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THE WATER-SOLUBLE VITAMIN NUTRITION OF THE GILTHEAD SEABREAM (*Sparus aurata* L.)

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**THE WATER-SOLUBLE VITAMIN NUTRITION OF
THE GILTHEAD SEABREAM (*Sparus aurata* L.)**

By

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

Doctor of Philosophy

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ABSTRACT

The Water-Soluble Vitamin Nutrition of the Gilthead Seabream (*Sparus aurata* L.)

By

Paul Christopher Morris

Given the paucity of information with regards the micronutrient requirements of the currently expanding number of cultured marine species, a series of investigations was undertaken to examine the water-soluble vitamin nutrition of the gilthead seabream (*Sparus aurata* L.). A preliminary study identified the qualitative requirement of this species for ascorbic acid, the B complex vitamins and choline for the maintenance of health and optimum productivity. Based on the observations of the preliminary experiment, a study was designed which allowed the development of a semi-purified diet and illustrated the qualitative requirement of the gilthead seabream for thiamin, niacin, riboflavin, pyridoxine and pantothenic acid. Using the semi-purified diet, the requirement for niacin and biotin were also established. As one of the vitamins for which the seabream exhibited a strong requirement, pyridoxine was chosen as the focus for an experiment which evaluated the benefit of the incorporation of this vitamin within a practical diet at levels which exceeded the minimum requirement. Thiamin is a vitamin which is known to exhibit a distinct relationship with carbohydrate metabolism, hence this vitamin was chosen as the focus of an investigation of the potential for the modification of vitamin requirements in response to major nutrient supply. Finally, having illustrated the requirement of gilthead seabream growers for ascorbic acid, an experiment was carried out to evaluate the efficacy of a series of vitamin C derivatives as feed supplements for this species. The current series of investigations has not only served to expand the present knowledge of the vitamin nutrition of the target species but has highlighted the potential for novel and applied research which remains within the field of micronutrients.

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Author's Declaration

I hereby state that this thesis has been composed by myself and is the result of my own investigations. It has neither been accepted or submitted for any other degrees. All the sources of information within this volume have been duly acknowledged.

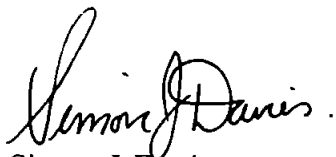
The husbandry and experimental work was performed in accordance with the Animals Scientific Procedures Act 1986 under the following Home Office license arrangements:

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Paul Christopher Morris.



Simon J Davies.
Principal Supervisor.

Chapter 1

General Introduction

1.1 Aquaculture

In 1991 the global output from aquaculture reached 16.6 million tons with a value of US\$ 28.4 billion therefore accounting for 15 % of the global aquatic harvest. Of this, the production of fish and shellfish was 12.7 million tons, representing a doubling in output since 1986. The 8.74 million tons of finfish produced in 1991 had a market value of US\$ 16.14 billion (Fish Farming International, 1993). These figures outline the importance of aquaculture in both the provision of food and its place in the world economy.

The demand for higher yields and faster growth in aquaculture has catalysed a rapid evolution in this field. Thus, emphasis has shifted from simple culture systems in which the fish rely on the natural food within the culture facility, towards supplementation of the natural feed supply in pond culture and the provision of nutritionally complete feeds for use in the intensive culture of fish (Lovell 1989). The latter produces the greatest yield of fish for a given volume of water and effort on the part of the fish farmer but relies on the provision of expensive, nutritionally complete feeds (Lovell 1889) and represents a considerable portion of the overall expenditure of most aquaculture units.

1.2. Overview of Aquatic Animal Nutrition.

A nutritionally balanced feed must, by definition, contain all the nutrients required by the fish for the provision of energy, growth and the supply of cofactors for

the utilisation of nutrients. The evaluation of the general principles of nutrition and the nutritional requirements of all domesticated species has been a fundamental objective since the maintenance of livestock began and hence, it is logical that similar efforts are directed towards the increasing number of cultured aquatic species.

In order to satisfy one of the primary objectives of animal production *ie.* optimised biomass gain for a given expenditure, many fish feed manufacturers provide a series of tables which delineate the amount of feed required for a given increase in live weight for the commonly cultured species. For example, well documented strategies defining optimised feeding rates in response to a wide range of variables such as feeding frequency (Grayton and Beamish 1977) are now well established for salmonid fish.

In terms of diet formulation, considerable attention has been directed towards feed quality and the selection of the raw materials and ingredients incorporated into feeds for commercial aquaculture. Together the raw materials furnish the essential nutritional components including proteins and amino acids, lipids and to some extent carbohydrates. Protein and amino acids are required for tissue repletion, growth and the provision of energy. The lipids provide non-protein energy and essential fatty acids for the maintenance of health and development while to varying extends the carbohydrates are used as a caloric source. In addition, the micro nutrients are deemed to be essential components in a nutritionally complete feed and these include the minerals and vitamins which are essential for the maintenance of optimum growth, feed efficiency and health. Inadequate micronutrient supply may be manifested in terms not just of sub-optimal growth but also as poor general health and clinical pathology (Cowey and Sargent 1972).

The research emphasis over the last two decades has focused on the specific requirements of fish for the major nutrients. Although critical views have been expressed

with regards the use of dietary concentration terminology *eg.* percent or weight of nutrient material in a given amount of feed, this approach is still in widespread use. The alternative approach is the expression of nutrient requirement with respect to the characteristics of the target species such as amount of nutrient required by a given weight of animal with respect to dietary energy level (Jobling 1983).

In keeping with the more commonly applied approach to nutrient requirements, it is generally accepted that most fish have a protein requirement in the region of 30 - 55 % of the ration dependent on developmental status. However, it should be noted that, as with other animals, fish do not have a true protein requirement but have a requirement for a balanced profile of essential and non-essential amino acids (Wilson 1989). In the main, the same ten amino acids are described as indispensable for most fish species and hence, must in some form or other, be included in the diet. The indispensable amino acids include; arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Ketola 1982).

As mentioned previously, the dietary lipids are essential for the provision of essential fatty acids (EFA), as a source of energy and in addition, are frequently utilised as carriers for the fat-soluble vitamins (NRC 1993). Fish may derive upto 85 % of their energy requirement from protein (Smith 1989) and an increasing volume of work has outlined the potential of dietary lipid as an energy source thus sparing an increased portion of ingested protein for growth. Consequently, the use of lipid as a source of energy in feeds for fish has been given great importance and as a result the dietary lipid content of fish feeds often reaches 30 % of the complete formulation. All animals have a capacity to synthesise fatty acids *de novo* to some extent but, in common with the amino acids, certain fatty acids are considered essential. Thus, the requirement of many

fish species for (n-3) poly unsaturated fatty acids (PUFA) have been established though the essentiality of the n-6 series remains to be quantified (Sargent *et al.* 1989). Unlike the amino acids, the fatty acids considered to be essential vary on a species by species basis and consequently the fatty acid requirements have been determined separately and are quoted in works such as NRC 1993. Hence, the lower limit for lipid supplementation in fish diets is determined by the supply of essential fatty acids as demonstrated for the rainbow trout (*Oncorhynchus mykiss*) by Yu and Sinnhuber (1976).

Carbohydrate may be utilised by most species of fin fish as a caloric source though the extent to which such materials may be included in the diet depends on the complexity and digestibility of the material (Cowey and Walton 1989) and varies on a species by species basis. Thus, a prime example of this variation was observed by Furuichi and Yone (1980) who recorded the ability of (*Cyprinus carpio*) to tolerate the incorporation of dextrin in the diet to levels of 30 - 40 % while red seabream (*Chrysophrys major*) and yellowtail (*Seriola quinqueradiata*) could only tolerate 20 and 10 % respectively. The pathology associated with the incorporation of excess starch in the diet of fish has been well documented and in the main is associated with hepatic glycogen accumulation and poor growth as demonstrated in the rainbow trout (*Oncorhynchus mykiss*) by Hilton and Atkinson (1982). Careful use of the carbohydrates allows their incorporation in the diets as a low cost filling ingredient which typically constitutes 10 to 30 % of the formulation having both a protein sparing effect (Degani and Viola 1987) and acting as an aid to binding in pelleted and extruded feeds.

The materials used in the manufacture of feeds for fish were classified in a review by Hardy (1989) into two broad classes. The first class of animal feed materials are the low value by-products of food production for human consumption which remain after the

high value materials have been sequestered. Thus, blood meal and meat and bone meal represent this class of feed stuffs being the by-products of the meat industry. The second class of feed materials may be produced directly for consumption by fish and higher vertebrates and include materials such as herring and anchovy meal (Pike *et al.* 1990).

While providing suitable and well characterised sources of the major nutrients, the provision of micro nutrients from the feedstuffs is highly variable and subject to major fluctuation in response to conditions of both supply and manufacture of the finished product effecting both the bioavailability and activity of the micronutrients. Consequently, the micronutrients are added to the feed in the form of vitamin and mineral pre-mixes which supplement the variable contribution from the component feedstuffs.

Despite the varied abilities of fish to derive minerals from the surrounding water, the minerals are essential nutrients in the diets of fish and their absence results in the manifestation of a wide variety of clinical pathologies (Lall 1989). In the main, the minerals are added to the diets in the form of pure chemicals which are combined into a pre-mix typically constituting between 1 and 5 % of the total diet formulation. The supplement is added at the time of manufacture and in general, the mineral pre-mix maintains its integrity and availability during feed manufacture (Hardy 1989).

The second group of micronutrients is represented by the vitamins. This group of micronutrients is highly diverse in all respects though a series of common features unite the vitamins. From an economic perspective, they are expensive to produce and hence, on a cost per unit weight basis, represent the most expensive components of the feed. However, since the entire vitamin input accounts for less than 3-5 and 5-7 % of the cost of fry and grower diets respectively, the majority of expenditure is accounted for by the bulk ingredients. The vitamins are seen as the most labile components of the feed and

thus losses of vitamins during feed manufacture have been the focus of a considerable research effort. Additionally, the vitamins frequently play key roles in intermediary metabolism and this forms the basis of the essentiality of these micronutrients.

Compared to the wealth of research which has been carried out to investigate the requirements of fish for the major nutrients, very little attention has been paid towards the vitamins. In the main, the research emphasis has been placed on vitamins C and E which have clearly identified roles in nutrition and in deficiency, frequently resulting in the appearance of characteristic pathology as reviewed by Halver (1989).

1.3. The Vitamins.

In summary, the vitamins are essential micro-nutrients which themselves are not utilised as metabolites for the provision of energy or as components in the construction of tissues but instead, act in synergism with the macronutrients often acting as cofactors within a wide diversity of biochemical pathways.

The vitamins are broadly classified according to their interaction with solvents, as either fat or water-soluble. The fat soluble vitamins are represented by the vitamins A, D, E and K and may be absorbed from the intestine along with dietary lipids (NRC 1993). Excess fat-soluble vitamins may be stored in the tissues when supply exceeds metabolic need and hence may lead to hypervitaminosis (Halver 1989). Unlike the water-soluble vitamins, none of the fat-soluble vitamins are associated with roles as coenzymes in metabolism, instead the vitamins in this group play well defined roles in an array of processes such as calcium homeostasis (vitamin D) and blood coagulation (vitamin K) (Cho *et al.* 1985). As a consequence of the role of vitamin E as an anti-oxidant and its interaction with the immune response, this vitamin has been the focus of considerably

more attention in fish nutrition research than the remaining fat soluble vitamins.

The larger division within this class of micronutrients comprises the water-soluble vitamins. A single sub-division exists within the water-soluble vitamins which contains a group of eight vitamins termed the B complex which are included in this group merely due to their abundance in materials of a similar nature. There is no further means of classification of the vitamins indeed; the immense diversity of structure, function, chemical and biological properties preclude the use of any rigid system of classification. The water-soluble vitamins are represented by the eight B complex vitamins (thiamin, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid and cyanocobalamin), ascorbic acid, choline and inositol. The water-soluble vitamins have a vast array of functions and unlike the fat-soluble vitamins, many of the water-soluble vitamins have clearly defined functions as coenzymes in intermediary metabolism. In addition, hypervitaminosis has not been recorded in fish maintained on high levels of water-soluble vitamins.

Presented here is a brief outline of the basic chemical nature and function of the water soluble vitamins. This has been compiled as a resumé of the reviews of McDonald *et al.* (1985), Halver (1989), Tacon (1991), Bender (1992) and NRC (1993) and is included simply to clarify aspects of the nature and function of the vitamins which will be raised in subsequent chapters. Hence, for more detailed descriptions of such matters the reader is referred to the said texts.

1.3.1 Thiamin

Thiamin is a water-soluble compound based on a pyrimidine and a thiazole ring as illustrated in Figure 1.1. Thiamin hydrochloride is a colourless, monoclinic crystalline, compound, which is heat tolerant and stable in acids but rapidly decomposed in neutral or alkaline solutions. Various derivatives of thiamin exist which appear to be more soluble in alkaline solution while maintaining biological activity *eg.* benzoylthiamin disulfide and dibenzoylthiamin. Thiamin hydrochloride and thiamin mononitrate are frequently used as vitamin supplements in animal feeds.

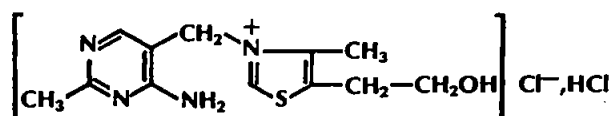


Figure 1.1: The structure of thiamin hydrochloride.

In animals, thiamin pyrophosphate (TPP), also referred to as thiamin di-phosphate (TDP) accounts for 80 % of the thiamin found within animal cells. Also present in smaller amounts are thiamin monophosphate (TMP) which accounts for around 15 % of the total thiamin content and thus the remaining portion is accounted for by the tri-ester, thiamin tri-phosphate (TTP) and free thiamin. Thiamin tri-phosphate plays an as yet ill defined role in nervous transmission while thiamin di-phosphate (TDP) is a key participant in intermediary metabolism acting as a coenzyme in glycolysis, the citric acid cycle and the pentose phosphate pathway.

In glycolysis, thiamin pyrophosphate participates in the decarboxylation of pyruvate to yield acetyl-CoA therefore committing the products of glycolysis to the TCA

cycle. TPP also plays a role within the TCA cycle as a participant in the oxidation and decarboxylation of alpha-ketoglutaric acid to yield succinyl-Co-A. Within the pentose phosphate pathway, TPP is active as the coenzyme of transketolase which catalyses the transfer of C_2 fragments and hence may lead to the formation of pentoses from hexoses and hence nucleotides via the intermediate ribose-5-phosphate or the formation of triose phosphates which may be utilised by glycolysis.

Thiamin is widely distributed in a variety feed materials. Rich sources of thiamin include brewers yeast, wheat middlings and bran, rice polishings and cotton seed meal. Animal sources include; egg yolk, kidney and pork muscle and dried fish solubles. Care is required when preparing diets as thiamin is easily hydrolysed under alkaline conditions. Well sealed dry pelleted feeds are usually stable but moist rations, especially those based on raw fish, have lower stability due to the presence of the vitamin antagonist thiaminase which inactivates the vitamin by damage to the thiazole ring. Such moist rations should be used quickly and cooking of fish based products will reduce thiaminase activity.

The symptoms associated with thiamin deficiency stem mainly from the importance of this vitamin in intermediary metabolism and hence are the result of the accumulation of metabolic intermediates. In animals deficiency symptoms include reduced appetite, emaciation, muscular weakness and dysfunction of the nervous system. The cause of muscular weakness is an accumulation of pyruvic and lactic acids resulting from impaired metabolism of key metabolites. Neurological damage may also become apparent due to the high requirement of neural tissue for carbohydrates. The metabolism of which is impaired during B_1 deficiency. The specific deficiency symptoms associated with fish are discussed in chapter 3.

1.3.2 Riboflavin

Riboflavin is a yellow coloured crystalline compound and consists of a dimethyl-isoalloxazine nucleus bound to ribitol. The vitamin is sparingly soluble in water and when in solution it exhibits a yellow-green fluorescence. Riboflavin is insoluble in most fat solvents but is soluble in alcohol. Under acid or neutral conditions the vitamin is heat stable. Riboflavin is inactivated under alkaline conditions and in the presence of U.V. yields the breakdown product lumiflavin.

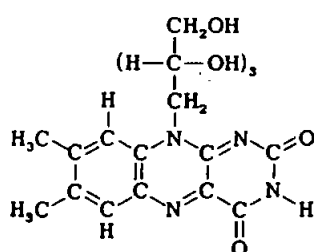


Figure 1.2: Structure of Riboflavin:

Riboflavin is a constituent of a group of proteins termed the flavoproteins and unlike many cofactors, the binding between the flavin coenzymes and the enzyme is continuous *ie.* after the catalysis the enzyme and cofactor remain bound. The flavoproteins are usually active as flavin mono nucleotide (FMN) or as flavin adenine di-nucleotide (FAD). The flavoproteins are involved in metabolism as electron carriers and may undergo reduction by either a single or double-electron step. Consequently, via enzymes such as cytochrome-C reductase, the flavoproteins participate in a wide variety of redox reactions and hence are central to many energy yielding processes including the mitochondrial transport chain. Riboflavin and pyridoxine cooperate in the conversion of tryptophan to niacin and vitamin B₂ is involved with the operation of the visual pigments.

In the main, riboflavin is present in most biological material bound to its specific

binding protein. This vitamin is synthesised by all green plants, yeasts, fungi and bacteria. However, the Lactobacilli are a notable exception and their inability to synthesise riboflavin forms the basis for the bioassay for this vitamin. Cereals provide a poor source of riboflavin while yeasts, leafy crops and soya are also rich in riboflavin. Milk and eggs contain relatively large amounts of free riboflavin while liver and lung meal and dried fish solubles are also sources of abundant riboflavin. However, deficiency syndromes may be commonly seen amongst domestic animals since their diets are composed mainly of cereals which contain little riboflavin.

UV. irradiation will promote the conversion of riboflavin to lumiflavin with resultant inactivation of the vitamin. Therefore diets must be stored under dark conditions. The exposure of the diet to light during manufacture does not usually result in significant vitamin inactivation provided that the riboflavin has a reasonable particle size.

1.3.3 Pyridoxine

The term vitamin B₆ (pyridoxine) refers to a series of vitamers which include pyridoxine, pyridoxamine and pyridoxal, the latter of which is the active form within metabolism and the vitamer to which the other forms may be converted. Pyridoxine is sensitive to damage by ultra violet irradiation while pyridoxal and pyridoxamine are labile to air, light and heat. Pyridoxine hydrochloride represents a stable form of these vitamers being readily soluble in water and thermo-stable in acid or alkaline solution, hence pyridoxine hydrochloride is commonly used as an animal feed supplement. Pyridoxine hydrochloride is freely soluble in water and is stable in acid or alkali solution.

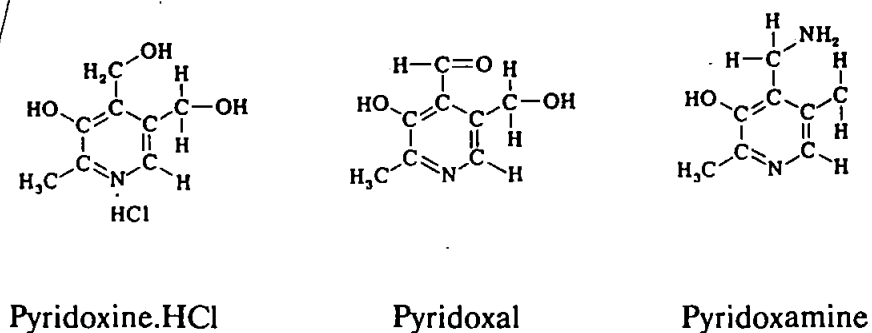


Figure 1.3: The structure of the B₆ vitamers.

Within nutritional biochemistry the function of pyridoxine is highly diverse due to the participation of this vitamin as a cofactor in the metabolism of proteins, fats and carbohydrates. The role most commonly associated with B₆ is in transamination as coenzyme co-decarboxylase which acts as a cofactor for the decarboxylation of amino acids. Pyridoxine is essential in the metabolism of glutamic acid, lysine, methionine, histidine, cysteine and lysine. Central to intermediary metabolism is the role of pyridoxal in the phosphorylation of desulphhydrase an enzyme catalysing the conversion of cysteine to lactic acid. Additionally B₆ is associated with the metabolic pathways of gluconeogenesis, while the role of pyridoxal-5-phosphate as a cofactor for glycogen phosphorylase has been recently evaluated. The metabolism of essential fatty acids and mRNA synthesis are both believed to require some input from this vitamin. Pyridoxine also acts as a cofactor in the synthesis of several neurohormones *eg.* the synthesis of serotonin via the decarboxylation of 5-hydroxy tryptophan, and it is also believed that this vitamin plays a role in steroid hormone receptor recycling.

Pyridoxine is widely distributed among most feed materials and rich sources of this vitamin are yeast, dried delactose whey, dried fish solubles wheat mill run and sunflower seed meal.

In animals, the general symptoms of deficiency are impaired amino acid metabolism causing changes in growth rates. The symptoms associated with a deficiency of this vitamin in fish are discussed in chapter 3 and the requirements of fish for B₆ are discussed in chapter 5.

1.3.4 Niacin

The generic term niacin is applied to nicotinic acid and its derivatives. This group of compounds includes three forms; niacin, nicotinamide (the biologically active form) and tryptophan which is an amino acid which may be utilised by some species as a niacin precursor.

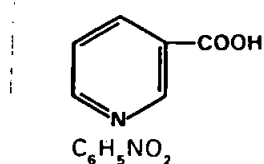


Figure 1.4: The structure of the niacin:

Niacin is a white crystalline odourless solid, soluble in water and alcohol. This vitamin is stable and is not easily inactivated by light, humidity, heating, acids, alkalies or oxidation and hence retains a large degree of activity during feed processing. As an acid, niacin reacts with metals and alkalies to yield salts.

Nicotinamide forms the basis of the coenzymes nicotinamide adenine di-phosphate (NAD) and its phosphorylated derivative (NADP) which function in the metabolic transfer of hydrogen. Hence niacin is central to the metabolism of proteins, fats and carbohydrates.

Niacin is involved in a large number of dehydrogenase reactions, for example, as a component of NAD and NADP, niacin participates in the removal of hydrogen from substrates and the transfer of hydrogen or electrons to another coenzyme in an electron or hydrogen transport chain. Such reactions are usually carried out via redox alternations between NAD and NADH or between NADPH and NADP. Niacin also participates in the TCA cycle facilitating the dehydrogenation of intermediates for the provision of energy. During respiration NAD and NADP are involved in the creation of high energy phosphate bonds by the coupling of NAD / NADP cycling to glycolysis, pyruvate metabolism or pentose synthesis.

Nicotinic acid is the predominant vitamer in feed stuffs of plant origin while nicotinamide predominates in feeds derived from animal sources. In raw materials niacin is generally stable as it is present in a bound state and hence the availability of the vitamin in the feed is often poor. Rich sources of niacin are rice polishings, yeast, wheat bran, dried fish solubles, liver and lung meal, and legumes. However, it would appear that within plant materials, the niacin in grains may be regarded as mainly unavailable while that from legumes is assumed to be free and almost all available. Supplemental niacin is stable during diet manufacture and storage.

Due to the ubiquitous nature of this vitamin and the ability of some animals to utilise tryptophan as a vitamin precursor, most diets for animals contain sufficient niacin for normal growth. However, the poor niacin content of maize may be problematic where its inclusion rate is high. Aspects of the effects of niacin deficiency in fish are discussed in chapter 3 while the requirement of fish for this vitamin is explored in chapter 4.

1.3.5 Pantothenic Acid

Pantothenic acid is a di-peptide derivative formed by linking di-hydroxymethyl butyric acid to β -alanine. The free acid takes the form of a yellow oil while the calcium salt is crystalline, freely soluble in water and insoluble in fat solvents. Calcium pantothenate is also resistant to mild redox reagents, dry heat and autoclaving, hot acids and alkalies, hence this derivative is commonly used as a feed supplement.

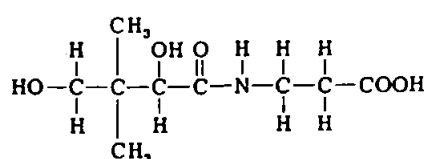


Figure 1.5: The structure of pantothenic acid.

Pantothenic acid forms part of the Coenzyme A molecule which plays an important role in the transfer of acyl groups in metabolism. Approximately 85 % of dietary pantothenic acid is present as acetyl CoA which represents the universal intermediate product in the metabolism of carbohydrates, fats and amino acids standing therefore, between glycolysis and the citric acid cycle. Pantothenic acid is also implicated in adrenal function, in steroidogenesis and long chain fatty acid synthesis.

Pantothenic acid is fairly common in feed stuffs and good sources of this vitamin are yeast, dried delactose whey, rice polishings, wheat bran, alfalfa, dried skim milk, groundnut meal, cane molasses and dried fish solubles. Calcium pantothenate is fairly stable when incorporated into dry or moist diets though heating should be minimised.

Deficiency symptoms rarely become apparent in domestic animals due to the high availability of this vitamin from dietary ingredients. The gut flora of ruminants

synthesises a large quantity of pantothenic acid and thus ruminants rarely suffer from deficiency.

1.3.6 Biotin

Biotin is a monocarboxylic acid the structure of which is shown in Figure 1.6. This vitamin is slightly soluble in water and insoluble in fats. Solutions of the biotin salts are stable to 100°C and are not deactivated by ultra violet irradiation. This vitamin is inactivated by acids, alkalies and oxidising agents. A reversible binding between avidin (an egg protein) and biotin will make the vitamin unavailable, though either pre-cooking the egg prior to incorporation into the feed or cooking the final product to denature the binding protein will maintain an available source of biotin.

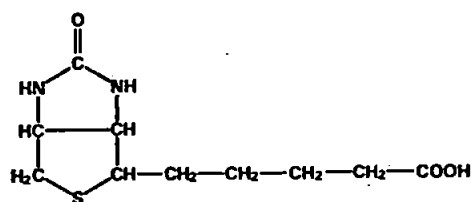


Figure 1.6: Structure of biotin.

Biotin serves as a cofactor for a series of enzymes catalysing the transfer of carbon dioxide *ie.* carboxylation and decarboxylation *eg.* pyruvate carboxylase which is central to intermediary metabolism and acetyl CoA carboxylase an enzyme essential in fatty acid synthesis. Biotin is also believed to be involved in the biosynthesis of citrulline.

Biotin is widely distributed in dietary ingredients and good sources of biotin include yeast products, rape and sunflower seed meals, rice polishings, ground-nut and soy bean meals, dried skim milk, fish meal and dried fish solubles, liver and lung meal and delactose whey. Diets must be protected from strong oxidising agents and, due to the

action of avidin, raw egg should not be included in formulations.

It has been thought that due to the abundance of biotin in feeds and the capacity of the gut flora of mammals to synthesise this vitamin, a deficiency of biotin would not be observed. However, deficiencies have become apparent in both mammals and fish, and hence the deficiency symptoms associated with this vitamin and the requirement of fish for biotin are discussed in chapter 4.

1.3.7 Folic Acid

Folic acid takes the form of yellow crystals which are soluble in water and inactivated by heat and light. Under conditions of low pH the vitamin is easily destroyed by heat and light or during prolonged storage.

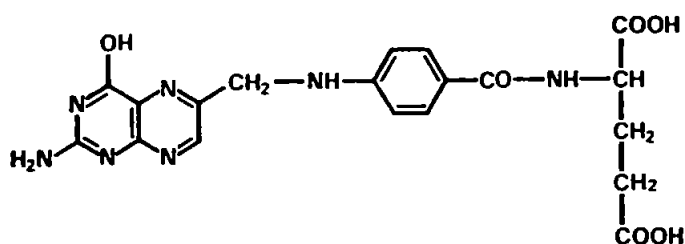


Figure 1.7: The structure of folic acid.

Folic acid functions as a coenzyme in the transfer one carbon groups eg. in serine - glycine interconversion, methionine - homocysteine synthesis and pyrimidine synthesis. This vitamin also plays a role in bone marrow transformation, normal blood cell formation, blood glucose regulation and cell membrane function.

The gut flora represents an important source of folic acid in animals while yeast products, liver and lung meal, full fat soya, wheat germ and bran, rape and sunflower seed meal and fish tissue provide good exogenous sources. Excessive sunlight may cause vitamin degradation.

1.3.8 Cyanocobalamin

Cyanocobalamin is a red crystalline compound which, when in solution, is stable to mild heating but is labile to acids and alkalis. The fact that this vitamin contains cobalt makes it unique and the central cobalt ion may in fact be replaced by other ions *eg.* by the hydroxide ion to produce hydroxocobalamin, vitamin B_{12b}. Several vitamers of B₁₂ have biological activity in bacteria and animals.

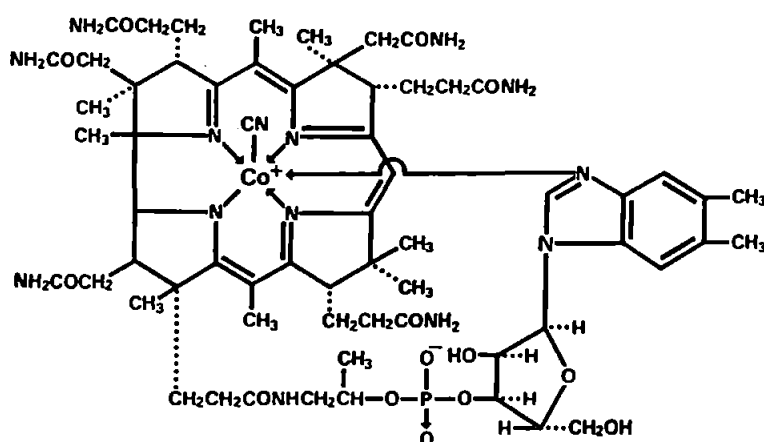


Figure 1.8: The structure of Cyanocobalamin

The cobamide coenzymes are involved in a wide variety of reactions. In ruminants cobamide coenzymes are involved in the transformation of methylmalonyl CoA to succinyl CoA by the process of reversible isomerisation during propionic acid metabolism. Cobamide coenzymes are involved in cholesterol metabolism and in purine and pyrimidine biosynthesis. In bacteria cyanocobalamin plays a role in methionine biosynthesis and is believed to act as a growth factor for certain animals.

Synergistic relationships exist between folacin and cyanocobalamin in both one carbon metabolism *eg.* the formation of labile methyl compounds, and in haemopoiesis.

The synthesis of Cyanocobalamin is due entirely to bacteria with no contribution

from any other source. Thus, its presence in animal tissue is due entirely to the consumption of feed containing B₁₂. The gut flora is responsible for a large proportion of cyanocobalamin intake though liver, kidney, fish and glandular tissues are rich sources of the vitamin. Any supplemental cyanocobalamin added to feeds must be carefully administered due to its sensitivity to low PH.

Cyanocobalamin was identified in the late 1940's as an anti pernicious anaemia factor though B₁₂ does not act alone in to this end. Juvenile animals depleted of cyanocobalamin show high mortality and suppressed growth but the essentiality of cyanocobalamin decreases with age. The requirement for cyanocobalamin of ruminants will normally be met by the gut flora provided a dietary source of cobalt is provided. Intrinsic factor (a low molecular weight mucoprotein found in the gut) is essential for B₁₂ absorption. Pernicious anaemia may result from an absence of I.F. Supplemental cyanocobalamin need usually only be supplied to juvenile farm animals.

1.3.9 Ascorbic Acid

This vitamin is also termed vitamin C and takes the form of a water soluble, colourless crystal with acidic and strong reducing properties. Ascorbic acid remains active in acidic solution but is inactivated under alkaline conditions and in the presence of light, heat and oxygen which becomes more potent when copper ions act in catalysis. L-ascorbic acid is the most biologically active form of this vitamin but a number of derivatives exist. These include dehydroascorbic acid (which has a lower biological activity but *in vivo* may be enzymatically reduced to form L-ascorbic acid) and L-ascorbate-2-sulphate.

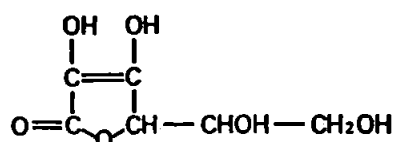


Figure 1.9: The structure of L-ascorbic acid

Most animals show no dietary requirement for ascorbic acid. In fact only man, a few other vertebrates, some fish and a small number of insects show a requirement for this vitamin. The enzyme L-gulonolactone oxidase catalyses the synthesis of ascorbic acid from glucose via glucuronic acid and gulonolactone and thus an absence of this enzyme imposes a dietary requirement for vitamin C on a species.

Ascorbic acid acts as a biological reducing agent for hydrogen transport and is involved in hydroxylation *eg.* in the hydroxylation of tryptophan and tyrosine. Vitamin C acts in the detoxification of aromatic drugs and is involved in the production of some hormones such as the catecholamines. Ascorbic acid is important in collagen metabolism and thus is essential for skin and bone growth and for wound repair.

Vitamins E and C act in synergism in the maintenance of intracellular anti-oxidants and free radical traps. Another example of synergism is the interaction between selenium and vitamins C and E. These three interact to maintain the activity of glutathione oxidase and superoxide dismutase which function within the immune response.

The sources of vitamin C are well known and include citrus fruits, green leafy vegetables and to a lesser extent liver, kidney and fresh insects. Feeds must be protected from aerobic oxidation and should not be contaminated with metals likely to catalyse the degradation of the ascorbic acid.

Most domestic animals are able to synthesise vitamin C and hence deficiency signs are rare. However, under stressful conditions, endogenous vitamin synthesis may be insufficient and supplementation may be necessary. The symptoms of ascorbic acid deficiency in fish and the efficacy of some of the presently available ascorbic acid derivatives in diets for fish is investigated in chapter 5.

1.3.10 Choline

Choline is one of the vitamins categorised by Bender (1992) as being of doubtful vitamin status due to the capacity of some animals to synthesise sufficient quantities of this vitamin to meet the metabolic requirement. Choline is a strong organic base and readily forms salts consequently forming a wide variety of derivatives which are widely distributed in organic materials. This vitamin is hydroscopic and highly soluble in water, stable to heat in acid conditions but not at high pH.

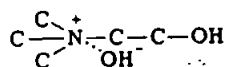


Figure 1.10: The structure of choline

Choline is found in biological systems as the basis of the phospholipids lecithin and sphingomyelin in cell membranes and neural tissues respectively. Additionally, acetyl choline is known as a neurotransmitter, as a methyl donor and is involved with the transport of lipids in the body.

A deficiency of choline in animals is un-common though in humans fed defined diets without supplemental choline some deficiency related pathology may become

apparent. This includes fatty infiltration of the liver with necrosis and haemorrhage in the kidney. Similarly, in fish the deficiency symptoms extend to the liver and kidneys and also include poor appetite and feed conversion.

A wide diversity of feed stuffs are considered to be good sources of this vitamin and include yeast products, wheat germ, soybean meal, rape seed meal, safflower seed meal, animal bi-product meals, white fish and shrimp meals. Choline is itself stable in vitamin pre-mixes but the degradative effect of this vitamin on other micronutrients means that choline is added to the diet as a separate ingredient.

1.3.11 Inositol.

Inositol is the common name for the compound hexahydroxycyclohexane which is a compound containing six carbon atoms arranged in a ring like structure as shown in Figure 1.11. Of the nine vitamers which exist *myo*-inositol is biologically active. Inositol is soluble in water and insoluble in lipid solvents.

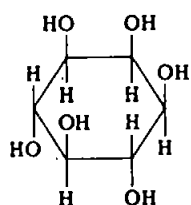


Figure 1.11: The structure of *myo*-inositol.

The main function of inositol is associated with phospholipids as phosphatidylinositol accounts for 5 - 10 % of the total membrane phospholipids. In addition, inositol is involved with the intracellular response to peptide hormones and neurotransmitters and participates as a component in the maintenance of cellular

structure.

Inositol is not recognised as an essential nutrient in humans and animals since the endogenous synthesis of this vitamin is believed to be sufficient to meet the requirement of most animals though in cases of diabetes impaired metabolism of sugars results in elevated plasma concentrations of this 'sugar-like' vitamin.

In keeping with its common name "muscle sugar" rich sources of this vitamin include animal tissues including muscle, heart and liver. Other good sources include yeast products, wheat germ and fishmeal. In plants, inositol is found as phytic acid which is believed to have some anti-nutritional activity *via* interference with mineral absorption in monogastric animals.

1.4 Vitamins in Feeds For Aquatic Animals.

The objectives of feeding both terrestrial and aquatic animals are the same *ie.* the provision of nutrients to meet an agreed standard of nutrition or productivity while maintaining optimum profitability (Lovell 1989). In the main, feeds for cultured aquatic animals are produced using some of the techniques used in the production of feeds for terrestrial animals *ie.* extrusion and pelleting. However, the provision of feeds for aquatic animals imposes an additional set of constraints *ie.* supply of the diet with minimum damage to the feed during the time spent in the water. Thus the vitamin content of a feed for aquatic animals is subjected to potential losses *via* two main routes *ie.* loss during manufacture and through leaching into the water.

1.4.1 Contribution of Vitamins From Feed Materials.

The vitamin content of many of the feed materials used in fish nutrition is well known and frequently quoted (NRC 1993), however, a wide variety of factors effect the scale and availability of the vitamin content of all feedstuffs. Factors beyond the control of the feed manufacturer include agronomic and harvesting conditions *eg.* amount and intensity of sunshine, rain fall, fertilisation, moisture content and method of pre-storage de-hydration (Scott 1982). The vitamin content of raw materials is subject to oxidation especially in products such as animal proteins which contain a high lipid content and hence, such materials must be of a high quality and stabilised with a suitable anti-oxidant to prevent damage or loss of the vitamin content (Scott 1982). Additionally, those materials with a high moisture content such as molasses are aggressive to the vitamin content of a feed (Gadient 1984).

Even where a high vitamin content is maintained in the feed materials, unlike the macronutrients such as protein, lipid and carbohydrates, there is a well recognised paucity of information with regards the availability to fish of the vitamins within feed ingredients. The difficulty in estimating the nutrient availability of water soluble vitamins to fish stems partly from the habitat in which the animals live. Estimations of nutrient digestibility by indirect methods rely on the collection of faeces in order to quantify the proportion of the material in question remaining undigested after passage through the digestive tract. After evacuation from the fish, the vitamin content of the faeces is subjected to leaching loss in the water while the vitamin content of the digesta is subject to further modification during digestion due to the actions of the gut microflora. Thus estimations of water soluble vitamin availability may not be evaluated by the well tested, classical methods of direct and indirect digestibility.

Despite the apparent lack of standardised techniques, experiments have demonstrated the ability of tilapia (*Oreochromis aureus* and *O. niloticus*) to grow adequately using rations produced without the addition of a vitamin supplement (Dickinson 1987) and growth of channel catfish (*Ictalurus punctatus*) in the absence of supplemental biotin (Lovell and Buston 1984). The availability of biotin to chickens from a variety of feed materials was evaluated by Frigg (1984) who observed a very wide range of availabilities ranging from totally unavailable (Rye) to completely available (Whey powder). Niacin was deemed to be freely bioavailable from processed soya products while by comparison the availability of this vitamin from corn was found to be much lower (Yen *et al.* 1977). However, these data were collected for other domestic animals and hence may not truly reflect the availability of vitamins to fish. Consequently, the availability of micronutrients to fish from carefully processed feed ingredients represents an avenue of potentially rewarding research but one which will be difficult to explore.

1.4.2 Manufacturing Losses

During the manufacture of feed, a wide variety of processes are undertaken not only for the manufacture of the product itself but to modify the nature of the constituents within the feed in order, for example, to improve digestibility and palatability or increase stability in storage (Ryley and Kajda 1994). While improving the quality of the feed in terms of macro nutrient supply these processes may decrease the availability and stability of the micro nutrients. Vitamins considered to be fairly un-stable during the production of animal feeds are A, D, K, thiamin, folic acid and vitamin C while niacin, biotin and vitamin E are regarded as more resilient vitamins (Gadient 1984).

During processing, loss of vitamins may occur during grinding and ripening during which coated vitamins may be physically damaged removing the protection of the coating and thus exposing the vitamins to oxidation (Gadient 1990). Pelleting involves the application of high temperature, pressure and moisture to the feed and hence, where insufficient anti-oxidant has been added, vitamins such as A E and K are particularly sensitive at this point (Scott 1982). Additionally, market forces demand feeds with a very low content of dust / fines which may only be achieved by the use of higher pressure (Gadient 1984) with consequent increases in vitamin loss. Measures which may be taken to reduce the loss of vitamin activity during pelleting include the use of expanded steam, a slower output of pellets from the die and the use of binders facilitating the use of reduced pressure during pelleting (Gadient 1984). Conversely, many vitamins such as niacin and cyanocobalamin are present in the feed materials in a bound state and the application of the conditions required for pelleting may indeed allow an increase in the available vitamin content (Scott 1982). Extrusion is also aggressive towards vitamins since the feed is placed under high pressure and then expanded, thus recorded losses of vitamins A and D are in the range of 50 and 20 % respectively (Gadient 1984).

Slinger *et al.* (1979) investigated the manufacturing losses encountered by vitamins during the production of fish feeds by pelleting and extrusion. Between 8 and 10 % of the pantothenic acid content was lost for steam pelleted and extruded feeds respectively after manufacture and 6 months of storage. However, doubling the amount of premix in the feed resulted in a three fold increase in manufacturing and storage losses for pantothenic acid. Under the same conditions, the loss of folic acid was 5 and 3 % for pelleted and extruded feeds respectively with a doubling of the premix size doubling the processing and storage losses in each case. For thiamin, negligible processing losses were

recorded by Slinger *et al.* (1979) indeed the loss of approximately 11 % was due solely to the storage period of 7 months. Pyridoxine was also shown to be stable during manufacture and storage for 10 months with minimal losses regardless of premix quantity and method of production. However, ascorbic was highly labile resulting in a 17 % loss via steam pelleting, a 32 % loss via extrusion and a combined storage and manufacture loss of approximately 95 % within 6 months of production.

Of all the vitamins incorporated into feeds for aquatic animals, ascorbic acid has been the focus of the majority of attention with regards manufacturing and storage losses. This highly labile vitamin has been subjected to both chemical modification and encapsulation with nutritive and non-nutritive coatings in an attempt to maintain adequate dietary levels of the vitamin while maintaining its bio-potency. The derivatives of ascorbic acid are the focus of an experiment in chapter 5 and hence will not be further discussed at this juncture.

1.4.3 Leaching Losses

Further to their investigation of manufacturing losses, Slinger *et al.* (1979) investigated the vitamin losses from extruded and steam pelleted rations for fish. The pellets were subjected to a 10 second immersion in aerated water and the loss of pantothenic acid, thiamin, folic acid, pyridoxine and ascorbic acid immediately determined. In all cases, the loss of vitamins by leaching was lower in extruded feeds than in steam pelleted rations. Taking the values recorded for a premix content of 2 % within the diet, minimal leaching losses were encountered for pyridoxine (3 and 6 % for steam pelleted and extruded rations respectively) while for thiamin, extruded feeds lost none of the vitamin content while the steam pelleted feeds lost around 17 %. Little of the pantothenic acid content in extruded feeds was lost via leaching (5 %) though the

loss in pelleted feeds was approximately 4 times greater. The folic acid content of steam pelleted feeds was reduced by 21 % by leaching while 30 % loss was encountered by leaching in extruded feeds. Very large leaching losses were encountered by ascorbic acid with 55 % of the vitamin lost in extruded feeds and 67 % of the ascorbic acid content lost in steam pelleted rations.

Leaching losses may primarily be minimised by good feeding practice *ie.* feed is supplied at such a rate that the animals quickly consume the ration and hence the time spent in the water is minimised. However, rations for crustaceans are often slowly consumed and hence the use of differing materials in the construction of the pellet was investigated by Goldblatt *et al.* (1979). Despite the fact that the ethyl cellulose encapsulated diets in this experiment exhibited very small losses of nutrients by leaching, the authors concluded that, due to the eating mechanism of crustaceans that there was no benefit in using encapsulated pellets since the integrity of the pellet is destroyed by the animal allowing the contents of the pellet to leach into the water. However, these data would indicate that for fin fish, where the pellet is consumed quickly and its entirety, encapsulation of the whole pellet may prove to be a worthwhile venture.

1.5 Factors Effecting Vitamin Requirements.

The overall vitamin requirements and level of vitamin supplementation required by fish are dictated by a combination of many factors the most important of which include; the scale and availability of the vitamin content within the native feed materials, modifications in the requirement for vitamins during fish development, synthesis of certain vitamins by the gut micro flora and finally the ability of some fish to synthesise vitamins from precursors.

1.5.1 Vitamin Requirements With Respect to Age.

Modifications in the vitamin requirements of fish with respect to developmental state and age have been investigated with regards only a small number of species and vitamins. Kitamura *et al.* (1965) observed differences in aspects of the vitamin C deficiency symptoms in the rainbow trout (*Oncorhynchus mykiss*) which appeared to be related to the size of the fish. This notion was further investigated by Sato *et al.* (1978) who investigated the ascorbic acid deficiency symptoms of rainbow trout fed vitamin C deficient diets from the age of six weeks or ten months. In short, after 24 weeks of feeding, the younger fish developed the symptoms of severe ascorbic acid deficiency including scoliosis, lordosis, exophthalmos, impaired wound healing and a fall in the hydroxyproline : proline ratio. Deficiency mediated pathology was not apparent in the older fish and the formation of the skin collagen proceeded normally. However, during wound repair experiments, the older fish fed vitamin deficient diets demonstrated a poorer healing response when compared to fish fed the ascorbic acid replete diets. Consequently Sato *et al.* (1978) concluded that the ascorbic acid requirement of trout decreases with age and thus corroborated the observations on growth in rainbow trout fed diets of varied vitamin C content recorded by Hilton *et al.* (1978).

In two separate experiments, Murai and Andrews 1975, 1979) demonstrated a marked decline in the requirement in the pantothenic acid requirement of channel catfish (*Ictalurus punctatus*). Thus, fry required 250 mg of pantothenate per kg of diet while fingerlings only required 15 mg kg⁻¹. However, these results were derived using crumbled feeds for the fry from which leaching losses were deemed to be considerable. Conversely, Kissil *et al.* (1981) defined a pyridoxine requirement for the gilthead seabream (2.7 g and 69.3 g initial weight) of 1.97 mg kg⁻¹ irrespective of fish size during

two experiments of 140 days duration.

1.5.2 Contribution of the Gut Microflora.

The vitamin synthesising ability of the micro flora inhabiting the gut of fish has been the subject of only a small volume of work. However, some attention has been afforded the microbial synthesis of vitamin B₁₂ in the gut of several aquatic species. Thus, cyanocobalamin production would appear to be strongly correlated with the presence of *Bacteroides* type A within the gut though many other microbial species including other obligate anaerobes eg. other members of the *Bacteroidaceae* and *Clostridium* also possess a degree of B₁₂ synthesising ability (Sugita *et al.* 1990 and 1991). Thus, it has been reported that *Tilapia niloticus* and *O. niloticus* x *O. aureus* fed diets containing a cobalt supplement maintain a gut flora with a cyanocobalamin producing capacity which negates dietary supplementation with this vitamin (Lovell and Limsuwan 1982, Shiau and Lung 1993). Cyanocobalamin production by gut microorganisms was also observed for the carp by Kashiwada *et al.* (1966, 1970) and for the channel catfish by Limsuwan and Lovell (1981). The latter demonstrated the availability of this vitamin to the fish and that channel catfish may derive sufficient B₁₂ from the gut flora for the provision of normal growth and health but not for the maintenance of normal vitamin levels in the liver. Overall, the maintenance of a gut flora comprising mainly anaerobes as demonstrated by the tilapia species, is correlated with a greater cyanocobalamin production when compared to that of the catfish, the gut flora of which, comprises a smaller number of anaerobes (Sugita *et al.* 1990).

Little information exists with regards the microbial contribution of other water soluble vitamins to the nutrition of fish, however, the observations of Kashiwada *et al.*

(1966) suggest that carp have only a limited capacity for *in vivo* microbial synthesis of nicotinic and pantothenic acid with a potential for elevated rates of pantothenic acid synthesis only after a long period of *in vitro* culture. Subsequently, Kashiwada *et al.* (1971) demonstrated the ability of the microflora of the carp to synthesise folic acid though carp fed diets containing this vitamin maintained a greater concentration of folate in the gut than those relying solely on microbial synthesis. Robinson and Lovell (1978) postulated the existence of a biotin synthesising microflora in the gut of the channel catfish as evidenced by the poorer performance of fish fed diets containing avidin (a biotin antagonist) when compared to those fed a diet deficient in biotin but otherwise nutritionally correct. Lovell and Buston (1984) subsequently determined the biotin content in the faeces of channel catfish as a proportion of the indigestible dry matter with reference to the dietary input. From such observations, Lovell and Buston (1984) deduced that the synthesising capacity of the catfish microflora for this vitamin was insignificant. Finally, microbial thiamin synthesis was postulated by Murai and Andrews (1978a) to be a factor contributing to the absence of histopathology in thiamin deficient channel catfish though no attempt was made to prove this postulate. The overwhelming wealth of the presently available literature indicates that in the majority of cases, the gut micro flora of fish is unable to produce sufficient quantities of vitamins to maintain adequate health and growth and that the synthesising ability which does exist varies on a species by species basis.

1.5.3 Vitamin Synthesis by Fish.

Finally, the ability of some species to synthesise vitamins from precursors has been investigated with regards niacin and vitamin C. The ascorbic acid synthesising ability of some species *eg.* the carp (*Cyprinus carpio*) has been known for some time (Ikeda and

Sato 1964). This ability relies on the presence within the fish of the enzyme L-gulonolactone oxidase which has been detected in the hepatopancreas, kidney and red muscle of carp by Yamamoto *et al* (1978). In addition, Soliman *et al.* (1985) using histochemistry detected this enzyme in the liver and kidney of common carp and in the kidney of the tilapias *Oreochromis spiluris* and *O. aurea*. The classification of niacin as a vitamin is questioned by virtue of the ability of many species to synthesise this micronutrient from the amino acid tryptophan (Bender 1992). In a study which compared the relative activities of the enzymes picolinic acid carboxylase (PC) and 3-hydroxyanthranilic acid oxygenase (3-HAA), Poston and DiLorenzo (1973) demonstrated only a poor conversion of tryptophan to niacin in the brook trout. Consequently, a high 3Haa:PC ratio was quoted by Chuang (1991) as indicative of the ability of fish to synthesise niacin from tryptophan. Chuang (1991) cited unpublished data which indicated that common carp, tilapia, red and black seabream milk fish and two species of fresh water shrimp maintain a significant potential for niacin production from tryptophan. The ability of some fish to synthesise niacin from tryptophan clearly demonstrates the close interaction between nutrition in terms of major nutrient supply and vitamin requirement.

In the main, the emphasis on micronutrient research has been placed on the commonly cultured cold water salmonid species such as the trout and various species of Atlantic and Pacific salmon. Of the warm water species studied, the carp and channel catfish have featured highly in expanding the present knowledge of vitamin nutrition in fish, however, little research has been carried out to examine the requirements of warm water marine species such as turbot, seabream and seabass. Given the recent interest in the culture of warm water marine fish and the paucity of information with regards the

vitamin requirements of such species, the gilthead seabream (*Sparus aurata*) was the chosen species for the present investigation.

1.6 The Gilthead Seabream (*Sparus aurata* L.)

1.6.1 General Biology.

The gilthead seabream belongs to the family *Sparidae* within the order of *Perciformes*. The body of the fish is laterally compressed, oval shaped and deepest at the anterior end as depicted in Plate 1. This species is quite common in the Mediterranean region though uncommon in the Black Sea. In the Atlantic ocean the distribution of this fish extends from the British Isles southward to the Canary Isles with the fish typically occupying coastal waters. The seabream is a hermaphroditic species thus, after two years a portion of the all male population becomes female. Male fish are sexually mature after two years having attained a body weight of 300 - 400 g while females mature at the age of two and a half years with a typical body weight of 400 - 500 g. The gilthead seabream is a carnivorous species and thus, in the wild the fish derives its nutriture from molluscs, crustaceans and gastropods. As outlined below, the gilthead seabream is a particularly resilient species and hence is well suited to intensive culture.

1.5.2 Culture of Seabream and Seabass

The gilthead seabream is a fish which adapts well to culture being tolerant of handling, wide fluctuations in temperature and salinity, though intolerant of low levels of dissolved oxygen (Tzoumas 1992). Poor growth is observed below 13°C and above 30°C thus, optimum growth is attained at 18°C and above. Additionally, due to the high

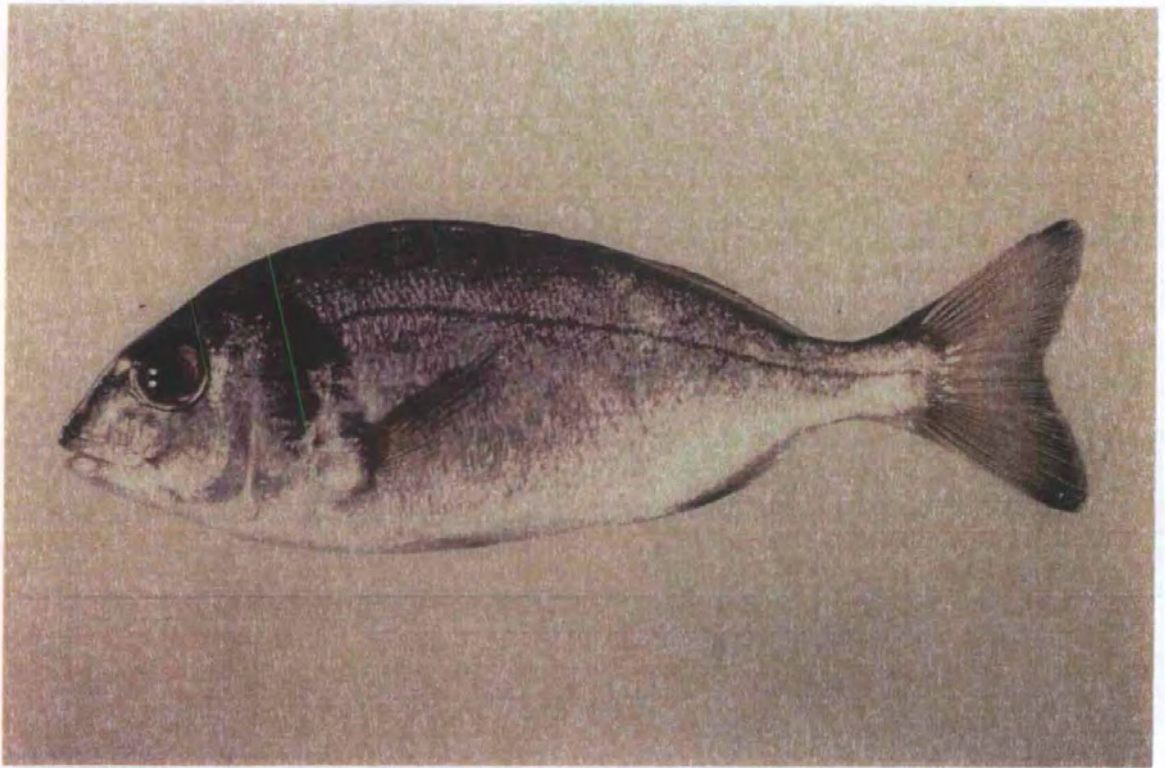


Plate 1: The Gilthead Seabream (*Sparus aurata* L.)

quality of its flesh and the short time (15 months) required for the fish to attain a marketable size of 350 g this fish demonstrates excellent potential as a cultured species (Mazzola and Rallo 1981).

In 1989 the three main producers of seabream were Greece (150 tons), Spain (100 tons) and Turkey (Kaushik 1989). These yields were lower than expected which was attributed by Kaushik (1989) to a poor supply of hatchery-produced juveniles though these yields were expected to increase by five fold over the subsequent 5 years. In 1991 the total Greek harvest of seabream (*Sparus aurata*) and seabass (*Dicentrarchus labrax*) was in the region of 4,000 tons with a hatchery production of 25 million juveniles (Tzoumas 1992). The Turkish yield of bream and bass for 1993 was expected to lie in the region of 3,000 to 5,000 tons and by the year 2000 the yield of these two species in Turkey is expected to exceed 20,000 tons (Wray 1993a). The spanish production of seabream in 1990 was 564 tons with a total of 16.1 million juveniles produced in spanish hatcheries in 1991 (Wray 1993b). Additionally, the Spanish seabream harvest was expected to increase to 7,500 tons for 1996 with a target for the year 2000 of 10,000 tons (Wray 1993b). Production of bream and bass is also carried out in Slovenia (200 tons), Monaco (80 tons), Malta (1,000 tons) while hatcheries have now been established in Tunisia (350,000 juveniles), Monaco (10 - 12 million bass and bream with a capacity to produce up to 20 million) and France (In excess of 1 million juveniles) (Wray 1993c). Thus, as this brief history of seabream and seabass culture demonstrates, the seabream now represents and will continue to represent a significant portion of the total production of finfish in Europe.

1.6.3 Known Vitamin Requirements of Bream and Bass.

Little information exists with regards the vitamin requirements of the gilthead seabream though Kissil (1981) published a speculative requirement of this species for pyridoxine and biotin which later was substantiated by Kissil *et al.* (1981) establishing a B₆ requirement of 1.97 mg kg⁻¹. Brooksbank (1993) investigated the activities of alanine aminotransferase in response to differing levels of supplemental B₆ though the activity of the enzyme appeared to be independent of the dietary levels selected. However, aspects of vitamin nutrition in other species of bream and bass have been studied and may be used in order to estimate the nutritional requirements of the gilthead seabream.

The pyridoxine requirement of the red seabream (*Chrysophrys major*) was determined on the basis of aminotransferase activity to lie in the region of 0.5 to 0.6 mg kg⁻¹ (Takeda and Yone 1971). Yone and Fujii (1974) illustrated the qualitative requirement of the red seabream for niacin, riboflavin, pyridoxine, niacin, pantothenic acid, cyanocobalamin, choline, inositol and ascorbic acid while biotin and folic acid were deemed not to be essential nutrients for this species. On the basis of growth and feed efficiency, the minimum requirement of the red seabream for choline and pantothenic acid were determined as 500 and 10 mg kg⁻¹ respectively (Yano *et al.* 1988). Additionally complement activity in this species was significantly enhanced by choline and pantothenic acid supplements of 8000 and 280 mg kg⁻¹ respectively. Furthermore, in the study of Yano *et al.* (1988), complement activity was unaffected by ascorbic supplements of up to 1 g kg⁻¹ of feed though haemagglutination was significantly improved at the higher level of supplementation.

Sargolia and Scarano (1984) did not define an exact ascorbic acid requirement for the seabass (*Dicentrarchus labrax*) though a supplement of 6 - 12 mg of ascorbic acid per kg of fish was recommended. Additionally Sargolia and Scarano (1984) proposed that

levels of less than 30 μg of ascorbic acid g^{-1} of liver tissue illustrated an insufficient supply of ascorbic acid. The distribution of ascorbic acid in the tissues of the seabass in response to dietary vitamin content was investigated by Alexis *et al.* (1989) and the protective effect of ascorbic acid against nitrite intoxication was investigated by Scarano *et al.* (1991). Carnitine administration to seabass fed high fat diets was shown by Santuli *et al.* (1988, 1990) to stimulate growth, improve protein deposition and decrease the fat content in the carcass.

The pyridoxine requirement of the Asian seabass (*Lates calcarifer*) was estimated to be 10.0 mg kg^{-1} by Wanakowat *et al.* (1989) while the minimum requirement of this species for ascorbic acid was estimated as 500 mg kg^{-1} by Boonyaratpalin *et al.* (1989). The qualitative requirement of the Asian seabass for thiamin, riboflavin, pantothenic acid and inositol was subsequently illustrated by Boonyaratpalin and Wanakowat (1991).

1.7 Aims and Objectives

The aim of the series of experiments constituting the present research programme was to examine the vitamin nutrition of the gilthead seabream (*Sparus aurata*) with regards qualitative and quantitative aspects of nutrition focusing not just on the growth and feed utilisation of the fish but also aspects of health and the clinical manifestations of water-soluble vitamin deficiency. Subsequently, based on the knowledge gained in such preliminary investigations, further studies served to examine the applied aspects of vitamin nutrition with a particular emphasis on elevated rates of vitamin supplementation, vitamin and gross nutrient interactions, and the use of vitamin derivatives as a means of overcoming the problems of ascorbic acid oxidation during feed manufacture and storage.

Chapter 2

General Materials and Methods

2.1.1 Experimental Animals.

The fish utilised in experiment 3.1 were obtained from the hatchery at Cefalonia Fisheries, Greece and had been acclimated to the culture conditions at the University of Plymouth in the six months prior to the start of the present study.

For the remaining nutrition studies, the experimental fish were supplied by the SIAM. hatchery (Montpellier, France). On reaching a weight between 0.3 and 0.5 g, approximately 500 fry were packed into 20 l wine casks filled with 5 l of hatchery water with the remaining volume filled with oxygen. The packages were then transported by air and the fish were introduced into the holding facility at the university within 18 hours of departure from the hatchery. On arrival, the fish were acclimated to the culture conditions within the facility for a period of at least 6 weeks prior to use in the nutrition trials.

2.1.2 Culture Facilities.

During the course of the present series of experiments, three types of recirculation system were used and designated, according to the volume of the tanks and the bio-filtration capacity, as a maxi, midi or mini system. In short, a maxi system comprised six square 400 l fibreglass tanks suspended over a 1200 l bio-filter while for the midi system 6 round 80 l polyethylene tanks were suspended over an 800 l bio-filter. Finally, the mini system was constructed from 8 round 15 l polyethylene tanks over a 60 l bio-filter. In order to assist the mechanical filtration provided by the bio-filter, a foam fractionation

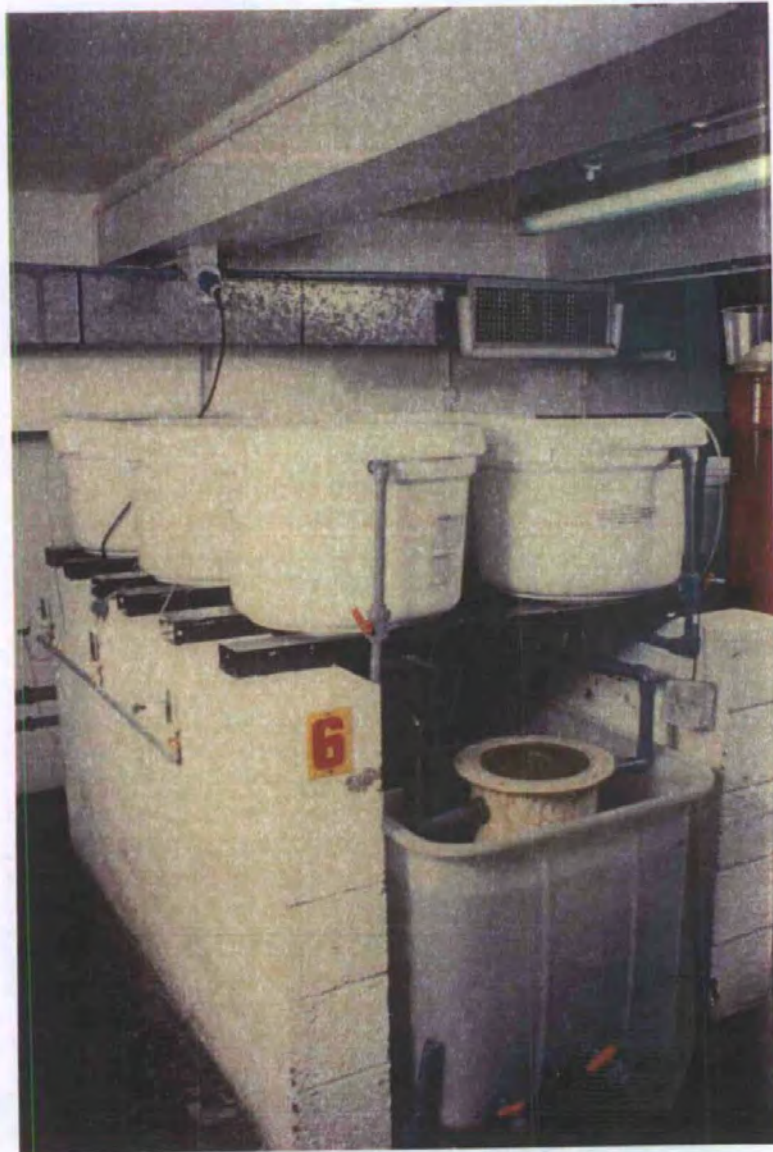


Plate 2: A Typical Midi Recirculation System

column was fitted to all three types of recirculation system. Between trials the recirculation systems were subjected to routine maintenance and development and hence details of the prevailing conditions within each system *eg.* heating, photoperiod and rates of water flow *etc* are outlined where appropriate.

2.1.3 Feeding.

During the acclimation period the fry were fed *ad libitum* three times daily at 09.00, 13.30 and 16.00 hrs. The fry were initially maintained on high energy salmon crumbles until reaching a suitable size for maintenance on a feed commercially produced for seabream culture by Paul's Aquaculture Ltd (PA). The larger stock fish were also presented with the (PA) diet once daily at 10.00 hrs.

In the course of the nutrition trials, the animals were fed as a percentage of the live weight three times daily. The ration size was determined on the basis of bi-monthly weighing and the percentage body weight fed during each of the experiments is outlined where appropriate.

2.2 The Test Diets.

2.2.1 Diet Formulation.

Throughout the present series of experiments, two diet formulations were applied which were modified to suit the needs of each nutrition trial. All the test diets were derived by modifications of either a practical type diet which was based on feedstuffs commonly applied in the manufacture of commercial diets or on a semi-purified model which contained materials believed to possess a minimal water soluble vitamin content.

The use of different feed materials as partial substitutes for fishmeal in diets for

the gilthead seabream have, in part been previously investigated in this laboratory by Nengas (1991). Consequently, the diets formulated by Nengas (1991) served as a model for the production of the feeds used in experiments investigating more applied aspects of fish nutrition. Thus, a practical type diet was formulated to provide 50 % crude protein from fishmeal and approximately 13 % lipid using cod liver oil to supplement the lipid component of the fishmeal. Carbohydrate was provided to a level of approximately 18 % in experiments 3.1 and 5.1 by the use of wheat feed. Experiment 5.2 required the use of carbohydrates of a greater digestibility and hence a mixture of dextrin and cornstarch (3:2) was used for the provision of carbohydrate within this particular study.

In order to examine aspects of vitamin nutrition without interference from vitamins contained within feed materials, the use of a purified ration is essential. Kissil and Koven (1987) investigated the suitabilities of the purified rations which had been formulated for use in studies on the sparid fish at that time. The main conclusion of that study was that optimal growth was attained using diets with an amino acid profile which matched that of the whole hens egg. On this basis, using a formulation package, a semi-purified ration was derived which would provide approximately 50 % crude protein using casein and gelatin in the ratio of 2:1 as the principle protein sources. A supplement containing nine amino acids was also included to produce an amino acid profile which matched that of whole hens egg. Cod liver oil and the dextrin : corn starch mixture were again employed as sources of non-protein energy with the former also providing the essential fatty acids required by the bream.

The mineral, fat soluble, B vitamin and macro vitamin premixes were formulated based on the vitamin and mineral requirements of salmonids quoted by Cho *et al* (1985). The vitamin premixes were maintained as separate dietary components allowing

modifications to the supplement levels to suit each experimental design. Thus given the diversity of the test diets, the exact formulation and measured proximate composition of each of the seven test diets is presented within the appropriate chapter.

2.2.2 Diet Materials.

The practical type diets were manufactured using white fish meal (Provimi 66), wheat feed, molasses and di-calcium phosphate purchased from a local feed merchant (Carne and Sons, Callington, Cornwall). The semi-purified diets were manufactured using casein, gelatin, corn starch, dextrin and α -cellulose supplied by the Sigma Chemical Company (Poole, Dorset). Seven Seas (Hull, Yorkshire) provided the cod liver oil while the minerals were purchased from BDH (Poole, Dorset). Colborne-Dawes (Heanor, Derbyshire), Lonza (Cheltenham, Gloucestershire) and The Takeda Chemical Company (Tokyo, Japan) supplied the vitamins.

2.2.3 Diet Manufacture and Storage.

Both the practical type and the semi-purified diets were manufactured in a similar way. Thus, the dry powdered ingredients *eg.* fish meal, casein, pre-mixes *etc.* were first thoroughly mixed using a beater in the bowl of a Hobart A101 food processor (Hobart Manufacturing Company Ltd, London) and the oil was then added. During further mixing, an appropriate dilution of the vitamin representing the focus of each experiment was prepared in 200 cm³ of distilled water and added to the mash. Finally, where appropriate, the molasses was quantitatively added to the mash using a minimum volume of distilled water. A further volume of distilled water, typically 200 cm³ per kg of dry matter, was then added during continuous mixing to yield a mash considered

sufficiently moist for extrusion.

Again, with regards the production of the pellets, both diet types were treated equally. Thus, using the extruder assembly of the Hobart processor, the diets were extruded through a series of holes of diameter 3/16". The practical type diets were then spread thinly onto trays and air dried at 45 °C in a fan assisted drying cabinet. The dried diets were then stored in black polythene bags inside air tight bins. The semi-purified diets were not dried but extruded into black polythene bags, sealed and immediately frozen at -20 °C. The moist diets remained in the freezer and the daily aliquot removed as necessary. Additionally, samples of both diet types were withdrawn immediately after manufacture and stored at -20 °C prior to analysis of proximate composition.

2.3 Analysis of Proximate Composition

2.3.1 Determination of Moisture Content.

The moisture content of feed and fish carcasses was determined as outlined in the A.O.A.C. handbook (1990). Thus, in summary, samples of feed materials, tissues or entire fish carcasses were weighed and dried to a constant final weight at 105 °C inside a fan assisted Pickerstone E 70F oven (R E Pickerstone Ltd, Thetford, Norfolk). 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 cm³ of concentrated H₂SO₄ (Sp.Gr. 1.84) and 2 Kjeldahl

catalyst tablets (2 X 3 g K₂SO₄, 105 mg CuSO₄.5H₂O and 105 mg TiO₂. Thompson and Capper Ltd, Runcorn, Cheshire). Digestion was carried out in a Gerhardt Kjeldatherm digestion block (C. Gerhardt Laboratory Instruments, Bonn, Germany) for 30 minutes at 250°C followed by a further 2 hours at 380°C with the acid fumes collected and neutralised by 15 % NaOH in a Gerhardt Turbosog unit.

After cooling, using a Gerhardt Vapodest 3S distillation unit, the sample was diluted with distilled water and neutralised with 40 % NaOH. The inorganic ammonia in the sample was then collected into 50 cm³ of saturated orthoboric acid (H₃BO₃) 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:

$$\% \text{ Crude Protein} = \frac{(\text{Titre sample (ml)} - \text{Titre Blank (ml)}) \times 0.25 \times 14 \times 6.25}{\text{Weight Sample (mg)}} \times 100 \%$$

Where:

- 0.25 = [HCl] in moles
- 14 = Relative molecular 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 Balckstock (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 cm³ of petroleum ether (40-60 fraction) for 40 minutes in the "recovery" mode. This was followed by a further 70 minutes of reflux 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:

$$\% \text{ Lipid} = \frac{\text{Mass of Lipid Residue Collected (g)}}{\text{Mass of Sample (g)}} \times 100 \%$$

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. Thus, 500 mg of dry material was weighed into a 50 cm³ Erlenmeyer flask to which 10 cm³ 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 filtered through a Whatman #2 filter into a test tube and the residue in the Erlenmeyer quantitatively removed using a further 10 cm³ of chloroform : methanol. Duplicate 5 cm³ aliquots were transferred to pre-weighed test tubes and the solvent evaporated at 60°C using a water bath. The weight gained by the test tube was proportional to the lipid content of the sample and hence the percentage of lipid in the dry material was calculated thus:

$$\% \text{ Lipid} = \frac{4 \times \text{Weight Gain of Tube (g)}}{\text{Weight of Sample (g)}} \times 100 \%$$

2.3.4 Determination of Ash Content.

The ash content of the dry material was determined as outlined in the A.O.A.C handbook (1990). Thus, 500 mg of dry sample were weighed into a crucible and heated for 8 hours at 525°C in a Carbolite GLM 11/7 furnace (Carbolite Furnaces Ltd, Bamford, Sheffield). The weight gained by the crucible was proportional to the ash content of the sample and hence the percentage of ash in the sample was calculated thus:

$$\% \text{ Ash} = \frac{\text{Weight gained by Crucible (g)}}{\text{Weight of sample (g)}} \times 100 \%$$

2.4 Determination of Glycogen.

Glycogen was determined using a method derived from that outlined in Plummer (1987). In summary, glycogen was liberated from the tissue by heating with KOH and then precipitated with ethanol using sodium sulphate as a coprecipitant to give a quantitative yield. The glycogen was then acid hydrolysed to liberate the glucose for determination by the glucose oxidase method.

1.5 g of tissue was weighed into a calibrated centrifuge tube containing 2 cm³ of KOH (300 g l⁻¹) and was heated for 20 minutes with occasional shaking in a boiling water bath. The tubes were cooled on ice and 200 µl of saturated Na₂SO₄ were added with shaking. 5.0 cm³ of ethanol (95 % v/v) was then added and following vortex mixing, the tubes were allowed to stand on ice for five minutes prior to centrifugation at 1500 X g. The supernatant was discarded and the pellet was then resuspended with gentle warming in 5 cm³ of distilled water before dilution to a final volume of 10 cm³.

Using a centrifuge tube calibrated to 10 cm³, 1 cm³ of the glycogen solution was added to an equal volume of HCl (1.2 mol l⁻¹) and heated in a boiling water bath for 2

hours. At the end of this period, the hydrolysate was neutralised with NaOH (0.5 mol l⁻¹) using phenol red as an indicator. The solution was then made up to a final volume of 5.0 cm³ and 25 µl was then withdrawn to determine the concentration of glucose in the hydrolysate by the glucose oxidase method as outlined in 2.5.4. The glycogen content of the tissue (g/g wet weight) was then determined thus:

$$\text{Weight glycogen (g/g wet weight)} = \frac{[\text{glucose}] \text{ in hydrolysate (mg ml}^{-1}\text{)} \times 50}{\text{Weight of Sample (g)}} \times 0.9^a$$

^a where, due to the difference in molecular weight, 0.9 is factor allowing the estimation of glycogen from the measured glucose content of the tissue.

2.5 Haematological Indices

2.5.1 Haematocrit.

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

$$\text{Haematocrit \%} = \frac{\text{Length of Packed Red Cell Column (mm)}}{\text{Total Length of Blood Column (mm)}} \times 100 \%$$

2.5.2 Total Haemoglobin.

Total haemoglobin in the whole blood was determined with a minimum of stasis by the cyanmethemoglobin method as described by the Sigma procedure No. 525. Thus, 20 µl of whole blood was added to 5.0 cm³ of Drabkins reagent, mixed thoroughly 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 Phillips PU 1800 U.V. Vis. spectrophotometer. The concentration of total haemoglobin in the blood was thus determined by reference to a calibration curve established using cyanmethemoglobin standard solutions in the range 0 - 18 g/dl.

2.5.3 Total Plasma Protein.

Total plasma protein was determined by the Biuret method as described by the Sigma procedure No 541. Thus, 20 μ l of bovine serum albumin (100 mg cm^{-3}), 20 μ l of distilled water and 20 μ l of plasma was added to 1.0 cm^3 of total protein reagent (Sigma Chemical Company) to produce the standard, blank and sample respectively. The reaction was allowed to proceed to completion (10 minutes at ambient temperature) and the absorbance of the sample was read against that of the blank at 540 nm using a Cecil Series 5000 U.V. Vis. spectrophotometer. Having demonstrated that the response of the assay was linear up to a protein concentration of 100 mg cm^{-3} the concentration of total protein in the sample was determined thus:

$$[\text{Total Protein}] (\text{mg cm}^{-3}) = \frac{\text{Absorbance of Test Solution}}{\text{Absorbance of Standard}} \times 100^a$$

^a Where [Standard] = 100 mg cm^{-3}

2.5.4 Plasma Glucose.

The concentration of glucose in the plasma was determined by the glucose oxidase method as described by the Sigma procedure No 541. For the test, standard and blank

25 μ l of sample, glucose standard (100 mg dl⁻¹) and water respectively were added to 0.5 cm³ of distilled water. 5.0 cm³ of combined enzyme colour reagent was added to all the tubes which were then incubated at 37 °C for 30 minutes. The absorbance of the sample was then read against that of the blank at 450 nm using a Cecil Series 5000 U.V. Vis. spectrophotometer. Having shown that the response of the assay was linear up to a glucose concentration of 300 mg dl⁻¹, the concentration of glucose in the sample was determined thus:

$$[\text{Plasma glucose}] (\text{mg dl}^{-1}) = \frac{\text{Absorbance of Test Solution}}{\text{Absorbance of Standard}} \times 100^a$$

^a Where [Standard] = 100 mg dl⁻¹

2.5.5 Plasma Triglyceride

The concentration of triglycerides in the plasma was determined by the enzymatic method as described by the Sigma procedure No 336. For the test, standard and blank 10 μ l of sample, triglyceride standard (250 mg dl⁻¹) and water respectively were added to 1.0 cm³ of triglyceride reagent. The reaction was allowed to proceed to completion at room temperature and the absorbance of the samples recorded against the blank at 500 nm using a Cecil Series 5000 U.V. Vis. spectrophotometer. The concentration of the triglycerides in the plasma was then determined thus:

$$[\text{Plasma Triglycerides}] (\text{mg dl}^{-1}) = \frac{\text{Absorbance of Test Solution}}{\text{Absorbance of Standard}} \times 250^a$$

^a Where [Standard] = 250 mg dl⁻¹

2.5.6 Plasma Pyruvate.

The concentration of pyruvate in the plasma was determined by the use of lactate dehydrogenase as described by the Sigma procedure No 726-UV. In summary, 0.5 cm³ of plasma was deproteinised in 1 cm³ of 8 % perchloric acid and centrifuged at 1500 X g retaining the supernatant. 667 µl of the prepared supernatant were then added to 167 µl of Trizma® base solution. This was mixed by inversion and 167 µl of NADH in Trizma® (0.455 mg ml⁻¹) were then added. Using water as a blank, the absorbance of this solution at 340 nm was recorded using a Cecil Series 5000 U.V. Vis. spectrophotometer. This value represented the initial absorbance. 16.7 µl of LDH (1000 U ml⁻¹) were then added to the cuvette and the reaction allowed to go to completion (change of less than 0.001 absorbance units min⁻¹). The final absorbance was determined at that point and the change in absorbance (ΔA_{340}) was then calculated. The concentration of pyruvate in the plasma was then determined with reference to a calibration curve derived by plotting ΔA_{340} against [pyruvate] using pyruvic acid standards in the range 0.6 - 3.0 mg dl⁻¹.

2.5.7 Plasma Lactate.

The concentration of lactate in the plasma was determined by the lactate oxidase method as described by the Sigma procedure No 735. For the test and standard 10 µl of sample or 10 µl of standard (40 mg dl⁻¹) respectively were added to 1.0 cm³ of lactate reagent. The tubes were incubated for 10 minutes and the absorbance then recorded against the blank (lactate reagent only) at 540 nm using a Cecil Series 5000 U.V. Vis. spectrophotometer.

The concentration of the lactate in the plasma was then determined thus:

$$[\text{Plasma Lactate}] (\text{mg dl}^{-1}) = \frac{\text{Absorbance of Test Solution}}{\text{Absorbance of Standard}} \times 40^a$$

^a Where [Standard] = 40 mg dl⁻¹

2.6 Determination of Ascorbic Acid.

The ascorbic acid (vitamin C) content of feeds and fish tissues was estimated by the method differential for L-ascorbic acid and ascorbyl sulphate developed by Tereda *et al.* (1978). Thus, for tissues, approximately 0.5 g of tissue was homogenised in 5.0 cm³ of 5 % H₂SO₄ and then centrifuged (5000 X g, 5 °C, 30 minutes), while for feeds, 2 g of material was added to 10 cm³ of 5 % H₂SO₄, agitated for 15 minutes and then centrifuged in a similar manner. For the determination of ascorbic acid in plasma, 100 µl of plasma was added to 4.9 cm³ of 5 % H₂SO₄ with thorough mixing followed by centrifugation.

After centrifugation, four 1 cm³ aliquots of the supernatant were transferred into 2 test tubes labelled KBr and a second pair labelled H₂O. 50 µl of 2,6-dichlorophenol-indophenol (DCPIP, 0.2 % w/v in distilled water) was added to all four tubes and incubated at room temperature for 5 minutes. 50 µl of distilled water was then added to the 2 tubes marked H₂O while an equal volume of potassium bromate (2 % w/v KBrO₃ in water) was added to the 2 tubes marked KBr. The tubes were then sealed and incubated in the dark for 1 hour. At the end of this period, 1 cm³ of thiourea (2 % thiourea w/v in 5 % w/w H₃PO₄) and 0.5 cm³ of dinitrophenyl hydrazine (2 % w/v

DNPH in 9 N H_2SO_4) were added to all tubes. After 3 hours incubation at 60°C the reaction was quenched by placing the tubes on ice and 2.5 cm^3 of H_2SO_4 (85 % w/v) was added to all tubes. For the blanks however, the DNPH was added immediately after and not prior to the 3 hour incubation. All tubes were then centrifuged ($5000 \times g$, 5°C , 15 minutes) and the absorbance recorded at 540 nm using a Phillips PU 1800 U.V. vis. spectrophotometer. The concentration of vitamin C in the sample was then determined by reference to a calibration curve constructed using L-ascorbic acid standards in the range $0 - 40\text{ }\mu\text{g ml}^{-1}$. The present assay allowed the differentiation between ascorbic acid and its derivative ascorbyl sulphate and thus, the concentration of ascorbyl sulphate in the tissue was determined from the differential in absorbance of the tubes marked KBr and H_2O .

2.7. Histological Examinations

The samples were prepared and examined by Mr D M Lowe (Plymouth Marine Laboratories, Citadel Hill, Plymouth). Hence, the methods employed are reproduced here by kind permission of Mr Lowe.

2.7.1. Sample Preparation.

At the termination of the growth studies, samples of liver (0.4 cm^3) were excised from the fish and prepared for examination as frozen sections. Frozen sections were prepared by quenching uniformly sized liver samples in liquid nitrogen cooled n-hexane (-70°C) followed by storage at -25°C prior to cutting. Sections of $10\text{ }\mu\text{m}$ thickness were cut using a Brights cryostat (Brights, Huntington, England), mounted and stained as

outlined below.

2.7.2. Haematoxylin and Eosin.

Fresh frozen sections were removed from the cryostat cabinet and fixed in cold (4 °C) Bakers formol calcium for fifteen minutes. The sections were washed in tap water, stained in Gill's haematoxylin (5 minutes) and then washed again in tap water to "blue" the stain. Following a rinse in distilled water, the sections were counter stained with eosin (1 % eosin in distilled water, 5 minutes), well washed in tap water and mounted in aqueous mounting media.

2.7.3. Oil Red O.

After sectioning, the frozen samples were fixed in Bakers formol calcium for 10 minutes, washed in water and placed into 60 % triethyl phosphate for 1 minute. The sections were then stained in Oil red O (Oil red O stock Solution : distilled water 3:2). Following a rinse in 60 % triethyl phosphate, the sections were washed in tap water and counter stained with Gill's haematoxylin (3 minutes). The sections were then washed in tap water followed by distilled water and finally mounted in aqueous medium.

2.7.4. Schmorl's Method for Lipofuscin.

Frozen sections were immersed in freshly prepared staining solution for 2 - 5 minutes (1 % ferric chloride 37.5 cm³, 1 % potassium ferricyanide 5.0 cm³, distilled water 7.5 cm³). The samples were then washed in tap water, immersed in 1 % acetic acid (2 minutes) and re-washed in tap water. After counter staining (2 minutes in 0.1 % neutral red), the sections were washed in tap water and then in distilled water. After mounting

in aqueous medium, the samples were immediately viewed in order to minimise the leaching losses of the counter stain. Consequently, lipofuscin was stained blue / dark blue, melanins were stained very dark blue and nuclei were red when counterstained.

2.7.5. Periodic Acid Schiff for Neutral Sugars.

Fresh frozen sections were fixed in Bakers formol calcium, rinsed in distilled water and incubated in 1 % periodic acid for 5 minutes. The sections were then washed in tap water and in distilled water prior to incubation with Schiff reagent (15 minutes, room temperature). The samples were then rinsed in tap water, incubated in 3N hydrochloric acid @ room temperature for 1 minute and thoroughly washed in tap water. The sections were then counterstained in Gill's haematoxylin (3 minutes) and after washing in tap and then distilled water the samples were mounted in aqueous medium.

2.7.6. Lysosomal N-acetyl- β -hexosaminidase (NAH).

Cryostat sections were fixed in BFC and incubated in the staining medium at 37°C for 30 minutes. The staining medium was prepared by dissolving 20 mg of AS-DI- β -D-N-acetyl glucosaminide in 2.5 cm³ of 2-methoxy ethanol to which 50 cm³ 0.1 M citrate buffer (pH 4.5 containing 2.5 % NaCl) and 5 g of polypep (low viscosity) were added. Post coupling was carried out by rinsing the sections in 0.1 M phosphate buffer (pH 7.5) and incubating the samples for 10 minutes at room temperature in phosphate buffer (pH 7.5) containing 1 mg ml⁻¹ fast violet B. The slides were rinsed in tap and then distilled water prior to mounting in aqueous medium.

2.8. Definitions, Terms and Related Equations.

Several nutritional parameters relevant to growth and feed utilisation efficiency were employed throughout the current programme of work and these are defined accordingly.

Specific Growth Rate

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

$$\text{SGR (\% d}^{-1}\text{)} = \frac{\ln w_2 - \ln w_1}{T} \times 100$$

where,

w2 = Final weight (g)
w1 = Initial weight (g)
T = Defined time period (days)

Feed Efficiency

Feed efficiency relates the ability of the feed to support weight gain with respect to the amount of feed consumed or put simply, the extent to which feed is utilised for growth. Feed efficiency may be expressed as the feed conversion efficiency (FCE) or as the feed conversion ratio (FCR). The latter term is widely accepted in practical fish and animal nutrition field trials.

$$\text{FCE (\%)} = \frac{\text{Live weight gain (g)}}{\text{Amount fed (g)}} \times 100$$

$$\text{FCR} = \frac{\text{Amount fed (g)}}{\text{Weight gain (g)}}$$

Protein Utilisation.

The utilisation of protein for growth may be expressed as either the protein efficiency ratio (PER) or the net protein utilisation (NPU). 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:

$$\text{PER} = \frac{\text{Live weight gain (g)}}{\text{Protein fed (g)}}$$

Net protein utilisation relates the utilisation of protein to its deposition in the carcass of the fish and hence indicates the efficiency of protein retention. Net protein utilisation may be determined thus:

$$\text{NPU} = \frac{\text{P(end)} - \text{P(start)}}{\text{P(fed)}}$$

Where,

P(End) = Weight (g) of protein in carcass at end of experiment

P(Start) = Weight (g) of protein in carcass at start of experiment

P(Fed) = Weight (g) of protein consumed by the fish

Chapter 3

Qualitative Requirements

3.1. The Necessity of Vitamin Supplementation in Practical Diets for the Gilthead Seabream (*Sparus aurata* L.)

3.1.1. Introduction.

As outlined in chapter one, the requirement of cultured fish for vitamins has long been recognised and consequently the vitamin requirements of large number of cultured species are now well established. Nutrient deficiency may become apparent especially in fish fed a semi-purified feed *i.e.* a defined diet within which the contribution of vitamins from the raw materials is minimal. Such diets are often utilised in order to characterise the minimum requirement of fish for essential nutrients such as indispensable amino acids, fatty acids and micro nutrients. The materials used in the manufacture of commercial diets *eg.* fishmeal, wheat, bloodmeal *etc.* contain vitamins at levels which are easily determined and consequently well documented (NRC 1993). However, the detection of a vitamin within a feed material gives no indication of its bio-availability though studies have been carried out to determine the bioavailability of vitamins from feed ingredients (Yen *et al* 1977, Frigg 1976 and 1984). Additionally, the modern processes involved in feed manufacture *eg.* thermal processing, pelleting and extrusion result in severe vitamin losses from feeds (Pickford 1968, Hilton *et al* 1977, Gadiant 1984, Soliman *et al.* 1987, Gadiant 1990, Ryley and Kajda 1994). Vitamin loss on storage is also well documented (Lovell and Lim 1978, Soliman *et al.* 1987) and finally losses of

vitamins due to leaching are particularly pertinent to feeds for aquatic animals (Goldblatt *et al.* 1979, Slinger *et al.* 1979).

As a consequence of all the above factors, the vitamin profile of an un-supplemented diet may fail to meet the minimum dietary requirement of farmed species. Thus, in order to ensure that the vitamin requirement of farmed fish is met, vitamin supplementation is common to most feed formulations. However, as a consequence of the factors outlined in chapter one which include the varied availability of vitamins from feedstuffs, the contribution of vitamins from the gut microflora and the capacity for synthesis of vitamins from precursors by the fish itself, the scale, content and even the requirement for a vitamin premix in fish feeds has been questioned.

A series of investigations have demonstrated the non-essentiality of supplemental cyanocobalamin in diets for channel catfish, *Ictalurus punctatus* (Limsuwan and Lovell 1981), and tilapias (Lovell and Limsuwan 1982, Shiau and Lung 1993) while a study by Lovell and Buston (1984) indicated that careful selection of feed materials could negate the use of a biotin supplement in practical diets for channel catfish. A study by Roem *et al.* (1990) indicated that in recirculating systems, blue tilapia (*Tilapia aurea*) maintained on purified diets appeared not to require a dietary supplement of either choline or pantothenate which the authors attributed to the grazing of the fish on the bacteria within the recirculation system. However, the bacterial vitamin contribution was insufficient for maintenance of blue tilapia fed diets entirely without a vitamin supplement. In an extreme example, a study by Dickinson (1987) demonstrated that careful selection of feed ingredients for intensively cultured tilapia (*Oreochromis aureus*) could negate the necessity for the inclusion of a vitamin premix within the diet.

The aim of the present, opening experiment was to investigate the necessity of the

fortification of practical diets for production sized seabream with ascorbic acid, choline and members of the B complex under conditions which closely matched those encountered in aquaculture. The vitamins selected for the present experiment have been the focus of a large volume of research in fish nutrition and represent the major classes of water soluble vitamins. The B complex represents a group of eight vitamins which are distributed in varied amounts with differing availabilities in feed materials and in the main, have essential roles in metabolism as cofactors. Ascorbic acid is an essential nutrient for fish, which with the possible exception of carp, are unable to synthesise from metabolic precursors. This vitamin plays a lesser role in intermediary metabolism but the consequences of a deficiency of this micronutrient are dramatic. Finally, choline was chosen to represent those vitamins which have a questionable and less well defined role in animal nutrition the deficiency of which does not always become apparent.

Thus, in the present experiment, the contribution of vitamins from commonly applied feed materials, the vitamin contribution of the gut microflora and the capacity for synthesis of vitamins by the fish themselves was quantified in terms of effects on fish growth and health in the absence of supplemental vitamins. The manifestations of vitamin depletion, in terms of deficiency syndromes, were also examined in order to characterise those pathologies associated with particular vitamin groups and thus highlight areas which merit further research.

3.1.2. Materials and Methods.

Forty gilthead seabream (*Sparus aurata*) fingerlings (initial weight 50 g) were stocked into each of five 400 l, self cleaning, fibreglass tanks over a 1200 l bio-filter within a closed recirculation system providing a parallel flow of seawater through the

tanks at 2.5 l min^{-1} . The water temperature was maintained within the desired range by the use of an integrated dip-cooler and immersion heater and while salinity was regulated between 33 and 36‰ saline respectively by the use of a freshwater inlet balanced to compensate for evaporative losses. The pH was maintained between 6 and 7.5 by the use of calcium carbonate buffering while ammonia and nitrite were held within ranges tolerated by this species. Daylight balanced fluorescent strip lamps maintained an 8hr light: 16hr dark regime.

Using a formulation package a test diet was designed providing approximately 48 % protein and 13 % oil from blended white fishmeal (Provimi 66) and marine oil respectively. This formulation was then modified to produce five test diets. Diets one, two and three contained no supplemental choline chloride (Ch-), B vitamins (B-) and ascorbic acid (ASA-) respectively. Diet 4 was a complete test diet (Con). Additionally a conditioning diet was prepared which was devoid of supplemental ascorbic acid and B vitamins. The diets were prepared as outlined in chapter 2 by mixing the dry ingredients, marine oil, molasses and water in a Hobart A101 mixer followed by cold extrusion through a 3/16" die. The resulting pellets were then air dried (40°C for 24 hrs) and stored in air tight bins. A commercially available seabream pellet manufactured by Pauls' Aquaculture Ltd. (PA), was fed to a second control group for comparison with the test formulation. The complete test diet formulation is presented in table 3.1.1. and the nutrient profile is summarised in table 3.1.2.

The feeding trial was sub-divided into three periods. Thus, the fish were weighed in bulk at the start of the preliminary period during which the fish were supplied with the conditioning diet at 0.5 % of the body weight per day and maintained at a water temperature of 15°C . After 8 weeks, the fish were individually weighed and the

conditioning diet was then supplied at 1.4 % of the body weight per day at a water temperature of 24 °C. From the 14th week of the growth trial until its termination, each of the test diets *ie.* diets 1 - 4 (con, ASA-, B- and Ch-) were supplied at 1.8 % of the body weight per day at a water temperature of 24 °C. Those fish fed the commercial diet PA were maintained on this ration throughout the whole of the trial and hence did not receive the conditioning diet. The diets were presented to the fish twice daily at 09:00 hrs and 16:00 hrs. Weighings were planned as a bi-monthly event however, due to the onset mortality after 14 weeks of feeding, the frequency of weighing was reduced to once monthly.

The weight gain, specific growth rate (SGR), feed intake, feed conversion ratio (FCR), feed conversion efficiency (FCE) and cumulative percentage mortality were monitored throughout the 24 weeks of the growth trial. The fish were also inspected daily for the onset of gross deficiency symptoms.

At the termination of the growth trial and following a 24 hour starvation period, the fish were desanguinated from caudal vein into heparinised syringes. The packed cell volume (PCV) was then immediately determined by micro haematocrit and the haemoglobin content of the whole blood was determined by the cyanmethemoglobin method using a diagnostic kit (Sigma Chemical Company, Procedure No. 525). The remaining sample was then centrifuged and the resulting plasma frozen at -70 °C. Sigma diagnostic kits were subsequently used to determine total serum protein by the Biuret method (Total Protein Reagent, Sigma 541-2) and plasma glucose by the glucose oxidase method (Sigma 510 A). Plasma L-ascorbate was determined by the DNPH method of Tereda *et al.* (1978).

Table 3.1.1: Composition of the complete diet from which, by the deletion of the vitamins ascorbic acid, choline and the B complex the 3 test diets were derived.

Ingredient	% Inclusion
Fish Meal (Provimi 66)	71.20
Fish Oil	6.30
Molasses	0.50
¹ Minerals	1.06
² Fat Soluble Vitamins	0.10
³ B Complex Vitamins	0.11
Choline Chloride	0.40
Inositol	0.04
Calcium Ascorbate	0.03
Wheat Feed	To 100 %

¹Contribution of minerals to diet (mg kg⁻¹ diet)

CaHPO₄ 2756.3, CaCO₃ 375.0, NaCl 1875.0, K₂SO₃ 2500.0, MgSO₄.7H₂O 2578.4, FeSO₄.7H₂O 87.5, MnSO₄.4H₂O 28.4, ZnSO₄ 64.5, CuSO₄.5H₂O 6.4, CoCl₂.6H₂O 3.3, KI 1.9, Na₂SeO₃ 0.3 .

²Contribution of fat soluble vitamins to diet (mg kg⁻¹ diet)

Vitamin A palmitate 2.060, Vitamin D₃ 0.0756, Vitamin E acetate 109.76, Vitamin K 10.02 .

³Contribution of B vitamins to diet (mg kg⁻¹ diet)

Biotin 2% 20.2, folic acid (88.8%) 5.631, niacin 150, calcium pantothenate 10.905, pyridoxine hydrochloride 24.31, riboflavin (96%) 20.83, thiamine hydrochloride 11.602, cyanocobalamin (95%) 0.021 .

Table 3.1.2: Proximate composition of the test and commercial control diets. Moisture is expressed as a percentage of the diet as fed. Protein, lipid and ash are expressed as a percentage of the dry matter. Nitrogen free extract (representing carbohydrate and fibre) resolved as the residual after the determination of protein, lipid and ash.

Diet	Moisture	Protein (%DM)	Lipid (%DM)	Ash (%DM)	Nitrogen Free Extract (%DM)
Test Diets	5.33	49.28	13.21	16.70	21.97
Commercial Diet	8.52	49.00	12.49	11.89	26.62

Immediately after manufacture, diet samples were frozen and later, along with the pooled carcasses of five fish from each dietary regime, subjected to proximate analysis. The carcasses were assayed for protein by the Kjeldahl (N X 6.25) method and lipid by a method derived from that of Barnes and Blackstock (1973). Moisture and ash were assayed according to the methods outlined in the Association of Analytical Chemists (A.O.A.C.) handbook (1990).

The final weights and haematological parameters were subjected to an analysis of variance where $p \leq 0.05$ was judged to be indicative of a significant difference. Where the ANOVA revealed significant differences Duncan's multiple range test (Duncan 1955) was applied in order to characterise the differences between the treatments.

3.1.3. Results

After 24 weeks there were no significant differences in the average weights of the test fish. However the biomass of the fish fed the deficient diets was smaller in all cases than that produced by the feeding of complete rations. Deficiency of B vitamins yielded the lowest biomass while the total biomass of the fish fed the commercially manufactured diet was marginally higher than that of the seabream fed the complete test diet.

In terms of survival, the fish depleted of B vitamins showed the highest cumulative mortality (30.8%), while those deficient in ascorbic acid and choline showed a lower cumulative mortality (15.0% and 12.2% respectively). The control group and those fish fed the commercial diet survived until the end of the experiment.

Deficiency induced pathology was manifested in the vitamin C depleted fish after 5 months of test diet feeding in the form of substantial scale loss on the lateral surfaces

(Plate 3) and a marked mono or bilateral exophthalmia (Plate 4) concomitant with a reduced appetite in the final two weeks of the nutrition trial. No externally visible pathology became apparent in any of the fish fed the remaining diets.

Table 3.1.3: Growth and dietary performance criteria of gilthead seabream fed diets deficient in choline (Ch-), B vitamins (B-), ascorbic acid (ASA-), complete diets (Con) and a commercially manufactured feed (PA) over the latter 100 days of the feeding period.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
	Ch -	B -	ASA -	Con	PA
Initial Mean Weight (g)	64.21	64.70	69.10	66.20	66.20
Final Mean Weight (g)	134.61	133.29	142.88	132.17	137.04
% Weight Gain	109.64	106.01	106.77	99.65	107.01
Cumulative Mortality (%)	12.20	30.77	15.00	0.00	0.00
Final Biomass (Kg)	4.73	3.69	4.86	5.29	5.48
SGR (%d ⁻¹)	0.73	0.72	0.72	0.68	0.72
Daily Feed Intake (gd ⁻¹)	1.17	1.23	1.39	1.12	1.14
Live Weight Gain (gd ⁻¹)	0.70	0.68	0.73	0.65	0.70
Feed conversion ratio	1.68	1.80	1.89	1.72	1.62
Feed Conversion Efficiency (%)	59.37	55.36	52.80	58.13	61.70

The haematology of the fish revealed a haematocrit significantly lower for the B vitamin and ascorbic acid depleted individuals when compared to that of the control and choline deficient groups ($p < 0.01$), however the packed cell volume observed for the fish fed the commercially manufactured diets was an intermediate of all the groups and hence was not significantly different to any dietary treatment. The haemoglobin content in the blood of the ascorbic acid deficient individuals was significantly lower than that of all treatments apart from that of the control ($p < 0.01$). The fish presented with diet 5, the commercial dietary regime, demonstrated a whole blood haemoglobin

concentration significantly higher than of the remaining groups. Total plasma protein showed no significant differences between the treatments ($p = 0.40$). An assay of plasma L-ascorbate revealed wide variation in average concentration with significantly lower levels of vitamin C in the plasma of the choline, B group and ascorbic acid deficient fish when compared to those of the bream fed the control and commercial diets. Within the deficient groups, the plasma ascorbate concentration was the lowest for the vitamin C deficient fish and was significantly lower than that observed during B complex deficiency.

Table 3.1.4: Haematological parameters of gilthead seabream fed diets deficient in choline (Ch-), B vitamins (B-), ascorbic acid (ASA-), complete (Con) and commercially manufactured (PA) diets for 100 days.
(Values on each row without or awarded similar superscripts are not significantly different from each other ($p < 0.01$).

		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
		Ch-	B-	ASA-	Con	PA
Haematocrit	Average	30.5 ^b	25.2 ^a	26.1 ^a	29.4 ^b	27.9 ^{ab}
(%)	(n)(s.d)	(10)(2.8)	(10)(2.58)	(19)(3.89)	(10)(3.70)	(10)(1.93)
[Haemoglobin]	Average	53.3 ^b	56.7 ^{bc}	36.7 ^b	43.3 ^{ab}	70.0 ^c
(mg/ml)	(n)(s.d)	(3)(0.58)	(3)(1.53)	(3)(0.58)	(3)(0.58)	(3)(0.00)
[Plasma L-Ascorbate]	Average	13.48 ^{ab}	23.50 ^{bc}	6.28 ^a	30.28 ^c	28.7 ^c
(µg/ml)	(n)(s.d)	(4)(7.59)	(4)(11.98)	(4)(23.29)	(4)(10.51)	(4)(3.55)
[Plasma Protein]	Average	33.8	32.8	29.3	33.3	34.5
(mg/ml)	(n)(s.d)	(4)(0.05)	(4)(0.43)	(3)(0.68)	(4)(0.22)	(4)(0.13)
[Plasma Glucose]	Average	0.373 ^a	0.331 ^a	0.940 ^b	0.340 ^a	0.442 ^a
(mg/ml)	(n)(s.d)	(4)(0.041)	(4)(0.035)	(3)(0.228)	(4)(0.069)	(4)(0.056)

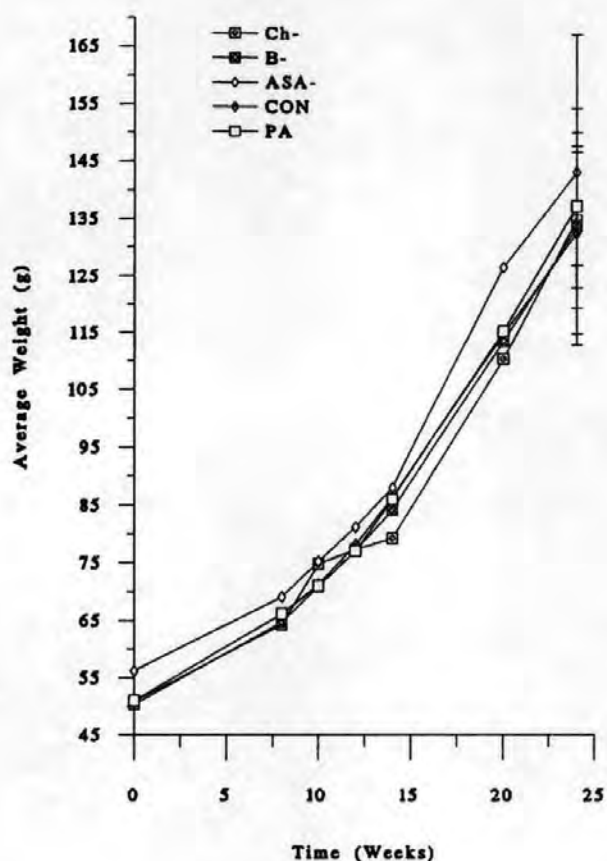
A deficiency of choline resulted in a plasma L-ascorbate concentration which was intermediate between that of the remaining deficient groups and consequently this was not significantly different from either ($p < 0.01$). Ascorbic acid deficiency resulted in a significant elevation in the concentration of plasma glucose above those of all the

remaining dietary regimes ($p < 0.001$)

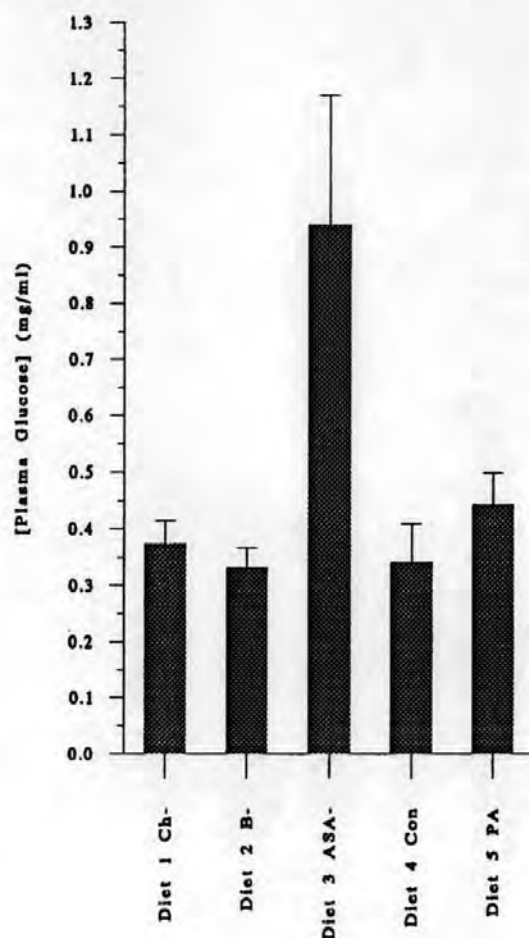
An analysis of the proximate composition revealed no obvious differences in the moisture, protein, lipid or ash content of the carcasses between the respective test fish as shown in table 3.1.5.

Table 3.1.5: Proximate composition of pooled carcasses of fish fed test diets for 100 days. Composition expressed as a percentage of the whole fish at the time of slaughter.

Diet	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Diet 1 Choline -	65.68	17.78	12.69	3.91
Diet 2 B-	66.68	16.90	12.48	3.70
Diet 3 ASA-	64.48	16.07	12.83	3.91
Diet 4 Control	65.75	17.28	11.94	3.81
Diet 5 PA	64.37	16.65	13.05	3.97



(a)



(b)

(a) Figure 3.1.1.

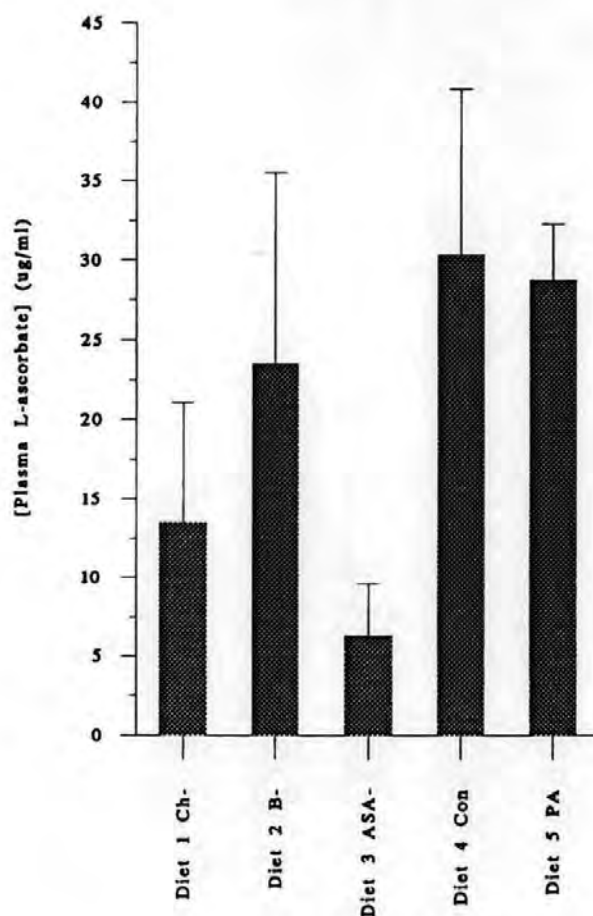
Growth of gilthead seabream fed diets deficient in choline, B complex vitamins and Vitamin C.

(b) Figure 3.1.2.

Plasma glucose concentration in gilthead seabream fed diets deficient in choline, B complex vitamins and Vitamin C.

(c) Figure 3.1.3.

Plasma ascorbic acid concentration in gilthead seabream fed diets deficient in choline, B complex vitamins and Vitamin C.



(c)



Plate 3: Scale loss in ascorbic acid deficient seabream.

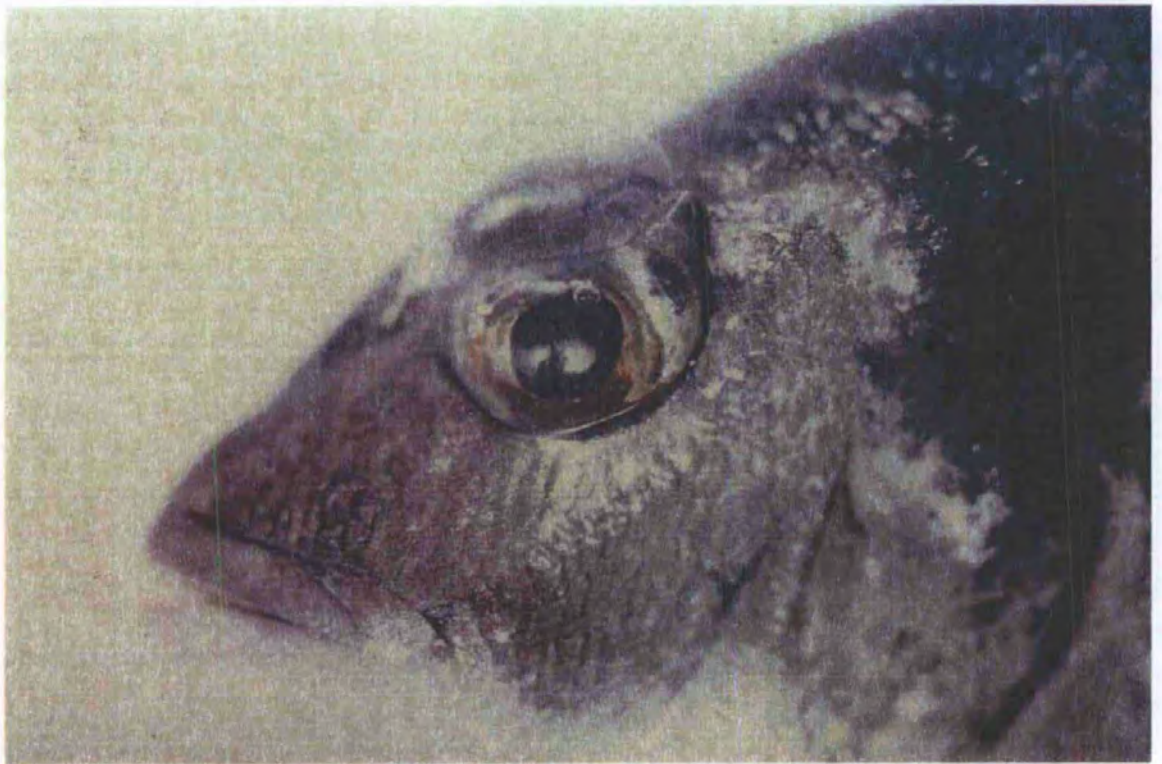


Plate 4: Exophthalmia in ascorbic acid deficient seabream.

3.1.4. Discussion.

The growth attained under the five dietary regimes revealed no significant differences between the treatments in terms of average weight though noticeable differences were revealed in terms of survivability and hence the biomass and quality of the fish produced.

In terms of survivability, the B vitamin deficient group suffered the highest mortality the distribution of which occurred in the six hours after the weighing events. The majority of the B vitamins are involved as cofactors in the metabolism of the major nutrients for example thiamin is essential for three enzyme catalysed reactions in the metabolism of carbohydrates, biotin for the decarboxylation of lipids and pyridoxine acts as a cofactor for the enzymes involved in both transamination within amino acid metabolism and for phosphorylase which is responsible for glycogenolysis. Thus, in many cases, specific metabolic lesions may be identified during the deficiency of a single B vitamin. Under resting conditions, the supply of these cofactors was sufficient to maintain the normal metabolism of the fish. However, under conditions of handling and weighing, elevations of the metabolic rate above that normally experienced may enforce a higher requirement for these cofactors in order to cope with the demands of increased metabolic activity associated with stress. The diets fed during the present experiment were deficient in all the B vitamins and hence metabolism would have been severely impeded, in this case to such an extent that mortality ensued.

The role of ascorbic acid in metabolism is far less rigidly defined than those of the B complex vitamins and this vitamin is not involved in intermediary metabolism to the same extent. Instead, ascorbic acid is associated with the activities of the iron and copper containing hydroxylases which participate in the post synthetic modification of

collagen during connective tissue metabolism and in the synthesis of the catecholamines from tyrosine (Bender 1992). Additionally, vitamin C acts as a reducing agent non specifically elevating the activity of a few enzymes (Bender 1992). Consequently in fish and other animals, ascorbic acid has been shown to be required for the metabolism of toxicants (Wagstaff and Street 1971, Mayer *et al.* 1978, Blanco and Meade 1980, Thomas *et al.* 1982, Scarano *et al.* 1991) and is involved with the normal function of the immune response as reviewed by Bendich (1990). With regards the present experiment, those mortalities induced by ascorbic acid deficiency were not attributable to any single metabolic perturbation but more likely a consequence of a wide variety of dysfunctions though scale loss with resultant fluid imbalance may have been a major contributory factor in the mortality of the fish.

Choline is a nutrient required for the formation of phospholipids both in cellular membranes (phosphotidylcholine) and in neurones (sphingomyelin) and also plays an important role in nervous transmission as acetyl choline (Bender 1992). In humans, phosphotidylcholine may be synthesised via a series of intermediates from phosphotidylserine and free choline may be generated by the hydrolysis of either dietary or endogenous phosphotidylcholine. Thus, the requirement of humans for choline is, at present, a matter of conjecture. Based on declining choline concentrations in the blood of humans fed diets low in this vitamin, the review of Zeisel (1990) concluded that choline was an essential nutrient in the diet of humans. Bender (1992), on the other hand, states that as a consequence of the high phosphotidylcholine (lecithin) content of biological membranes and in the absence of deficiency symptoms, there is no requirement for pre-formed choline in the diet of humans maintained on diets of sufficient quality.

Choline has long been recognised as an essential nutrient in the diets of fish and hence deficiency symptoms (mainly associated with perturbations of hepatic lipid metabolism) have been recorded in choline deficient chinook salmon (*Oncorhynchus tshawytscha*) (Halver 1957), rainbow trout (*Oncorhynchus mykiss*) (McLaren *et al.* 1947, Kitamura *et al.* 1967), common carp (*Cyprinus carpio*) (Ogino *et al.* 1970a), white sturgeon (*Acipenser transmontanus*) (Hung 1989) and red seabream (*Chrysophrys major*) (Yone and Fujii 1974).

The observations of the present experiment indicate that the fish fed diets without supplemental choline were indeed deficient in this vitamin as evidenced by the mortality and low plasma ascorbate content of these individuals. However, none of the other parameters evaluated denoted a deficiency of this vitamin. Some fish species maintain an ability to synthesise choline from precursors, thus, lake trout (*Salvelinus namaycush*) may utilise methyl- and dimethylaminoethanol as precursors for the production of this vitamin (Ketola 1976) and channel catfish (*Ictalurus punctatus*) may utilise methionine to spare dietary choline (Wilson and Poe 1988). This metabolic pathway was not observed in the rainbow trout which is unable to form choline from methionine (Rumsey 1991). However, in the same experiment Rumsey (1991) demonstrated that betaine could approximately halve of the choline requirement rainbow trout. Thus it was postulated that half the dietary requirement of rainbow trout for choline must be met by preformed vitamin but the remaining portion may be satisfied by betaine. Consequently, the results of the present experiment may be attributed to either an almost sufficient supply of phosphatidylcholine within the diet or an ability of the gilthead seabream to synthesise choline from precursors.

Only the fish fed diets deficient in ascorbic acid demonstrated externally visible

pathology which was characterised by mono and bilateral exophthalmia and a severe loss of skin and scales exposing the musculature of the fish. However scoliosis and lordosis, often considered to be characteristic of severe ascorbic acid deficiency, did not become apparent in the test animals.

Fish display symptoms of ascorbic acid deficiency which are attributed to a decline in the activity of the proline and lysine hydroxylases and hence frequently described as scurvy. Exophthalmia has also been recorded in rainbow trout (Halver *et al.* 1975), Asian seabass (*Lates calcarifer*) (Boonyaratpalin *et al.* 1989) and *Cichlasoma urophthalmus* (Chavez De Martinez 1990a) during ascorbic acid deficiency and is a consequence of the non uniform deposition of support cartilage in the eye resulting in distortion of the eye ball (Halver *et al.* 1975). The loss of skin and scales within the present experiment has also been recorded along with an erosion and malformation of the fins in ascorbic acid deficient Nile tilapia (*Oreochromis niloticus*) (Soliman *et al.* 1994), channel catfish (Lim and Lovell 1978), Indian major carp (*Cyprinus mrigla*) (Mahajan and Agrawal 1979), *Tilapia aurea* (Stickney *et al.* 1984) and *Cichlasoma urophthalmus* (Chavez De Martinez 1990). In a similar vein, the observations of Ashley *et al.* (1975) and Jauncey *et al.* (1985) have respectively demonstrated an impairment of the wound repair mechanisms in the skin of ascorbic acid deficient coho salmon (*Oncorhynchus kisutch*), rainbow trout and tilapia (*Oreochromis niloticus*).

Scoliosis and lordosis have frequently been observed during ascorbic acid deficiency in species such as coho salmon and rainbow trout (Halver *et al.* 1969, Hilton *et al.* 1978), plaice (*Pleuronectes platessa*) (Rosenlund *et al.* 1990), Indian major carp (Mahajan and Agrawal 1980), channel catfish (Wilson and Poe 1973, Miyazaki *et al.* 1985), snakehead (*Channa punctatus*) (Mahajan and Agrawal 1979), *Tilapia aurea*

(Stickney *et al.* 1984) and *Cichlasoma urophthalmus* (Chavez De Martinez 1990a). Thus, since these conditions were not observed during the deficiency of any of the other vitamins scoliosis and lordosis have been ascribed as diagnostic features of inadequate ascorbic acid supply.

Halver *et al.* (1975) proposed that for growing salmonids, a ten fold increase in weight was required in order that ascorbic acid deficiency became severe enough to become apparent as spinal deformity. Additionally, as outlined in chapter one, the ascorbic acid requirement of teleosts declines as the fish increase in weight (Hilton *et al.* 1978, Sato *et al.* 1978,). Drawing together the factors of an absence of spinal malformation and the appearance of severe degeneration in the skin of the fish within the present experiment, it becomes apparent that in gilthead seabream approaching the market size, ascorbic acid deficiency manifests itself as a dermatopathy without the onset of spinal malformation. The present experiment utilised fish of a large initial weight (approximately 70 g) and the final weight was only double that of the initial. Consequently, in the light of this small weight gain coupled to the presumed lowering of the ascorbic acid requirement of the larger fish, the absence of spinal deformity is not unexpected.

Haematological modifications have frequently been associated with deficiencies of the B vitamins in fish and are considered in a subsequent chapter which examines the consequences of the absence of individual B vitamins in the diet of the gilthead seabream. In summary however, modifications of the haematological parameters associated with erythrocytes have been recorded for fish deficient in; riboflavin (Tunison *et al.* 1942, Soliman and Wilson 1992), niacin (Andrews and Murai 1978), pantothenic acid (Murai and Andrews 1979, Roem *et al.* 1991, Soliman and Wilson 1992), pyridoxine

(Halver 1957, Smith *et al.* 1974, Adron *et al.* 1978, Agrawal and Mahajan 1983b, Vel *et al.* 1990), biotin (Halver 1957), folic acid (Halver 1957, Kitamura *et al.* 1967, Smith 1968, Smith and Halver 1969, John and Mahajan 1979, Duncan *et al.* 1993) and cyanocobalamin (Halver 1957, John and Mahajan 1979). Thus, in the present case the reduction in packed cell volume associated with the depletion of the B vitamins agrees with the findings for many other species. Additionally, simultaneous depletion of B vitamins results in a quicker and more significant lowering of the packed cell volume as evidenced by the observations of John and Mahajan (1979).

In the present experiment, a significant depression of the haematocrit was associated with vitamin C deficiency. Anaemia is frequently associated with ascorbic acid deficiency in most animals and is believed to be a consequence of interactions of this vitamin with iron and or folate (Bender 1992). The aetiology of the ascorbic acid - folate relationship is uncertain though the role of ascorbate in the maintenance of the body pool of reduced folates appears plausible since none of the reactions of folate itself are believed to be ascorbate dependent (Bender 1992). Ascorbic acid is recognised as a factor involved in the absorption of inorganic metals *eg.* calcium (Mahajan and Agrawal 1980) and iron (Dabrowski and Köck 1989) and in the mobilisation of iron reserves (Lipschitz *et al.* 1971), thus a relationship between vitamin C deficiency and anaemia immediately becomes apparent as evidenced in the present case by the low haemoglobin content in the blood of the ascorbate deficient fish. Reductions of the haematocrit and or low haemoglobin content have also been recorded in the blood of ascorbic acid deficient Atlantic salmon (*Salmo salar*) (Maage *et al.* 1990) rainbow trout (Hilton *et al.* 1978, Akand *et al.* 1987), channel catfish (Lovell and El Naggar 1989), plaice (Rosenlund *et al.* 1990), Nile tilapia (Soliman *et al.* 1994) and hybrid tilapia (*Oreochromis niloticus*

X *O. aureus*) (Shiau and Jan 1992).

Under the vitamin C deficient regime, the concentration of circulating ascorbic acid was demonstrated to be significantly lower than those of the control groups since, not surprisingly, ascorbic acid deficiency is always associated a depression of the tissue pools of this vitamin. The plasma ascorbate concentration under the choline depleted regime was also significantly lower than that recorded for the control groups. A well known relationship exists between the anti-oxidant vitamins C and E in that Vitamin C is oxidised in order to recycle vitamin E from its radical (Bender 1992) and it is also known that vitamin E plays a key role in the maintenance of membrane integrity (Niki *et al.* 1989). Thus, given the role of choline as a molecule involved in the formation of phospholipid membranes, it is not unreasonable to postulate at this point that choline deficiency may indirectly enforce a greater requirement for ascorbic acid in order to maintain adequate levels of reduced vitamin E and hence maintain membrane integrity.

In fish, environmental and handling conditions represent non specific stressors which invoke a stress response. This may be sub-divided into primary, endocrinal effects and secondary, metabolic responses (Mazeaud *et al.* 1977). In simple terms, the endocrinal effects include elevations in circulatory catecholamines and or the corticosteroid hormones. These hormonal changes, either directly or indirectly, induce a metabolic response the nature of which depends on the stressor but may include significant increases in the levels of circulatory glucose or free fatty acids. In the present case, those individuals fed diets without supplemental ascorbic acid developed a plasma glucose concentration which was significantly higher than that observed under any other regime. Such elevations in plasma glucose concentration have frequently been observed in fish subjected to stress (Mazeaud *et al.* 1977, Hunn and Greer 1991, Hopkins and

Cech 1992) and given the poor condition of the ascorbic acid deficient fish, it is not unreasonable to postulate that the observed elevation in plasma glucose is a response to the poor ascorbic acid status of the fish.

The overall observations of the present experiment are not just a factor of the inclusion or absence of certain vitamins from the pre-mix incorporated into the diets but also a combination of several factors related to vitamin nutrition which include; the scale and availability of the vitamin content of the raw feed materials, modifications in the requirement for vitamins with respect to fish size, tissue retention of vitamins in the fish and finally, synthesis of certain vitamins by the gut micro flora and the ability of some fish to synthesise vitamins from precursors. These factors were reviewed in chapter one, and must all make a contribution to the pattern of the results observed. However, the relative contribution of each of these to the observations of the current study is not easily quantified and outside the scope of the present experiment.

The current study indicates that production sized seabream are able to maintain a capacity for growth using the supplies of vitamins which are available from the feed materials and or synthesised by the gut flora. However, for the maintenance of optimum health, survivability and diet performance the use of a vitamin pre-mix is essential. Based on these observations, further experiments will focus on aspects of the water-soluble vitamin nutrition of the gilthead seabream including qualitative and quantitative aspects of B vitamin supply from both purely academic and applied perspectives. Such investigations will examine qualitative aspects of ascorbic acid source, quantitative and qualitative aspects of B vitamin supply and the relationship between dietary macronutrients and vitamin requirements.

3.2. The Qualitative Requirement of the Gilthead Seabream

(Sparus aurata L.) For B Vitamins.

3.2.1. Introduction.

Due to the wide distribution of the B vitamins within feed materials, their chemical diversity, and the complexity of their roles in metabolism, the deficiency syndromes associated with a paucity of individual B vitamin supply are not commonly seen separately. Furthermore the clinical manifestation of a single vitamin deficiency often results in pathologies apparently similar to those of other vitamins. Consequently, studies of the results of poor vitamin supply with respect to individual vitamins are difficult. However, under unusual circumstances, for example poor storage conditions of feed or feed ingredients, individual vitamin deficiencies may become apparent due to specific losses or degradation of one vitamin within the formulation. In such events pathologies may become apparent within the stock and thus a knowledge of the deficiency symptoms displayed by each species during vitamin deficiency will allow prompt remedial action.

By the application of semi-purified diets, the pathologies associated with individual B vitamin deficiencies have been characterised for a range of important species in aquaculture. At present, there exists a vast array of literature which outlines the deficiency related pathologies and qualitative requirements for vitamins of many cultured species, with suitable reviews written by; Halver (1989), Steffans (1989) and Tacon (1991).

Little information exists regarding the B vitamin requirements or the deficiency associated pathology of the gilthead seabream though studies include an estimation of

the Pyridoxine requirement of the species (Kissil 1981, Kissil *et al* 1981) and a preliminary evaluation of the biotin requirement of this species (Kissil 1981). The qualitative water-soluble vitamin requirements of a series of similar species have however been elucidated. For example, the pyridoxine requirement and associated deficiency related pathologies were elucidated in the red seabream (*Chrysophrys major*) by Takeda and Yone in 1971. Later the qualitative requirement of this species for thiamin, riboflavin, niacin, choline and ascorbic acid was demonstrated by Yone and Fujii (1974), however, under the test conditions applied Yone and Fujii (1974) found no requirement for biotin or folic acid. Yano *et al* (1988) defined the quantitative requirement of the red seabream for pantothenic acid and inositol and additionally investigated the relationship between these vitamins and serum complement activity.

The quantitative requirements of the Asian seabass (*Lates calcarifer*) for vitamin C and pyridoxine have been determined by Boonyaratpalin *et al.* (1989) and Wanakowat *et al.* (1989) respectively, while the qualitative requirements of this species for thiamin, riboflavin, pantothenic acid and inositol were demonstrated by Boonyaratpalin and Wanakowat (1991).

The aim of the present experiment was to characterise the pathologies associated with dietary deficiencies of thiamin, riboflavin, pyridoxine, niacin and pantothenic acid with reference to a complete test diet in order to assess the relative importance of each in terms of growth, feed utilisation and general health in the nutrition of the gilthead seabream. The vitamins targeted for the present study all, to various extents, play key roles in intermediary metabolism (as detailed in chapter 1) and hence were expected in their deficiency to produce marked effects on the fish within a reasonable time scale *i.e.* less than 12 weeks.

3.2.2. Materials and Methods.

Sixty Seabream fry (Initial Weight 8 g) were stocked into each of six 400 l, self cleaning, fibreglass tanks over a 1200 l bio-filter within a closed recirculation system providing a parallel flow of seawater through the tanks at 2.8 l min^{-1} . Water temperature and salinity were maintained at 24°C and 33 - 36‰ saline respectively, the former by an integrated dip-cooler and immersion heater, the latter by the use of a freshwater inlet balanced to compensate for evaporative losses. The pH was maintained between 6 and 7.5 by the use of calcium carbonate buffering while ammonia and nitrite were held within ranges tolerated by this species. Daylight balanced fluorescent strip lamps maintained an 8 hr light: 16 hr dark regime.

Using a feed formulation package, a test diet was designed with casein, gelatin and crystalline amino acids providing approximately 50% crude protein and 11% lipid provided by cod liver oil. Six test diets were manufactured differing with respect to the vitamins deleted from the B vitamin pre-mix. Diet 1 was a complete test diet (Con) while the remaining diets were devoid of supplemental riboflavin (Ribo-), niacin (Niacin-), pantothenic acid (Panto-), pyridoxine (Pyri-) and thiamin (Thia-). The diets were formulated as shown in table 3.2.1 by mixing the dry ingredients, cod liver oil and sufficient water to produce a moist dough in a Hobart A101 mixer followed by cold extrusion through a 3/16" die. The resultant pellets were then frozen (-18°C) and stored in air tight bags.

The fish were presented with frozen, moist pellets three times daily (09:00, 13:00 and 16:00 hrs) at up to 2% of the live weight. The moisture content of each diet (approx 30 - 35 %) was determined in order that equivalent amounts of dry matter was presented to the fish. The weight of the daily ration was re-calculated on the basis of bi-monthly

Table 3.2.1: The Semi-purified Test Diet.

Ingredient	% Inclusion
Casein (Vitamin Free)	31.95
Gelatin (225 Bloom)	15.00
Corn Starch	19.70
Cod Liver Oil	11.84
Mineral Pre-mix ¹	10.00
Fat Soluble Vitamin Pre-mix ²	0.50
B Vitamin Pre-mix ³	0.50
Macro Vitamin Pre-mix ⁴	2.00
Amino Acid Pre-mix ⁵	5.18
Dietary Marker (CrO ₃)	0.50
Di-Calcium Phosphate	2.88

¹Contribution of minerals to diet (mgkg⁻¹ Diet)

CaHPO₄ 22050, CaCO₃ 3000, NaCl 15000, K₂SO₃ 20000, MgSO₄·7H₂O 20627, FeSO₄·7H₂O 700, MnSO₄·4H₂O 227.3, ZnSO₄ 515.6, CuSO₄·5H₂O 160.0, CoCl₂·6H₂O 26.0, KI 15.0, Na₂SeO₃ 2.5, α-cellulose 17676.6

²Contribution of the fat-soluble vitamins to diet (mgkg⁻¹ Diet)

Vitamin A Palmitate 4.2, Vitamin D₃ (99%) 0.046, Vitamin E Acetate 439, Menadione Sodium Bisulphite 780, α-cellulose 3776.75 (Contribution from cod liver oil (cm⁻³: Vitamin A 400 I.U., Vitamin D 40 I.U., Vitamin E 1.0 I.U.)

³Contribution of the B vitamins to the diet (mgkg⁻¹ Diet)

Thiamin Hydrochloride 69.9, Riboflavin (96%) 208.3, Pyridoxine Hydrochloride 48.6, Niacin 800, Calcium Pantothenate 305.3, Biotin (2%) 300, Folic Acid (88.8%) 16.9, Cyanocobalamin (95%) 0.01, α-cellulose 3250.99

⁴Contribution of the macro vitamins to the diet (gkg⁻¹ Diet)

Calcium Ascorbate 2.216, Choline Chloride 10.62, Inositol 4.00, α-cellulose 3.164

⁵Contribution of the amino acids to the diet (gkg⁻¹ Diet)

D-L-Methionine 4.80, L-Tryptophan 4.70, L-Threonine 8.70, L-Phenyl Alanine 5.80, L-Histidine 1.40, L-Arginine 7.00, L-Isoleucine 4.80, L-Leucine 5.00, L-Valine 9.10

Table 3.2.1: Nutrient profile of the test diets. Moisture is expressed as a percentage of the diet as fed. Protein, lipid, ash and residual expressed as a percentage of the dry matter.

Moisture (%)	Protein (%DM)	Lipid (%DM)	Ash (%DM)	Residual (%DM)
37.89	49.54	7.20	6.55	36.71

weighing of the fish. Where feed refusal was apparent, the fish were fed *ad libitum* up to 2 % of the body weight and the actual amount of feed consumed was accurately recorded.

The weight gain, specific growth rate (SGR), feed intake, feed conversion ratio (FCR), feed conversion efficiency (FCE) and cumulative percentage mortality were monitored throughout the trial. The fish were inspected daily for the appearance of gross deficiency symptoms.

At the termination of the growth trial, samples of whole blood were collected by caudal section into heparinised micro haematocrit tubes and the packed cell volume was immediately determined by micro haematocrit. The hepatopancreas and spleen were excised and the hepato and splenosomatic indices determined at this point. Additionally, samples of liver were quenched in liquid cooled n-hexane (-70°C). The sections then maintained at -25°C prior to sectioning and staining as outlined in chapter 2.

Immediately after manufacture, diet samples were frozen and later, along with the carcasses of fish sampled at the beginning of the trial and the pooled carcasses of five fish from each dietary regime sampled at the end of the experiment, subjected to proximate analysis. Crude protein was determined by the Kjeldahl method, lipid by soxhlet extraction using petroleum ether and moisture and ash as outlined by the A.O.A.C (1984). Based on the proximate composition of the diets and fish, the protein efficiency ratio (PER) and net protein utilisation (NPU) were calculated.

The data were subjected to analysis of variance where $P \leq 0.05$ was judged to be indicative of a significant difference. Where the ANOVA revealed significant differences Duncan's multiple range test (Duncan 1955) was applied in order to characterise and quantify the differences between the treatments.

3.2.3. Results.

After 14 weeks of feeding there were significant differences in the average weights of the fish under the six regimen. An examination of the percentage weight gains showed that only the control group doubled their initial weight and was the only treatment to display continuous growth throughout the entire fourteen week period. Thus, those fish fed a complete diet were significantly larger ($P < 0.01$) than any of the fish fed any of the vitamin depleted diets. Those fish maintained on a diet deficient in pyridoxine were significantly smaller ($P < 0.01$) than those maintained on diets deficient in pantothenic acid and thiamin but not significantly smaller than those fish fed niacin and riboflavin free diets. The final average weights of those individuals depleted of niacin, riboflavin, pantothenic acid and thiamin were not significantly different from each other.

Weight loss became apparent under the pyridoxine, niacin and riboflavin deficient regimen at 4, 6 and 8 weeks respectively. A cessation of weight gain was observed in those individuals deficient in pantothenic acid and thiamin. Comparison of specific growth rate (SGR) can only be applied to the control, pantothenic acid and thiamin depleted treatments since under the remaining regimen the fish lost weight. On this basis the rate of growth of the control fish was approximately 40% higher than that observed for the thiamin and pantothenic acid deficient fish.

Cumulative percentage mortality for the control group was the lowest (1.7%) with mortality for the pyridoxine deficient fish the highest (18.3%). The remaining deficiencies resulted in mortalities of 6.7% for thiamin and riboflavin and 13.3% for niacin and pantothenic acid.

Table 3.2.3: Growth and dietary performance criteria of gilthead seabream fed six dietary treatments deficient in individual members of the B complex. Values on the same row carrying similar superscripts are not significantly different from each other ($P < 0.01$)

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
	Control	Ribo -	Niacin -	Panto-	Pyri -	Thia -
Initial Mean Wt (g)	7.96	7.98	8.00	8.05	7.68	7.86
Final Mean Wt (g)	17.70 ^c	11.22 ^a	11.82 ^a	14.18 ^b	9.33 ^a	13.71 ^b
% Weight Gain	122.36	40.60	40.25	76.15	21.48	74.43
Specific Growth Rate (SGR) (%d ⁻¹)	0.82	0.35	0.40	0.58	0.20	0.57
Daily Feed Intake (mgd ⁻¹)	214.70	126.00	119.40	137.40	109.90	155.20
Live Weight Gain (mgd ⁻¹)	99.40	33.10	39.00	62.50	18.10	59.70
Feed Conversion Ratio (FCR)	2.16	3.81	3.06	2.20	6.53	2.60
Feed Conversion Efficiency (FCE) (%)	46.28	26.23	32.99	45.50	15.32	38.46
Protein Efficiency Ratio (PER)	1.07	1.89	1.54	1.11	3.22	1.30
Net Protein Utilisation (NPU) (%)	13.58	7.25	9.21	12.15	1.93	9.66
Mortality (%)	1.67	6.67	13.33	13.33	18.33	6.67

Table 3.2.4: Haematocrit, hepatosomatic index (HSI) and splenosomatic index (SSI) of gilthead seabream fed six dietary treatments deficient in individual members of the B complex. Values on the same row carrying similar superscripts are not significantly different from each other ($P < 0.05$).

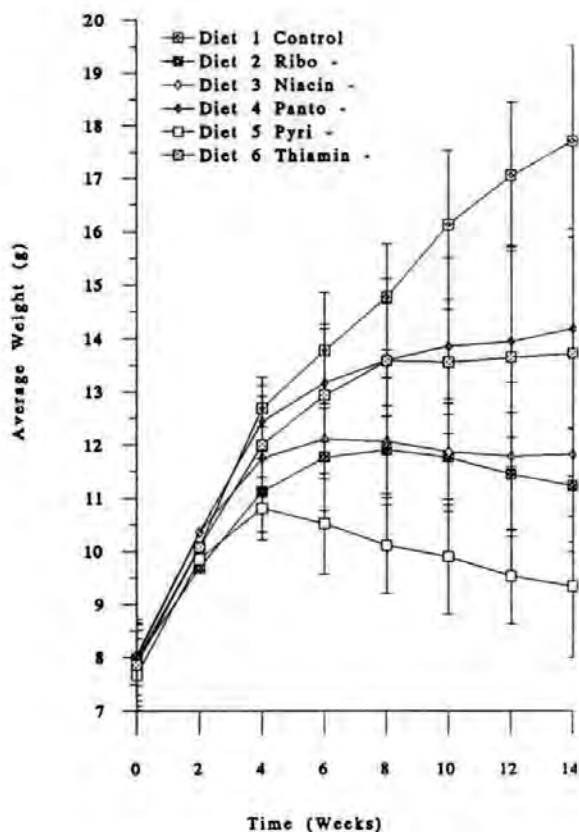
		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Parameter		Control	Ribo -	Niacin -	Panto -	Pyri -	Thia -
Haematocrit	n	10	10	10	10	10	10
	Average	38.78 ^c	19.93 ^a	26.77 ^b	28.01 ^b	27.96 ^b	21.63 ^a
	S.E.	1.10	1.41	0.78	1.06	1.99	0.91
HSI	n	21	19	18	19	20	19
	Average	1.01 ^a	0.72 ^b	0.74 ^b	0.75 ^b	0.73 ^b	0.65 ^b
	S.E.	0.07	0.06	0.08	0.06	0.05	0.05
SSI	n	10	9	8	10	10	9
	Average	0.070	0.060	0.064	0.056	0.056	0.042
	S.E.	0.009	0.009	0.010	0.006	0.005	0.006

Appetite for the control group remained adequate throughout the fourteen week period while that of the pyridoxine, niacin and riboflavin deficient fish became suppressed after three weeks of feeding. A reduction in the appetite of those individuals deficient in pantothenic acid and thiamin became apparent after six weeks.

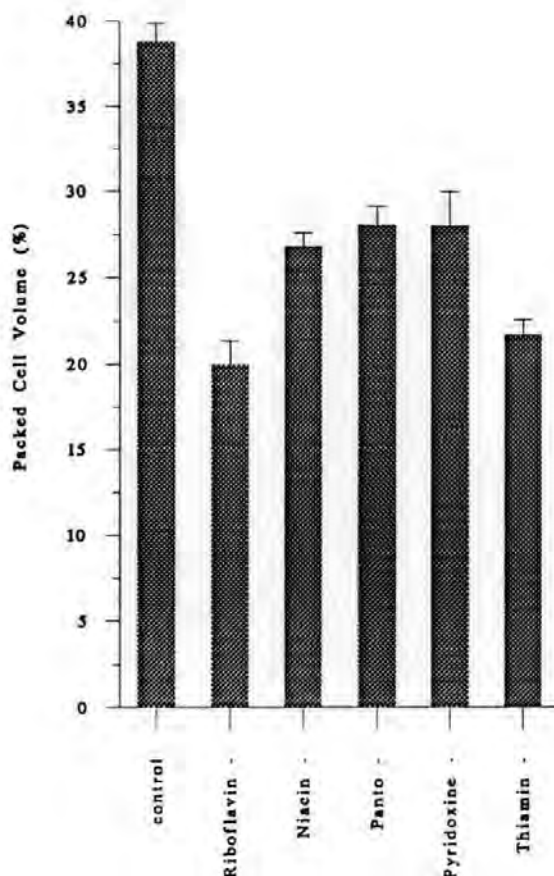
In terms of feed conversion efficiency (FCE), the control, pantothenic acid and thiamin deficient groups showed similar values (46.28, 45.5 and 38.46% respectively) while those individuals depleted of riboflavin and niacin again demonstrated similar FCE (26.23 and 32.99% respectively). The poorest FCE (15.32 %) was attained for those fish depleted of pyridoxine.

Due to the small size of the fish at the end of the experiment, pooled samples were utilised for the determination of the proximate composition of the fish. Consequently, the proximate composition of the carcasses could not be subjected to a statistical analysis. Despite this, it becomes apparent that the protein and ash content of the fish under each of the deficient regimen was maintained to within 1 % of the control values. However, the moisture and lipid content were subject to wide variation during vitamin deficiency. Thus the deficient individuals demonstrated a reduction in the lipid content concomitant with an elevation in the carcass moisture. This relationship was not strictly quantitative *ie.* the carcass moisture content was not elevated in direct response to reductions in the lipid component however, the modifications in the proportions of the remaining components (protein and ash) were small. To summarise, the deficient fish demonstrated a much reduced lipid content and an elevated carcass moisture when compared with the fish fed the complete ration.

Protein efficiency ratio and net protein utilisation were adversely affected during vitamin deficiency. Thus in terms of net protein utilisation, the fish fed the complete diet



(a)



(b)

(a) Figure 3.2.1.

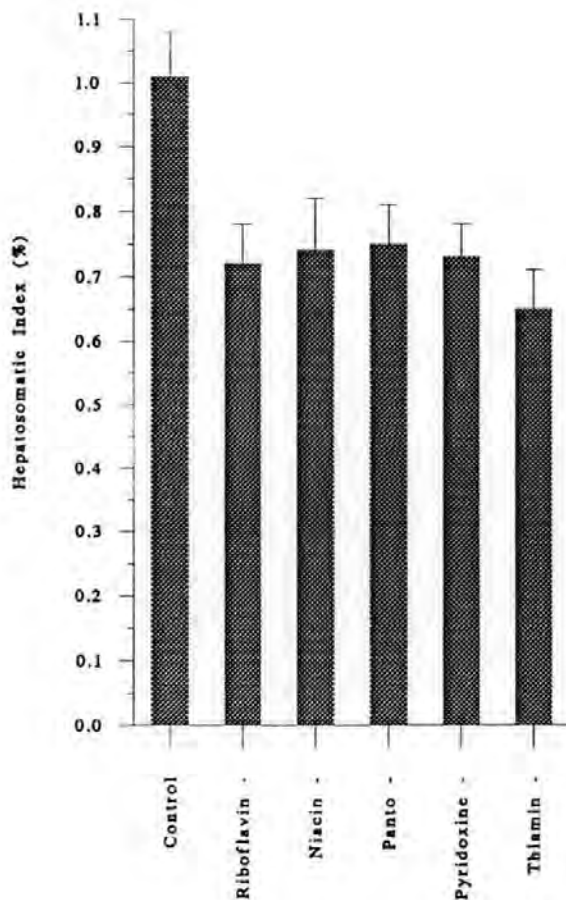
Growth of gilthead seabream fed diets deficient in thiamin, riboflavin, niacin, pyridoxine and pantothenic acid.

(b) Figure 3.2.2.

Haematocrit of gilthead seabream fed diets deficient in thiamin, riboflavin, niacin, pyridoxine and pantothenic acid.

(c) Figure 3.2.3.

Hepatosomatic index of gilthead seabream fed diets deficient in thiamin, riboflavin, niacin, pyridoxine and pantothenic acid.



(c)

performed the best scoring values of 13.58 % and 1.07 for NPU and PER respectively. NPU did not closely reflect growth in all cases, thus, despite an adequate rate of growth the thiamin deficient fish demonstrated a comparatively poor NPU (9.66 %). As expected, the deficiency of pyridoxine resulted in extremely poor values for both PER and NPU (1.93 and 3.22 % respectively).

Table 3.2.5: Proximate composition of initial carcasses and carcasses of experimental animals after 14 weeks of test ration feeding. Carcass composition represents the nutrient profile of five pooled individuals on the as is basis *i.e.* as a proportion of the live fish.

	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Initial Fish	72.34	16.27	5.44	4.77
+ve Control	71.73	15.35	7.02	4.98
Riboflavin -	74.89	15.53	3.10	5.21
Niacin -	75.34	15.60	3.10	5.32
Pantothenate -	75.25	15.08	2.91	5.32
Pyridoxine -	77.56	14.49	1.05	5.68
Thiamin -	75.03	14.69	4.03	5.03

In comparison with the packed cell volume of control group (38.78 %), all treatments exhibited a significant suppression of the haematocrit ($p < 0.05$). Riboflavin and thiamin deficiency induced the greatest suppression (19.93 and 21.63%), while the remaining treatments induced smaller reductions in haematocrit (niacin 26.77, pantothenic acid 28.01, pyridoxine 27.96%).

Comparison of the hepatosomatic index (HSI) of the control group with those of the deficient groups shows an HSI for the control group significantly higher than that of

the remaining treatments ($p < 0.05$). Thiamin and riboflavin deficiency resulted in a reduced splenosomatic index in comparison with the remaining treatments.

A low power examination of the haematoxylin and eosin stained liver sections (Plates 5 and 6) revealed considerable disturbances in the normal architecture of this organ in those fish fed diets deficient in riboflavin, niacin and pyridoxine. Overall, the deficient groups exhibited atrophy of the pancreas while the hepatocytes were either stained intensely and uniformly or pale and diffuse. The pancreas of the fish presented with the control diet was intact though the acinar cells appeared to be agranular. The pancreas of the riboflavin and niacin deficient fish exhibited a less specific staining reaction and a degree of atrophy was exhibited, while those of the fish deficient in pyridoxine, thiamin and pantothenic acid were very atrophic. With regards the hepatocytes, both types of staining reaction were observed in the control fish. The hepatocyte staining was patchy in the riboflavin and niacin deficient fish though the extent of atrophy was greater in the latter. The fish maintained on diets deficient in pantothenic acid, pyridoxine and thiamin exhibited extensive intra and inter animal variability though, in general, the hepatocytes were stained homogeneously but patchy.

The livers of the fish maintained on all diets exhibited very little PAS positive material with the more intense staining exhibited by the macrophages associated with the pancreas and those distributed among the hepatocytes. Large melanomacrophage aggregates associated with the pancreas were observed in all dietary groups apart from the control. In the case of the pantothenic acid, thiamin and pyridoxine deficient fish, a number of pigmented granules were also observed in close association with the melanomacrophage aggregates.

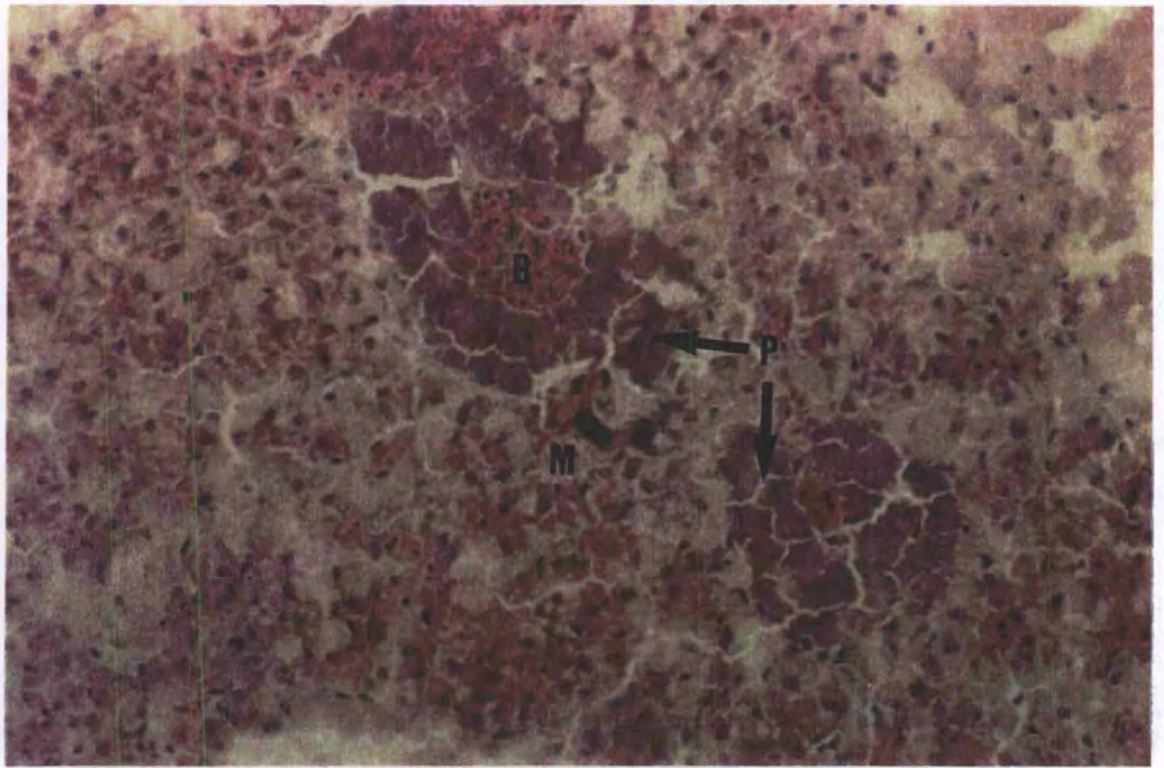
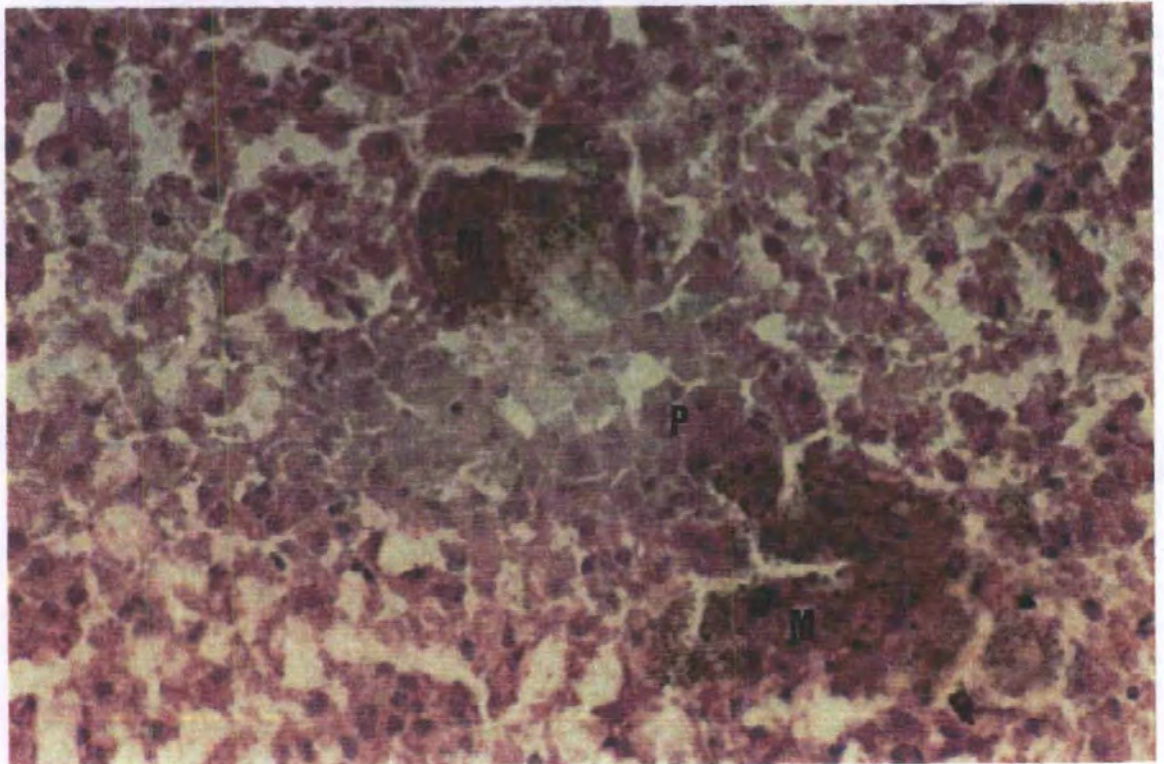


Plate 5: Melanin accumulation (M) and increased blood cell activity (B) associated with intact pancreas (P) of riboflavin deficient gilthead seabream (H and E, X 160).

Plate 6: Melanin accumulation (M) associated with atrophic pancreas (P) of thiamin deficient gilthead seabream. (H and E, X 160).



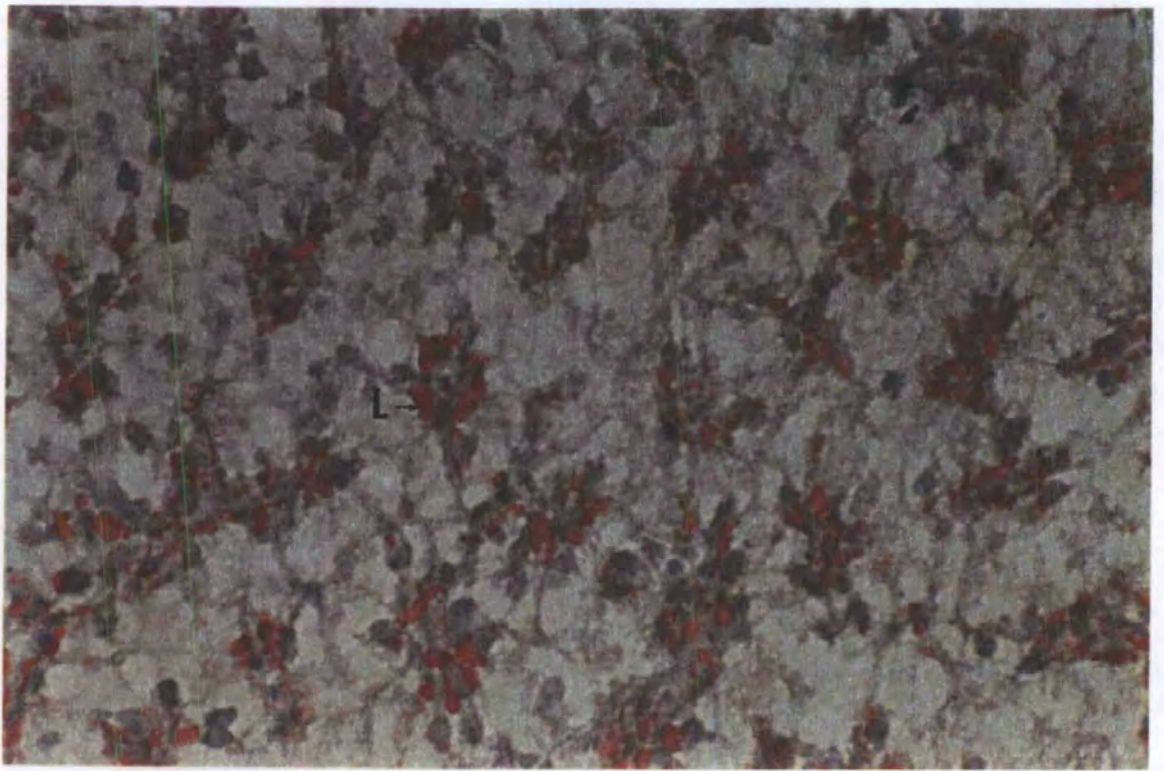


Plate 7: Distribution of neutral lipid (L) in liver parenchyma of gilthead seabream fed complete diets (Oil red O, X 100)

Plate 8: Distribution of neutral lipid (L) in liver parenchyma of gilthead seabream fed riboflavin deficient diets (Oil red O, X 100)



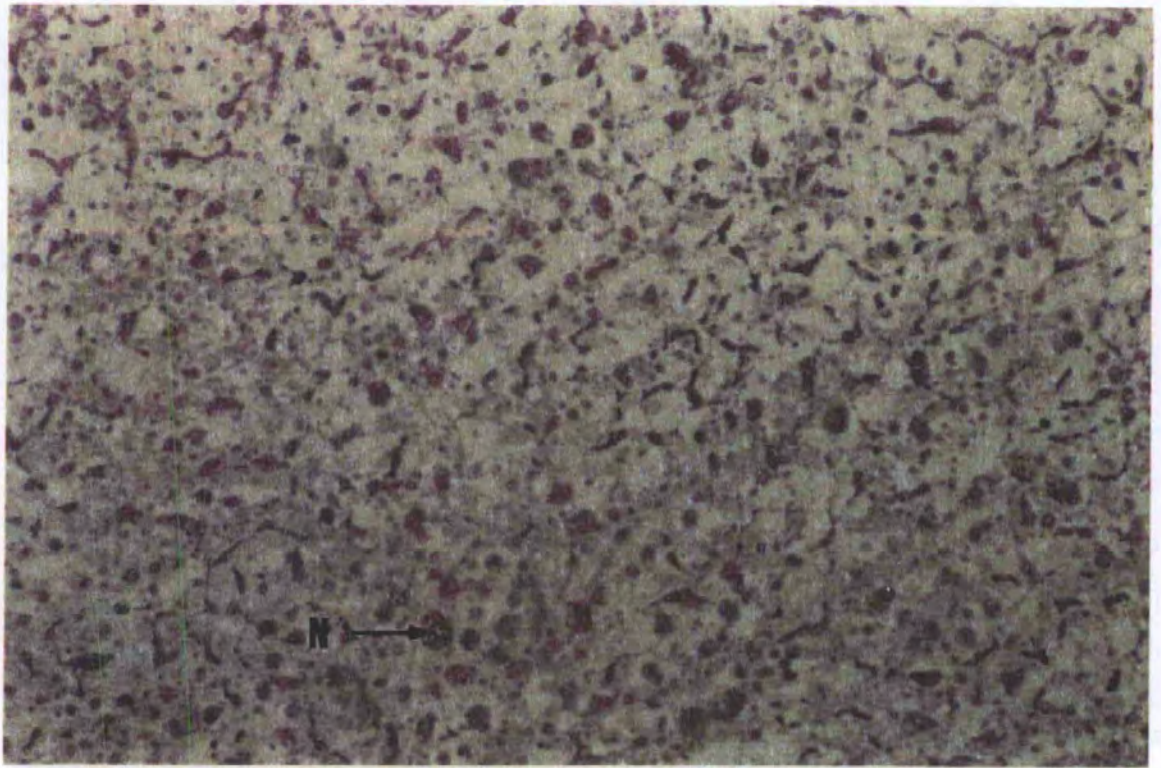
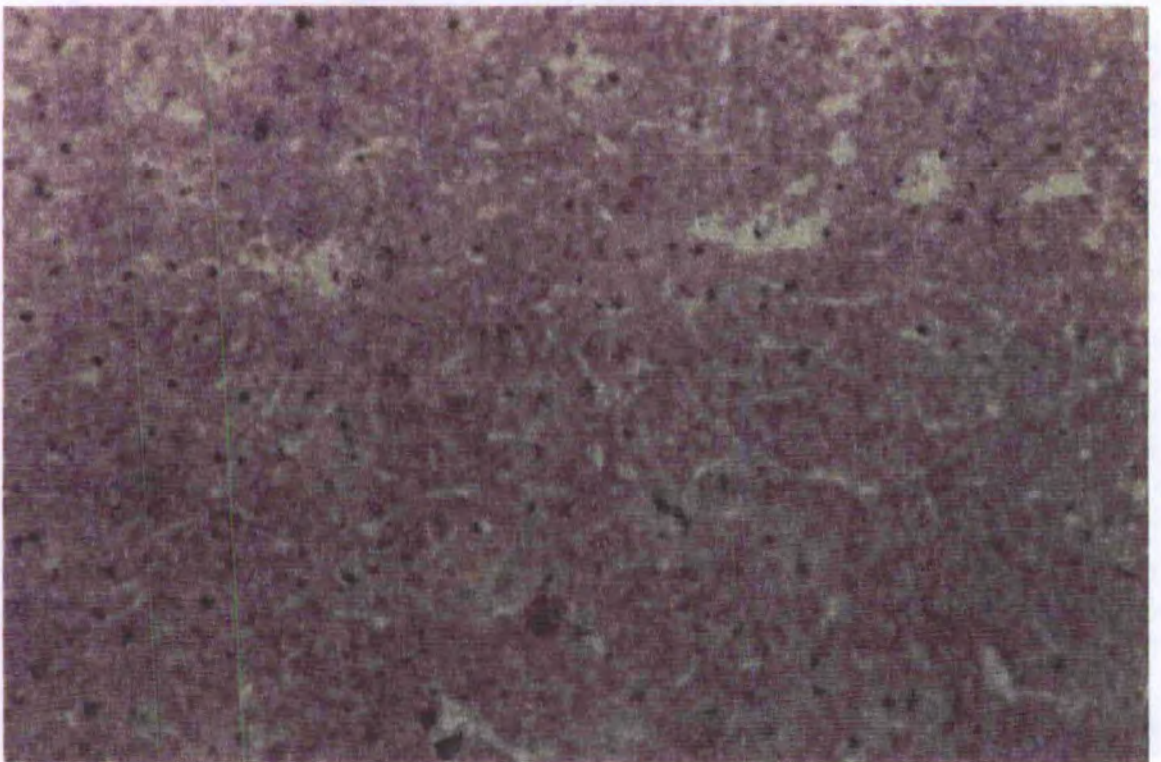


Plate 9: Distribution of lysosomal NAH (N) in the liver parenchyma of gilthead seabream fed vitamin adequate diets (X 100).

Plate 10: Distribution of lysosomal NAH in the liver parenchyma of gilthead seabream fed pyridoxine deficient diets (X 100).



A stereological examination of the Oil red O stained liver sections indicated that there was significantly less neutral lipid in the livers of the control fish (Plate 7) than in those of the riboflavin (Plate 8) and niacin deficient individuals. However, the large inter animal variability among the vitamin deficient individuals meant that only the pyridoxine deficient fish exhibited a liver lipid content which was lower than that of the riboflavin deficient group.

The fish maintained on the complete diet exhibited a distribution of lysosomal N-acetyl β D hexosaminidase (NAH) which appeared to be meshlike *ie.* small grains joined by interconnecting strands of reaction product (Plate 9). Additionally, the hepatocytes among the fish in this group were lightly and diffusely stained with reaction product. With regards the vitamin deficient fish, a reduction in the overall intensity of staining, a change in the organisation of the reaction product and a high degree of intra and inter animal variability (particularly in the pantothenic acid and thiamin deficient fish) were observed. Consequently, reaction product was distributed as larger granules and not grains in the thiamin, niacin and riboflavin deficient fish while the aggregation was totally absent from those fish fed pyridoxine (Plate 10) and pantothenic acid deficient diets and hence replaced by a diffuse background staining.

After nine weeks under the pyridoxine deficient regime one individual displayed abnormal, spiral swimming behaviour. Additionally, by fourteen weeks 12 % of the fish surviving under this regime displayed randomly distributed orange, pustular lesions of approximately 3 mm diameter on the body surface. All the remaining pyridoxine fish also appeared thin and emaciated developing a 'pin-head' appearance. At the end of the feeding trial, several instances were recorded where the eyes of the riboflavin deficient fish appeared to protrude forward in the head. This did not appear to be an

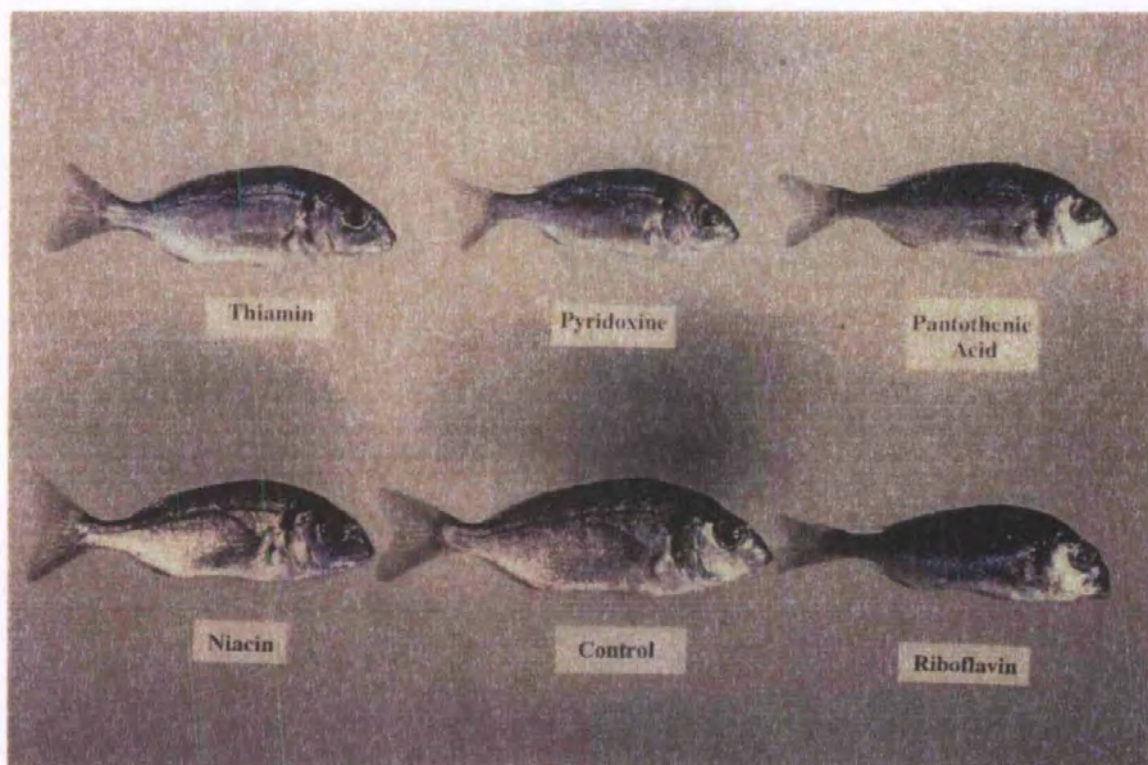


Plate 11: Appearance of gilthead seabream fed diets deficient in B vitamins.

exophthalmos but more a change in the shape of the head of the fish. Damage to the cornea was observed in two of the riboflavin deficient fish though these were probably the result of mechanical damage. The external appearance of the majority of the remaining fish was normal except that they were very obviously smaller than the control individuals as shown in Plate 11.

3.2.4. Discussion.

The observations of the present experiment confirm the qualitative requirement of the gilthead seabream for five B vitamins and provide an insight into the deficiency symptoms associated with the selected deletion of these major vitamins from the diet. The control diet *i.e.* the complete test formulation, proved adequate in its supply of nutrients as evidenced by the nutritional parameters and apparent absence of histopathology. Hence, the suitability of this diet for use in subsequent nutrition trials with the gilthead seabream was demonstrated. In order to maintain the clarity of these findings each of the vitamin deficiencies will be discussed separately.

Riboflavin.

As a consequence of the absence of riboflavin in the diet, the seabream performed significantly less well with respect to all the measured nutritional performance criteria when compared to the fish fed the complete diet after 14 weeks. Thus riboflavin was associated with reduced weight gain and rate of weight gain (SGR), reduction of feed intake concomitant with poorer feed conversion (FCR and FCE) and inefficient utilisation of protein. Reduction in feed intake was recorded by Halver (1957) for chinook salmon (*Oncorhynchus tshawytscha*). Lim *et al.* (1991b) observed a rapid onset

of riboflavin deficiency indicated by poor growth and anorexia in red hybrid tilapia. For the channel catfish (*Ictalurus punctatus*), Murai and Andrews (1978) observed poor growth and feed efficiency in fish fed riboflavin supplements of less than 9 mg kg⁻¹ after 20 weeks. A similar time period was shown by Hughes and Rumsey (1981) to be required for the development of significant riboflavin deficiency mediated growth retardation in the rainbow trout (*Salmo gairdneri*) since, after 14 weeks no growth differences were apparent though feed efficiency worsened as a result of feed refusal after 10 weeks. However, Woodward (1983) observed a more rapid onset of deficiency in rainbow trout with growth retardation apparent after 6 weeks and reduced feed efficiency apparent after 8 weeks of feeding diets deficient in riboflavin. Similarly, Takeuchi *et al.* (1980) recorded a growth suppression and poor feed conversion in rainbow trout after 8 weeks of maintenance on diets containing less than 4.0 mg of riboflavin per kg of feed. Common carp (*Cyprinus carpio*), red seabream (*Chrysophrys major*) and Asian seabass (*Lates calcarifer*) demonstrated deficiency related growth retardation after 4, 4 and 12 weeks respectively (Aoe *et al.* 1967a, Yone and Fujii, 1974, Boonyaratpalin and Wanakowat, 1991) while the fish of the present experiment developed a reduced weight gain within 6 weeks. Growth retardation is not always associated with deficiency of this vitamin, for example, Butthep *et al.* (1983) and Soliman and Wilson (1992) working with the walking catfish (*Clarias batrachus*) and blue tilapia (*Oreochromis aureus*) respectively, observed no suppression of growth, however, feed conversion was adversely affected in the latter study.

Due to the small size of the fish at the end of the experiment, carcass analysis was carried out using pooled samples and hence statistical analysis of the proximate composition of the fish could not be performed. However is evident that the lipid content

of the riboflavin deficient fish is lower than that of the control animals. Given the feed refusal observed during deficiency, it is not unreasonable to postulate that this modification in carcass lipid is the result of feed refusal or starvation as a consequence of which deposited lipid was used as a source of metabolites by the fish. Takeuchi *et al.* (1980) also observed a decline in the carcass lipid concomitant with a rise in carcass moisture content in common carp fed diets containing less than 5.0 mg of riboflavin per kg of diet however, Takeuchi *et al.* (1980) did not observe a similar trend in rainbow trout.

Mortality for the fish fed riboflavin deficient diets was almost 7 % which, higher than the control, was not the largest observed mortality. Butthep *et al.* (1983) observed mortalities in riboflavin deficient catfish (*Clarias batrachus*) after 5 weeks of feeding and within 12 weeks mortality was significantly higher than that of the vitamin adequate group. Red hybrid tilapia demonstrated mortality after 6 weeks of deficiency in the study of Lim *et al.* (1993) while rainbow trout showed mortality after 11 weeks of receiving riboflavin deficient rations (Woodward 1983). Mortality accounting for 30 and 23 % of the test animals was recorded by Takeuchi *et al.* (1980) in common carp and rainbow trout respectively, while mortalities of upto 80 % were observed in riboflavin deficient carp within 8 weeks by Aoe *et al.* (1967a). The mortality observed in the present experiment were not large and hence correlate with the low mortality rate of red seabream (*Chrysophrys major*) observed in the study of Yone and Fujii (1974), though a continuation of the present feeding regime may have resulted in a greater incidence of deficiency induced mortality.

Apart from the reduction in appetite, little modification in the behaviour of the riboflavin deficient fish was apparent. However as graphically shown in Plate 11,

externally visible pathological signs of vitamin deficiency were evident after 14 weeks of feeding. The fish appeared slightly darker than the control animals though the integrity of the fins was maintained. Modifications in the skin colour and integrity have been observed in riboflavin deficient walking catfish (Butthep *et al.* 1983), blue tilapia (Soliman and Wilson 1992), common carp (Aoe *et al.* 1967a, Takeuchi *et al.* 1980), chinook salmon (Halver 1957) and rainbow trout (Kitamura *et al.* 1967, Woodward 1984). Necrosis of the fins was also recorded during riboflavin deficiency in the latter 5 studies.

Cataracts have frequently been associated with riboflavin deficiency (Halver 1957, Takeuchi *et al.* 1980, Hughes *et al.* 1981, Butthep *et al.* 1983, Boonyaratpalin and Wanakowat 1991, Soliman and Wilson 1992, Lim *et al.* 1991b) and consequently cataract formation has been ascribed as a diagnostic feature of deficiency of this vitamin. However cataracts were not observed during the course of the present experiment and given the absence of cataractogenesis in common carp (Aoe *et al.* 1967a, Takeuchi *et al.* 1980) and rainbow trout (Woodward 1984) the value of this feature as a deficiency indicator for all species is called into question.

One of the most striking consequences of deficiency was the apparent impression that the eyes were protruding over the snout. The observed forward projection of the eyes was markedly different from the exophthalmos observed during vitamin C deficiency during the previous study and hence it may be postulated that this symptom may not be as a consequence of exophthalmos but due to short body dwarfism (SBD) which has been observed in red hybrid tilapia (Lim *et al.* 1991b), channel catfish (Murai and Andrews 1978) and Asian seabass Boonyaratpalin and Wanakowat, 1991). Lim *et al.* (1991b) and Murai and Andrews (1978) used X-radiographic techniques to demonstrate the cause of

short body dwarfism which appears to be attributable to a cessation of longitudinal growth in the vertebrae. Murai and Andrews (1978) postulated that hypothyroidism was causative of this arrested longitudinal growth of the vertebrae as a consequence of the relationship between thyroid activity and riboflavin status. Condition factor was applied to rainbow trout by Hughes *et al.* (1981) in order to diagnose short body dwarfism. In this case SBD was not apparent which was attributed by the authors to be a consequence of the large initial weight of the fish at the start of the experiment. The application of condition factor (a length body weight relationship) to the present experiment would probably fail as an indicator of SBD due to the poor weight gain of the fish.

The ventral surface of the fish appeared to be wasted between the pectoral fins and the anal vent which was probably a consequence of the reduced feed intake of the riboflavin deficient individuals and not a deficiency symptom *per se*.. This lends credibility to the argument against the use of condition factor for the diagnosis of short body dwarfism.

The haematocrit of the riboflavin deficient fish was significantly smaller than that observed for the fish fed the complete diet and in addition the suppression of packed cell volume was greater than that observed in niacin, pantothenate and pyridoxine deficiency. Anaemia has also been recorded for riboflavin deficient brook trout, *Salvelinus fontinalis*, (Tunison *et al.*, 1947) and blue tilapia (Soliman and Wilson 1992) however reductions in packed cell volume has not been reported elsewhere though this may be due to a failure of other authors to include this observation in their work.

As with all the deficient groups, deficiency of riboflavin was associated with a lower hepatosomatic index when compared to the control animals. At the histological level, riboflavin deficiency was associated with considerable lipid accumulation in the

hepatopancreas, degeneration of the pancreas with melano-macrophage accumulation, atrophy of the hepatocytes and modified distribution of the lysosomal enzyme NAH. Aoe *et al.* (1967a) characterised the histopathology associated with riboflavin deficiency in common carp. In summary, histological damage was recorded as haemorrhage in the myocardium with necrosis of the urinary tubules and epithelial degeneration in the anterior and posterior kidney respectively. Lim *et al.* (1992) and Murai and Andrews (1978) have searched for histopathological lesions in the red hybrid tilapia and the channel catfish respectively but have failed to detect any abnormality apart from cataractogenesis. Butthep *et al.* (1983) did observe the incidence of pallid liver and gills in the walking catfish while Kitamura *et al.* (1967) also recorded necrosis of the gills in riboflavin deficient rainbow trout, however none of these authors carried out histological investigations. In considering the hepatosomatic index it must be emphasised at this point that the control animals were significantly heavier than the fish fed all the remaining test diets and consequently the effect of size and overall condition of fish must be taken into account.

With regards the present experiment, the PAS reaction and Oil red O staining for glycogen and neutral lipid respectively, indicated very little glycogen but significant lipid accumulation in the livers of the riboflavin deficient fish. In addition, the degeneration of the pancreas and the modified distribution of the lysosomal enzyme NAH clearly emphasise the importance of riboflavin in the metabolism of the seabream. The extensive lipid deposit and absence of glycogen accumulation in the hepatopancreas indicates a severe metabolic imbalance. It would appear that the pancreas maintained its capacity for carbohydrate modulation as evidenced by the low glycogen content of the liver, though the poor pancreatic staining specificity (haematoxylin and eosin) and

accumulation of melano-macrophages around this organ indicate that the pancreas was in an advanced state of degeneration. The lysosomal enzyme N-acetyl β D hexosaminidase (NAH) is responsible for the removal of the terminal N-acetylgalactosamine residue from the lipid ganglioside and a reduced NAH activity is associated with lysosomal lipid accumulation (Stryer 1988). Hence, the change in distribution of NAH may account for some of the lipid accumulation in the hepatopancreas. As outlined above, fatty infiltration in the livers of riboflavin deficient fish has not previously been recorded in fish though the accumulation of triglycerides in the livers of riboflavin deficient rats was observed by Duerdan and Bates (1985). The effect of riboflavin deficiency on lipid metabolism was briefly reviewed by Bender (1992). In summary, lipid accumulation is a consequence of impaired acyl coA dehydrogenase activity and hence other, less efficient pathways are utilised for fatty acid oxidation. This may, in part, be the cause of the lipid accumulation in the hepatopancreas.

Niacin.

Niacin deficiency was clearly associated with a mortality rate of 13 %, poor growth, reduced feed efficiency and protein utilisation when compared with the fish fed the niacin adequate diet. This is in broad agreement with the findings for the red seabream, *Chrysophrys major* (Yone and Fujii, 1974), rainbow trout (McLaren *et al.*, 1947, Poston and Wolfe, 1985), common carp (Aoe *et al.* 1967c), channel catfish (Andrews and Murai, 1978) and walking catfish (Butthep *et al.* 1983). In the present experiment, the rate of growth was lowered between 4 and 6 weeks with a cessation of weight gain after the 6th week. Yone and Fujii (1974) also observed a rapid onset of niacin deficiency characterised by poor growth and loss of appetite after 16 days of feeding diets devoid

of this vitamin but, even after 100 days of deficiency, mortality remained absent. Conversely, within 140 days, Andrews and Murai (1978) recorded a mortality of 100 % in channel catfish fed niacin deficient diets. After 12 weeks of feeding a niacin inadequate diet, walking catfish exhibited a reduction in appetite and weight gain (Butthep *et al.* 1983) while rainbow trout were slower to develop overt signs of deficiency (16 weeks) in the form of retardation of growth and loss of appetite when fed diets with a niacin content of less than 10 mg kg⁻¹ (Poston and Wolfe 1985). Additionally, the fish maintained on the deficient diets again exhibited an absence of mortality.

Once again, the proximate composition of the pooled animals demonstrated a reduction in the lipid content of the deficient fish after 14 weeks. This contradicts the findings of Poston and Wolfe (1985) who also observed no changes in the proximate composition of rainbow trout, and in part contradicts the observations of McLaren *et al.* (1947) who observed an increase in the liver lipid content at the expense of the moisture component in niacin deficient rainbow trout when compared to individuals maintained on niacin fortified diets. Within the current study, the protein utilisation for the niacin deficient individuals was better than that of the riboflavin and pyridoxine deficient fish. However, once again the small size of the fish at the end of the trial necessitated the use of pooled samples and hence precluded a statistical analysis. Again, when examining changes in the proximate composition in response to niacin status, the overriding consideration is the feed refusal of the fish which probably indicates depletion in stored lipid as a consequence of starvation.

Under the present dietary regime, the seabream developed neither external or behavioural signs of deficiency related pathology. This corroborates the observations of Phillips and Brockway (1947) who observed no pathology or mortality at levels of

supplemental niacin lower than the minimum required for maximal liver storage in brook (*Salvelinus fontinalis*), brown (*Salmo trutta*) and rainbow trout. However, other studies have highlighted outward signs of deficiency. Thus, during niacin deficiency chinook salmon (*Oncorhynchus tshawytscha*) demonstrated muscle spasms, jerky or difficult motion, lesions of the colon and edema of the stomach and colon (Halver 1957). As a consequence of niacin deficiency, walking catfish (*Clarias batrachus*) exhibit muscle spasms, loss of equilibrium, lethargy, haemorrhage under the skin and fins, and exophthalmia (Butthep *et al.* 1983). Similarly, niacin deficient channel catfish exhibited deficiency symptoms including; skin and fin lesions with haemorrhage and exophthalmia (Andrews and Murai, 1978), while haemorrhage under the skin was also observed in niacin deficient common carp (Aoe *et al.* 1967c). Rainbow trout developed swollen but not clubbed gills when fed diets depleted of niacin which was postulated by McLaren *et al.* (1947), to be the cause of the mortalities observed during that study.

The most commonly studied deficiency symptom associated with niacin is susceptibility of salmonids to sunburning during exposure to ultra violet irradiation. Allinson (1960), in studies on the lake trout (*Salvelinus namaycush*), investigated the use niacin supplements which were 8 times higher than the requirement of the species (the actual requirement was not stated). In short, niacin was seen to retard the onset and speed the recovery of the fish from the U.V. induced lesions though total protection was not afforded at high doses of ultra violet. Poston and Wolfe (1985) carried out a thorough investigation of the relationship between niacin and sunburning in the rainbow trout. In summary, dietary niacin supplements of less than 10 mg kg⁻¹ failed to prevent the appearance of U.V. induced dermopathy characterised depigmentation of the fins, desquamation of the epidermis around the head and snout and severe erosion of the

caudal peduncle. At the histological level, the number of mucous producing goblet cells was seen to decline in proportion to the severity of niacin deficiency. Poston and Wolfe (1985) proposed that niacin in the form of NAD was used in order to regenerate the physiological reductants *eg.* peroxidases used to reduce U.V. generated radicals and consequently, niacin deficiency resulted in an increased susceptibility to sunburning.

When compared to the fish fed the complete diet, the haematocrit of the niacin deficient fish in the current study was significantly lower. Suppression of the packed cell volume has also been recorded in the channel catfish during niacin deficiency (Andrews and Murai, 1978). No single metabolic lesion has yet been specifically attributed to niacin deficiency (Bender 1992) though it is not surprising that packed cell volume should be reduced during deficiency of this vitamin which plays such a key role in intermediary metabolism.

The hepatosomatic index was also significantly lower than that observed for the control animals though probably as a size or developmental effect and not as a consequence of deficiency *per se*. At the histological level, niacin deficiency was associated with pancreatic degeneration and melanomacrophage accumulation though without glycogen deposition, lipid accumulation, hepatocyte atrophy and an absence of NAH aggregation. This strongly contrasts with the observations of Andrews and Murai (1978) who observed an absence of histopathology in niacin deficient channel catfish. Central to the whole question of both the qualitative and quantitative niacin requirement of animals, is the ability to utilise tryptophan as a precursor for the formation of niacin. Hence, little work has been carried out on the effects of niacin deficiency since, for most animals, tryptophan is a suitable niacin precursor. Thus, where apparent, niacin deficiency is compounded by the poor tryptophan intake of the animal (Bender 1992).

Consequently, there appears to be no histopathological evidence to compare with the present observations.

As with the riboflavin deficiency, the accumulation of lipid in the hepatopancreas and degeneration of the pancreas, indicates a metabolic imbalance. However, unlike the deficiency of riboflavin, a reduction in the activity of a single enzyme or group of enzymes has been not attributed to niacin deficiency. Through the coenzymes NAD and NADP, niacin plays a key role in hydrogen transfer and hence this vitamin is implicitly linked to the citric acid cycle and the respiratory chain (Blum 1991). Hence, it is not unreasonable to postulate that the accumulation of lipids is a consequence of the reduced efficiency of these key energy yielding pathways for which, in fish, lipid derivatives are significant substrates. Alternatively, given the diffuse nature and lack of aggregation of NAH stain product, it is not unreasonable again to postulate that niacin deficiency is responsible for lipid accumulation as a consequence of low NAH activity.

The present data show, that under the present dietary conditions, the gilthead bream has a qualitative requirement for dietary niacin the deficiency symptoms of which rapidly become apparent when compared with rainbow trout and walking catfish.

Pantothenic Acid.

A deficiency of pantothenic acid was causative of a reduction in weight gain within 6 weeks and resulted in a cessation of weight gain after 10 weeks. Though significantly smaller than the fish fed the complete diets, the pantothenate deficiency was not as marked in terms of its effect on growth as niacin, riboflavin or pyridoxine. Suppression of growth as a consequence of pantothenic acid deficiency has also been observed in the red seabream (Yone and Fujii, 1974), common carp (Ogino 1967), Asian seabass

(Boonyaratpalin and Wanakowat, 1991), walking catfish (Butthep *et al.* 1983), blue tilapia (Roem *et al.*, 1991, Soliman and Wilson, 1992) and Channel catfish (Wilson *et al.*, 1983, Murai and Andrews, 1975, 1979).

Feed conversion efficiency and net protein utilisation for the pantothenate deficient fish was very high, particularly in the light of the poor growth response of these individuals. Reductions in feed conversion efficiency are frequently associated with a paucity of dietary pantothenic acid as observed for the blue tilapia (Roem *et al.*, 1991), Asian seabass (Boonyaratpalin and Wanakowat, 1991), and the Channel catfish (Murai and Andrews, 1979, Wilson *et al.*, 1983). In the light of the lack of differences between the treatments in terms of proximate composition, an analysis of the feed input of the pantothenate deficient fish when compared to the control animals, demonstrates a very low appetite which could conceivably account for the observed high feed conversion efficiency. This loss of appetite is in keeping with the observations of Halver (1957) for the chinook salmon (*Oncorhynchus tshawytscha*), Yone and Fujii (1974) for the red seabream (*Chrysophrys major*) and of Butthep *et al.* (1983) for the walking catfish (*Clarias batrachus*). However, these studies made no evaluation of feed efficiency or protein utilisation and hence, comparison may only be extended in terms of growth and appetite.

Apart from their smaller size, no differences were immediately apparent in terms of behaviour and external pathology when comparing the control and pantothenic acid deficient fish. This contrasts with the findings for many species which show a plethora of deficiency symptoms. The symptoms of an inadequate dietary supply of pantothenic acid became apparent within 2 weeks for the Asian seabass (*Lates calcarifer*) in the study of Boonyaratpalin and Wanakowat (1991) as evident by the development of dark colouration, loss of equilibrium, abnormal swimming behaviour, haemorrhage of the

lower jaw, operculum, isthmus and pelvic fin. Roem *et al.* (1991) observed severe fin erosion in pantothenic acid deficient blue tilapia (*Tilapia aurea*). Additional deficiency symptoms for this species were observed by Soliman and Wilson (1992) which included haemorrhage in the skin and a general sluggish behaviour. A diverse group of deficiency symptoms were reported by Butthep *et al.* (1983) for the walking catfish which included subcutaneous haemorrhage, fragile fins with swelling at the base of the pectoral fins, edema, eroded barbels and rapid breathing. Marked external pathology was observed by Murai and Andrews (1975, 1979) in the channel catfish which included anorexia, sluggish swimming and erosion of the skin, lower jaw and of the barbels. Anorexia and emaciation were also observed by Poston and Page (1982) in lake trout (*Salvelinus namaycush*), while exophthalmos and haemorrhage of the body surface were recorded in pantothenic acid deficient common carp (*Cyprinus carpio*) by Ogino (1967).

In the present case it is possible that the time period selected (14 weeks) was insufficient for the development of gross outward deficiency signs, however the occurrence of a substantial mortality indicated that the health of the fish was poorer than that of the control animals and hence nutritional pathology had developed but was outwardly unapparent.

Undoubtedly the most studied deficiency symptom associated with pantothenic acid is the clubbing of the gills. Tunison *et al.* (1942) recorded this effect in rainbow trout and conclusively attributed this type of gill disease to a deficiency of pantothenic acid. Subsequently, gill pathology associated with pantothenate deficiency has been observed in chinook salmon (Halver 1957) lake trout (Poston and Page, 1982), rainbow trout (Karges and Woodward, 1984, Masumoto *et al.*, 1993), walking catfish (Butthep *et al.*, 1983), Channel catfish (Murai and Andrews, 1975, 1979, Wilson *et al.*, 1983), blue tilapia

(Soliman and Wilson, 1992, Roem *et al.*, 1991), Asian seabass (Boonyaratpalin and Wanakowat, 1991) and *Cichlasoma urophthalmus* (Chavez De Martinez *et al.*, 1990b).

The extent, aetiology and rapidity of onset of this deficiency symptom varies from species to species though in general, this pathology is characterised by a fusion and hyperplasia of the lamellae which, initiated at the distal end of the gill progresses proximally (Karges and Woodward, 1984). The fusion may for some species, for example *Cichlasoma urophthalmus*, progresses to such an extent that adjacent filaments become fused (Chavez De Martinez *et al.*, 1990b). Hence overall the gills appear 'clubbed' and in addition deficiency mediated pathology is characterised by the exudation of a large quantity of mucous. Given the role of pantothenic acid in the metabolism of lipids, Masumoto *et al.* (1991) investigated the lipid content of pantothenate deficient rainbow trout. In short modifications in the qualitative and quantitative aspects of lipid distribution in the gill were not observed and hence perturbations in lipid metabolism were not apparent. Consequently the observed gill pathology was not attributable to lipid metabolism in this organ. Furthermore, the ability of the pantothenate deficient trout to osmoregulate during a seawater challenge was unaffected though Masumoto *et al.* (1991) did not rule out the use of other organs for osmoregulation.

Once again the packed cell volume was decreased during deficiency of this vitamin when compared to the control. Anaemia has also been recorded in pantothenic acid deficient common carp (Ogino 1967) and channel catfish (Murai and Andrews 1979). However, packed cell volumes would appear to be a point of conjecture with respect to pantothenic acid deficiency in the blue tilapia. Thus Roem *et al.* (1991) observed decreased packed cell volume only in moribund fish while Soliman and Wilson (1992) routinely observed a reduction in haematocrit. There two postulates, both

physiological and biochemical, which may explain the observations of the present experiment. The former may be attributed to a failure of the osmoregulatory mechanisms of the fish due to hyperplasia of the gills with consequent haemo-concentration evident as an increase in the packed cell volume. This would however, contradict the observations of Masumoto *et al.* (1991) who, as outlined above, noted that rainbow trout maintained osmoregulatory ability despite significant damage to the gill integrity.

As was the case for all the deficient groups, the hepatosomatic index in the pantothenate deficient fish was smaller than that of the vitamin replete individuals, while at the histological level, pantothenic acid deficiency was associated with severe pathology in the liver. By comparison with niacin, the effects of pantothenic acid deficiency have been extensively studied at the histological level in fish. With regards the present study, the haematoxylin and eosin stained sections revealed a marked intra and inter animal variability though in general, the individual hepatocytes were stained homogeneously though the distribution of stained cells was not uniform. Little PAS positive material was observed in the hepatocytes which contrasts with the observations of Poston and Page (1982) and Chavez De Martinez *et al.* (1990) who recorded large hepatic infiltrations of PAS positive material in lake trout (*Salvelinus namaycush*) and *Cichlasoma urophthalmus* respectively.

In the present experiment, the test diet contained 20 % corn starch and the absence of large amounts of PAS positive material and intact pancreas of the control fish indicate that this was an appropriate source of carbohydrate for the seabream. The diets of Poston and Page (1982) contained a carbohydrate content of 20 % (10 % dextrin and 10 % sucrose) while those of Chavez De Martinez *et al.* (1990b) contained 27 % starch (9 % dextrin and 18 % starch). The relationship between dietary carbohydrate content,

digestibility, complexity, and glycogen deposition has been well characterised (Furuichi and Yone 1980, Kaushik *et al.* 1989, Hung and Fynn-Aikins 1991) and the lower level of glycogen accumulation in the pantothenic acid deficient fish within the present experiment may be attributable to the type and suitability of dietary carbohydrate incorporated in the diet. Consequently, had the pantothenic acid deficient seabream in the present experiment been supplied with a more simple, more digestible carbohydrate source *eg.* glucose then more marked glycogen deposition in the hepatopancreas may have occurred.

The lipid content in the hepatopancreas of the bream in the present experiment was marginally, though not significantly, higher than that of the control fish while conversely, Poston and Page (1982) found that during pantothenate deficiency in the lake trout, fatty infiltration of the liver was lowered by comparison to fish supplied a complete diet. Cytoplasmic lipid globules in the hepatocytes of pantothenic acid deficient blue tilapia (*Tilapia aurea*) were observed by Roem *et al.* (1991) while Chavez De Martinez *et al.* (1990b) observed ceroid deposition in the liver and spleen in *Cichlasoma urophthalmus* fed pantothenic acid deficient diets.

As a component of acetyl CoA, pantothenic acid is essential in the normal progress of fatty acid metabolism since the carboxylation of acetyl CoA to malonyl CoA is the first step in fatty acid synthesis (Stryer 1988), hence it appears that modifications in the distribution of lipids in the fish is not unexpected. In the present case, dietary lipid was supplied as cod liver oil comprising 7.2 % of the test ration as determined by chemical assay. This is a lower lipid input than used by Chavez De Martinez *et al.* (1990b) (8 % fish oil, 4 % corn oil) and Poston and Page (1982) (10 % soy oil). Hence, the absence of significant lipid accumulation in response to pantothenic acid deficiency

in the present study, may be in response to both the low lipid content of the diet and the fatty acid profile of the cod liver oil. Consequently, an examination of the qualitative nature of the fatty acids in the livers of both the present study and those of Poston and Page (1982) and Chavez De Martinez *et al.* (1990b) may have revealed an accumulation of shorter chain fatty acids in the latter experiments due to perturbations of fatty acid synthesis.

Within the present experiment, haematoxylin and eosin staining depicted pancreatic tissue which was highly atrophic and consequently staining with PAS demonstrated the presence of large melanomacrophage aggregates and the production of pigmented granules. As a consequence of pantothenic acid deficiency, Chavez De Martinez *et al.* (1990b) observed pancreatic necrosis in *Cichlasoma urophthalmus* while Poston and Page (1982) observed atrophic, vacuolated acini cells in the pancreas of lake trout. In addition, the cytoplasm appeared to be granular and a loss of cellular definition was observed. Thus, the present study is in close agreement with those of Poston and Page (1982) and Chavez De Martinez *et al.* (1990b) though again, no direct relationship exists between pantothenic acid supply and pancreas function. However, it is not unreasonable to postulate that the absence of dietary pantothenic acid results in modifications in the homeostatic process which are responsible, at least in part for the degeneration of the pancreas.

In common with the haematoxylin and eosin staining, the distribution of NAH highlighted a much increased inter and intra animal variability amongst the fish maintained on pantothenic acid diets though no direct relationship between NAH and pantothenic acid is evident.

One of the more outstanding observations of the present experiment was the

relatively high mortality of pantothenic acid deficient individuals which contrasted with their good feed conversion and level of growth. Given that a deficiency of pantothenic acid is so clearly associated with nutritional gill disease it is not unreasonable to postulate that hypoxia as a consequence of gill hyperplasia, was in part responsible for the high mortality rate. This conclusion was also proposed by Soliman and Wilson (1992) in order to explain the high mortality observed in blue tilapia. However, the extensive histopathology in the liver must also have played a significant role in the onset of mortality and, given a longer feeding period, externally visible pathology may have become evident.

Pyridoxine.

Of the five deficiencies examined, the deletion of pyridoxine from the diet resulted in the most dramatic effects on growth. Consequently within four weeks a marked suppression of weight gain was observed followed by continued weight loss until the termination of the experiment after 14 weeks of feeding. This closely resembles the observations for the Asian seabass (*Lates calcarifer*) of Wanakowat *et al.* (1989) in which, deficiency resulted in a retardation of growth within 2 weeks followed by sustained weight loss there after. Additionally, Ogino (1966) observed weight loss in B₆ deficient carp within 4 to 6 weeks of the start of the feeding period. Similarly, retardation of weight gain was recorded by Sakaguchi *et al.* (1969) and Yone and Fujii (1974) in the yellowtail (*Seriola quinqueradiata*) and red seabream (*Chrysophrys major*) although without apparent weight loss. Butthep *et al.* (1983) observed a reduction in the rate of growth of walking catfish (*Clarias batrachus*) after 3 weeks of deficiency without weight loss within the duration of the trial (24 weeks). The manifestation of pyridoxine

deficiency with respect to growth in the snakehead (*Channa punctatus*) was markedly slower, thus, in the study of Agrawal and Mahajan (1983a) a reduced growth rate only became apparent after 120 days. For the turbot (*Scophthalmus maximus*), Adron *et al.* (1978) recorded only a cessation of weight gain without weight loss after 8 weeks of feeding diets supplemented with less than 1.0 mg kg⁻¹ of pyridoxine. A similar period of time was shown by Andrews and Murai (1979) to be required for the onset of deficiency mediated growth retardation in the channel catfish (*Ictalurus punctatus*). In the study carried out by Kissil *et al.* (1981) a retardation of growth in the gilthead seabream (*Sparus aurata*) only became apparent after almost 6 weeks of feeding pyridoxine deficient diets. However the pyridoxine content of the deficient diet was estimated to be 1.15 mg kg⁻¹ which was probably causative of the slower onset of deficiency.

The poor growth observed during the present experiment was reflected by the low values for feed conversion efficiency and net protein utilisation. Once again, with respect to lipid and moisture, the proximate composition of the deficient fish was markedly different. Thus, the deficient individuals demonstrated a low lipid content which was offset by an elevation in the carcass moisture component. However, it should be noted that the feed refusal and low nutrient intake of these fish must represent a major factor contributing to the observed modification in the proximate composition. Poor feed conversion also became apparent for pyridoxine deficient seabream and seabass in the studies of Kissil *et al.* (1981) and Wanakowat *et al.* (1989) respectively. Conversely, for the channel catfish, feed conversion (measured as the gain/feed ratio) was unaffected by the B₆ content of the diet (Andrews and Murai, 1979). However, unlike the present study none of these examples characterised the proximate composition and hence protein utilisation of the fish. Given the importance of pyridoxine in the metabolism of protein,

the high protein content of the test diet and the central role of protein in the metabolism of this species, the present observations were not unexpected. The relationship between pyridoxine and the metabolism of protein is further discussed in the successive chapter (5.1) which focuses on this vitamin and hence the underlying mechanisms of pyridoxine deficiency and alterations in feed performance will not be discussed at this point.

Gross deficiency symptoms became apparent for the pyridoxine deficient fish during the present experiment. Thus, deficient individuals appeared anorexic though, unlike the riboflavin deficient fish, the normal body form and positioning of the eyes was maintained. One case of abnormal, spiral swimming was observed after 9 weeks though this was an isolated case and hence was regarded as not representative of the surviving fish maintained on this diet. Between weeks 12 and 14, 12 % of the surviving fish within this dietary regime developed randomly distributed lesions over the surface of the body. Typically, these pustular lesions were an orange colour with an average diameter of 3-4 mm. It is postulated that these lesions were not a direct consequence of pyridoxine deficiency but more likely the result of an opportunistic bacterial infection exacerbated by the poor nutritional state of the pyridoxine deficient individuals.

The pathological effects of pyridoxine deficiency have been extensively studied in fish. For salmonids, common gross deficiency symptoms include; anorexia, epileptiform fits, erratic swimming and ataxia (Halver 1957, Smith *et al.* 1974, Hardy *et al.* 1987). Andrews and Murai (1979), in a study on the channel catfish, observed anorexia, tetany, abnormal body colouration and nervous disorder in deficient individuals. The gross deficiency symptoms associated with pyridoxine deficiency for the snakehead (*Channa punctatus*), were elucidated by Agrawal and Mahajan (1983a). These include; anorexia, poor growth, ataxia, hyper irritability, muscular spasms, scale loss with associated fluid

imbalance and opacity of the lens leading to blindness. Kissil *et al* (1981) observed irritability, erratic swimming and degeneration of the peripheral nerves in pyridoxine deficient gilthead seabream (*Sparus aurata*). Similarly Takeda and Yone (1971) observed hyperirritability and epileptiform fits in the red seabream. Sakaguchi *et al.* (1969) observed deficiency symptoms in the yellowtail which included anorexia, neuropathy, exophthalmia, injury to the mouth scoliosis which is un-characteristic of pyridoxine deficiency and more frequently associated with poor ascorbic acid supply. Wanakowat *et al.* (1989) determined the requirement of the Asian seabass for pyridoxine. Once again the common symptoms of deficiency were observed including neuropathy and behavioural changes. Ogino (1966) used the pyridoxine antagonist deoxypyridoxine to enhance the deficiency symptoms associated with pyridoxine deficiency in the common carp. Thus, low dietary B₆ levels were associated with edema of the peritoneal cavity, exophthalmia and a range of neuropathological symptoms including hyperirritability and epileptiform fits. On application of the dietary antagonist, the range of symptoms was extended to include edema and haemorrhage of the whole body, poor appetite, sluggishness and an un-classified disturbance of the skin.

After 14 weeks of test diet feeding the cumulative mortality for the pyridoxine deficient fish accounted for 18 % of the original population. This value is exclusive of those individuals which had developed the surface lesions since these individuals were humanely terminated prior to the end of the 14 week period and hence did not represent true deficiency induced mortalities. The 18 % mortality observed in the present experiment correlates strongly with the 16 % mortality recorded by Yone and Fujii (1974) for the red seabream within the same time period. After 140 days of feeding pyridoxine deficient diets to gilthead seabream, a 93 % mortality was attained by Kissil

et al. (1981) while the Asian seabass in the study of Wanakowat *et al.* (1989) demonstrated a more rapid onset of mortality during pyridoxine deficiency, thus only 50 % of the population survived after 4 weeks with only a survival of only 25 % after 8 weeks. Similarly, in the study of Andrews and Murai (1979) a mortality of 100 % was attained within 12 weeks for channel catfish.

The haematocrit of the pyridoxine deficient fish was significantly depressed compared to that observed for the individuals fed the complete diet. A normocytic normochromic anaemia, characterised by reductions in plasma protein, haematocrit and haemoglobin content, was observed by Smith *et al.* (1974) in rainbow trout. An uncharacterised anaemia was also observed in chinook salmon (*Oncorhynchus tshawytscha*) fed diets of insufficient pyridoxine content by Halver (1957). In a haematological study on pyridoxine deficient snakehead, Agrawal and Mahajan (1983b) observed a hypochromic microcytic anaemia and leucopenia. Similarly, in the study of pyridoxine deficiency in carp (*Cyprinus carpio*) Vel *et al.* (1990) observed anaemia and leucopenia characterised by reduced red and white cell counts, lowered haematocrit and haemoglobin concentration, and reductions in the mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). With respect to the erythrocyte population, it was observed that the proportion of mature cells fell with respect to the proportion of immature cells and additionally a series of pathological disorders were observed in the haematopoietic tissues of the fish. Similarly, Kissil *et al.* (1981) observed a destruction of red blood cells in haematopoietic regions of the kidney. For the Asian seabass, Wanakowat *et al.* (1989) observed that where pyridoxine deficiency was marginal, lower lymphocyte and higher neutrophil counts were recorded. However, only those turbot (*Scophthalmus maximus*) fed diets containing the pyridoxine

antagonist, 4-deoxypyridoxine hydrochloride, demonstrated a reduced haematocrit in the study of Adron *et al.* (1978). In contradiction of the majority of the literature, Andrews and Murai (1979) did not observe anaemia in cases of deficiency, instead, those fish fed more than 20 mg of B₆ kg⁻¹ diet developed microcytic normochromic anaemia.

In common with all the remaining treatments, the hepatosomatic index of the pyridoxine deficient fish was significantly lower than that of the control group. At the histological level, the pyridoxine deficient seabream of the present experiment exhibited a series of pathological conditions some of which have been observed in previous studies with this species (Kissil *et al.* 1981) and in B₆ deficient rainbow trout (Smith *et al.* 1974). However, despite the severity of the gross morphological deficiency symptoms reported by Andrews and Murai (1979), pyridoxine deficient channel catfish did not display any deficiency symptoms at the histological level in the hepatopancreas, kidney, lateral muscle or gill.

Haematoxylin and eosin stained liver sections revealed a considerable alteration in the architecture of the livers of the pyridoxine deficient seabream in the present experiment. Thus, a high degree of inter and intra animal variability was observed and in general the hepatocytes were homogeneously stained though the distribution of stained cells was irregular. PAS staining revealed a general absence of accumulated neutral sugars while the distribution of Oil red O stained material indicated a lipid retention which was highly variable. Stereology indicated that though significantly lower than that of the riboflavin deficient fish, the lipid retention in the B₆ deficient animals was not significantly lower than that of the control. To some extent, this is in accordance with Smith *et al.* (1974) who observed an absence of the normal glycogen and fat vacuolation in the hepatocytes of pyridoxine deficient rainbow trout. The low lipid content in the

livers of the fish in the present experiment is probably attributed to the low feed intake of the pyridoxine deficient individuals and is reflected in the very low lipid content of the whole carcass. In addition the low lipid content of the fish may relate to the low level of NAH activity.

In the present study, significant pancreatic degeneration was in progress as evidenced by the development of severely atrophic pancreatic cells and the appearance of large aggregations of melano-macrophages with deposition of pigmented granules around this organ. An accumulation of eosinophilic granule cells in the adipose tissue surrounding the pancreas was observed by Kissil *et al.* (1981) in the gilthead seabream, while in pyridoxine deficient rainbow trout necrosis and haemorrhage of the pancreatic acinar cells was observed by Smith *et al.* (1974). However, neither Smith *et al.* (1974) or Kissil *et al.* (1981) attempted to explain the reason for such degeneration.

Kissil *et al.* (1981) working with the gilthead seabream also observed extensive melanin dispersal and an apparent lack of melanomacrophage cells was observed in the haematopoietic tissues, a congestion of the spleen and meninges was recorded and finally an infiltration of the submucosa of the intestine by inflammatory cells became apparent. The nervous disorders observed by Kissil *et al.* (1981) were attributed to the degeneration and necrosis of the peripheral nerve fibres with an associated inflammatory response. A further series of histopathologies were recorded by Smith *et al.* (1974) in pyridoxine deficient rainbow trout which included; a swelling of the proximal convoluted tubules, shrinkage of the glomeruli and the formation of calcium oxalate crystals in the kidney, a sloughing off of the mucosal epithelium of the intestine and pyloric caeca and finally, necrosis in the adipose tissue surrounding the pyloric caecae. At the histological level the symptoms associated with pyridoxine deficiency in the snakehead were briefly

described by Agrawal and Mahajan (1983a) and shown to include nodule formation in the kidney and spleen.

Estimations of pyridoxine requirement and evaluations of the deficiency states associated with poor dietary supply of pyridoxine make frequent use of the transaminases. These enzymes are dependent on an adequate supply of Pyridoxal-5-phosphate which acts quantitatively as a cofactor for transaminase function. Thus, the pyridoxine requirements and deficiency symptoms of rainbow trout, turbot and the gilthead seabream have been evaluated using the activity of the transaminases as indicators of pyridoxine status (Ogino 1966, Smith *et al.* 1974, Jürss 1978, Adron *et al.* 1978, Kissil *et al.* 1981, Hardy *et al.* 1987). In order to avoid repetition, the transaminases and their role in the metabolism of protein and their relationship with B₆ will be further discussed in chapter 5 which includes an evaluation of pyridoxine super supplementation in diets for the gilthead seabream.

Thiamin.

A deficiency of thiamin was characterised in the present experiment by a marked suppression of growth when compared to the control group thus, the rate of growth decreased between weeks 2 and 8 with very little growth there after. In the study on the red seabream (*Chrysophrys major*) of Yone and Fujii (1974), deficiency induced growth suppression by comparison to fish fed thiamin replete diets, became significant after 79 days. Turbot (*Scophthalmus maximus*) supplied with thiamin at less than 0.19 mg kg⁻¹ exhibited a cessation of growth after 12 weeks of feeding and only after 14 weeks were these fish smaller than fish fed thiamin adequate diets (Cowey 1975). Poor growth was also observed by Hashimoto *et al.* (1970), Murai and Andrews (1978a) and by Morito *et*

al. (1986) in thiamin deficient eels (*Anguilla japonica*), channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*) respectively. Conversely, Butthep *et al.* (1983) and Boonyaratpalin and Wanakowat (1993), in studies on the walking catfish (*Clarias batrachus*) and Asian seabass (*Lates calcarifer*) respectively, observed no differences in the average final weights of fish fed thiamin deficient diets. Aoe *et al.* (1967c) did not observe growth suppression in carp maintained on thiamin deficient low carbohydrate diets however, in a later paper, Aoe *et al.* (1969) described a suppression of growth induced by an undisclosed elevation of the carbohydrate content of the diet and by the use of dietary thiamin antagonists.

In the present experiment, feed conversion efficiency and net protein utilisation mirrored the growth response of the thiamin deficient fish. Additionally the similar growth response of the thiamin and pantothenic acid deficient individuals was reflected in the similarity of their feed utilisation performances. The small difference in feed conversion efficiency between the thiamin and pantothenate deficient individuals is probably attributable to the reduced feed intake and slightly larger final weight of the pantothenic acid deficient fish. However, the lowered net protein utilisation observed for the thiamin deficient individuals is not just a factor of final weight and feed intake but is also attributable to the low protein content of the carcasses of the thiamin deficient individuals. Given the role of thiamin in intermediary metabolism and the use of protein as the primary source of energy in fish, it is thus proposed that the thiamin deficient individuals spared a reduced amount of protein for carcass deposition and utilised a greater proportion of the ingested protein for the provision of energy. This hypothesis is further expanded in a later chapter (5.2). However, the overriding consideration when comparing the performance of the deficient groups to that of the control is the reduction

in appetite and the very low lipid content of the carcasses of the vitamin deficient fish. Poor appetite and reduced efficiency of nutrient utilisation during thiamin deficiency have been recorded for the red seabream (Yone and Fujii, 1974), chinook salmon (Halver, 1957), rainbow trout (Morito *et al.*, 1986), the turbot (Cowey *et al.*, 1975), channel catfish (Murai and Andrews, 1978a) and the Asian seabass (Boonyaratpalin and Wanakowat, 1991).

For the thiamin deficient fish, throughout the course of the present experiment, a low cumulative mortality was recorded and the outward appearance and behaviour of the fish remained normal. Deficiency mediated mortality has been recorded in thiamin deficient rainbow trout (Morito *et al.*, 1986, Masumoto *et al.*, 1987), channel catfish (Murai and Andrews, 1978a) and Asian seabass (Boonyaratpalin and Wanakowat, 1991). However, for the walking catfish, high rates of survival were maintained despite an absence of thiamin in the diet (Butthep *et al.*, 1983) and additionally in the study of Cowey *et al.* (1975) mortalities only became apparent in turbot fed the thiamin antagonist, pyriithiamin.

The absence of deficiency related pathology in the present experiment contrasts strongly with the observations for many species. Salmonids exhibit deficiency mediated pathology including; premortem convulsions and loss of equilibrium (Halver 1957), anorexia, irritability, instability, ataxia and dark pigmentation (Morito *et al.*, 1986, Masumoto *et al.*, 1987). Similarly, in the study of Boonyaratpalin and Wanakowat (1991), a deficiency of thiamin for the Asian seabass was manifested in the form of neurological disorder including convulsions and sensitivity to shock. Anorexia and dark pigmentation without overt neurological disorders were observed by Murai and Andrews (1978a) in the channel catfish and dark pigmentation was the only pathology recorded by Butthep

et al. (1983) for walking catfish fed diets devoid of thiamin. Hashimoto *et al.* (1970) recorded the appearance of severe flexion of the trunk in eels which has subsequently been termed "trunk winding syndrome" while additional thiamin deficiency related pathologies in this species included poor appetite, haemorrhage in the fins and ataxia. The turbot, in the study of Cowey *et al.* (1975), failed to develop overt deficiency symptoms though the authors attributed this to the benthic nature of the fish since neurological and hence behavioural changes more usually become apparent as modifications in the swimming ability of the fish. Aoe *et al.* (1967c) recorded a complete absence of pathology in carp fed diets without a thiamin supplement though pathology was later induced by the use of a diet containing either a greater carbohydrate content or a thiamin antagonist (Aoe *et al.* 1969). Consequently, the thiamin deficiency symptoms recorded by Aoe *et al.* (1969) included anorexia, fading body colour, fin disorders and ecchymotic congestion of the skin. The role of thiamin in nervous transmission is currently not well understood though the nervous disorders observed in the present experiment and by previous researchers are probably attributed to this relationship.

A severe depression of the haematocrit was observed during thiamin deficiency in the present experiment. This contrasts with the majority of the presently available literature since reductions in the packed cell volume have only been recorded in thiamin deficient tilapia (*Oreochromis mossambicus* x *Urolepis hornorum*) by Lim *et al.* (1991a). The appearance of a reduction in haematocrit is surprising only in that it has not previously been observed more widely. At the haematological level, the concentrations of plasma lactate and serum pyruvate have been seen to increase in thiamin deficient rainbow trout (Morito *et al.*, 1986), and the activity of the thiamin dependent erythrocyte transketolases and the plasma concentration of their cofactor (thiamin pyrophosphate)

are frequently used as diagnostic indicators of thiamin status in fish (Morito *et al.*, 1986, Masumoto *et al.*, 1987, Cowey *et al.*, 1975).

Once again the hepatosomatic index of the deficient fish was significantly smaller than that of the control group. The histopathology of thiamin deficient rainbow trout and channel catfish has been examined by Morito *et al.* (1986) and Murai and Andrews (1978a) respectively. In both cases the heart, hepatopancreas (catfish), liver (trout), kidney, lateral muscle, gastrointestinal tract and gills were free of deficiency related pathologies. These observations contrast strongly with those of the present experiment. In general, the normal architecture of the liver was markedly different to that of the control fish. Hence, the pattern of staining with haematoxylin and eosin reflected that observed for the pyridoxine and pantothenic acid deficient fish *ie.* the distribution of stained hepatocytes was very irregular. Stereological examination of the Oil red O stained sections indicated that the hepatic lipid content of the thiamin deficient fish was not significantly lower than that of the individuals fed the complete diet. However, the intra and inter fish variation meant that a large proportion of the deficient individuals exhibited a lipid retention in the hepatopancreas much lower than that of the control fish.

Once again, the pancreas of the deficient fish exhibited severe degeneration with the accumulation of melanomacrophage aggregates and deposition of pigmented granules. Thiamin is implicitly involved with carbohydrate metabolism and acts at three places in intermediary metabolism (in the TCA cycle, pentose phosphate pathway and as a cofactor for pyruvate dehydrogenase). Consequently, given the role of the pancreas in glucose homeostasis, it was not unexpected that this organ should be so severely effected by thiamin deficiency. However, a direct relationship between thiamin deficiency

and pancreas degeneration is unlikely to become apparent.

General Conclusions.

The present study unequivocally demonstrates the qualitative requirement of the gilthead seabream for riboflavin, niacin, pantothenic acid, pyridoxine and thiamin. All of these vitamins are required in as yet ill defined quantities, for the provision of adequate growth, feed efficiency and for the prevention of mortality in the gilthead seabream.

The diversity of the deficiency symptoms observed in the present case emphasises the complexity of the roles of the B vitamins in nutrition and nutrient metabolism. Many of the pathologies observed during the deficiency of one vitamin became apparent in deficiency of other nutrients. Thus, despite the wide variety of deficiency related pathologies, few may be considered indicative of any single vitamin deficiency. This is particularly true of those vitamins which play roles in a multiplicity of metabolic pathways *eg.* riboflavin and niacin, and consequently, the metabolism of several nutrients is disturbed resulting in pathologies which are not easily be attributed to the function or metabolism of the deficient vitamin. Consequently, nutritionists have further investigated the metabolism of the vitamins in order to define more specific indicators of deficiency which are attributable to specific metabolic lesions induced by inadequate supply of a single vitamin. However, despite the simplicity of the present study, a series of investigative pathways have been highlighted which will prove invaluable in expanding the present knowledge of the B vitamin requirements and metabolism of the gilthead seabream.

In the present experiment, a deficiency of pyridoxine produced the most dramatic effects on all the parameters measured thus underlining the importance of this vitamin

in the nutrition of this species and demonstrating the potential for further research on this vitamin. Protein (the metabolism of which is inextricably linked with B₆) represents the major nutrient supplied to cultured fish. The interaction between the metabolism of protein and pyridoxine status has previously been investigated with respect to both quantitative (Bai *et al.* 1991, Hardy *et al.* 1979) and qualitative (Fisher *et al.*, 1984) aspects of the protein supply. Additionally, the present experiment outlined the dependence of this species on a dietary supply of pyridoxine for the maintenance of health. The relationship between vitamin B₆ and health has been the focus of a great deal of attention in mammalian nutrition and medicine (Chandra and Sudhakaran 1990, Lakshimi *et al.* 1991, Arnadottir *et al.* 1993, Folkers *et al.*, 1993). Presently the antioxidant vitamins, supplied in the feed in amounts in excess of the minimum requirement, have been the focus of a great deal of attention with regards the effect on health and stress tolerance in cultured fish. Thus, given the high protein component in the feed of farmed fish, the stressors involved in aquaculture, the potential health dividends of pyridoxine super supplementation and the reliance of this species on a dietary source of pyridoxine, further experiments with this vitamin are warranted. Consequently, this line of enquiry will be evaluated in chapter 5 in which pyridoxine super supplementation in diets for the gilthead seabream is investigated.

Under the conditions of macro nutrient supply in the present test formulation, a definite requirement for thiamin was demonstrated. The use of least cost formulation in the derivation of cost effective feeds for fish often results in quite major modifications in the proportions of the macro nutrients in the finished product. The use of carbohydrate in fish feeds has been extensively investigated as a low cost energy source thus in part, replacing oil and protein for the provision of energy. Thiamin is a vitamin

which, in its deficiency, is characterised by lesions in the metabolism of carbohydrates and thus is very obviously required by fish for the maintenance of normal carbohydrate metabolism. Given the potential for change in the proportion of carbohydrate in the diet and the very obvious relationship between thiamin and carbohydrate, it is not unreasonable to postulate that the requirement for this vitamin may be altered by elevations in the carbohydrate content of the diet as observed by Ogino (1967c, 1969) in the carp. This relationship will be investigated in chapter 5 in which the thiamin requirement of gilthead seabream fed diets of varied lipid to carbohydrate ratios is examined.

Given the overall observations with regards histopathology, it was immediately apparent that in all deficient groups the degeneration of the pancreas was a common feature. It is unlikely that in all cases a specific relationship exists between pancreas function and vitamin deficiency though some vitamin hormone interactions exist *eg.* pyridoxine is involved with steroid hormone receptor activity and ascorbic acid is a cofactor in the production of catecholamines (Bender 1992). Additionally, with regards the exocrine pancreas, B vitamin deficiency was associated with poor feed intake and hence the relationship between exocrine pancreas secretion, vitamin status and digestive function should be investigated. Hence, the role of the pancreas in vitamin deficient fish and the root causes of pancreatic degeneration appear to be avenues of research which exhibit considerable potential.

The histology of the liver of fish is recognised as different to that of mammals in that the hepatocytes are not organised into cords or lobules (Roberts 1989), indeed an apparent lack of lobular organisation was exhibited by the striped bass (*Morone saxatilis*) by Groman (1982) and the large mouth bass (*Micropterus salmoides*) by Hinton *et al.*

(1972). This was adequately demonstrated by the uniform staining of the hepatopancreas in the control fish within the present study. During vitamin deficiency, this apparently uniform distribution of stain was not evident particularly with regards the lysosomal enzyme NAH. Assuming that a lobular structure is a pre-requisite of functional differentiation and that a uniform distribution of stain in the control individuals indicates an absence of zonation, then the increased intra animal variability observed in the vitamin deficient individuals may indicate a collapse of the functional homogeneity. The reason for such alterations in functional distribution may be a consequence of changes in the distribution of vitamin or vitamin derived metabolites within the liver. Hence, an investigation of the root causes of such pathology may prove worthwhile.

Chapter 4

Requirement Studies

4.1. The Requirement of the Gilthead Seabream (*Sparus aurata* L.) for Niacin.

4.1.1. Introduction.

In chapter 3, the qualitative aspects of B vitamin supply were investigated and the essentiality of thiamin, riboflavin, pyridoxine, niacin and pantothenic acid was evaluated. An absence of niacin in the semi-purified diets fed to gilthead seabream was associated with a suppression of weight gain within 28 days, a lowering of the haematocrit, poor nutrient utilisation and eventual mortality. Indeed, within that experiment, a deficiency of niacin resulted in one of the more significant retardations of growth thus highlighting the importance of this vitamin in the nutrition of the gilthead seabream. The effects of niacin deficiency in other aquatic species were previously discussed in detail and hence will not be reiterated at this point suffice to say that niacin has so far been designated as an essential nutrient in diets for the rainbow trout (*Oncorhynchus mykiss*), chinook salmon (*Oncorhynchus tshawytscha*), channel catfish (*Ictalurus punctatus*), carp (*Cyprinus carpio*) and the red seabream (*Chrysophrys major*).

Niacin is a white crystalline solid and due to its high stability when subjected to high temperatures, light, heat and humidity is regarded as one of the more stable vitamins incurring very little loss of activity during feed manufacture and storage (Blum 1991). The niacin content of many feed materials has been determined (NRC 1993), though frequently the niacin within feed materials is unavailable to animals. Additionally a well known pathway exists allowing the synthesis of niacin from dietary tryptophan. The availability of vitamins from feed materials and synthesis of vitamins by fish was

discussed in chapter 1 and hence will not be repeated at this point suffice to say that the niacin content of major feedstuffs and bio-synthesis by fish may be largely ignored when determining niacin requirements of fish.

Niacin functions as a precursor of the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) which catalyse the transfer of hydrogen between metabolites. Consequently, via these coenzymes, niacin is central to both the synthesis and catabolism of amino acids, fatty acids and carbohydrates (Blum 1991). Additionally, the coenzymes NAD and NADP by their frequent interaction with the intermediates in the citric acid cycle, play a central role in energy metabolism.

Little information exists with regards the requirement for this vitamin of many cultured aquatic species and the literature which does exist is often contradictory establishing markedly different requirements for the same or similar species. For example McLaren *et al.* 1947, Phillips and Brockway 1947 and Poston and Wolfe 1985 established requirements for the rainbow trout (*Oncorhynchus mykiss*) ranging from 5 up to 95 mg kg⁻¹. An upper level for niacin supplementation (10,000 mg kg⁻¹) has been delineated for the brook trout (*Salvelinus fontinalis*) by Poston (1969) which was derived on the basis of altered lipid metabolism attributed by Poston to the utilisation of choline by the trout for the detoxification of excess niacin.

The essentiality of niacin in the nutrition of the gilthead seabream was highlighted in chapter 3. Given the absence of an established quantitative dietary requirement for niacin, the following experiment was designed to determine the optimum dietary inclusion level for the promotion of growth and efficient nutrient utilisation in the gilthead seabream.

4.1.2. Materials and Methods.

Twenty five seabream fingerlings (initial weight $21.68 \text{ g} \pm 0.38 \text{ S.E.}$) were stocked into each of six 80 l, self cleaning, polyethylene tanks over an 800 l bio-filter within a closed recirculation system providing a parallel flow of seawater through the tanks at 2.4 l min^{-1} . Water temperature and salinity were maintained at 24°C and 33 - 36‰ saline respectively, the former by an immersion heater, the latter by the use of a freshwater inlet balanced to compensate for evaporative losses. The pH was maintained between 6 and 7.5 by the use of calcium carbonate buffering while ammonia and nitrite were held within ranges tolerated by this species. Daylight balanced fluorescent strip lamps maintained an 8 hr light : 16 hr dark regime.

The complete semi-purified diet formulation employed in the evaluation of qualitative B vitamin requirements was again used to provide a basal diet. In order to improve the palatability of the feed, desiccated squid mantle tissue was included in the formulation. Thus six test diets differing only with respect to their niacin content were manufactured. Diets 1 to 6 were supplemented with niacin in the range 5.0 to 200 mg kg^{-1} ascending thus; 5.0, 25.0, 50.0, 100.0, 150.0 and 200.0 mg kg^{-1} . The complete diet formulation is presented in table 4.1.1 and the proximate composition is displayed in table 4.1.2. The conditions for diet manufacture and storage were as outlined in chapter two.

The test diets were presented to the fish three times daily (09:00, 13:00 and 16:00 hrs). On the basis of dry matter, the diet was supplied at 2.5 % of the body weight per day for 6 weeks and the ration was supplied *ad libitum* up to 2.5 % of the live mass for the remaining 6 weeks of the growth trial. The moisture content of each diet (approx. 30 - 35 %) was determined in order that equivalent amounts of dry matter was presented to the fish.

Table 4.1.1: The semi-purified test diet used to determine the niacin requirement of the gilthead seabream

Ingredient	%Inclusion
Casein (Vitamin Free)	31.95
Gelatin (225 Bloom)	15.00
Corn Starch/Dextrin (2:3)	19.70
Cod Liver Oil	11.84
Mineral Pre-mix ¹	10.00
Fat Soluble Vitamin Pre-mix ²	0.50
B Vitamin Pre-mix ³	0.45
Niacin in α -Cellulose ^a	0.05
Macro Vitamin Pre-mix ⁴	2.00
Amino Acid Pre-mix ⁵	5.18
Squid meal	0.50
Di-Calcium Phosphate	2.88

¹Contribution of minerals to diet (mg kg⁻¹ diet)

CaHPO₄: 22,050, CaCO₃: 3,000, NaCl: 15,000, K₂SO₃: 20,000, MgSO₄·7H₂O: 20,627, FeSO₄·7H₂O: 700.0, MnSO₄·4H₂O: 227.3, ZnSO₄: 515.6, CuSO₄·5H₂O: 160.0, CoCl₂·6H₂O: 26.0, KI: 15.0, Na₂SeO₃: 2.5, α -cellulose: 17676.6

²Contribution of fat soluble vitamins to diet (mg kg⁻¹ diet)

A Palmitate: 4.20, Rovimix D-50 SD: 3.68, Rovimix E-50 SD: 1,000.00, Rovimix Menadione: 780.00, α -Cellulose: 3,212.12

³Contribution of water-soluble vitamins to diet (mg kg⁻¹ diet)

(niacin free B vitamin pre-mix)

Thiamin HCl: 69.90, Riboflavin (96%): 208.30, Pyridoxine HCl: 48.60, Calcium Pantothenate: 305.30, Biotin (2%): 300.00, Folic Acid (88.8%): 16.90, Cyanocobalamin (95%): 0.01, α -Cellulose: 3,550.99.

⁴Contribution of macro vitamins to the diet (mg kg⁻¹ diet)

Ascorbic Acid (Rovimix Stay C): 2000, Choline Chloride: 5000, Inositol: 2000, α -cellulose: 11000

⁵Contribution of amino acids to the diet (g kg⁻¹ diet)

D-L-Methionine: 4.80, L-Tryptophan: 4.70, L-Threonine: 8.70, L-Phenyl Alanine: 5.80, L-Histidine: 1.40, L-Arginine: 7.00, L-Isoleucine: 4.80, L-Leucine: 5.00, L-Valine: 9.10

^aNiacin in α -cellulose

Represents the space allowed within the formulation for niacin. Supplemental niacin was supplied at 5, 25, 50, 100, 150 and 200 mg kg⁻¹ of diet and the remaining space within the formulation filled with α -cellulose.

Table 4.1.2: Proximate composition of the test diet. Moisture is expressed as a percentage of the diet as fed. Protein, lipid and ash are expressed as a percentage of the dry matter. Nitrogen free extract (including carbohydrate and fibre) was resolved as the residual of the dry matter analysis.

Moisture (%)	Protein (%)	Lipid (%)	Ash (%)	Nitrogen Free Extract (%)
34.55	53.75	10.78	9.02	44.45

The fish were weighed twice monthly in order to re-calculate the appropriate ration size and follow the growth parameters. The weight gain, specific growth rate (SGR), feed intake, feed conversion ratio (FCR), feed conversion efficiency (FCE) and cumulative percentage mortality were monitored throughout the trial. The fish were also inspected daily for the appearance of gross deficiency symptoms.

After 12 weeks of feeding, five individuals from each tank were selected for proximate analysis. Each carcass was individually analysed for protein, lipid, ash and moisture content. Crude protein was determined by the Kjeldahl ($N \times 6.25$) method and lipid by a method derived from that of Barnes and Blackstock (1973). Moisture and ash were assayed according to the methods outlined in the A.O.A.C. Handbook (1990). Based on the growth data, nutrient analysis of the test diet and the proximate composition of fish sub-sampled at the beginning of the trial, the net protein utilisation (NPU) and protein efficiency ratio (PER) were calculated.

Additionally, at the end of the growth study, five individuals were selected from each tank and blood was withdrawn into heparinised syringes by cardiac puncture. Following centrifugation, the plasma was decanted into micro centrifuge tubes and stored at -70°C until required. Diagnostic kits were subsequently used to determine total plasma protein by the Biuret method (Total Protein Reagent, Sigma No. 541-2) and

plasma glucose by the glucose oxidase method (Sigma, procedure No. 510 A). At this point the weight of the liver with respect to complete carcass mass for five fish in each tank was recorded and the hepatosomatic index (HSI) determined.

The data were subjected to analysis of variance where $P \leq 0.05$ was judged to be indicative of a significant difference. Where the ANOVA revealed significant differences Duncan's multiple range test (Duncan 1955) was applied in order to characterise and quantify the differences between then dietary regimes.

4.1.3. Results.

Within six weeks of the start of the nutrition trial, those fish fed the diet supplemented with niacin at 5.0 mg kg^{-1} were significantly smaller ($p < 0.01$) than the fish fed any other diet. After 10 weeks of feeding, the fish fed the diet containing 5.0 mg of niacin per kg of diet were significantly smaller than all the fish fed any of the remaining diets while those fed a supplement of 25 mg kg^{-1} were significantly smaller than those fish fed diets containing a niacin supplement of 100 mg kg^{-1} or greater. The fish fed the diets supplemented with niacin at 50 mg kg^{-1} were neither significantly smaller than those fed a niacin supplement of 100 mg kg^{-1} or significantly larger than those fed a supplement of 50 mg kg^{-1} . Between weeks 10 and 12, the mean weight of the fish fed diet 1 decreased while those fish fed diet 2 failed to gain weight. Thus, at the end of the growth study, the final mean weights of the fish reflected the *status quo* observed after 10 weeks of feeding.

A low cumulative mortality (12 %) was recorded for all dietary treatments apart from those supplemented with niacin at 25 and 200 mg kg^{-1} for which, at the end of the study, the cumulative mortality was 20 and 0 % respectively. Apart from the reduced

feed consumption of the fish fed diets containing less than 100 mg kg⁻¹ there were no apparent behavioural changes and visible pathology was not observed in the deficient fish.

The proximate composition of the fish fed diets containing the lowest level of supplemental niacin (5.0 mg kg⁻¹) was significantly different from that of all the remaining dietary treatments with regards both lipid and moisture ($p < 0.05$). Thus, in these fish, carcass moisture content was significantly elevated in response to the declining carcass lipid component. The protein and ash content of these fish was also marginally increased when compared to the fish fed the remaining diets though this is likely to be a consequence of the lowered lipid content and not a factor of niacin status *per se*.

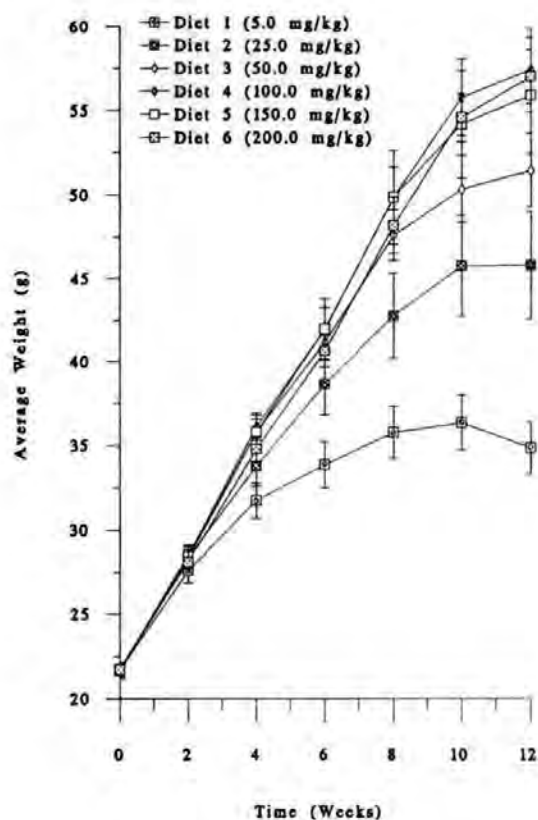
Table 4.1.3: Performance characteristics of gilthead seabream fed diets of varied niacin content. Values in each row carrying similar superscripts are not significantly different from each other ($p < 0.001$).

	Diet 1 5.0 mg kg ⁻¹	Diet 2 25.0 mg kg ⁻¹	Diet 3 50.0 mg kg ⁻¹	Diet 4 100.0 mg kg ⁻¹	Diet 5 150.0 mg kg ⁻¹	Diet 6 200.0 mg kg ⁻¹
Initial mean weight (g)	21.66	21.65	21.58	21.68	21.66	21.73
Final mean weight (g)	34.85 ^a	45.78 ^b	51.42 ^{bc}	57.37 ^c	55.85 ^c	56.96 ^c
Weight gain (%)	60.90	111.45	138.28	164.62	157.85	162.13
Specific growth rate (%d ⁻¹)	0.57	0.89	1.03	1.16	1.13	1.15
Daily feed intake (mgd ⁻¹)	531.4	608.2	598.2	771.9	712.0	723.6
Feed conversion ratio (FCR)	3.38	2.18	1.68	1.82	1.75	1.73
Feed conversion efficiency (FCE)	29.55	47.23	59.38	55.04	57.16	57.96
Protein efficiency ratio (PER)	1.82	1.14	0.91	0.98	0.94	0.93
Net protein utilisation (NPU %)	10.11	15.56	19.98	18.71	19.39	20.31
Mortality (%)	12.0	20.0	12.0	12.0	12.0	0.00

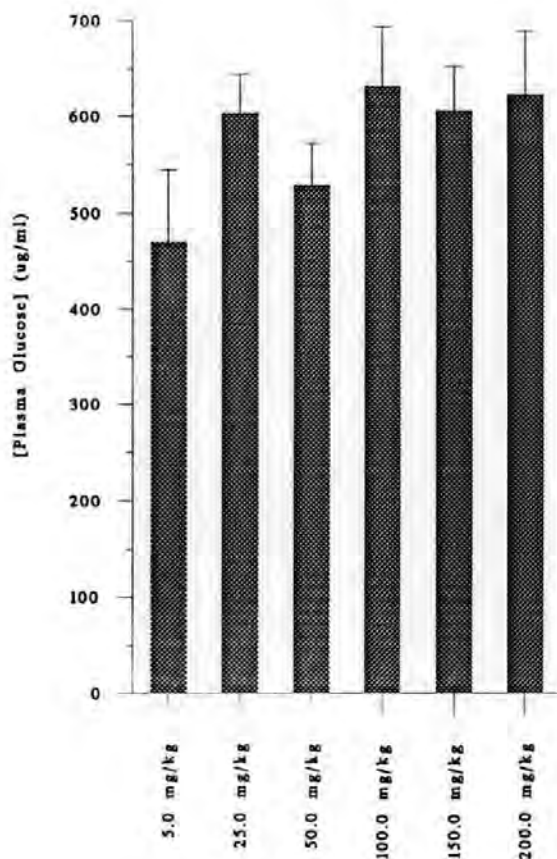
Table 4.1.4: Proximate composition of initial carcasses and those of test animals fed diets of varied niacin content. Average nutrient composition of five individuals is expressed as a percentage of the live weight. Values in each column bearing similar superscripts are not significantly different from each other ($p < 0.05$).

Diet		Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Initial Fish		69.63	19.57	10.78	3.99
1. 5.0 mg kg ⁻¹	Mean	71.97 ^a	19.13	6.76 ^a	4.69
	S.E.	0.47	0.30	0.44	0.10
2. 25.0 mg kg ⁻¹	Mean	68.37 ^b	18.59	11.09 ^b	4.51
	S.E.	0.85	0.21	0.11	0.08
3. 50.0 mg kg ⁻¹	Mean	68.32 ^b	18.70	10.83 ^b	4.32
	S.E.	1.41	0.25	1.50	0.10
4. 100.0 mg kg ⁻¹	Mean	66.61 ^b	18.79	12.62 ^b	4.30
	S.E.	1.66	0.07	0.57	0.03
5. 150.0 mg kg ⁻¹	Mean	67.43 ^b	18.75	11.44 ^b	4.49
	S.E.	1.66	0.29	1.60	0.17
6. 200.0 mg kg ⁻¹	Mean	67.04 ^b	19.12	11.66 ^b	4.37
	S.E.	0.78	0.35	0.75	0.04

The feed conversion efficiencies and protein utilisation values recorded for the fish fed diets containing niacin supplements of 5 and 25 mg kg⁻¹ were very poor and reflected the pattern observed for growth. Consequently, the fish fed the lowest dietary supplement were the least efficient with regards nutrient utilisation. Where the niacin supplement in the diet was 100 mg kg⁻¹ or greater, the nutrient utilisation in terms of feed conversion efficiency and net protein utilisation, was marginally improved with each increase in niacin supplement. However, all nutrient utilisation parameters except NPU



(a)



(b)

(a) Figure 4.1.1.

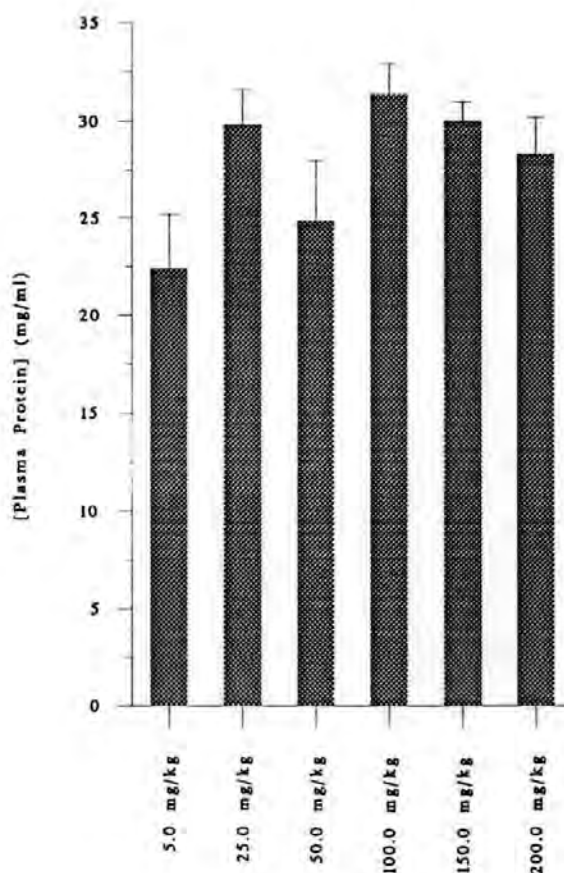
Growth of gilthead seabream fed diets of varied niacin content

(b) Figure 4.1.2.

Plasma glucose concentration in gilthead seabream fed diets of varied niacin content.

(c) Figure 4.1.3.

Plasma protein concentration in gilthead seabream fed diets of varied niacin content.



(c)

(which was optimised in the fish fed diet 6) appeared to be optimal for those fish fed diets containing a niacin supplement of 50 mg kg⁻¹. Given the poor growth of these individuals, this is probably a consequence of the low feed intake of these fish and not indicative of an optimum level of niacin supplementation.

Table 4.1.5: Hepatosomatic index of gilthead seabream fed diets of varied niacin content. Values represent the mean for five individuals and those bearing similar superscripts are not significantly different from each other ($p < 0.05$).

		Diet 1 5.0 mg kg ⁻¹	Diet 2 25.0 mg kg ⁻¹	Diet 3 50.0 mg kg ⁻¹	Diet 4 100.0 mg kg ⁻¹	Diet 5 150.0 mg kg ⁻¹	Diet 6 200.0 mg kg ⁻¹
Hepatosomatic	Mean	0.76 ^a	1.01 ^{ab}	1.22 ^b	1.10 ^b	1.30 ^b	1.17 ^b
Index (%)	S.E.	0.07	0.08	0.12	0.09	0.14	0.08

The hepatosomatic index (HSI) of the fish fed the diet supplemented with niacin at the lowest level was significantly lower than that of the fish fed diets with a niacin content of 100 mg kg⁻¹ or more, while the HSI of the fish maintained on diet 2 (25.0 mg kg⁻¹) was not significantly different from that recorded for any of the remaining dietary regimes.

The plasma glucose concentration of the fish maintained with a niacin supplement of 5.0 mg kg⁻¹ was the lowest amongst all the dietary regimes. However the wide variation in glucose concentration between individuals in this group meant that the mean could not be considered to be significantly lower than that recorded for any of the other treatments. Similarly, the plasma protein concentration of the fish maintained on the diet with the least niacin concentration was the lowest of all the dietary regimes. However,

as indicated by the results of the ANOVA this value was only just considered to be significant.

Table 4.1.6: Mean plasma protein and glucose and protein concentration (n=5) of gilthead seabream fed diets of varied niacin content. Plasma protein concentrations bearing similar superscripts are not significantly different from each other (p = 0.0493).

		Diet 1 5.0 mg kg ⁻¹	Diet 2 25.0 mg kg ⁻¹	Diet 3 50.0 mg kg ⁻¹	Diet 4 100.0 mg kg ⁻¹	Diet 5 150.0 mg kg ⁻¹	Diet 6 200.0 mg kg ⁻¹
[Plasma Glucose]	Mean	470.53	602.68	527.68	629.46	605.36	622.32
(μg cm ⁻³)	S.E.	73.99	40.15	43.45	62.44	45.71	66.16
[Plasma Protein]	Mean	22.41 ^a	29.84 ^b	24.86 ^{ab}	31.37 ^b	30.00 ^b	28.27 ^{ab}
(mg cm ⁻³)	S.E.	2.79	1.73	3.11	1.51	0.97	1.88

4.1.4. Discussion.

The present experiment demonstrates that optimum growth, both in terms of weight gain and specific growth rate, was attained in seabream fed a niacin supplement of 100 mg kg⁻¹ or above. Though the final mean weight of those fish maintained on a diet supplemented with niacin at 50 mg kg⁻¹ was not significantly smaller than those fed a larger supplement, the poor growth of these fish during the last four weeks of the growth study would indicate that a supplement of 50 mg kg⁻¹ would eventually result in growth retardation. The relationship between niacin deficiency and growth retardation is discussed in chapter 3 which deals with the qualitative aspects of niacin supply. However, in summary, reduction of weight gain has been observed during niacin deficiency by Yone and Fujii (1974) in red seabream (*Chrysophrys major*), in common carp (*Cyprinus*

carpio) by Aoe *et al.* 1967b, by McLaren *et al* (1947) and Poston and Wolfe (1985) in the rainbow trout (*Oncorhynchus mykiss*), in chinook salmon (*Oncorhynchus tshawytscha*) by (Halver 1957), by Andrews and Murai (1978) in channel catfish (*Ictalurus punctatus*) and in the walking catfish (*Clarias batrachus*) by Butthep *et al.* (1983).

Given the key role of niacin in metabolism, it is not surprising that feed utilisation should be severely suppressed during niacin deficiency. Despite the decline in appetite, for diets 1 and 2 (5 and 25 mg kg⁻¹), the feed conversion efficiency and net protein utilisation reflected the level of niacin supplementation and growth of the fish. Similarly, with niacin supplements of 100 mg kg⁻¹ or more, feed conversion efficiency and net protein utilisation reflected the level of supplemental vitamin. Additionally, the high feed intake of the fish fed the niacin supplement of 100 mg kg⁻¹ was coupled to the poorest feed utilisation efficiency within the 3 groups of fish fed the diets containing the highest levels of supplemental niacin.

The best feed conversion efficiency (FCE) recorded for any of the fish within the present experiment was observed amongst those fed the diets supplemented with niacin at 50 mg kg⁻¹. The net protein utilisation (NPU) of the fish fed this diet was also superior to that of the fish fed diets 4 and 5 (100 and 150 mg kg⁻¹). The apparent improvement in feed utilisation is probably an artefact of the means by which these figures are calculated. Thus, feed or protein intake act as the denominators in both the feed conversion and protein utilisation calculations where weight gain and protein deposition respectively act as the numerators. Consequently, a poor feed or protein intake will artificially inflate the efficiency of nutrient utilisation since, for the determination of NPU and PER a small denominator is divided into a comparatively large numerator.

If, due to the poor growth of the fish fed diet 3 the high feed conversion efficiency

of these fish is ignored, then a dietary supplement of 200 mg kg⁻¹ was associated with optimum nutrient utilisation though this was only marginally better than that observed for the fish fed supplements of 150 mg kg⁻¹.

Feed conversion in rainbow trout (*Oncorhynchus mykiss*) was shown by Poston and Wolfe (1985) to be proportional to niacin supplementation up to niacin levels of 10 mg kg⁻¹, while Aoe *et al* (1967b) also recorded poor feed conversion in carp (*Cyprinus carpio*) fed diets with a niacin content below 28 mg kg⁻¹. Feed gain ratio in the channel catfish (*Ictalurus punctatus*) was shown to be sub-optimal when fed a niacin supplement of less than 25 mg kg⁻¹ in the studies of Andrews and Murai (1978).

In common with the earlier experiment which highlighted the qualitative requirement of the gilthead seabream for niacin, the deficient fish demonstrated a significant decline in the carcass lipid content. Thus, a niacin supplement of 25 mg kg⁻¹ or above was sufficient for the maintenance of normal carcass composition and hence the changes observed with regards net protein utilisation are in the main, a consequence of changes in growth and appetite and not the result of modified carcass composition. In keeping with the present experiment, Poston and Wolfe (1985) observed no alteration in any aspect of carcass composition in rainbow trout fed diets of varied niacin content.

In an earlier study, a mortality of 13 % with an absence of externally visible pathology was associated with niacin deficiency. Thus, the observations of the present experiment are in broad agreement with the former study in that no pathology became apparent though it would appear that a dietary niacin supplement of 5.0 mg kg⁻¹ was sufficient to prevent a significant mortality. However, the high incidence of mortality in the fish fed 25 mg kg⁻¹ remains unexplained. The symptoms of niacin deficiency and its effect on mortality have been discussed in chapter 3 which deals with aspects of

qualitative B vitamin requirements and hence will not be further discussed at this juncture.

Once again, niacin deficiency was associated with a reduction in the hepatosomatic index, indeed, the values recorded for HSI were remarkably similar to those observed in the former experiment (0.74) with the present study yielding a value of 0.76. The modifications in the hepatosomatic index may reflect the carcass composition since the deficient fish demonstrated a lower carcass lipid content which may result in lower lipid retention in the liver and a consequent reduction in the liver weight. The observations of Poston and Wolfe (1985) would support this argument since in that study those rainbow trout fed 5.0 mg kg^{-1} or less exhibited a significantly decreased hepatic lipid content when compared to those fed niacin replete diets.

An examination of the plasma glucose concentrations reveals that the fish fed diets containing 5.0 mg of niacin per kg of diet exhibited a low mean glucose content however, a large amount of variation was observed between the individuals maintained on this diet. Additionally, a large degree of variation with an absence of any trend was also apparent in the mean glucose concentrations of the fish fed the remaining diets and hence, it is unwise to attempt to make any recommendations from these observations. However, given the importance of niacin in metabolism, it would appear that the maintenance of normal plasma glucose concentration may be compromised when the fish are maintained on a diet supplemented with less than 25 mg kg^{-1} . Similarly, the plasma protein concentrations reflected those obtained for plasma glucose and again the wide variation both within and between dietary groups means that little information may be obtained by an analysis of this data.

As stated in chapter 3, the requirement of animals for dietary niacin relates to the

ability of each species to synthesise this vitamin from tryptophan. The niacin synthesising capacity of an animal may be determined by measurements of the activity of the enzyme which regulates the conversion of tryptophan-niacin intermediates to niacin (3-hydroxyanthranilic acid oxygenase, 3HAA) compared to that which participates in the degradation of such intermediates to CO₂ via the glutaryl-CoA pathway (picolinic acid carboxylase, PC). On the basis of the 3HAA/PC ratio, Poston and DiLorenzo (1973) determined that brook trout (*Salvelinus fontinalis*) were unable to synthesise significant amounts of niacin from tryptophan. In a study summarised by Chuang (1991), 10 of the 38 species of aquatic animal tested demonstrated 3HAA/PC ratios which indicated some capacity for niacin synthesis. The finfish exhibiting a capacity for niacin synthesis included the common carp, tilapia (*Tilapia spp.*), red seabream (*Chrysophrys major*), black seabream (*Mylio macrocephalus*), and the milkfish (*Chanos chanos*) though no indication was given with regards the extent to which these species may rely on tryptophan as a niacin source. Despite the high protein content and ready supply of crystalline L-tryptophan (4.7 g kg⁻¹) in the present diets, a definite requirement for preformed niacin was demonstrated by the fish. This would indicate that if any niacin is formed by the fish from tryptophan, it is insufficient to meet the minimum requirement of the gilthead seabream and indeed a significant dietary supplement is still required.

In conclusion, the results of the present experiment indicate that optimal growth may be attained in the gilthead seabream with a niacin supplement of 100 mg kg⁻¹ though a supplement of 150 mg kg⁻¹ provided a small additional benefit with regards feed utilisation performance. This value is higher than the 28 mg kg⁻¹ determined by Aoe *et al.* (1967b) for the carp and the 14 mg kg⁻¹ recommended by Andrews and Murai (1978) for the channel catfish, though the observations of Chuang (1991) would indicate some

niacin synthesising capability in the former species. For the rainbow trout (*Oncorhynchus mykiss*) the requirement for niacin was determined to lie between 1 and 5 or at 10 mg kg⁻¹ by McLaren *et al* (1947) and Poston and Wolf (1985) respectively while Halver (1989) places the requirement of this species somewhere between 120 and 150 mg kg⁻¹. The former values are much lower than the 95 mg kg⁻¹ determined by Phillips and Brockway (1947) as the requirement of brook (*Salvelinus fontinalis*), brown (*Salmo trutta*) and rainbow (*Salmo gairdneri*) trout, though the study of Phillips and Brockway used maximal liver storage to define the niacin requirement. The niacin requirement of salmon (*Salmo spp.*) is quoted as 150 to 200 mg kg⁻¹ by Halver (1989) and represents one of the highest published requirements.

Thus, the presently available literature exhibits a large degree of confusion with regards niacin requirement of fish even for the same species. However, the present observations define the requirement of the gilthead seabream as lying in the region of 100 to 150 mg kg⁻¹ and depend on whether growth or feed efficiency assessments are chosen to delineate the vitamin requirement.

4.2. The Requirement of the Gilthead Seabream (*Sparus aurata* L.) for Biotin.

4.2.1. Introduction.

In chapter 3 the qualitative aspects of B vitamin requirement and deficiency were examined for the gilthead seabream. These preliminary experiments were further developed to quantify the requirement of the gilthead seabream for niacin, a vitamin for which this species clearly demonstrated a dietary requirement. Biotin is a vitamin which, like niacin, plays a key role in intermediary metabolism and hence would also be expected to act as an essential dietary micronutrient.

The role of biotin in metabolism has been well defined and, in the main, the symptoms associated with a deficiency of this vitamin are well characterised. For animals the importance of biotin centres around its involvement as a cofactor for the enzymes catalysing the transfer of carbon atoms from more complex molecules *i.e.* carboxylation. Consequently biotin is involved in a diverse group of metabolic pathways including gluconeogenesis, the catabolism of certain amino acids and the modification of fatty acids via chain elongation and desaturation (Bender 1992). Biotin is also involved in carboxylation and transcarboxylation in microorganisms but these roles are outside the scope of the present study and hence will not be discussed at this point.

The biotin requirement of some cultured species has already been determined and the range of requirements extends from less than 0.1 mg kg⁻¹ in the rainbow trout (*Oncorhynchus mykiss*) as determined by Woodward and Frigg 1989 up to 2.5 mg kg⁻¹ for the carp (*Cyprinus carpio*) as determined by Günther and Meyer-Burgdorff 1990. However, the essentiality of this vitamin has also been questioned since the red seabream (*Chrysophrys major*) failed to demonstrate a requirement for biotin (Yone and Fujii 1974).

The availability of this vitamin from feed materials has been shown to be highly variable (Frigg 1976, 1984) though the studies of Castledine *et al.* (1978) and Lovell and Buston (1984) have demonstrated that the biotin content of carefully selected feed materials was sufficient to meet the requirements of rainbow trout and channel catfish (*Ictalurus punctatus*) respectively. While evidence exists which suggests that synthesis of biotin by the gut flora of humans negates the need for a dietary input (Bender 1992), the lack of a significant biotin content in the faeces of channel catfish was assumed by Lovell and Buston (1984) to be indicative of an absence of biotin synthesising capacity.

The aim of the present experiment was to demonstrate a qualitative requirement of the gilthead seabream for biotin, determine a quantitative requirement for this vitamin and subsequently characterise any apparent symptoms of deficiency which may arise as a consequence of inadequate biotin supply.

4.2.2. Materials and Methods.

Batches of 25 seabream fingerlings (initial weight 38.05 g \pm 0.33 g S.E.) were stocked into each of six 400 l, self cleaning fibreglass tanks over a 1200 l bio-filter within a closed recirculation system. A parallel flow of sea water was maintained through the tanks at 10.8 l min⁻¹. Water temperature and salinity were maintained at 24 °C and 33-36 ‰ saline respectively, the former by the use of an immersion heater with an integrated dip-cooler the latter by the use of a fresh water inlet balanced to compensate for evaporative losses. The pH was maintained between 6 and 7.5 by the use of magnesium and calcium carbonate buffering. Weekly water changes amounted to 5 % of the system volume. Daylight balanced fluorescent discharge lamps maintained a 12 hr light / dark photoperiod.

The complete semi-purified diet formulation employed in chapter 3 and 4.1 was again used to provide a basal diet. In order to improve the palatability of the feed, oven dried squid mantle tissue was added to the formulation. Thus, six test diets differing only with respect to their biotin content were manufactured. Diet 1 was a biotin free formulation while diets 2 to 6 were supplemented with biotin in the range 0.5 to 2.5 mg kg⁻¹ ascending in increments of 0.5 mg kg⁻¹. The complete diet formulation is presented in table 4.2.1 and the proximate composition is presented in table 4.2.2. The conditions for diet manufacture and storage were as outlined in chapter two.

The test diets were presented to the fish three times daily (09:00, 13:00 and 16:00 hrs). The diet was supplied at 2.0 % of the body weight per day for 10 weeks and the ration was supplied *ad libitum* up to 2.0 % of the live mass for the remaining 2 weeks of the growth trial. The moisture content of each diet (approx. 30 - 35 %) was determined in order that equivalent amounts of dry matter was presented to the fish.

The fish were weighed twice monthly in order to re-calculate the appropriate ration size and to follow the growth parameters. The weight gain, specific growth rate (SGR), feed intake, feed conversion ratio (FCR), feed conversion efficiency (FCE) and cumulative percentage mortality were monitored throughout the trial. The fish were also inspected daily for the appearance of gross deficiency symptoms.

After 12 weeks of feeding, five individuals from each tank were selected for proximate analysis. Each carcass was individually analysed for protein, lipid, ash and moisture content. Crude protein was determined by the Kjeldahl (N x 6.25) method and lipid by a method derived from that of Barnes and Blackstock (1973). Moisture and ash were assayed according to the methods outlined in the A.O.A.C. Handbook (1990).

Table 4.2.1: Composition of the semi-purified diet.

Ingredient	%Inclusion
Casein (Vitamin Free)	31.90
Gelatin (225 Bloom)	15.00
Corn Starch/Dextrin (2:3)	19.70
Cod Liver Oil/Linseed oil	11.84
Mineral Pre-mix ¹	10.00
Fat Soluble Vitamin Pre-mix ²	0.50
B Vitamin Pre-mix ³ (Biotin Free)	0.45
Biotin in α -Cellulose ^a	0.05
Macro Vitamin Pre-mix ⁴	2.00
Amino Acid Pre-mix ⁵	5.18
Squid meal	0.50
Di-Calcium Phosphate	2.88

¹Contribution of minerals to diet (mg kg⁻¹ diet)

CaHPO₄; 22,050, CaCO₃; 3,000, NaCl; 15,000, K₂SO₃; 20,000, MgSO₄·7H₂O; 20,627, FeSO₄·7H₂O; 700.0, MnSO₄·4H₂O; 227.3, ZnSO₄; 515.6, CuSO₄·5H₂O; 160.0, CoCl₂·6H₂O; 26.0, KI; 15.0, Na₂SeO₃; 2.5, α -cellulose; 17676.6

²Contribution of fat soluble vitamins to diet (mg kg⁻¹ diet)

A Palmitate; 4.20, Rovimix D-50 SD; 3.68, Rovimix E-50 SD; 1,000.00, Rovimix Menadione; 780.00, α -Cellulose; 3,212.12

³Contribution of water soluble vitamins to diet (mg kg⁻¹ diet)

Thiamin HCl; 69.90, Riboflavin (96%); 208.30, Pyridoxine HCl; 48.60, Calcium Pantothenate; 305.30, Niacin; 200.00, Folic Acid (88.8%); 16.90, Cyanocobalamin (95%); 0.01, α -Cellulose; 3,650.99

⁴Contribution of macro vitamins to the diet (mg kg⁻¹ diet)

Ascorbic Acid (Rovimix Stay C); 2000, Choline Chloride; 5000, Inositol; 2000, α -cellulose; 11000

⁵Contribution of amino acids to the diet (g kg⁻¹ diet)

D-L-Methionine; 4.80, L-Tryptophan; 4.70, L-Threonine; 8.70, L-Phenyl Alanine; 5.80, L-Histidine; 1.40, L-Arginine; 7.00, L-Isoleucine; 4.80, L-Leucine; 5.00, L-Valine; 9.10

^aBiotin in α -cellulose.

Denotes the maximum allowable limit for biotin incorporation within the formulation. Diets supplemented in the range 0 - 2.5 mg of biotin kg⁻¹ of diet with the remainder of the space in the formulation filled with α -cellulose.

Table 4.2.2: Proximate composition of the test diets.
Moisture is expressed as a percentage of the diet as fed. Protein, lipid and ash are expressed as a percentage of the dry diet while nitrogen free extract was derived from the unresolved component of the dry matter.

Moisture (%)	Protein (%)	Lipid (%)	Ash (%)	Nitrogen Free Extract (%)
34.27	54.45	9.92	12.54	23.09

Based on the growth data, nutrient analysis of the test diet and the proximate composition of fish sub-sampled at the beginning of the trial, the net protein utilisation (NPU) and protein efficiency ratio (PER) were calculated.

Additionally, at the end of the growth study, five individuals were selected from each tank and blood was withdrawn into heparinised syringes by cardiac puncture. The packed cell volume of the blood was immediately determined by micro haematocrit while the haemoglobin content was determined by the cyanmethemoglobin method using a diagnostic kit (Sigma Chemical Company, Procedure No. 525 A). Following centrifugation the plasma was decanted into micro centrifuge tubes and stored at -70°C until required. Diagnostic kits were subsequently used to determine total plasma protein by the Biuret method (Total Protein Reagent, Sigma No. 541-2), plasma triglycerides (Sigma, procedure No. 336-10), plasma glucose by the glucose oxidase method (Sigma, procedure No. 510 A) and plasma lactate by the lactate oxidase method (Sigma, procedure No. 735). At this point the weight of the liver with respect to complete carcass mass for five fish in each tank was recorded and the hepatosomatic index determined.

The data were subjected to analysis of variance where $P \leq 0.05$ was judged to be

indicative of a significant difference. Where the ANOVA revealed significant differences Duncan's multiple range test (Duncan 1955) was applied in order to characterise and quantify the differences between then dietary regimes.

4.2.3. Results.

After 12 weeks of feeding, those fish fed the diet devoid of supplemental biotin were significantly smaller ($p < 0.05$) than those fed diets fortified with the vitamin at 1.0 mg kg⁻¹ or more but not significantly smaller than those supplemented with biotin at 0.5 mg kg⁻¹. The fish fed diets fortified with biotin at 0.5 mg kg⁻¹ were not significantly smaller than the fish maintained on rations with a biotin content of 1.0 mg kg⁻¹ or more.

Table 4.2.3: Performance characteristics of gilthead seabream fed diets of varied biotin content. Diet performance characteristics are calculated using feed inputs corrected for moisture content. Values in each row carrying similar superscripts are not significantly different from each other ($p < 0.05$).

	Diet 1 0.0 (mgkg ⁻¹)	Diet 2 0.5 (mgkg ⁻¹)	Diet 3 1.0 (mgkg ⁻¹)	Diet 4 1.5 (mgkg ⁻¹)	Diet 5 2.0 (mgkg ⁻¹)	Diet 6 2.5 (mgkg ⁻¹)
Initial mean weight (g)	38.15	38.01	38.07	37.92	38.23	37.91
Final mean weight (g)	95.56 ^a	104.31 ^{ab}	109.69 ^b	107.37 ^b	111.21 ^b	107.10 ^b
Weight gain (%)	150.48	174.43	188.13	183.15	190.90	182.51
Specific growth rate (%d ⁻¹)	1.09	1.20	1.26	1.24	1.27	1.24
Daily feed intake (gd ⁻¹)	0.958	1.051	1.125	1.074	1.108	1.065
Feed conversion ratio (FCR)	1.40	1.33	1.32	1.30	1.28	1.29
Feed conversion efficiency (FCE)	71.37	75.10	75.76	76.96	78.42	77.34
Protein efficiency ratio (PER)	0.76	0.73	0.72	0.71	0.69	0.70
Net protein utilisation (NPU %)	27.75	29.50	29.36	29.79	30.78	29.96
Mortality (%)	0.00	0.00	8.00	0.00	0.00	4.00

Throughout the 12 weeks of the feeding period, a very low cumulative mortality was recorded (8 % for diet 3 and 4 % for diet 6) which appeared to be independent of dietary biotin content. No deficiency mediated pathology became evident apart from a reduced appetite in the fish fed the diets without supplemental biotin.

The values for feed conversion ratio and feed conversion efficiency reflected those of growth, hence only those fish fed the un-supplemented diets exhibited markedly poorer values for the feed utilisation criteria.

Table 4.2.4: Proximate composition of initial carcasses and those of the fish fed diets of varied biotin content. Carcass composition represents the nutrient profile of five individuals within each dietary regime and is expressed as proportion of the live fish.

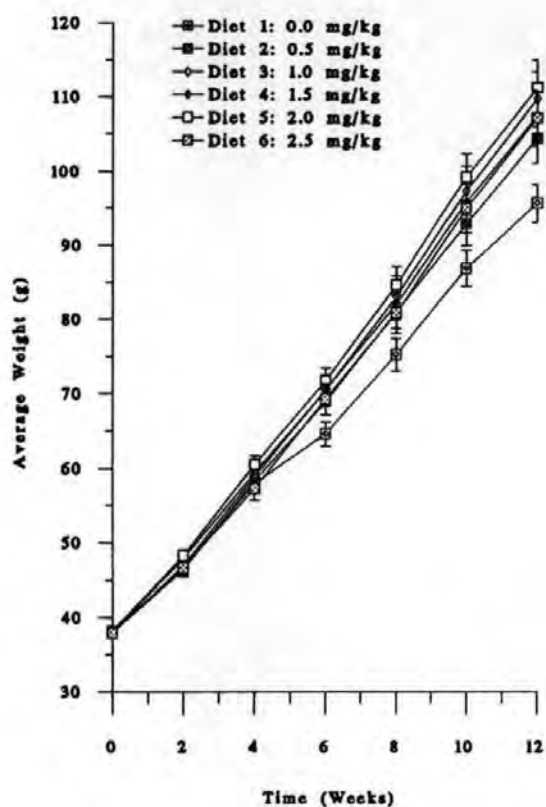
Diet		Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Initial Fish		70.22	19.38	10.21	2.69
1. 0.0 mgkg ⁻¹	Mean	64.67	20.46	13.49	4.13
	S.E	1.27	0.18	1.24	0.19
2. 0.5 mgkg ⁻¹	Mean	65.40	20.66	13.36	4.08
	S.E	0.54	0.08	0.72	0.08
3. 1.0 mgkg ⁻¹	Mean	64.44	20.50	12.23	3.83
	S.E	0.28	0.09	1.27	0.06
4. 1.5 mgkg ⁻¹	Mean	64.57	20.48	14.61	3.83
	S.E	0.86	0.86	0.55	0.20
5. 2.0 mgkg ⁻¹	Mean	64.75	20.69	14.58	3.92
	S.E	0.88	0.22	1.15	0.08
6. 2.5 mgkg ⁻¹	Mean	65.03	20.48	13.78	3.85
	S.E	0.49	0.10	0.66	0.05

The proximate composition of the carcasses revealed no trends or significant differences between the dietary treatments with regards moisture, lipid or protein though a marginally higher ash content was recorded for those fish fed diets containing biotin at 0.5 mg kg⁻¹ or less. Consequently the values attained for net protein utilisation (NPU) and protein efficiency ratio (PER) reflected the growth data and thus the poorer NPU of the deficient fish was more likely the result of impaired growth and not modified carcass composition. The hepatosomatic index of the test animals demonstrated neither trend or significant differences between the dietary regimes.

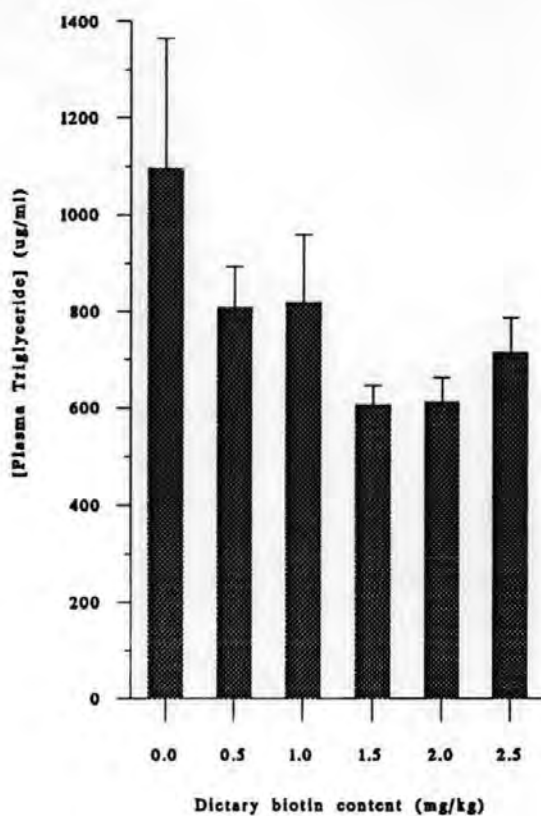
Table 4.2.5: Hepatosomatic index (HSI) of gilthead seabream fed diets of differing biotin content. (n = 5)

		Diet 1 0.0 mg kg ⁻¹	Diet 2 0.5 mg kg ⁻¹	Diet 3 1.0 mg kg ⁻¹	Diet 4 1.5 mg kg ⁻¹	Diet 5 2.0 mg kg ⁻¹	Diet 6 2.5 mg kg ⁻¹
HSI (%)	Mean	0.952	1.024	0.900	0.908	1.091	0.921
	(S.E)	0.087	0.062	0.070	0.055	0.072	0.029

The haematology of the fish at the end of the present experiment exhibited values which were in accordance with those recorded elsewhere in this volume. However, within the present experiment, no significant differences between the dietary regimes were apparent though a number of trends did become apparent. The fish fed diets without supplemental biotin exhibited the highest concentration of triglycerides in the plasma though the large range of values within this group meant that the fish maintained on this dietary regime were not significantly different from any of the other groups. The lowest plasma glucose concentration was recorded for those fish fed diets supplemented with biotin at 0.5 mg kg⁻¹ or less. The highest value was recorded in fish fed a biotin



(a)



(b)

(a) Figure 4.2.1.

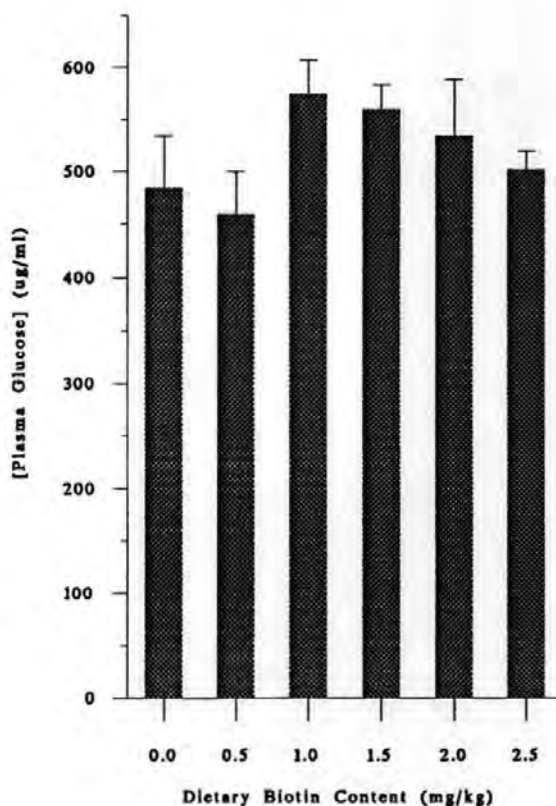
Growth of gilthead seabream fed diets of varied biotin content.

(b) Figure 4.2.2.

Plasma triglyceride concentration in gilthead seabream fed diets of varied biotin content.

(c) Figure 4.2.2.

Plasma glucose concentration in gilthead seabream fed diets of varied biotin content.



(c)

supplement of 1.0 mg kg^{-1} with declining plasma glucose concentrations observed with each incremental increase in biotin input thereafter. A similar pattern was observed with regards the plasma protein concentration though again the differences between the treatments were not significant. A very wide variation in the mean plasma lactate concentration was recorded which reflected the large within treatment variation. No trend or significant difference was apparent with regards this haematological parameter and due to the large variation within each dietary regime.

Table 4.2.5: Haematological characteristics of gilthead seabream fed diets supplemented with biotin at various levels ($n = 5$).

		Diet 1 (0.0)	Diet 2 (0.5)	Diet 3 (1.0)	Diet 4 (1.5)	Diet 5 (2.0)	Diet 6 (2.5)
[Plasma Protein] (mg ml^{-1})	Mean (S.E)	29.05 (2.85)	25.52 (1.88)	32.55 (0.66)	31.10 (1.54)	27.33 (3.28)	26.88 (0.96)
[Plasma Glucose] ($\mu\text{g ml}^{-1}$)	Mean (S.E)	484.08 (50.27)	459.14 (40.87)	574.47 (32.74)	559.50 (28.58)	533.60 (54.65)	501.63 (17.56)
[Plasma Triglyceride] ($\mu\text{g ml}^{-1}$)	Mean (S.E)	1096.50 (268.18)	808.75 (85.38)	819.69 (139.49)	606.56 (39.33)	611.82 (50.30)	713.79 (71.62)
[Plasma Lactate] ($\mu\text{g ml}^{-1}$)	Mean (S.E)	29.15 (12.23)	65.08 (20.58)	116.70 (35.90)	54.61 (9.82)	98.79 (41.71)	52.57 (16.03)

4.4. Discussion.

Supplementation of the semi-purified diet with biotin made a significant impact on the growth of the fish within the present experiment. Thus, those fish fed the un-supplemented diet were significantly smaller than those fed diets fortified with biotin at a level of 1.0 mg kg^{-1} . Biotin supplementation at 0.5 mg kg^{-1} resulted in a final mean weight which lay between that of the un-supplemented group and those fed the higher levels of supplemental biotin. By the use of Duncan's multiple range test, the mean

weight of these fish was shown not to be significantly different from that recorded for any of the remaining dietary treatments though the slightly poorer growth response did indicate that a biotin supplement of 0.5 mg kg^{-1} was insufficient for optimum growth.

In the study of Ogino *et al.* (1970b), carp (*Cyprinus carpio*) fed semi-purified diets without a biotin supplement grew adequately for the first 60 days of the growth trial but within 80 days the deficient carp were significantly smaller than those fed diets supplemented with biotin at 0.5 mg kg^{-1} . Similarly, in studies on the carp, Günther and Meyer-Burgdorff (1990) observed a reduced rate of growth for individuals maintained on a purified ration without supplemental biotin. The importance of biotin supplementation in diets for the chinook salmon (*Oncorhynchus tshawytscha*) was outlined in the study of Halver (1957) during which the salmon grew adequately for 70 days when fed biotin deficient diets but thereafter a sustained weight loss was recorded. Similarly, during the study of Poston and McCartney (1974), brook trout (*Salvelinus fontinalis*) demonstrated a reduced weight gain within 6 weeks of feeding biotin deficient diets followed within 14 weeks, by a period of sustained weight loss. These observations were later substantiated in a study by Poston (1976) in which biotin deficient lake trout (*Salvelinus namaycush*) grew significantly less well than vitamin replete individuals over a 20 week period. In a further study on the lake trout (Poston and Page 1982), growth retardation became significant after 14 weeks of feeding on biotin deficient purified diets. Suppression of growth and reduced feeding was observed by Woodward and Frigg (1989) during biotin deficiency in rainbow trout (*Oncorhynchus mykiss*) when fed to appetite and by Walton *et al.* (1984) in rainbow trout fed restricted rations, indeed maximal weight gain was achieved in the former study with biotin supplements of 0.08 mg kg^{-1} and above. The incorporation of avidin from egg white into practical diets and biotin supplemented

purified diets significantly suppressed the growth of rainbow trout in the study of Castledine *et al.* (1978). Furthermore, in that study un-supplemented practical diets (biotin contribution from ingredients 0.50 mg kg^{-1}) and purified diets containing a biotin supplement of 0.25 mg of biotin per kg of feed, appeared to be sufficient for optimal growth.

The values recorded for feed conversion ratio, conversion efficiency and net protein utilisation reflected the observations of the present experiment with regards growth. Thus nutrients were less efficiently utilised by those fish fed diets containing a biotin supplement of less than 1.0 mg kg^{-1} but above this level there was little difference between the dietary regimes. Reduced feed efficiency was recorded in biotin deficient brook trout and lake trout by Poston and McCartney (1974) and by Poston (1976) respectively. Poor feed efficiency was also observed in biotin deficient rainbow trout fed avidin treated diets by Castledine *et al.* (1978). Günther and Meyer-Burgdorff (1990) observed optimum feed conversion and protein utilisation in carp maintained on diets with a biotin supplement of 0.8 mg of biotin per kg of purified diet.

The present experiment was characterised by a very low rate of mortality and those mortalities which did occur were only observed among the fish fed 1.0 and 2.5 mg of biotin per kg of diet and hence, were considered to be independent of the level of supplemental vitamin. Additionally, there was an absence of externally apparent pathology and a small reduction in the feed intake of the fish fed the diets without supplemental biotin was the only behavioural change observed throughout the present experiment.

In common with the present study, a suppression of growth was recorded in rainbow trout, carp and red seabream (*Chrysophrys major*) fed semi-purified diets without

a biotin supplement by Kitamura *et al.* (1967), Ogino *et al.* (1970b), Günther and Meyer-Burgdorff (1990) and Yone and Fujii (1974) respectively. In addition, at the gross, level deficiency related pathology and mortality remained absent in all of these studies. However, at the histological level, Ogino *et al.* (1970b) observed atrophy of the of the acinous cells of the pancreas, glycogen deposition in the muscle and urinary epithelia with damage to the gills including shortened / fattened lamellae and hypertrophy of the respiratory epithelia. The rainbow trout in the study of Walton *et al.* (1984) also failed to exhibit externally apparent deficiency symptoms though the authors attributed this to the use of restricted ration size and the short duration (12 weeks) of the experiment. In common with the present experiment, biotin deficient chinook salmon demonstrated a loss of appetite (Halver 1957), however a range of more severe deficiency symptoms were also apparent which also included lesions of the colon, muscular atrophy and spastic convulsions. In the study of Poston and McCartney (1974) biotin deficient brook trout refused feed, became anorexic and developed pale livers. Additionally, in the study of Poston and McCartney (1974), the swimming stamina of biotin deficient brook trout was equal to that of the fish fed biotin adequate/fat deficient diets but significantly poorer than that of nutrient replete individuals and hence, the availability of lipid in the diet masked the effect of biotin deficiency with regards swimming stamina. In a further study, Poston (1976) again demonstrated a reduction in the swimming stamina of lake trout when fed diets containing dietary biotin at less than 1.0 mg kg^{-1} . The lake trout in the study of Poston and Page (1982) failed to demonstrate a mortality caused by biotin deficiency despite the onset of a number of deficiency symptoms at the gross and histological levels. The rainbow trout fed 0.05 mg kg^{-1} of semi-purified diet in the study of Woodward and Frigg (1989) also exhibited marked deficiency symptoms

which manifested themselves within 4 weeks by the onset of feed refusal and progressed to include lethargy, emaciation, light skin colouration and eventual mortality. In addition, rapid movement of the operculae was observed which was probably as a consequence of the shortening and thickening of the gill lamellae and epithelial hyperplasia. Castledine *et al.* (1978) described the gill pathology associated with biotin deficiency in rainbow trout. Thus, at the gross level, the gills appeared pale, protruded outside the operculum and the arches were covered in a mucinous coating. At the histological level, a shortening and thickening of the lamellae was described with hypertrophy of the respiratory epithelium. These observations were later substantiated in biotin deficient lake trout by Poston and Page (1982). Castledine *et al.* (1978) could not attribute this pathology to a single biochemical failure and hence postulated that a biotin - nutrient interaction was responsible.

A series of biotin dependent enzymes exist which are involved with the transfer of CO₂ in a variety of carboxylation, decarboxylation and transcarboxylation reactions. The latter groups of enzymes are mainly associated with the metabolism of microorganisms while the biotin dependent carboxylating enzymes, of which there are four, have been studied in higher vertebrates and some species of fish. These four biotin dependent carboxylases all play key roles in intermediary metabolism thus; acetyl CoA carboxylase is the first and rate-limiting step in fatty acid synthesis. Pyruvate carboxylase catalyses the carboxylation of pyruvate to oxaloacetate and permits the repletion of TCA cycle intermediates and propionyl CoA carboxylase catalyses the carboxylation of propionyl CoA to methylmalonyl CoA thus providing a pathway into the TCA cycle for some amino acids and odd-chain fatty acids. Finally, methylcrotonyl CoA carboxylase is involved in the generation of acetyl CoA and acetoacetate from leucine (Bender 1992).

Thus overall, the metabolic symptoms of biotin deficiency include impaired gluconeogenesis, with the accumulation of lactate, pyruvate and alanine and impaired lipogenesis with accumulation the of acetyl CoA. Lactate and pyruvate accumulation is believed to be induced by reduced efficiency of the enzyme pyruvate carboxylase and impaired propionyl CoA carboxylase activity (Bender 1992).

The study of Arinze and Mistry (1971) demonstrated that in rats and chicks, biotin deficiency reduced the activity of the biotin dependent enzymes acetyl CoA carboxylase, pyruvate carboxylase and propionyl carboxylase within an experimental period of 28 days and additionally, within six weeks the residual activities of these enzymes reflected the tissue levels of biotin. However, the activity of hepatic acetyl CoA carboxylase was reduced to a far smaller extent (approx 50 - 60 % residual activity) than that of the other enzymes which retained less than 20 % of the activity of the biotin replete individuals. Arinze and Mistry (1971) attributed the higher retention of acetyl CoA carboxylase activity in the liver when compared to adipose tissue to a slower degradation of this enzyme in the liver and the higher lipogenic activity of the adipose tissues. In the rainbow trout, Woodward and Frigg (1989) observed that the dietary level which furnished maximal activity of hepatic pyruvate carboxylase and acetyl CoA carboxylase was lower (0.05 mg kg^{-1}) than that required for maximal vitamin storage (0.08 mg kg^{-1}) however, in the muscle maximal enzyme activity was only attained at the much higher level of 0.14 mg kg^{-1} . Arinze and Mistry (1971) also demonstrated a decline in the rate of gluconeogenesis in biotin deficient rats as evidenced by lowered incorporation of radio-labelled alanine into the blood. This was attributed to reduced pyruvate carboxylase activity which was subsequently exacerbated by a lack of reducing equivalents in the cytosol. The activity of pyruvate carboxylase was shown by Whitehead and

Bannister (1980) to be a suitable indicator of biotin status in broilers and highlighted the stability of this vitamin to steam-pelleting and the microbial synthesis of biotin in the litter.

The interplay between dietary fat, lipogenesis and biotin supply was examined in chicks by Mason and Donaldson (1972). In brief, the authors concluded that during biotin deficiency the activity of acetyl CoA carboxylase was limited and hence lipogenesis was suppressed. However, the incorporation of fat in the diet suppressed the lipogenic pathways and hence dietary fat could mask the effects of biotin deficiency. The brook trout in the study of Poston and McCartney (1974) exhibited declining activities of the enzymes acetyl CoA carboxylase and pyruvate carboxylase when fed biotin deficient diets with or without dietary lipid. In the presence of a biotin supplement, those brook trout fed diets containing lipid demonstrated significant suppressions in the activities of these two enzymes while in the absence of biotin, brook trout fed lipid free diets only exhibited a decline in the activity of acetyl CoA carboxylase. Robinson and Lovell (1978) examined the essentiality of biotin for channel catfish fed lipid free and lipid supplemented diets. Thus, the activity of pyruvate carboxylase was suppressed during biotin deficiency in channel catfish fed lipid free diets while the inclusion of lipid and a biotin supplement in the diet failed to lower the activity of this enzyme. Walton *et al.* (1984) observed a reduction in the activity of the enzymes pyruvate carboxylase and acetyl CoA carboxylase during biotin deficiency in rainbow trout fed diets of varied lipid and carbohydrate content. In summary the authors proposed that trout fed diets containing carbohydrate did not rely on gluconeogenesis for the maintenance of blood glucose and hence a lowering of the activity of pyruvate carboxylase made little impact on the fish. However, those fish maintained with a dietary absence of starch did not deplete their reserves of

hepatic glycogen in response to perturbations in the gluconeogenic pathways. The authors attributed this factor to either the maintenance of slightly higher rates of pyruvate carboxylase activity in the fish fed the carbohydrate free diets or a utilisation of gluconeogenic precursors which did not require the pyruvate carboxylase step. Additionally, despite the lowered activity of acetyl CoA carboxylase and the low fat content of the diet, Walton *et al.* (1984) observed little change in the rate of lipogenesis in the muscle of biotin deficient rainbow trout though the authors did admit that muscle is not a key lipogenic tissue.

In the present experiment, the haematology of the fish revealed no significant differences between the treatments with regards any of the measured parameters, however several trends did become apparent. The plasma triglyceride concentration of the fish fed the un-supplemented diet was elevated above that of the remaining dietary regimes. However, the large variation within this group which, may itself be indicative of a marginal deficiency, meant that the plasma triglyceride concentration of this group was not significantly different from any of the remaining groups. In the study of Arinze and Mistry (1971) the levels of free fatty acids (FFA) in the plasma of biotin deficient rats fell markedly during the experimental period and thus within 5 weeks the levels of FFA in the plasma of the deficient rats was significantly lower than that of the biotin replete individuals. The free fatty acids may be utilised for the provision of energy and hence it is not unreasonable to postulate that due to the reduced activity of pyruvate carboxylase activity observed by Arinze and Mistry (1971) that free fatty acids assumed added importance in the provision of energy. In the present experiment, the diets contained a significant amount of available carbohydrate and hence the demand for glucose produced by gluconeogenesis was low and thus may account for the absence of

any significant differences in the triglyceride concentration in the plasma of the bream in the current study.

The plasma glucose concentration was lowest for those fish fed diets either deficient in biotin or supplemented with this vitamin at 0.5 mg kg^{-1} . The highest plasma glucose concentration was recorded for those fish fed supplemental biotin at 1.0 mg kg^{-1} and the glucose concentration fell with each incremental rise in biotin supplement thereafter. As stated above, given the high content of available carbohydrate in the diets fed during the present experiment, it is unlikely that gluconeogenesis played a significant role in the maintenance of plasma glucose levels. However, the lower levels of glucose in the plasma of the fish fed diets either devoid of biotin supplement or fortified at the lowest level with this vitamin would indicate a marginal perturbation in carbohydrate metabolism possibly as a consequence of reduced efficiency of the TCA intermediate repletion normally mediated via the enzyme pyruvate carboxylase. The pattern of plasma glucose concentration was also reflected in the plasma protein concentrations though the significance of this is as yet inexplicable.

In the present case, the lowest concentration of plasma lactate was recorded for those fish fed the diets devoid of supplemental biotin and the wide range of plasma lactate concentrations recorded for the fish fed diets supplemented with biotin at 1 and 2 mg kg^{-1} prevented the appearance of any discernable trend. Given that lactate accumulation is frequently associated with biotin deficiency, it was expected that the concentration of lactate in the plasma of the biotin deficient fish in the present experiment should have risen. Lactate accumulation was observed in the livers of biotin deficient rainbow trout when fed diets containing potato starch (Walton *et al.* 1984). The lack of any significant differences or trend with regards plasma lactate accumulation

within the present experiment means that no inference may be made from this haematological parameter.

Fragmentation of the erythrocytes was observed in biotin deficient chinook salmon (Halver 1957), an increased propensity towards haemolysis during osmotic shock was observed in biotin deficient rainbow trout (Castledine *et al.* 1978) and decreased haematocrits were observed in biotin deficient lake trout (Poston and Page 1982) and channel catfish (Robinson and Lovell, 1978). Additionally, during deficiency of this vitamin Ogino *et al.* (1970b) observed an increase in the proportion of immature erythrocytes when compared to that of vitamin replete individuals though the total count remained constant. The haematology of the biotin deficient rainbow trout in the study of Woodward and Frigg (1989) remained normal despite the onset of other pathological conditions during biotin deficiency.

The proximate composition of the fish revealed no significant differences between the dietary regimes, indeed, the average protein content of the carcasses was highly consistent between the groups. Hence, the differences recorded for net protein utilisation are more likely a factor of the poor growth of the deficient groups when compared to the vitamin replete individuals and the availability of lipid and carbohydrate within the diet which allowed a significant sparing of protein for deposition in the carcass regardless of biotin supplement. There were no apparent trends with regards the lipid or moisture content of the carcasses though a marginally greater ash content was observed for those fish fed the un-supplemented diets and those supplemented with biotin at 0.5 mg kg⁻¹.

In the study on brook trout by Poston and McCartney (1974), biotin supplementation was associated with significantly improved protein deposition when compared to that of biotin deficient individuals while in the study on lake trout by

Poston (1976), biotin supplements of 0.5 mg kg^{-1} or less resulted in significant lipid deposition in the carcass. A significant interplay was observed between biotin supplement and carcass composition by Günther and Meyer-Burgdorff (1990). Thus, carp fed diets with a biotin supplement less than 0.4 mg kg^{-1} , exhibited a high moisture, low lipid and high protein content though above this level no further significant differences were apparent.

The hepatosomatic index of the fish fed the differing levels of biotin supplement within the present experiment demonstrated no significant differences or trends. A marked accumulation of glycogen was observed in the livers of biotin deficient rainbow trout and lake trout by Castledine *et al.* (1978) and Poston (1976) respectively, which the former attributed to a lowered rate of glycolysis resulting from an accumulation of lactate in response to the lowered activity of pyruvate carboxylase. An abnormal infiltration of fat was observed in the hepatocytes of biotin deficient lake trout by Poston and Page (1982) though the livers of both the control and deficient fish exhibited a high glycogen content. Günther and Meyer-Burgdorff (1990) recorded a trend towards lowered hepatic lipid content in carp fed diets containing less than 0.8 mg of biotin per kg of diet. Subsequently, Günther and Meyer-Burgdorff (1990) determined the carbohydrate content of the livers as the residual in their proximate analysis and demonstrated a trend towards glycogen accumulation with decreasing biotin supplement. The lipid content in the livers of biotin deficient channel catfish was un-effected in the study of Robinson and Lovell (1978). Additionally, the composition of the livers of rainbow trout were unaffected by differing biotin supplements in the study of Walton *et al.* (1984), indeed in that case the glycogen content was not altered even when the proportion of starch in the diet was elevated.

Based on the growth, feed conversion and net protein utilisation data, the present experiment demonstrates that the requirement of the gilthead seabream for biotin lies between 0.5 and 1.0 mg kg⁻¹ of diet. This range is proposed since the fish fed diets supplemented with 0.5 mg of biotin kg⁻¹ of diet were not significantly larger than those fed diets devoid of supplemental biotin though not significantly smaller than those supplemented at the higher levels. Despite the amount of research which has been directed towards biotin, few authors have defined a minimum requirement for many species. The requirement derived for the gilthead seabream by the present experiment compares favourably with those of other species. The observations agree closely with those of Kitamura *et al.* (1967), who on the basis of maximal hepatopancreatic biotin storage, defined the biotin requirement of carp as 1 mg of biotin kg⁻¹ of diet. The same minimum value was proposed for carp by Günther and Meyer-Burgdorff (1990) based on growth and feeding performance though the higher range of 2.0 to 2.5 mg kg⁻¹ was described as suitable for the maintenance of optimum liver and plasma levels. Woodward and Frigg (1989) observed maximal liver storage of biotin at the much lower level of 0.08 mg of biotin kg⁻¹ of diet in the rainbow trout which additionally furnished maximal weight gain, maximal activity of the enzymes pyruvate carboxylase and acetyl Co-A carboxylase in the liver and prevented mortality. This value is lower than the 0.25 mg kg⁻¹ demonstrated by Castledine *et al.* (1978) to be required by the same species.

Several experiments have questioned the value of the supplementation of practical diets with biotin due to the biotin content of the feed materials themselves. Thus, using a practical diet, Castledine *et al.* (1978) could only induce biotin deficiency by the application of avidin to the diet and additionally, having used a purified diet to determine the biotin requirement of the rainbow trout (0.25 mg kg⁻¹), concluded that

the minimum biotin requirement of this species could indeed be met by the practical diet with its biotin content of 0.5 mg kg^{-1} . In the study of Lovell and Buston (1984) an un-supplemented practical diet with a biotin content of $0.3 - 0.4 \text{ mg kg}^{-1}$ appeared to provide sufficient vitamin for normal growth and maximal biotin dependent enzyme activity in the channel catfish. Hence, in the absence of a significant biotin content in the faeces, the authors declared that biotin synthesis by the gut microflora was minimal but that the un-supplemented feed ingredients represented an adequate source of vitamin. The observations of Woodward and Frigg (1989) defined the requirement of rainbow trout for optimal biotin dependent enzyme activity as $0.14 \text{ mg of biotin kg}^{-1}$ of diet and this value was approximately double the minimum requirement for optimal growth. The authors thus questioned the scale of the recommended supplement which at the time of publication was 1.0 mg kg^{-1} and proposed the much lower value of 0.15 mg kg^{-1} which should provide a sufficient safety margin given the known variation in the available biotin content of feed materials (Frigg 1976, 1984). However, the present data tend to reinforce the arguments for the use of a biotin supplement in diets for the gilthead seabream since the requirement of this species appears to lie between 0.5 and 1.0 mg kg^{-1} which is in excess of the values recorded elsewhere for the biotin content of an un-supplemented practical diets.

The present experiment defined the requirement of the gilthead seabream for biotin under a single set of conditions of major nutrient supply. The availability and cost of feed materials are subject to wide fluctuations and hence diet formulations may vary widely in their composition. It has been clearly demonstrated that vitamin requirements of animals may vary in response to the macro nutrients supplied in the diet. (Aoe *et al.*, 1967c, 1969, Hardy *et al.* 1979, Fisher *et al.* 1984, Bai *et al.* 1991) and thus a vitamin such

as biotin, with its key role in intermediary and fatty acid metabolism, should be the focus of attention with regards aspects of lipid supply. As outlined above, many authors have focused on the relationship between quantitative aspects of lipid supply and biotin requirement but to date none have examined the relationship between this vitamin and more qualitative aspects of lipid supplementation in feeds.

The requirement of the gilthead seabream for the fatty acids 20:5 ω 3 and 22:6 ω 3 was demonstrated by Koven and Kissil (1984) in an experiment in which capelin oil (rich in these two fatty acids) was either partly or wholly replaced by corn oil (rich in 18:2 ω 6/linoleic acid). However, the authors postulated that the essentiality of these fatty acids be investigated by the use of suitable precursors *eg* 18:3 ω 3 (linolenic acid) and therefore examine the ability of the fish to de-saturate and elongate such precursor molecules.

The role of biotin in the elongation and de-saturation of fatty acids is reviewed by Greene and Selivonchick (1987) but in short, a reduced capacity for the manufacture of the elongation products becomes apparent in biotin deficient rat hepatocytes (Puddu *et al.* 1967), chicks (Watkins and Kratzer 1987) and fish (Castledine *et al.* 1978).

Given the role of biotin in the decarboxylation reactions of fatty acid metabolism, it would appear logical that the supply of a diet low in long chain, highly unsaturated fatty acids but rich in suitable precursors for example 18:3 ω 3 (linolenic acid) would place a greater emphasis on lipogenesis and therefore elevate the biotin requirement of the fish in order to maintain maximal activity of the lipogenic enzymes. Hence, a suitable experiment to investigate this relationship, would involve the partial substitution of the cod liver oil used in the present experiment with linseed oil which is rich in linolenic acid but would provide only a poor supply of the longer, less saturated fatty acids.

Chapter 5

Applied Studies

5.1. Pyridoxine Super Supplementation in Practical Diets for the Gilthead Seabream (*Sparus aurata* L.)

5.1.1. Introduction.

The term vitamin B₆ (pyridoxine) refers to a series of vitamers which include pyridoxine, pyridoxamine and pyridoxal, the latter of which is the active form within metabolism and the vitamer to which the other forms are transformed. Pyridoxine hydrochloride represents a stable form of these vitamers being readily soluble in water and thermo-stable in acid or alkaline solution hence, pyridoxine hydrochloride is commonly used as an animal feed supplement.

Within nutritional biochemistry the function of pyridoxine is very diverse due to the participation of this vitamin as a cofactor in the metabolism of proteins, fats and carbohydrates. The role most commonly associated with B₆ is in transamination as coenzyme co-decarboxylase which acts as a cofactor for the decarboxylation of amino acids. Pyridoxine is essential in the metabolism of glutamic acid, lysine, methionine, histidine, cysteine and lysine. Central to intermediary metabolism, is the role of pyridoxal in the phosphorylation of desulphydrase an enzyme catalysing the conversion of cysteine to lactic acid, additionally B₆ is associated with the metabolic pathways of gluconeogenesis. The role of pyridoxal-5-phosphate as a cofactor for glycogen phosphorylase has been recently evaluated (Palm *et al.* 1990), while the metabolism of essential fatty acids and mRNA synthesis are both believed to require some input from this vitamin.

Pyridoxine also acts as a cofactor in the synthesis of several neuro hormones *eg.* the synthesis of serotonin via the decarboxylation of 5-hydroxy tryptophan, and it is also believed that this vitamin plays a role in steroid hormone receptor recycling.

The effects of pyridoxine deficiency in the gilthead seabream were discussed in chapter 3 and have been extensively studied in other fish species. For salmonids, common gross deficiency symptoms include; anorexia, erratic swimming and ataxia (Halver 1989). In studies on rainbow trout (*Oncorhynchus mykiss*) by Hardy *et al.* (1987) and Smith *et al.* (1974) the activities of the pyridoxine dependent transaminases were evaluated. In the former study, aspartate aminotransferase activity was shown to be thirty percent lower for deficient individuals by comparison with a control group. Additionally, the potential for elevation of transaminase activity (by the addition of exogenous pyridoxal-5-phosphate to the enzyme assay) was greater among the deficient fish. Smith *et al.* (1974) observed lowered muscle and erythrocyte glutamate pyruvate transaminase (GPT) activity in B₆ deficient individuals, however an elevation of activity of this enzyme was observed in the livers of pyridoxine deficient and starved individuals. Jürss (1978) examined the possibility of using aspartate and alanine aminotransferases as indicators of B₆ status in rainbow trout. Aminotransferase activity in the white muscle appeared to be a more sensitive indicator of deficiency since the activity of these enzymes fell more quickly than that observed in the liver.

Andrews and Murai (1979), in a study on the channel catfish (*Ictalurus punctatus*), observed anorexia, tetany, abnormal body colouration and nervous disorder in deficient fish. In contradiction of the majority of the literature, anaemia was not observed in cases of deficiency, instead those fish fed B₆ at more than 20 mg kg⁻¹ diet developed microcytic normochromic anaemia. The gross deficiencies associated with pyridoxine deficiency for

the snakehead (*Channa punctatus*), were elucidated by Agrawal and Mahajan (1983a). These include; anorexia, poor growth, ataxia, hyper irritability, muscular spasms, scale loss with associated fluid loss, nodule formation in the kidney and opacity of the lens leading to blindness. Additionally, in a second study (Agrawal and Mahajan 1983b), the haematology of B₆ deficient snakeheads was studied. Deficiency symptoms included a hypochromic microcytic anaemia and leucopenia. With respect to the erythrocyte population it was observed that the proportion of mature cells fell with respect to the proportion of immature cells.

The pyridoxine requirement of the turbot (*Scophthalmus maximus*) was determined by Adron *et al.* (1978). In terms of gross deficiency, those individuals on an inadequate diet showed smaller weight gains. With respect to liver and muscle aspartate aminotransferase and liver alanine aminotransferase, the activity of these enzymes was proportional to dietary B₆ content up to 2.5 mg kg⁻¹ diet. Again the potential for elevation of aminotransferase activity was greater in deficient individuals.

Wanakowat *et al.* (1989) determined the requirement of the seabass (*Lates calcarifer*) for pyridoxine. Once again the common symptoms of deficiency were observed including poor survival, feed conversion and growth, neuropathy and behavioural changes. Where the deficiency was marginal, lower lymphocyte counts were recorded.

An extensive study was carried out by Kissil *et al* (1981) in order to evaluate the pyridoxine requirement of the gilthead seabream (*Sparus aurata*). Deficiency resulted in poor growth and feed conversion, high mortality, irritability, erratic swimming and degeneration of the peripheral nerves. The alanine aminotransferase activity again showed a greater potential for pyridoxal-5-phosphate mediated elevation where the dietary pyridoxine supplement failed to meet the requirement of the fish.

The typical B₆ requirement range for salmonids lies between 10 and 15 mg kg⁻¹ diet (Halver 1982). Hardy *et al.* (1979) in investigations on pyridoxine requirement and dietary protein level, demonstrated a B₆ requirement of over 10 mg kg⁻¹ for chinook salmon (*Oncorhynchus tshawytscha*) for the conference of lowered disease susceptibility. Additionally, Hardy *et.al.* (1987) demonstrated that B₆ below 2.8 mg kg⁻¹ diet was insufficient for the maintenance of adequate transaminase activity in rainbow trout. Ogino (1966) evaluated the pyridoxine requirement of common carp (*Cyprinus carpio*) at 5.4 mg kg⁻¹. This was further substantiated by Vel *et al.* (1990) who observed impairment of the normal haematology of carp at dietary pyridoxine levels below 5 mg kg⁻¹ diet. Adequate growth and maximal alanine aminotransferase activity were observed in turbot (*Scophthalmus maximus*) fed 2.5 mg of B₆ per kg of diet by Adron *et al.* (1978). Andrews and Murai (1979) observed a dichotomy in the pyridoxine requirement of the channel catfish (*Ictalurus punctatus*) thus 2.2 mg of B₆ per kg of diet proved sufficient for normal health while improved growth was seen in response to dietary levels of 3 mg kg⁻¹. Wanakowat *et al.* (1989) in studies on the Asian seabass (*Lates calcarifer*) determined that levels of 10 mg of pyridoxine per kg of semi-purified diet furnished the fish with normal growth, good feed conversion and a normal lymphocyte population. The dietary pyridoxine requirement of the gilthead seabream (*Sparus aurata*) has been determined by Kissil *et al.* (1981). Thus, on the basis of normal growth and optimum activity of alanine amino transferase this value was found to be 1.97 mg of B₆ per kg of diet.

Given the role of pyridoxine as a cofactor in the transamination of amino acids and the results of the study by Hardy *et al.* (1979) which demonstrated that elevation of dietary protein resulted in an increased pyridoxine requirement for chinook salmon, it would appear that increased supplementation of this vitamin may improve feed efficiency

(with respect to protein utilisation) with possible improvement in product / carcass quality.

As a consequence of the involvement of B₆ in hormone metabolism and activity, this vitamin has recently been applied in doses which exceed the minimum recommended dose for the treatment of carpal tunnel syndrome, homocystinuria, galactorrhea and premenstrual syndrome in humans (Bender 1992). Aquaculture is an environment in which fish are influenced by a multitude of stressors and the development of dietary treatments to minimise the effects of stressors would be beneficial. Due to its role in steroid hormone regulation, elevation of dietary pyridoxine therefore represents a potential means of stress mediation.

The aim of the present study was to evaluate the effect of four supplementary levels of pyridoxine supplied as part of a practical diet formulation in order to assess the effect of the fortification of diets with levels of pyridoxine which fall short of, matched and exceeded the minimum dietary requirement of the species. Measurement of suitability of the dietary levels was in terms of growth, feed utilisation, general health and response to an acute stress.

5.1.2. Materials and Methods.

Duplicate batches of thirty seabream fingerlings (initial weight 39.13 g \pm 0.33 g SE) were stocked into each of six 400 l, self cleaning fibreglass tanks over a 1200 l bio-filter within a closed recirculation system. A parallel flow of sea water was maintained through the tanks at 10.8 l min⁻¹. Additionally 20 fry (initial weight 2.80 g \pm 0.03 g SE) were stocked in duplicate into each of eight 15 l round, polyethylene tanks over a 60 l bio-filter within a second closed recirculation system providing a parallel flow of seawater

to the tanks at 2.3 l min^{-1} . Water temperature and salinity were maintained at 24°C and 33-36 ‰ saline respectively, the former by the use of immersion heaters with an integrated dip-cooler provided for the larger system, the latter by the use of a fresh water inlet balanced to compensate for evaporative losses. The pH was maintained between 6 and 7.5 by the use of magnesium and calcium carbonate buffering. Weekly water changes amounted to 5% of the system volume. Daylight balanced fluorescent discharge lamps maintained a 12 hr light / dark photoperiod.

Using a feed formulation package, a test diet was designed with fishmeal and cod liver oil providing approximately 48% crude protein and 13.4% lipid. Based on this formulation (presented in table 5.1.1). Four test diets were designed differing with respect to the level of pyridoxine supplementation and manufactured as outlined in chapter 2. Diets 1, 2, 3 and 4 were supplemented with 0.5, 5.0, 50.0 and 100.0 mg B_6 per kg of diet. Immediately after manufacture samples were withdrawn for proximate analysis while additional samples were assayed for B_6 content courtesy of F. Hoffmann La Roche AG (Basel, Switzerland). The pyridoxine content of the diets is shown in table 5.1.2 while the proximate composition is displayed in table 5.1.3. All four diets were fed to the fry while diets 1, 2 and 4 only were fed to the fingerlings.

The test diets were supplied to the fish three times daily (09:00, 13:00 and 16:00 hrs). For the larger fish the diet was supplied at 2.0% of the body weight per day for the first 16 weeks followed by a ration of 1.8% of the live mass per day for the remaining 10 weeks. The fry were fed at 5% of the live weight per day for the first 8 weeks, at 4.5% during weeks 9 to 12 inclusive, and the ration was supplied *ad libitum* up to 3.5% of the live mass for the remaining 6 weeks of the study. The fish were individually weighed at bi-monthly intervals in order to follow growth parameters and to determine

Table 5.1.1: Composition of the test diets used to investigate the pyridoxine nutrition of the gilthead seabream

Component	% Inclusion
Fish Meal (Provimi 66)	71.20
Cod Liver Oil	6.30
Wheat Feed	18.80
Molasses	0.50
Minerals ¹	1.50
Fat Soluble Vitamins ²	0.20
B Complex Vitamins (B ₆ Free) ³	0.42
Pyridoxine in α -cellulose ^a	0.08
Macro Vitamins ⁴	1.00

¹ Mineral pre-mix (mg kg⁻¹ diet):

CaHPO₄, 2756.3; CaCO₃, 375.0; NaCl, 1875.0; K₂SO₃, 2500.0; MgSO₄·7H₂O, 2578.4; FeSO₄·7H₂O, 87.5; MnSO₄·4H₂O, 28.4; ZnSO₄, 64.5; CuSO₄·5H₂O, 6.4; CoCl₂·6H₂O, 3.3; KI, 1.9; Na₂SeO₃, 0.3; α -cellulose, 4723.0

² Fat-soluble vitamin pre-mix (mg kg⁻¹ diet):

Vit A Palmitate, 4.2; Vit D₃, 0.046; Vit E Acetate, 439.8; Menadione Sodium Bi-Sulphite, 78.0; Maize Gluten Meal, 1,477.95

³ Pyridoxine-free B Vitamin pre-mix (mg kg⁻¹ diet):

Biotin 2%, 300.0; Folic Acid 88.8%, 16.9; Niacin, 48.6; Calcium Pantothenate, 305.3; Riboflavin 96%, 208.3; Thiamine Hydrochloride, 69.9; Cyanocobalamin 95%, 0.01; α -cellulose, 3250.99

⁴ Macro vitamin pre-mix (mg kg⁻¹ diet):

PolyPhosphorylated Ascorbic Acid, 2000; Choline Chloride, 5000; Inositol, 2000; Maize Gluten Meal, 1000

^a Pyridoxine in α -cellulose.

Pyridoxine hydrochloride supplied at 0.5, 5.0, 50.0, and 100.0 mg kg⁻¹ of diet using α -cellulose as a filler to comprise a total of 0.08 % of the complete formulation.

Table 5.1.2: The pyridoxine content of the test diets, declared and actual values after manufacture.

Diet	Declared Pyridoxine (mgkg ⁻¹ Diet)	B ₆ in diet (Assay) (mgkg ⁻¹ Diet)
1	0.5	1.75
2	5.0	5.40
3	50.0	51.50
4	100.0	103.00

Table 5.1.3: Proximate composition of the test diet. Moisture is expressed as a percentage of the diet as fed while protein, lipid and ash are expressed as a percentage of the dry matter. Carbohydrate and fibre calculated by difference.

Carbohydrate and Fibre(%)	Moisture (%)	Crude Protein (%)	Lipid (%)	Ash (%)
13.31	7.01	48.30	13.43	17.96

appropriate ration size. The production sized fish were starved in the 24 hours prior to weighing.

At the end of both growth studies, the individual weights of all fish were recorded and these weights were subjected to an analysis of variance, where $p < 0.05$ was judged to be indicative of a significant difference. Duncan's multiple range test (Duncan 1955) was then used to characterise any significant differences between the treatments. Additionally, specific growth rate (SGR), feed conversion efficiency (FCE) and feed conversion ratio (FCR) were calculated based upon the recorded feed input over the trial period.

After 26 weeks of feeding for the fingerlings, five individuals from each tank were selected for carcass analysis. Each carcass was individually analysed for protein, lipid, ash and moisture content as outlined in chapter 2. Crude protein was determined by the Kjeldahl ($N \times 6.25$) method and lipid by a method derived from that of Barnes and Blackstock (1973). Moisture and ash were assayed according to the methods outlined in the A.O.A.C. Handbook (1990). These data were subsequently subjected to an analysis of variance with respect to proximate composition where the number of fish considered under each dietary regime was 10. Subsequently, Duncan's multiple range test (Duncan

1955) was applied where significant differences were observed between the treatments. Due to the small size of the fry carcasses, four individuals were pooled for proximate analysis from each tank and hence the number replicates in this case was two since each treatment was duplicated. Based on the growth data, nutrient analysis of the test diet and the proximate composition of fish sub-sampled at the beginning of the trial the net protein utilisation (NPU) and protein efficiency ratio (PER) were calculated.

At the end of the growth study involving the production sized fish, five individuals were selected from each tank and blood was withdrawn into heparinised syringes from the caudal vein. A second group of five fish from each tank was subjected to a short term, acute stress by catching each fish five times followed by a thirty minute recovery period. At the end of this time the fish were again recaptured and de-sanguinated from the caudal vein. The packed cell volume of the blood was determined by micro haematocrit while the haemoglobin content was determined using a cyanmethemoglobin method (Sigma Total Haemoglobin Test, Sigma No. 525 A). Following centrifugation the plasma was decanted into micro centrifuge tubes and stored at -70°C until required. Diagnostic kits were subsequently used to determine plasma protein by the biuret method (Total Protein Reagent, Sigma No. 541-2) and plasma glucose by the glucose oxidase method (Glucose Test Kit, Sigma No. 510 A). For all treatments, including stressed and un-stressed groups, haematological indices were subjected to analysis of variance with the number of individuals sampled from each treatment equal to ten for both stressed and un-stressed fish. Where significant differences were apparent, Duncan's multiple range test was applied in order to characterise and quantify the differences between treatments. At this point the weight of the liver with respect to complete carcass mass for five fish in each tank was recorded and the hepatosomatic index determined.

In addition an investigation of the effect of pyridoxine supplementation on the activity of alanine amino transferase was carried out according to the method outlined in Brooksbank (1993).

5.1.3. Results

In terms of growth, no significant differences were observed between the treatments *i.e.* the level of dietary pyridoxine supplementation caused no change in either weight gain or the rate of weight gain for the fry or production sized fish, though the rate of weight gain for the fry was quicker. The diet performance characteristics, in terms of the FCR, FCE, PER and NPU with respect to both size classes, were not significantly altered by the extent of pyridoxine supplementation in the formulation.

Table 5.1.4: Growth and dietary performance criteria for production size seabream fed diets containing varied pyridoxine supplement over 26 weeks.

	Diet 1 0.5 mg kg ⁻¹	Diet 2 5.0 mg kg ⁻¹	Diet 3 100 mg kg ⁻¹
Initial mean weight (g)	39.13	39.11	39.15
Final mean weight (g)	252.22	250.29	248.07
Weight gain (%)	544.34	540.05	533.71
SGR (%d ⁻¹)	1.02	1.02	1.02
Daily feed intake (gd ⁻¹)	1.91	1.91	1.86
Live weight gain (gd ⁻¹)	1.17	1.16	1.15
FCR	1.63	1.65	1.62
FCE (%)	61.35	60.87	61.71
PER	1.27	1.26	1.28
NPU (%)	23.69	23.40	24.00

Table 5.1.5: Growth and dietary performance criteria for seabream fry fed diets containing varied pyridoxine supplement over 18 weeks.

	Diet 1 0.5 mg kg ⁻¹	Diet 2 5.0 mg kg ⁻¹	Diet 3 50 mg kg ⁻¹	Diet 4 100 mg kg ⁻¹
Initial mean weight (g)	2.80	2.80	2.80	2.81
Final mean weight (g)	49.45	49.45	41.16	49.91
Weight gain (%)	1666.07	1669.64	1369.82	1676.04
SGR (%d ⁻¹)	2.30	2.28	2.13	2.29
Daily feed intake (gd ⁻¹)	0.487	0.470	0.423	0.490
Live weight gain (gd ⁻¹)	0.370	0.370	0.311	0.374
FCR	1.32	1.27	1.39	1.31
FCE (%)	76.02	78.77	71.97	76.29
PER	1.57	1.63	1.49	1.58
NPU (%)	26.93	26.74	25.46	26.97

With respect to all components, the proximate composition of the fry carcasses showed no significant differences between treatments. An increase in carcass lipid proportional to dietary B₆ dose was observed in the fry but, due to the small sample sizes available, insufficient replicates were available to allow statistical analysis of this data. However, with respect to the production sized fish, a significant difference was observed with those fish receiving 100 mg B₆ kg⁻¹ diet demonstrating a moisture content significantly higher than that of the 0.5 mg kg⁻¹ group (P = 0.049).

Table 5.1.6: Proximate composition of production size carcasses (n = 10) expressed as a percentage of the live mass. Values without or carrying similar superscripts within each column are not significantly different from each other (p < 0.05). The initial carcasses have been excluded from the ANOVA.

Dietary B ₆ (mgkg ⁻¹)		Moisture (%)	Crude Protein (%)	Lipid (%)	Ash (%)
0.5	Average	61.50 ^a	18.32	17.60	3.52
	SE	1.00	0.25	0.86	0.06
5.0	Average	63.20 ^{ab}	18.24	15.05	3.54
	SE	0.60	0.29	0.76	0.10
100.0	Average	64.11 ^b	18.44	17.55	3.64
	SE	0.53	0.21	0.94	0.07
Initial Fish	Average	69.80	16.47	10.48	4.18
	SE	0.87	0.11	0.78	0.16

Table 5.1.7: Proximate composition of n = 2 pooled fry expressed as a percentage of the live mass. Values without or carrying similar superscripts within each column are not significantly different from each other. The carcass composition of the initial fish has been excluded from the ANOVA.

Dietary B ₆ (mgKg ⁻¹ Diet)		Moisture (%)	Crude Protein (%)	Lipid (%)	Ash (%)
0.5	Average	73.84	17.04	6.55	3.60
	s.d.	1.19	0.02	0.43	0.21
5.0	Average	71.93	17.35	6.95	3.45
	s.d.	0.46	0.20	1.41	0.14
50.0	Average	72.71	17.00	7.14	3.25
	s.d.	0.12	0.54	1.13	0.16
100.0	Average	71.73	17.01	7.52	3.46
	s.d.	0.87	0.21	0.95	0.13
Initial Fish	Average	72.84	15.90	8.32	3.73
	s.d.	0.54	0.16	0.51	0.10

The hepatosomatic indices of all groups showed no significant differences between the dietary regimes.

Table 5.1.8: Hepatosomatic indices of the production size fish after 26 weeks of test diet feeding.

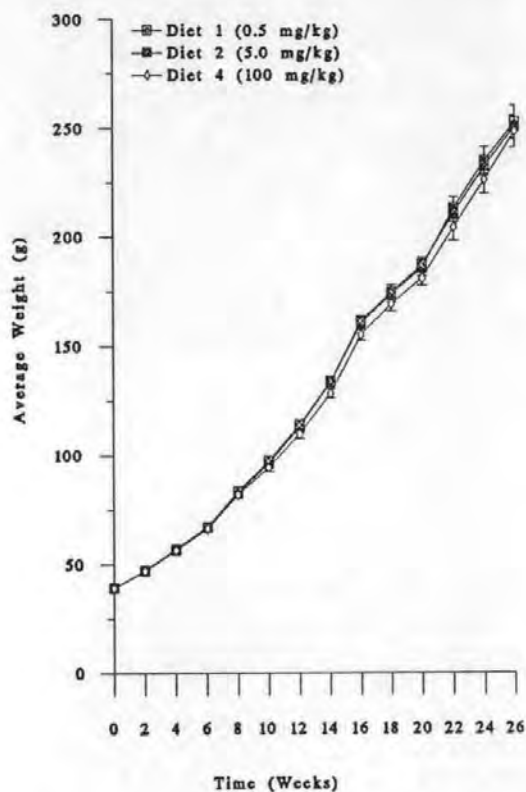
	Diet 1 0.5 mg kg ⁻¹	Diet 2 5.0 mg kg ⁻¹	Diet 4 100.0 mg kg ⁻¹
Average	1.01	1.10	1.04
n	10	10	10
SE	0.09	0.07	0.08

The haematocrit of the un-stressed fish showed no significant differences between treatments, however, after being subjected to an acute, short term stress those fish fed the diet supplemented with the lowest level of B₆ exhibited a significant elevation of the packed cell volume ($P < 0.001$). The haemoglobin concentration exhibited in the blood of all fish showed no significant differences either between dietary regimes or under stressed / un-stressed conditions. Due to the small sample volumes obtained from the stressed fish, the plasma protein assay could not be carried out, however, in the absence of a stressor, the plasma protein concentration showed no significant variation between treatments. The plasma glucose concentration of the test fish showed a series of significant differences ($P < 0.001$) which were highlighted using Duncan's multiple range test as shown below. Prior to stress, under all three dietary regimes, the plasma glucose concentration of the fish was not significantly different. Where the fish had been fed diets fortified at either the 5.0 or the 100.0 mg kg⁻¹ level, the plasma glucose concentration observed in the stressed fish was significantly higher than that of the

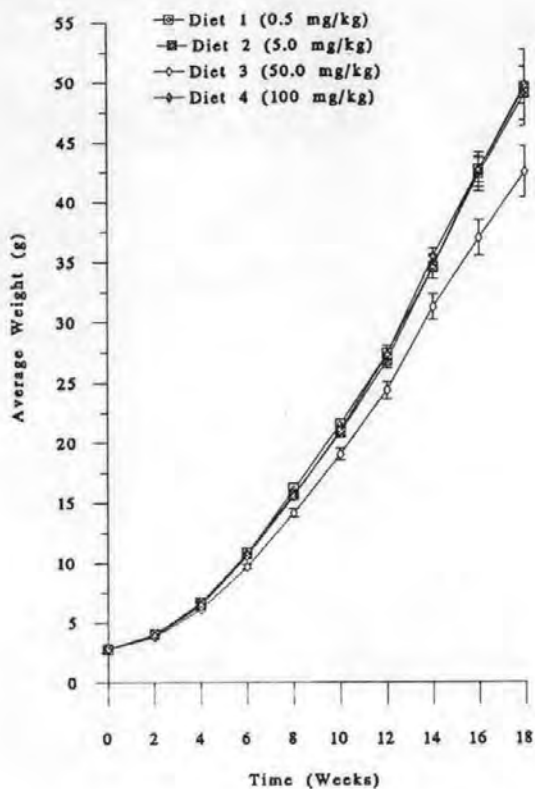
Table 5.1.9: Haematological parameters. Values in parentheses represent those recorded for stressed fish. Values within parameter columns bearing similar superscripts are not significantly different from each other ($p < 0.01$)

Dietary B ₆		Haematocrit	[Haemoglobin]	[Plasma Glucose]	[Plasma Protein]
(mg kg ⁻¹)		(%)	(mg cm ⁻³)	(mgcm ⁻³)	(mgcm ⁻³)
0.5	Average	35.54^a (52.60)^b	75.0 (77.0)	0.658^{ab} (0.911)^{bc}	30.09
	SE	3.42 (2.03)	5.00 (8.32)	0.019 (0.115)	1.55
	n	7 (7)	10 (10)	10 (7)	10
5.0	Average	38.45^a (40.43)^a	74.4 (71.1)	0.527^a (1.034)^c	25.21
	SE	2.50 (2.58)	3.37 (7.53)	0.049 (0.191)	3.45
	n	7 (7)	9 (9)	10 (7)	10
100.0	Average	34.40^a (41.06)^a	68.0 (56.0)	0.529^a (1.534)^d	30.01
	SE	2.07 (4.61)	5.72 (6.80)	0.035 (0.163)	2.36
	n	9 (6)	10 (5)	9 (8)	10

control individuals. The post stress plasma glucose concentration of the fish fed at the lowest dietary B₆ level was not significantly higher than that of the respective control group. Those fish fed the diets fortified with pyridoxine at the 5.0 and 100.0 mg kg⁻¹ exhibited a plasma glucose concentration which was significantly higher than that of the respective control individuals under both regimes. With respect to dietary supplement level, the post stress glucose concentrations were significantly different to each other, *i.e.*,



(a)



(b)

(a) Figure 5.1.1.

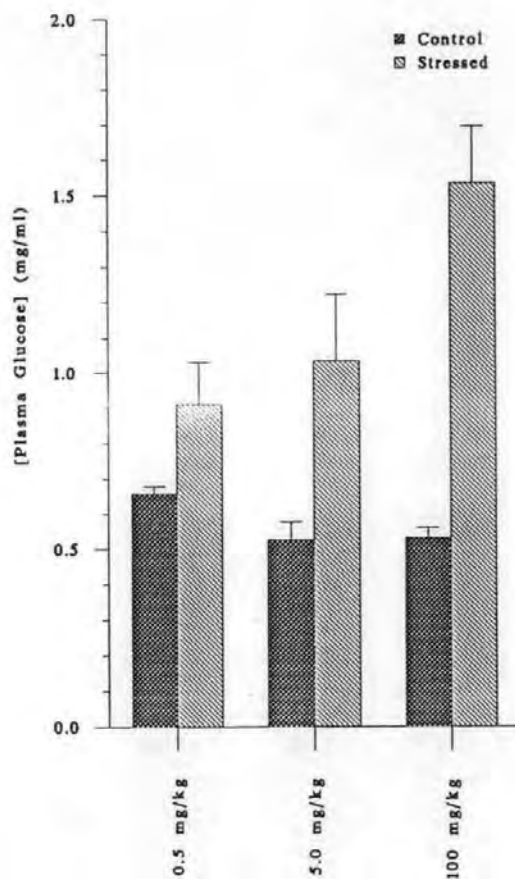
Growth of production size seabream fed diets of varied pyridoxine content.

(b) Figure 5.1.2.

Growth of gilthead seabream fry fed diets of varied pyridoxine content.

(c) Figure 5.1.3.

Plasma glucose concentration in control and stressed production size seabream fed diets of varied pyridoxine content.



(c)

the 0.5 and 5.0 mg kg⁻¹ supplemented groups were not significantly different from each other but the 100 mg kg⁻¹ was significantly higher than both .

Table 5.1.10: Percentage increase in alanine aminotransferase activity after pre-incubation with pyridoxal-5-phosphate for fry production sized groups. (Brooksbank 1993)

Production sized Fish				
Amount of B ₆ in Diet (mg kg ⁻¹)	0.5	5.0	50.0	100.0
Sample Size	4	4	-	4
Mean	44.24	32.99	-	31.58
Standard Deviation	10.11	8.50	-	18.97
Fry				
Amount of B ₆ in Diet (mg kg ⁻¹)	0.5	5.0	50.0	100.0
Sample Size	5	6	4	6
Mean	48.46	54.26	37.40	45.96
Standard Deviation	14.74	10.86	8.24	11.54

Analysis of the data recorded by Brooksbank (1993) shows that for the production sized fish, a pyridoxal-5-phosphate (PLP) mediated increase in glutamate pyruvate transferase (GPT) activity was attained at all dietary levels of pyridoxine. The increase in activity was greatest for those fish which had been fed B₆ at the lowest level (0.5 mg kg⁻¹) while diets 2 and 4 demonstrated changes in activity which were not similar to each other but lower than that of diet 1. Overall however, these differences were shown to be not significantly different from each other ($p > 0.05$). For the fry, again pre-incubation

with PLP resulted in an elevation in GPT activity above the normal level for all treatments. In this case the degree of stimulation appeared to be independent of dietary vitamin B₆ level and no treatment was significantly different from another ($p > 0.05$). The summary data are presented in table 5.1.10.

5.1.4 Discussion.

The studies performed on both the fry and the production sized fish yielded no significant differences with respect to either growth or feed utilisation. The minimum pyridoxine requirement of gilthead seabream, *ie.* that which supplied sufficient vitamin for growth and the prevention of mortality, was determined by Kissil *et al.* (1981) to be 1.97 mg kg⁻¹ diet. Analysis of the test diet with respect to B₆ showed that the diet contained 1.75 mg kg⁻¹ diet, almost sufficient pyridoxine to satisfy the minimum requirement of the species. Hardy *et al.* (1979) working with trout did not observe a pyridoxine dose dependency for growth within diets of equal protein content, however feed conversion rate and protein efficiency ratio did show improvements with respect to dietary pyridoxine content. A notable conclusion derived from that study was that the requirement for pyridoxine was elevated at higher dietary protein levels. It is quite possible that the protein content of the diet fed during the present study was insufficient to command a higher pyridoxine requirement and hence the fortification of the diet with B₆ at 100 mg kg⁻¹ at the protein level supplied was neither beneficial or detrimental. Increasing the protein content of the diet from 48% to 60% or above may enforce a greater B₆ requirement but the provision of surplus amino acids may not necessarily result in growth or improved protein deposition rather effect detrimental changes in

carcass composition. With respect to aminotransferase, all treatments showed a potential for increased activity when the assay was spiked with pyridoxal-5-phosphate (PLP). The increase in activity showed no correlation with dietary dose for the fry, however with respect to the production sized fish, those individuals supplemented with the lowest dietary pyridoxine showed the largest PLP mediated elevation in transaminase activity. This effect was however, found not to be statistically significant ($P > 0.05$). However, with respect to maximal alanine aminotransferase activity, Kissil *et. al.* (1981) recommended dietary supplements of 3.0 and 5.06 mg kg⁻¹ diet for fry and fingerlings respectively, levels which exceeded the pyridoxine content of the diet supplemented with pyridoxine at the lowest level. In the study by Hardy *et al.* (1979) serum glutamic oxaloacetic acid transaminase (SGOT) activity was found to be greatest amongst the chinook salmon fed the low protein diet (30 % crude protein) for all levels of pyridoxine supplementation. Additionally, SGOT activity was higher than that observed for all fish fed the high protein diet (65% cp) regardless of pyridoxine supplementation except those presented with pyridoxine at the level of 40 mg kg⁻¹. These data may be summarised thus; at low levels of protein, the activity of the SGOT was dependent on the availability of the protein, where protein was supplied to excess the rate determining factor was the availability of B₆ supplied by the diet. Applying this to the data obtained from the present study, it may be postulated that the small potential for PLP mediated elevation in GPT activity observed for the production sized fish demonstrated that the pyridoxine supplement was close to that required for the saturation of the transaminase system. Hence, it may be concluded that the diet provided sufficient pyridoxine for maximal GPT activity, in contradiction of the results of kissil *et. al.* (1981) and hence the supply of exogenous cofactor could not effect a change in GPT activity.

Tables 5.1.6 and 5.1.7 both show that the deposition of protein in the carcasses of the fish was independent of dietary pyridoxine content and, given that neither net protein utilisation or protein efficiency ratio showed a dose dependent effect, it may be assumed that sufficient pyridoxine was available for adequate protein utilisation. The lipid concentration within the carcasses of the fingerlings showed a dose dependent effect with those fish maintained on the lowest dietary level of pyridoxine exhibiting the lowest levels of carcass lipid. The diets employed in this investigation were all isocalorific and isonitrogenous and hence the deposition of fat can only be related to the B₆ intake. On the basis that protein can only be deposited to comprise a maximal proportion of the carcass, then the products of the metabolism of excess dietary amino acids must be catabolised. Thus after deamination in the liver, excess amino acids are metabolised to form α -keto acids which are converted either into glycogen or lipid (Cowey and Walton 1989). Observation of the carcass composition of the fry shows that the potential for improved protein utilisation via transamination was not realised, hence it may be suggested that, excess calories were deposited as fat and not as protein as a consequence of pyridoxine mediated deamination and subsequent α -keto acid formation. The significant differences with respect to carcass moisture observed between production sized fish fed at dietary doses of 0.5 and 100 mgkg⁻¹ did not extend to any of the other parameters and thus these changes were considered un-important in terms of product quality.

Given the recent clinical application of pyridoxine to the treatment of a variety of medical conditions, the safety of megadose vitamin B₆ supply has been examined in humans and animals. A review of the literature by Cohen and Bendich (1986), delineates the dietary levels at which detrimental effects become apparent. In humans the

recommended maximum supplement is 500 mgkg^{-1} body weight per day. Studies on dogs and rats show an absence of sensory neuropathy below 150 and 50 mgkg^{-1} body weight per day respectively. Based on the maximum B_6 content of the diet and the daily feed intake of the fish, the daily pyridoxine intake was calculated to be $4.03 \text{ mgkg}^{-1}\text{day}^{-1}$. This was far smaller than those levels deemed dangerous in mammals and hence the absence of pathology was not un-expected.

With respect to haematocrit, only those individuals fed the lowest dietary pyridoxine exhibited a packed cell volume which was significantly higher than that recorded for all other groups. It is well documented that stress in fish may result in elevation of haematocrit (Wedemeyer and Yasutake 1977) and that handling stress may result in haemo concentration (Fletcher 1975). Given these two hypotheses, it is fair to assume that handling stress was the cause of the increased packed cell volume observed in those fish fed the diet fortified with the lowest B_6 level. The relationship between pyridoxine and stress moderation in humans and mammals is still little understood and hence a mechanism for pyridoxine mediated alleviation of the symptoms of handling stress, in this case manifested as haemo concentration, is open to speculation.

The diet independent results of the plasma glucose assay showed that under normal conditions the fish were not stressed as a result of changes in the dietary pyridoxine dose. However, upon the application of an acute stress, the dietary pyridoxine made a significant impact upon the response of the individual to that stressor. A dose response was observed thus, those fish fed the lowest level of B_6 incurred the least elevation in plasma glucose concentration. However, this was not significantly higher than that observed for the control group under the same dietary regime. The plasma glucose concentration recorded in the stressed fish after feeding the greatest level of dietary

vitamin was significantly higher than that observed for any other group. The results observed may be examined with respect to the role of pyridoxine as a cofactor in the action of glycogen phosphorylase. The role and mode of action of this vitamin in glycogen phosphorylase activity was elucidated by Palm *et.al.* (1990) and should conditions arise, where only a lack of the cofactor prevents activity of glycogen phosphorylase at its full rate, for example under stressful conditions, then a dose effect would be observed in terms of liberation of glucose from glycogen. Additionally as stated above, the α -keto acids formed as a consequence of the supply of amino acids excess to requirement, may be stored as glycogen in the livers of those fish supplied with adequate supplies of pyridoxine and would, if catabolised during or after stress, provide a large source of glucose.

In the light of the present study, given the wealth of evidence for the role of pyridoxine in the moderation of stress and the role of this vitamin in health, a considerable scope for further research exists within the framework of fish nutrition. Aquaculture represents an environment in which fish are placed under extremes of handling conditions and stocking densities. Under such conditions the individuals are placed under stresses which at times, may be quite severe, often resulting in mortality or increased susceptibility to disease. Pyridoxine plays a series of roles in both the moderation of stress and in the normal function of the immune system. The study carried out by Hardy *et. al.* (1979) included an examination of the effects of pyridoxine on the resistance of trout to vibriosis. In summary; serum antibody levels were independent of dietary protein or pyridoxine dose following vaccination with heat killed *Vibrio anguillarum*. However, when challenged with live, virulent *V. anguillarum* those fish receiving the high protein diet coupled with a pyridoxine dose of 40 mgkg⁻¹ showed a

significantly higher survivability than all other treatments. A recent review of the role of B₆ in the function of the immune system was written by Chandra and Sudhakaran (1990). The findings based on research on humans and animals, show that pyridoxine deficiency may result in atrophy of lymphoid tissue, decreasing lymphoid cell counts, suppression of primary and secondary antibody responses to vaccines and decreased cytotoxicity of the lymphocytes. Additionally Lakshimi *et al.* (1991) observed that pyridoxine deficiency in rats was responsible for an enhancement of the inflammatory response. With respect to fish immunology, little work apart from the study by Hardy *et al.* (1979) has been carried out concerning the role of pyridoxine in disease resistance in fish and hence the relationship between pyridoxine and cellular immunity remains a promising area of research. The steroid hormones are of considerable importance in aquaculture since many of the actions of the glucocorticoids result in an effect which is deleterious to the individual (Pickering 1981). Such effects may include poor or restricted growth, poor feed efficiency and reduced fertility in broodstock. Given the role of pyridoxine in steroid hormone activity this too represents a potentially rich field of research for the alleviation of aquaculture derived stress.

5.2. Thiamin Nutrition in Gilthead Seabream (*Sparus aurata* L.)

Fed Diets With Two Oil : Carbohydrate Ratios

5.2.1 Introduction.

Thiamin (Vitamin B₁) is a water soluble vitamin with the empirical formula C₁₂H₁₈ON₄SCl₂. The compound, for which a large number of derivatives exist, is composed of a pyrimidine ring covalently linked to a thiazole moiety. The crystalline material is relatively stable in dry heat but unstable in neutral or alkaline solutions. Of particular interest to fish nutritionists is the hydrolysis of the pyrimidine ring which may be induced by thiaminases which are present in high concentrations in fish flesh. The commonly applied derivatives of thiamin are the industrially synthesised mono nitrate and hydrochloride forms which maintain a high degree of biological activity despite their increased stability in feeds.

The roles of thiamin are organised into two major functions. One function of this vitamin is associated with neural transmission which at present, is not well understood in higher animals. The primary and best understood role of thiamin is within intermediary metabolism and this aspect was the focus of the present experiment. In the main, the B₁ content in the body is present as the compound thiamin pyrophosphate (TPP or co-carboxylase) which acts as a cofactor for enzymes at three points in intermediary metabolism. Thus TPP is a cofactor for the enzymes involved in the pentose phosphate pathway, the citric acid cycle and for the decarboxylation of pyruvate to acetyl-CoA. Consequently, thiamin deficiency may result in a plethora of metabolic dysfunctions which may or may not be interactive with respect to the gross nutrition of the gilthead seabream.

Deficiency and Requirements.

The symptoms associated with thiamin deficiency are discussed in chapter 3 which outlined the qualitative requirements of the gilthead seabream for the B complex vitamins and hence will not be further described at this point.

One of the first aquatic species for which the thiamin requirement was accurately determined was the rainbow trout. Using data derived from growth and feeding efficiency parameters, McLaren *et al.* (1947) determined that the thiamin requirement of the rainbow trout (*Salmo gairdneri*) lay between 0.1 and 1.0 mg kg⁻¹ of diet. This value was later substantiated by the observations of Morito *et al.* (1986) who, having studied growth and a wide variety of biochemical indices including the activity of transketolase (a TPP dependent enzyme), determined that the B₁ requirement of rainbow trout was adequately met by the provision of 1.0 mg of thiamin per kg of feed. Using semi-purified diets, the thiamin requirement of the turbot (*Scophthalmus maximus*) was determined by Cowey *et al.* in 1975. Using growth, feed utilisation data and via an examination of the potential for increased activity of the transketolase enzymes in the presence of exogenous thiamin pyrophosphate, the requirement of the turbot for thiamin was defined as lying between 0.6 to 2.6 mg kg⁻¹ feed. Murai and Andrews in 1978a demonstrated that for optimal growth and feed utilisation, the requirement of the channel catfish (*Ictalurus punctatus*) for thiamin was 1.0 mg kg⁻¹ feed. The qualitative thiamin requirement of the red seabream was demonstrated by Yone and Fujii in 1974 and for the Asian seabass in 1991 by Boonyaratpalin and Wanakowat, however neither of these studies gave an indication of the quantitative thiamin requirement of these species.

Dietary Energy and Metabolism in Fish.

Since thiamin is implicitly involved in the key pathways of intermediary metabolism in animals, the interaction of the major nutrients with each other and their place in metabolism must be reviewed before their relationship with thiamin is examined.

When compared to terrestrial vertebrates the net energy requirements of fish are relatively low with fish having maintenance energy requirements in the region of 10 % of those of birds and mammals (Smith 1989). The reason for this low maintenance energy requirement has been extensively examined in the reviews of both Cowey and Sargent (1979) and Smith (1989). In summary these authors state that, as poikilotherms in an aquatic environment, fish do not expend energy on the maintenance of body temperature and expend little energy in supporting or locomoting the body. Additionally, as a consequence of the passive excretion of ammonia from the gills expend minimal energy on the detoxification and excretion of the nitrogenous waste products of protein metabolism. As a consequence of these factors, nearly 85 % of ingested feed remains available for growth making the rearing of fish very efficient when compared to the production of domesticated terrestrial livestock.

Analysis of the proximate composition of the majority of fish species clearly shows that the carcass consists of moisture, protein, lipid and ash in proportions dependent on both intrinsic and dietary factors. The remaining fraction of the carcass is attributed to the stored carbohydrate component of the fish in the form of glycogen. Thus, it immediately becomes apparent that the carbohydrate intake of wild, carnivorous species is very low. Consequently, the entire metabolism of carnivorous fish has evolved to derive energy from protein and lipid with the efficiency of carbohydrate utilisation being, at best, quite poor when compared to that of mammals.

Protein.

Of the protein ingested by fish, some 70 % is utilised to provide energy (Walton and Cowey 1982) following conversion into intermediates which may be channelled into the citric acid cycle at entry sites characteristic for each amino acid derivative allowing oxidation into carbon dioxide and water (Walton and Cowey 1982). The metabolism of amino acids is closely regulated by coarse and fine controls. The former equates to changes in the amount of enzyme available for metabolism in response to dietary input of amino acids, while the latter is mediated by modifications in the substrate affinity (K_m value) of the enzymes (Cowey and Sargent 1979, Walton and Cowey 1982).

Once the energy requirement of the fish has been satisfied, excess dietary protein may not be stored in a form resembling the substrate material un-like carbohydrate and lipid. Instead, surplus protein is deaminated and oxidised via the TCA cycle (Walton and Cowey 1982) or after utilisation for lipogenesis or gluconeogenesis is stored as fat or glycogen respectively (Cowey and Sargent 1979). After the maintenance requirement of a species for protein has been attained, the inclusion of higher levels of protein within the diet for a given energy content results in reduced utilisation of protein for growth. Conversely, insufficient energy for a given dietary protein content results in lowered protein deposition as more protein is used to satisfy the energy requirement of the fish (Cowey and Sargent 1979).

Lipids.

During the process of digestion, complex lipids are reduced to free fatty acids prior to absorption from the gut. Post prandial absorption and assimilation of the fatty acids leads to their reformation into triglycerides for transportation and consequent

metabolism or storage. Dietary essential fatty acids are usually deposited in proportion to their intake in the visceral or intramuscular tissues (Cowey and Sargent 1979). However, the incorporation of certain essential fatty acids into the polar phospholipid fraction of tissues are subjected to metabolic transformation as a consequence of biotic and especially abiotic factors such as temperature and salinity (Sargent *et al.* 1989). Fish also have the capacity to produce lipids *de novo* in the liver from carbohydrates and proteins via acetyl-CoA which, after a series of carboxylations, condensations and reductions may result in the formation of fatty acyl-CoA. Consequently glycerol-1-phosphate is formed by the esterification of glycerol from tri-glycerides with 3 fatty acyl molecules as a bi-product (Walton and Cowey 1982). However, given the substantial lipid input which fish receive from natural feed, the lipogenic capacity of fish is not large and in a farmed situation probably represents a mechanism for the deposition of excess energy derived from lipid.

The catabolism of lipids proceeds in mitochondria and peroxisomes mainly in the liver and kidney of fish (Walton and Cowey 1982, Sargent *et al.* 1989). Triglycerides of dietary and tissue origin may be utilised with equal efficiency by fish for the provision of energy (Cowey and Sargent 1979). Having entered the cell, triglycerides are converted into fatty acyl-CoA prior to transport across the mitochondrial membrane as fatty acylcarnitine. Once re-generated the fatty acyl-CoA is subjected to beta oxidation with the product, acetyl-CoA, oxidised in the citric acid cycle yielding ATP. The peroxisomal route uses beta oxidation to produce acetyl-CoA in the peroxisome which is then free to cross the mitochondrial membrane for oxidation via the TCA cycle. The mitochondrial pathway is believed to represent the normal pathway of fatty acid metabolism in fish while the peroxisomal route is thought to be an adaptive mechanism by which fish

respond to modifications in dietary lipid supply (Sargent *et al.* 1989).

The supply of excess lipid arising from either route results in the formation of ketone bodies which arise from acetoacetyl-CoA (Sargent *et al.* 1989) and the deposition of lipid within the viscera.

Carbohydrates.

As a consequence of the greater emphasis on protein and lipid as the major caloric sources, for most fish the catabolism of carbohydrates is awarded a lesser significance than that attributed by mammals in terms of energy contribution from the diet. However, certain tissues *eg.* neural tissues, show a particular requirement for glucose and in addition, processes important for the provision of essential metabolites required by the remaining metabolic pathways *eg.* the pentose phosphate pathway which provides NADPH for lipogenesis, are still of great significance.

In the main, the pathways involved in the derivation of energy from carbohydrates are present in teleosts but overall, the metabolism of carbohydrate in fish proceeds at a slow rate which was attributed by Cowey and Sargent (1979) to be a consequence of the low activity of hexose kinase. Additionally, the use of other substrates for the provision of energy in preference to glucose indicates a poor capacity for glucose utilisation.

Gluconeogenesis resulting in the formation of glucose by the metabolism amino acids was believed by Cowey and Sargent (1979) to provide the majority of the glucose required by fish to maintain the normal glucose content of the blood with approximately 1 mole of glucose produced by gluconeogenesis from 2 moles of amino acids (Walton and Cowey 1982). Glycogen may be formed in the livers of teleosts by the conversion of glucose-6-phosphate to glucose-1-phosphate which, mediated by the enzymes of glycogen

synthesis, is then added to a glycogen primer . However, fish are known to have little ability to utilise glycogen for the provision of glucose for catabolism which has been attributed to either a deficiency of or strict hormonal control of phosphorylases. (Cowey and Sargent 1979, Walton and Cowey 1982).

Despite these factors many fish do have the ability to utilise dietary carbohydrate for the provision of energy, though the amount of carbohydrate which may be incorporated into the diet varies widely. This depends mainly upon the source and complexity of the carbohydrate which affects both digestibility and assimilation (Kaushik *et al.* 1989, Hung and Fynn-Aikins 1991).

Nutrient Interaction.

Given that fish derive the majority of their energy requirement from protein and lipid, it is well known that where a suitable non-protein energy source is made available, protein may be partially spared for growth (Cowey and Sargent 1979, Walton and Cowey 1982, Degani and Viola 1987). Thus the supply of non-protein energy is beneficial in terms of improvements in growth rates but also in terms of cost since lipid and carbohydrate are more economic in terms of the energy supplied per unit mass fed to the fish. Naturally, there is a point where the benefits of increased supplementation of the diet with non-protein energy become deleterious to the growth and health of the fish. The threshold at which cost considerations and productivity are balanced varies with respect to species, developmental state and environmental conditions *eg.* season, salinity and water temperature, Consequently the use of materials involved in the provision of non-protein energy has been a focus of a great deal of attention in fish nutrition research.

Rationale.

Under the dietary conditions chosen for the determination of the qualitative B vitamin requirements of the gilthead seabream, it was observed that an absence of thiamin within the diet resulted in significant suppression of growth of the fish when compared to individuals fed a thiamin adequate formulation. Given this qualitative requirement and the relationship between thiamin and the metabolism of carbohydrates, the potential for changes of the quantitative requirement in response to modifications in the gross composition of the diet were envisaged. This experiment represented an opportunity to examine vitamin nutrition from an applied perspective given that carbohydrate is frequently incorporated into the diets of carnivorous fish as both a potential source of non-protein energy and as a convenient filler in the manufacture of extruded diets.

The aim of the present experiment was explore the relationship between thiamin, carbohydrate and lipid utilisation in order to evaluate the suitability of elevations in dietary carbohydrate at the expense of lipid as an energy source in iso-proteinaceous diets of similar metabolisable energy. The suitability of each lipid to carbohydrate ratio was measured in terms of the protein spared for growth in the absence of pathology related to excess carbohydrate with an investigation of the potential for modifications in thiamin requirement as a consequence of elevated carbohydrate.

5.2.2 Materials and Methods.

Batches of twenty five seabream fingerlings (initial weight $66.3 \text{ g} \pm 0.06 \text{ g S.E.}$) were stocked into each of six 400 l, self cleaning fibreglass tanks over a 1200 l bio-filter within a closed recirculation system. A parallel flow of sea water was maintained through the tanks at 10.8 l min^{-1} . Water temperature and salinity were maintained at 24°C and 33-36 ‰ saline respectively, the former by the use of an immersion heater with an integrated dip-cooler, the latter by the use of a fresh water inlet balanced to compensate for evaporative losses. The pH was maintained between 6 and 7.5 by the use of magnesium and calcium carbonate buffering. Weekly water changes amounted to 5% of the system volume. Daylight balanced fluorescent discharge lamps maintained a 12 hr light / dark photoperiod.

For the present experiment, the practical type diet used for the experiment in chapter 3 which investigated the importance of vitamin supplementation in diets for the gilthead seabream was further developed. Thus, two basal formulae were designed to be iso-proteinaceous and equal in terms of metabolisable energy while differing with respect to lipid and carbohydrate content. Using the data supplied by Davies 1993 (Personal communication), the ratio of metabolisable energy derived by gilthead seabream from equivalent masses of oil and starch (dextrin / corn starch 3:2) is 9:4. Thus, two basal formulae were derived with diets 1, 2 and 3 (high oil) containing supplemental cod liver oil at 6.30 % and starch (dextrin / cornstarch 3:2) at 10 %. Diets 4, 5 and 6 (low oil) contained cod liver oil at 2.4 % and starch at 20 %. Alpha-cellulose at 8.80 % and 2.70 % was used as a filler for the high oil and low oil diets respectively. The basal formulation was subsequently fortified with thiamin hydrochloride at 0.5, 5.0 and 10.0 mg kg^{-1} (as hydrochloride) for diets 1 and 4, 2 and 5, 3 and 6 respectively. The test diets

Table 5.2.1: Composition of the low and high oil diets

Component	High Oil Diets 1, 2 and 3 % Inclusion	Low Oil Diets 4, 5 and 6 % Inclusion
Fish Meal (Provimi 66)	71.20	71.20
Cod Liver Oil	6.30	2.40
Dextrin/Corn Starch (3:2)	10.00	20.00
α -Cellulose	8.80	2.70
Molasses	0.50	0.50
Minerals ¹	1.50	1.50
Fat Soluble Vitamins ²	0.20	0.20
B Complex Vitamins (B ₁ Free) ³	0.45	0.45
Thiamin in α -cellulose	0.05	0.05
Macro Vitamins ⁴	1.00	1.00

¹ Mineral mix (mg/kg diet):

CaHPO₄ 2756.3; CaCO₃ 375.0; NaCl 1875.0; K₂SO₃ 2500.0; MgSO₄·7H₂O 2578.4; FeSO₄·7H₂O 87.5; MnSO₄·4H₂O 28.4; ZnSO₄ 64.5; CuSO₄·5H₂O 6.4; CoCl₂·6H₂O 3.3; KI 1.9; Na₂SeO₃ 0.3; Maize Gluten Meal 4723.0

² Fat Soluble Vitamin Mix (mg/kg diet):

Vit A Palmitate, 4.2; Vit D₃ (Rovimix D-50 SD); Vitamin E (Rovimix E-50 SD) 1000; Menadione Sodium Bi-Sulphite, 780.0; Maize Gluten Meal 3776.75

³ Thiamin Free B Vitamin mix (mg/kg diet):

Biotin(2%) 300.0; Folic Acid (88.8%) 16.9; Niacin 48.6; Calcium Pantothenate 305.3; Riboflavin (96%) 208.3; Pyridoxine Hydrochloride 50.0; Cyanocobalamin (95%) 0.01; Maize Gluten Meal 3570.89

⁴ Macro Vitamin mix (mg/kg diet):

Ascorbic Acid (Rovimix Stay C), 2000; Choline Chloride, 5000; Inositol, 2000; Maize Gluten Meal, 1000

Table 5.2.2: Nutrient profile of the test diets. Moisture content is expressed as a percentage of the diet as presented to the fish. Protein, lipid and ash are expressed as a percentage of the dry matter.

	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)	Nitrogen Free Extract (%)
Diets 1, 2 and 3 (High Oil)	4.53	50.62	14.16	16.48	14.21
Diets 4, 5 and 6 (Low Oil)	5.48	50.76	10.09	16.55	17.12

Table 5.2.3: The thiamin and α -cellulose content of the test diets.

Diet	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
	Oil	Oil	Oil	CHO	CHO	CHO
	0.5	5.0	10.0	0.5	5.0	10.0
Thiamin (mgKg ⁻¹)	0.5	5.0	10.0	0.5	5.0	10.0
α -Cellulose (mgKg ⁻¹)	495.5	495.0	490.0	495.5	495.0	490.0

were manufactured as outlined in chapter two while the formulation, proximate composition and thiamin content of each is displayed in tables 5.2.1, 5.2.2 and 5.2.3.

The test diets were supplied to the fish three times daily at 09:00, 13:00 and 16:00 hrs. For the first 10 weeks the ration was supplied at 2.0 % of the live mass per day, 1.8 % for weeks 11 to 24 inclusive and at 1.4 % for weeks 25 and 26. (Feeding was *ad libitum* up to the prescribed ration from week 19 onwards). Following a 24 hour starvation period, the fish were individually weighed at bi-monthly intervals in order to follow growth parameters and to recalculate the appropriate ration size.

At the end of the growth study, the individual weights of all fish were recorded, the specific growth rate (SGR), feed conversion efficiency (FCE) and feed conversion ratio (FCR) were calculated based upon the recorded feed input over the trial period.

After 26 weeks of feeding and 48 hours starvation, five individuals from each tank were selected for proximate analysis. Each carcass was individually analysed for protein, lipid, ash and moisture content. Crude protein was determined by the Kjeldahl (N x 6.25) method and lipid by a method derived from that of Barnes and Blackstock (1973). Moisture and ash were assayed according to the methods outlined in the A.O.A.C.

Handbook (1990). Based on the growth data, nutrient analysis of the test diet and the proximate composition of fish sub-sampled at the beginning of the trial, the net protein utilisation (NPU) and protein efficiency ratio (PER) were calculated.

At the end of the growth study following 24 hours of starvation, five individuals were selected from each tank and blood was withdrawn by cardiac puncture into heparinised syringes. The packed cell volume of the blood was determined by micro haematocrit while the haemoglobin content was determined by the cyanmethemoglobin method (Sigma Total Haemoglobin, Sigma 525 A). Following centrifugation, the plasma was decanted into micro centrifuge tubes and stored at -70°C until required. Sigma diagnostic kits were subsequently used to determine; plasma protein by the biuret method (Total Protein Reagent, Sigma 541-2), plasma glucose by the glucose oxidase method (Sigma, 510 A), plasma triglycerides (Sigma, 336-10), plasma pyruvate by the use of lactate dehydrogenase (Sigma, 726 UV) and lactate by the lactate oxidase method (Sigma, 735).

At the time of slaughter, the weight of the liver with respect to complete carcass mass for 10 fish in each tank was recorded and the hepatosomatic index (HSI) determined. These livers were subsequently frozen prior to assay for glycogen. The method outlined in Plummer (1987) was used to isolate and hydrolyse the glycogen with the resultant glucose assayed by the glucose oxidase method. In order to quantify glycogen on the dry matter basis, the livers were dried and the desiccated tissue then assayed for lipid by the method of Barnes and Blackstock (1973).

The final weights ($n=25$), proximate composition ($n=5$), hepatic and haematological parameters ($n=5$) were subjected to analysis of variance, where $p < 0.05$ was judged indicative of a significant difference. Where the ANOVA revealed significant

differences Duncan's multiple range test (Duncan 1955) was applied in order to characterise and quantify the differences between treatments.

5.2.3 Results.

After 26 weeks of feeding, only diet 3, (380.57 percent live weight gain) showed a significantly larger final weight than that of the remaining regimes which increased their original weight by between 320 and 334 percent ($p < 0.001$). The final weights could, however, be sub-divided according to dietary oil content with those fish fed the high lipid diet demonstrating a higher final weight than those fed the high carbohydrate diets.

With respect to feed conversion ratio and feed conversion efficiency, diet 3 (Oil 10.0) appeared to be the most effective with values of 1.80 and 55.45 % for FCR and FCE respectively. Fish fed the remaining diets scored similar FCR and FCE values in the range 2.03 to 1.96 and 51.08 to 49.21 % respectively. Feeding efficiency showed no dependence on either lipid level or thiamin supplement for all diets with the exception of diet 3.

In terms of efficiency of protein utilisation (NPU), diet 3 again was the most effective scoring an NPU of 23.03 %. For the remaining diets, NPU values ranged between 21.46 and 20.17 %. Again NPU for all diets except number 3 was independent of dietary lipid and carbohydrate. These values were reflected in the carcass composition with no significant differences observed between all groups with respect to moisture, lipid or protein content on the live weight basis. However, carcass ash was significantly lowered ($P < 0.05$) for those fish fed the high oil diet supplemented at the lowest level of thiamin. Lipid content for these individuals was seen to be lowered concurrent with

elevations in carcass moisture, however, given the variation within treatments the differences between treatments were not significant.

Table 5.2.4: Performance characteristics of gilthead Seabream fed diets of varied oil : carbohydrate ratios supplemented with three levels of thiamin. Values in each row carrying similar superscripts are not significantly different from each other ($p < 0.001$).

	Diet 1 Oil 0.5	Diet 2 Oil 5.0	Diet 3 Oil 10.0	Diet 4 CHO 0.5	Diet 5 CHO 5.0	Diet 6 CHO 10.0
Initial mean weight (g)	66.44	66.27	66.28	66.34	66.33	66.26
Final mean weight (g)	288.52 ^a	286.80 ^a	318.52 ^b	284.80 ^a	272.67 ^a	278.49 ^a
Weight gain (%)	334.26	332.78	380.57	329.30	311.09	320.30
Specific growth rate (%d ⁻¹)	0.81	0.80	0.86	0.80	0.78	0.78
Daily feed intake (gd ⁻¹)	2.39	2.38	2.50	2.36	2.30	2.30
Feed conversion ratio (FCR)	1.96	1.96	1.80	1.97	2.03	1.98
Feed conversion efficiency (FCE)	51.08	50.96	55.45	50.79	49.21	50.62
Protein efficiency ratio (PER)	1.01	1.31	1.09	1.00	1.03	0.98
Net protein utilisation (NPU %)	21.46	20.53	23.03	21.17	20.17	21.14
Mortality (%)	4.0	4.0	0.0	0.0	0.0	4.0

The hepatosomatic indices (HSI) of fish on all dietary regimes were not significantly different, however, HSI did show a trend towards larger values in response to dietary thiamin within those fish fed the high lipid diet.

The glycogen content in the livers of those fish fed diets 5 and 6 (Low oil 5.0 and 10.0) was significantly greater ($p < 0.001$) than that observed for all four of the remaining dietary regimes. All fish fed the high carbohydrate diets accumulated more glycogen in the liver than those fed high oil diets. However, diet 4 accumulated glycogen at levels which, though greater than that observed for the diets 1, 2 and 3, was still significantly smaller than that of diets 5 and 6. Liver lipid showed no significant

differences between the dietary regimes except for diet 5 (CHO 5.0) which demonstrated a significantly ($p < 0.01$) lower lipid content than all other diets.

Table 5.2.5: Characteristics of seabream livers following 26 weeks of feeding diets of two oil : carbohydrate ratios supplemented with three levels of thiamin. HSI (hepatosomatic index) was determined as a percentage of the live weight at slaughter ($n=10$). Glycogen was determined using whole tissue and later corrected for moisture ($n=5$). Hepatic lipid was determined using dried material ($n=5$). Means on each row bearing similar superscripts are not significantly different from each other ($p < 0.001$ liver glycogen), ($p < 0.01$ liver lipid).

		1 Oil 0.5	2 Oil 5.0	3 Oil 10.0	4 CHO 0.5	5 CHO 5.0	6 CHO 10.0
Hepatosomatic index (%)	Mean	1.03	1.06	1.17	1.28	1.17	1.15
	S.E.	0.06	0.06	0.08	0.08	0.07	0.08
Hepatic Glycogen (mg g ⁻¹ dry weight)	Mean	31.36 ^a	29.00 ^a	50.59 ^a	70.55 ^a	123.05 ^b	120.78 ^b
	S.E.	1.81	6.40	9.73	14.44	21.08	17.44
Hepatic lipid (mg g ⁻¹ dry weight)	Mean	516.23 ^a	552.84 ^a	595.31 ^a	491.57 ^a	363.33 ^b	504.80 ^a
	S.E.	41.95	35.51	26.65	45.68	17.11	27.05

Table 5.2.6: Proximate composition of the initial carcasses and carcasses of test animals after 26 weeks of feeding presented as percentage of the whole fish at the time of slaughter. Values in each column bearing similar superscripts are not significantly different from each other ($p < 0.05$).

		Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Initial Fish		73.59	17.81	6.02	3.85
1. Oil 0.5	Mean \pm S.E.	64.89 \pm 0.90	20.47 \pm 0.40	14.43 \pm 0.92	3.28 \pm 0.08 ^a
2. Oil 5.0	Mean \pm S.E.	63.27 \pm 0.73	19.84 \pm 0.30	15.91 \pm 0.64	3.94 \pm 0.16 ^b
3. Oil 10.0	Mean \pm S.E.	62.76 \pm 0.58	20.35 \pm 0.31	15.99 \pm 0.57	3.74 \pm 0.11 ^b
4. CHO 0.5	Mean \pm S.E.	62.97 \pm 0.82	20.38 \pm 0.33	15.19 \pm 1.00	3.70 \pm 0.11 ^b
5. CHO 5.0	Mean \pm S.E.	62.78 \pm 0.78	20.08 \pm 0.33	15.31 \pm 0.76	3.80 \pm 0.11 ^b
6. CHO 10.0	Mean \pm S.E.	62.43 \pm 1.05	20.39 \pm 0.12	15.16 \pm 1.08	3.78 \pm 0.17 ^b

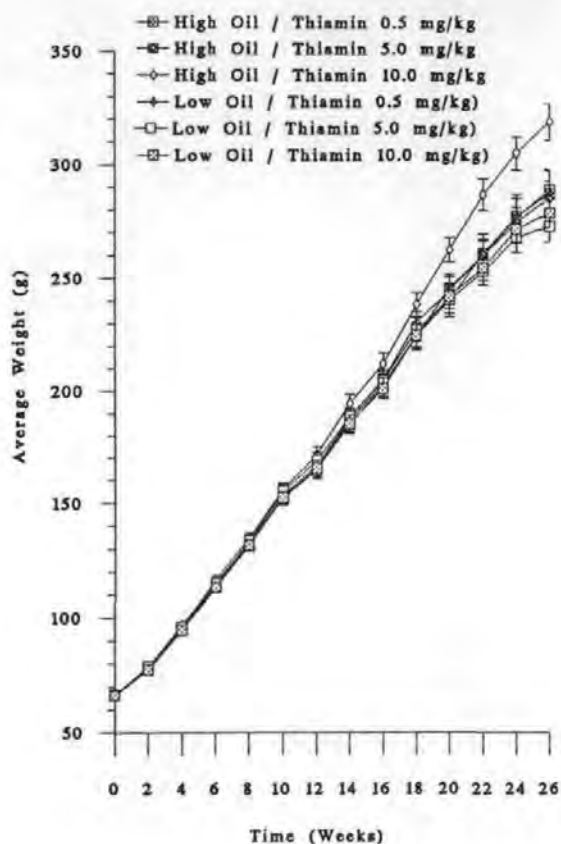
No significant differences were observed with respect to the haematocrit of the test fish though those individuals fed diet 3 (Oil 10.0) demonstrated a packed cell volume of 24.32 % which was lower than that observed for the remaining diets with values typically between 32 and 27.4 %.

Plasma protein concentrations were not significantly different though at both oil levels elevations of the thiamin content resulted in a slight elevation in plasma protein content.

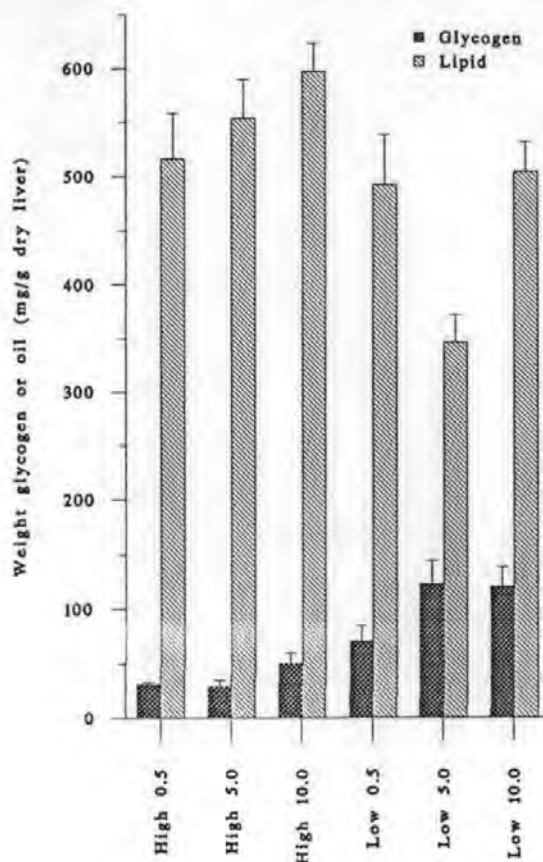
Glucose and triglyceride concentration in the plasma again yielded no significant differences between dietary regimes however, the latter parameter demonstrated two trends. Those fish fed the high lipid diet in general supported slightly greater levels of plasma triglycerides than those fed the low lipid diet. Additionally, at the high oil level, incremental increases in thiamin supplement resulted in a dose dependent elevation in triglyceride concentration. The converse was observed for those fish fed the low oil diet thus, higher thiamin supplement resulted in lowered concentrations of plasma triglycerides.

There were no observable trends with respect to either dietary thiamin or lipid content for plasma lactate. Plasma pyruvate was elevated in those fish fed the lowest thiamin diet within the high oil regime. Conversely, those fish maintained under the low oil regime, the highest plasma pyruvate levels were recorded at the highest level of supplemental thiamin. There were no observable trends with respect to plasma pyruvate concentration in response to modified dietary lipid content.

Mortalities were recorded in fish fed diets 1, 2 and 6 representing 4% (1/25) of the fish in the tank. These fish exhibited no outward signs of deficiency or diet induced trauma.



(a)



(b)

(a) Figure 5.2.1.

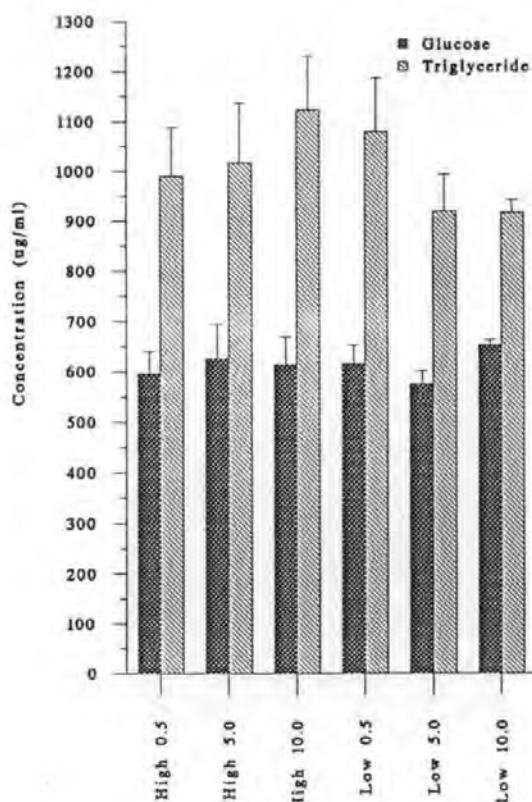
Growth of gilthead seabream fed diets differing in oil : carbohydrate ratio supplemented with thiamin at 3 levels.

(b) Figure 5.2.2.

Hepatopancreatic glycogen and lipid content of gilthead seabream fed diets differing in oil : carbohydrate ratio supplemented with thiamin at 3 levels.

(c) Figure 5.2.3.

Plasma glucose and triglyceride concentration in gilthead seabream fed diets differing in oil : carbohydrate ratio supplemented with thiamin at 3 levels.



(c)

Table 5.2.7: Haematological parameters of gilthead seabream fed diets of two oil : carbohydrate ratios supplemented with three levels of thiamin. Means on each row without or bearing similar superscripts are not significantly different from each other. The values recorded for plasma lactate and pyruvate were not subjected to ANOVA.

	Diet 1 Oil 0.5	Diet 2 Oil 5.0	Diet 3 Oil 10.0	Diet 4 CHO 0.5	Diet 5 CHO 5.0	Diet 6 CHO 10.0
Haematocrit (%)	32.55	31.29	24.32	30.95	28.98	27.74
(n) (S.E.)	(7) (2.68)	(10) (2.28)	(8) (1.92)	(9) (1.53)	(7) (1.85)	(8) (2.50)
[Total Haemoglobin] (mgcm^{-3})	70.20	71.32	73.50	70.50	77.50	72.90
(n) (S.E.)	(8) (5.23)	(8) (3.25)	(9) (7.70)	(8) (6.97)	(9) (4.40)	(9) (6.53)
[Plasma Protein] (mgcm^{-3})	44.17	38.63	39.15	40.43	38.59	38.14
(n) (S.E.)	(5) (0.90)	(5) (0.96)	(5) (2.91)	(5) (1.52)	(5) (1.52)	(5) (1.71)
[Plasma Triglycerides] (μgcm^{-3})	990.54	1017.02	1122.46	1078.49	918.20	915.37
(n) (S.E.)	(4) (96.95)	(5) (119.20)	(5) (107.53)	(5) (107.45)	(5) (74.81)	(5) (25.91)
[Plasma Glucose] (μgcm^{-3})	599.00	625.50	614.17	616.26	575.11	652.94
(n) (S.E.)	(5) (42.74)	(5) (67.70)	(5) (55.40)	(5) (36.68)	(5) (25.79)	(5) (9.45)
[Plasma Lactate] (μgcm^{-3})	122.42	30.83	234.29	171.51	67.18	96.80
(n) (S.E.)	(5) (29.82)	(2) (2.50)	(4) (86.40)	(5) (47.13)	(5) (13.06)	(5) (13.02)
[Plasma Pyruvate] (μgcm^{-3})	4.34	1.45	2.05	2.67	2.73	3.76
(n) (S.E.)	(5) (0.88)	(4) (0.44)	(3) (0.76)	(3) (0.82)	(3) (0.50)	(5) (0.40)

5.2.4 Discussion.

In terms of growth and feed efficiency, the present data indicated only one discernable difference between all six diets. Seabream fed rations supplemented with dietary lipid at the higher level and thiamin at 10 mg kg^{-1} grew to a final weight significantly larger than that of the remaining treatments concomitant with an improved feed efficiency.

With respect to the presentation of a low carbohydrate diet, the absence of a vitamin dependent growth curve, prevents the accurate determination of the thiamin requirement for the species at this level of supplemented oil. However, within this group, the failure of those fish fed the diets supplemented with thiamin at the level of 5.0 mg

kg⁻¹ to grow as large as those fed B₁ at the greatest level would lead to the tentative conclusion that the requirement for this vitamin of the seabream is greater than 5.0 mg kg⁻¹. This value is higher than the 1.0 mg kg⁻¹ determined by McLaren *et al.* (1947) and Morito *et al.* (1986) for rainbow trout (*Salmo gairdneri*) and for channel catfish (*Ictalurus punctatus*) by Murai and Andrews (1978a). Cowey *et al.* (1975) did not define an exact thiamin requirement for the turbot (*Scophthalmus maximus*) but suggested a value between 0.62 and 2.6 mg kg⁻¹ while Lim *et al.* (1991) proposed a thiamin requirement of 2.5 mg kg⁻¹ for the tilapia (*O. mossambicus* X *O. urolepis hornorum*). Only the observations of Halver (1982) place the B₁ requirement of finfish at over 10 mg kg⁻¹ which subsequently disagreed with the findings of Morito *et al.* (1986).

The inclusion of higher levels of available carbohydrate in the diets, resulted in growth and feed efficiency values, in general, lower than those of the high oil supplemented group and in addition none of the included levels of thiamin resulted in improvements in growth, feed conversion and NPU within this group. The growth response of fish fed diets of varied lipid and carbohydrate content has been extensively studied for many species and in general, the ability of each species to utilise different non-protein energy relates to both the metabolism and nutritional habit of the fish. Thus carnivorous species such as rainbow trout, yellowtail (*Seriola quinqueradiata*) and cod (*Gadus morhua*) show poor growth response where starch is added in significant amounts in the diet (Hilton and Atkinson 1982, Furuichi and Yone 1980, Hemre *et al.* 1989). Other fish demonstrate a more flexible ability to utilise dietary carbohydrate thus, striped bass (*Morone saxatilis*) utilise lipid and carbohydrate interchangeably up to lipid a content of 17 % of the diet (Berger and Halver 1987), while *Tilapia zillii* show a similar interchangeability of lipid and carbohydrate in accordance with their respective metabolic

fuel values (El-Sayed and Garling 1988). Differences in the nutritive habit of three selected species and the ability of each to utilise carbohydrate was examined by Furuichi and Yone (1980). Consequently the authors demonstrated that omnivores (Carp, *Cyprinus carpio*), semi-carnivores (red seabream *Chrysophrys major*), and carnivores (yellowtail, *Seriola quinqueradiata*) may utilise carbohydrate at up to 30, 20 and 10 % of the diet respectively. However, for the present experiment, an extension of the range of thiamin supplement may have resulted in an improvement in the growth performance of the fish fed the high carbohydrate diets if indeed elevations in dietary carbohydrate do enforce higher requirements for vitamin B₁.

The proximate composition of the fish receiving all diets showed no significant differences with respect to moisture, protein or lipid. The findings of Berger and Halver (1987) are in broad agreement with the findings of the current investigation in that striped bass receiving diets of equal calorific value with carbohydrate and supplemental oil constituting up to 33 % and 17 % of the diet respectively, demonstrated no significant alterations in proximate composition. Conversely El-Sayed and Garling (1988) fed diets of equal metabolisable energy but varied lipid to carbohydrate ratio to *Tilapia zillii*. The latter authors observed that carcass lipid content and net protein utilisation increased in proportion to increases in dietary lipid when supplied at the expense of carbohydrate. Cowey *et al.* (1975) demonstrated a positive correlation between lipid content and protein sparing in plaice (*Pleuronectes platessa*) which concurs with the findings of Ellis and Reigh (1991) who also observed that fat was deposited in the carcass of red drum (*Sciaenops ocellatus*) in proportion to the dietary lipid input. However the findings of these authors indicate that in terms of protein sparing ability, for all levels of gross energy, lipid was more effective than carbohydrate. Hilton and Atkinson (1982), feeding

rainbow trout diets of equal protein and energy values observed increases in carcass lipid content with increasing inclusion of carbohydrate in the diet. The findings of Hemre *et al.* (1989) indicated that cod fed diets of equal energy where lipid and carbohydrate were substituted for protein in variable amounts, ate to satisfy a minimum protein input with the level of ingested energy dependent on the amount of diet consumed to attain this protein requirement. However, carcass composition and hence net protein utilisation (NPU) was un-effected by the ratio of lipid to carbohydrate in the feed consumed.

Considering the present experiment, at the end of which the carcass composition showed no significant differences between the treatments, the improvement in feed efficiency and protein utilisation observed for those fish fed the high oil diet fortified with thiamin at 10 mg kg^{-1} would appear to be a result of the improved growth of these fish. Within the high oil diets, the improved growth of the fish fed diet 3 (Oil 10.0) was as a consequence of thiamin supplementation. Hence, the observed improvement in protein utilisation was not considered a consequence of the proximate composition of the fish but again a result of improved growth.

Comparisons of the suitability of increased carbohydrate content are tentative unless thiamin adequacy is assured. If indeed 10 mg kg^{-1} thiamin supplement were proved to meet the requirement of the seabream at both oil levels, then lipid may be described as a more suitable energy source from a growth and feed efficiency perspective. This conclusion is given additional credibility by analysis of the observed values of net protein utilisation where those fish fed low oil diets demonstrated poorer values for NPU as a consequence of the utilisation of protein for energy and not for growth.

In the present experiment, at both levels of oil and even at the lowest supplemental level of thiamin (0.5 mg kg^{-1}) there was no external evidence of pathology and the mortalities which did occur (representing one fish from each treatment) showed

a distribution which was independent of dietary regime. The pathologies associated with deficiency are discussed in chapter 3 which examined the qualitative requirements of the gilthead seabream for B vitamins and hence will not be dealt with here suffice to say, that in the present investigation, thiamin hydrochloride supplied at 0.5 and 5.0 mg kg⁻¹ maintained an absence of significant gross deficiency symptoms but was insufficient to furnish sufficient vitamin for optimal growth and feed efficiency.

At the haematological level, the packed cell volumes of the tested fish were not significantly different but the trend towards lower haematocrit would appear to positively correlate with thiamin supplement for fish fed diets of both oil levels.

Measurements of plasma lactate in the present experiment demonstrated a large degree of variability and were high in comparison to the range shown by Masumoto *et al.* (1987) to be indicative of thiamin deficiency in the rainbow trout. Thiamin pyrophosphate is a cofactor for the enzyme pyruvate dehydrogenase and hence a deficiency of thiamin would be expected to generate an accumulation of pyruvate and lactate (Bender 1992). However, given the large variability of the lactate concentrations in the present experiment no conclusion on the thiamin status of the fish may be drawn from them.

As a consequence of the role of TPP in the activity of pyruvate dehydrogenase, plasma pyruvate was also shown by Masumoto *et al.* (1987) to be indicative of thiamin deficiency. As with the lactate concentrations in the present experiment, the plasma pyruvate concentration shows an extreme degree of variability between the treatments. Taking first the fish fed the high oil diets, the plasma pyruvate concentration of the fish fed thiamin at 0.5 mg kg⁻¹ was significantly higher than that of the fish fed the remaining levels of thiamin supplement, which, would not be un-expected if indeed diet 1 (Oil 0.5) did not match the thiamin requirement of the species. Diets 4, 5 and 6 (the high

carbohydrate diets) demonstrate pyruvate concentrations which are not significantly different from each other and which also are not significantly different from diet 1. If indeed pyruvate concentration were indicative of thiamin deficiency then this would lend credibility to the assumption that diets 4, 5 and 6 were deficient in this vitamin. However, plasma taken from fish fed diets 4 and 5 did not contain concentrations of pyruvate significantly greater than that observed in the proposed thiamin adequate diets 2 and 3. Thus overall, the use of pyruvate concentration in the plasma of the fish fed the low oil diets gives no indication whether the fish are thiamin adequate at the level of carbohydrate supplied.

For all the test fish, there were no significant differences in the hepatosomatic index, however the quantitative distribution of metabolites did vary between the dietary regimes. Thus, the hepatopancreatic glycogen content was seen to increase significantly for those fish fed the diet containing 20 % corn starch and dextrin when fortified with thiamin at more than 5 mg kg⁻¹. However, the hepatic glycogen content of the fish fed the lowest thiamin supplement at low levels of oil, despite being higher, was not significantly different from those fed all three levels of thiamin and high oil diets.

The findings of the present experiment agree in part with the observations of many researchers who, as a consequence of feeding high carbohydrate diets have observed increases in glycogen deposition (Hunn and Fynn-Aikins 1991, Hilton and Atkinson 1982, Cowey *et al.* 1975, Nagai and Ikeda 1971a). Qualitative aspects of carbohydrate supply also influence glycogen deposition (Hunn and Fynn-Aikins 1991, Furuichi and Yone 1971). In fact, elevations in dietary lipid content are rarely associated with glycogen deposition. Thus, the findings of Berger and Halver (1987) who observed increased hepatosomatic index and glycogen deposition in response to elevations of dietary lipid within iso-proteinaceous diets for the striped bass are an exception to the

rule. In a similar vein, as in the case of Hemre *et al.* (1989) who studied the cod, if lipid and protein were supplied in varied ratios at the expense of protein hepatic glycogen content remained un-effected.

The more detailed investigations of Hilton and Atkinson (1982), Cowey *et al.* (1975) and Nagai and Ikeda (1971b) examined the metabolic and biochemical consequences of the incorporation of carbohydrate in the diets of fish. Hilton and Atkinson (1982) in studies on the rainbow trout, determined the activities of key glycolytic, lipogenic and gluconeogenic enzymes and followed the fate of C¹⁴-glucose in response to carbohydrate inclusion in the diet. In summary, Hilton and Atkinson (1982) concluded that the absence of high pyruvate kinase activity indicated that the rate of glycolysis was not increased and hence glycogenesis was the preferred route for ingested carbohydrate. Additionally, the rate of glucose utilisation and gluconeogenesis were un-effected by dietary carbohydrate content. Cowey *et al.* (1975), observed that plaice (*Pleuronectes platessa*) fed carbohydrate as the principle non-protein energy source, incorporated significantly greater amounts of C¹⁴-glucose into hepatic glycogen than when lipid alone was the sole source of non-protein energy. In 1971 Nagai and Ikeda (1971b) examined the fate of C¹⁴-glucose administered to carp (*Cyprinus carpio*) fed very high carbohydrate rations (90 % CHO), very low carbohydrate rations (10 % CHO) or rations of 50 % protein in lipid free formulations. The metabolic rate of the fish was significantly elevated in those animals fed the high carbohydrate diet as indicated by the greater portion of the labelled carbon excreted by the fish as carbon dioxide. Where the protein content of the diet was elevated to 50 % at the expense of carbohydrate, significantly more labelled carbon was found in hepatic glycogen and the total body pool of glucose.

The hepatic lipid content and plasma triglyceride concentration of the fish fed the

high oil diet in the present experiment showed no significant differences within this group though a trend towards increasing lipid retention in these fish, in terms of hepatic lipid deposit and plasma triglyceride concentration, correspondent with thiamin supply was observed. The lipid content in the livers of those fish fed the low oil diet was only significantly lower than that observed in the high oil diet where thiamin was supplemented in the diet at 5.0 mg kg⁻¹. Plasma triglyceride concentration in this group showed no significant differences however, those fish supplemented with thiamin at 0.5 mg kg⁻¹ demonstrated a concentration which was slightly higher than the remaining two treatments.

Modifications in hepatic lipid content in response to carbohydrate input for the yellowtail, red seabream and the carp were investigated by Furuichi and Yone in 1980. Carp showed the greatest ability to regulate hepatic lipid in response to reductions in dietary fat while the red seabream and the yellowtail were less able to regulate hepatic lipid when faced with similar dietary modifications. Both qualitative and quantitative aspects of carbohydrate input were seen by Hung and Fynn-Aikins (1991) to influence the plasma triglycerol content in sturgeon (*Acipenser transmontanus*). Thus the presentation of glucose and maltose or the supply of carbohydrate above 14 % of the diet, elevated plasma triglycerol concentration to around twice that observed in fish fed other forms of carbohydrate or fed starch comprising 7 % or less of the diet.

In the present experiment, plasma glucose and protein concentrations showed no trends between any of the dietary regimes. Thus, the homeostatic mechanisms of the fish appear to be capable of dealing with a wide range of lipid to energy ratios under various conditions of thiamin supply. For the seabream, it has thus been demonstrated that when carbohydrate is supplied at different levels in the diet, glucose concentration in the plasma appears to be regulated to within the normal range of the species by

combinations of changes in metabolism via glycogenesis, gluconeogenesis or glycolysis and only the findings of Hung and Fynn-Aikins (1991) would appear to contrast with the present data.

The observations of Nagai and Ikeda (1971b) for carp and those of Kohla *et al.* (1992) for *Colossoma macropomum*, demonstrate that little stored glycogen is used to maintain plasma glucose levels and that compared to visceral fat deposits glycogen is little used as an energy source when fish are adequately nourished.

Given the importance of thiamin in intermediary metabolism and the consequences of its deficiency on the overall nutrient utilisation of a carnivorous species which derives the majority of its nutriture from protein and lipid, the construction of an overall model which adequately explains the findings of the present experiment with respect to both alterations in thiamin supplement and carbohydrate load is a complex task.

Considering first the fish fed the low carbohydrate diet, the results observed are easily attributable to the thiamin content of the diet with those fish fed the thiamin adequate diet (Oil 10.0) growing to a significantly larger final weight and utilising dietary protein more efficiently for growth. For the remaining two treatments within this group, the impairments introduced into intermediary metabolism as a consequence of thiamin deficiency are immediately apparent given the elevations in plasma pyruvate concentration for the fish fed diet 1 (Oil 0.5), which is as a consequence of lowered activity of pyruvate decarboxylase an enzyme for which thiamin pyrophosphate is a cofactor. Thus, thiamin deficiency may have reduced the efficiency of carbohydrate metabolism with the fish to depending more on other metabolites for the provision of energy. The plasma glucose concentration and hepatic glycogen content showed no significant differences between the treatments at the end of the experiment. Lipid and

protein are known to provide gluconeogenic precursors and the utilisation of these materials by the thiamin inadequate fish for the maintenance of circulatory metabolites becomes apparent when the plasma triglyceride content and hepatic lipid content are examined. Hence attributing a model to explain the findings of the present experiment for those fish fed the high oil diet is more in line with our current understanding of intermediary metabolism in fish.

Extending the model outlined above to the fish fed the high carbohydrate diet is not so simple given the pattern of the observed liver parameters. In terms of growth and utilisation of nutrients, none of the fish fed the low lipid diets performed significantly differently from each other and hence the involvement of thiamin and its deficiency in the metabolism of these fish is less obvious.

Analysis of the hepatopancreatic glycogen content of those animals maintained on a high carbohydrate diet shows that glycogen was deposited above the level of that observed in the fish fed the high oil diet only at levels of thiamin supplementation higher than 0.5 mg kg^{-1} . If at low levels of lipid input, carbohydrate may be utilised as an energy source and if for those fish fed the diet supplemented with thiamin at the lowest level, the perturbation of carbohydrate metabolism resulted in a lowered efficiency of the utilisation of carbohydrate, less of the material remained available for deposition as glycogen. At the supplemental level of 5.0 mg kg^{-1} the glycogen deposition in the livers of the fish fed the high carbohydrate diet increased significantly above that of the fish fed the lowest thiamin supplement. However, at this point hepatic lipid retention was significantly reduced when compared to that of the remaining dietary regimes.

None of the known functions of thiamin can be used to explain this difference in lipid content especially as the hepatic lipid content of the fish fed the low oil, low thiamin supplemented diets was not significantly different from those fed the highest

thiamin supplement. Of note with respect to this enigma, are the fact that diet 5 (CHO 5.0) demonstrated a poorer NPU value than diets 4 and 6 and that plasma triglyceride concentration demonstrated the reverse trend of that observed for the high oil diets with in this case, the greatest plasma triglyceride content correlating with the lowest level of hepatic glycogen deposition. Given the data observed for those fish fed diet 5 (CHO 5.0), it is not unreasonable to assume that 5.0 mg kg⁻¹ when fed in conjunction with high carbohydrate rations induced a marginal deficiency and consequently the metabolism of the fish was disturbed therefore enforcing a greater dependence on alternative metabolic pathways. This may in part explain why the findings for the fish fed the high carbohydrate diet do not adhere to the model outlined for those fish fed the high oil diet.

Overall, the present experiment demonstrates that for the gilthead seabream carbohydrate may be incorporated to 20 % the diet without deleterious effects. However for maximal growth and feed efficiency lipid is a more suitable non-protein energy source than carbohydrate and additionally these two non protein energy sources are not mutually exchangeable on the basis of metabolisable energy.

Considerable potential for research has been highlighted by the present experiment. Of particular interest is the relationship between thiamin status and the utilisation of dietary components during vitamin B₁ deficiency. Given the close regulation of blood glucose, questions which need to be addressed include; which mechanisms allow this close regulation and what are the controlling factors which regulate transitions between for example gluconeogenesis, glycogenesis and glycogenolysis. It is not unreasonable to assume that the utilisation of all these pathways and the relative importance of each will depend not only on the macro nutrient supply but also on the vitamin status of the animal.

5.3. An Evaluation of the Performance of Ascorbic Acid Derivatives as Feed Supplements in Diets for the Gilthead Seabream (*Sparus aurata* L.)

5.3.1. Introduction.

The significance of ascorbic acid (vitamin C) in animal nutrition with an emphasis on finfish is reviewed in chapter one and subsequently expanded in chapter three. The preliminary study which investigated the consequences of the omission of ascorbic acid from a practical diet served to highlight the importance of this vitamin in the nutrition of the seabream. Consequently, a further experiment was designed to investigate the ascorbic acid nutrition of this species with special emphasis on an applied topic *ie.* qualitative aspects of vitamin supply.

Ascorbic acid is well known to feed manufacturers for its instability due to oxidative loss within feed materials. The labile nature of vitamin C is a consequence of the high potential for sequential oxidation of the second and third hydroxyl groups yielding firstly monodehydro ascorbic acid and subsequently dehydroascorbic acid (Bender 1992). Both of these products have *in vivo* activity as a factor of their potential for *in vivo* reduction and hence regeneration of ascorbate. However, further oxidation of dehydroascorbate in the presence of oxygen, under alkaline conditions and in the presence of metal ions yields dioxogulonic acid which has no biological activity (Bender 1992). This last oxidation is not reversible by *in vivo* mechanisms and hence represents an irreversible loss of vitamin within the feed material.

The factors effecting the oxidative loss of ascorbic acid in feed have been the focus of a great deal of attention within the field of fish nutrition. Thus oxidative loss of ascorbic acid during feed processing is exacerbated by high pH, high temperature, and

moisture either as steam or liquid (Hilton *et al.* 1977, Gadiant 1990). Poor conditions of storage such as high temperature and humidity and the duration of storage also result in significant losses of unprotected vitamin C from the diet (Soliman *et al.* 1987). Finally, water soluble vitamin supplements in feeds for aquatic animals are subject to loss via leaching, thus in extreme cases, between 97 and 99 % of an ascorbic acid supplement may be lost from the feed by the combined actions of processing, storage and leaching (Slinger *et al.* 1979). As a result, there exists a need to develop products with the anti-scorbutic effects of ascorbic acid but which are less easily oxidised. A large number of commercial products are currently available and these are divided into two categories based upon the type of technology employed in their manufacture. Thus, vitamin C may be either coated with nutrient or non-nutrient materials or chemically modified resulting in a series of analogues of the vitamin.

Chemical modification may be carried out by substitution on either one or both of the second and third hydroxyl groups of L-ascorbic acid inhibiting oxidation of the vitamin and resulting in up to a threefold increase in the oxidative stability of the vitamin (Wang and Seib 1990). During digestion, the blocking group is removed resulting in a vitamin which is thus available to the animal. A wide variety of ascorbic acid derivatives have been synthesised by substitutions to the second and third hydroxyls and have been employed not just to stabilise the vitamin but for the study of metabolites and for the production of radio labelled molecules. A broad range of phosphate and sulphate esters of vitamin C are presently in existence and these derivatives are frequently the focus of attention in nutrition research. The alternative to chemical modification is to encapsulate the vitamin within a protective coat which confers resistance to oxidation while the feed is manufactured and stored but which may be removed from the ascorbic acid during

digestion. A diverse group of coatings exist of differing thicknesses and resistances to pressure *etc.* though the resistance to oxidation must often be offset against bioavailability.

Ascorbate-2-sulphate (Vitamin C₂) represents a derivative of ascorbic acid the stability of which in fish feeds has been proven by Soliman *et al.* (1987) and by Schüep *et al.* (1989). However, much conjecture surrounds the suitability of this analogue in animal nutrition. Ascorbyl sulphate is a metabolite occurring in the urine of primates, guinea pigs and fish (Baker 1971) and this derivative was shown by Halver *et al.* (1975) to be distributed throughout carcass and excreted in the urine of salmonids while Dabrowski and Hinterleitner (1989) failed to detect this derivative in the tissues of roach (*Rutilus rutilus*), common carp (*Cyprinus carpio*) or rainbow trout (*Oncorhynchus mykiss*). Machlin *et al.* (1976), in studies on the rhesus monkey, declared ascorbate-2-sulphate to be biologically unavailable. As a result of the conjecture caused by ascorbyl sulphate, a great wealth of research has been carried out in order to evaluate the suitability of this derivative as a feed supplement for fish.

The phosphorylation of ascorbic acid improves the resistance of this vitamin to oxidation and as with the sulphate esters, numerous derivatives have been synthesised and utilised in animal nutrition. The stability of this vitamin in feeds has been demonstrated by Dabrowski (1990), Gädient (1990), and Gabaudan *et al.* (1990) and the processing and storage losses of this vitamin are significantly lower than that of L-ascorbic acid. During digestion, gut hydrolases in fish release the ascorbic acid from its bound state allowing the uptake of the free ascorbate (Buddington *et al.* 1993, Miyasaki *et al.* 1992). Consequently, the phosphate esters of ascorbic acid have been shown to be bioavailable to guinea pigs (Imai *et al.* 1967), chickens (Broz and Ludwig 1990), pigs

(Schulze *et al.* 1990) and an ever increasing number of aquatic species. Thus, the phosphate esters of ascorbic acid are considered to be highly suitable as sources of vitamin C.

The alternative to chemical modification of the vitamin is encapsulation. Thus a variety of coating materials including silicon, ethyl-cellulose, synthetic polymers, fats and waxes have all been developed and tested (Hilton *et al.* 1977, Soliman *et al.* 1987, Skelbaek *et al.* 1990, Gadiant 1990). The observations of these series of experiments were summarised by Gadiant (1990) thus, the coated forms are susceptible to mechanical damage but are relatively stable during the manufacture and storage of mash and pelleted feeds but not in extruded feeds which are considered more aggressive towards the coating materials.

The aim of the present experiment was to compare the anti-scorbutic properties of four ascorbic acid analogues when supplied at equimolar levels with respect to and in reference to L-ascorbate in a series of diets for the gilthead seabream. Hence the treatments derived compared the activity of calcium ascorbate, ascorbate-2-sulphate, polyphosphorylated ascorbic acid (Rovimix Stay-C) and fat coated ascorbic acid to a diet supplied with L-ascorbic acid at 250 mg kg⁻¹ and an ascorbic acid free diet.

5.3.2. Materials and Methods.

Forty Seabream fry (initial Weight 8.0 g) were stocked into each of six 80 l, self cleaning, polyethylene tanks over a 800 l bio-filter within a closed recirculation system providing a parallel flow of seawater through the tanks at 2.4 l min⁻¹. Water temperature and salinity were maintained at 24 °C and 33 - 36‰ saline respectively, the former by an

immersion heater, the latter by the use of a freshwater inlet balanced to compensate for evaporative losses. The pH was maintained between 6 and 7.5 by the use of calcium carbonate buffering while ammonia and nitrite were held within ranges tolerated by this species. Daylight balanced fluorescent strip lamps maintained an 8hr light: 16hr dark regime during the 18 weeks of the feeding trial.

The complete semi-purified diet formulation developed for use in the experiment which examined the qualitative requirements of the gilthead seabream was again employed to provide a basal diet. This was further modified to produce a series of test diets differing only with respect to their ascorbic acid source. In all, six test diets were formulated comprising ascorbic acid free (AAF), L-ascorbate (AA), fat coated ascorbic acid (FCAA), calcium ascorbate (CaAA), poly phosphorylated ascorbic acid (PA) and ascorbate-2-sulphate (AS). The ascorbic acid derivatives were included at levels which supplied 250 mg of L-ascorbate or equivalent activity per kg of dry diet. The source, ascorbic acid equivalence and residual ascorbic acid content after diet manufacture for each of these derivatives is shown in table 5.3.3. The conditions for diet manufacture, storage and presentation to the fish were as outlined in chapter 2 while the formulation and nutrient profile of the diet is presented in tables 5.3.1 and 5.3.2.

The weight gain, specific growth rate (SGR), feed intake, feed conversion ratio (FCR), feed conversion efficiency (FCE) and cumulative percentage mortality were monitored throughout the trial. The fish were also inspected daily for the appearance of gross deficiency symptoms.

Table 5.3.1: The Semi-purified test diet employed to evaluate the efficacy of L-ascorbic acid and four of its derivatives.

Ingredient	% Inclusion
Casein (Vitamin Free)	31.95
Gelatin (225 Bloom)	15.00
Corn Starch	19.70
Cod Liver Oil	11.84
Mineral Pre-mix ¹	10.00
Fat Soluble Vitamin Pre-mix ²	0.50
B Vitamin Pre-mix ³	0.50
Macro Vitamin Pre-mix ⁴	2.00
Amino Acid Pre-mix ⁵	5.18
Dictary Marker (CrO ₃)	0.50
Di-Calcium Phosphate	2.88

¹ Contribution of minerals to diet (mg kg⁻¹ Diet)

CaHPO₄ 22050, CaCO₃ 3000, NaCl 15000, K₂SO₃ 20000, MgSO₄·7H₂O 20627, FeSO₄·7H₂O 700, MnSO₄·4H₂O 227.3, ZnSO₄ 515.6, CuSO₄·5H₂O 160.0, CoCl₂·6H₂O 26.0, KI 15.0, Na₂SeO₃ 2.5, α-cellulose 17676.6

² Contribution of the fat soluble vitamins to diet (mg kg⁻¹ Diet)

Vitamin A Palmitate 4.2, Vitamin D₃ (99%) 0.046, Vitamin E Acetate 439, Menadione Sodium Bisulphire 780, α-cellulose 3776.75 (Contribution from Cod Liver Oil (CM³; Vitamin A 400 I.U., Vitamin D 40 I.U., Vitamin E 1.0 I.U.)

³ Contribution of the B vitamins to the diet (mg kg⁻¹ Diet)

Thiamin Hydrochloride 69.9, Riboflavin (96%) 208.3, Pyridoxine Hydrochloride 48.6, Niacin 800, Calcium Pantothenate 305.3, Biotin (2%) 300, Folic Acid (88.8%) 16.9, Cyanocobalamin (95%) 0.01, α-cellulose 3250.99

⁴ Contribution of The macro vitamins to the diet (g kg⁻¹ Diet)

Ascorbic acid and α-cellulose 2.216, Choline Chloride 10.62, Inositol 4.00, α-cellulose 3.164

⁵ Contribution of the amino acids to the diet (gkg⁻¹ Diet)

D-L-Methionine 4.80, L-Tryptophan 4.70, L-Threonine 8.70, L-Phenyl Alanine 5.80, L-Histidine 1.40, L-Arginine 7.00, L-Isoleucine 4.80, L-Leucine 5.00, L-Valine 9.10

Table 5.3.2: Proximate composition of the test diets. Moisture is expressed as a percentage of the diet as fed. Protein, lipid and ash are expressed as a percentage of the dry matter.

Moisture (%)	Protein (%DM)	Lipid (%DM)	Ash (%DM)	Nitrogen Free Extract (%)
37.77	48.57	7.54	8.95	34.94

Table 5.3.3: Ascorbic acid derivatives included in test diets.

Derivative in diet	Code	Source	Equivalence to L-ascorbate (%)	Retention in diets (mg kg ⁻¹)
Ascorbic acid free	AAF			14.46
L-ascorbic acid	AA	Takeda Chemical Co., Japan	100.00	178.37
Fat coated ascorbic acid	FCAA	Takeda Chemical Co., Japan	91.20	192.83
Calcium ascorbate	CaAA	Takeda Chemical Co., Japan	90.24	207.30
Ascorbyl-2-sulphate	AS	K.I. Chemical Co., Japan	52.35	145.43
Poly phosphorylated ascorbic acid	PA	Hoffmann La Roche, Switzerland	15.00	153.46

At the termination of the growth trial, whole blood samples were collected by caudal section into heparinised micro haematocrit tubes. The packed cell volume (PCV) was then immediately determined by micro haematocrit. The livers of five fish from each treatment were excised and at this point the hepatosomatic index was determined. The livers were then pooled, frozen at -70 °C and along with samples of each test diet the ascorbic acid content subsequently determined by the method of Tereda *et al.* (1978). Additional liver samples were prepared for histological analysis by quenching in liquid nitrogen cooled n-hexane (-70 °C) prior to examination as frozen sections as outlined in chapter 2.

Immediately after manufacture, diet samples were frozen and later, along with the carcasses of five fish sampled at the beginning of the trial and the pooled carcasses of five fish from each dietary regime sampled at the end of the experiment, subjected to an

analysis of proximate composition. Crude protein was determined by the Kjeldahl method, lipid by soxhlet extraction, moisture and ash as outlined by the A.O.A.C. (1990). Based on the proximate composition of the diets and fish, the protein efficiency ratio (PER) and the net protein utilisation (NPU) were calculated for the period of the growth trial.

The data were subjected to analysis of variance where $P \leq 0.05$ was judged to be indicative of a significant difference. Where the ANOVA revealed significant differences Duncan's multiple range test (Duncan 1955) was applied in order to characterise and quantify the differences between each dietary regime.

5.3.3. Results.

After eighteen weeks of feeding, the average weights of the test fish demonstrated significant differences between the treatments ($P < 0.01$). Those fish fed L-ascorbic acid tripled their initial weight and were significantly larger than those fed any other vitamin derivative. Those fish fed ascorbate-2-sulphate, the only treatment which failed to furnish a doubling of the initial weight, were significantly smaller than all other individuals except those fed calcium ascorbate. The fish fed ascorbic acid free diets showed average final weights which were not significantly different to those attained by the fish fed calcium ascorbate (CaAA) or fat coated ascorbic acid (FCAA). Fortification of the diets with polyphosphorylated vitamin C (PA) resulted in significantly larger fish than those fed diets devoid of supplemental ascorbic acid or supplemented with CaAA, AS or FCAA.

A comparison of overall specific growth rate (SGR) may not be extended to include the treatments AS and AAF since these dietary regimes induced a period of

adequate weight gain followed by weight loss. Overall, AA supplementation resulted in the fastest rate of growth with PA, FCAA and CaAA furnishing lower rates of weight gain in that order.

The cumulative mortality for diets 3 and 5 (AAF and AS) was high (47.5% and 40.0% respectively), while mortality resulting from AA and PA supplementation was low (0% and 2.5% respectively). Losses amongst those individuals fed diets supplemented with calcium ascorbate and fat coated ascorbic acid were 12.5% and 10% respectively.

Table 5.3.4: Growth and dietary performance criteria for gilthead seabream fed ascorbic acid free (AAF), L-ascorbic acid supplemented (AA) and diets fortified with ascorbic acid derivatives (AS, FCAA, PA and CaAA). (Values on each row assigned similar superscripts were not significantly different from each other ($p < 0.01$). Feed input, efficiency and utilisation values were calculated on a dry matter basis.

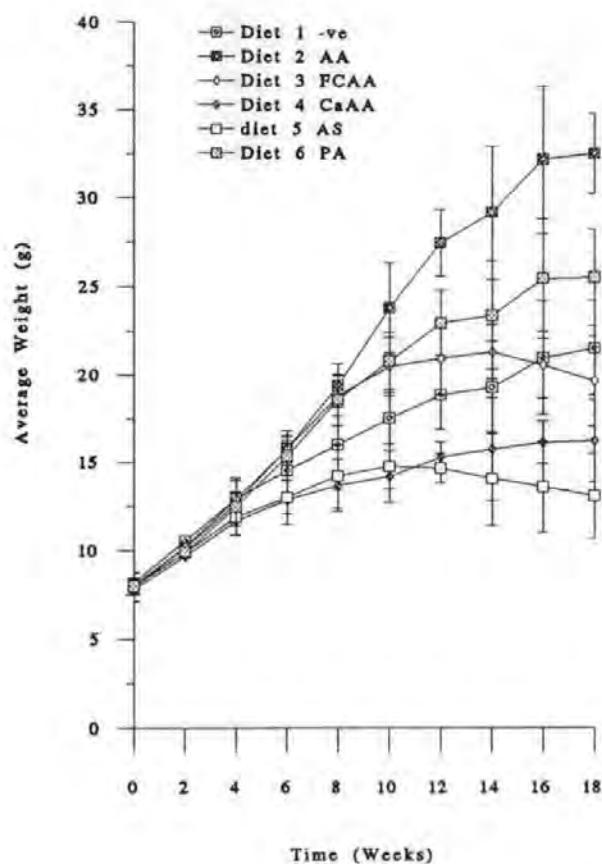
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
	FCAA	AA	AAF	CaAA	AS	PA
Initial Mean Wt (g)	8.17	8.06	7.89	7.77	7.92	7.97
Final Mean Wt (g)	21.52 ^{cd}	32.49 ^e	19.63 ^{bc}	16.21 ^{ab}	13.07 ^a	25.49 ^d
% Weight Gain	163.40	303.10	148.80	108.62	65.03	219.82
SGR (%d ⁻¹)	0.77	1.11	0.72	0.58	0.40	0.92
Daily Feed Intake (mgd ⁻¹)	244.00	331.00	256.00	196.0	158.00	271.00
Live Weight Gain (mgd ⁻¹)	106.00	194.00	93.00	67.0	41.00	139.00
Feed Conversion Ratio (FCR)	2.30	1.70	2.75	2.93	3.86	1.94
Feed Conversion Efficiency (FCE) (%)	43.37	58.66	36.35	34.10	25.89	51.34
Protein Efficiency Ratio (PER)	0.91	1.21	0.75	0.70	0.53	1.05
Net Protein Utilisation (NPU) (%)	13.61	19.05	10.46	9.84	8.08	16.43
Mortality (%)	10.00	0.00	47.50	12.50	40.00	2.50

Table 5.3.5: Haematocrit, hepatosomatic indices (HSI) and hepatic ascorbic acid content for the test fish after 18 weeks of test diet feeding. Values bearing similar superscripts are not significantly different from each other ($P < 0.05$)

Parameter		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
		FCAA	AA	AAF	CaAA	AS	PA
	n	5	5	5	5	5	5
Haematocrit (%)	Average	30.62 ^{ab}	32.90 ^b	28.30 ^{ab}	31.16 ^{ab}	20.10 ^a	29.16 ^{ab}
	S.E.	4.96	1.96	1.82	2.36	2.28	4.23
	n	7	10	9	10	8	10
HSI (%)	Average	1.01	0.98	0.83	1.00	0.89	0.92
	S.E.	0.16	0.11	0.08	0.14	0.10	0.07
[Ascorbic Acid] ($\mu\text{g g}^{-1}$ Wet Weight)		28.53	59.92	16.17	14.88	15.48	46.48

The feed intake and feed conversion efficiency parameters also reflected the overall growth performance and final weights of the fish. Feed intake was highest for the fish fed the diets supplemented with L-ascorbic acid and lowest for those fish fed diets containing ascorbate-2-sulphate.

An analysis of the proximate composition of the carcasses sampled at the end of the experiment shows that the protein content of the fish fed the ascorbic acid free diets was markedly lower than that of the fish fed the remaining diets. Additionally, the moisture and fat content of the fish were subject to modification. Thus, the lipid and moisture content of the pooled carcasses reflected the weight gains of the fish with the lowest lipid and highest moisture content observed in those fish which grew the least. The fact that the proximate analysis was carried out on pooled samples however, precluded any statistical analysis. Finally the proximate composition indicated that net protein utilisation and protein efficiency ratio also reflected the values observed for growth, FCR and FCE.



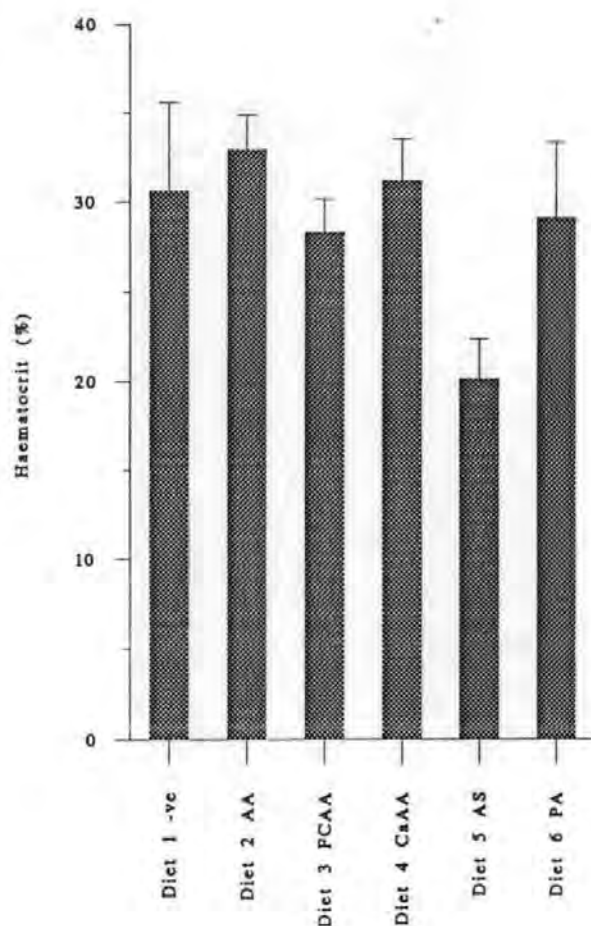
(a)

(a) Figure 5.3.1.

Growth of gilthead seabream fed diets supplemented with ascorbic acid derivatives.

(b) Figure 5.3.2.

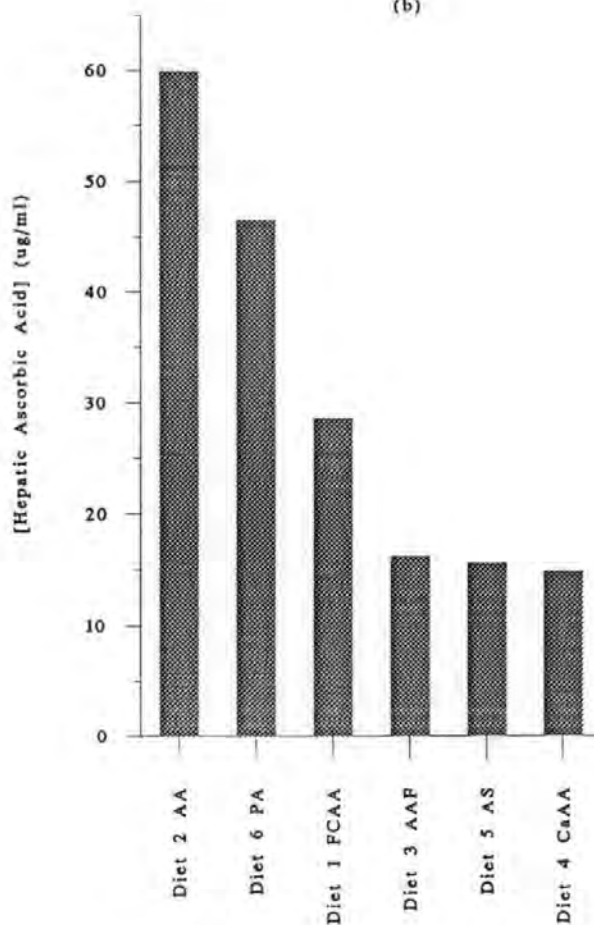
Haematocrit of gilthead seabream fed diets supplemented with ascorbic acid derivatives.



(b)

(c) Figure 5.3.3.

Concentration of ascorbic acid in the pooled livers of gilthead seabream fed diets supplemented with ascorbic acid derivatives.



(c)

Table 5.3.6: Proximate composition of initial carcasses and carcasses of test animals fed ascorbic acid free, L-ascorbic acid supplemented and diets fortified with vitamin C derivatives. Nutrient composition for five pooled individuals per diet is expressed as a percentage of the live carcass.

	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Initial fish	72.34	16.27	5.44	4.77
Diet 1. Fat Coated Ascorbic Acid (FCAA)	72.22	15.42	7.03	4.62
Diet 2. L-Ascorbic Acid (AA)	70.94	15.89	8.37	4.54
Diet 3. Ascorbic Acid Free (AAF)	73.07	14.91	6.27	5.01
Diet 4. Calcium Ascorbate (CaAA)	72.36	15.13	6.57	4.76
Diet 5. Ascorbate-2-Sulphate (AS)	74.26	15.25	4.56	5.60
Diet 6. Poly Phosphorylated Ascorbic Acid (PA)	70.17	15.88	8.29	4.45

The ascorbic acid content of the diets demonstrated that the present conditions of manufacture were suited to this type of experiment in that little loss of L-ascorbic acid was encountered. The diet designated as ascorbic acid free contained very low levels of ascorbic acid while the retention of activity for the calcium ascorbate and fat coated vitamin C were in keeping with their known stabilities. The residual levels of ascorbate-2-sulphate and poly phosphorylated vitamin C were poorer than the majority of the literature would suggest. The ascorbic acid content of the pooled livers reflected the patterns of growth and feed utilisation. Thus the hepatic ascorbic acid content was highest for those fish fed the diets supplemented with polyphosphorylated, fat coated or L-ascorbic acid. The lowest levels of vitamin storage were associated with the supplementation of the diets with ascorbate-2-sulphate and calcium ascorbate or an absence of ascorbic acid in the diet.

The individuals fed diets supplemented with ascorbate-2-sulphate were the only

fish to exhibit a packed cell volume which was significantly lower than that of the negative control (AAF) or the AA supplemented regime ($P < 0.05$). The hepatosomatic index demonstrated no significant differences between the regimen however, a series of histopathologies were observed in the liver.

In the present case, haematoxylin and eosin staining revealed some evidence of degeneration in the exocrine pancreas and changes in the general architecture of the hepatocytes in some dietary groups. Thus, for the fish fed diets containing calcium ascorbate (CaAA) the acinar cells were the largest and least modified of all dietary groups and, in addition, there was some evidence of "fat cells" and an accumulation of zymogen granules (Plate 12). The acinar cells of the fish supplied with diets 1, 2 and 3 (FCAA, AA and AAF) were also slightly atrophic. While there was evidence of atrophy in the acinar cells of the fish fed diets supplemented with poly-phosphorylated ascorbic acid (PA), the most extensive degeneration was associated with those supplied with ascorbate-2-sulphate (Plate 13). The general architecture of the livers was consistent between all the dietary treatments apart from diet 5 (AS) for which a loss of structural integrity was observed.

With the exception of those fish fed diet 5 (AS), the glycogen content of the hepatopancreas appeared to be normal for the species though the most intense reaction occurred in the livers of fish fed diets 1 (FCAA) and 5 (AS). Thus, those fish supplied with ascorbate-2-sulphate supplemented diets exhibited a very patchy distribution of glycogen. Using stereology, the neutral lipid content in the livers of all the fish demonstrated no significant differences between the treatments.

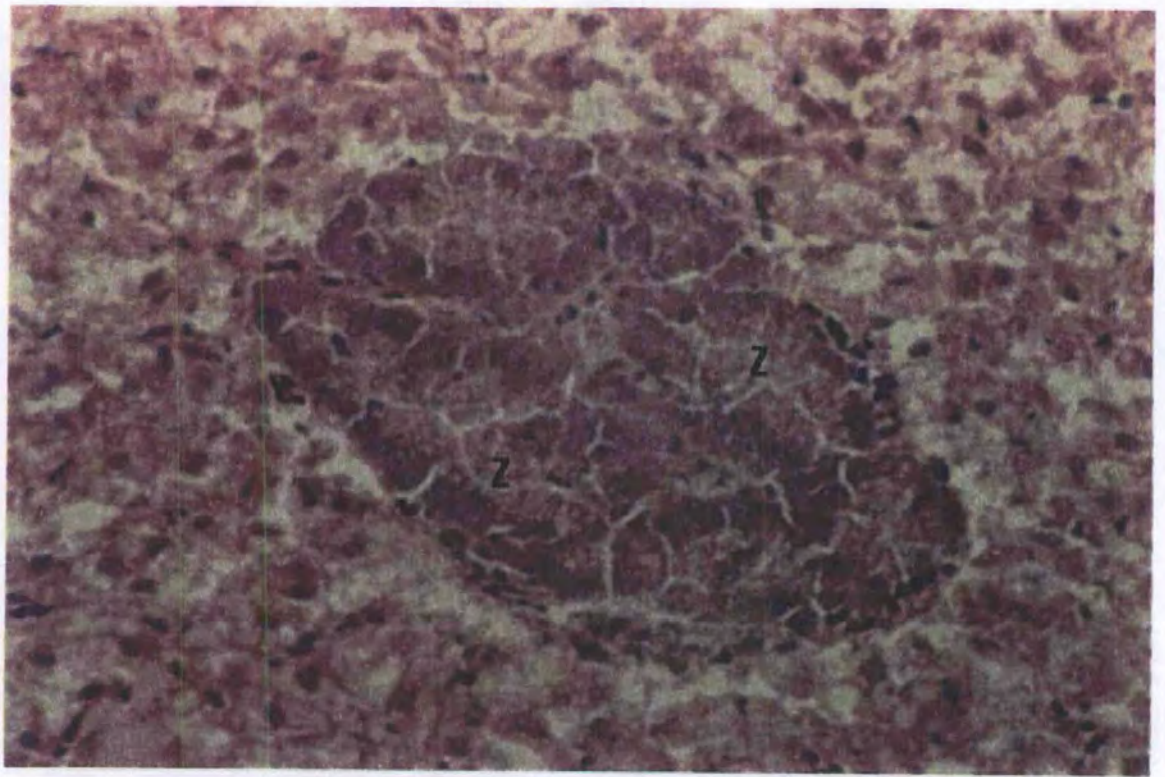


Plate 12: Accumulation of zymogen granules (Z) within intact pancreas of a gilthead seabream presented with a diet containing calcium ascorbate. (H and E, X 160)

Plate 13: Atrophy and increased blood cell activity (B) in the pancreas of gilthead seabream presented with a diet supplemented with ascorbate-2-sulphate. (H and E, X 160)

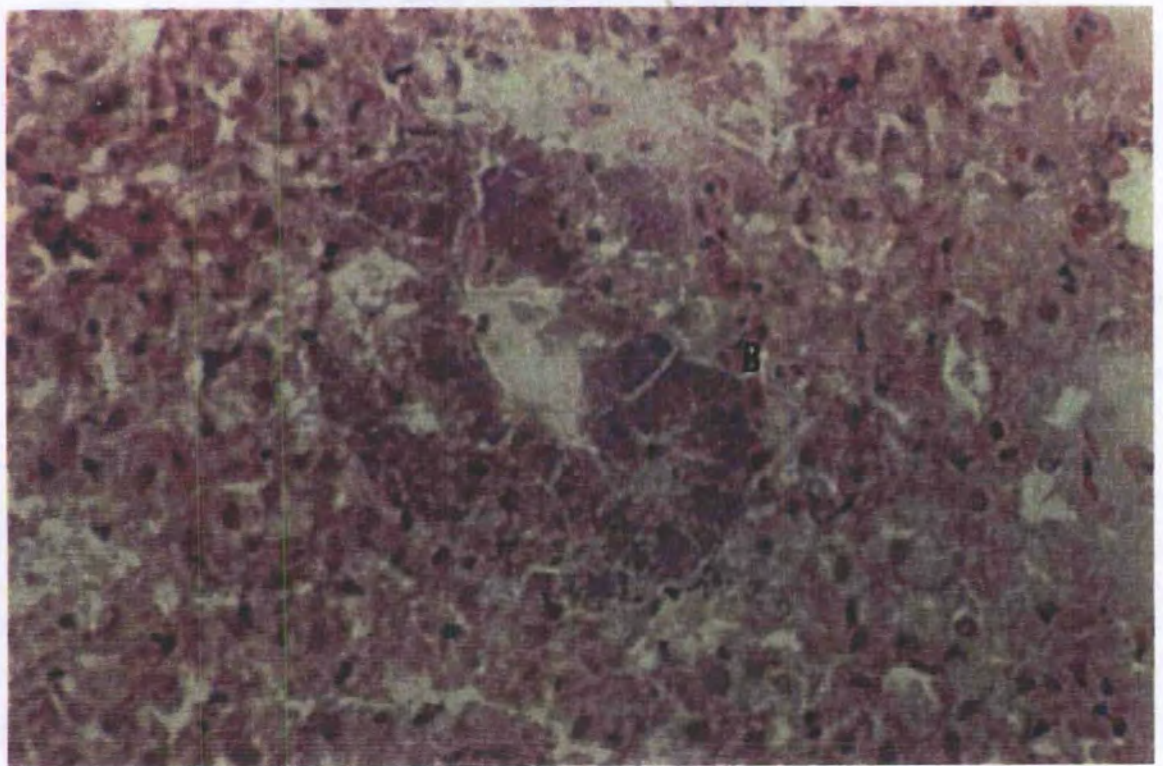




Plate 14: Extent of inter animal variability for two gilthead seabream supplied with a diet containing ascorbate-2-sulphate. (NAH, X 100). The fish on the left exhibits the pattern of staining observed amongst the control animals in 3.2, while the fish on the right clearly shows a uniform and diffuse distribution of NAH.

Using the schmorl reaction for lipofuscin, little or no reaction product was observed for those fish fed L-ascorbic acid or the fat coated vitamin C. For the remaining diets, little staining was observed in the hepatocytes though some lipofuscin was seen the tissues adjacent the macrophages surrounding the exocrine pancreas.

Those fish presented with diets containing calcium ascorbate (CaAA) exhibited the greatest lysosomal N acetyl β D-hexosaminidase (NAH) activity with both a high level of cytoplasmic staining and numerous intracellular clumps of activity. The livers of the fish fed diets containing L-ascorbic acid exhibited the least NAH activity both in terms of overall staining and the aggregation of stained material into clumps. To a lesser extent, the uniform distribution of lysosomal NAH observed for diet 4 (CaAA) was seen in the livers of the fish fed diets devoid of vitamin C or supplemented with fat coated ascorbic acid. However, the intracellular clumps were not observed in fish fed the AAF and FCAA supplemented diets. In general, the distribution of stain in the livers of fish fed diets 1 - 4 was homogeneous, however, where the diet was supplemented with ascorbate-2-sulphate or polyphosphorylated ascorbic acid, a high degree of inter and intra animal variation became apparent (Plate 14).

After eight weeks for the ascorbate-2-sulphate supplemented group, nine weeks under the ascorbic acid free regime and thirteen weeks for the fish fed Calcium ascorbate, a loss of scales became apparent and the external appearance of individuals under these regimes maintaining a full complement of scales was generally poor. Those fish fed diets devoid of ascorbic acid began to haemorrhage at the base of the fins after thirteen weeks. Scorbutic symptoms did not become apparent in the fish fed the diets supplemented with fat coated, polyphosphorylated or L-ascorbic acid.

5.3.4. Discussion.

L-ascorbic acid proved to be the most effective source of vitamin C activity with respect to all the parameters studied, providing after manufacture, sufficient activity to furnish an adequate rate of growth with acceptable feed conversion efficiency, an absence of deficiency related pathology and a complete absence of mortality throughout the 18 week trial. Under the conditions of manufacture and storage applied to the production of moist, semi-purified diets *ie.* cold mixing and extrusion (without the use of steam) followed by immediate freezing, little loss of activity was encountered by the L-ascorbate and thus the loss of activity normally associated with L-ascorbic acid supplementation was avoided. Consequently, under these conditions, L-ascorbic acid was a suitable vitamin source for this species.

Within the present experiment, ascorbic acid deficiency was characterised by poor weight gain and eventual weight loss, inferior utilisation of nutrients and a very high rate of mortality. As a consequence of the ability of ascorbic acid to donate one or two electrons, this vitamin is essential for the normal function of enzymes involved in hydroxylation reactions and in the metabolism of many key nutrients and pharmacological agents (Moser and Bendich 1991). Thus, ascorbic acid is essential for the normal function of the iron and copper containing hydroxylases, operates as a non specific reducing agent for a variety of enzymes, performs a variety of functions as an electron donor and acts as a reducing agent and oxygen radical quencher (Bender 1992). Consequently, ascribing a specific metabolic lesion to explain the poor performance of the ascorbic acid deficient fish is difficult. However, in all cases where vitamin deficiency has become apparent, a retardation of growth associated with a decrease in feed utilisation efficiency and an increase in mortality has been observed.

Scurvy is the symptom most commonly ascribed in animals as a manifestation of ascorbic acid deficiency. This condition arises as a consequence of the role of vitamin C as a cofactor in the activity of the proline and lysine hydroxylases which are involved in the post-synthetic modification of collagen (Bender 1992). Scorbutic pathology (scurvy) in fish becomes evident as skin and scale loss, exophthalmia, distortion of the gill filaments and in extreme cases, scoliosis and lordosis. The pathologies associated with connective tissue metabolism are frequently observed during ascorbic acid deficiency in the vast majority of fish species (Halver 1989). In the present experiment gross morphological pathologies indicative of scurvy became evident, thus within nine weeks of the start of the experiment a loss of scales became apparent and within thirteen weeks haemorrhage at the base of the fins of the ascorbic acid deficient fish was observed. However, the exophthalmia which characterised the ascorbic acid deficiency of the fish in the preliminary experiment (3.1) which investigated vitamin fortification in practical diets for the gilthead seabream was notable, in this case, by its absence.

In common with the preliminary experiment, scoliosis and lordosis did not become apparent even after 18 weeks of deficiency. These features have frequently been ascribed as diagnostic of extreme ascorbic acid deficiency and have been characterised in coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) (Halver *et al.* 1969, Hilton *et al.* 1978), plaice (*Pleuronectes platessa*) (Rosenlund *et al.* 1990), Indian major carp (*Cirrhina mrigala*) (Mahajan and Agrawal, 1980), *Tilapia aurea* (Stickney *et al.*, 1984) and *Cichlasoma urophthalmus* (Chavez De Martinez, 1990a). However, scoliosis and lordosis have not been recorded in all cases of deficiency hence these two symptoms were not apparent in ascorbic acid deficient Asian seabass (*Lates calcarifer*) (Boonyaratpalin *et al.* 1989), tilapia (*Oreochromis niloticus*) (Soliman *et al.* 1994) and

hybrid tilapia (*O. niloticus* X *O. aureus*) (Shiau and Jan 1992). Indeed, spinal abnormality was observed by Andrews and Murai (1975) in vitamin C deficient channel catfish (*Ictalurus punctatus*) fed practical diets but not when the fish were maintained on semi-purified diets.

A ten fold increase in weight was postulated by Halver *et al.* (1975) to be necessary for the generation a total ascorbic acid deficiency in rainbow trout. However, the present growth, nutrient utilisation and liver storage data, all of which were poorer for the fish fed ascorbyl sulphate and calcium ascorbate, would suggest that the fish were not wholly vitamin deficient despite the retardation of growth, mortality and appearance of scorbutic pathology.

The observations of the present experiment demonstrate that the mixture of mono, di and triphosphate esters of ascorbic acid (Rovimix stay-C) provided sufficient vitamin C activity to maintain growth, prevent the appearance of scorbutic symptoms and allow the maintenance of adequate levels of vitamin C in the hepatopaneas. However, drawing comparisons between the efficacy of the phosphorylated derivatives and crystalline ascorbic acid are complicated by the contrasting results for vitamin storage and the dietary performance criteria. Thus, when vitamin C is supplied as the phosphorylated derivative, the growth and feed utilisation of the fish was significantly poorer than when crystalline L-ascorbic acid was supplied. However, in contrast with these data, the ascorbic acid concentration in the pooled livers appeared higher for those fish fed ascorbyl phosphates compared to those fish maintained under any of the remaining regimes. The small amounts of liver available for vitamin assay at the end of the trial precluded the use of statistical interpretation for the quantification of these differences with respect to liver storage. If indeed, the growth and feed utilisation

parameters are indicative of vitamin efficacy then ascorbyl phosphate does not have a potency equal to that of crystalline L-ascorbic acid for the gilthead seabream under the present circumstances.

Anggawati-satyabudhy *et al.* (1989) demonstrated that for the rainbow trout, sodium ascorbate-2-phosphate provided an amount of vitamin activity sufficient for normal growth, FCR and liver storage at ascorbic acid equivalents of 20, 80 and 320 mg kg⁻¹ diet, while Dabrowski (1990) concluded that for the rainbow trout, L-ascorbate-2-monophosphate was as available as L-ascorbic acid and hence represents a highly suitable vitamin C source.

L-ascorbyl-2-polyphosphate was shown by Wilson *et al.* (1989) to be a very effective vitamin C source in the nutrition of the channel catfish resulting in rates of growth, feed efficiency and tissue storage levels greater than those observed when ascorbyl sulphate and coated ascorbic acid were supplied. Lovell and El Naggar (1989) demonstrated an equal activity for L-ascorbyl-2-phosphate Mg and L-ascorbic acid when supplied to channel catfish at levels equimolar with respect to ascorbic acid over a 14 week period. Additionally, in keeping with the findings of the present experiment, ascorbic acid retention in the liver and kidney of the channel catfish was higher where the phosphorylated derivative represented the dietary source of vitamin.

Further experiments have demonstrated the suitability of the phosphate esters of ascorbic acid in the nutrition of other aquatic species including; rainbow trout (Gabaudan *et al.* 1990, Verlhac and Gabaudan 1990), Atlantic salmon (*Salmo salar*) (Roem and Oines 1991), channel catfish (Brandt *et al.* 1985), yellowtail (*Seriola quinqueradiata*) (Kanazawa *et al.* 1992), Japanese flounder (*Paralichthys olivaceus*) (Teshima *et al.* 1991) and shrimp (Schigueno and Itoh 1988) though these studies have

not compared the activities of the derivatives applied with that of crystalline L-ascorbic acid.

Despite their availability to fish, the phosphate esters of ascorbic acid have been shown to be resistant to losses incurred during diet manufacture and storage (Gabaudan *et al.* 1990, Gadiant 1990, Roem and Oines 1991). Consequently, had the conditions of diet manufacture and storage within the present experiment, been more aggressive towards vitamin C, the high losses incurred by L-ascorbic acid and the higher residual activity of PAA may have established the poly phosphorylated derivative as a superior vitamin source when compared to L-ascorbate.

With respect to all the performance criteria measured in the present investigation, fat coated ascorbic acid performed poorly as a source of vitamin C. Thus by comparison to both phosphorylated and even un-protected L-ascorbic acid, this source of vitamin failed to supply sufficient activity to maintain adequate growth and feed utilisation though the appearance of scorbutic pathology was prevented. Additionally, the hepatopancreatic vitamin content was very low when compared to that of fish receiving the diets supplemented with L-ascorbic acid and polyphosphorylated vitamin C.

The coated forms of ascorbic acid have demonstrated anti-scorbutic activity and a degree of resistance to oxidation during diet manufacture and storage. The relative stabilities of silicon, glyceride and polymer coated ascorbic acid were investigated by Gadiant (1990). During pelleting alone, approximately 20 % of the ascorbic acid was lost from silicon and glyceride coated material while the polymer coated vitamin was more stable by a factor of 1.14. However, after 3 months of storage the loss of activity for the glyceride and silicon coated derivatives had reached 50 %. The polymer coated form was 1.5 times more resistant to oxidation during storage. Thus for pelleted feeds, the Gadiant

(1990) concluded that coating of the vitamin affords adequate protection during pelleting and storage but where more aggressive processes and conditions prevail then more resistant materials such as the polymer coating return a higher dividend in terms of ascorbate retention. Extrusion, which involves the use of higher temperatures and pressures than conventional pelleting, was shown by Gadiant (1990) to be more aggressive towards the coated forms of vitamin C. Thus, immediately after extrusion only 40 % of the original vitamin activity was retained demonstrating the susceptibility of the coating materials to physical damage.

Skelbaek *et al.* (1990), observed only a 19 % decrease in the activity of polymer coated vitamin C after warm pelleting contrasting with a 29 % loss for crystalline ascorbic acid. Additionally, after 6 weeks of storage at room temperature, loss of the coated form was only an eighth of that of the un-protected derivative. In the same study, both forms were shown by Skelbaek *et al.* (1990), to have equal bioavailability for rainbow trout. For the channel catfish, Murai *et al.* (1978), showed that over 20 weeks, a dose of 25 mg of ethyl-cellulose coated vitamin C per kg of diet prevented the appearance of spinal abnormality. However, a dose above 50 mg kg⁻¹ diet furnished maximum growth and raised blood and liver ascorbate concentrations in proportion to dietary vitamin input. Thus, ethyl-cellulose coated vitamin C demonstrated a similar bioavailability to L-ascorbate for the channel catfish. Similarly, Wilson *et al.* (1989) demonstrated the suitability of ethylcellulose coated vitamin C as an ascorbic acid source for the channel catfish though typically, carcass levels of the vitamin were significantly (approximately 50 %) lower than those observed where an equal amount of vitamin was supplied in the form of ascorbyl-2-polyphosphate

The availability of glyceride coated vitamin C to the tilapia was shown by Soliman

et al. (1986a) to be equal to that of L-ascorbate. Subsequently, Soliman *et al.* (1987) investigated the stabilities of a series of vitamin C derivatives and concluded that glyceride coated ascorbic acid was a highly suitable form of the vitamin being resistant to oxidation during manufacture and storage and, due to the hydrophobic nature of the glyceride coat, resistant to damage during the addition of water to the diet.

In the light of the high vitamin retention in the diet and the wealth of literature concerning the suitability of the coated forms of ascorbic acid in animal nutrition, the observations of the present experiment present rather a conundrum. The fish fed the coated ascorbic acid derivative during the course of the present study exhibited a low but sustained rate of growth throughout the 18 weeks of the trial resulting in a final weight which was not significantly different from that of the ascorbic acid deficient fish. However, the fish fed diets supplemented with the fat coated vitamin failed to exhibit scorbutic pathology and retained more vitamin C in the hepatopancreas than those fed the ascorbic acid deficient diets. One question which the present experiment could not resolve is the availability to the fish of the vitamin C within the fat coat. The study of Soliman *et al.* (1986a) demonstrated that a warm water carnivorous fish such as the tilapia has the ability to utilise coated vitamin C. In the present case it would appear that at least some vitamin C may be derived by the seabream from the diets as evidenced by the lack of scorbutic pathology, maintained growth and hepatopancreatic vitamin content of the fish, however the amount of available vitamin was insufficient to furnish optimal growth and nutrient utilisation. An investigation of the digestibility and absorption of coated vitamin C in the gilthead seabream would prove useful in resolving this question.

Of the four ascorbic acid derivatives tested during the present experiment, calcium ascorbate was the second poorest in terms of growth, mortality, feed utilisation, liver

storage and scorbutic pathology. The enhanced stability of the calcium salt when compared to L-ascorbic acid was apparent as illustrated by the improved retention of calcium ascorbate in the diet when compared to that of the free acid. This correlates with the observations of Soliman *et al.* (1987) who demonstrated an improved stability for sodium ascorbate when compared to L-ascorbic acid.

The present observations contradict the findings of Soliman *et al.* (1986a) who observed an equal nutritive value for both L-ascorbic acid and another metal salt of ascorbic acid (sodium ascorbate) in the nutrition of the tilapia (*Oreochromis niloticus*). Additionally, Teshima *et al.* (1991) illustrated the suitability of calcium ascorbate as a vitamin C source as evidenced by a 15 fold increase in weight and absence of nutritional pathology in Japanese flounder (*Paralichthys olivaceus*). In the light of the poor growth response of the fish and the low levels of ascorbic acid retention in the hepatopancreas, the ability of this species to absorb ascorbic acid derived from its calcium salt is obviously in question. Given that the uptake of dehydroascorbate from the intestine of rainbow trout has been proven (Dabrowski and Köck 1989) and the high retention of calcium ascorbate in the diets, it would not be unreasonable to postulate that the high affinity of the calcium for the ascorbate which protects the vitamin from oxidation also prevents dissociation of the ligand from the ascorbate in the gut, therefore decreasing the availability of the ascorbate for passive uptake across the intestinal mucosa.

The results of the present study indicate, that for the gilthead seabream, dietary supplementation with ascorbate-2-sulphate (vitamin C₂) at 250 mg kg⁻¹ with respect to vitamin C, had no positive effect on any of the measured nutritional parameters resulting in poor growth and eventual weight loss, a reduction in feed efficiency and excessive mortality. Additionally, those fish fed diets supplemented with ascorbate-2-sulphate

developed symptoms characteristic of ascorbic acid deficiency including a loss of scales, a reduced haematocrit and very low residual levels of ascorbic acid in the hepatopancreas.

The suitability of the sulphated derivatives of ascorbic acid as feed supplements for fish has been a source of some conjecture within fish nutrition. An extensive series of experiments by Halver *et al.* (1975) proposed the anti-scorbutic activity of ascorbate-2-sulphate (Vitamin C₂) for the rainbow trout and an ability of this species to store excess vitamin C. Subsequent publications (Tucker and Halver 1984, 1986, Halver *et al.* 1991) have postulated that approximately 70 % of an ascorbate-2-sulphate supplement may be absorbed from the diet by rainbow trout providing on a basis equimolar for ascorbic acid, equal anti-scorbutic activity. Additionally post absorption, an enzyme mediated interchange between C₂ (believed to be the storage form of the vitamin) and free ascorbate ensures that adequate levels of ascorbic acid are maintained within the fish.

Conversely, extensive studies on the rainbow trout (Dabrowski and Köck 1989, Dabrowski *et al.* 1990a, 1990b) have questioned the suitability of ascorbate-2-sulphate as a dietary vitamin C source for this species. Firstly, when compared to ascorbic acid, the absorption of ascorbate-2-sulphate from the gut of rainbow trout was much lower (Dabrowski and Köck 1989). Secondly, despite the negligible absorption of C₂ by rainbow trout when fed this derivative, the concentration of ascorbic acid in key tissues was higher than that of individuals fed diets devoid of vitamin C. Additionally, in direct contradiction of the findings of Tucker and Halver (1984 and 1986) and of Navarre and Halver (1989), ascorbyl sulphate appeared only in the tissues of those trout fed diets containing this derivative and was absent from the tissues of fish fed diets deficient in or supplemented with L-ascorbic acid (Dabrowski *et al.* 1990a). Finally the findings of

Dabrowski *et al.* (1990b) indicated that the tissue storage of ascorbic acid in rainbow trout fed ascorbyl sulphate was only one sixth of that recorded for fish fed diets of equal vitamin C content where L-ascorbic was the supplemented form of the vitamin.

Further investigations of the suitability of ascorbyl sulphate as a vitamin supplement have been carried out for a variety of species for example; channel catfish (Murai *et al.* 1978, Brandt *et al.* 1985, Lovell and El Naggar 1989, Wilson *et al.* 1989), Atlantic salmon (Maage *et al.* 1990, Sandes *et al.* 1990) and tilapia (Soliman *et al.* 1986a). The present study demonstrates that, if indeed ascorbate-2-sulphate has some anti-scorbutic activity for the seabream, on an equimolar basis with respect to L-ascorbate, vitamin C₂ does not possess vitamin C activity equal to that of crystalline ascorbic acid. This would corroborate the observations of Lovell and El Naggar (1989), who estimated the efficacy of ascorbate-2-sulphate in channel catfish to be one fifteenth of that of crystalline L-ascorbic acid and demonstrated a failure of this analogue to furnish normal growth below 130 mg kg⁻¹ of diet. Previously, Murai *et al.* (1978) had for the same species, observed that for maximal growth, 200 mg of ascorbate-2-sulphate kg⁻¹ of diet was required where only 25 mg kg⁻¹ of crystalline or coated ascorbic acid would suffice. Ascorbate-2-sulphate was also shown by Sandes *et al.* (1990) to be a less effective source of vitamin C activity than ascorbic acid for the Atlantic salmon since at reasonable supplemental levels, ascorbate-2-sulphate failed to provide sufficient activity to maintain normal physiological function indicated by biochemical and haematological indices. Additionally, in corroboration with Dabrowski *et al.* (1990b) and Murai *et al.* (1978) but contradictory to the observations of Tucker and Halver (1984, 1986), Navarre and Halver (1989) and Wilson *et al.* (1989) ascorbate-2-sulphate was not detected in any of the tissues examined.

In common with the low ascorbic acid content of the hepatopancreas observed in the present experiment for fish fed ascorbate-2-sulphate, Soliman *et al.* (1986a) working with tilapia (*Oreochromis niloticus*), Wilson *et al.* (1989) and Murai *et al.* (1978) with the channel catfish, all recorded lower storage levels of ascorbic acid in the tissues excised from fish maintained under an ascorbyl sulphate supplemented regime when compared to fish fed diets supplemented with any of the other derivatives tested. However, the tilapia in the study of Soliman *et al.* (1986a) and the channel catfish in the studies of Wilson *et al.* (1989) and Brandt *et al.* (1985) grew adequately and failed to exhibit any outward signs of ascorbic acid deficiency.

Dabrowski *et al.* (1990b) attributed the apparent anti-scorbutic activity of ascorbyl sulphate not to the properties of the material itself but to the product of its hydrolysis. Thus during storage ascorbate-2-sulphate is hydrolysed liberating free ascorbic acid which may, given suitably large dietary levels and sufficient storage time, be sufficient to maintain an absence of deficiency mediated pathology but insufficient to provide the high tissue concentrations observed when other derivatives are supplied.

One of the outstanding features of the performance of the fish fed diets containing calcium ascorbate and ascorbyl-2-sulphate is primarily their poor growth and feed utilisation when compared to the individuals maintained on diets devoid of supplemental ascorbic acid and secondly the similarity of the hepatic vitamin content of the fish fed ascorbic acid free diets or diets supplemented with calcium ascorbate and ascorbyl sulphate. Additionally, only those fish fed ascorbate-2-sulphate demonstrated a significant suppression in the packed cell volume. Anaemia has been recorded in ascorbic acid deficient rainbow trout (Hilton *et al.* 1978) channel catfish (Andrews and Murai, 1975) tilapia (Soliman *et al.* 1986a, Soliman *et al.* 1994), hybrid tilapia (Shiau and Yen,

1992) and plaice (Rosenlund *et al.* 1990). However, in the main, the severity of the pathology and extent of the mortality was greater in the ascorbate depleted fish.

After 14 weeks of feeding, a marked reduction in the average weight of the fish was observed for those individuals fed the ascorbic acid free diet. At this point it would appear that the vitamin requirement for growth had been breached resulting in a cessation of weight gain amongst these fish. Mortality and scorbutic pathology became apparent in those fish fed vitamin free diets and those supplemented with calcium ascorbate and ascorbyl sulphate. It would thus appear that a threshold exists such that the dietary input of a vitamin C supply of poor efficacy is of less value than a total absence of ascorbate from the diet. Halver *et al.* (1975), proposed that in very young rainbow trout, a ten fold increase in weight was required to deplete the total body store of ascorbic acid and thus allow the onset of acute deficiency related pathology. In the present case for the fish fed the ascorbate free diet, a weight gain of 2.5 to 3 fold was required for the manifestation of deficiency in terms of weight loss and externally apparent pathology. A reduced rate of weight gain was apparent for those fish fed the calcium ascorbate and ascorbyl sulphate supplemented diets for the first 10 weeks of the growth trial, thereafter a sustained weight loss became apparent for those fish maintained on diets containing ascorbyl sulphate. In the light of the proposition that the administration of an ascorbic acid derivative may interfere with the normal metabolism of this vitamin (Halver *et al.* 1975), it is not unreasonable to postulate that the administration of these two vitamin C derivatives (calcium ascorbate and ascorbate-2-sulphate) interfere with either the mobilisation and utilisation of stored ascorbate or the endogenous synthesis of ascorbate which for this species has not been conclusively refuted. Hence a thorough investigation of the pathways associated with and the

regulation of the metabolism of stored ascorbate and its interaction with exogenous or dietary vitamin C would prove both beneficial and enlightening.

Histological examinations of the gills of ascorbic acid deficient coho salmon (Halver *et al.*, 1969), rainbow trout (Gabaudan *et al.* 1990, Dabrowski *et al.* 1990a) and channel catfish (Lim and Lovell 1978, Wilson *et al.* 1989) have highlighted the necessity of vitamin C for the maintenance of structural integrity. Furthermore, the role of ascorbic acid in collagen biosynthesis in other tissues has been quantified at the biochemical level in channel catfish (Wilson and Poe 1973) and rainbow trout (Sato *et al.* 1978, Gabaudan *et al.* 1990) and characterised at the microscopic level in coho salmon (Halver *et al.* 1969, Ashley *et al.* 1975), rainbow trout (Sato *et al.* 1978, Dabrowski *et al.* 1990a, Gabaudan *et al.* 1990), channel catfish (Miyazaki *et al.* 1985) and *Oreochromis niloticus* (Soliman *et al.* 1985). However, little research has previously been carried out with regards the histopathology of ascorbic acid deficiency with respect to parameters other than connective tissue formation.

In the present case, a degree of damage to the architecture of the liver was observed amongst those fish fed diets supplemented with ascorbate-2-sulphate. Such degenerations in the structure of the hepatic parenchyma are likely to relate to the function of ascorbic acid as a cofactor in the hydroxylation of proline and lysine during connective tissue formation. Indeed, the loss of scales amongst this group would reinforce this notion. However, structural changes in the hepatic parenchyma amongst the fish fed unsupplemented diets was not apparent despite the significant damage to the skin. Consequently, the structural integrity of the liver may not be used as an advanced indicator of ascorbic acid deficiency.

Atrophy of the acinar cells of the pancreas was greatest amongst those fish fed

diets containing ascorbate-2-sulphate and poly-phosphorylated ascorbic acid though the diets containing L-ascorbic acid (AA and FCAA) also exhibited a lesser degree of pancreatic damage. Additionally, the schmorl reaction demonstrated the accumulation of lipofuscin in the tissues surrounding the macrophages associated with the exocrine pancreas for all the diets except those containing either crystalline or fat coated L-ascorbic acid. For the fish maintained on these two diets, the ascorbic acid was supplied in a form which required no chemical modification, while for the remaining derivatives *ie.* ascorbyl sulphate, ascorbyl phosphate and calcium ascorbate, some kind of chemical modification was required before these derivatives were in a suitable format for use in metabolism.

Miyasaki *et al.* (1992) demonstrated that orally administered ascorbyl-2-phosphate was hydrolysed to yield L-ascorbic acid in the pyloric caecae and intestine of rainbow trout (*Oncorhynchus mykiss*) prior to absorption. The authors postulated that an alkaline phosphatase with a low substrate specificity was responsible for such hydrolysis. Additionally, Miyasaki *et al.* (1992) observed the rapid hydrolysis of the phosphate ester administered by the intraperitoneal route to yield ascorbic acid and attributed this conversion to the use of acid phosphatases which are present in both the blood and within the lysosomes of cells. Similar observations were made by Buddington *et al.* (1993) for the channel catfish (*Ictalurus punctatus*). Thus, gut hydrolases were required for the removal of the phosphate group to liberate L-ascorbic acid which was then transported across the gut epithelium by Na^+ dependent carriers. Tucker and Halver (1984) proposed that the enzyme ascorbate-2-sulphate sulfohydrolase (AAS sulphatase) isolated from rainbow trout liver was the enzyme responsible for the modulation of circulatory vitamin C levels in fish by the liberation of ascorbic acid from ascorbate-2-sulphate. Dabrowski

et al. (1990a) detected no AAS sulphatase activity in the pyloric caecae of rainbow trout and the activity of this enzyme in the liver was not significantly modified when the fish were presented with diets containing ascorbate-2-sulphate or L-ascorbic acid. In addition Dabrowski *et al.* (1990a) observed lower tissue concentrations of ascorbic acid in trout maintained on diets containing an equimolar level of vitamin from ascorbate-2-sulphate and ascorbyl sulphate was only detected in the tissues of fish supplied with this derivative. Thus, the absence of AAS sulphatase activity in the pyloric caecae of rainbow trout, the presence of this derivative only in the livers of fish fed diets containing ascorbate-2-sulphate and the low levels of vitamin C in the fish fed the sulphated derivative indicates a mechanism by which some AAS is absorbed in an unmodified format.

Apart from the fish fed diets supplemented with AS and PA, the distribution of N acetyl β hexosaminidase (lysosomal NAH) in the liver appeared to be no different from that observed for the fish fed diets containing L-ascorbic acid. Consequently those animals maintained on diets containing the ascorbic acid esters AS and PA exhibited the widest inter and intra animal variation with respect the distribution of this lysosomal enzyme. In addition, the most intense NAH activity was associated with diets supplemented with the calcium salt of ascorbic acid (CaAA) and the least activity associated with L-ascorbic acid.

Given the degeneration observed in the pancreas (Haematoxylin and eosin and the Schmorl reaction for lipofuscin) and the changes in distribution and activity of lysosomal NAH in the livers of those fish fed diets containing vitamin C in a chemically modified form, it is not unreasonable to propose a relationship between hepatopancreas function and efficacy of the chemically modified ascorbic acid derivatives. Hence the role

of the hepatopancreas with regards both the production of enzymes required for the uptake of ascorbic acid derivatives and the post absorptive modification of ascorbic acid should be further investigated.

In conclusion, the present experiment demonstrated that in a moist, semi-purified diet, 250 mg of L-ascorbic acid, poly phosphorylated vitamin C or fat coated ascorbic acid per kg of diet were sufficient to prevent the onset of gross scorbutic pathology. However, it would appear that the efficacy of fat coated and poly phosphorylated vitamin C is low when compared to that of L-ascorbic acid. Thus, the manufacture of the test diets in the present experiment resulted in residual levels of L-ascorbic acid which satisfied the requirement of the seabream for this vitamin. However had the experiment been carried out using more aggressive diet manufacturing processes in for example a practical diet, the losses incurred by L-ascorbic acid may have resulted in a lower level of presented vitamin. Consequently, under more rigorous conditions the benefits of ascorbyl phosphate and coated ascorbic acid supplementation would be evaluated.

Finally, the poor performance of those fish fed calcium ascorbate or ascorbate-2-sulphate when compared to those fed ascorbic acid free diets highlights the potential for further research with regards the utilisation of ascorbic acid and the interaction of dietary ascorbate with endogenous vitamin stores.

Chapter 6

General Discussion

The observations arising from the present series of experiments underline the importance of the water-soluble vitamins in the nutrition of the gilthead seabream (*Sparus aurata*) and highlight the scope for further work in this area.

In chapter one, the qualitative aspects of vitamin nutrition were examined and a preliminary experiment demonstrated that in a situation employing practical type diets, on growing fish derive sufficient vitamins from the feed materials for the maintenance of adequate growth and efficient feed utilisation. However, for the maintenance of health, the contribution of vitamins from the feed materials was clearly shown to be insufficient over a long period.

Extending the scope of the preliminary experiment into an investigation of the qualitative B vitamin requirement of seabream fingerlings, clearly demonstrated the dietary essentiality of thiamin, riboflavin, pyridoxine, niacin and pantothenic acid. Under the conditions of major nutrient supply applied in that study, an insight was gained with regards the relative importance of these five vitamins with respect to growth, feed utilisation and health. Consequently, a deficiency of pyridoxine was responsible for the most serious suppression of growth as would be expected given the high protein content of the test diet and the key role of B₆ in the metabolism of protein.

The relationship between pyridoxine and the quality and quantity of protein supplied in diets for animals including fish has already been the focus of some research (Hardy *et al.* 1979, Fisher *et al.* 1984, Bai *et al.* 1991) though little attention has been paid to the relationship between B₆ and health in domestic animals. Ascorbic acid has

already been the subject of a great deal of attention with regards aspects of pathology and immunology in many species of fish (Li and Lovell 1985, Navarre and Halver 1989, Hardie *et al.* 1990, 1991, Verlhac *et al.* 1990) and given the recent attention paid to pyridoxine in human health and nutrition (Chandra and Sudhakaran 1990, Ockhuizen *et al.* 1990, Lakshimi *et al.* 1991, Arnadottir *et al.* 1993, Folkers *et al.* 1993) this vitamin appeared to be a suitable focus for novel research in fish nutrition. Thus, a study was designed which investigated the effect of B₆ on the potential for improved protein utilisation and examined the effect of high dietary doses of pyridoxine on the response of fish to acute stress. In summary, this experiment demonstrated that given a single dietary protein level, the provision of pyridoxine in excess of the minimal requirement (established for the seabream under conditions of similar protein supply by Kissil *et al.* in 1981) did not improve protein deposition but highlighted a potential for further research with regards the response of cultured fish to stress.

Pyridoxal phosphate is involved as a cofactor in the release of steroid hormone-receptor complexes from their tight nuclear binding and hence in the regulation of the transcription induced by the binding of such complexes (Bender 1992). Consequently, during pyridoxine deficiency, the response of an animal to steroid hormone action may be elevated due to prolonged hormone-receptor complex and DNA interaction. Conversely, the provision of additional B₆ may be beneficial in terms of increasing the rate of steroid-receptor complex release from its nuclear binding and hence down regulating the response of the animal to the steroid hormone. Thus, the supply of pyridoxine and its role in the response of fish to stress represents a field of potentially rewarding research.

The relationship between pyridoxine and immune function has been examined in

human and animal medicine and it would appear that cellular immunology may be highly responsive to pyridoxine status (Lakshimi *et al.* 1991). Only a small amount of research has been carried out to examine the relationship between B₆ and the immunology of fish (Hardy *et al.* 1979) and, given the role of B₆ in protein metabolism, aspects of the immune response which may be particularly responsive to pyridoxine status are cellular proliferation, cytotoxicity and the inflammatory response.

Returning to the experiment which demonstrated the essentiality of B vitamins in the nutrition of the gilthead seabream, a definite requirement for thiamin was exhibited though one which was not as stringent as that for pyridoxine. Thiamin, like pyridoxine but unlike niacin and riboflavin, is a vitamin which has a distinct involvement with the metabolism of a single major nutrient group *ie.* the carbohydrates. Currently, much emphasis is placed on the use of non-protein energy sources in feeds for fish and, given the relationship between thiamin and carbohydrate metabolism, the effect of elevations in the carbohydrate content in the diet represented an opportunity to investigate vitamin nutrition from an applied perspective. In summary, this experiment demonstrated that highly digestible carbohydrate could comprise upto 20 % of the feed without deleterious effects on the health of the fish. However, oil and starch were not mutually interchangeable on the basis of their metabolic fuel values. Thus, growth was optimised where lipid was used as the primary non-protein energy source while for those fish fed the high carbohydrate diet, the effect of supplemental thiamin input appeared inconclusive.

The former experiment raised at least two questions which need to be addressed. Firstly, the response of the fish to thiamin at the higher level of oil supplementation was obvious and hence the vitamin requirement appeared to exceed 5.0 mg kg⁻¹. However at

the lower level of oil, the growth and overall nutrient utilisation of the fish was independent of thiamin supplementation though a greater accumulation of hepatic glycogen was observed where the ration contained 5.0 mg of thiamin kg⁻¹ diet or more. Thus, the question of thiamin adequacy at this level of dietary carbohydrate is raised. Secondly, the haematology of the fish, especially with regards plasma glucose, was remarkably similar irrespective of dietary starch and thiamin content. These observations suggest that an investigation of the homeostatic mechanisms controlling the levels of metabolites in the blood merit further investigation with regards the effects of dietary carbohydrate load and thiamin status on key metabolic pathways such as glycolysis, gluconeogenesis and glycogenesis.

Having delineated the quantitative requirement of the gilthead seabream for biotin this merits the initiation of a further series of experiments to investigate the relationship between biotin and aspects of lipid supply. The relationship between biotin and the quantity of lipid in the diet has previously been investigated (Poston and McCartney 1974, Robinson and Lovell 1978) and to a limited extent the relationship between biotin and aspects of fatty acid chain length and saturation value has been investigated in the rainbow trout (*Oncorhynchus mykiss*) by Walton *et al.* (1984). However, such investigations have not been extended to the seabream with the eventual aim of incorporating oils of differing qualities eg. plant oils into practical rations for this species.

Ascorbic acid, due to its essentiality in the diets of most species of fish, has been the focus of a great deal of attention in fish nutrition. Despite the amount of research which has been directed towards the development of the ascorbic acid derivatives and the determination of their efficacy, no attention has been directed towards an

investigation of the interaction of dietary vitamin C and stored ascorbic acid. In the current work, the efficacy of several ascorbic acid derivatives as feed supplements in diets for the seabream was investigated. One of the outstanding observations was the apparently superior growth of the fish maintained on diets without vitamin C supplementation when compared to those presented with diets containing ascorbate-2-sulphate and calcium ascorbate. These data would lead to the inference that the supply of an ascorbic acid analogue with a low efficacy in some way interferes with the ability of the fish to utilise stored ascorbate for the maintenance of normal body function. Thus, an understanding of the mechanisms by which the fish may utilise stored ascorbic acid and the conditions necessary for such activities may promote a methodological approach to the development of ascorbic acid derivatives in diets for fish.

The histological examinations carried out on the B vitamin deficient fish and those supplied with the ascorbic acid derivatives exhibited a series of extensive pathologies. One of the more striking observations was the sensitivity of the pancreas to vitamin status. It is not unreasonable to postulate that the metabolic lesions induced during vitamin deficiency effect the function of the pancreas itself. For example pyridoxine deficiency may severely impair the ability of the exocrine pancreas to produce digestive enzymes for secretion into the gut. Additionally, the "work load" of this organ for the maintenance of homeostasis may be increased. For example, during thiamin deficiency the perturbations caused within carbohydrate metabolism may enforce a greater necessity for the pancreatic hormones such as insulin and glucagon. Thus, the role of the pancreas during and in response to vitamin deficiency should be examined more closely.

In terms of general liver function, vitamin deficiency was associated with an increase in the number of melano-macrophage centres which were frequently in close

association with the pancreas. Increases in the number and size of melano-macrophage centres in teleosts have been associated with the tissue catabolism which occurs during starvation (Agius and Roberts 1981, Micale and Perdichizzi 1990). However, Micale and Perdichizzi (1990) observed a small increase in the number of splenic melano-macrophage centres in fed *Diplodus annularis* during adaptation to laboratory conditions. Hence, Micale and Perdichizzi (1990) postulated that the occurrence of such centres was not just associated with tissue catabolism but also with adaptation to new environmental or physiological changes. In the present case, the association of the centres with the atrophic pancreas would suggest an association between the melano-macrophages and the degeneration of this organ. However, given the low feed intake of the vitamin deficient fish, it is also not unreasonable to assume that the increased number of centres is a response to starvation.

The current series of experiments could not address all aspects of vitamin nutrition and hence a vast potential for research still remains pertaining to the vitamins and their role in the nutrition of aquatic animals.

Relationship With the Major nutrients.

The water-soluble vitamins are implicitly involved in intermediary metabolism and given the role of vitamins as coenzymes for many in the reactions participating in the metabolism of the major nutrients, it is not unreasonable to postulate that modifications in major nutrient supply will influence the vitamin requirements.

The potential for modification in the requirements for thiamin, pyridoxine and biotin in response to changes in the amount and quality of single nutrients has briefly

been discussed. However, modifications of vitamin requirement with regards protein to energy ratio is a field which has been paid little attention thus far. It would seem logical however, that increasing the proportion of energy derived from protein would increase the demand for pyridoxine while increasing the non-protein energy component within the diet should elevate the requirement for vitamins associated with the catabolism of lipids and carbohydrates eg. thiamin and biotin. The relationship between major nutrient supply and vitamin requirements should be addressed in order not just to optimise vitamin supply but also to improve the utilisation of the major nutrients.

It is an established fact that, in humans, niacin deficiency cannot be induced without addressing the synthesis of niacin from tryptophan (Bender 1992). Most fish are believed to have a poor capacity for this conversion though a few species are believed to exhibit the potential for such operations (Chuang 1991) though the extent of this capacity is, as yet, unquantified. Hence, for species which possess the ability to synthesise niacin, the relationship between the sparing action of dietary tryptophan on the niacin requirement is worthy of investigation.

Choline has been shown to be an essential micronutrient in the diets of rainbow trout (*Oncorhynchus mykiss*), chinook salmon (*Oncorhynchus tshawytscha*), common carp (*Cyprinus carpio*) red seabream (*Chrysophrys major*), and white sturgeon (*Acipenser transmontanus*) by McLaren *et al.* (1947), Halver (1957), Ogino *et al.* (1970), Yone and Fujii (1974) and Hung (1989) respectively. However, some attention has also directed towards the sparing effects on the requirement for this vitamin of components in the feed such as lecithin, methionine and betaine (Ketola 1976, Wilson and Poe 1988, Rumsey 1991, Poston 1990 a,b, 1991 a,b). The extent to which these materials may act as choline precursors is discussed in chapter 3 and in summary, varies widely on a species by species

basis. Hence, the relationship between aspects of choline requirement and major nutrient supply require some scrutiny, especially where products such as soy beans which have a low methionine content, are incorporated in the diet. In addition, due to its hydroscopic nature, choline chloride is a material known to be potentially aggressive towards the components of the vitamin pre-mix (NRC 1993) and hence any efforts to reduce the level of choline supplementation in the diet would be welcomed by the manufacturers of feeds for fish.

Expression of Vitamin Requirements.

In the main, the dietary vitamin requirements of fish are expressed in concentration terms of *ie.* amount of vitamin in a known weight of feed *eg.* as mg of vitamin per kg of feed. Consequently the daily vitamin intake of the fish will vary in accordance with the total feed intake of the animal. In some respects, this terminology is adequate for the water-soluble vitamins since they act as coenzymes in many of the processes of metabolism. However, the amount of energy ingested by the animal depends on the density of metabolisable energy in the feed which is a factor of a number of variables such as the digestibility of the feed components and the protein : energy ratio of the entire feed. Hence, the requirement for those vitamins implicitly involved with intermediary metabolism will be influenced by the energy intake of the animal. Some vitamins *eg.* cyanocobalamin and folic acid function independently of intermediary metabolism in processes such as erythropoiesis and hence the requirement for these vitamins is less dependent of the overall metabolic activity of the animal. In such cases vitamin requirements would be adequately expressed in terms of daily intake with regards the body weight of the animal. However, for those vitamins implicitly involved

with intermediary metabolism, it would appear to be logical to develop the idea of relating vitamin requirement to the energy and major nutrient intake of the animal. This strategy would allow the supply of the vitamins essential for the maintenance of optimum performance under certain conditions of metabolic demand without over fortification with those which exhibit requirements independent of the metabolism of the fish. Prime examples of vitamins which could be supplied with reference to energy intake are thiamin, pyridoxine and biotin, the requirement for which would be expected to relate to caloric density.

Factors Effecting Vitamin Requirements.

As poikilotherms in an aquatic environment, other variables which potentially influence the vitamin requirements of aquatic animals are abiotic factors such as temperature, salinity and concentration of pollutants. The former pair indirectly influence vitamin requirements via metabolic changes while the latter has been investigated to a limited extent. Ascorbic acid supplementation has been positively correlated with improved tolerance of nitrite toxicosis in the steelhead trout (*Salmo gairdneri*) and the seabass (*Dicentrarchus labrax*) by Blanco and Meade (1980) and Scarano *et al.* (1991) respectively. Thomas *et al.* (1982) demonstrated a 60 % decline in the hepatic concentration of ascorbic acid in mullet (*Mugil cephalus*) exposed to chronic cadmium poisoning when compared to unexposed individuals and in addition the stores of ascorbate in the gill and brain were also depleted. However, the stored ascorbate in the kidney was largely unaffected by cadmium poisoning and an analysis of the cadmium content of the organs was positively correlated with ascorbic acid depletion. However, Lano *et al.* (1985) did not record any protective effect against dietary copper toxicity in

the rainbow trout (*Oncorhynchus mykiss*). During ascorbic acid deficiency, the guinea pigs in the study of Wagstaff and Street (1971) failed to maintain the activity of microsomal hydroxylative enzymes in response to organochlorine pesticide poisoning. Similarly Mayer *et al.* (1978) observed improvements of the detoxification mechanisms of channel catfish (*Ictalurus punctatus*) fed ascorbic acid supplements during exposure to the organochlorine pesticide, toxaphene and in addition, stored ascorbate was depleted during toxicant exposure. Thus, given the potential for improved tolerance of toxicants, the apparent decline in stored ascorbate in the organs of fish, and breakdown of the detoxification mechanisms of animals exposed to chronic poisoning, the relationship between ascorbic acid and detoxification should be further explored.

As briefly outlined in chapter one, a number of biotic factors including age, developmental state, the capacity for vitamin synthesis from precursors and the microbial gut flora effect the vitamin requirement of fish. One other factor which should be addressed is the vitamin requirements of broodstock fish with regards not just the performance of the adult but with respect the survival and growth of the fry.

Sandes (1987), reviewed the role of vitamin C in fish reproduction at three levels *ie.* broodstock supplementation, hatching performance and larval development. In broodstock fish, the simultaneous accumulation of ascorbic acid and elevated rate of steroidogenesis in the ovaries, was believed to be indicative of a relationship between ascorbic acid and the production of the steroid hormones associated with reproduction (Sandes 1987). The role of ascorbic acid in the synthesis of the catecholamines is well known (Bender 1992), however, the possible role of ascorbic acid in the steroid hydroxylating systems is unclear and hence, in fish, the relationship between ascorbic acid and steroidogenesis should be further investigated.

With respect to hatching performance, the effect of supplementing the feed of broodstock rainbow trout (*Salmo gairdneri*) on egg hatchability was investigated by Sandes *et al.* (1984). In summary, this experiment demonstrated that for optimal hatchability, the broodstock should be in receipt of a diet which yielded an ascorbic acid content in the eggs of 20 μg per gram. Finally, with respect to survival of the progeny, Soliman *et al.* (1986b) demonstrated that elevated supplementation of broodstock feeds with vitamin C (1250 mg kg^{-1}) significantly improved both the hatchability and survival of tilapia (*Oreochromis mossambicus*) fry.

At the time of maturity, the carotenoid content in the reproductive organs of fish is significantly elevated by both redistribution of tissue carotenoids and an increased deposition from dietary sources. However, the relative contribution from both these sources varies widely (Choubert 1987). Despite the accumulation of the carotenoids in the gonads, the function of these pigments is not yet understood. Consequently, Choubert (1987) points to literature which both proposes and denies the involvement of the carotenoids in the timing of maturation and fertility of broodstock, fertilisation of the ovules, survival of the eggs and in the development of the embryo.

A series of studies have investigated the role of specific carotenoids in reproduction in fish. Harris (1984) observed no alteration in the fecundity of three year old rainbow trout which were maintained on a canthaxanthin supplemented diet in the 6 month pre-spawning period. Torrissen (1984) demonstrated that the supplementation of diets for Atlantic salmon (*Salmo salar*) with astaxanthin and cantaxanthin failed to effect embryo survival and increased the photosensitivity of the eggs. However, the incorporation of these two pigments in the first feeding rations significantly improved the growth of the fry when compared to unsupplemented individuals. Watanabe and Miki

(1991) illustrated that astaxanthin significantly improved egg quality in the red seabream (*Pagrus major*) while β -carotene did not. This was attributed by Watanabe and Miki (1991), to the superior free-radical trapping capacity of astaxanthin.

Given the diverse chemical structures and biological functions of vitamin C and the carotenoids, the anti-oxidant and free-radical quenching abilities of these micronutrients appears to be a unifying feature. Hence, the potential for improved reproductive capacity and fry survival in response to the anti-oxidant micronutrients warrants further investigation.

Processing Stability and Bioavailability.

The instability of vitamins in feedstuffs, during feed manufacture and in the finished product is well known. Since little scope exists for the modification of the feed manufacturing processes, a major research effort has been directed towards the development of products resistant to processing damage. As described in chapter 5, ascorbic acid represents a prime example of such efforts which, in simple terms, have progressed along two pathways *ie.* encapsulation and chemical modification.

Encapsulation allows the use of vitamins which are known to be biologically active for the target species and easily quantified by existing analytical methods. However, the coating materials are frequently damaged by the aggressive processes of feed manufacture resulting in vitamin inactivation. Consequently, some effort has been directed towards the development of more resilient coatings however, the scope for such development is limited by the ability of the target species to access the vitamin within the coating.

The alternative approach is the synthesis of chemical derivatives which have

superior resistance to the rigors of diet manufacture. However, the derivatised vitamins frequently have unquantified availability and potency thus, the development of new derivatives is a constant source of new potential for research. Derivatisation of the vitamins also presents a problem at the stage of analysis. Thus, analyses based on bio-assay which allow the determination of vitamin availability, depend on the ability of the microorganism to metabolise the derivative as efficiently as the non-derivatised form. Additionally, chemical analysis relies on the ability of the analyst to manipulate the vitamer in such a way as to overcome the stability of the product prior to detection by the currently available means. In summary, the manufacture of vitamin derivatives generates potential for research not just in terms of evaluation of vitamin efficacy but also in terms of detection and quantification in order to keep pace with the development of the vitamin derivatives

The post-manufacture stability of the vitamins during both storage and at the time of presentation to the fish could be improved by the encapsulation of the entire pellet within a suitable hydrophobic material. This is very obviously demonstrated by an examination of the vitamin requirements determined for fry which are much higher than those reported for fingerlings and growers. Undoubtedly, a major factor in the difference in requirement may be attributed to the differences in metabolism of the differing size classes. However, the smaller surface to volume ratio of crumbled feeds must account for a significantly increased rate of vitamin loss via leaching. Though the technology is currently in existence for the encapsulation of small particles as exemplified by the coated forms of amino acids and vitamins which are currently available, the manufacturers of feeds are, at present, unable to adequately encapsulate particles as large as those required in the fingerling and grower stages of fish production.

Vitamins as a Means of Promoting Stress Tolerance and Oxidative Stability.

Intensive aquaculture is a practice involving the culture of very large numbers of fish in a relatively small area maintained entirely on artificial feeds. Consequently fish are subjected to a variety of non specific stressors related to high stocking density *eg.* lowered oxygen tension and crowding. Additionally stock management, including weighing and grading of the animals to maintain optimum growth and feed efficiency, are themselves stressful events. The potential for further research with regards vitamin B₆ in the moderation of stress has already been discussed though another vitamin which has been investigated with regards stress in fish is ascorbic acid.

The relationship between hormones, stress and vitamin C was first studied in fish by Wedemeyer in 1969. To summarise these results, during a non specific stress event applied to coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*), serum cortisol was elevated while interrenal ascorbate levels fell, however serum cortisol levels were maintained at the elevated concentration even after ascorbate levels had returned to normal. Mazeaud *et al.* (1977) reviewed the findings to that date and stated that the consequences of non specific stress in salmonids could be sub-divided into primary, endocrinal effects and secondary, metabolic responses. Endocrinal effects included elevations in circulatory catecholamines and corticosteroids while metabolic responses include pronounced elevations in the levels of circulatory glucose and free fatty acids. Such metabolic responses were maintained over a long period even where the duration of stress was relatively short and hence may prove to be reliable, easily measured indicators of stress.

The influence of salinity, temperature and capture stress on ascorbate distribution in the mullet (*Mugil cephalus*) were evaluated by Thomas in 1984. All three treatments

effected changes in ascorbate concentration in the organs studied. Elevations in gill ascorbate concentration were observed when the fish were placed in a hypo-osmotic medium, however in accordance with the findings of Wedemeyer (1969) ascorbate concentrations fell after capture. Both changes in salinity and temperature resulted in a lowering of the ascorbate content of the kidney though ascorbate concentration in the brain remained constant after capture. Long term reductions in the vitamin C content of the liver were not apparent. Thomas (1984) suggested an involvement of ascorbate in osmo-regulatory functions in the gills, salinity and thermal adaptation mechanisms and the response of renal tissue to adverse conditions.

Dabrowski and Ciereszko (1993) examined the relationship between fish size, origin and stress in rainbow trout (*Oncorhynchus mykiss*) and the distribution of ascorbic acid within the tissues. To summarise their findings; considerable variation was observed in the hepatic ascorbic acid concentration with respect to origin and size of the tested fish. In fish subjected to hauling stress liver and kidney ascorbate concentrations increased, however, dehydroascorbate levels significantly decreased when variations in fish size were included in the analysis. The authors thus recommended that the lowered ascorbate concentrations in the vital organs of older fish be borne in mind when handling and grading such individuals.

The relationship between vitamin C status and the tolerance of channel catfish to aquaculture related stressors was investigated by Mazik *et al.* (1987) drawing the conclusion that deficient fish were more susceptible to mortality induced by poor water quality with respect to lowered dissolved oxygen and increased ammonia concentration. The consequences of non-specific stress applied to ascorbic acid deficient turbot (*Scophthalmus maximus*) was examined by Gouillou-Coustans and Guillaume (1991).

This species does not exhibit the classical scorbutic symptoms associated with vitamin C deficiency and thus tissue ascorbate concentrations and tyrosine metabolism are used as symptoms of deficiency. The results showed that only hypertyrosinaemia was susceptible to change with respect to ascorbic acid status during periods of repeated stress.

As this brief review indicates, there is considerable scope for research with regards the role of vitamin C in aquaculture as a means of improving the resistance of fish to the stresses involved with aquaculture.

Given the observed changes in the ascorbic acid concentration in the tissues of fish exposed to stress, it is not unreasonable to expect changes in the ascorbic acid content of fish tissues during harvesting. Vitamin E, like vitamin C, is an anti-oxidant, and is believed to prevent oxidative damage in the tissues of animals. Hence the relationship between vitamin E and aspects of the oxidative status and keeping qualities of meat products has been thoroughly investigated in domestic animals (Buckley and Morrissey 1992). The potential for improved meat quality in pigs given an ascorbic acid supplement has been clearly demonstrated by Mouro *et al.* (1990) hence, the anti-oxidant properties of ascorbic acid warrant further investigation in terms of pre-harvest vitamin supplementation and stability of the fish flesh in the post-harvest period.

Overall, the current series of experiments have served to expand the present knowledge of the water-soluble vitamin nutrition of the gilthead seabream, not only in terms of requirement levels but also with regards the role of vitamins in the health of the fish. In addition, a number of areas which merit further investigation have been highlighted with respect to most species of fish though, vitamin nutrition still remains a largely un-explored field.

7.0. References

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