry diet)(mean±s.e.)
0	7.86 ± 0.41
5	9.61 ± 0.68
15	15.27 ± 1.32
30	28.719 ± 0.12
45	38.45 ± 2.88
60	50.24 ± 2.82
75	63.99 ± 1.91
100	80.68 ± 2.17

Table 4.4 Total lipid fatty acid composition of dietary oil (cod-liver/corn oil (1:1)). Values are expressed as % contribution of each fatty acid to the total chromatogram area (n=1).

fatty acids	area %
14:0	2.7
16:0	11.6
16:1 n7	3.4
18:0	2.4
18:1 n9	18.7
18:1 n7	1.9
18:2 n6	31.5
18:3 n3	1.0
18:4 n3	1.3
20:0	0.3
20:1 n9+11	1.2
20:1 n7	0.1
20:2 n6	0.2
20:3 n6	0.1
20:4 n6	0.5
20:3 n3	0.0
20:4 n3	0.5
20:5 n3	7.7
22:0	0.2
22:1 n11+13	0.5
22:1 n9	0.2
21:5 n3	0.3
22:4 n6	0.2
22:5 n3	1.1
22:6 n3	6.3
others	6.1
ΣSFA †	17.2
ΣMUFA ‡	26.0
ΣPUFA §	50.7
n3:n6	0.56

† ΣSFA = total saturates
‡ ΣMUFA = total mono-unsaturates
§ ΣPUFA = total polyunsaturates

4.3 Results

Varying dietary α-tocopheryl acetate concentration did not result in any differences in measured growth parameters (table 4.5). Treatment mean final weights were not significantly different ($P>0.05$) at the end of the 12 week feeding period. Other indices of growth were not tested statistically due to their derivation from pooled treatment data, though no trends could be discerned.

Hepatosomatic indices (HSIs) of *Clarias* (table 4.6) yielded significant inter-treatment differences ($P<0.05$) although recorded values did not correlate to dietary vitamin E inclusion level.

Table 4.7 presents haematological values and demonstrates that dietary vitamin E was not influential on measured parameters. Neither plasma haemoglobin, nor % of whole-blood haemoglobin contained within plasma demonstrated any significant trends ($P>0.05$). Haematocrits were observed to increase slightly with increased dietary tocopherol dose above the inclusion level of 15 mg all-rac-α-tocopheryl acetate kg^{-1} dry diet. Linear regression analysis of haematocrits from fish from treatments fed vitamin E at above this level dose demonstrated an R-squared of 0.71 ($n=48$). In fish fed the unsupplemented diet, haematocrits also increased significantly ($P<0.05$) from values obtained for *Clarias* fed slightly higher doses.

In all tissues assayed, significant inter-treatment differences were recorded in α-tocopherol concentration ($P<0.001$) (table 4.8). Tissue α-tocopherol concentration increased with respect to dietary dose of all-rac-α-tocopheryl acetate in muscle, liver and plasma (R-Sq = 0.93 for muscle (figure 4.1); 0.94 for liver and plasma (figure 4.2 and 4.3 respectively)).

Determination of concentration of thiobarbituric acid reactive substances (TBARS) within *Clarias* tissues (table 4.9) revealed that for muscle and liver, TBARS concentration was significantly affected by dietary regime ($P<0.001$) with lowest recorded TBARS values

coming from tissues of *Clarias* fed higher tocopherol diets. Muscle and liver responded similarly to dietary tocopherol dose (figure 4.4), though values for liver TBARS were more consistent and as such were subjected to break-point analysis (figure 4.5). From the break-point observed it can be suggested that at dietary vitamin E concentrations less than 34 mg kg^{-1}, TBARS concentration within hepatic tissues of cafish increased in response to further decreased dietary vitamin E status. Assayed plasma TBARS yielded no significant differences between treatments ($P > 0.05$).

Table 4.5 Growth related performance and nutrient utilization of catfish fed experimental diets after 84 days. Values are expressed as means, with standard errors (±s.e.) where appropriate.

	mg all-rac-α-tocopheryl acetate per kg dry diet							
	0	5	15	30	45	60	75	100
Initial weight (g)	10.23 ±0.36	10.25 ±0.41	10.23 ±0.33	10.34 ±0.33	10.25 ±0.39	10.22 ±0.43	10.25 ±0.35	10.26 ±0.41
Final weight (g)	125.33 ±8.09	123.44 ±10.89	120.99 ±8.18	119.11 ±7.43	127.19 ±11.04	122.44 ±8.24	119.2 ±11.70	118.28 ±8.43
% change in body weight	1125	1104	1083	1047	1141	1098	1063	1053
Specific growth rate (% d^{-1})	2.98	2.96	2.94	2.91	2.99	2.96	2.92	2.91
Feed Efficiency	1.29	1.29	1.29	1.26	1.29	1.25	1.27	1.32

No significant differences exist with respect to any growth index ($P > 0.05$).

Table 4.6 Hepatosomatic indices (%) of *Clarias* after 84 days of feeding test diets.

	mg all-rac-α-tocopheryl acetate per kg dry diet							
	0	5	15	30	45	60	75	100
Mean	1.42^{abc}	1.51^{a}	1.43^{abc}	1.31^{c}	1.45^{abc}	1.47^{ab}	1.33^{bc}	1.40^{abc}
s.e.	0.15	0.17	0.17	0.11	0.18	0.11	0.18	0.13
n	12	10	10	8	8	8	8	8

^{abc} Values carrying common superscripts are not significantly different ($P > 0.05$).

Table 4.7 Haematocrit (% PCV) and plasma haemoglobin (Hb) (mg cm^{-3}) of *Clarias* after 84 days of feeding test diets. Values are means of 8 determinations ± s.e.

	mg all-rac-α-tocopheryl acetate per kg dry diet							
	0	5	15	30	45	60	75	100
Haematocrit (%)	27.93^{ab}	n.d.§	26.02^{c}	26.67^{bc}	27.47^{abc}	26.59^{bc}	27.19^{abc}	28.46^{a}
	±0.62	-	±0.30	±0.65	±0.37	±0.77	±0.59	±0.72
Plasma haemoglobin (Hb) (mg cm^{-3})	67.0^{b}	90.0^{ab}	72.0^{b}	79.0^{ab}	84.0^{ab}	93.0^{ab}	103.1^{a}	78.0^{ab}
	±8.1	±13.0	±12.8	±4.9	±8.1	±17.0	±15.7	±7.7
% haemoglobin in plasma ¶	0.33^{c}	0.51^{ab}	0.41^{bc}	0.43^{abc}	0.48^{ab}	0.52^{ab}	0.58^{a}	0.40^{abc}
	±0.04	±0.08	±0.08	±0.02	±0.05	±0.08	±0.10	±0.04

[ab] Values carrying common superscripts are not significantly different ($P > 0.05$).

¶ (plasma haemoglobin/whole-blood haemoglobin) x 100

§ n.d. not determined

Table 4.8 Assayed α-tocopherol in *Clarias* muscle, livers and blood-plasma after 84 days of feeding test diets. Values are means $\pm$ s.e. (n=5 or 6 duplicate extractions).

	0	5	15	30	45	60	75	100
	α-tocopherol content ($\mu g\ g^{-1}$tissue or ml^{-1}plasma)							
Muscle	0.63^{a}	0.64^{a}	1.39^{b}	2.16^{c}	3.52^{d}	3.92^{de}	4.55^{cf}	5.11^{f}
	± 0.08	± 0.12	± 0.15	± 0.05	± 0.35	± 0.19	± 0.33	± 0.16
Liver	7.99^{a}	11.91^{b}	30.29^{c}	48.24^{d}	81.24^{e}	95.53^{ef}	132.02^{fg}	157.25^{g}
	± 0.61	± 0.55	± 1.63	± 4.74	± 3.00	± 7.03	± 13.29	± 7.87
Plasma	2.83^{a}	3.66^{a}	5.12^{b}	7.49^{c}	9.58^{d}	11.99^{e}	12.34^{e}	15.83^{f}
	± 0.13	± 0.15	± 0.34	± 0.24	± 0.49	± 0.59	± 0.63	± 0.74

^{abcdefg} Within rows, values sharing common superscripts are not significantly different ($P > 0.05$).

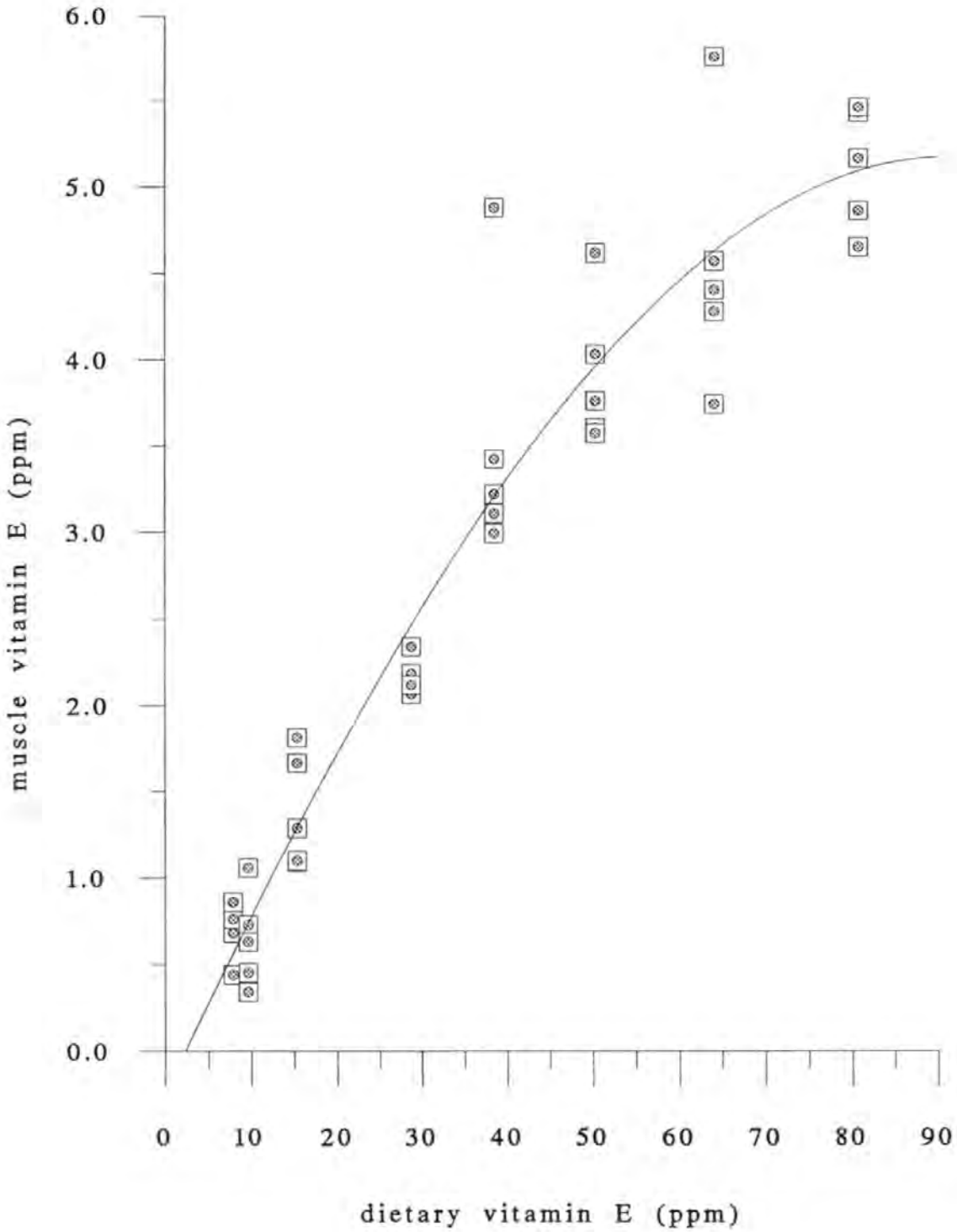


Figure 4.1 Assayed muscle α-tocopherol (μg g^{-1}) from *Clarias* fed diets varying in α-tocopheryl acetate inclusion, for twelve weeks.

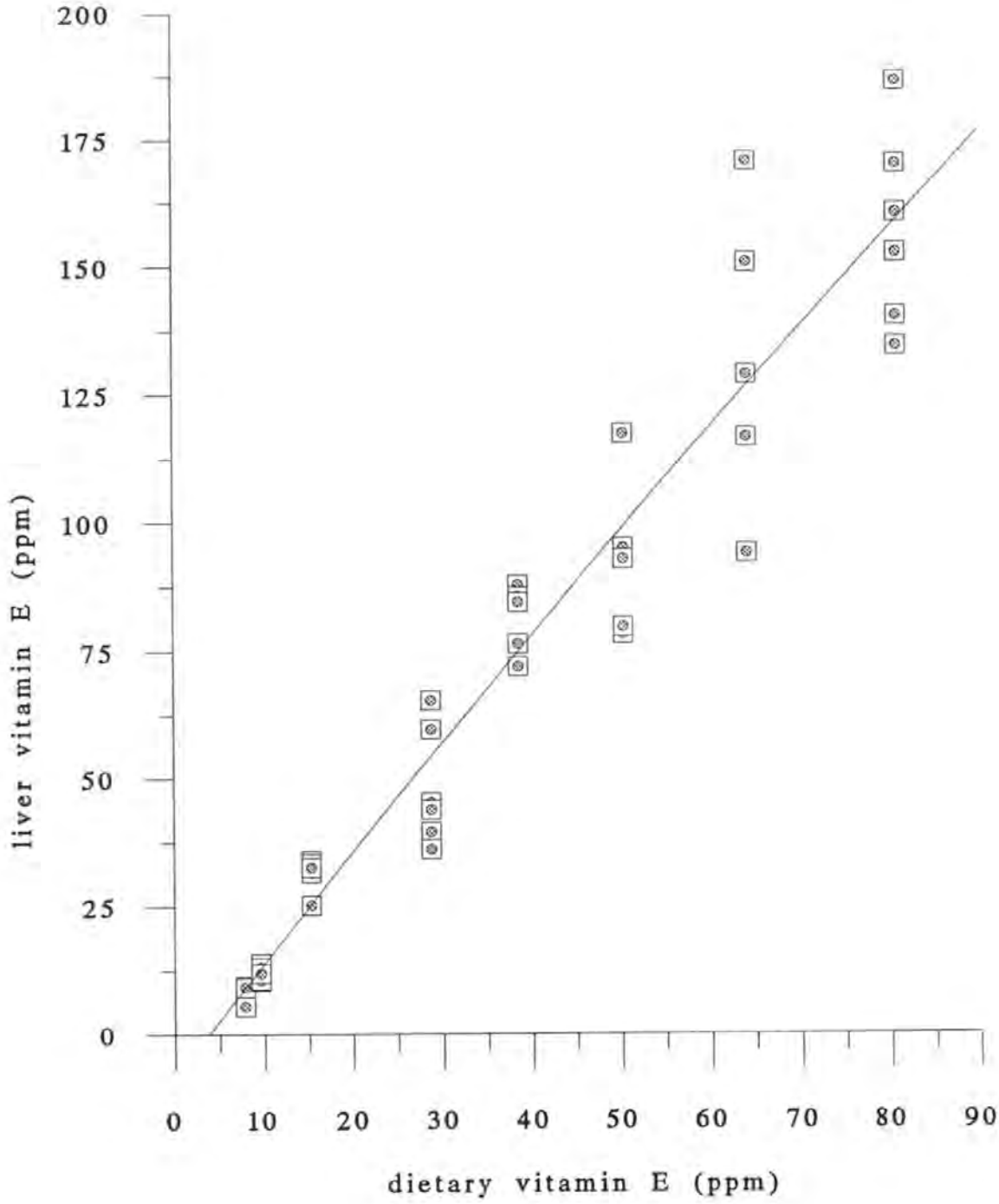


Figure 4.2 Assayed liver α-tocopherol ($\mu g\ g^{-1}$) from *Clarias* fed diets varying in α-tocopheryl acetate inclusion, for twelve weeks.

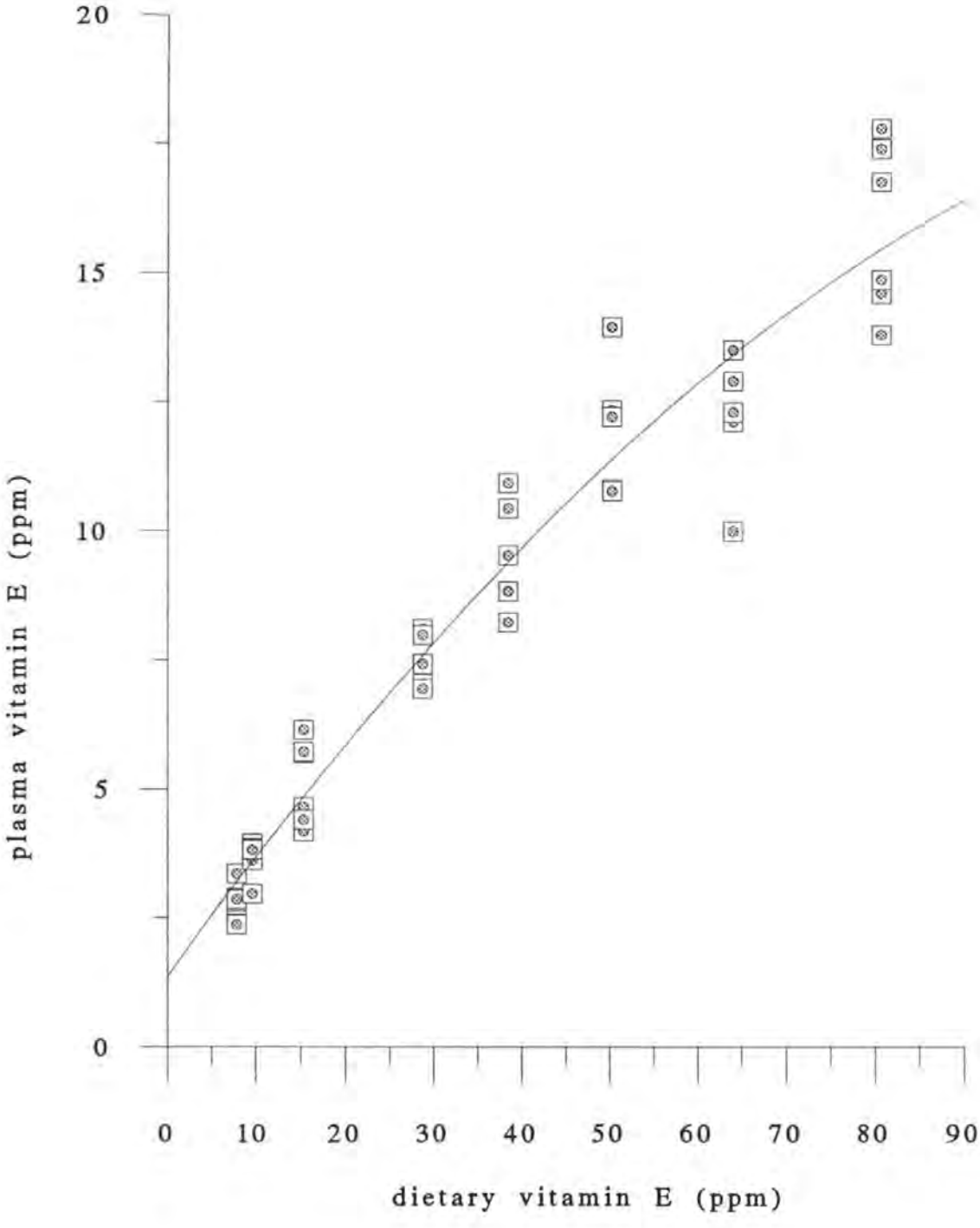


Figure 4.3 Assayed plasma α-tocopherol (μg ml^{-1}) from *Clarias* fed diets varying in α-tocopheryl acetate inclusion, for twelve weeks.

Table 4.9 TBARS in *Clarias* muscle, livers and blood-plasma after 84 days of feeding test diets. Values are means ± s.e. (n=5 or 6).

	0	5	15	30	45	60	75	100
	TBARS (nmoles MDA equivalents g^{-1}tissue or ml^{-1}plasma)							
Muscle	44.03^{b}	43.44^{b}	40.10^{b}	23.66^{a}	20.64^{a}	18.73^{a}	20.58^{a}	18.63^{a}
	±4.48	±8.97	±6.10	±2.93	±2.27	±3.38	±5.05	±3.53
Liver	47.76^{d}	38.15^{cd}	30.81^{bc}	21.68^{ab}	18.79^{a}	17.66^{a}	17.81^{a}	18.49^{a}
	±6.87	±6.87	±6.84	±2.34	±2.15	±2.07	±2.10	±1.93
Plasma	7.88	7.47	7.23	6.97	7.02	7.23	7.26	7.16
	±0.32	±0.13	±0.21	±0.35	±0.40	±0.26	±0.29	±0.61

^{abcd} Within rows, values sharing common superscripts, or possessing none, are not significantly different ($P>0.05$).

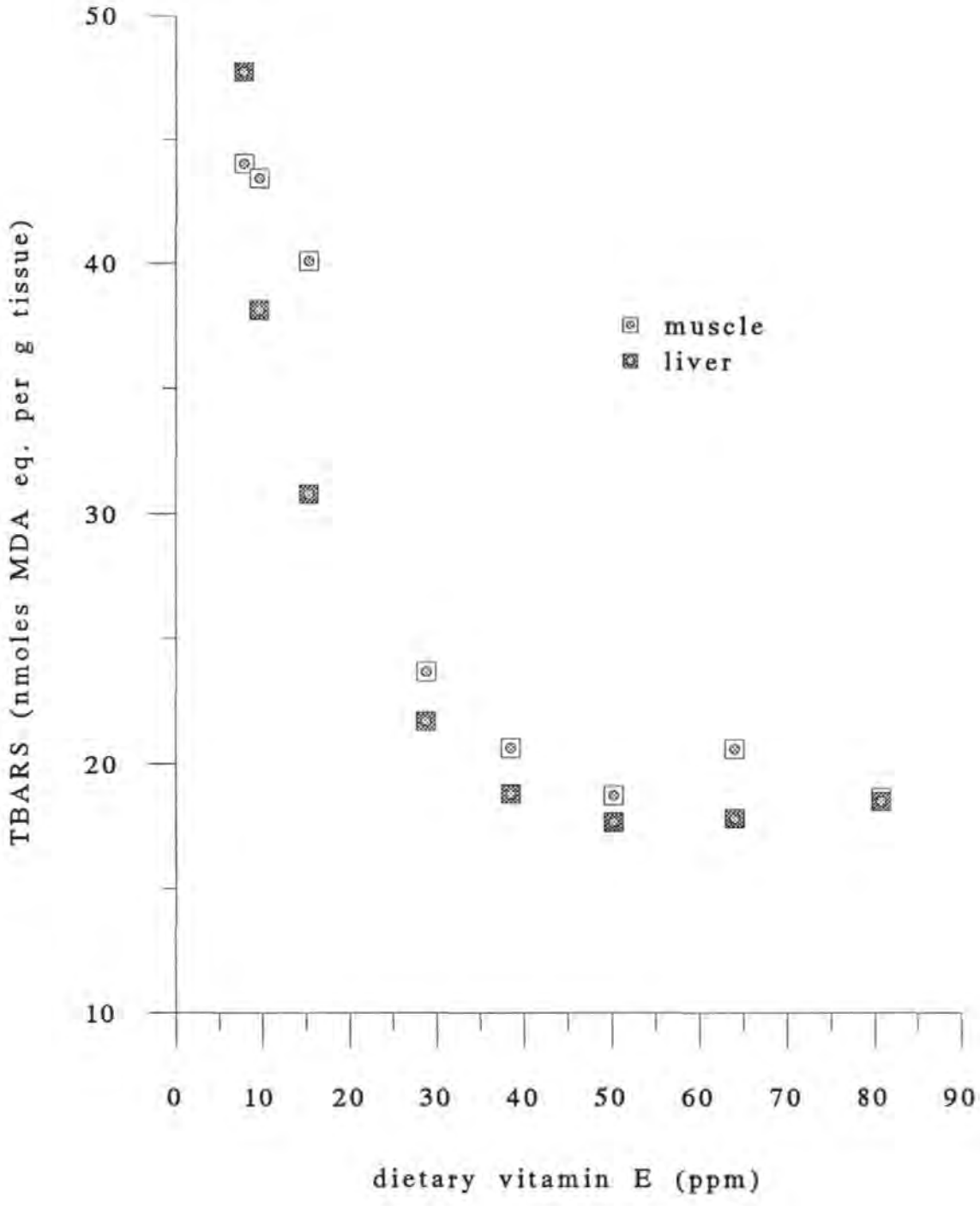


Figure 4.4 Response similarity between muscular and hepatic TBARS. Points represent means of six determinations, with variances omitted for clarity.

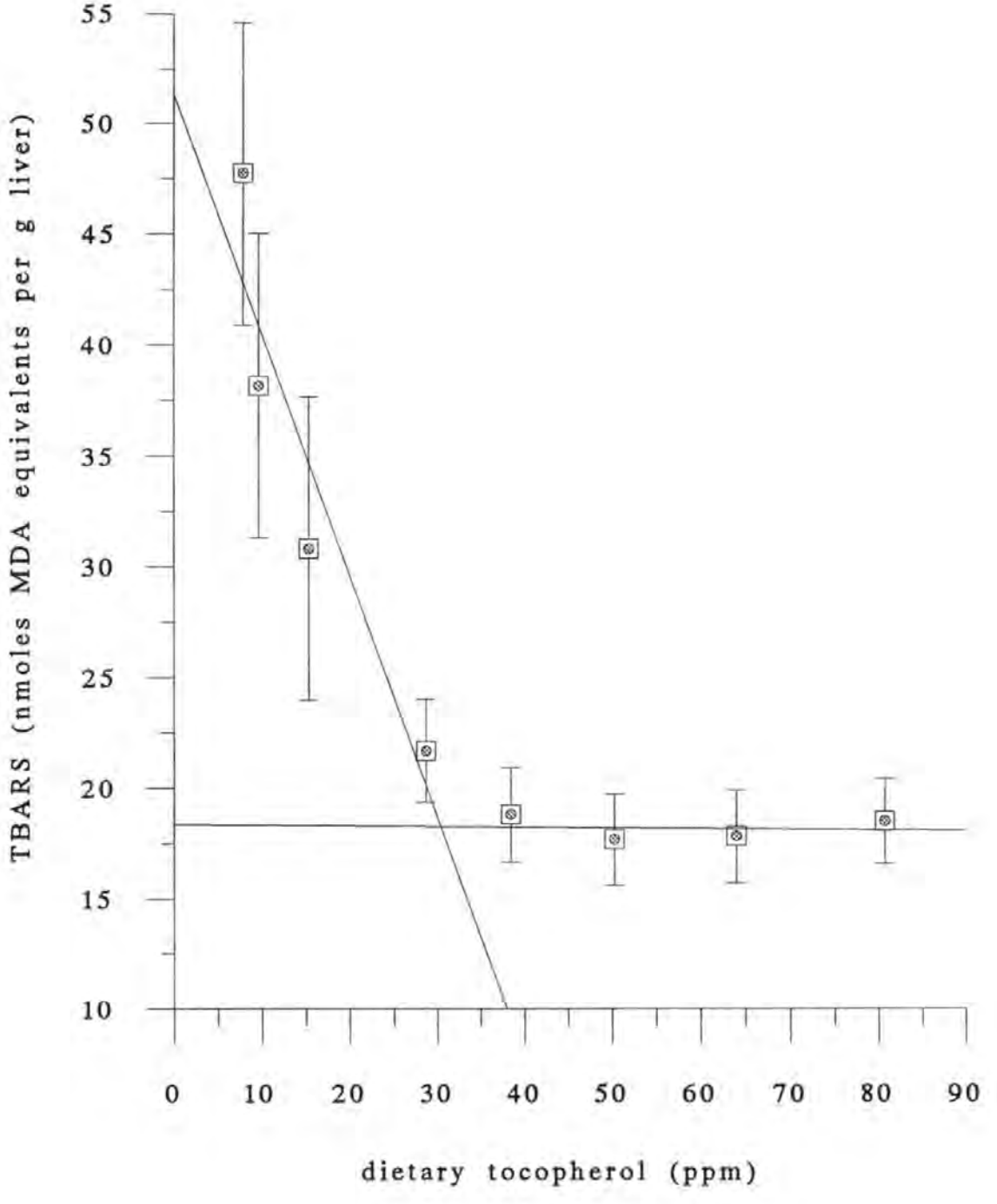


Figure 4.5 Hepatic TBARS concentration in *Clarias* fed diets varying in α-tocopheryl acetate inclusion for twelve weeks, and application of break-point analysis to determine the minimum dietary α-tocopherol concentration providing adequate *in vivo* suppression of TBARS generation. Points are means of six determinations ± s.e.

4.4 Discussion

Contrary to the findings of Roem *et al.* (1990) and Satoh *et al.* (1987) in tilapia (*Oreochromis aureus* and *O.niloticus* respectively) and those of Hamre and Lie (1995) in Atlantic salmon fry (*Salmo salar*), no significant differences in growth performance of *Clarias gariepinus* resulted from feeding varying amounts of all-rac-α-tocopheryl acetate in fresh diets. This supports work by Cowey *et al.* (1981) and Hung *et al.* (1981) on rainbow trout (*Oncorhynchus mykiss*), and work by Murai and Andrews (1974) on channel catfish (*Ictalurus punctatus*), and is also confirmatory of earlier findings of the author (chapter 3; Baker and Davies, 1996), although in that instance catfish were maintained on experimental diets for only eight weeks and starting weights were also higher in that investigation (approx. 60 g previous; approx. 10 g current), thereby furnishing the fish with a large hepatic tocopherol reservoir. In the current investigation, all fish were reared for twelve weeks during which fish increased in body-weight by a factor of 12, therefore one may have expected the tocopherol pool to have been turned over a number of times. This notion is supported to some extent upon comparison of hepatic α-tocopherol concentrations between the two studies. In the present experiment, liver concentrations of α-tocopherol are five times less than previously reported (chapter 3), yet no depression in any growth parameter, or appearance of pathology, were noted. In Atlantic salmon, Poston and co-workers (1976) noted pathologies when liver tocopherol levels were assayed at 45 μg per g, this value being attained in catfish fed 30 mg α-tocopheryl acetate per kg dry diet. Hamre and Lie (1995) declared a minimum requirement for growth in Atlantic salmon fry of 60 mg α-tocopheryl acetate per kg dry diet and incidentally, the hepatic tocopherol of these fish was found to be about 48 $\mu g\ g^{-1}$. Based on the above, it may be suggested that African catfish are fairly resilient to oxidative stresses, in that vitamin E may be omitted from a practical type diet without detriment to growth performance, and without appearance of pathology. Additionally this investigation has demonstrated that

haematological indices of health status were unaffected by dietary regime on the whole, despite blood-plasma α-tocopherol concentration having been demonstrated to be directly related to dietary dose of the vitamin. Although assessment of erythrocyte haemolysis is widely used in the evaluation of vitamin E status of vertebrates ranging from humans (Clark *et al.*, 1985) to rainbow trout (Cowey *et al.*, 1984), no trends were noted in data from the present work. Due to heightened membrane fragility (see Chapter 1), vitamin E deficiency should result in increased haemolysis of erythrocytes, either spontaneously or induced by an appropriate stressor (e.g. osmotic stress, detergent lysis (Pillai *et al.*, 1992)). For speed and ease of analysis, values presented within this chapter resulted from the determination of plasma haemoglobin since it may be assumed that increased spontaneous haemolysis would result in raised free haemoglobin within plasma. This method did not rely on erythrocyte 'wash steps', precise time-bases or multiple chemical dispensing and therefore, combined with the colorimetric stability of the reagent/haemoglobin chromophore, offered the advantage that all samples could be processed simultaneously. Despite these virtues, the assay could not discern any reasonable differences between treatments and therefore could not be used as the basis for establishing a minimum requirement of α-tocopherol in the catfish under test. Although it may be argued that this method would be dependent on changes in whole-blood erythrocytes (number or size), expressing the plasma haemoglobin proportional to whole-blood haemoglobin should modulate potential variability, though no trends were apparent employing this method either.

With respect to recorded haematocrit values of experimental catfish blood, it was evident that values increased (sometimes significantly ($P<0.05$)) either side of that obtained for the fish fed 15 mg α-tocopheryl acetate per kg dry diet. Regression of haematocrit data from fish fed diets supplemented with 15-100 mg α-tocopheryl acetate per kg dry diet clearly showed a decrease in haematocrit with lowered dietary vitamin E status (R-

sq=0.71). This is contrary to the findings presented in chapter 3, though agrees up to a point, with most other published works in fish nutrition and haematology. Results of Lovell *et al.* (1984) in channel catfish, and Cowey *et al.* (1984) and Moccia *et al.* (1984) in rainbow trout, show this phenomenon in cultured fish species, though inter-treatment differences were much more pronounced than the differences observed in this investigation. Additionally, the increased haematocrit noted in the catfish fed the lowest tocopherol diet conforms to previous findings in this species (chapter 3). A similar mechanism as was described in chapter 3 is again proposed.

As in chapter 3 (and Baker and Davies, 1996), tissue levels of α-tocopherol demonstrated that increasing dietary dose causes increased tissue deposition of vitamin E, and this may affect lipid peroxidation within these tissues.

From extrapolation of regression plots of tissue α-tocopherol concentrations relative to dietary dose, it may be possible to ascertain some of the mechanisms involved in vitamin E uptake and mobilization. The intercept of the regression line in figures 4.1 and 4.2 may indicate that at extremely low tocopherol doses, both muscle and liver tissues mobilize the vitamin into the plasma for transport into tissues more susceptible to peroxidation. This may be inferred since even at a dietary concentration of 4 μg α-tocopherol per kg diet, the tissue concentration is 'zero' with respect to the vitamin. Figure 4.3 however demonstrates that in plasma, even in a theoretical deficient diet, circulating α-tocopherol may be present, the pool being maintained by mobilization from tocopherol rich tissues as previously postulated. It must be noted that these hypotheses are speculative and require ellucidation through testing by feeding of purified test diets. Ideally, a feeding trial where fish are reared until the point of development of serious pathology is encountered should be undertaken, to ensure that the data set is extensive enough to cover tissue tocopherol levels below those obtained in the current investigation.

Both liver and muscle tissue responded similarly in terms of susceptibility of *in vivo* peroxidation (quantified as TBARS) in accordance to the results previously obtained in *Clarias* (chapter 3). Muscle and liver TBARS decreased concomitant with an increase in accretion of tissue α-tocopherol up to a point, and this reponse forms the basis for declaration of a minimum requirement of α-tocopherol in this species. Lipid peroxidation status (TBARS and exhaled hydrocarbons) has also recently been used successfully in determining vitamin E requirements of young pigs (Wang *et al.*, 1996). Discussion on this topic will follow shortly.

Plasma TBARS was found not to respond to dietary dose of vitamin E despite plasma α-tocopherol concentration increasing in line with vitamin intake, and therefore plasma-TBARS could not be used as an index of peroxidation status in the fish under study. Although this may be unexpected based on the response from other tissues, Duthie *et al.* (1989) also noted that plasma vitamin E is not always closely associated with actual lipid peroxidation values.

In agreement with Cowey and co-workers (1981) on work in rainbow trout, the only parameter enabling declaration of a minimum requirement of α-tocopherol in the catfish under study, is an index of lipid peroxidation. Although Cowey *et al.* (1981) relied upon the formation of malonaldehyde by hepatic sub-celluar fractions (microsomes and mitochondria), there are surprising similarities with results from the present investigation, which were based on hepatic TBARS. From the results obtained, Cowey and his team (1981) stated that the minimum requirement of rainbow trout for α-tocopherol is 20-30 mg α-tocopherol per kg diet. The current investigation demonstrates by broken-line analysis, that the minimum requirement of α-tocopherol for the prevention of *in vivo* lipid peroxidation in the African catfish is about 35 mg per kg and this agrees closely with estimated requirements for many salmonids and tilapia (NRC, 1993). It must be noted, that on comparison with data from chapter 3, despite muscle and liver *in vivo* TBARS in this

study reaching an apparent 'minimum peroxidation' plateau, it would be possible to stabilize tissue against lipid peroxidation by supplementation of higher concentrations of vitamin E into diets. Indeed, this forms the basis for improvement of flesh quality for fish destined for retail and also explains why organoleptic studies on fresh products fail to differentiate fillets from fish fed different tocopherol doses. It may be expected that similar studies on stored products would yield different results as off-flavours would appear later in fillets from fish fed a high tocopherol diet.

In conclusion, this investigation has established a minimum dietary level of α-tocopherol required for the suppression of lipid peroxidation in tissues from African catfish juveniles (*Clarias gariepinus*). Employing practical-type diets containing 12 % lipid (approx. 4.4 % PUFA >18:2), 2.34 mg selenium kg^{-1} diet and 500 mg ascorbic acid kg^{-1}, juvenile catfish required 30-40 mg α-tocopherol kg^{-1} dry diet in order to minimize peroxidation of tissue lipids *in vivo*, during a twelve week feeding period in which fish increased in body weight by twelve times.

Future research must examine effects of other nutrients on tissue status of vitamin E in order to establish the impact of nutritional interactions on this important antioxidant vitamin. On this theme, the following chapter will investigate the role played by oxidised dietary oils with respect to antioxidant status of African catfish.

CHAPTER 5

MODULATION OF THE NUTRITIONAL STRESS IMPOSED BY OXIDISED DIETARY OIL, BY THE SUPPLEMENTATION OF ALL-RAC-α-TOCOPHERYL ACETATE INTO DIETS FOR THE AFRICAN CATFISH.

5.1 Introduction

Oxidative modification of dietary lipid sources, whether by exposure to atmospheric oxygen or by chemical or thermal means, is known to have detrimental effects on organoleptic and nutritive value of feeds for humans and animals. Free radical mediated lipid peroxidation in dietary oils or tissue lipids, gives rise to hydroperoxides (Halliwell and Gutteridge, 1993) which are in turn susceptible to secondary oxidation or scission to produce further lipid radical species, or products such as aldehydes, ketones, acids and alcohols. The latter group of compounds are known to be central in the development of aroma and off-flavours in oils and meat products (Vercellotti *et al.*, 1992), and can be detected by various means in order to determine the extent of lipid peroxidation in either oils or living tissue.

It is known that products of lipid peroxidation can be digestively assimilated in the rat (Draper *et al.*, 1984) and that alterations in dietary fatty acids are usually reflected in tissues. Therefore, one can assume that an oxidised lipid incorporated into animal feed, will result in modifications of tissue fatty acid profiles and heightened oxidative stress in the organism receiving the diet. Indeed this has been observed in most studies involving rancid animal feeds.

Incorporation of antioxidants into feeds has been applied in many food-systems in order to minimize or negate oxidative damage of oils. Addition of synthetic antioxidants such as butylated hydroxy -toluene or -anisole, or ethoxyquin into diets prevents oxidation of lipids *in situ*. Similarly, natural antioxidants including the tocopherols (E vitamers),

ascorbate and retinol are capable of preventing the peroxidative damage of dietary oils. Alternatively, it is possible to afford protection to the organism by increasing the concentration of *in vivo* antioxidants. This offers scope for nutritional manipulation of feeds to ensure higher tissue antioxidant levels and thus confer a resistance against potential free radical peroxidative challenge.

Previous studies by Sheehy *et al.* (1993b, 1994), Hung *et al.* (1980, 1981), Stephan *et al.* (1993) and Sakaguchi and Hamaguchi (1969) on poultry, rainbow trout (*Oncorhynchus mykiss*), seabass (*Dicentrarchus labrax*) and yellowtail (*Seriola quinqueradiata*) respectively, have successfully shown the beneficial effect of vitamin E supplementation in feeds containing oxidised oils. It should be noted that in all of these studies, a stable α-tocopherol ester (α-tocopheryl acetate) was employed, meaning that little or no protection was provided to the dietary oil, since the ester functions as an antioxidant only upon acid hydrolysis (as encountered in the gut). The alcohol (α-tocopherol) is then able to quench free radical reactions *in vivo*.

Although it is clear that prevention of dietary oxidation would be a better strategy than protection of tissues upon exposure to the autoxidised lipid, there exists great consumer pressure directed to minimization or avoidance of the use of synthetic antioxidants in animal tissues destined for human consumption (Bailey and Um, 1992). Inclusion of natural, unprotected antioxidants in diets is not cost effective due to the relative instability of these compounds and therefore their short shelf-life. This latter factor is of considerable importance in tropical countries, where feeds may be stored under conditions likely to promote oxidation. Indeed, oxidised dietary components have already been implicated in the aetiology of jaundice in the tropical food fish *Clarias gariepinus* (African catfish) (Pearson, 1993).

Supplementation of α-tocopheryl acetate into animal diets would result in increased tissue resistance to oxidative stresses and this would manifest as prevention of pathologies related

to lipid-peroxidation and the improvement of tissue stability, even *post mortem*. Obviously this is beneficial when considering the quality of muscle-products. This is particularly poignant in fish culture, since fish tissues are relatively high in polyunsaturated fatty acids and these are particularly sensitive to free radical attack.

The present investigation aims therefore to study the effect of all-rac-α-tocopheryl acetate inclusion in fresh and oxidised diets for the African catfish (*Clarias gariepinus*), with respect to growth performance, selected health criteria, tissue levels of α-tocopherol and fatty acids, and muscle product-quality (stability against peroxidation, and membrane integrity).

5.2 Materials and Methods

5.2.1 Chemicals

All chemicals used were of ANALAR grade or better, and purchased from Sigma Chemical Company Limited, Poole, Dorset, U.K., and Merck Limited, Poole, Dorset, U.K.. All-rac-α-tocopheryl acetate (Rovimix E-50 SD) was a generous gift from Roche Products, Heanor, Derbyshire, U.K.

5.2.2 Fish stock, Experimental facilities and Diets

African catfish (*Clarias gariepinus*) of mixed sexes were spawned in-house according to the procedure described in section 2.9, and grown to 15 g. These juveniles, of mixed sexes, were stocked into 80 litre tanks of a freshwater closed recirculation system. System water temperatures were maintained at 27^{0}C, the photoperiod held at 12 hours light:12 hours dark, and water quality parameters measured and adjusted as appropriate.

A low-tocopherol, basal practical diet (table 5.1) was formulated as described in section 2.1.1, but modified as follows:

Cod-liver/ corn oil (1:1) was included at 6 % of the formulation in order to increase the energy density of the feed, to maximise growth potential. So as to create rancid test diets, a portion of blended cod-liver-/ corn- oil (1:1) was oxidised by aeration for 30 days at room temperature. Oils were assayed for oxidative state prior to incorporation into the diet, yielding anisidine values (section 2.5.4)) of 26.99 $\pm$ 0.06 (mean $\pm$ s.e.) for the fresh oil and 133.09 $\pm$ 6.07 for the air-oxidised oil. Determination of oil malondialdehyde by HPLC was carried out upon modification of the procedure for tissues (section 2.5.3). An aliquot of an oil solution (in 2:1:0.001 chloroform/methanol/BHT) was assayed in the same way as the homogenate in the tissue procedure. Values for fresh and oxidised oils were 24.327 $\pm$ 0.238 and 496.887 $\pm$ 5.099 nmoles MDA g^{-1} oil respectively.

Levels of dietary vitamin E (supplied as all-rac-α-tocopheryl acetate) were set in relation to levels reported by other workers in a variety of fish species. Inclusion levels of Rovimix E-50 SD (donated by Roche Products Ltd., Heanor, Derbyshire, U.K.) were selected to provide either 20 or 100 mg α-tocopheryl acetate per kg of air-dried feed, for both the fresh and oxidized diets. Diets were allocated codes reflecting their oxidative state and vitamin E content. Thus, Fr-20 and Fr-100 represent the fresh-oil diets with 20 and 100 mg α-tocopheryl acetate kg^{-1} dry diet, and Ox-20 and Ox-100 represent the rancid equivalents of those diets. Manufacture of the diets proceeded as detailed in section 2.1.2.

5.2.3 Dietary analyses

Proximate analysis (AOAC, 1990; described in section 2.3) of the completed test diets revealed that diets contained 47.07 % ± 0.24 crude protein, 11.64 % ± 0.06 lipid and 16.04 % ± 0.12 ash, on a dry matter basis, and 5.96 % ± 0.14 moisture. These data are presented in table 5.2.

HPLC determination of α-tocopherol content of the pelleted feeds (according to the method in section 2.4.1) revealed that the actual values of α-tocopherol were 22.25 ± 0.90 mg α-tocopherol kg^{-1} for both low vitamin E diets and 112.12 ± 4.44 mg α-tocopherol kg^{-1} for the high vitamin E diets.

GC-MS determination of dietary fatty acid profiles was carried out as described in section 2.4.3 and values reported in table 5.3.

5.2.4 Feeding regime and Nutrition trial protocol

The fish were uniformly graded and assigned, twenty per tank, into the eight tanks (four treatments duplicated) of the experimental system.

Clarias were fed three times daily for 56 days, at a ration level of up to 3 % body weight per day (calculated on a dry matter basis). Rations dispensed were recalculated weekly to

allow for body-weight changes.

On completion of the feeding period, individual fish weights were recorded. Six fish per treatment (3 per tank) were desanguinated by caudal venipuncture (section 2.6.1), haematocrits determined and the bloods centrifuged to enable plasma storage at -80^{0}C for future analysis. Tissues of these fish were excised and subsamples frozen at -80^{0}C prior to analysis. These catfish were selected on the basis of their weight, individuals whose body weights were nearest to the tank mean being considered representative of the treatment.

Twelve fish from treatments Fr-20 and Fr-100 were reallocated to tanks for use in a depletion study. Thus, both sets of catfish were fed oxidised-oil/low-tocopherol diets (Ox-20) at 3 % body weight for 4 weeks. From previous work (Chapter 3) it was concluded that hepatic tissue was a sensitive indicator of vitamin E status. As such, liver tissues from six fish per treatment were sampled for analysis at two weekly intervals.

5.2.5 Growth and nutrient utilization parameters

The following parameters were calculated, according to the formulae in section 2.2.5, from data acquired from the feeding trial; percent change in mean body weight, specific growth rate and feed efficiency. In addition, protein efficiency ratio was calculated after proximate analysis of test diets.

5.2.6 Histopathological and Haematological techniques

Additional to the final body weights on termination of the trial, the liver weights of six fish per treatment were recorded in order to allow calculation of the hepatosomatic index (% contribution of liver weight to the total body weight).

Plasma samples (stored at -80^{0}C) were subjected to the cyanmethemoglobin method for analysis of haemoglobin (possibly indicative of spontaneous haemolysis)(section 2.6.4), and

determination of plasma α-tocopherol (section 2.4.1).

5.2.7 Analytical methods

α-tocopherol was extracted from muscle, liver, heart, spleen and blood-plasma samples, and quantified using the method outlined in section 2.4.1. In this way, six samples from each treatment (three fish per tank) were analysed in duplicate (n=6).

Thiobarbituric acid reactive substances (TBARS) were quantified spectrophotometrically by determination of equivalents of the lipid peroxidation product, malondialdehyde (MDA) after exposure to varying degrees of oxidative attack (duration of incubation with iron/ascorbate solution).

Of the samples described in section 5.2.4, samples of muscle tissue from each treatment were analysed in duplicate in accordance with the method in section 2.5. Incubation times were set at 0, 50, 100 and 200 minutes and colour intensity of the MDA-TBA adduct measured spectrophotometrically at 535 nm.

Duplicate extractions of fatty acids from muscle and liver tissues from six fish per treatment (see section 5.2.4), proceeded as in section 2.4.3.1..

Two extractions of each diet were carried out in a different manner (section 2.4.3.2).

Methyl-esterification of the fatty acids was carried out as directed in section 2.4.3.3, and these separated chromatographically and identified as previously described (section 2.4.3.4).

5.2.8 Exudative moisture loss

Gravimetric evaluation of the moisture lost from a fillet upon freezing and subsequent thawing proceeded as detailed in section 2.7. Six fish from each treatment (3 per tank)

were filleted by hand and one fillet per fish used as directed.

5.2.9 Statistical analysis

After variance checks and log(10) or arcsine data transformations where appropriate, groups were compared using either two-way ANOVA (all factorial designed procedures) or one-way ANOVA for the depletion study (two treatments only). Duncan's multiple range test (Steel and Torrie, 1960) was applied to data in order to allocate superscripts. Differences between treatments were considered significant when $P<0.05$. Linear regression ($P<0.05$) was used in the determination of relationship between liver α-tocopherol and hepatosomatic index. Analyses were performed using the statistical software package 'Statgraphics' (Manugistics Incorporated, Rockville, MD, U.S.A.).

Table 5.1 Composition of practical test diets for the African catfish, *Clarias gariepinus*.

Ingredient	%inclusion
Fishmeal (Chilean)	60.00
Meat and bone meai	10.00
Bloodmeal	2.00
Cod-liver oil	3.00
Corn oil	3.00
Corn starch:Dextrin (3:2)	15.35
Molasses	1.00
Mineral premix[1]	5.00
B-complex vitamin premix[2]	0.10
Fat-soluble vitamin premix[3]	0.05
Macro-vitamin premix[4]	0.50

[1] Mineral salt inclusion (g kg^{-1}dry diet):
Calcium orthophosphate ($CaHPO_4.2H_2O$) 12.000, Magnesium sulphate ($MgSO_4.7H_2O$) 4.8450, Sodium chloride (NaCl) 2.2800, Potassium chloride (KCl) 1.9000, Iron sulphate ($FeSO_4.7H_2O$) 0.9500, Zinc sulphate ($ZnSO_4.7H_2O$) 0.2090, Manganese sulphate ($MnSO_4.4H_2O$) 0.0960, Copper sulphate ($CuSO_4.5H_2O$) 0.0298,Cobalt sulphate ($CoSO_4.7H_2O$) 0.0181, Calcium iodate ($CaIO_3.6H_2O$) 0.0112, Chromic chloride ($CrCl_3.6H_2O$) 0.0048, Sodium selenite (Na_2SeO_3) 0.0025, Filler (α-cellulose) 27.6536.

[2] B-vitamin inclusion (mg kg^{-1} dry diet):
B1-Thiamine hydrochloride 50, B2-Riboflavin (feed grade-96%) 52.1, B6-Pyridoxine hydrochloride 40, Calcium pantothenate 100, Niacin 200, H2-Biotin (2%) 300, Folic acid (90%) 16.7. Note B12 added separately at 0.05 mg kg^{-1} dry diet.Filler (α-cellulose) 241.2.

[3] Fat-soluble vitamin inclusion (mg kg^{-1} dry diet):
A-Vitamin A palmitate (1.7×10^6 I.U. g^{-1}) 2, D-Rovimix D3-500 (5×10^5 I.U. g^{-1}) 20, K-Menadione sodium bisulphite (51%) 80.Filler (α-cellulose) 398.

[4] Macro-vitamin inclusion (mg kg^{-1} dry diet):
Inositol 200, Choline chloride 2000, Ascorbic acid 500, Filler (α-cellulose) 2300.

Table 5.2 Proximate composition of basal practical test diet (bracketed values depict theoretical composition as calculated from NRC (1993))

	Moisture (%)	Protein (% DM)	Lipid (% DM)	Ash (% DM)	Residual
Mean	5.96	47.01 [49.35]	11.64 [11.87]	16.04 [14.14]	19.35
S.E.	0.139	0.238	0.063	0.118	
n	12	8	12	12	

n= number of determinations from a single diet.

Table 5.3 Dietary total lipid fatty acid composition. Values are expressed as % contribution of each fatty acid to the total chromatogram area (n=2 extractions).

fatty acids	Fr-20	Fr-100	Ox-20	Ox-100
14:0	4.35	4.46	4.65	4.77
16:0	15.57	15.31	16.69	16.89
16:1 n7	5.57	5.71	5.82	6.03
18:0	9.37	8.54	9.75	9.22
18:1 n9	14.14	13.99	15.86	14.95
18:1 n7	2.35	2.36	2.66	2.59
18:2 n6	13.78	13.41	14.33	14.63
18:3 n3	0.82	0.82	0.67	0.74
18:4 n3	1.56	1.61	1.26	1.40
20:0	0.34	0.33	0.38	0.39
20:1 n9+11	1.42	1.43	1.60	1.59
20:1 n7	0.20	0.22	0.23	0.23
20:2 n6	0.23	0.24	0.23	0.24
20:3 n6	0.12	0.13	0.13	0.13
20:4 n6	0.60	0.63	0.49	0.54
20:3 n3	0.17	0.15	0.14	0.12
20:4 n3	0.49	0.51	0.37	0.43
20:5 n3	9.86	10.30	7.54	8.55
22:0	0.19	0.22	0.17	0.18
22:1 n11+13	0.76	0.78	1.01	0.97
22:1 n9	0.22	0.23	0.26	0.26
22:4 n6	0.17	0.17	0.12	0.14
22:5 n3	1.50	1.57	1.11	1.28
22:6 n3	6.61	6.87	4.11	4.85
others	9.56	10.00	10.43	8.85
ΣSFA †	29.8	28.9	31.6	31.5
ΣMUFA ‡	24.7	24.7	27.4	26.6
ΣPUFA §	36.5	37.0	30.9	33.6
n3:n6	1.5	1.5	1.0	1.1

† ΣSFA = total saturates
‡ ΣMUFA = total mono-unsaturates
§ ΣPUFA = total polyunsaturates

5.3 Results

From figure 5.1 it can be seen that measured statistical differences in body weight were detected after only 4 weeks of feeding. Weekly thereafter, treatment Ox-20 was observed to perform least well with respect to growth. Diet Ox-100 produced a tailing-off in growth performance from week 5. Fresh-oil fed fish grew equally well irrespective of tocopherol dose, although at the end of week 8 the rate of weight gain of Fr-20 seemed to be declining. Table 5.4 reveals that significant differences ($P<0.001$) existed between final weights of fish on various dietary treatments. Superscript allocation following ANOVA demonstrates that *Clarias* fed fresh oil diets (Fr-20 and Fr-100) did not differ significantly ($P=0.1765$) in mean final weight, irrespective of dietary tocopherol inclusion. For fish fed the oxidised-oil diets (Ox-20 and Ox-100) mean weights were lower than the fresh-oil equivalents. In addition, increased dietary α-tocopheryl acetate in the oxidised-oil diets resulted in an appreciably larger final mean body weight ($P=0.0008$). No interaction between the nutrients was detected by 2-way ANOVA ($P=0.1815$). Despite being unable to test other growth related parameters statistically, due to values being obtained from mean treatment data, further general trends emerged. Values for % change in mean body weight, specific growth rate (SGR), feed efficiency (FE) and protein efficiency ratio (PER) followed the same trend as already noted for mean final body weights, with fish fed Ox-20 performing least well, Ox-100 performing better and both fresh-oil treatments resulting in the greatest performance.

Hepatosomatic indices (HSI) (table 5.5) of fish fed low levels of α-tocopheryl acetate, irrespective of dietary oil quality, were significantly larger ($P=0.0027$) than fish receiving the higher tocopherol dose. Oil quality was seen to have no effect ($P=0.8188$) on liver weights relative to body weight.

Table 5.6 presents mean results for haematological data obtained at the end of the 56 day feeding trial. Haematocrits (%) were lower in fish fed fresh oils although this was only statistically significant ($P=0.0001$) for Fr-20 as compared to all other treatments. Haematocrits from fish fed Fr-100 were not statistically different to values for fish fed oxidised-oil diets ($P=0.1765$), yet analysis of main effects showed oil state to be the main factor governing recorded values ($P=0.0015$). Plasma haemoglobin values provided no significant trends ($P=0.1512$).

With respect to tissue levels of α-tocopherol (table 5.7), it was obvious that dietary regime influenced tissue-tocopherol status markedly ($P<0.001$). In all cases lowest assayed levels of α-tocopherol were associated with the feeding of oxidised-oil diets, low in α-tocopheryl acetate. Tocopherol levels for tissues from fish fed the various dietary treatments increased in the order Ox-20, Fr-20, Ox-100, Fr-100. On comparing the different tissues assayed, it was apparent that the liver contained the highest concentration of α-tocopherol. Two-way ANOVA revealed that in all tissues both oil state and dietary vitamin E dose affected observed tissue tocopherol levels, and that an interaction between these nutrients occurred in muscle, liver and spleen ($P<0.01$).

From table 5.8 it is evident that switching from fresh- to oxidised- oil diets resulted in significantly ($P<0.05$) lower hepatic tocopherol values after 4 weeks of feeding. Livers from fish formerly fed Fr-100 and Fr-20 responded significantly to changed diet within two weeks. After maintaining catfish on experimental diets for four weeks only the liver α-tocopherol concentration from fish fed Fr-100 dropped significantly from week 2 values.

Determination of muscle TBARS (table 5.9 and figure 5.2) revealed that for fresh-oil fed fish, time=0 values were significantly lower ($P=0.0131$) for fish fed the highest α-tocopheryl acetate level, though the significance was lost later on in the incubation. No

significant differences were seen at any time ($P=0.1723$) between oxidised-oil fed fish (Ox-20 and Ox-100). Differences were present between fresh- and oxidised- oil treatments later in the incubation time-base, with MDA equivalents being greater in the muscle from fresh oil fed fish. It was evident from 2-way ANOVA that oil state was the main factor influencing recorded TBARS values. Vitamin E was only significantly influential in the first 50 minutes of peroxidation incubation, although a tocopherol-oil interaction was evident throughout the analysis ($P<0.05$).

Tables 5.10 and 5.11 portray the fatty acid profiles of muscle and liver tissue respectively. The muscle tissue fatty acid profile (table 5.10) from *Clarias* fed diet fresh-oil diets did not differ to any extent. The profile obtained for Ox-20 was different to all other treatments. Relative concentrations of hexadecaenoic acid (16:0, palmitic), octadecaenoic acid (18:0, stearic), octadecaenoic acid (18:1 n-9 (oleic) and n-7), eicosaenoic acid (20:1 n-11 and n-9), eicosadienoic acid (20:2 n-6), eicosatrienoic acid (20:3 n-6), eicosatetraenoic acid (20:4 n-6), docosaenoic (22:0) and docosaenoic acid (22:1 n-11+13) all were increased in this treatment as compared to fresh-oil fed treatments. The increases described above were accompanied by relative decreases in octadecadienoic acid (18:2 n-6), octadecatrienoic acid (18:3 n-3), octadecatetraenoic acid (18:4 n-3), eicosatetraenoic acid (20:4 n-3), eicosapentaenoic acid (20:5 n-3, EPA) docosapentaenoic acid (22:5 n-3) and docosahexaenoic acid (22:6 n-3, DHA). Values from treatment Ox-100 were closer to those from the 'Fr-' treatments, though many values lay between those for fresh-oil fed fish and treatment Ox-20. Non-identified integrated peaks accounted for 19.29% of the total area in treatment Ox-100, but between 14.87 and 16.15 % of other chromatograms. The fatty acid profile of *Clarias* livers after feeding the test diets is presented in table 5.11.

Fish fed oxidised oil/low tocopherol diets possessed relatively higher hepatic concentrations

of eicosatetraenoic acid (20:4 n-6), docosatetraenoic acid (22:4 n-6) and DHA (22:6 n-3), but lower concentrations of all eighteen carbon fatty acids (except 18:1 n-7 and n-9), mono-unsaturated eicosaenoates (20:1) (except 20:1 n-9), eicosatetraenoic acid (20:4 n-3), docosaenoic acid (22:1 n11+13) and docosapentaenoic acid (22:5 n-3), when compared to treatments Fr-20 and Fr-100. Levels of DHA were significantly correlated to dietary regime ($P=0.0004$) with marked significant differences ($P<0.001$) existing between all treatments. Liver concentration of eicosatrienoic acid (20:3 n-6) was high in treatment Ox-20 relative to other treatments. Feeding oxidised oils also resulted in the disappearance of eicosanoic acid (20:0), octadecatetraenoic acid (18:4 n-3), eicosaenoic acid (20:1 n-7) and the saturated and mono-unsaturated docosaenoates. Comparisons between Ox-20 and Ox-100 reveal that relative abundances of octadecanoic acid (18:0) and eicosatetraenoic acid (20:4 n-3) are lower in Ox-20, but eicosatrienoic acid (20:3 n-6 and n-3) and eicosatetraenoic acid (20:4 n-6) are higher in this treatment. Slight differences were noted between hepatic abundances of fatty acids in fish fed fresh-oil diets. Fr-20 livers contained proportionally less octadecaenoic acid (18:0), but higher eicosatrienoic acid (20:3 n-3) and docosaenoic acid (22:1 n-11+13). DHA was significantly less abundant ($P<0.01$) in fish fed the higher tocopherol dose.

Again, application of two-way ANOVA revealed the extent to which dietary factors influenced relative abundance of individual fatty acids. Where statistical differences were observed between treatments, oil quality was most frequently recorded to be the main affecting factor. Vitamin E alone was only demonstrated to be the main factor in hepatic octadecaenoic and eicosatrienoic acid abundances. Dietary vitamin E exerted effects on many other fatty acid abundances, although these mainly occurred concurrently with oxidised oil effects. Nutrient interactions were also evident.

Non-identified integrated peaks accounted for 13.90% of the total area from chromatograms of treatment Ox-20. Other treatments were observed to yield between 7.01

and 9.51% of un-identified chromatogram area.

Determination of the % area of an unknown peak (table 5.12) revealed a significant treatment dependence ($P < 0.01$) linked to tissue oxidative stress. Values obtained for the compound increased in the order Fr-100, Fr-20, Ox-100, Ox-20. An equivalent chain length (ECL) of 13.1 relative to saturated fatty acid methyl esters was calculated.

Values obtained for the post-thaw exudative moisture loss from *Clarias* fillets under two different temperature regimes are presented in table 5.13.

After a thawing period of 48 hours at 6^0C, it was shown that fish from treatments fed fresh-oil diets (Fr-20 and Fr-100) had lost less moisture than fish on diets Ox-20 and Ox-100, though this was only statistically significant ($P < 0.05$) when comparing Fr-100 with oxidised-oil treatments. Exudative moisture loss was significantly less ($P = 0.0002$) in Ox-100 than in Ox-20. At this time, both oxidised oil and dietary vitamin E exerted an influence on drip loss as demonstrated by 2-way ANOVA ($P < 0.05$).

The above trends were not apparent after 96 hours of thawing. Statistical differences ($P < 0.05$) existed between Ox-20 and the two higher-tocopherol diets (Fr-100 and Ox-100). Ox-20 had lost the most moisture, although Fr-20 was not significantly different ($P > 0.05$). Exudative moisture loss from muscle was least in treatments Fr-100 and Ox-100. After 96 hours thawing, dietary tocopherol was seen to have the main effect on drip loss ($P < 0.005$).

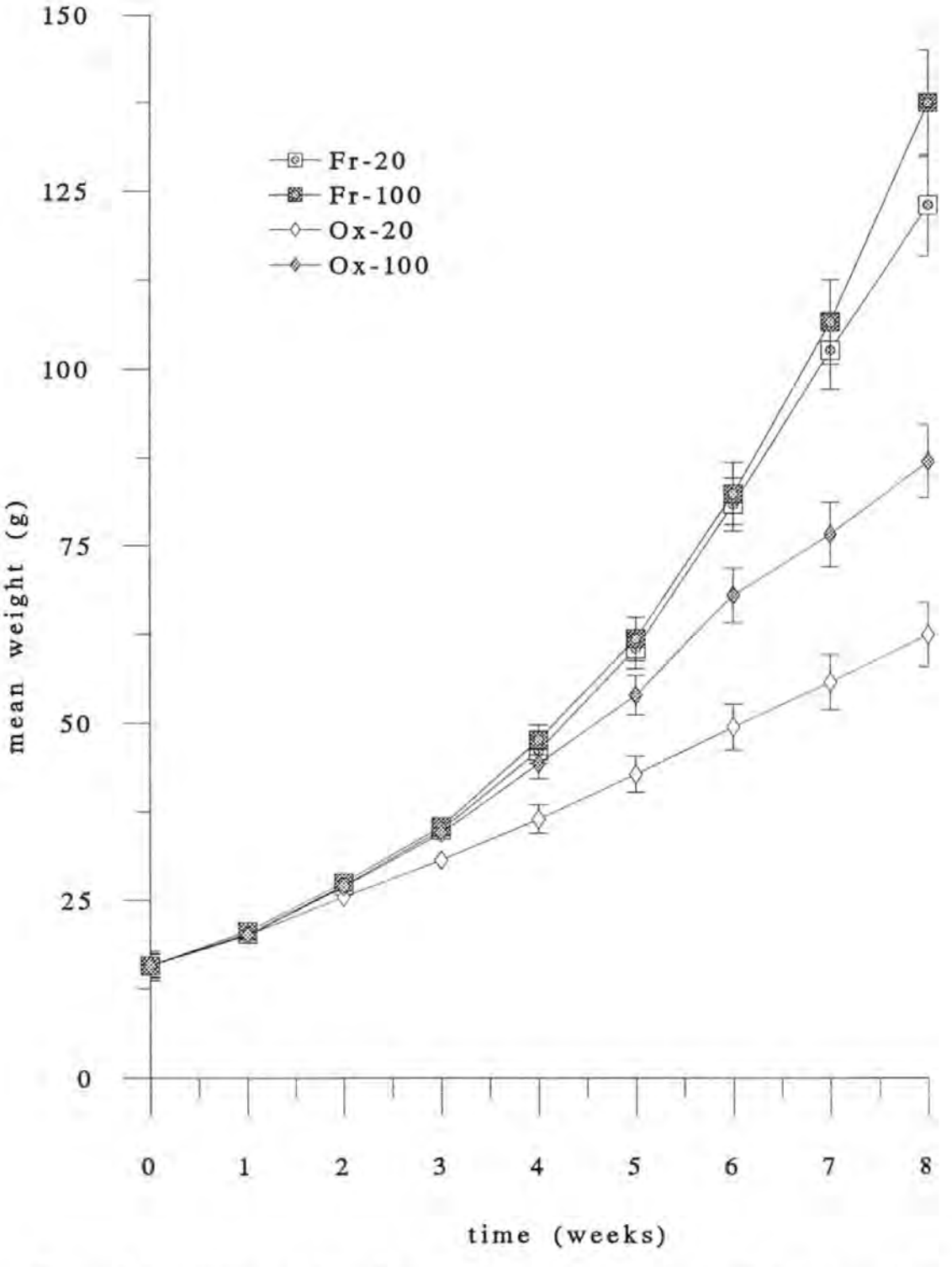


Figure 5.1 Mean weights of *Clarias* fed fresh- or oxidised- oil diets at two levels of α-tocopheryl acetate inclusion. Values are presented as means ± s.e.

Table 5.4 Growth related performance and nutrient utilization of catfish fed experimental diets after 56 days.

Diet Code †	Fr-20	Fr-100	Ox-20	Ox-100	Main Effect ¶
Mean initial weight (g)	15.77 ±0.25	15.75 ±0.27	15.75 ±0.29	15.75 ±0.35	
Mean final weight (g)	122.87[a] ±7.09	137.32[a] ±7.67	62.42[c] ±4.54	86.89[b] ±5.20	O, E
% change in mean weight	679.71	771.80	296.28	451.28	
Specific Growth Rate (% d^{-1})	3.73	3.94	2.50	3.10	
Feed Efficiency	155.49	152.64	113.80	135.66	
Protein Efficiency Ratio	3.35	3.22	2.42	2.87	

† Diet code:
Fr= Fresh dietary oil
Ox= Oxidised dietary oil
20 or 100 reflect α-tocopheryl acetate inclusion in diets (mg kg^{-1} dry diet)

[abc] Values carrying common superscripts are not significantly different ($P>0.05$).

¶ Determination of main effects by 2-way ANOVA ($P<0.05$). O=oil state; E=vitamin E; OxE=interaction.

Table 5.5 Hepatosomatic indices (%) of *Clarias* after 56 days of feeding test diets.

Diet code †		Fr-20	Fr-100	Ox-20	Ox-100	Main effects ¶
	Mean	1.01^{a}	0.82^{b}	1.02^{a}	0.81^{b}	E
	S.E.	0.035	0.019	0.087	0.072	
	n	6	6	6	6	

† Diet code:
Fr= Fresh dietary oil
Ox= Oxidised dietary oil
20 or 100 reflect α-tocopheryl acetate inclusion in diets (mg kg^{-1} dry diet)

[ab] Values carrying common superscripts are not significantly different ($P>0.05$).

¶ Determination of main effects by 2-way ANOVA ($P<0.05$). O=oil state; E=vitamin E; OxE=interaction.

Table 5.6 Haematocrit (% PCV) and plasma haemoglobin (Hb) (mg cm^{-3}) of *Clarias* after 56 days of feeding test diets. Values are means of six determinations per treatment ± standard errors.

Diet Code †	Fr-20	Fr-100	Ox-20	Ox-100	Main Effect ¶
Haematocrit (%)	28.69^{a}	30.61^{b}	32.26^{b}	32.16^{b}	O
	±0.64	±0.40	±0.85	±0.79	
Plasma Hb (mg cm^{-3})	0.339	0.232	0.417	0.205	
	±0.06	±0.03	±0.01	±0.03	

† Diet code:
Fr= Fresh dietary oil
Ox= Oxidised dietary oil
20 or 100 reflect α-tocopheryl acetate inclusion in diets (mg kg^{-1} dry diet)

[ab] Values carrying common superscripts are not significantly different ($P > 0.05$).

¶ Determination of main effects by 2-way ANOVA ($P < 0.05$). O=oil state; E=vitamin E; OxE=interaction.

Table 5.7 Assayed α-tocopherol in *Clarias* muscle, livers, heart, spleen and blood-plasma after 56 days of feeding test diets.

Diet code †		Fr-20	Fr-100	Ox-20	Ox-100	
		α-tocopherol content (μg g^{-1}tissue or ml^{-1}plasma)				Main effect ¶
Muscle	mean	1.45^{a}	8.25^{c}	1.09^{a}	3.53^{b}	O, E, OxE
	s.e.	0.12	0.76	0.10	0.45	
	n	6	6	5	6	
Liver	mean	18.03^{b}	271.68^{d}	2.34^{a}	25.44^{c}	O, E, OxE
	s.e.	1.03	20.01	0.61	2.59	
	n	5	5	5	6	
Plasma	mean	1.82^{b}	8.53^{d}	0.68^{a}	4.51^{c}	O, E
	s.e.	0.23	0.76	0.08	0.49	
	n	6	6	6	6	
Heart	mean	2.56^{b}	13.34^{d}	0.98^{a}	5.32^{c}	O, E
	s.e.	0.26	1.46	0.12	0.72	
	n	6	6	6	6	
Spleen	mean	5.50^{b}	28.63^{d}	2.36^{a}	11.66^{c}	O, E, OxE
	s.e.	0.32	1.24	0.23	0.54	
	n	6	6	6	6	

† Diet code:
Fr= Fresh dietary oil
Ox= Oxidised dietary oil
20 or 100 reflect α-tocopheryl acetate inclusion in diets (mg kg^{-1} dry diet)

n= number of duplicate extractions performed per treatment.

^{abcd} Within rows, values sharing common superscripts are not significantly different ($P>0.05$).

¶ Determination of main effects by 2-way ANOVA ($P<0.05$). O=oil state; E=vitamin E; OxE=interaction.

Table 5.8 Liver α-tocopherol concentration of fish previously fed fresh-oil diets at two levels of α-tocopheryl acetate inclusion on feeding an oxidised-low-tocopherol (Ox-20) diet.

		μg α-tocopherol g^{-1} liver at time		
Previously fed		0 weeks	2 weeks	4 weeks
Fr-20•	Mean	18.03^{c}	7.96^{d}	7.17^{d}
	S.E.	1.03	0.79	0.36
	n	5	6	6
Fr-100†	Mean	271.68^{a}	41.75^{b}	17.92^{c}
	S.E.	20.01	5.05	2.05
	n	5	6	6

• Fr-20 Fresh oil diet / 20 mg α-tocopheryl acetate kg^{-1} dry diet
† Fr-100 Fresh oil diet / 100 mg α-tocopheryl acetate kg^{-1} dry diet

^{abcd} Within rows, values sharing common superscripts are not significantly different ($P > 0.05$).

Table 5.9 TBARS (nMol MDA equivalents mg^{-1} tissue)(n=6 determinations per treatment) in *Clarias* muscle after iron/ ascorbate induced lipid peroxidation.

Diet code †	time (mins)	TBARS (nMol MDA mg^{-1} tissue)	S.E.
Fr-20	0	0.468^{a}	0.065
	50	1.136^{a}	0.067
	100	1.300^{a}	0.113
	200	1.605^{a}	0.153
Fr-100	0	0.215^{b}	0.032
	50	0.613^{b}	0.130
	100	0.824^{ab}	0.204
	200	1.269^{a}	0.142
Ox-20	0	0.138^{bc}	0.025
	50	0.385^{b}	0.027
	100	0.425^{b}	0.030
	200	0.521^{b}	0.036
Ox-100	0	0.097^{c}	0.009
	50	0.354^{b}	0.044
	100	0.476^{b}	0.076
	200	0.624^{b}	0.035

† Diet code:
Fr= Fresh dietary oil
Ox= Oxidised dietary oil
20 or 100 reflect α-tocopheryl acetate inclusion in diets (mg kg^{-1} dry diet)

^{abc} Values from common incubation times sharing similar superscripts are not significantly different ($P > 0.05$).

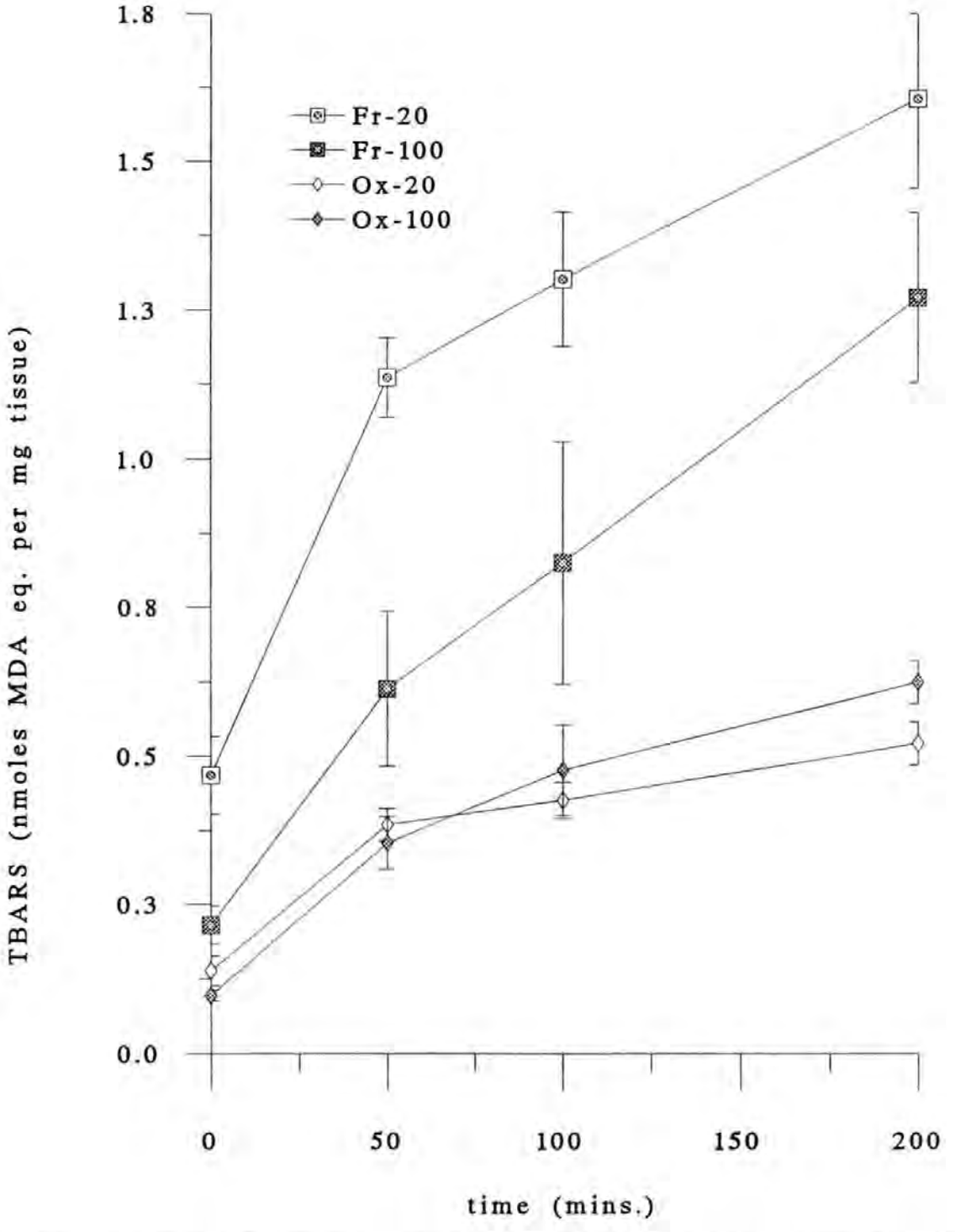


Figure 5.2 Iron-ascobate induced Thiobarbituric acid rective substances (TBARS) in muscle from *Clarias* fed fresh or oxidised diets supplemented with 20 or 100 mg α-tocopheryl acetate kg^{-1} air-dried diet. Values are presented as means $\pm$ s.e. (n=6).

Table 5.10 Muscle total lipid fatty acid profile of catfish fed diets containing fresh or oxidised oil at two levels of α-tocopheryl acetate inclusion. Values are expressed as % contribution of each fatty acid to the total chromatogram area ± s.e. (n=6 fish per treatment).

fatty acid	Fr-20	Fr-100	Ox-20	Ox-100	Main effects¶
14:0	2.90 ±0.10	3.11 ±0.12	2.89 ±0.31	2.84 ±0.18	
16:0	16.43^{a} ±0.29	16.15^{a} ±0.29	19.70^{b} ±0.68	17.61^{ab} ±0.87	O
16:1 n7	3.64 ±0.08	3.79 ±0.10	3.71 ±0.30	3.61 ±0.16	
18:0	8.73^{a} ±0.12	8.43^{a} ±0.13	9.82^{b} ±0.06	8.88^{a} ±0.48	O, E
18:1 n9	13.67^{a} ±0.16	13.69^{a} ±0.35	17.23^{c} ±0.69	14.64^{b} ±0.37	O, E, OxE
18:1 n7	3.04^{a} ±0.05	3.03^{a} ±0.07	3.96^{b} ±0.16	3.36^{a} ±0.17	O, E, OxE
18:2 n6	9.42^{c} ±0.27	9.42^{c} ±0.23	6.63^{a} ±0.17	8.02^{b} ±0.42	O, E, OxE
18:3 n3	0.45^{b} ±0.03	0.45^{b} ±0.02	0.21^{a} ±0.01	0.28^{a} ±0.02	O
18:4 n3	0.78^{c} ±0.04	0.84^{c} ±0.04	0.34^{a} ±0.02	0.42^{b} ±0.03	O
20:0	0.25 ±0.01	0.24 ±0.01	0.25 ±0.03	0.23 ±0.01	
20:1 n11	0.24^{a} ±0.01	0.24^{a} ±0.01	0.35^{b} ±0.02	0.27^{a} ±0.02	O, OxE
20:1 n9	1.15^{a} ±0.05	1.17^{a} ±0.04	1.87^{c} ±0.02	1.44^{b} ±0.09	O, E, OxE
20:1 n7	0.17^{a} ±0.01	0.19^{ab} ±0.01	0.20^{b} ±0.02	0.17^{a} ±0.01	OxE
20:2 n6	0.18^{a} ±0.02	0.20^{a} ±0.02	0.28^{b} ±0.02	0.19^{a} ±0.01	O, OxE
20:3 n6	0.27^{a} ±0.01	0.27^{a} ±0.01	0.47^{c} ±0.02	0.37^{b} ±0.02	O, E, OxE
20:4 n6	0.66^{a} ±0.02	0.62^{a} ±0.02	0.78^{b} ±0.05	0.66^{a} ±0.05	O, E
20:3 n3	0.60 ±0.10	0.59 ±0.09	0.78 ±0.16	0.41 ±0.09	
20:4 n3	0.41^{b} ±0.01	0.46^{b} ±0.01	0.34^{a} ±0.06	0.39^{ab} ±0.03	O
20:5 n3	6.61^{c} ±0.16	6.82^{c} ±0.12	3.21^{a} ±0.13	4.27^{b} ±0.27	O, E, OxE
22:0	0.14^{a} ±0.01	0.12^{a} ±0.01	0.23^{b} ±0.02	0.31^{b} ±0.02	O, E
22:1 n11+13	0.50^{a} ±0.04	0.50^{a} ±0.02	0.77^{b} ±0.06	0.54^{a} ±0.06	O, E, OxE
22:1 n9	0.15 ±0.01	0.14 ±0.01	0.17 ±0.01	0.13 ±0.01	
22:4 n6	0.22 ±0.01	0.22 ±0.01	0.27 ±0.05	0.21 ±0.03	
22:5 n3	2.62^{b} ±0.07	2.52^{b} ±0.06	1.68^{a} ±0.14	2.13^{ab} ±0.19	O, OxE
22:6 n3	10.61^{b} ±0.28	10.56^{b} ±0.27	9.00^{a} ±0.97	9.34^{ab} ±0.74	O
others	16.15	16.15	14.87	19.29	
ΣSFA	28.38^{a} ±0.46	28.05^{a} ±0.52	32.89^{b} ±0.86	29.86^{ab} ±1.51	O
ΣMUFA	22.52^{a} ±0.36	22.74^{a} ±0.56	28.25^{c} ±0.90	24.17^{b} ±0.82	O, E, OxE
ΣPUFA	33.51^{b} ±0.37	33.66^{b} ±0.44	25.14^{a} ±1.29	27.47^{a} ±1.59	O
Σn6	10.73^{b} ±0.28	10.72^{b} ±0.23	8.48^{a} ±0.11	9.46^{ab} ±0.48	O
Σn3	22.08^{b} ±0.35	22.23^{b} ±0.35	15.55^{a} ±1.23	17.32^{a} ±1.23	O

^{abc} within rows, values sharing superscripts are not significantly different ($P>0.05$). Where superscripts are absent, no significant differences exist between groups ($P>0.05$).

¶ Determination of main effects by 2-way ANOVA ($P<0.05$). O=oil state; E=vitamin E; OxE=interaction.

† ΣSFA = total saturates

‡ ΣMUFA = total mono-unsaturates

§ ΣPUFA = total polyunsaturates

Table 5.11 Liver total lipid fatty acid profile of catfish fed diets containing fresh or oxidised oil at two levels of α-tocopheryl acetate inclusion. Values are expressed as % contribution of each fatty acid to the total chromatogram area ± s.e. (n=6 fish per treatment).

fatty acid	Fr-20	Fr-100	Ox-20	Ox-100	Main Effects ¶
14:0	1.34 ±0.02	1.46 ±0.01	1.50 ±0.18	1.60 ±0.06	
16:0	20.10 ±0.20	20.32 ±0.08	19.81 ±0.39	21.41 ±1.18	
16:1 n7	3.31^{b} ±0.06	3.45^{b} ±0.03	2.64^{a} ±0.39	2.98^{a} ±0.28	O
18:0	13.93^{a} ±0.33	15.02^{b} ±0.13	13.04^{a} ±0.40	15.68^{b} ±0.96	E
18:1 n9	20.60 ±0.89	22.02 ±0.36	17.24 ±2.15	19.58 ±2.22	
18:1 n7	3.43 ±0.09	3.57 ±0.04	3.12 ±0.16	3.28 ±0.17	O
18:2 n6	9.08^{b} ±0.27	9.34^{b} ±0.11	4.25^{a} ±0.17	5.38^{a} ±0.23	O, E
18:3 n3	0.29^{b} ±0.01	0.29^{b} ±0.01	0.00^{a} ±0.00	0.03^{a} ±0.03	O
18:4 n3	0.19^{b} ±0.01	0.19^{b} ±0.01	0.00^{a} ±0.00	0.00^{a} ±0.00	O
20:0	0.17^{b} ±0.02	0.20^{b} ±0.01	0.00^{a} ±0.00	0.00^{a} ±0.00	O
20:1 n11	0.26^{b} ±0.01	0.29^{b} ±0.01	0.06^{a} ±0.05	0.09^{a} ±0.05	O
20:1 n9	1.44^{b} ±0.10	1.73^{b} ±0.04	1.33^{ab} ±0.12	1.31^{a} ±0.14	O
20:1 n7	0.15^{b} ±0.01	0.18^{b} ±0.01	0.00^{a} ±0.00	0.00^{a} ±0.00	O, E, OxE
20:2 n6	0.30^{ab} ±0.01	0.31^{b} ±0.01	0.34^{b} ±0.02	0.28^{a} ±0.01	OxE
20:3 n6	0.69^{a} ±0.02	0.74^{a} ±0.01	1.32^{b} ±0.14	0.63^{a} ±0.04	O, E, OxE
20:4 n6	0.76^{a} ±0.03	0.74^{a} ±0.01	2.23^{c} ±0.23	1.37^{b} ±0.19	O, E, OxE
20:3 n3	0.37^{b} ±0.23	0.24^{a} ±0.09	0.37^{b} ±0.06	0.25^{a} ±0.06	E
20:4 n3	0.39^{c} ±0.06	0.41^{c} ±0.02	0.14^{a} ±0.05	0.26^{b} ±0.03	O
20:5 n3	3.49 ±0.24	3.08 ±0.10	3.30 ±0.41	3.88 ±0.88	
22:0	0.03 ±0.02	0.03 ±0.01	0.00 ±0.00	0.00 ±0.00	
22:1 n11+13	0.14^{c} ±0.01	0.10^{b} ±0.01	0.00^{a} ±0.00	0.00^{a} ±0.00	O, E, OxE
22:1 n9	0.02 ±0.01	0.03 ±0.01	0.00 ±0.00	0.00 ±0.00	
22:4 n6	0.12^{a} ±0.01	0.13^{a} ±0.01	0.36^{c} ±0.01	0.22^{b} ±0.01	O, E, OxE
22:5 n3	2.42^{c} ±0.27	2.04^{bc} ±0.11	1.39^{a} ±0.16	1.47^{ab} ±0.28	O
22:6 n3	7.64^{b} ±0.12	7.13^{a} ±0.25	13.67^{c} ±1.71	10.77^{c} ±1.88	O,E,OxE
other	9.38	7.01	13.90	9.51	
ΣSFA	35.57^{a} ±0.34	37.03^{ab} ±0.79	34.35^{a} ±0.36	38.69^{b} ±2.14	E
ΣMUFA	29.33 ±1.05	31.38 ±1.31	24.37 ±2.81	27.22 ±2.80	
ΣPUFA	26.22 ±1.03	25.01 ±1.72	27.87 ±2.47	24.76 ±3.28	
Σn6	10.94^{b} ±0.27	11.26^{b} ±0.34	8.50^{a} ±0.32	7.87^{a} ±0.38	O
Σn3	14.80^{ab} ±0.90	13.33^{a} ±1.44	18.85^{c} ±2.33	16.51^{bc} ±2.99	O, E, OxE

^{abc} within rows, values sharing superscripts are not significantly different ($P>0.05$). Where superscripts are absent, no significant differences exist between groups ($P>0.05$)

¶ Determination of main effects by 2-way ANOVA ($P<0.05$). O=oil state; E=vitamin E; OxE=interaction.

† ΣSFA = total saturates

‡ ΣMUFA = total mono-unsaturates

§ ΣPUFA = total polyunsaturates

Table 5.12 Concentration of unknown compound (ECL= 13.1) in muscle and liver of *Clarias* fed test diets for 56 days. Values are expressed as area % means of six determinations per treatment. Within rows, common superscript allocation denotes no significant difference (P>0.05).

Diet code †	Fr-20	Fr-100	Ox-20	Ox-100	Main Effect ¶
Muscle	0.82^{a}	0.74^{a}	1.41^{b}	1.11^{ab}	O
Liver	0.64^{b}	0.43^{a}	2.26^{d}	1.10^{c}	O, E, OxE

† Diet code:
Fr= Fresh dietary oil
Ox= Oxidised dietary oil
20 or 100 reflect α-tocopheryl acetate inclusion in diets (mg kg^{-1} dry diet)

¶ Determination of main effects by 2-way ANOVA (P<0.05). O=oil state; E=vitamin E; OxE=interaction.

Table 5.13 Exudative moisture loss (%) from *Clarias* fillets after freezing at -20°C for 24 hours and subsequent thawing at 6°C for 48 or 96 hours. Values are means of 5 or 6 determinations (bracketed) ± standard errors.

Diet code †	Fr-20	Fr-100	Ox-20	Ox-100	Main Effect ¶
48 hour thaw	7.518^{ab}	7.074^{a}	9.332^{c}	8.308^{b}	O, E
	0.26	0.31	0.36	0.26	
	(6)	(6)	(6)	(5)	
96 hour thaw	11.133^{ab}	10.181^{a}	12.035^{b}	10.105^{a}	E
	0.47	0.41	0.58	0.30	
	(6)	(6)	(6)	(5)	

† Diet code:
Fr= Fresh dietary oil
Ox= Oxidised dietary oil
20 or 100 reflect α-tocopheryl acetate inclusion in diets (mg kg^{-1} dry diet)

^{abc} Within rows, values carrying common superscripts are not significantly different ($P>0.05$).

¶ Determination of main effects by 2-way ANOVA ($P<0.05$). O=oil state; E=vitamin E; OxE=interaction.

5.4 Discussion

Varying dietary all-rac-α-tocopheryl acetate inclusion and oxidation of dietary oils in practical catfish diets resulted in effects on growth performance, selected health criteria and tissue status of α-tocopherol, fatty acids and products of lipid peroxidation, as well as tissue membrane integrity.

Growth was adversely affected by oxidation of the dietary lipid source ($P<0.001$), with fish fed oxidised (rancid) low-tocopherol diets (22 mg assayed α-tocopherol kg^{-1} dry diet) attaining only half of the mean final weight of the equivalent fresh diet and performing relatively poorly with respect to feed utilization parameters. It is possible that palatability was an influencing factor governing feed intake and thus growth, since the diets made with oxidised oils possessed strong rancid aromas. Although feed intake differences between treatments were minimised by feeding a restricted ration, catfish fed oxidised diets tended to repeatedly take in and expel the pellets from their buccal cavity, thus allowing pellet disintegration in the water.

Inclusion of 100 mg kg^{-1} all-rac-α-tocopheryl acetate into rancid diets was able to significantly improve all growth parameters, though not to the level of either of the fresh-oil diets. Nevertheless, factorial analysis revealed dietary vitamin E to be significantly influential on final body weight ($P<0.001$). Differences in performance resulting from lower dietary α-tocopheryl acetate inclusion into fresh diets were not significant, although projection of the growth curve (figure 5.1) may lead one to assume that α-tocopherol was becoming limiting. This is to be discussed at the appropriate juncture.

Data for hepatosomatic indices (HSI) of *Clarias* fed test diets for 56 days demonstrates that livers of fish fed diets containing 100 mg kg^{-1} all-rac-α-tocopheryl acetate contributed significantly less to the body weight than those of fish fed the low-tocopherol diets. This trend was irrespective of oil quality. On comparison of the present data with data from

Chapter 3 (see also Baker and Davies (1996)) it should be noted that all HSIs were smaller than values previously obtained for this species, though making direct comparisons may be unwise due to the differing oil contents of the diets employed. Fatty infiltration into liver cells of fish deficient in vitamin E may account for enlarged livers in the low tocopherol -fed fish. This would explain the hepatic size difference between treatments and is supported by preliminary results of a histological examination (not presented). These findings contradict the previous findings from Chapter 3 (also Baker and Davies, 1996) when it was shown that elevated dietary tocopherol levels resulted in larger livers. Determination of liver MDA levels in that study provided evidence that the hepatic enlargement was not due to a pro-oxidant action of α-tocopherol at high doses, since lipid peroxidation (TBARS) was lower in livers from fish fed 500 mg kg^{-1} all-rac-α-tocopheryl acetate. Vitamin E concentrations in the liver (to be described later) did not correlate well with observed HSI (R-sq= 0.59, n=24) yet must have played some role in the regulation or depositional control of hepatic lipids in *Clarias* under these experimental conditions. Fatty acid profiles revealed relatively high eicosatrienoic acid (20:3 n3) in liver of fish fed low tocopherol diets, irrespective of oil oxidation state. No mechanism may be offered to explain this phenomenon and additionally, vitamin E has as yet not been implicated in the control of lipid metabolism.

Further assessment of 'health' status involved the determination of haematocrit (%PCV) and plasma haemoglobin concentration. The latter was used as an indicator of haemolysis, since increased incidence of erythrocyte lysis (either from *in vivo* membrane damage or from mechanical damage during centrifugation) would result in the presence of greater levels of haemoglobin in the plasma. Haematocrit values were significantly lower in fish fed fresh-oil/low-tocopherol diets (relative to other treatments and relative to similar sized stock fish (31.32 $\pm$ 0.42 %, n=38)) as has been described in vitamin E deficient fish previously (Lovell *et al.*, 1984; Moccia *et al.*, 1984). One would have expected the low-

tocopherol/oxidised oil treatment to have had a lower haematocrit due to the combined action of increased dietary lipid-peroxides and the absence of adequate vitamin E, thus decreasing membrane fluidity and heightening the possibility of membranal disruption.

Muscle, liver and plasma α-tocopherol were seen to increase significantly ($P<0.05$) concomitant with an increase in dietary dose of all-rac-α-tocopheryl acetate, confirming the earlier findings described in Chapter 3. In addition, heart and spleen levels of the vitamin were also found to respond to dietary dose. The effect of oxidised dietary oils in reducing tissue α-tocopherol status was noticed in all tissues with the exception of skeletal muscle in the fish fed diets containing the lower α-tocopheryl acetate inclusion level. The oxidised oil from treatment Ox-100 caused a significant ($P<0.05$) drop in tissue α-tocopherol concentration from that obtained in the fresh oil equivalent treatment (Fr-100). These findings are in agreement with those of Sheehy and co-workers (1993b, 1994) in their work on poultry, and the findings of Hung *et al.* (1980, 1981) and Stephan *et al.* (1993) in rainbow trout and seabass respectively. The mechanism responsible may be due to the role of α-tocopherol in modulating free radical induced peroxidation. To these ends, α-tocopherol may have been more rapidly utilized in tissues of catfish, challenged by the oxidative stress imposed by the feeding of rancid diets. Alternatively, lipid peroxidation products from the rancid dietary oil may have hindered intestinal absorption of the free alcohol, or merely oxidised the vitamin prior to digestive assimilation.

Possibilities to account for the similarity in muscular tocopherol amongst the low tocopherol fed treatments (Fr-20 and Ox-20), include the fact that at the low tissue α-tocopherol concentrations, alternative antioxidant mechanisms may have operated, thereby sparing the vitamin E to a certain extent, although this was not observed in other tissues. Alternatively, the muscle tocopherol may not have been depleted sufficiently due to reserves of hepatic vitamin E, accumulated prior to the feeding trial. α-tocopherol levels

in fish tissues at the start of the experiment were 7.80 ± 0.34 μg g^{-1} muscle (n=8) and 192.20 ± 14.04 μg g^{-1} liver (n=8), these representing sizeable pools of the vitamin (comparable to the levels in the fresh oil/ higher tocopherol diet). It is possible that liver α-tocopherol was mobilized to the muscle when the tissue was oxidatively stressed. Had the feeding trial duration been extended, it is likely that muscle vitamin E levels in *Clarias* fed fresh-diets low in tocopherol, would have decreased further and manifested as suppressed growth as well as changes in other measured parameters.

On combining data collected during the investigation, liver vitamin E status was re-expressed as μg α-tocopherol per liver per μg α-tocopherol ingested. It was felt that this parameter was more indicative of the vitamin E pool since hepatic tissues are the major storage site of α-tocopherol. Additionally, use of this crude index should minimise differences due to different feed intake levels. Remarkably, values for Fr-20, Fr-100, Ox-20 and Ox-100 respectively were 0.015, 0.034, 0.0016 and 0.003 μg α-tocopherol per liver per μg α-tocopherol ingested, thereby demonstrating the tenfold increment between fresh and oxidised dietary treatments of equi-molar tocopherol concentrations in absolute terms. In other words, the rancid oil used in the manufacture of the current test diets was mediating oxidation of α-tocopherol pre- or post- assimilation at ten times the rate of the fresh-oil equivalent diets. No statistics could be performed on this index due it being derived from mean treatment values, though the index deserves further attention and validation when known individual feed intakes can be quantified.

Upon changing from fresh-oil diets at two levels of dietary α-tocopheryl acetate, to an oxidised:low-tocopherol diet it was apparent that oil status exerted a dramatic influence on hepatic vitamin E concentrations, especially in catfish previously fed a high tocopherol dose. In this treatment it was observed that α-tocopherol values decreased at a mean rate of 16.42 μg per g liver per day over the first two weeks, compared to 0.72 μg α-tocopherol per g liver per day in the fish previously fed the lower tocopherol diet. Since

feed intake was similar in both groups of fish and assimilation of α-tocopherol must have also been similar (same diet), it can be assumed that differences in the rate of hepatic α-tocopherol decline must be due to a decreased rate of tocopherol tissue-replenishment, coupled to a rapid turn-over of the vitamin in oxidatively stressed catfish. Since α-tocopherol concentrations in the *Clarias* previously fed Fr-20 were deemed to be low before the dietary change-over, it may have been difficult to deplete the tissues further, hence the relative stability of these tissues with respect to vitamin E.

It is possible that diet induced oxidation of α-tocopherol in the intestinal lumen may not have been a very prominent factor in modulation of tissue vitamin E concentrations, since switching to an oxidised-oil diet of similar tocopherol content had little effect in the liver α-tocopherol content. More detailed studies would have to explore this however, due to the possible protective effects of antioxidant enzymes, thereby sparing vitamin E in these tissues.

Inclusion of additional dietary vitamin E for catfish would be necessary in order to maintain tissue tocopherol levels at those of fish fed fresh diets. Indeed, in situations where dietary vitamin E levels are only marginally above requirement levels of the vitamin, the antagonistic nature of oxidised dietary oils may lead to the appearance of vitamin E deficiency symptoms, or heightened free radical tissue insult in cultured fish. Evidently this is of particular importance in countries where feeds may become oxidised due to climatic conditions.

TBARS determination in catfish muscle gave rise to somewhat surprising results. Although within fresh-oil treatments, as expected, TBARS formation was suppressed with increased dietary vitamin E, it would appear that the feeding of oxidised-oil diets, lessened measurable TBARS even after additional stimulated peroxidation. This finding is contrary to most published works in animal nutrition, since it is assumed that the increased

oxidative stress associated with rancid feeds would heighten TBARS formation. Additionally, one would expect increased dietary tocopherol to reduce TBARS concentration within these stressed treatments. However, this was not observed, leading speculation into possible mechanisms for TBARS suppression in oxidatively challenged catfish. Perhaps free radical modification of tissue lipids *in vivo* had previously caused a depletion in those fatty acids responsible for MDA production. This would seem possible, although the hypothesis is not borne out by the fatty acid profiles of muscle tissue. Arachidonic acid is thought to be the primary substrate for MDA production (Simic *et al.*, 1992) and levels of this fatty acid are in fact slightly elevated in the oxidised/ low-tocopherol treatment. Alternatively, it could be viewed that tissue-PUFA levels in 'rancid' treatments were low therefore the oxidative potential was much less. The possibility of a TBA-MDA adduct blocking mechanism could also account for the data. Such a mechanism would rely on competition for TBA- or MDA- binding by an alternative lipid peroxidation product. As will be mentioned later, GC-ms revealed the presence of an aliphatic compound whose concentration was correlated to oxidative damage. Whatever mechanism operated, the current investigation's results cast some doubt on the efficacy of using TBARS as an indicator of peroxidation status when applied to tissues of dissimilar fatty acid profiles. Nolan *et al.* (1993) have also stated that TBARS did not reflect degree of peroxidative challenge in the porcine tissues from their investigations. Determination of TBARS will continue to be used as an indicator of lipid peroxidation due to the assays relative speed and ease, although investigators ought to be cautious in interpretting results from studies where tissue-lipid status may be affected.

Oxidised dietary oil was seen to influence the fatty acid profiles of tissue total lipids. Saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) accounted for a significantly greater ($P < 0.05$) proportion of the profile in muscle from fish fed the rancid,

low-tocopherol diet compared to fresh oil diets. This may have resulted from decreased abundance of polyunsaturated fatty acids (PUFA) through peroxidation, as already described by Sheehy and co-workers (1993, 1994), thus elevating the relative SFA and MUFA components. For treatments fed fresh oil diets, no beneficial effect was seen by vitamin E supplementation. Hepatic fatty acid totals did not yield similar trends as for muscle, possibly due to a regulatory ability since liver is the primary organ of synthesis. However, feeding oxidised oils resulted in proportionally less hepatic n-6 fatty acid totals, either due to an oxidant mechanism, affected fatty acid uptake, heightened use, or to artefactual decrease caused by proportional increase of other fatty acids. Alternatively, it cannot be discounted that fresh oil fed catfish selectively conserved these PUFA within their tissues.

Relative amounts of linoleate (18:2 n-6) and linolenate (18:3 n-3) in both muscle and liver were lower on feeding the rancid diets, despite dietary sources of these essential fatty acids (EFA) being similar in all four diets. This effect may have been due to utilization of the EFAs in the synthesis of longer chain fatty acids, as a response to increased radical attack of existing tissue PUFAs. Indeed, muscle eicosadienoic acid (20:2 n-6) was elevated in Ox-20, possibly demonstrating the addition of the two-carbon unit on to the linoleate molecule. The n-6 eicosatetraenoic acid (arachidonate) was also more abundant in 'Ox-' treatments indicating the action of desaturase enzymes in PUFA synthesis, or a greater conservation of arachidonate from the diet. At this stage it should be noted that muscle levels of this fatty acid were unlikely to be due to *in situ* synthesis, but to mobilization of arachidonate from the liver; the major organ responsible for fatty acid synthesis in fish. Not surprisingly, the raised arachidonate abundances as a result of oxidised oil ingestion, were most marked in the liver. Abundances of further chain elongation products are apparent on considering liver tissue data. Significantly increased abundance of docosahexaenoic acid (22:6 n-3, DHA) ($P=0.0004$) in livers from fish fed oxidised oil

could be attributed to the combined action of elongases and desaturases on linolenate. It is worthy of note that both hepatic -linoleate and -linolenate were depleted in 'Ox-' treatments. Combining the evidence, it would seem possible that hepatic pathways were directed to the production of DHA, although selective retention of selective fatty acids from the diet cannot be discounted. Nevertheless, increased DHA production would explain the depleted linolenate levels. Total liver n-3 fatty acids were greater in oxidised treatments, although the reverse trend is true in muscle tissue. A mobilization of shorter chain n-3 fatty acids from the muscle, for hepatic conversion to DHA would account for this. The augmented production of DHA in oxidatively stressed livers has not been previously described in fish, though may be explained by a number of hypotheses.

Firstly, high DHA levels may be due to the compensatory overproduction of this fatty acid as a result of its peroxidation by free radicals and this was proposed as the mechanism responsible for similar findings in vitamin E and selenium deficient rats (Buttriss and Diplock, 1988).

Increased production may have also been due to increased activity of elongase enzymes as experienced in tissues from fish fed low lipid diets (Greene and Selivonchick, 1987). Although the test diets used in the present investigation were lipid and essential fatty acid (EFA) adequate, oxidised-oil diets may have been nutritionally poor due to an autotoxic effect and this may govern lipid synthetic pathways.

Alternatively the response may be a protective antioxidant mechanism. It is known that oestrogen hormones can inhibit microsomal lipid peroxidation (Ruiz-Larrea *et al.*, 1994) and that, though fatty acid hydroperoxides are highly reactive pro-oxidants, they have been recorded to inhibit superoxide production (Kapus *et al.* 1994). Additionally, DHA may be oxidatively converted into an eicosanoid type hormone, since lipoxygenase is capable of converting DHA into its acyl-hydroperoxide (German and Kinsella, 1985). Despite the inactivity of hepatic lipoxygenases, perhaps DHA was being modified through a related

enzyme pathway, thus shifting the equilibrium towards generation of more DHA to fuel the lipoxygenase route of eicosanoid production. Although eicosatetraenoic acid (arachidonate) is the primary substrate for lipoxygenase in most fish and mammal tissues (Johnson *et al.*, 1983), other fatty acids may be utilised in this manner. Bell *et al.* (1993) reported that eicosapentaenoic acid (EPA) was the predominant substrate for lipoxygenase in salmon gill. Under times of oxidative stress *Clarias* may utilize DHA, and this may be re-inforced by the fact that when vitamin E was supplemented into rancid diets, DHA production was suppressed to levels closer to those from fresh-oil type diets.

A further possibility exists to explain liver DHA values. It is known that n-3 fatty acids are antithetic with arachidonic acid (Li *et al.*, 1994). Increased concentration of tissue arachidonic acid in response to oxidised oil consumption was observed in the present study. Since eicosanoids derived from arachidonic acid are responsible for, amongst other things, an inflammatory response, it would seem possible that incorporation of large amounts of DHA into tissue membranes, from either heightened synthesis or selective retention from the dietary source, could serve to modulate arachidonic acid use, and thus inflammation. Further research must quantify tissue eicosanoid production in response to oxidative stress in order to elucidate the true mechanism underlying the increased DHA abundance in *Clarias* fed oxidised-oil diets. This type of eicosanoid suppression by tocopherol has been observed by Reddanna *et al.* (1989) who further stated that the effect was not due to α-tocopherol's antioxidant role, but to an enzyme binding mechanism displayed by tocopherol *in vitro*.

It must be noted that although the above hypotheses offer possible explanations for increased PUFA synthesis in oxidatively stressed catfish, the possibility of selective conservation of PUFA or efficient uptake from dietary sources is clearly feasible. In order to demonstrate conclusively that catfish had produced DHA in response to oxidative stress, it would be necessary to either radio-label potential DHA precursors, or to remove DHA

from the diet to preclude this source.

Modification of PUFA profile in *Clarias* tissues was apparent on considering n-6 fatty acids in muscle and liver. Total n-6 fatty acid values were consistently lower in tissues from rancid- low tocopherol treatments compared to those from fresh oil fed fish. Therefore, although n-6 PUFA synthesis may have occurred and may have been greater in tissues from 'Ox-' treatments, a slight loss of n-6 fatty acids must also have taken place. Reasons explaining the decrease in n-6 fatty acid abundance are similar to those provided earlier for n-3 utilization. The lessened degree of severity of n-6 fatty acid loss when compared to n-3 losses may illustrate the fact that n-3 fatty acids are more prone to oxidation.

Evidence of peroxidative modification of fatty acids was established on examination of chromatographic data. Calculation of area % of a peak from a compound with a retention time of approximately 36 minutes, revealed a strongly significant (muscle: $P=0.0096$, liver: $P<0.001$) treatment dependence. For both muscle and liver tissues, the area % of the compound increased in the order Fr-100, Fr-20, Ox-100, Ox-20, indicating that tissue concentrations increased concomitant with increased oxidative stress (see table 5.11). Unfortunately, the database of mass spectra associated with the GC software was not extensive enough to identify the compound conclusively. However, characteristic ion fragments were present enabling a tentative identification as a straight-chain alkene with an equivalent chain length (ECL (Stransky *et al.*, 1992)) of 13.1 relative to saturated fatty acid methyl esters. It has been shown previously that aliphatic compounds are produced on peroxidation of fatty acids (Simic *et al.*, 1992). For improved identification it would be necessary to further isolate the compound in sufficient quantity to perform biochemical assays, and also re-evaluate mass spectra based on the monitoring of selected ion

fragments. Whatever future analysis reveals, it seems highly likely that the alkene is a remnant of a peroxidized longer chain fatty acid and it would be interesting if this is the causal factor in reduced TBARS in muscle from fish fed oxidised oil.

With respect to exudative moisture losses from thawing *Clarias* muscle fillets, it was apparent that both the tissue tocopherol and the dietary oil oxidative state exerted their influence on tissue moisture retention. After a 48 hour thawing period, the oil quality appeared to be the key factor governing membrane permeability, with both fresh oil diets (Fr-20 and Fr-100) losing less moisture to the absorbent pad. This trend was significant ($P < 0.05$) when comparing Fr-100 with the two rancid treatments, and demonstrated the possibility that lipid-peroxyl formation had not occurred *in vivo* to any great extent in fish fed fresh diets.

The beneficial effect of vitamin E supplementation was in evidence on comparing the values from fish fed rancid diets. In this case, elevated all-rac-α-tocopheryl acetate supplementation significantly ($P < 0.05$) curtailed drip-loss, as has been demonstrated by Asghar *et al.* (1991) and Cheah *et al.* (1995) in work on pork, and Monahan *et al.* (1994) in work lamb. *Post mortem* effects of α-tocopherol on tissue stability were noticeable after 96 hours of thawing. After this time, tissue oxidation by atmospheric air had obviously further damaged the muscle tissue membranes. Tissues possessing the higher α-tocopherol concentrations (Fr-100 and Ox-100) displayed lower moisture loss values. α-tocopherol in these tissues was therefore maintaining membrane stability, even in the treatment oxidatively stressed by rancid dietary oil. Both the fresh- and oxidized- low tocopherol diets were unsuccessful in lowering exudative losses after 96 hours. No significant improvement ($P > 0.05$) in stability was provided by the fresh oil, after this time.

The results of this investigation have highlighted the importance of vitamin E, and the

antagonistic effect of oxidized dietary oils, with respect to growth performance, feed utilization, tissue α-tocopherol and fatty acid profiles, haematology and exudative losses of moisture from catfish muscle. Based on the test diets employed in the present study, lipid stability against the oxidative stresses imposed on tissues by oxidized dietary oils, is significantly improved through the supplementation of vitamin E in diets for the African catfish. Additionally, that stabilizing effect is maintained *post mortem*.

CHAPTER 6

INVESTIGATING THE α-TOCOPHEROL - ASCORBATE SYNERGY: THE EFFECT OF SUPPLEMENTATION OF VITAMIN C, IN *CLARIAS* DIETS CONTAINING VITAMIN E AT ADEQUATE AND SUPRA-NUTRITIONAL LEVELS

6.1 Introduction

Previously (chapter 5) it has been established that consumption of oxidised dietary oil by *Clarias* was able to modulate the antioxidant defence systems within this species. Dietary supplementation of alternative antioxidants may be capable of boosting the free radical trapping mechanisms thus improving tissue health of the African catfish.

Synergy between the membrane-bound, lipid-soluble antioxidant α-tocopherol and various aqueous phase antioxidants has been demonstrated *in vitro* and in the case of certain enzymes, also *in vivo*.

McCay (1985) and Niki (1987) have shown an ascorbate-tocopherol interaction and both suggested that this was due to ascorbate's recycling role. Ascorbate (vitamin C) has been observed to regenerate α-tocopherol from the reacted tocopheroxyl radical (McCay, 1985). As already described (section 1.4.2), at the membrane-cytosol interface, ascorbate donates a hydrogen ion to the tocopheroxyl radical (see figure 1.6), which can in-turn be recycled back to the reducing form (ascorbate) by reducing agents in the aqueous phase.

Theoretically, sparing of α-tocopherol could also be achieved by ascorbate, by directly reducing reactive radical species prior to bio-membrane attack. Alternatively, high membrane concentrations of α-tocopherol could function to lower the requirement of ascorbate under conditions of oxidative stress by sparing vitamin C the need to recycle vitamin E.

By whatever mechanism, scope may exist for nutritional manipulation of tocopherol and

ascorbate tissue concentrations, in order to optimise tissue condition.

Within physiological concentrations of vitamins C and E deemed to be healthy, few studies have demonstrated any synergy between these antioxidants in any vertebrate model. Robertson *et al.* (1991) could not find evidence of synergy between the vitamins with regard to protection from cataract formation in humans. In research on the house cricket (*Acheta domesticus*), McFarlane (1992) stated that even at high doses of dietary ascorbate, spermiogenesis still required the presence of vitamin E, although in this case a specific role of vitamin E in spermiogenesis is implied.

Chen *et al.* (1980) and Chen and Chang (1978) demonstrated some sparing of vitamin E by ascorbate in vitamin E deficient rats and guinea-pigs respectively. Furthermore, Chakraborty and co-workers (1994) recorded the ability of ascorbate to protect guinea-pig tissues against lipid peroxidation, a role normally fulfilled by α-tocopherol.

Of the research carried out in fish, results focus principally on sparing by one nutrient when the other is approaching deficiency status. Frischknicht *et al.* (1994) observed some mutual sparing of vitamins C and E, in that mortalities resulting from tocopherol deficiency in rainbow trout were reduced by high ascorbate doses and *vice-versa*. Also, vitamin C was able to prevent myodegeneration resulting from tocopherol deficiency, and vitamin E prevented the negative effects of ascorbate on numerous haematological indices. So far, little evidence of an *in vivo* synergy between ascorbate and tocopherol in healthy fish- tissues has come to light, despite a large volume of work carried out on these nutrients with regard to fish immune status (Verlhac *et al.*, 1993).

In the case of the African catfish (*Clarias gariepinus*) little is known of quantitative vitamin requirements, let alone the influence of vitamin interactions. Qualitatively, vitamin C has been known to be essential for the prevention of deficiency syndromes (scoliosis, lordosis, external haemorrhaging, fin erosion and skin darkening) in Asian catfish (*Clarias batrachus* Linnaeus)(Butthep *et al.*, 1983), although in that study growth suppression was

not observed. At present, the minimum ascorbate requirement of the African catfish in agricultural by-product based diets is defined as 60 mg per kg diet (Mgbenka, 1991), this value being satisfactory for prevention of pathology and maximal growth under normal culture conditions.

This study aims therefore to examine the effect of α-tocopherol supplementation in *Clarias* fry feeds varying in ascorbate inclusion, on growth performance, tissue vitamin retention and oxidative state. Additionally the experimental design allows assessment of the effect of dietary ascorbate dose on tissue ascorbate deposition in two size-classes of *Clarias*.

6.2 Materials and Methods

6.2.1 Chemicals

All chemicals used were ANALAR grade obtained from Sigma Chemical Company Limited, Poole, Dorset, U.K., and Merck Limited, Poole, Dorset, U.K.. All-rac-α-tocopheryl acetate (Rovimix E-50 SD) and polyphosphorylated ascorbic acid (Rovimix Stay-C 25%) were kindly donated by F. Hoffmann- La Roche, Basel, Switzerland. All other vitamins were supplied by Roche Products Limited, Heanor, Derbyshire, U.K..

6.2.2 Fish stock, Experimental facilities and Diets.

0.6 g fry of mixed sexes of the African catfish, *Clarias gariepinus*, were spawned in-house (according to section 2.9) and stocked into five tanks of the culture facility described in section 2.2. System water temperatures were maintained at 26^{0}C, the photoperiod held at 12 hours light:12 hours dark, and water quality parameters measured and adjusted as appropriate.

A basal practical diet was formulated as described in section 2.1.1., following the diet composition from chapter 5 (table 5.1), except for the fact that vitamin C was not supplied in pre-mix form.

Vitamins (excluding vitamins C and E) and minerals were added to diets in the form of the previously defined premixes (section 2.1.1). Tocopherol and ascorbate sources (Rovimix E-50 SD and Rovimix Stay-C 25% respectively) were added to the powdered ingredients and the diets manufactured following the procedure outlined in section 2.1.2. Compared to calculated values, proximate analysis of the test diets (section 2.3) confirmed levels of macronutrients in the feeds. These data are presented in table 6.1.

Inclusion levels of vitamin C and vitamin E (supplied as all-rac-α-tocopheryl acetate) were set in order to create low and high ascorbate diets (25 or 200 mg kg^{-1}) at adequate or high tocopherol levels (150 or 500 mg kg^{-1}). In addition, a tocopherol adequate-ascorbate

deficient diet was designed to provide information on scorbutic lesions in *Clarias*. Dietary codes were allocated to reflect inclusion levels of the vitamins. As such, C_0, C_1 and C_2 represent deficient, low and high ascorbate diets respectively, and E_1 and E_2 represent tocopherol- adequate and supra-supplemented diets.

Dietary analyses of vitamin levels in the dried feeds were performed prior to commencement of the feeding trial in accordance with sections 2.4.1 and 2.4.2..

Table 6.2 shows the results of the DNPH colorimetric assay for ascorbic acid and HPLC determination of tocopherol content, these characterizing the feed vitamin status as fed.

6.2.3 Feeding regime and nutrition trial protocol

The *Clarias* fry were uniformly graded and assigned, initially 150 per tank, into five tanks. Fish were weighed in groups of 10 at the start of the feeding trial. ANOVA revealed that there were no significant differences ($P > 0.05$) between tanks.

A food ration level of up to 4% body weight per day was employed, with three times daily feeding, this ration calculated on a dry matter basis. All fish were weighed weekly in tens, in order to determine feed totals for the proceeding weeks. At this stage, fish populations were rotated to the proceeding tank in order to combat any potential tank effect. With the exception of fish from the ascorbate free (C_0E_1) treatment, all fish populations were reduced at week six so as to prevent appearance of density dependent effects on growth. This was carried out by removal of those fish greater, or less than two standard deviations from the mean fish weight in the tank. On termination of the 84 day feeding trial, individual fish weights were recorded and appropriate statistical analyses applied.

6.2.4 Growth and nutrient utilization parameters

The following parameters were calculated (according to section 2.2.5) from data acquired from the feeding trial; percent change in mean body weight, daily feed intake, specific

growth rate and feed efficiency.

6.2.5 Sampling of fish tissues

After the feeding trial had been completed, fish were maintained on the appropriate feeding regime, and sacrificed upon attainment of a critical size. Size classes were defined as follows: 60-80g and 80-100g. With the exception of treatment 1 (C_0E_1), that never attained even the lowest weight-class, muscle and liver from six individuals were sampled from each treatment for different size classes. Fish tissues destined for ascorbate determination were kept on ice prior to being processed in accordance with the procedure in section 2.4.2.1.

Muscle tissue from 60-80 g fish was frozen at -80 ^{0}C for future determination of α-tocopherol and thiobarbituric acid reactive substances (TBARS)(described in section 6.2.6).

6.2.6 Analytical methods

The proximate composition of diets was performed using the standard AOAC (1990) chemical procedures for moisture, crude protein (N X 6.25), and ash (section 2.3).

Lipid determination was carried out gravimetrically after using the Folch cold lipid extraction as described by Barnes and Blackstock (1973).

Determination of the ascorbic acid and ascorbyl-2-sulphate concentrations in selected tissues was performed as outlined in section 2.4.2.

α-tocopherol was extracted and assayed from samples of feed and muscle tissue by the method described in section 2.4.1. Values obtained were expressed as μg α-tocopherol g^{-1} dry feed or fresh tissue.

Quantification of *in vivo* TBARS in muscle tissue proceeded as outlined in section 2.5, except that the iron/ ascorbate incubation stage was omitted. Instead, 400 μl of distilled water was added to the tubes.

6.2.7 X-radiography and photography

Four *Clarias* individuals from the vitamin C deficient diet (C_0E_1) were removed from the final population. These were anaesthetized and subjected to X-radiography in order to determine the presence of scorbutic skeletal deformities. The exact procedure is described in section 2.8. Photography of the fish described above, allowed further assessment of the general condition of the *Clarias* fry.

6.2.8 Statistical analyses

ANOVA ($P<0.05$) and Duncan's new multiple range (Steel and Torrie, 1960) test were performed wherever possible using the statistical software package 'Statgraphics'.

Linear multiple regression (using 'Cricketgraph') was carried out in order to assess the effects of various factors on final tissue ascorbate levels. Models were derived and R-squared values obtained to determine the 'fit' of the model.

Table 6.1 Proximate composition of basal practical test diet (bracketed values depict theoretical composition as calculated from NRC (1993))

	Moisture (%)	Protein (% DM)	Lipid (% DM)	Ash (% DM)	Residual
Mean	4.75	50.36 [49.35]	11.67 [11.87]	15.02 [14.17]	18.20
S.E.	0.292	0.391	1.070	0.179	
n	6	12	12	12	

n= number of determinations from a single diet.

Table 6.2 Assayed total ascorbate and α-tocopherol in test diets (μg g^{-1} dry weight). Expected values based on inclusion are bracketed.

Diet code¶		C_0E_1	C_1E_1	C_2E_1	C_1E_2	C_2E_2
Total ascorbate	mean	6.72 [0.00]	24.98 [25.00]	195.23 [200.00]	22.45 [25.00]	157.26 [200.00]
	S.E.					
	n	1	1	1	1	1
α-tocopherol	mean	109.01 [150.00]	152.02 [150.00]	113.41 [150.00]	549.58 [500.00]	521.27 [500.00]
	S.E.	3.59	7.90	2.09	9.12	17.04
	n	3	3	3	3	3

¶ Diet codes:

C_0E_1 Ascorbic acid free + 150 ppm α-tocopheryl acetate
C_1E_1 25ppm polyphosphorylated ascorbic acid + 150ppm α-tocopheryl acetate
C_2E_1 200ppm polyphosphorylated ascorbic acid + 150ppm α-tocopheryl acetate
C_1E_2 25ppm polyphosphorylated ascorbic acid + 500ppm α-tocopheryl acetate
C_2E_2 200ppm polyphosphorylated ascorbic acid + 500ppm α-tocopheryl acetate

6.3 Results

Growth data presented in table 6.3 reveals that only those fish on the ascorbate-free diet attained a significantly lower final weight than the remaining treatments ($P<0.001$). Amongst the fish fed ascorbate supplemented diets, those on feeds containing the lower vitamin C levels performed less well with respect to percent change in body weight and specific growth rate than higher ascorbate treatments. However, no significant difference existed between body weights ($P=0.1912$). Dietary α-tocopheryl acetate inclusion did not affect any growth, or feed utilization parameter.

X-radiography revealed vertebral lateral distortion (lordosis) in 6 % of fish fed ascorbate deficient diets, as shown in plate 6.1. Cranial deformities were also observed in these fish (see also plate 6.2). Although no 'control' fish were X-rayed (due to the vast size differences), the author observed that the frontal-cranial region was stunted, resulting in catfish with comparatively rounded heads ('Spoon-head syndrome').

Photography of those fish subjected to X-radiography (plates 6.3 and 6.4) confirmed head deformation, as well as demonstrating possible scoliosis (plate 6.4). Caudal fin erosion, or malformation, can also be observed, indicating the general poor condition of the fish.

From table 6.4 it is evident that elevating the dietary ascorbate concentration increases the total ascorbate in tissues measured.

Irrespective of dietary ascorbate dose, L-ascorbic acid concentration of muscle and liver tissues decreased (often significantly ($P<0.05$)) with increase in fish size.

Linear multiple regression analysis of both dietary ascorbate dose and fish size with respect to muscle or liver total ascorbate was performed. R-squared values for the fitted lines were 0.62 and 0.66 respectively. Regression models were as follows:

Muscle ascorbate = 17.317 + (0.0705 X dietary ascorbate dose) + (-0.1304 X fish weight)

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Photography of those fish subjected to X-radiography (plates 6.3 and 6.4) confirmed head deformation, as well as demonstrating possible scoliosis (plate 6.4). Caudal fin erosion, or malformation, can also be observed, indicating the general poor condition of the fish.

From table 6.4 it is evident that elevating the dietary ascorbate concentration increases the total ascorbate in tissues measured.

Irrespective of dietary ascorbate dose, L-ascorbic acid concentration of muscle and liver tissues decreased (often significantly ($P<0.05$)) with increase in fish size.

Linear multiple regression analysis of both dietary ascorbate dose and fish size with respect to muscle or liver total ascorbate was performed. R-squared values for the fitted lines were 0.62 and 0.66 respectively. Regression models were as follows:

Muscle ascorbate = 17.317 + (0.0705 X dietary ascorbate dose) + (-0.1304 X fish weight)

Plate 6.3 (Opposite) Vitamin C deficient *Clarias gariepinus* (dorsal view). Note the atypical head shape ('Spoon-head syndrome').

Plate 6.4 (Opposite) Vitamin C deficient *Clarias gariepinus* (lateral view). Note the 'stunted' head appearance and possible scoliosis (**S**).

Liver ascorbate = 73.778 + (0.3461 X dietary ascorbate dose) + (-0.7301 X fish weight)

Similar models, allowing for dietary α-tocopheryl acetate inclusion, were not accurate predictors of tissue vitamin C status.

Irrespective of size class, increased dietary tocopherol was associated with a subtly elevated deposition of bound-ascorbate (possibly as ascorbyl-sulphate, the supposed storage form of vitamin C (Tucker and Halver, 1984)) in fish fed adequate amounts of vitamin C. The tissue concentrations of 'bound ascorbate' (ascorbyl-sulphate or another analogue), can be seen to vary with dietary treatment. Amongst treatments containing higher concentrations of ascorbate, the ascorbate/ascorbic acid differential in muscle tissues is never significantly less ($P > 0.05$) in the larger size class of fish, in contrary to the muscular and hepatic L-ascorbic acid discussed above. In C_2E_2 the ascorbate/ascorbic acid differential in muscle is significantly ($P < 0.05$) higher in larger fish. This size effect was not seen in low ascorbate treatments.

Hepatic concentrations of the compound responsible for the ascorbate/ascorbic acid differences in low vitamin C treatments were not significantly different ($P > 0.05$) from liver concentrations of L-ascorbic acid. In other treatments however, the liver L-ascorbic acid was far more abundant ($P < 0.05$) than the bound vitamer.

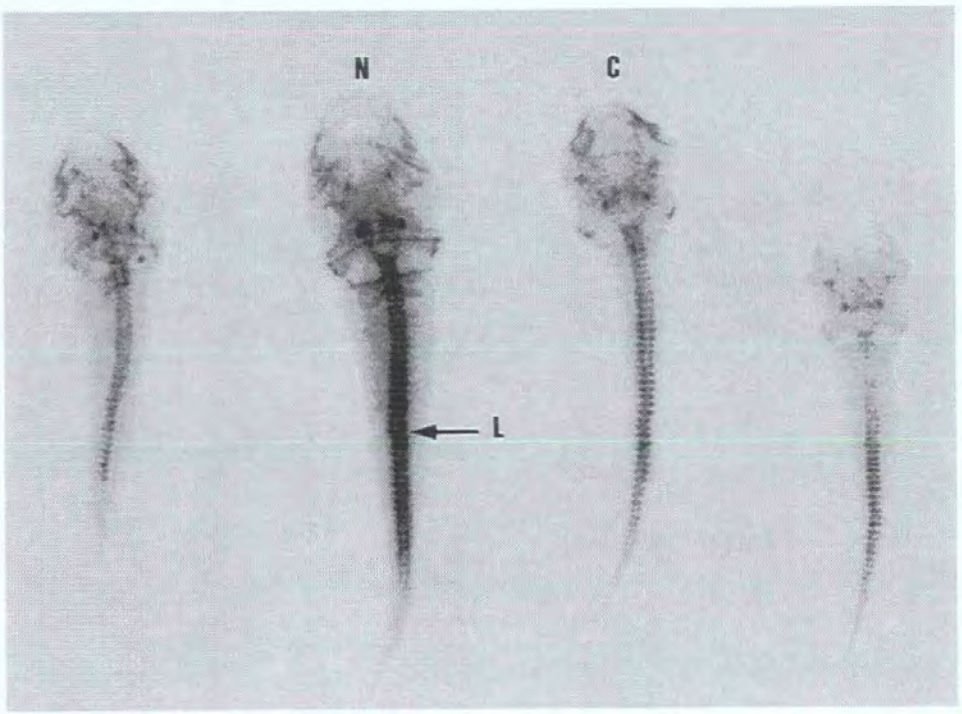
In 60 - 80 g fish, muscular α-tocopherol concentration was seen to be correlated to dietary vitamin E dose, with those fish fed higher concentrations of vitamin E exhibiting significantly greater tissue concentrations of the vitamin ($P < 0.001$) (see table 6.5).

Evaluation of *in vivo* TBARS formation in muscle of 60 - 80 g catfish (table 6.5) revealed

that the extent of lipid peroxidation was significantly less (P=0.0011) as a result of increased dietary tocopherol dose. Ascorbate dose was not seen to significantly influence TBARS formation in either tocopherol -adequate or -super-supplemented diets (P=0.5541 and 0.3515 respectively).

Plate 6.1 (Opposite) X-radiographs of vitamin C deficient *Clarias gariepinus* (dorsal view). **L** apparent lordosis. **C** cranial deformation (roundness). **N** normal cranial shape.

Plate 6.2 (Opposite) X-radiographs of vitamin C deficient *Clarias gariepinus* (lateral view). **S** apparent scoliosis. **C** cranial deformation (roundness). **N** normal cranial shape.


N
C
L

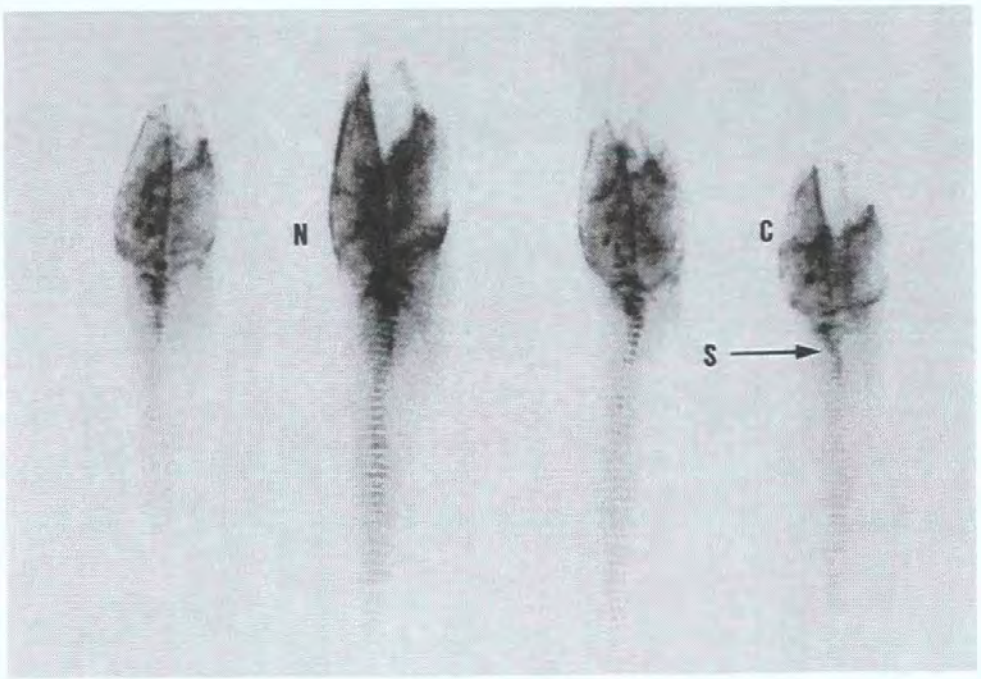
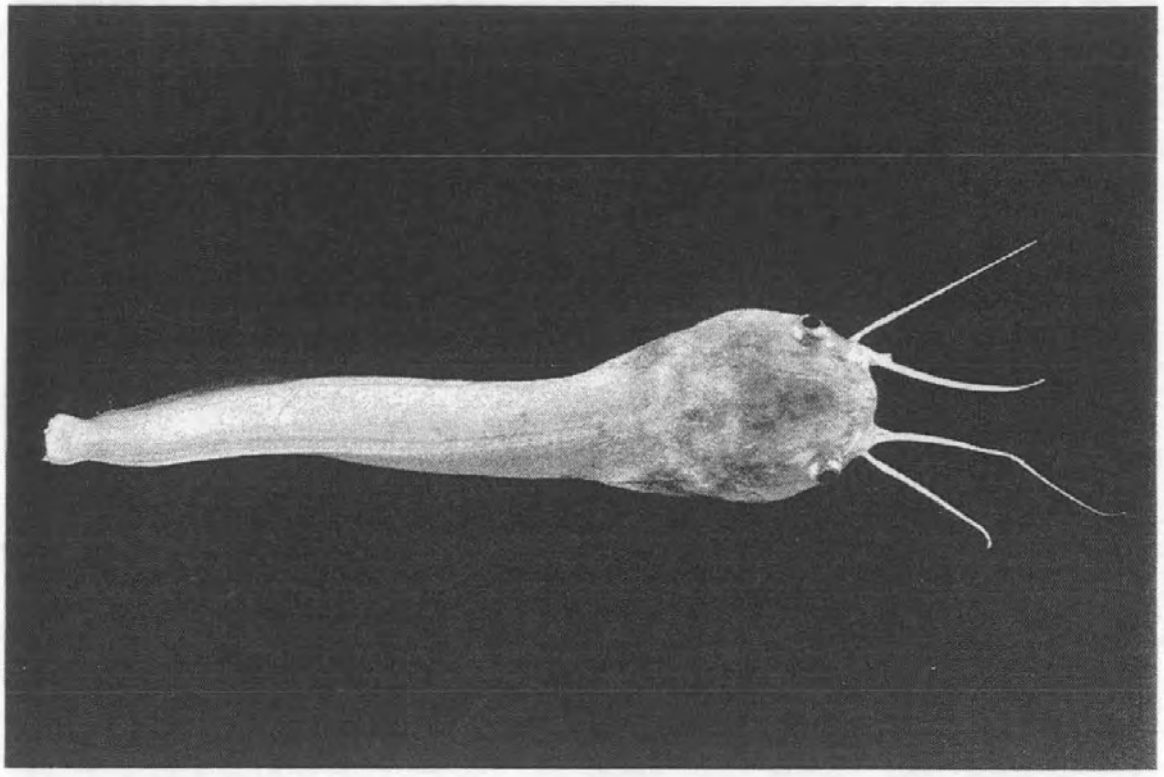

N
C
S

Plate 6.3 (Opposite) Vitamin C deficient *Clarias gariepinus* (dorsal view). Note the atypical head shape ('Spoon-head syndrome').

Plate 6.4 (Opposite) Vitamin C deficient *Clarias gariepinus* (lateral view). Note the 'stunted' head appearance and possible scoliosis (**S**).



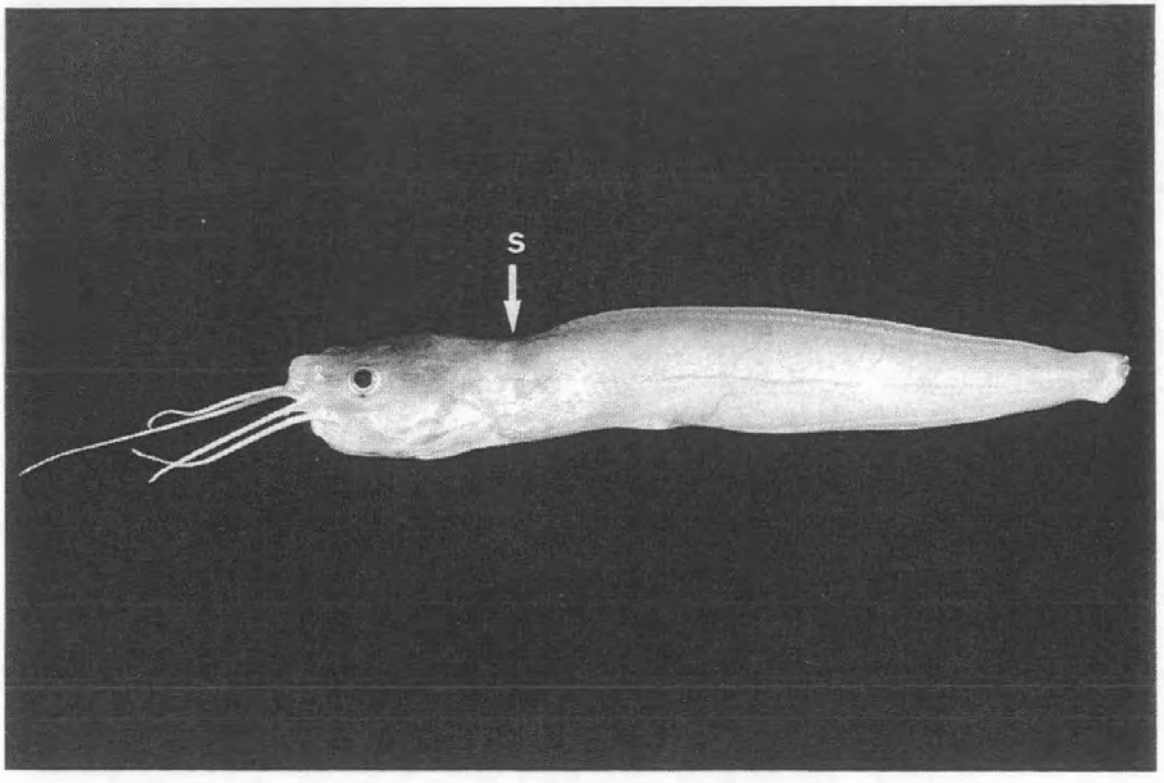

S

Table 6.3 Growth related performance and nutrient utilization of catfish fed experimental diets after 84 days.

Diet Code ¶	C_0E_1	C_1E_1	C_2E_1	C_1E_2	C_2E_2
Mean initial weight (g)	0.61	0.63	0.63	0.57	0.63
Mean final weight (g)	1.64[a]	31.14[b] ±4.82	32.78[b] ±3.73	26.07[b] ±3.53	36.71[b] ±3.69
% change in mean weight	168.9	4842.9	5103.2	4473.7	5727.0
Daily feed intake (g)	0.005	0.212	0.242	0.199	0.257
Specific Growth Rate ($\%d^{-1}$)	1.18	4.64	4.70	4.55	4.84
Feed Efficiency	2.34	1.72	1.58	1.52	1.67

¶ Diet codes:

C_0E_1 Ascorbic acid free + 150 ppm α-tocopheryl acetate
C_1E_1 25ppm polyphosphorylated ascorbic acid + 150ppm α-tocopheryl acetate
C_2E_1 200ppm polyphosphorylated ascorbic acid + 150ppm α-tocopheryl acetate
C_1E_2 25ppm polyphosphorylated ascorbic acid + 500ppm α-tocopheryl acetate
C_2E_2 200ppm polyphosphorylated ascorbic acid + 500ppm α-tocopheryl acetate

[ab] Mean values sharing common superscripts (or possessing none) are not significantly different ($p > 0.05$).

Table 6.4 Assayed L-ascorbic acid and total Ascorbate in muscle and liver tissues of *Clarias* of varying size classes. Each value is a mean of six replicates ± standard error (±se). Values within each column sharing common superscripts are not significantly different ($P > 0.05$).

Diet assay				Muscle vitamin C (μg/g)		Liver vitamin C (μg/g)	
Diet Code ¶	Total ascorbate (μg/g)	α-tocopherol (μg/g)	Size Class (g)	L-ascorbic acid	Total ascorbate	L-ascorbic acid	Total ascorbate
C_1E_1	24.98	152.02	60-80	7.177^{b} ±0.762	13.17^{bc} ±0.926	11.380^{a} ±0.749	24.51^{a} ±0.862
			80-100	4.556^{ab} ±1.182	5.40^{a} ±0.701	7.444^{a} ±1.302	18.35^{a} ±3.645
C_2E_1	195.23	113.41	60-80	17.560^{de} ±1.522	18.99^{de} ±1.155	73.927^{e} ±4.021	93.63^{d} ±6.243
			80-100	13.024^{bc} ±2.253	16.09^{cd} ±1.428	56.623^{bc} ±6.498	67.75^{c} ±5.163
C_1E_2	22.45	549.58	60-80	6.044^{ab} ±0.583	10.11^{b} ±1.791	20.544^{a} ±5.548	29.75^{a} ±3.886
			80-100	4.186^{a} ±0.455	5.53^{a} ±0.459	6.405^{a} ±1.883	17.26^{a} ±2.529
C_2E_2	157.26	521.27	60-80	20.737^{e} ±0.969	23.14^{f} ±1.498	64.642^{e} ±7.469	97.16^{d} ±4.218
			80-100	16.379^{cd} ±0.999	23.26^{f} ±1.839	38.277^{b} ±4.541	52.32^{b} ±7.709

¶ Diet codes:

C_1E_1 25ppm polyphosphorylated ascorbic acid + 150ppm α-tocopheryl acetate
C_2E_1 200ppm polyphosphorylated ascorbic acid + 150ppm α-tocopheryl acetate
C_1E_2 25ppm polyphosphorylated ascorbic acid + 500ppm α-tocopheryl acetate
C_2E_2 200ppm polyphosphorylated ascorbic acid + 500ppm α-tocopheryl acetate

Table 6.5 Assayed Ascorbate analogues, α-Tocopherol and *in vivo* TBARS in muscle tissue of *Clarias* of size 60-80g. Each value is a mean of six replicates ± the standard error (±se). Values within each column sharing common superscripts are not significantly different ($P > 0.05$).

	Diet assay		Muscle assays				
Diet Code ¶	Total ascorbate (μg/g)	α-tocopherol (μg/g)	L-ascorbic acid (μg/g)	Ascorbyl-sulphate (μg/g)	Total Ascorbate (μg/g)	α-tocopherol (μg/g)	*in vivo* TBARS †
C_1E_1	24.98	152.02	7.177^a ±0.762	5.993^b ±0.740	13.169^a ±0.926	4.611^a ±0.515	0.079^b ±0.008
C_2E_1	195.23	113.41	17.560^b ±1.522	3.005^{ab} ±1.234	18.996^b ±1.155	4.763^a ±0.635	0.070^b ±0.010
C_1E_2	22.45	549.58	6.044^a ±0.583	4.069^{ab} ±1.343	10.111^a ±1.791	13.880^b ±0.938	0.044^a ±0.002
C_2E_2	157.26	521.27	20.737^b ±0.969	2.574^{ab} ±1.109	23.141^b ±1.498	14.592^b ±0.944	0.050^a ±0.008

¶ Diet codes:

C_1E_1 25ppm polyphosphorylated ascorbic acid + 150ppm α-tocopheryl acetate
C_2E_1 200ppm polyphosphorylated ascorbic acid + 150ppm α-tocopheryl acetate
C_1E_2 25ppm polyphosphorylated ascorbic acid + 500ppm α-tocopheryl acetate
C_2E_2 200ppm polyphosphorylated ascorbic acid + 500ppm α-tocopheryl acetate

† TBARS (nmoles MDA equivalents/ mg muscle tissue)

6.4 Discussion

In this instance, investigation into potential tocopherol-ascorbate synergy shall be examined from the vitamin C perspective. In other words, dissemination of results from this study will be addressed primarily in terms of tissue ascorbate concentration. It is hoped that this will help to highlight the fairly one-sided relationship between the two antioxidant vitamins, with vitamin C status having more impact on tissue vitamin E than the converse, due to ascorbate being a 'first-line' antioxidant defense and vitamin E acting as a free radical reaction chain terminator; adequate tissue concentrations of vitamin C should prevent chain initiation, whereas even high tocopherol concentrations may not prevent free radical oxidant damage in the aqueous phase.

With respect to growth related indices, data obtained for feed efficiency (FE) are rather misleading. The observed improvement in feed utilization by catfish fed low dietary ascorbate may be accounted for by the low daily feed intakes resulting in greater utilization of the food ingested, as has been observed by Henken and co-workers (1985) in *Clarias gariepinus*. This may not have been the only influencing factor however, since the tocopherol supplemented/ low vitamin C diet (C_1E_2) possessed a surprisingly low FE value despite a low daily feed intake. Evidently, the fact that tissue weight has increased out of line with food ingested, reflects a deposition of large amounts of water within tissues of catfish from C_0E_1.

The classical scorbutic symptoms noted in fish fed an ascorbate deficient diet indicated the dietary essentiality of vitamin C for *Clarias* fry. Growth suppression was marked, as was the appearance of lordosis and scoliosis (horizontal or vertical spinal distortion respectively) in a number of individuals. These clinical signs are already documented in fish such as the channel catfish (*Ictalurus punctatus*)(Wilson and Poe, 1973; Lim and Lovell, 1978; Miyazaki *et al.*, 1985), coho salmon (*Oncorhynchus kisutch)*(Halver *et al.*, 1969), rainbow trout (*Oncorhynchus mykiss*)(Kitamura *et al.*, 1965; Halver *et al.*, 1969),

Asian catfish (*Clarias batrachus*)(Butthep *et al.*, 1983) and the African catfish (*Clarias gariepinus*)(Mgbenka, 1991).

Elevating the dietary ascorbate concentration to 25 mg ascorbate per kg (as assayed) was able to prevent all of the scorbutic effects over the twelve week trial, since tissue accretion increased with dietary dose. However, Mgbenka (1991) recorded pathologies in *Clarias* fed diets supplemented with 30 mg L-ascorbic acid per kg, though losses of this vitamin from the feed may have been severe since the unprotected crystalline vitamin is susceptible to oxidation and rapid leaching (Slinger *et al.*, 1979) and the diets in that study were wood-fire dried. It is unfortunate that assayed ascorbate levels were not cited in Mgbenka's investigation, thus comparison with the current results would appear impossible.

In the present study, the available vitamin C pool was boosted by increased dietary intake of the vitamin, since L-ascorbic acid represents the mobile vitamin C reserve.

The significant ($P < 0.05$) decrease in muscle and liver L-ascorbic acid concentration with increase in fish size may be explained in terms of the reduction of the proportion of lean tissue as a result of an increase in lipoid tissue in larger fish (Shearer, 1994), though in this study this can only be implied since proximate analysis was not undertaken. Decreasing the proportion of lean tissue may diminish the potential carrying capacity of the tissue with respect to the water-soluble vitamin. Regression models demonstrated that tissue vitamin C levels were regulated only by ascorbate intake and fish size. Models, allowing for tocopherol inclusion, were not accurate predictors of vitamin C status, suggesting that the dietary vitamin E levels used in the present study did not significantly affect tissue vitamin C levels. The possible sparing role of vitamin E on ascorbate (Hilton, 1989) was therefore not immediately evident in this experiment. Possibilities of some ascorbate sparing by vitamin E cannot be excluded however. Elevated dietary α-tocopherol inclusion resulted in increased tissue α-tocopherol levels as has been seen previously by

the author (Chapters 3, 4 and 5; Baker and Davies, 1996). In 60-80 g fish, this resulted in the values for liver L-ascorbic acid and total ascorbate being greater where vitamin C was limiting (C_1E_2), than in the treatment where lower levels of vitamin E had been furnished. Although this trend was not statistically significant ($P>0.05$), some further weak evidence in support of this theory was apparent when considering the liver ascorbate/ ascorbic acid differential from both fish size-classes, in fish fed adequate amounts of vitamin C. Increased dietary tocopherol may have resulted in subtly elevated deposition of bound-ascorbate (possibly as ascorbyl-sulphate, the supposed storage form of vitamin C (Tucker and Halver, 1984)). The mechanism for this being that vitamin E, being a non-specific antioxidant (albeit fat-soluble), is able to spare the tissue ascorbic-acid which is then surplus to requirement and therefore stored as ascorbyl sulphate.

The significantly higher muscle ascorbate/ascorbic acid differential in 80-100 g fish fed C_2E_2, would indicate the possible storage of ascorbate in a form other than L-ascorbic acid in the muscle tissues of these fish.

Furthermore, hepatic concentrations of the compound responsible for the ascorbate/ascorbic acid differences in low vitamin C treatments were not significantly different ($P>0.05$) from liver concentrations of L-ascorbic acid, whereas in other treatments liver L-ascorbic acid was far more abundant than the bound vitamer. Evidently, this may suggest that in low ascorbate diets, the bound form is conserved whilst mobile L-ascorbic acid is being continuously utilized, thus preventing the ascorbic acid concentration from increasing out of line with the bound reserves. In the vitamin C adequate diets the L-ascorbic acid concentration is able to increase due to repletion of mobile reserves through dietary means. One may postulate that at further elevated dietary ascorbate doses, increased deposition of ascorbate as a bound analogue may occur.

So far it has been tentatively suggested that, employing the current test diets, increased α-

tocopherol in the tissues of *Clarias* fry resulted in some degree of sparing of vitamin C. Ascorbate, however, was not able to significantly spare α-tocopherol in fish fed the experimental rations. This statement is based on the concentration of thiobarbituric acid reactive substances from muscle tissue of catfish from the different treatments. It is speculated that had vitamin E been spared by ascorbate, there would have been a detectable decrease in measurable TBARS resulting from either an efficient recycling of tocopherol by vitamin C, allowing enhanced prevention of lipid peroxidation, or that vitamin C would itself lower tissue-lipid peroxidation by direct antioxidant action at the membrane-cytosol interface. Indeed, Chakraborty *et al.* (1994) reported that vitamin C could lessen lipid peroxidation in guinea pig tissues. At the present dietary α-tocopherol concentrations used however, it is felt that little scope existed for a sparing effect since tocopherol was not limiting. In order to observe a tocopherol-sparing effect by vitamin C, it would probably be necessary to drop the dietary vitamin E concentration nearer to (or below) the requirement levels of the vitamin, since in the present investigation vitamin E is presented to the fish well in excess of the declared requirement for most species (NRC, 1993).

Future work on the ascorbate-tocopherol synergy must examine these antioxidants and their functioning at a molecular level. That is to say, research into oxidation of lipoproteins may offer a more accurate view of oxidant processes and the protective roles of vitamins C and E. At present, much of this type of work is carried out *in vitro* or *ex vivo*, where isolated lipoproteins are examined in artificial media in which metal-ion concentrations and levels of metabolites and nutrients are manipulated (see Frei and Gaziano (1993) for example). Ideally, oxidative condition of lipoproteins *in vivo* would be derived, though this would rely on arresting plasma oxidant status and extracting, isolating and assessing lipoproteins for peroxidative damage. This could then be correlated to numerous other measurable

parameters such as concentrations of antioxidant vitamins (C and E, and possibly β-carotene), antioxidant enzymes (glutathione enzymes, catalase, superoxide dismutase, etc.), pro-oxidant enzymes (lipoxygenase, etc.), metal-ions (iron, copper, manganese, etc), polyunsaturated fatty acids, as well as other aqueous antioxidants such as urea (Ma *et al.*, 1994) and bilirubin (Stryer, 1988). Although it is felt that the experimental approach outlined above is beyond the scope of the current research project, nutritional aspects of a pro-oxidant (iron) will be investigated at tissue level, in the next chapter.

CHAPTER 7

PRO-OXIDATION OF LIPIDS BY DIETARY IRON: THE IMPACT ON CATFISH GROWTH PERFORMANCE, AND TISSUE CONCENTRATIONS OF α-TOCOPHEROL AND MALONDIALDEHYDE (MDA).

7.1 Introduction

Peroxidation of tissue lipids can occur by numerous mechanisms, broadly classified as either metal-ion dependent or metal-ion independent (Frei and Gaziano, 1993). The involvement of transition metals in free radical mediated oxidation reactions has been recognised for a considerable period (Halliwell and Gutteridge, 1993). Of these metals, iron has attracted particular attention, due to its involvement *in vivo* with various oxygen transport mechanisms. Characteristically, iron is able to exist at different valencies, and this allows the ionic form to accept or donate electrons and thus participate in redox reactions including those involved in oxygen carriage. Additionally, iron may be liganded to various proteinaceous matrices to produce metallo-enzymes, which may further catalyse oxidation processes.

Iron is of particular importance in cellular respiration through its redox activity and associated electron transport. Iron exists in the body mainly in complex form bound to proteins such as haem compounds (haemoglobin or myoglobin), as haem enzymes (mitochondrial and microsomal cytochromes, catalase and peroxidase), or as non-haem compounds (transferrin, haemosiderin and ferritin) (Underwood, 1971). The non-haem proteins haemosiderin and ferritin are used as a means of storing iron in the spleen, liver, kidney and bone marrow (Underwood, 1971). Transferrin is the principal carrier of iron in the blood and regulates the distribution of iron within the body.

It has been demonstrated that dissolved iron can be taken up by fish via the gills (Steffens,

1989) though little is known about the precise mechanisms of dietary iron absorption through this route. It is believed that most iron is absorbed by the intestinal mucosal cells where iron is then converted to ferritin. Once the cells become saturated with ferritin, further absorption is inhibited until iron is released from ferritin and transferred to the plasma. It has been documented that most of the iron absorbed remains in the mucosal cells which are lost to the gut when sloughed from the intestinal villi (Underwood, 1971). Supplemental ascorbic acid in the diet is also known to increase iron absorption especially under conditions of iron deficiency (Underwood, 1971). This may be due to ascorbate's reduction of ferric ions to the ferrous state. Indeed, Lall (1979) documented that in fish, ferrous iron (Fe^{2+}) is absorbed more readily than ferric iron (Fe^{3+}).

Chronic iron toxicity is not commonly observed amongst fish since iron does not usually reach sufficiently high levels in the environment. However, toxic levels of iron in the aquatic environment can arise from coal mining as a result of water percolating through the exposed rocks and leaching the heavy metals (Gonzalez *et al.*, 1990). Recently it has also come to the author's attention, that hatchery-water from beach bore-holes may also be high in iron, although at present no literature has documented associated problems. Chronic iron overload in humans has also been related to genetic defects such as hereditary haemochromatosis, to metabolic alterations such as defective erythropoiesis, chronic liver disease or as a result of blood transfusions (Thurnham, 1995; Omara and Blakley, 1993). Symptoms of dietary iron overload have been reported in fish. Desjardins *et al.* (1987) working with rainbow trout (*Oncorhynchus mykiss*) observed suppressed growth, poor feed utilisation, feed refusal, diarrhoea, increased mortality and histopathological damage to hepatocytes. Omara and Blakley (1993) have also shown that excess dietary iron in mice causes the accumulation of iron in the liver resulting in an increase of the relative liver weight. Most notably however, it is well known that iron can enhance lipid peroxidative

damage of tissues through free radical mediated chain reactions, with long-term consequences to health in humans and animals.

Iron is able to initiate peroxidative processes through interaction with non-radical species, as has been well observed at normal partial pressures of oxygen *in vitro* (Pokorný, 1987), within meat products (Decker and Hutlin, 1992) and *in vivo* within the rat (Bristow *et al.* (1991).

By way of the Fenton reaction, ferrous iron causes production of the reactive hydroxyl radical from hydrogen peroxide.

$$H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^- + Fe^{3+}$$

Alternatively, via the Haber-Weiss reaction, superoxide anions and hydrogen peroxide may react to form hydroxyl radicals in the presence of a transition metal catalyst such as iron.

$$O_2^- + H_2O_2 \xrightarrow{\text{iron-salt catalyst}} O_2 + \bullet OH + OH^-$$

Hydroxyl free radicals, though in the aqueous phase may initiate lipid peroxidation deep within biomembranes through an intermediary protein radical (Halliwell and Gutteridge, 1993).

It is evident that tissue α-tocopherol would combat these effects due to the radical trapping action of the molecule, though as a consequence vitamin E would be depleted. It may therefore be stated that dietary iron is an antagonist to vitamin E status by its pro-oxidative mechanism and as such, this study shall evaluate the impact of nutritional iron on tissue iron levels, vitamin E concentration and concentrations of malondialdehyde in tissues of the African catfish. This of particular relevance when considering that *C.gariepinus* is typically cultured in earth-ponds, and these may be high in dissolved iron content.

Additionally, catfish may consume mud-burrowing organisms to supplement their diet, with incidental associated silt consumption, and therefore further metal loading.

7.2 Materials and Methods

7.2.1 Reagents

All reagents used were ANALAR grade obtained from Sigma Chemical Company Limited, Poole, Dorset, U.K., and Merck Ltd., Poole, Dorset, U.K.. All-rac-α-tocopheryl acetate (Rovimix E-50 SD) was a generous gift from F. Hoffmann- La Roche, Basel, Switzerland. All other vitamins were kindly donated by Colborn-Dawes, Heanor, Derbyshire, U.K.

7.2.2 Fish stock, experimental facilities and diets.

African catfish, *Clarias gariepinus*, juveniles were spawned in-house (according to section 2.9) and grown to approximately 30g, on commercial trout feeds, prior to commencement of the trial. Forty juveniles of mixed sexes and of mean weight 32.25 $\pm$ 0.64 g (mean $\pm$ s.e.) were stocked into two tanks of the culture facility described in section 2.2.2.

A low-tocopherol, basal practical diet was formulated as described in section 2.1.1, with the following modifications.

Lipid was delivered in the form of a cod-liver oil: corn oil mix (1:1) at a level of 6% providing a sufficient balance of n-3 and n-6 fatty-acids for this species.

The constituents and respective dietary inclusion levels for the basal diet are presented in table 7.1 and dietary manufacture was carried out as detailed in section 2.1.2.

Twenty-five grams of crystalline iron sulphate heptahydrate ($FeSO_4.7H_2O$) were added to the experimental diet at the expense of cornstarch and dextrin yielding a ferrous iron supplement of 5000mg kg^{-1}. The amount of iron (Fe) in the control diet, present in the mineral premix and bloodmeal was adequate to provide a nutritionally balanced diet, according to requirements for warm-water fish (NRC, 1993). For ease of identification, diets were allocated nominal codes of 'control' and 'ferrous' reflecting supplemental crystalline iron supplementation.

Compared to calculated values, proximate analysis of the finished test diets confirmed the

values of macronutrients in the feeds. These data are presented in table 7.2.

Dietary vitamin E dose (supplied as all-rac-α-tocopheryl acetate) was set at 100 mg kg^{-1} dry diet, a level selected to be above the minimum requirement for this species. HPLC determination of α-tocopherol content, displayed in table 7.3 confirmed the actual values of α-tocopherol in the diets to be very close to that intended. Additionally, total iron concentrations were determined as described in section 2.4.4 and also reported in table 7.3, revealing that control diets contained 663.53 μg Fe g^{-1} and that iron dosed diets contained 6354.36 μg Fe g^{-1}. Supplemental iron sulphate resulted in slight oxidation of the diets prior to ingestion, although no differences existed between diets with respect to sensory properties (smell). Oxidative state was assessed by determination of malondialdhyde production in diets according to section 2.5.3 and values presented in table 7.3. It should be noted that this was measured on termination of the feeding trial, thus diets may not have differed initially.

7.2.3 Feeding regime and nutrition trial protocol

The *Clarias* were uniformly graded and assigned, 20 per tank, into two tanks, and fish weighed individually at the start of the feeding trial.

A food ration level of up to 2% body weight per day was employed, with three times daily feeding, this ration being calculated on a dry matter basis. All fish were weighed individually, weekly in order to determine feed totals for the proceeding week. On termination of the five week feeding trial, individual fish weights were recorded and six fish from each treatment were desanguinated by caudal venipuncture (section 2.6.1) in order to determine haematocrits (% packed cell volume). After centrifugation, plasma samples were frozen at -80^{0}C prior to further analysis. Samples of skeletal muscle, liver and heart were excised and also stored at -80^{0}C for determination of malondialdehyde and, in the case of liver, α-tocopherol.

7.2.4 Growth and nutrient utilization parameters

The following parameters were calculated (according to the formulae described in section 2.2.5) from data acquired from the feeding trial; percent change in mean body weight, specific growth rate (SGR) and feed efficiency (FE).

Additional to the final body weights on termination of the trial, liver weights were recorded in order to allow calculation of the hepatosomatic index (% contribution of liver weight to the total body weight).

7.2.5 Analytical methods

α-tocopherol was extracted and determined from diets and liver using the method of Buttriss and Diplock (1984) as outlined in section 2.4.1. Values were obtained from four duplicate extractions of diets (n=4) and six duplicate extractions of liver tissue per treatment (n=6).

Determination of malondialdehyde (MDA) was achieved upon following the procedure set-out in section 2.5.3. Six samples per treatment of skeletal muscle, liver and heart tissues were assayed accordingly.

Total iron in muscle, liver and plasma was determined by flame atomic absorption spectrometry, as directed by section 2.4.4. Six samples were analysed per treatment.

7.2.6 Statistical analysis

ANOVA ($P<0.05$) were performed in order to facilitate inter-treatment comparisons. Analyses were performed using the statistical software package 'Statgraphics'.

Table 7.1 Composition of practical test diets for the African catfish, *Clarias gariepinus*.

Ingredient	%inclusion	
Fishmeal (Chilean)	60.00	
Meat and bone meal	10.00	
Bloodmeal	2.00	
Cod-liver oil	3.00	
Corn oil	3.00	
Molasses	1.00	
Mineral premix*	5.00	
B-complex vitamin premix†	0.10	
Fat-soluble vitamin premix‡	0.05	
Macro-vitamin premix§	0.50	
all-rac-α-tocopheryl acetate	0.01	
	control	**ferrous**
iron sulphate heptahydrate ($FeSO_4.7H_2O$)	0.00	2.50
Corn starch:Dextrin (3:2)	18.00	15.50

* Mineral salt inclusion (g kg^{-1}dry diet):
Calcium orthophosphate ($CaHPO_4.2H_2O$) 12.000, Magnesium sulphate ($MgSO_4.7H_2O$) 4.8450, Sodium chloride (NaCl) 2.2800, Potassium chloride (KCl) 1.9000, Iron sulphate ($FeSO_4.7H_2O$) 0.9500, Zinc sulphate ($ZnSO_4.7H_2O$) 0.2090, Manganese sulphate ($MnSO_4.4H_2O$) 0.0960, Copper sulphate ($CuSO_4.5H_2O$) 0.0298,Cobalt sulphate ($CoSO_4.7H_2O$) 0.0181, Calcium iodate ($CaIO_3.6H_2O$) 0.0112, Chromic chloride ($CrCl_3.6H_2O$) 0.0048, Sodium selenite (Na_2SeO_3) 0.0025. Filler (α-cellulose) 27.6536.

† B-vitamin inclusion (mg kg^{-1} dry diet):
B1-Thiamin hydrochloride 50, B2-Riboflavin (feed grade-96%) 52.1, B6-Pyridoxine hydrochloride 40, Calcium pantothenate 100, Niacin 200, H2-Biotin (2%) 300, Folic acid (90%) 16.7. **Note** B12 added separately at 0.05 mg kg^{-1} dry diet. Filler (α-cellulose) 241.2.

‡ Fat-soluble vitamin inclusion (mg kg^{-1} dry diet):
A-Vitamin A palmitate ($1.7x10^6$ I.U. g^{-1}) 2, D-Rovimix D3-500 ($5 x10^5$ I.U. g^{-1}) 20, K-Menadione sodium bisulphite (51%) 80. Filler (α-cellulose) 398.

§ Macro-vitamin inclusion (mg kg^{-1} dry diet):
Inositol 200, Choline chloride 2000, Ascorbic acid 500. Filler (α-cellulose) 2300.

Table 7.2 Proximate composition of basal practical test diet (bracketed values depict theoretical composition as calculated from NRC (1993))

	Moisture (%)	Protein (% DM)	Lipid (% DM)	Ash (% DM)	Residual
Mean	5.96	47.01 [49.35]	11.64 [11.87]	16.04 [14.14]	19.35
S.E.	0.129	0.238	0.063	0.118	
n	12	8	12	12	

Table 7.3 Assayed α-tocopherol, total iron and oxidative state (MDA) in test diets

	Control	Ferrous
α-tocopherol (mg /kg DM)	102.20 $\pm$ 2.49 (n=4)	103.70 $\pm$ 1.79 (n=4)
iron (μg Fe/ g DM)	663.53 $\pm$ 56.35 (n=2)	6354.36b $\pm$ 70.25 (n=2)
MDA (μmoles g^{-1})†	0.93 $\pm$ 0.13 (n=3)	1.74 $\pm$ 0.01 (n=3)

† MDA measured at end of trial.
n= number of duplicate determinations per diet.

7.3 Results

From table 7.4, it is apparent that after 5 weeks of feeding, fish fed the 'ferrous' diet were significantly smaller than those fed the reference diet ($P=0.0284$). Accordingly, the iron supplemented diet was having a detrimental effect on specific growth rate and feed efficiency of fish, though these parameters were not subjected to statistical analysis due to the fact that they were derived from pooled mean treatment data.

Hepatosomatic index and haematocrit values were not significantly influenced by the dietary treatment ($P>0.05$) (table 7.5).

Iron concentration in livers, muscle and plasma of fish (table 7.6) were not significantly affected by dietary iron intake ($P=0.163$, 0.656 and 0.291 for liver, plasma and muscle respectively).

HPLC determination of malondialdehyde (MDA) in tissues (table 7.7) revealed there was significantly more MDA in the livers and hearts of fish fed high iron diets ($P<0.0001$ and $P=0.0174$ for liver and heart respectively). No significant difference was found in skeletal muscle MDA concentrations between the two treatments ($P=0.231$).

There was significantly less α-tocopherol in the livers of fish fed the iron supplemented diet than fish fed a control diet ($P=0.0003$) (table 7.8).

Table 7.4 Growth related performance and nutrient utilization of catfish fed experimental diets after 5 weeks.

Diet descriptor	Control	Ferrous	*
Mean initial weight (g)	33.35[a]	31.21[a]	±0.64
Mean final weight (g)	86.67[a]	73.52[b]	±4.64
% change in mean weight	159.88	135.56	
Specific Growth Rate ($\%d^{-1}$)	2.73	2.45	
Feed Efficiency	1.39	1.25	

* ± SEM (Standard error of their pooled means)

[ab] Within rows, values allocated different superscripts are significantly different ($P < 0.05$)

Table 7.5 Hepatosomatic indices (%) and Haematocrit of *Clarias* after 5 weeks of feeding test diets. Values are expressed as means of six fish ± s.e.

	Contol	Ferrous
Hepatosomatic index (%)	0.93 ±0.10	1.06±0.08
Haematocrit (%)	30.77±3.30	32.64±2.10

No significant differences exist between treatments ($P > 0.05$).

Table 7.6 Muscle, liver and blood-plasma iron concentrations resulting from feeding test diets for 5 weeks. Values are expressed as means of six determinations per treatment ± s.e.

Tissue	Control	Ferrous
Liver (μg Fe/g)	523.55 ± 71.04	745.43 ± 125.72
Muscle (μg Fe/g)	251.22 ± 10.02	238.77 ± 4.55
Plasma (μg Fe/cm^3)	59.80 ± 1.90	61.76 ± 3.72

No significant differences exist between treatments ($P > 0.05$).

Table 7.7 Concentration of malondialdehyde (MDA) in muscle, liver and heart of *Clarias* fed diets for 5 weeks. Values are expressed as means of six determinations per treatment ± s.e.

Tissue	Control	Ferrous
Muscle (nmoles/g)	$3.26^{a} \pm 0.43$	$3.98^{a} \pm 0.36$
Liver (nmoles/g)	$3.43^{a} \pm 0.21$	$8.35^{b} \pm 0.22$
Heart (nmoles/g)	$4.61^{a} \pm 0.78$	$7.86^{b} \pm 0.83$

[ab] Within rows, values allocated different superscripts are significantly different ($P < 0.05$)

Table 7.8 Assayed α-tocopherol in *Clarias* livers after 5 weeks of feeding test diets. Values are expressed as means of six determinations per treatment $\pm$ s.e.

	Control	Ferrous
Liver (μg/g)	$130.95^{a} \pm 15.92$	$43.86^{b} \pm 3.74$

[ab] Values sharing common superscripts are not significantly different ($P > 0.05$).

7.4 Discussion

It is apparent from the current data that dietary iron inclusion at levels well above those declared as requirements, had a significant impact on growth indices as well as on antioxidant status of *Clarias* juveniles.

The recorded growth suppression in catfish fed the high iron diet may have been expected since assayed dietary iron concentrations of over 6 g kg^{-1} were fed to the *Clarias* and this level is twenty times over the conservative figure quoted for red sea bream (150 mg kg^{-1})(Sakamoto and Yone, 1978). Even the currently employed basal diet contained twice as much iron as suggested by Sakamoto and Yone (1978), though no suppression in growth was observed in fish fed this control diet and fish performed well with respect to specific growth rate and feed utilization (FE). Consumption of the high iron diets led to comparatively poorer growth rates and decreased efficiency of food utilization, implying some degree of toxicity of iron at this high level. Indeed, investigations into toxic effects of iron to rainbow trout have demonstrated reduced growth as being symptomatic of chronic iron overload (Desjardins *et al.*, 1987). Although direct iron toxicity may be detrimental to growth in fish, a further possibility cannot be discounted. It is known that iron can instigate lipid peroxidation in oils (Pokorný, 1987) and that oxidised dietary lipids can suppress growth in *Clarias* (Chapter 5). It is therefore possible that the observed slight increase in dietary lipid peroxidation (measured as MDA) in the high iron diet led to a decrease in the nutritive value of the feed. This may result in feed refusal due to off-tastes (rancidity), leading to lowered feed intake, or in heightened assimilation of pre-formed lipid hydroperoxides (autotoxicity), presenting a source of exogenous free radicals.

Contrary to work on rats (Lucesoli and Fraga, 1994), mice (Omara and Blakley, 1993), rainbow trout (*O. mykiss*) (Desjardins *et al.*, 1987) and Atlantic salmon (*S. salar*) (Bjornevik and Maage, 1993), tissue iron levels were not seen to be significantly related

to dietary intake of the metal, demonstrating the possibility of efficient regulation of iron status by African catfish. This may be an adaptation to living in rivers in tropical climates, where environmental conditions may fluctuate markedly. Additionally, this may be advantageous in *Clarias* culture, especially since catfish are typically cultured in unlined earth-ponds (Haylor, 1990) and these may be subject to greatly fluctuating mineral loads. Iron concentrations of all tissues assayed did not differ significantly ($P > 0.05$) irrespective of dietary iron dose, indicating that either excess iron was not taken up from the gut, or that the body's iron pool was effectively regulated and surplus iron excreted. The present data suggest that even the basal diet was sufficient to saturate iron stores (transferrin). Had this not been the case, an increase in dietary iron would have resulted in further accretion of the metal into protein chelates, which would have promoted higher tissue iron concentrations. Regulation by intestinal mucosal cells cannot be discounted, although Singh and Ferns (1978) postulated that regulatory mechanisms involve an increased rate of loss rather than a reduced rate of uptake of excess metals. The hypothesis is further borne-out by results from the determination of malondialdehyde in tissues and by assayed levels of hepatic α-tocopherol.

Strict pre-digestive maintenance of the iron-pool within catfish would preclude possibility of heightened *in vivo* peroxidative challenge resulting from iron's involvement in Fenton or Haber-Weiss reactions (see section 7.1).

Catfish tissues from the current investigation, subjected to analysis for the lipid peroxidation product malondialdehyde (MDA), revealed higher concentrations of MDA, thus increased peroxidative challenge, in tissues from fish consuming the 'ferrous' diet. This was significant ($P < 0.05$) in the case of liver and heart tissue, though not quite for muscle ($P > 0.05$). Values of MDA concentration were always higher in liver tissue comparative to other tissues, since hepatic tissue is lipid-rich making the liver a target organ for lipid peroxidation. Additionally, iron may accumulate in this organ, bound

within haem-proteins and transferrin. The relative lack of response in skeletal muscle may have resulted from the decreased abundance of PUFAs within this tissue, and these findings are consistent with those of Desjardins *et al.* (1987). Indeed, in chapter 5, it was demonstrated that muscle tissue was less prone to oxidative fatty-acid changes than liver. Nevertheless, elevated feed-iron can be implicated in initiation of oxidative insult, even if it is subsequently removed from the organism. This transient lipid-damage instigation may also be responsible for modulating tissue α-tocopherol concentrations as is demonstrated by the significantly lower ($P<0.05$) hepatic α-tocopherol concentration from fish fed the diet high in ferrous sulphate. As already discussed in chapter 5 with respect to the pro-oxidative action of oxidised lipids, vitamin E may be oxidised pre-assimilation, upon hydrolysis of the acetate ester of tocopherol. Although this cannot be ruled out, it would seem more reasonable to assume that differences in α-tocopherol status were linked to a depletion of the vitamin resulting from its use in combatting the free radical mediated lipid attack. Similarly to chapter 5, calculation of an α-tocopherol tissue retention index further indicated inter-treatment differences. Re-expressing data as μg α-tocopherol per liver per μg α-tocopherol ingested, negated effects caused by differences in feed intake and yielded values of 0.027 and 0.0097 for 'control' and 'ferrous' diets respectively, indicating depletion of the vitamin E pool by increased iron uptake. Evidently this result emphasises the pro-oxidant capability of iron, since despite identical dietary tocopherol contents and allowing for varied feed intake, livers from high iron-fed fish contained much less of this antioxidant vitamin, and since the liver is the major storage organ for vitamin E, decreased hepatic α-tocopherol content reflects the vitamin E pool.

The α-tocopherol retention value for 'ferrous' diet agreed closely with the value obtained in chapter 5 for catfish on comparable treatments. Diet 'Fr-100' from chapter 5 and the present test diet were formulated identically (fresh oil, 100 mg kg^{-1} α-tocopherol) and this gave rise to values of 0.034 and 0.027 μg α-tocopherol per liver per μg α-tocopherol

ingested, respectively. The small difference between these values may be attributed to differences in fish size (137 g chapter 5, compared to 87 g current investigation) reflecting varied feeding duration and feed-level, and also to assayed levels of dietary vitamin E (102 mg α-tocopherol kg^{-1} diet in the current study, compared to 112 mg α-tocopherol kg^{-1} diet in chapter 5). This parameter appears relatively reliable however, and merits further attention for use as an index of vitamin E status, or alternatively oxidative status.

The current observations support the findings of Diplock *et al.* (1994), who performed *in vitro* evaluations of the effect of iron in liver cell fractions and observed consumption of vitamin E in preparations subjected to iron induced peroxidation, and production of malondialdehyde was seen to increase concomitant with this vitamin E depletion. Fukuzawa *et al.* (1987) observed the same effect in synthetic charged micelles. Furthermore, Kirchin *et al.* (1992) recorded this phenomenon *in vivo* in mussels (*Mytilus edulis*) exposed to the transition metal, copper.

In summary, levels of dietary iron considered toxic in most fish species, compromised antioxidant status and growth performance of juvenile catfish during a five week feeding trial, though no obvious pathologies were noted. This latter point may require histological confirmation at a later date. Lipid peroxidation in catfish tissues was heightened by dietary iron as demonstrated by increased production of malondialdehyde in several major organs and tissues. Additionally peroxidation resulted in the partial depletion of hepatic α-tocopherol, probably due to increased free radical trapping by the tocopherol molecule. The author believes that depletion of tissue α-tocopherol in fish by nutritional iron, has not been previously demonstrated. Further research into the role of vitamin E in preventing damage caused by nutritional iron overload in catfish is warranted.

It is hoped that the present study has served to emphasise the importance of dietary α-tocopherol in the modulation of free radical induced lipid peroxidation by pro-oxidant

metal species such as iron.

CHAPTER 8

GENERAL DISCUSSION

It has been the objective of this series of investigations to examine the antioxidant role of α-tocopherol in the nutrition of the African catfish, *Clarias gariepinus* Burchell. Dietary uptake of the vitamin by *Clarias* was established and furthermore all tissues assayed responded positively to vitamin E supplementation with respect to tissue concentrations of the vitamin. These findings have been in line with published works in terrestrial vertebrates (Asghar *et al.*, 1991; Engeseth *et al.*, 1993) and cultured fish species such as common carp, *Cyprinus carpio* (Watanabe *et al.*, 1970b), channel catfish, *Ictalurus punctatus* (Bai and Gatlin, 1993), Nile tilapia, *Oreochromis niloticus* (Satoh *et al.*, 1987), blue tilapia, *O. aureus* (Roem *et al.*, 1990), Atlantic salmon, *Salmo salar* (Hamre and Lie, 1995) and rainbow trout, *Oncorhynchus mykiss* (Boggio *et al.*, 1985).

Although the author could not establish any pathologies associated with feeding diets deficient (or extremely low) in vitamin E, a minimum requirement for *Clarias* was implied based on the prevention of lipid peroxidation within hepatic tissues of this species. Employing practical-type diets containing 12 % lipid (approx. 4.4 % PUFA >18:2 (estimated from dietary fatty acid profiles from chapter 5)), 2.3 mg selenium kg^{-1} diet and 500 mg ascorbic acid kg^{-1}, this was declared as 30-40 mg α-tocopherol kg^{-1} dry diet for juvenile catfish and agrees closely with many stated requirements for farmed fish (NRC, 1993). Although most cited requirement levels have been established on the basis of growth performance, the author's findings and criterion for declaration of the minimum α-tocopherol requirement are supportive of work by Cowey *et al.* (1981). It is felt that indices of lipid peroxidation may allow for accurate determination of vitamin E requirement and as such it would be of great interest if values obtained from growth

studies would correlate with determined values of hepatic TBARS, or MDA concentrations of sub-cellular fractions.

As already intimated, heightened tissue vitamin E status led to parallel increases in the stability of catfish tissues to lipid peroxidation (assessed by determination of TBARS). This was noted in hepatic and muscular tissues, but not in blood-plasma, though this phenomenon has been previously encountered (Duthie *et al.*, 1989). Suppression of TBARS formation in response to oxidant stress has obvious commercial implications in the quality of fish-flesh products destined for human consumption, since delaying the on-set of oxidative rancidity would lengthen the time that fish fillets could be stored. Additionally, protection of tissue polyunsaturated fatty acids *post mortem* can improve structural properties and eating quality of fillets. Indeed, elevated α-tocopherol supplementation into *Clarias* diets reduced the severity of post-thaw exudative moisture loss ('drip') as has been demonstrated in pork (Cheah *et al.*, 1995) and lamb (Monahan *et al.*, 1994). With respect to structural properties, it has already been established that vitamin E deficiency in fish may result in muscular dystrophy (Watanabe *et al.*, 1970a), and therefore increased supply of dietary α-tocopherol, in situations where vitamin E supply is sub-optimal with regard to free radical degradation, may confer benefits to muscle tissues in relation to muscle-fibre arrangement and density. It must also be realised that these factors will contribute to texture of fillets and this is important to the consumer. With modern microscopic and image analysis techniques, there exists a powerful tool to evaluate muscle-fibre dimensions and hence some indicators of flesh texture, and these may be employed in future studies examining the role of vitamin E in fish-meat quality. Coupled to appropriate staining and sectioning processes, these examinations may also reveal sites of lipid deposition and/or oxidation, and these too may prove useful evaluators of tissue condition.

Antagonism of the antioxidant defence system in *Clarias* by oxidised (rancid) dietary oil and by ingested iron was noted in the present studies. Both of these pro-oxidant nutrient classes caused appreciable depletion in hepatic tocopherol and were also responsible for increases in selective indices of peroxidation. Transition metals are known pro-oxidants both *in vitro* and *in vivo* (Halliwell and Gutteridge, 1993) and in the mussel (Kirchin *et al.*, 1992) oxy-radical generation by transition metals has been recorded to deplete tissue vitamin E to a certain extent. Tissue vitamin E levels are also widely reported to be depleted upon consumption of oxidised oils (Hung *et al.*, 1981; Cowey *et al.*, 1984; Stephan *et al.*, 1993).

In the case of iron, increased consumption of this metal was responsible for augmented levels of hepatic MDA (measured as MDA-TBA adduct by HPLC). Although determination of muscular-TBARS gave rise to paradoxical results on feeding oxidised-oil diets, gas chromatographic separation of fatty acids followed by mass-spectroscopic determination, revealed a compound with equivalent chain length 13.1 whose concentration increased with oil oxidation, and was modulated by elevated dietary α-tocopherol. This investigation also provided the first record of increased docosahexaenoic acid (22:6 n-3, DHA) in livers of fish, in response to feeding oxidised oil. Possible mechanisms are discussed in chapter 5, although it suffices to say that this phenomenon appears to be the result of increased production of this fatty acid. Again, dietary vitamin E was able to temper the production of DHA.

Before leaving this subject, it is necessary to state that possibility exists for oxidation of vitamin E within the intestinal lumen prior to digestion and this may influence availability of the vitamin for uptake. In addition, products of lipid peroxidation have been known to be assimilated in vertebrates (Draper *et al.*, 1984), thereby compounding the problem of interpretation, since measured levels of lipid peroxidation product may not equate to *in situ* peroxidation, but may relate to dietary concentrations of lipid degradation compounds.

Further work is merited within this field in order to establish the site of oxidation of free α-tocopherol. It is likely that this would require radio-isotopic studies to determine the fate of labelled α-tocopherol (or α-tocopheryl acetate) from a test diet containing rancid oil.

Due to the high dietary concentrations of α-tocopherol used in the study on ascorbate/tocopherol synergism, results did not indicate any significant vitamin C and E relationship. Although these two antioxidants are known to be mutually sparing *in vitro* (McCay, 1985), and when one of the nutrients is approaching deficiency *in vivo* (Chen and Chang, 1978; Chen *et al.*, 1980; Frischknicht *et al.*, 1994), only tentative statements could be made with respect to the current work. Namely, increased α-tocopherol in tissues of *Clarias* fry resulted in subtly elevated abundance of tissue ascorbate, thus implying some degree of sparing. A further consideration is that in the present work, ascorbate polyphosphate was employed as dietary source, therefore the vitamin existed mostly in the free-ascorbate form within tissues. Since ascorbic acid and tocopherol exist in aqueous and lipid phases respectively, physical constraints limit the extent to which these antioxidants may relate. To wit, reduction of α-tocopheroxyl radical by ascorbate relies on interaction at the membrane-cytosol interface. Recent work by Bruun-Jensen and co-workers (1995) has established that in processed meats, presence of a fatty acid ester of vitamin C, ascorbyl-palmitate, in place of ascorbic acid can increase the effectiveness of tocopherol's functioning with respect to TBARS suppression. An improved recycling of vitamin E by the ascorbyl-palmitate would account for these findings and this may be explained in terms of the fact that this molecule resides partially within the membrane, thus decreasing the physical distance between vitamins C and E. Although one may assume ascorbyl-palmitate to be the better feed-supplement (on comparison with ascorbic acid yielding vitamin C forms), it is necessary to consider extrinsic factors such as cost and feed-mill stability when making such judgements. Furthermore, since ascorbyl-palmitate may be hydrolysed

within the intestinal lumen prior to uptake, it may prove futile to employ this vitamin source since ascorbyl-palmitate may not reach the tissues intact. Perhaps delivery of the vitamin C-palmitate via an alternative route (*intra peritoneal, intra muscular*), or protection of the vitamin from digestive action may prove useful. The former is unlikely however, due to the practicality and stresses involved with repeated injections of the nutrient.

Until further investigations establish the relationship between vitamins C and E, it may be impossible to ascertain whether these antioxidants act synergistically due to a recycling mechanism or to merely additive effects.

In order to obtain an accurate overview of fish status with respect to these nutrients, it would be necessary to evaluate antioxidant processes at a molecular level. Assessment of concentration of a battery of antioxidants from plasma (or tissues) arrested at a particular state, would unravel the complex biochemical oxidative processes proceeding within biological material, and provide more realistic information than that currently being derived from isolated cell fraction work. Problems arise from the complexity of living systems with respect to antioxidation since interactions may occur within the fat-soluble class of antioxidants (tocopherols, retinols, carotenoids, ubiquinone, flavonoids), within aqueous phase antioxidants (ascorbate, reduced glutathione, bilirubin, uric acid, numerous enzymes) or between classes. Some progress is currently being made in this area since researchers are now beginning to examine the process of antioxidation holistically. For example, Chen and Tappel (1996) have evaluated the influence of supplementation of combinations of antioxidants on TBARS suppression in rat liver homogenates, and although the approach of these authors is still rather crude, it represents a significant step towards the ideal scenario postulated above.

Despite having established a sound framework of knowledge on α-tocopherol nutrition of

the African catfish, *Clarias gariepinus*, it is felt that many areas of research require further attention and some of these have been mentioned earlier. Additionally, the author has so far not examined the effects of size, sex or life-stage on vitamin E utilisation of this species and this needs clarification. As well as changes in carcass composition with ontogenic development (Shearer, 1994), specific requirements for vitamin E may change periodically in response to spawning demands, migratory behaviour and/or food type. Although the latter two points are of negligible concern in culture situations, where confined fish are manipulated so as not to spawn, the primary point must be addressed since growth stage almost certainly influences requirement for vitamin E. In Atlantic salmon, for example, fry were observed to require 60 mg α-tocopherol kg^{-1} diet (Hamre and Lie, 1995) although adult fish require half of this dose (NRC, 1993) to support optimal growth. This lends support to those who suggest that requirements ought to be expressed relative to body weight gain (amount of vitamin required to support 'x' g of growth) and may be valid where requirements for growth are established. In situations where response criteria other than growth are measured, coupling required vitamin doses to growth may be invalid due to the fact that attainment of the desired level of any parameter may have been reached in much shorter period than that time needed to demonstrate growth suppression. Put simply, in the current studies, hepatic-TBARS levels measured may have been attained relatively quickly due to rapid tissue vitamin E equilibration. If this had occurred over two weeks, then fish may only have grown by 20 g. However, had TBARS been determined at week 4, then fish would have grown by 50 g but possessed similar hepatic TBARS, and therefore requirement declared differently (*note*: these figures are fictional but illustrative). Although the current system of expressing nutrient requirements in terms of dietary concentration may not be ideal in situations where fish growth between studies is not comparable, it has provided some degree of uniformity of results between studies. As such this system is acceptable (if over-simplified) for the

purposes of evaluating optimal nutrient feed-concentrations for use in finfish culture. With respect to tissue deposition of nutrients, since duration of feeding is known to influence vitamin E status of channel catfish (Bai and Gatlin, 1993), and it may be assumed that other fish respond similarly, it would be of interest to examine the kinetics of α-tocopherol uptake in *C. gariepinus* in order to establish optimum feeding strategies for culture situations. When fillet quality is of primary importance to a producer, optimisation of dietary tocopherol feeding strategy may involve the supply of minimal doses of vitamin E to fish, until a short while before harvest. Then super-doses of the vitamin supplied over an intensive period may allow for satisfactory tissue deposition of the vitamin, yet minimise feed-production cost overall. Although this strategy is proven to work in channel catfish (Bai and Gatlin, 1993), it must also be noted that this would negate any possible health benefits accrued from a continued high vitamin E intake. In this respect, fish nutritionists await irrefutable evidence supporting the role of supra-supplementation of α-tocopherol in strengthening the immune response of fish.

Of the many factors affecting antioxidant nutrition in catfish, interactions between vitamin E and alternative antioxidant defences in *Clarias* tissues (such as reduced glutathione and associated enzymes) have not been covered within this thesis, and these are of obvious importance, especially when considering that compensatory mechanisms may exist with respect to enzymic removal of free radicals. Since the author states that this tropical air-breathing species may be resilient to oxidative stresses, as characterised by the absence of growth suppression in low tocopherol diets, a survey of antioxidant defenses of this species is warranted. Although preliminary work from this laboratory (unpublished) demonstrates the presence of glutathione-peroxidase in catfish tissues, no further research has been reported in this species. It would be of interest if the aforementioned enzyme, and other antioxidant systems, were present in higher amounts (or more active) in *Clarias* than in

other cultured fish species. In addition, it remains to be seen if dietary vitamin E supplementation would modulate these systems as has been observed previously in salmonids (Cowey, 1986).

Given that *Clarias* are cultured as a fresh-water species, they are subjected to a multiplicity of potential free radical initiating stressors including temperature fluctuations, variable water-flow (thus swim-rate) and ultra-violet radiation (resulting from shallow water culture, as in ponds). Elevated culture temperatures would necessitate increased respiratory rate, thus increased through-put of oxygen in fish. Evidently, this would also accelerate formation of oxy-radicals, with leakage of free radicals from specific pathways, leading to initiation of the lipid peroxidation cascade with far reaching consequences on cellular integrity and functioning, and thus tissue condition. In a similar manner, higher rates of exercise (swim-rate) would heighten metabolic activity and oxygen consumption, thus once again applying an oxidant stress to the fish. The mode of action of U.V. light is quite different from the two aforementioned stressors. Fish cultured in shallow ponds would be affected by incident U.V. light, this causing fragmentation of certain molecules, potentially giving rise to two reactive free radicals. Other potential sources of free radicals include any redox reaction involved in modifications of substrate for growth and energy requirements. As such, the provision of high energy diets to cultured fish may not only cause increased carcass fat deposition, but may also augment incidental leakage of lipid peroxy radicals (for example) and this may stimulate the lipid peroxidation cascade. Generation of exogenous free radicals may contribute to oxidative processes within fish tissues. As it is known that vitamin E functions non-specifically to trap damaging radicals, and that many aquatic-pollutants exert free radical damaging effects, a further area of interest in today's climate of environmental concern is the impact of toxicants on cultured fish health status. Chapter 7 may be seen to provide evidence that metal pollution will

affect tissue vitamin E levels and demonstrates that determination of this vitamin in fish tissues may provide information on pollution levels within systems. It should be noted that such information could only be supported by assayed levels of the vitamin far from expected values, since slight deviations from the norm may result naturally. Strictly speaking, use of vitamin E as a biomarker is highly unlikely because of the variability in tissue tocopherol concentrations between individuals in the wild (per. obs.). In controlled situations where vitamin E intakes are known however, such as in aquaria, differences between treatments could be attributed to the effect of an applied pollutant, and as such compounds could be tested for their likely impact on the antioxidant status of fish.

As outlined above, under conditions of aquaculture related stress, so-called optimum nutrient requirements may well satisfy growth and performance criteria but fall short of inclusion levels associated with maximised product quality and health. Only a broader understanding of the factors modulating tissue antioxidant status will lead to the production of effective, cost-efficient diets for cultured fish species. Despite the fact that presently, intensive *Clarias* culture relies on the feeding of salmonid type diets, there exists scope to develop specialised feeds for this species, and formulation of these diets will benefit from continued research in catfish nutrition. Additionally, knowledge obtained in the field of *Clarias* nutrition may be applied to other species of finfish and this is especially true with respect to vitamin E, since the vitamin functions non-specifically in free radical degradation.

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