VITAMIN E NUTRITION AND PANCREAS DISEASE IN FARMED ATLANTIC SALMON (*SALMO SALAR* L.)

William J. Roy, B.Sc.(hons.), M.Sc.

A thesis submitted to the University of Stirling
for the degree of Doctor of Philosophy

Institute of Aquaculture
University of Stirling
Stirling, Scotland

October 1991

To My Parents

ACKNOWLEDGEMENTS

I would like to thank BP Nutrition Ltd. who provided my studentship on very fair terms. Their staff, and in particular Mr. Bob Watret and Dr. John Roberts, gave me generous support and advice. My supervisors, Dr. Kim Jauncey, Professor Allen Matty and Dr. Alistair Bullock, also have my sincere thanks for their guidance, wisdom and patience during the course of this study. The work described here was conducted at the Dunstaffnage Marine Laboratory, Oban, and I am grateful to the Director and staff for the use of their facilities. I would also like to thank the Director and staff of the Institute of Aquaculture, University of Stirling, who provided funds for the construction of the experimental tank system and considerable other material and technical support for this project.

I am grateful to many people who gave information and assistance. The advice of Mr. Gordon Bell in establishing the biochemical procedures, and the expertise of Ms. Mairi Beveridge, Ms. Laura Sinclair and Mr. Stuart Millar in preparing histological material, were especially valuable. I would also like to acknowledge the generosity of the salmon producers who provided fish for this study, in particular, Messrs. Colin Blair, David Gervis, Tim O'Hara, Ian Keen-Smith, David Mitchell and Jim McCartney.

Dr. Roy Clarke and Messrs. Brian Clarke, Brian Howie, John Joyce and Drew Milroy deserve especial thanks for their help in the design and construction of the experimental tank system, as do Mr. John FitzGerald and Mr. Mark Willcox, among others, who helped to look after the fish.

My wife, Jocelyn, helped in every way and she has my love and thanks for her support. Thanks also to my family, friends and fellow students for their encouragement and interest in this study.

ABSTRACT

Tissue vitamin E concentrations in farmed Atlantic salmon (*Salmo salar* L.), fed commercial diets, were measured by high-performance liquid chromatography. In healthy fish, fed diets containing about 20 mg 100 g^{-1} supplementary *all-rac*-α-tocopheryl acetate, vitamin E (α-tocopherol) accumulated in the liver and plasma. Over a 20 month period, beginning four months prior to seawater transfer, mean α-tocopherol concentrations ranged between 51 and 754 μg g^{-1} in liver and 7 and 68 μg ml^{-1} in plasma. Sharp increases in the α-tocopherol content of these tissues were related to a rise in the proportion of lipid as polyunsaturated fatty acid in response to transfer to seawater. There was no marked difference between tissue vitamin E concentrations in farmed and wild salmon.

In contrast, vitamin E depletion was found to be a consistent feature of pancreas disease (PD) in farmed salmon. The degree of depletion was found to vary from case to case. A serial study indicated that the onset of vitamin E depletion occurred early in the course of the outbreak. Some information was collected with regard to the cause and possible pathological effects of vitamin E depletion in PD, but definitive studies are still required. Parenteral administration of *all-rac*-α-tocopherol in molten cocoa-butter reduced plasma pyruvate kinase activity in affected fish from one outbreak of PD, suggesting that muscle degeneration in PD may respond to treatment with vitamin E. However, no effects on the histological signs of PD were noted. In a second outbreak, supplementation of the diet with 100 mg *all-rac*-α-tocopherol 100 g^{-1} produced no statistically significant elevation of plasma vitamin E or reduction in plasma pyruvate kinase activity. In a third case, dietary supplementation with a water-miscible preparation of *all-rac*-α-tocopheryl acetate produced no significant rise in plasma vitamin E concentrations and no histological evidence of any therapeutic effect was obtained.

Relationships between husbandry practices and vitamin E status were examined experimentally. Acute handling and crowding had no observable effect on plasma α-tocopherol concentrations. Chronic crowding stress produced a

slight, but statistically insignificant, reduction in plasma and liver α-tocopherol concentrations. Chronic crowding stress did not precipitate signs of vitamin E deficiency in salmon fed diets with no supplementary source of α-tocopherol.

Starvation caused a significant depletion of α-tocopherol within 3 weeks in the plasma and liver of post-smolts in experimental tanks, but had no effect on tissue vitamin E concentrations in one sea-winter salmon held in sea-cages over a 4 week period. Differential feeding rates of 1-3% body weight day^{-1} over a six week period had no measurable effect on plasma vitamin E concentrations.

The dietary level of α-tocopheryl acetate had no effect on the magnitude of the cortisol response to acute handling stress and no beneficial effect of vitamin E supplementation was determined in fish under conditions of chronic crowding.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 PANCREAS DISEASE

1.1.1 Introduction

Pancreas disease (PD) was first described by Munro *et al.* (1984) as an epizootic disease of farmed Atlantic salmon, *Salmo salar* L., causing loss of growth and characterised by degeneration of the exocrine pancreas. Its aetiology is unknown, but the condition has been responsible for serious production losses in commercial salmon farming operations.

1.1.2 Pathology

1.1.2.1 Structure and function of the normal pancreas

The structure of the exocrine pancreas in Atlantic salmon was described by Munro *et al.* (1984). The exocrine pancreas is diffuse, being composed of strands of tissue embedded between the pyloric caeca and around the stomach, bile duct, anterior spleen and upper intestine. Adipose cells, the size and number of which vary with the season and state of maturity of the fish, lie both within and around these strands. The endocrine glands are situated amid exocrine tissue and consist of large, principal islets (Brockmann bodies) and smaller accessory islets (Brinn 1973). Blood vessels, protected by a collagenous sheath, ramify within the organ. The exocrine tissue consists of numerous small lobules or acini separated by a connective stroma, one cell thick, composed of elongate epithelial cells. The acinar cells are pyramidal and radially orientated around a central lumen. In histological sections, zymogen granules can be readily observed in the apical region of these cells, some in the process of discharge into the central lumen. The lumina converge into ducts lined with columnar epithelium which eventually lead to a single large duct opening into the duodenum in close proximity to the bile duct. Particularly in post-smolts and older fish, the larger ducts are protected within a sheath of connective tissue surrounded by fibrocytes and collagen. Solute flow along the ductal system is probably generated by secretion from bicarbonate-water cells (Bencosme and Lechago 1971). In histological sections

the acinar cytoplasm is densely basophilic, whilst the endocrine islets are less so (fig.1.1).

Using electron microscopy, Munro *et al.* (1984) observed two different forms of acinar cell. Form A contained a large, spherical or ovoid nucleus with distinct chromatin, situated in the distal third of the cell. In the region of the nucleus were large amounts of rough endoplasmic reticulum (ER) in the form of sheets or plates. The rough ER became progressively more fragmented so that towards the proximal end of the cell it consisted of small spheres, the cisternae of which contained granular material. In addition, a well-defined Golgi apparatus, autophagous vacuoles, numerous large, round mitochondria with distended cristae, and zymogen granules, were situated proximally in the cell. Form B was irregular in shape, with a similar or smaller nucleus than A, and rough ER condensed into sheets or whorls throughout the cell. No Golgi body, autophagous vacuoles or zymogen granules were observed regularly, but this cell type typically contained elongate mitochondria with narrow cristae. Cell form A was regarded to be active in protein (zymogen) synthesis and release, whilst form B was inactive, possibly undergoing regeneration.

1.1.2.2 Histopathology and diagnosis

Some controversy exists over the histopathology typical of PD, there being disagreement over which structural changes may be considered primary effects of the condition (Ferguson *et al.* 1986*b*; McVicar and Munro 1989). In the absence of more accurate diagnostic methods it is not possible to resolve this question at present. The situation is further complicated by the wide variety of secondary changes which may be associated with PD.

Exocrine pancreatic degeneration

Munro *et al.* (1984) described a sequence of histopathological changes in the exocrine pancreas which they regarded as characteristic of PD. These they related to the clinical progression of the condition (table 1.1). In the pre-acute phase, they described minor changes in the exocrine tissue including an increase, soon after transfer to seawater, in the proportion of form B

exocrine cells. Under the light microscope this was barely discernible as an increase in basophilia, loss of zymogen and possible hypoplasia of the exocrine tissue. Later, immediately prior to the acute phase, they observed the formation of syncytia of acinar cells and cloudy swelling, further loss of zymogen and cytoplasmic vacuolation pending necrosis of these cells. These changes preceded any gross signs of PD. The acute stage featured an acute and generalized (often haemorrhagic) pancreatic necrosis coinciding with the expression of gross signs of the condition (overt PD). Within individual fish, pancreatic necrosis appeared to progress rapidly, leading to the complete loss of the exocrine tissue within a period of hours or days (McVicar 1987). Remnants of the exocrine tissue could be discerned within the gut and, later, infiltration of the pancreatic tissue by lymphocytes and fibrocytes was also noted. The original description of the post-acute phase as a chronic recovery phase was modified by McVicar and Munro (1989) to portray three possible sequelae to the acute phase: rapid, slow or no recovery. In the first, regeneration was rapid occurring within 2-4 weeks and fish made a good recovery. In the second, regeneration of acinar tissue was delayed, occurring within 1-3 months. The persistence of small foci of degeneration in such fish suggested a continual necrosis of regenerating elements. Recovery was delayed and over this period peri-pancreatic fat deposits became depleted. In the third, fibrotic replacement of exocrine and adipose tissue was observed and a proliferation of blood capillaries noted on some occasions. Fish so affected were unlikely to show a complete recovery.

Ferguson *et al.* (1986*a*) also described a marked reduction in pancreatic tissue, with loss of acini, stromal condensation, depletion of fat together with some haemorrhage and congestion, and fibrosis. An increase in cellularity indicative of mild inflammation was also noted in this study. Similarly, Kent and Elston (1987) reported a diffuse pancreatic necrosis leading to severe pancreatic atrophy and fibrosis. Classically, the exocrine tissue is highly liquified or shows fibrotic replacement, but other components (ie. islet tissue, exocrine tubules, blood vessels, etc.) are largely intact. In the present study this lesion has been termed exocrine pancreatic degeneration (EPD) (fig. 1.2). Identification of EPD is currently the principal means for the diagnosis of pancreas disease. Total absence of exocrine tissue has been

Table 1.1. Proposed sequential pathology of PD (after McVicar and Munro 1989)

1. Pre-acute :		Early predominance of inactive form of acinar cell, possibly hypoplasia of acinar tissue Prior to acute: focal syncytia formation, cloudy swelling and vacuolation of all acinar cells.	
2. Acute :		Acute generalized (haemorrhagic) necrosis and later infiltration with leucocytes and fibrocytes	Inappetence and listlessness. Haemorrhage between caecae and erythema of peri-pancreatic fat. Gut contains necrotic elements
3. Post-acute:	a. Rapid recovery	Acinar cell regeneration rapid (2-4 weeks)	Resumption of growth
	b. Slow recovery	Acinar cell regeneration slow (up to 3 months)	Inappetance, emaciation, fat depletion, muscle atrophy
	c. No recovery	Fibrotic replacement of exocrine tissue and peri-pancreatic fat. Proliferation of blood capillaries	Dark, eel-like. Extreme muscle wasting

the criterion used in critical experimental studies of the condition (McVicar 1987), but clinically, pancreatic necrosis may be patchy and less than complete (Munro *et al.* 1984; McVicar 1987).

Cardiac, oesophageal and skeletal myopathies

Degenerative polymyopathy associated with PD was first described by Ferguson *et al.* (1986*a*). In this study, the hearts of fish with PD showed coagulative necrosis of the ventricular myocardium in both spongy and compact layers, with loss of striation, increased eosinophilia and granular and vacuolar change. Some fibres contained macrophages and showed a proliferation of satellite-type sub-endocardial cells giving the appearance of increased cellularity (fig.1.3). Atrial myocardium was often also affected and mural thrombi were sometimes present on the endocardium. Similar changes were found in the skeletal muscle around the lateral line, particularly in red muscle fibres, but also in the rest of the body musculature. Some fish showed a

fibres, but also in the rest of the body musculature. Some fish showed a similar degeneration of oesophageal muscle. Kent and Elston (1987) noted a mild, diffuse epicarditis in fish with PD from the western United States and Palmer (1988) recorded pericarditis and cellular infiltration of the myocardium associated with a number of cases of PD from Ireland. In Norway, Poppe *et al.* (1989) reported severe degeneration of the inner spongious myocard and extensive degeneration of skeletal muscles, together with a mild to severe epicarditis, in fish with PD in coincidence with an infectious pancreatic necrosis virus infection. T.Murphy (personal communication 1991) observed epicarditis followed by an increase in cellularity of the myocardium, due to the accumulation of lymphocytes or proliferation of intercardial cells, particularly at the juncture of the spongious and compact myocardial tissues. In their mild form these were discrete, but they became merged in more severe cases. Thrombus formation and, in red skeletal muscle, hyaline degeneration of muscle fibres was also observed. Ferguson *et al.* (1986*a*) noted that polymyopathy provided a good explanation for the gross effects of PD. Cardiac and red muscle lesions would be expected to impair swimming ability, whilst a poor peripheral circulation, compounded by net damage, could explain injury to the skin and fins.

Cardiac, oesophageal and skeletal myopathies in association with exocrine pancreatic degeneration were not recorded by Munro *et al.* (1984) and, in a further study, Bell *et al.* (1987*b*) detected no histological evidence of cardiac myopathy in PD. McVicar and Munro (1989) regarded muscle degeneration as a feature of the post-acute phase representing a condition secondary to PD. However, Ferguson *et al.* (1986*a*,*b*) proposed that severe myocardial degeneration developed simultaneously with pancreatic necrosis and was the most significant lesion of PD. There is little doubt that histological signs of muscle degeneration, however common, are not evident in all outbreaks of PD (A.McVicar, personal communication 1987). Biochemical methods have been used to detect such changes with greater sensitivity and objectivity (section 1.1.2.5), but it remains unclear whether myopathy presents itself to a greater or lesser degree in every case of PD and is an integral part of the condition, or whether it occurs in some but not all cases and should be regarded as a complicating factor.

Changes in the structure of the heart have been noted in smolts immediately after transfer to seawater (H.Rodger, E.Branson, personal communications 1990) and, although mild and infrequent, were also observed in the hearts of apparently healthy salmon during studies on PD (Ferguson *et al.* 1986*a*,*b*). The pathological significance of such changes is uncertain. The fish heart is known to undergo rapid and large scale structural changes during the course of normal growth, (Farrell *et al.* 1988), in response to changes in temperature (Farrell 1987), diet (Farrell *et al.* 1986) sustained or increased exercise (Totland *et al.* 1987; Houlihan *et al.* 1988), and during maturation (Robertson *et al.* 1961; Farrell *et al.* 1986), so it may be difficult to distinguish between physiological and pathological responses.

Recent clinical observations suggest that myopathies can be associated with all phases, preceding, accompanying or succeeding EPD (T.Murphy, E.Branson, personal communications 1991). In routine samples where myopathy was noted prior to exocrine pancreatic degeneration its severity invariably increased during the period of pancreatic necrosis (T.Murphy, personal communication 1991). That the severe changes observed in association with PD are of pathological significance is suggested by the increased level of mortality noted during a number of PD outbreaks with accompanying cardiomyopathy (Palmer 1988). 'Sudden death syndrome' was recently recognised on a number of farms in Ireland (T.Murphy, personal communication 1991), and also in Scotland (E.Branson, personal communication 1991). This condition typically affects fish about 8 weeks after an outbreak of PD and results in mortalities among actively feeding fish in good condition. On histopathological examination, affected fish showed regeneration of acinar tissue with focal pancreatic fibrosis, only moderate cardiomyopathy, extensive red skeletal muscle degeneration and lesions in the muscles of the pectoral fins and gill covers (T.Murphy, personal communication 1991). Polymyopathy in the absence of exocrine pancreatic degeneration occurs rarely in Ireland and Scotland (H.Rodger, E.Branson, personal communications 1991). In Norway, a condition known as 'Cardiomyopathy syndrome' (CMS) has been described (Amin and Trasti 1988; Ferguson *et al.* 1988, 1990). In CMS, severe degeneration of the cardiac and skeletal muscle leads to high mortality typically among older (1 or 2 sea-winter) fish with no history or evidence of EPD. Poppe *et al.*

(1989) and Ferguson *et al.* (1990) compared cardiac myopathies associated with PD to those of CMS. Although these conditions are both clinically and pathologically distinct, a common aetiology cannot yet be ruled out (Ferguson *et al.* 1990).

Whether the degenerative changes in pancreas and muscle have a common cause, or whether they are due to independent factors and result from different disease processes is not known. Therefore, in the absence of pancreatic necrosis, histological evidence of muscle degeneration is probably not a sufficiently specific feature of PD on which to base a diagnosis. Nevertheless, whether or not muscle degeneration is a consistent feature of PD, the occurrence of severe muscle degeneration may affect the prognosis of an outbreak and should therefore be routinely evaluated.

Other histopathological changes

A wide variety of secondary pathologies, presumably resulting from the effects of metabolic change, starvation and intercurrent disease, have been reported in association with PD. For example, injuries to the liver, gills, eye and intestine have repeatedly occurred together with PD on individual sites (Munro *et al.* 1984; Ferguson *et al.* 1986*b*; McVicar 1987; Kent and Elston 1987). McVicar (1987) regarded these as atypical. Munro *et al.* (1984) described large eosinophilic bodies within hepatocytes of fish with PD, and Ferguson *et al.* (1986*b*) reported multi-focal to focally extensive liver damage in fish with PD, but neither attached any pathological significance to these changes. Intestinal degeneration associated with PD was noted by Kent and Elston (1987). Degeneration of the intestinal collagen layer has been observed previously in studies on fish with PD (P.Southgate, personal communication 1991), but is not a consistent finding (R.Reece, W.Roy, unpublished observations). Munro *et al.* (1984) observed moderate leucocyte hyperplasia in the head and mid-kidney, occurring towards the end of the acute phase of PD, possibly in response to pancreatic degeneration. Poppe *et al.* (1989) described a severe hypoplasia of the respiratory epithelium, goblet cell proliferation and fusion of secondary lamellae in the gills of fish collected during an outbreak of PD in Norway. Characteristic 'sunburn cells' have also been observed in fish with PD suffering UV-induced integumental

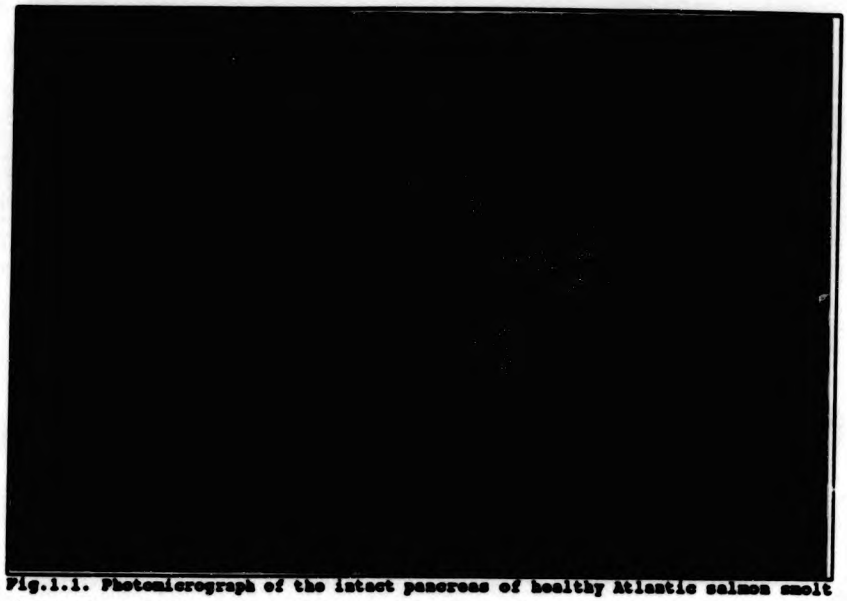

Fig.1.1. Photomicrograph of the intact pancreas of healthy Atlantic salmon smolt showing densely basophilic acinar tissue (H&E x 50).

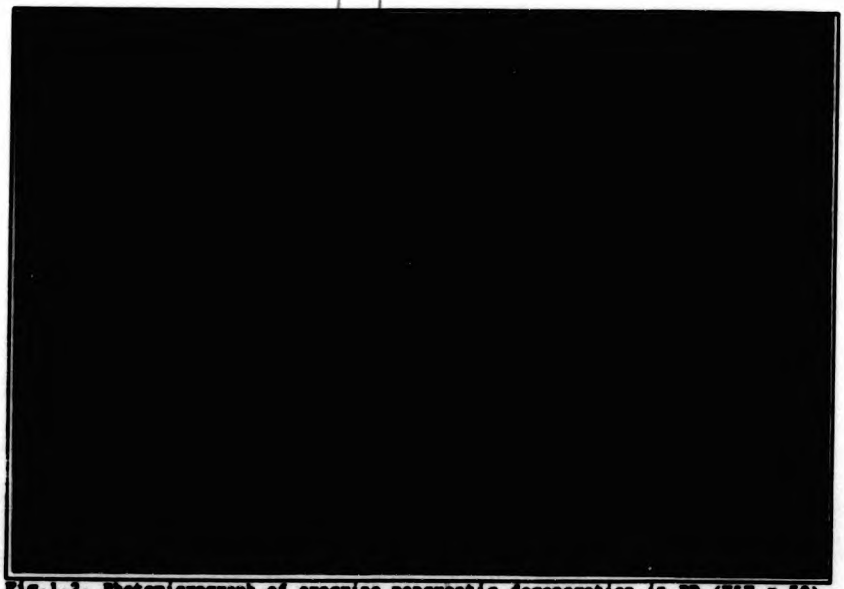

Fig.1.2. Photomicrograph of exocrine pancreatic degeneration in PD (H&E x 50). Note total absence of basophilic acinar cells.

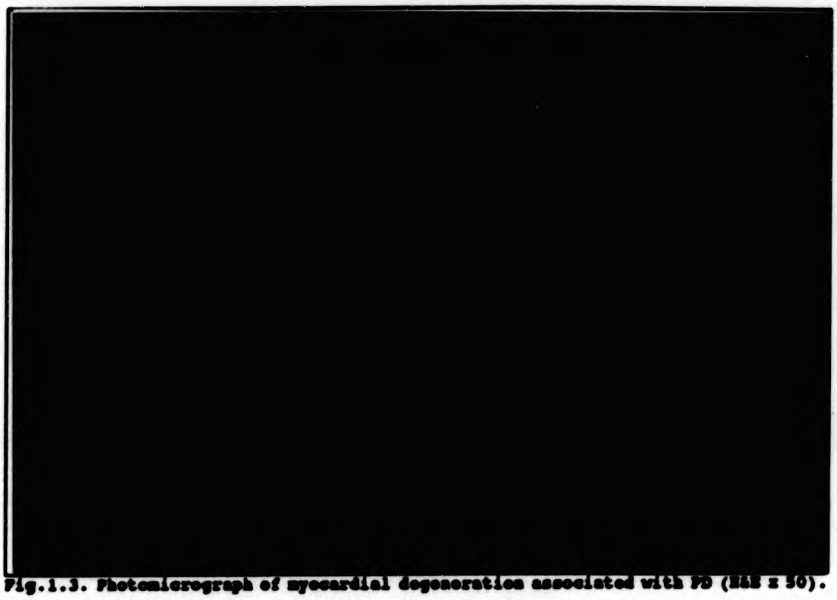

Fig.1.3. Photomicrograph of myocardial degeneration associated with PD (H&E x 50). Note areas of increased eosinophilia and vacuolation.

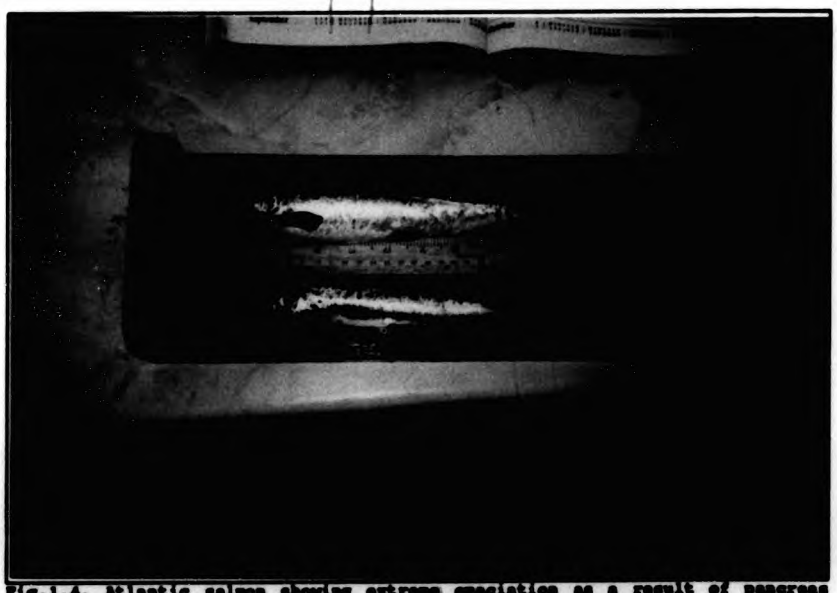

Fig.1.4. Atlantic salmon showing extreme emaciation as a result of pancreas disease (below) in comparison with an apparently healthy fish (above).

necrosis (so called 'summer lesion syndrome') (T.Murphy, personal communication 1991).

1.1.2.3 Gross clinical signs

Mortality

The level of mortality resulting directly from PD was regarded by Munro *et al.* (1984) to be low, and McVicar and Munro (1989) considered morbidity as a result of increased susceptibility to secondary disease to be variable (see section 1.1.5.1). Exceedingly high mortality/morbidity rates have been connected with PD enzootics in Ireland and Norway (Poppe *et al.* 1989; H.Rodger, T.Poppe, personal communications 1991), but whether as a direct result of PD or due to secondary factors is not clear. Ferguson *et al.* (1986*a*) indicated that affected fish had poor stress tolerance in that they died quickly when netted out of the cage. Such fish entered *rigor mortis* rapidly.

Behaviour and external appearance

In individual fish, the gross manifestations of PD typically include anorexia, dark coloration and listlessness (Munro *et al.* 1984), but not all fish with exocrine pancreatic degeneration show these signs (Ferguson *et al* 1986*b*; Kent and Elston 1987). Anorexia occurs early in the acute phase and, within affected cages, a decrease in feeding activity is often the first indication of PD. Increased jumping behaviour has been noted at this time (Munro *et al.* 1984). Subsequently a number of non-feeding fish cease to shoal and become lethargic, gathering near the surface in the down-current corners of the cage. Such fish are unable to maintain either their position or orientation and, although they may capture food pellets, they then egest them. Emaciation is a common feature of outbreaks where recovery is delayed and becomes apparent approximately 1 month after the cessation of feeding (D.Mitchell, personal communication 1991). Non-recovers become severely emaciated and appear dark and eel-like (fig. 1.4). Fish with gross signs of PD often suffer eroded and ulcerated skin and fins, an increased lice burden and secondary disease.

Internal appearance

Internal examination of moribund fish typically reveals a gut empty of food but containing white 'sloughings'. White faecal casts are easily observed in small cages and can give an indication of otherwise barely noticeable PD. In the acute phase, affected fish may show a line of haemorrhage between the pyloric caeca (Munro *et al.* 1984) and erythema of the pancreas and peri-pancreatic fat with petechial haemorrhage in these tissues (Bell *et al.* 1987*b*; Kent and Elston 1987). Frank haemorrhages extending through the intestine were noted by Kent and Elston (1987) in a small proportion of affected fish. Atrophy of visceral fat occurs if recovery is delayed. Resumption of normal feeding and locomotory activity, accumulation of fat and an increase in weight and condition factor are signs of recovery.

1.1.2.4 Haematology

Munro *et al.* (1984) reported that, in fish with PD, haematocrits and erythrocyte morphology were apparently normal.

1.1.2.5 Biochemistry

Pancreatic enzymes

Pancreatic enzyme assays have been used to detect PD on fish farms without recourse to histology (T.O'Hara, personal communication 1989). Trypsin determinations on homogenized pyloric caeca have shown a correlation between the occurrence of EPD and very low trypsin activity. This observation supports the view that exocrine pancreatic degeneration is complete in affected fish, but such tests do not provide an early indication of PD (D.Houlihan, personal communication 1991). Normal trypsin activities are achieved with only sparse regeneration of pancreas (McVicar 1986).

Enzyme indicators of myodegeneration

Ferguson *et al.* (1986*b*) reported an increase in plasma creatine phosphokinase (CPK) activity in fish showing chronic degeneration and repair of the heart and skeletal muscle in association with PD. Bell *et al.* (1987*b*)

reported that plasma pyruvate kinase (PK) activity was also increased in an outbreak showing no histological evidence of cardiac myopathy. Both CPK and PK have been used as indicators of muscle degeneration in various species (Hyldgaard-Jensen 1977; Machlin *et al.* 1978; Chen and Lin 1980; Lang 1981; Chen *et al.* 1983). Ferguson *et al.* (1986*b*) interpreted plasma CPK elevation in PD to be an indication of the specific cardiac and red skeletal muscle lesions observed histologically and, in favour of this, CPK activities appeared to be elevated to similar degrees in fish showing polymyopathy regardless of whether or not they exhibited gross signs of PD (Ferguson *et al.* 1986*b*). However, Bell *et al.* (1987*b*) regarded the increase in plasma PK to be a sign of the incipient, general loss of muscle bulk during the process of emaciation, although fish tested were in the early stages of PD. Evidence to support this interpretation was gathered from electrophoretic PK isozyme analysis. The results indicated that the bulk of plasma PK was of the M-type isozyme emanating primarily from skeletal muscle (both white and red). However, the electrophoretic isozyme profile typical of heart muscle may have been masked rather than absent (Bell *et al.* 1987*b*).

Other indicators of tissue damage

The activity of serum glutamate dehydrogenase, a mitochondrial-bound enzyme released during tissue damage, was consistently elevated in farmed fish with PD, but not in experimental fish with induced PD. Serum C-reactive protein activities were variable and showed no consistent change in fish with PD (A.McVicar, personal communication 1987).

Nutritional status

There is no published information on the body composition of fish with PD and most information on the nutritional status of affected fish comes from biochemical analysis of the plasma and liver. Plasma protein concentrations are consistently reduced in fish with acute PD. Munro *et al.* (1984) found that losses were primarily in the electrophoretically fast-moving, small molecular weight, albumin fraction, whilst Ferguson *et al.* (1986*b*) found that both albumin and globulin fractions were depleted. Plasma protein concentrations were not reduced in feeding fish with exocrine pancreatic degeneration and

polymyopathy, indicating that plasma protein depletion may be due to anorexia associated with PD (Ferguson *et al.* 1986*b*). However, Munro *et al.* (1984) suggested that these losses were greater than could be explained by anorexia. Faecal protein concentrations may increase in fish with acute PD (T. O'Hara, personal communication 1987), presumably as a result of malabsorption due to pancreatic insufficiency.

Vitamin E concentrations have been found to be reduced in the plasma and liver (Ferguson *et al.* 1986*b*) and also the white muscle (Bell *et al.* 1987*b*) of fish with PD. Ferguson *et al.* (1986*b*) indicated that liver fatty acid profiles were unchanged, which suggests that increased concentrations of polyunsaturated fatty acid are not responsible for vitamin E depletion. Further consideration to the role of vitamin E in PD is given in chapter 4. Ferguson *et al.* (1986*b*) also found selenium concentrations to be reduced in the plasma and liver of fish with PD. The seleno-enzyme glutathione peroxidase (GPX) is a functional indicator of selenium status (Rotruck *et al.* 1973). Erythrocyte GPX activities were reduced in fish with overt PD, but not in fish with no gross clinical signs, and may therefore be due to anorexia (Ferguson *et al.* 1986*b*). Bell *et al.* (1987*b*) found plasma GPX activity to be normal, but liver GPX was significantly lower in fish with PD.

Mineral balance

Plasma concentrations of calcium, magnesium and phosphate were little changed in affected fish (Ferguson *et al.* 1986*b*), suggesting no loss of osmoregulatory ability in PD.

Endocrine involvement

Liver glycogen concentrations are reduced in fish with PD and emaciation occurs rapidly. These features may simply be as a result of starvation. Alternatively, it has been suggested that they are indicative of a diabetic state since the pancreatic hormone insulin is known to be involved in hepatic glycogen deposition and other catabolic processes (Christiansen and Klungsøyr 1987). However, there is no consistent histological evidence of change in the endocrine pancreas and plasma insulin concentrations are not reduced in PD (R.Raynard, personal communication 1991).

1.1.3 Epizootiology

Some epizootiological information has been derived from case studies (McVicar 1987). Since records of clinical disease may not reflect sub-clinical trends these findings must be treated with caution.

1.1.3.1 Geographical distribution and prevalence

PD was first recognised in Scotland in 1976 (McVicar 1987), but it may have been present since at least 1972 as 'over-winter runting syndrome' (Anon 1988). The condition has also been described in fish from Ireland (Palmer 1988), Norway (Poppe 1987), North America (Western seaboard) (Kent and Elston 1987), France (Atlantic coast) (F.Baudin-Laurencin, personal communication 1991), Spain (Galicia) (J.Barja, personal communication 1990) and England (A.McVicar, personal communication 1990). Within Scotland, cases have been recorded from all parts of the north and west coasts (Northern Isles and Hebrides to Clyde) (McVicar 1987). The number of cases increased steadily between 1982 and 1987 in line with the growth in number of sites over this period (table 1.2). In Ireland, the condition was first recorded in 1984. Its incidence increased dramatically between 1984 and 1987 (table 1.2) and between 1987 and 1990 an estimated 75-95% of sites, in all areas, became affected each year (T.Murphy, personal communication 1991). In Norway, PD was first recognised in 1985 (Poppe 1987) and is now widespread. Its earlier presence may have been masked by the extensive occurrence of 'Hitra' disease (Egidius *et al.* 1981) (T.Poppe, personal communication 1991). PD was recognised in North America in 1986 (Kent and Elston 1987) and is believed to have occurred in France since 1986 and in Spain since 1989.

1.1.3.2 Species and strain susceptibility

PD commonly occurs in farmed Atlantic salmon. No cases have been recorded in wild Atlantic salmon or feral sea-trout (*Salmo trutta*), or in rainbow trout (*Oncorhynchus mykiss*), either wild or farmed, even when held on the same site as affected Atlantic salmon. Recently, however, the condition

Table 1.2. Known cases of PD in Scotland and Ireland 1976-1990

Year	Scottish cases	Irish cases
1976	1	
1977	2	
1981	3	17
1984	-	1
1985	25 (19%)	4 (33%)
1986	35	11 (69%)
1987	43	16 (73%)
1988		(75-95%)
1989		(75-95%)
1990		(75-95%)

Note: Figures in parentheses show numbers of PD outbreaks as a percentage of the total number of operational marine salmon farms.

has been reported in *Salmo trutta* farmed in sea-cages in France (F.Baudin-Laurencin, personal communication 1991). Palmer (1988) suggested that some epizootiological data indicated differences in strain susceptibility, but this has yet to be verified.

1.1.3.3 Relationship to marine environment

Under natural conditions, PD appears to have been restricted to the seawater growth phase and is absent from freshwater hatcheries and smolt production sites (McVicar 1987) and indeed from freshwater ongrowing (landlocked salmon) operations. However, PD has been induced experimentally in Atlantic salmon parr in freshwater (see section 1.1.4.3) and there is some concern that it may be introduced into freshwater farms through movements of affected broodstock.

1.1.3.4 Relationship to season and age of fish

Ongoing PD has been recognised throughout the year, but McVicar (1987) found that the majority of new cases occurred between 3 and 10 months after transfer to seawater. New outbreaks are rare between November and March, but

this absence may be due to a lack of detection, the appetite of healthy fish being low at this time of year. The precise timing of an outbreak has on some occasions been related to the date of transfer to seawater. The results of a survey of outbreaks at several sites suggested that the severity of PD was linked to its timing (D.Mitchell, personal communication 1991). Outbreaks earlier in the year showed quick recovery, whilst outbreaks in the late autumn or winter were protracted.

Cases are most frequent among fish in their first year in seawater, but one and even two sea-winter fish have become affected, particularly on the first outbreak at a site. Under experimental conditions, parr appear to be more susceptible to exocrine pancreatic degeneration than smolts (section 1.1.4.3), but some anecdotal evidence suggests that larger, faster growing fish are more prone to PD.

1.1.3.5 Temporal and spatial variability

McVicar (1987) indicated that the first outbreak at a site appeared to be a random occurrence in old or newly established sites, although the risk of an outbreak was increased if other farms in the vicinity were affected. Repeat sampling showed that PD developed in newly-stocked smolt populations each year thereafter, exceptions having been sites which have operated a fallow period before the introduction of new stock and an onshore tank site where fish were held separately and tanks disinfected. This study recorded that PD did not recur in the previously affected population and also indicated that the severity of outbreaks, in terms of their duration and the number of affected fish, showed considerable site to site and year to year variability.

McVicar (1987) also noted that clinical disease affected different cage populations on any one site to different degrees and at different times, and proposed that the cage rather than the site or water body could be regarded as an epidemiological unit. McVicar and Munro (1989) found that, within a cage population, all fish showed signs of exocrine pathology, but fewer showed complete exocrine pancreatic degeneration. Other studies have found that the majority of fish in an affected cage showed EPD, but fewer showed gross signs of PD (Munro *et al.* 1984; Ferguson *et al.* 1986*a*,*b*).

In an affected cage, many fish may develop signs of PD rapidly and synchronously and may also recovery together, alternatively individuals may develop and recover from the effects of the condition in progression. Similarly, different cage populations on a farm may develop PD in unison or at different times. However, some cages may remain unaffected whilst others become affected.

1.1.3.6 Relationship with features of site and husbandry practices

The possible importance of secondary factors and in particular of the role of stress in susceptibility to, and the expression, duration and severity of an outbreak of PD, is apparent from data obtained from case studies. Environmental factors which may affect the course of an outbreak are numerous and include exposure to strong currents, low salinity flushes, storms, predators, pathogens and net-fouling organisms (McVicar 1986, 1987; McVicar and Munro 1989). Husbandry-related factors may be equally important. Delay in cleaning nets, treating disease or deterring predators, handling, exposure to toxins (eg. TBT-based antifoulants), and the use of poor quality feed and oversized pellets, have all been implicated in recent outbreaks of PD (McVicar 1986, 1987; McVicar and Munro 1989). Kent and Elston (1987) identified transport trauma and *Cytophaga-Flexibacter* infection as possible stressors precipitating an outbreak of PD in the United States. In Scotland, use of the sea-lice treatment 'Aquaguard' has been linked to the expression of overt disease (D.Mitchell, personal communication 1991).

Overfeeding has been linked to outbreaks of PD. Farms which fed fish either continuously, or more than 6 times per day, at rates greater than the that required to maximise the specific growth rate, were more seriously affected by the condition than sites where feeding was more closely regulated (T.O'Hara, personal communication 1987). Anecdotal evidence suggests that, although strong currents can exacerbate PD, sites which have good water exchange may escape the condition in some years.

Husbandry-related factors may explain geographical variability in the prevalence of PD. In Ireland the use of more exposed, offshore sites, higher water temperatures, the practice of holding several year classes on one site,

use of higher stocking densities and feeding rates and of 'high energy' diets throughout the first sea-year may be linked to the greater frequency and severity of outbreaks (T.Murphy, personal communication 1991).

1.1.3.7 Relationship with physiological state of fish

Experimental evidence of the importance of stress in PD is lacking. Treatment of fish with the synthetic corticosteroid dexamethasone, prior to challenge, had no significant effect on the prevalence of experimentally-induced EPD (R.Raynard, unpublished observations 1989). However, a relationship with nutritional state has been demonstrated. Raynard *et al.* (1991) showed that fish with low tissue vitamin E concentrations were significantly more susceptible to experimentally-induced EPD than fish with higher tissue vitamin E concentrations, and in the same study, an increased susceptibility in fish fed a diet low in polyunsaturated fatty acids was also demonstrated. The mechanisms of this action were unknown.

1.1.4 Aetiology

Munro *et al.* (1984), Ferguson *et al.* (1986*a*,*b*) and McVicar (1987) considered possible causes of exocrine pancreatic degeneration in PD. Munro *et al.* (1984) noted that whilst the epizootiological data was consistent with an infectious aetiology, the pathological findings were more typical of a toxic effect or nutritional deficiency.

1.1.4.1 Toxins

Munro *et al.* (1984) noted that diffuse pancreatic necrosis is a common response to toxins, both natural and man-made. Toxins causing pancreatic necrosis in fish include diesel oil (Solangi and Overstreet 1982; Poirier *et al.* 1986), whilst in homeotherms, numerous toxic agents are known or suspected to induce acinar cell damage or necrosis (Longnecker 1982). However, the once only occurrence of PD within individual fish is not typical of toxicity.

1.1.4.2 Nutrition

Starvation may result in pancreatic atrophy in some fish species (O'Connell 1976). Hess (1935) observed (sometimes complete) pancreatic degeneration in rainbow trout fed 100% beef liver or 100% pig spleen, and Donaldson (1943) recorded pancreatic degeneration in chinook salmon (*Oncorhynchus tschawytscha*) fingerlings fed meat-based diets containing 20% salmon oil, but in both of these studies the factor responsible was not determined. In homeotherms, both copper deficiency and zinc toxicity result in pancreatic necrosis (Dubick *et al.* 1989; Lü and Combs 1988). These factors have not been examined in relation to the development of pancreas disease in salmon.

Ferguson *et al.* (1986*a*,*b*) indicated that both muscle degeneration and pancreatic necrosis were typical of vitamin E-selenium deficiency. Gries and Scott (1972) induced atrophy and fibrosis of the exocrine pancreas (nutritional pancreatic atrophy, NPA) in chicks by feeding selenium-deficient, (but vitamin E-adequate) experimental diets, to birds hatched from eggs of selenium-depleted brood hens. However, Smart *et al.* (1985) were unable to induce NPA in selenium-deficient chicks without selenium depletion *in ovo*. NPA in chicks was considered to be purely a selenium-related condition, until Whitacre *et al.* (1987) demonstrated that it was also prevented by high-level vitamin E supplementation, and presented further results consistent with the hypothesis that the condition was due to peroxidative membrane degeneration.

Exocrine pancreatic degeneration has also been noted in rats fed vitamin E-selenium deficient diets (Skjaerlund 1989), but pancreatic necrosis has not been recorded in experimental studies on the effects of simple vitamin E deficiency in Atlantic salmon parr or smolts (see section 1.2.5.2 and chapter 4), or vitamin E-selenium deficiency in parr (Bell, McVicar, Mitchell and Cowey, unpublished). Selenium deficiency was not reported to cause pancreatic atrophy in rainbow trout (Hilton *et al.* 1980; Bell *et al.* 1986) or in Atlantic salmon in freshwater (Poston *et al.* 1976), and although Bell *et al.* (1987*a*) noted ultrastructural changes in the pancreas of selenium-deficient Atlantic salmon parr, these in no way resembled the lesion characteristic of PD. The effects of selenium, or combined vitamin E-selenium, deficiency in salmon

farmed in the marine environment has not been examined. Selenium deficiency may be difficult to induce in seawater since salmonid fish can accumulate waterborne, inorganic selenium (Hodson *et al.* 1986). Bell and Cowey (1989) studied the digestibility and bioavailability of dietary selenium for Atlantic salmon post-smolts from various sources and concluded that fishmeal based diets would generally provide sufficient selenium to satisfy the nutritional requirements of salmon. This is supported by the field data of Ferguson *et al.* (1986*b*) and Bell *et al.* (1987*b*) which indicated that tissue selenium concentrations and glutathione peroxidase activities were not diminished, even in fish with PD.

1.1.4.3 Infection

A.McVicar and colleagues (Marine Laboratory, Aberdeen) have gathered data from case studies, transmission trials and laboratory tests which indicate that exocrine pancreatic degeneration in PD is due to an infectious agent, probably a virus (McVicar 1986, 1987, 1990; McVicar and Munro 1987, 1989). However, the extent to which this agent is involved in the pathogenesis of PD is unknown.

Epizootiology

McVicar (1987) regarded several features of the epizootiology of PD to indicate the involvement of an infectious agent. The annual recurrence of PD on previously affected sites, and the increased risk of PD in fish held in the vicinity of affected or recovered stock (for example different year classes on a farm, different sites in a water body), is consistent with transmission of an infectious agent from carrier populations. Indeed in one study, the pattern of development of PD at five sites within a single sea-loch appeared to show the spread of PD from a single site (G.Rae, personal communication 1987). This interpretation is supported by the apparent introduction of PD to some farms by stock movements and the effectiveness of fallowing as a means of elimination on some sites (McVicar and Munro 1987). The importance of stress in the precipitation of overt PD is typical, but not exclusively a feature, of infectious disease. Finally, the apparent lack of recurrence of

PD in previously affected individuals may indicate acquired protection towards an infectious agent.

Since all seawater sites are believed to be equally at risk, there being no apparent correlation between occurrences of the condition and source of smolt or any aspect of the freshwater phase of production (McVicar 1987), PD has been suggested to involve an infectious agent of natural and cosmopolitan marine origin (McVicar 1987; Kent and Elston 1987).

Transmission

In 1985, the induction of exocrine pancreatic degeneration in experimental stock was achieved by transmission, via effluent water, from fish with PD (McVicar 1986, 1987). Similar results were later obtained by cohabiting affected fish in tanks with experimental fish stock. Although the prevalence of EPD was variable and recovery rapid, successful transmission strongly supported the hypothesis of an infectious aetiology and was also an important step towards the development of experimental models of the condition.

McVicar and Munro (1988) and McVicar (1990) described a procedure for the induction of EPD by injection of material from affected farm populations into experimental stock. Using this procedure, EPD was successfully induced on at least 8 separate occasions (McVicar and Munro 1988). Although there was no history of natural PD at the experimental site, parr in freshwater were routinely used as the recipient animals in these studies to eliminate the possibility that a water-borne agent was responsible for the apparent effects of treatment. In addition, challenges were repeated to verify the findings using post-smolts in seawater. Kidney tissue from farmed fish with PD was collected aseptically, homogenised in sterile phosphate-buffered saline (1:10) and sieved through a 100 µm mesh to remove fibrous material (McVicar 1990). Kidney from donor fish in the early stages of the acute phase of PD was found to most successfully induce EPD (McVicar and Munro 1987). A standard dose of 0.02 ml of kidney homogenate was injected intra-peritoneally into parr and 0.1 ml into smolts. Control groups were challenged with kidney homogenate prepared from healthy fish. Typically, 200 fish were used in each trial and 10 parr or 3-5 smolts examined at weekly intervals for evidence of EPD. Positive results

were recorded only when EPD was judged total and complete (McVicar and Munro 1988; McVicar 1990). More recently a standardized dose, based on the protein content of the homogenate, has been utilized. Using this method, a dose-response study showed that the proportion of fish developing EPD could be related to the size of challenge, but that the response was highly variable, particularly at high doses (R.Raynard, personal communication 1991).

Initial studies of the time course of experimentally-induced pancreatic necrosis demonstrated the rapid induction of this condition. Some fish developed EPD within 6-10 days of challenge, and 40-100% of the challenged fish (both parr and smolts) became affected within 20-30 days. Pancreatic regeneration was recorded in all smolts within 1-3 months, but in parr outbreaks were more protracted with 30-50% being still affected 3-5 months after challenge (McVicar and Munro 1988, 1989; McVicar 1990). Successful passage between experimental fish has been achieved by injection and by cohabitation in freshwater and seawater (McVicar and Munro 1988).

McVicar and Munro (1988) also examined the transmissibility of EPD at different stages in the development of the condition. In parr, passage by injection could be achieved 10-28 days after the initial challenge, but at 34 days this was unsuccessful. In smolts, passage was possible at 14, but not 15 or 26, days. Passage by cohabitation was achieved in freshwater up to 16, but not at 48 or 68, days (McVicar and Munro 1988). These results indicate that transmissibility is reduced later in the course of disease. Temperature effects were also studied by McVicar and Munro (1988). At 13.5°C, 50% of fish challenged developed EPD at 10 days and 100% by 15 days; at 9°C, 10% developed EPD at 12 days and 60% at 26 days; whilst at 6.5°C none had developed EPD by 29 days and 30% at 45 days. This temperature dependent progression could explain the apparent absence of new cases of PD during the winter months. The activity of the infective material was found to be retained after freezing in liquid nitrogen (McVicar and Munro 1988) and this has allowed the collection of a pool of kidney homogenate enabling the challenge to be standardized. Filtration through 0.22 μm mesh was carried out to remove bacteria, but the filtrate retained its activity (McVicar and Munro 1988).

Important features of the experimentally induced pancreatic condition which indicate that this condition is the same as occurs in PD are that:

a) it occurred in fish challenged with material from fish with PD, or induced EPD, but not in controls injected with either saline or kidney homogenate from healthy animals; b) the generalized pancreatic necrosis was histologically identical to the pancreatic lesion characteristic of PD; c) electron microscopy has visualized similar virus-like inclusions in the cytoplasm of pancreatic tissue from affected farm stock and in kidney tissue from experimental fish with induced EPD (McVicar and Munro 1989).

Inconsistencies between the experimental and clinical conditions which remain to be resolved are that: a) gross clinical signs may not develop, although emaciation has been noted in prolonged studies; b) no associated lesions have been consistently observed (in particular of the heart and skeletal muscle); c) no evidence of any infectious agent is regularly seen in histological preparations examined by light microscopy; d) no pathology is observed in the kidney although the agent may be in residence.

Control

There is some evidence that experimental fish, once challenged, acquire resistance to subsequent challenge (G.Houghton, personal communication 1991). This establishes the possibility that vaccines may be developed to prevent PD.

Identity of the proposed infectious agent

Munro *et al.* (1984) discounted the possibility of any aetiological relationship between PD and other known viral conditions causing pancreatic pathology in fishes, due to differences in the pathology, microbiology and epizootiology (Roberts 1989).

Examination of electron micrographs showing the presence of virus-like particles indicated that these were 60-62 nm in diameter, probably icosahedral in shape and located within large cytoplasmic vacuole-like structures. No areas of replication were located and the cells in which the particles were found were altered to such an extent that it was not possible to indicate which cell type was involved (McVicar and Munro 1989).

Despite many attempts, no agent has been repeatedly isolated from fish with PD, characterised, cultured, demonstrated to reproduce the condition, and then re-isolated from experimentally-affected fish (Munro *et al.* 1984;

M.Frerichs, personal communication 1987; D.Smail, personal communication 1991; T.Murphy, personal communication 1991). Thus the consistent occurrence of any agent in PD cannot be demonstrated and its primary role in the development of the condition cannot be verified.

From the information summarized above, McVicar (1987) proposed a model for the pathogenesis of PD on the basis that the condition was primarily infectious. This model proposed that PD required the introduction of an infectious agent from an unknown marine source. However, despite extensive investigations, evidence regarded as proof of an infectious aetiology (ie. fulfilment of Koch's postulates) has not been forthcoming. The aetiology of the condition thus remains uncertain.

1.1.5 Economic effects and management

Practical information on the possible economic and commercial effects of an outbreak of PD can be provided to farmers, but methods of prevention and treatment cannot be recommended at present. This is an area where progress is urgently required. Economically, PD is one of most important diseases of farmed salmon in Scotland, Ireland and Norway. In Ireland in 1988, losses due to PD were valued at I£5.63 million, equivalent to some 25% of annual production (Anon 1988).

1.1.5.1 Mortality

Munro *et al.* (1984) regarded the level of mortality associated with PD to be typically about 5%. Additional losses due to culling of non-recoverers amounted to approximately 15%. Morbidity, as a result of increased susceptibility to stress and secondary disease is difficult to predict, but is likely to be related to the timing, duration and severity of the PD outbreak. Since affected fish fail to feed, difficulties in administering antibiotics via the food are likely to lead to relatively high losses from easily controllable bacterial diseases. Palmer (1988) described high mortalities in fish with PD and associated cardiomyopathy. In some recent cases of PD with associated polymyopathy, mortality levels have reached 90%.

Kent and Elston (1987) reported a mortality rate of 27% over a period of 13 weeks in salmon with pancreatic necrosis and mild epicarditis and were unable to detect any associated infection. PD may interact synergistically with diseases such as IPN (Munro 1988; McVicar and Munro 1989; Poppe *et al.* 1989). The severity of pathology and associated losses in such cases may be greater than expected as an independent combination of these conditions.

1.1.5.2 Lost growth

Lost production associated with lack of growth, weight loss and emaciation depends on the course of the post-acute phase, which varies from case to case (McVicar and Munro 1989), and also the age/size of fish affected. Outbreaks may have particularly severe effects if they occur during the prime growing season or second sea-year. If regeneration of the acinar tissue is rapid (2-4 weeks), lost growth is minimal and indeed there is some evidence of a subsequent compensatory increase in specific growth rate. However, if regeneration of acinar tissue is slow (up to 3 months), considerable weight loss may occur. Such fish may resume feeding and growth but their size and consequently their value at harvest is reduced, alternatively they require a longer period to reach market size with increased costs in capital, feed and labour.

Differences in growth rates may cause husbandry difficulties. Affected or recovered fish may require a different feeding regime (quantity and size of food required) and the wide variation in weight creates difficulties at harvest. In addition, affected stocks may show reduced grilse rates leading to overcrowding in the second sea-year. Smaller fish may be graded out after recovery or during the grilse grade and grown on in separate cages. Alternatively, these fish may be culled. Fish showing no signs of regeneration of exocrine pancreas and resumption of feeding within 3 months are unlikely to recover (McVicar and Munro 1989). Such fish are generally culled since they provide a reservoir for infection and are likely to be unmarketable.

1.1.5.3 Management

Ideally, elimination of the primary factor is the preferred method of control, but this is currently difficult given that the aetiology of PD is uncertain. Since no infectious agent can be detected, the source of any natural reservoir cannot be identified and culling of carriers cannot be performed with any certainty. However, in view of the proposed infectious nature of PD, practical and economic methods of control may include measures to minimize the possibility of exposure, such as fallowing of sites, the use of an all-in, all-out policy, separation of year-classes, avoiding the importation of affected/exposed stock onto a site, disinfection, and the maintenance of distance between sites. Legislation to restrict the movement of affected fish may be introduced in the future, but is unlikely to lead to the elimination of any infectious agent if, as suspected, there is a natural, marine reservoir.

Measures to reduce the impact of PD may allow it to be tolerated without its elimination. Identification and alleviation of stress factors, for example by the rapid and effective control of intercurrent diseases, the use of low stocking densities, regular cleaning of nets, minimum handling or moving, effective predator deterrence, etc. may minimize the severity of an outbreak. Careful control of feeding has reduced the impact of PD on some farms and has the added benefit of improving cost efficiency (Thorpe *et al.* 1990). Food must be readily available, but feeding rates should not be above those required to maximise the specific growth rate (Crampton and Jackson 1981; Austreng *et al.* 1987; T.O'Hara, personal communication 1987). Several distinct meals are preferable to a constant input of feed, and hand feeding allows finer control over the distribution and rate at which pellets are fed. Changes in feeding practice may also promote recovery from PD. Some farmers starve fish in affected cages for a short period to avoid disturbance to sick fish by actively feeding fish from below. Reduced feeding rates and the use of smaller pellets may also promote recovery. Pre-digested or enzyme supplemented fat and protein sources and more digestible, high-quality fish meals and oils in order to overcome pancreatic dysfunction have been used in some cases. Diets containing extra fish oil and other attractants have also been used to

encourage fish to resume feeding. Vitamin supplements to boost disease resistance and prevent deficiency, and high lipid diets to provide additional energy, may also be beneficial (Roy 1990).

Such techniques are inappropriate for use when large numbers of fish refuse to feed. Careful monitoring of stock is essential in order to recognise early signs of PD. If PD is suspected, laboratory verification of the diagnosis confirms the nature of the problem, allows an exchange of information and draws attention to any problems which may affect the severity of an outbreak.

1.1.5.4 Research

Future research into the aetiology of PD is likely to be closely linked with the development of the transmission model, to achieve consistent results and thereafter to describe the pathogenesis of the experimental condition under defined conditions. This model may be useful to study the influence of various diets and husbandry practices on the severity of PD outbreaks. It is important to identify any transmissible agent in order to improve diagnosis and confirm the aetiology of PD. The development of vaccines and alternative treatments may eventually be successful in reducing the problem. Alternatively, there may be some basis for genetic selection to improve resistance to PD.

It is important to ascertain the cause of lesions of the muscle in association with PD and to determine any relationship with other conditions affecting farmed salmon, involving similar muscle pathologies. These include cardiomyopathy syndrome (Ferguson *et al.* 1990), Hitra disease (Fjelstad and Heyeraas 1985; Poppe *et al.* 1986) and sudden death syndrome, all conditions which cause significant losses in commercial operations. It is in relation to the development of muscle damage that the vitamin E nutrition of farmed salmon is of considerable interest.

1.2 VITAMIN E NUTRITION

1.2.1 Historical

The discovery of vitamin E in 1922 was due to H.M.Evans and K.S.Bishop (University of California) who partially characterised a component of vegetable oils essential for fertility in female rats (Evans and Bishop 1922). Sure (1924) named this factor vitamin E. Vitamin E deficiency was later shown to be responsible for a wide variety of pathological signs in different species under different conditions (section 1.2.5). Cumings (1942) appears to have been among the first to examine the effects of vitamin E deficiency in fish. In this study guppies, *Lebistes reticulatus*, were reared on synthetic diets treated with iron to remove vitamin E by oxidation. Growth was inhibited and a form of muscular dystrophy ensued. In males the testes failed to develop or became degenerate. Injury to the liver, pancreas and spleen was also observed, marked histologically by clumping of chromatin and the formation of signet-ring nuclei. Damaged areas were replaced by a characteristic 'reticular' tissue, and this same tissue developed in the gut sometimes leading to occlusion of the lumen. The severity of injury was related to the age at which the use of the test diet was begun, and could be prevented by supplementation of the diet with vitamin E.

1.2.2 Chemistry of vitamin E

1.2.2.1 Chemical structure

Early studies leading to the characterization, identification and synthesis of vitamin E were described by Scott (1978). Evans *et al.* (1936) adopted the name α-tocopherol for a compound derived from wheat-germ oil with very high vitamin E activity. The chemical structure of α-tocopherol, illustrated in fig. 1.5, was first correctly postulated by Fernholz (1938).

Eight naturally occurring compounds with vitamin E activity have been isolated and chemically characterized (table 1.3). These are believed to be formed successively during biosynthetic processes in plants (Morton 1968;

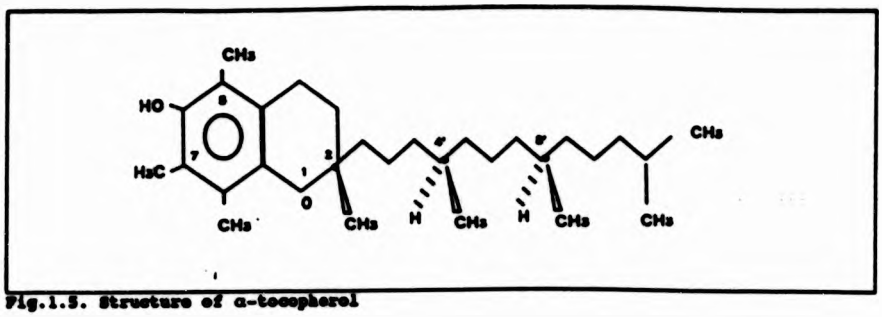

Fig.1.5. Structure of α-tocopherol

Draper 1980*b*). Standard nomenclature for this series of compounds is given by the IUPAC-IUB Commission on Biochemical Nomenclature (1974). *RRR*-α-tocopherol (natural α-tocopherol) is an abbreviated form for 2R,4'R,8'R-5,7,8-trimethyltocol, where 'tocol' is the accepted term for the basic two-ring structure within all vitamin E compounds. Its epimer, 2-*epi*-α-tocopherol, is prepared synthetically and has the configuration 2S,4'R,8'R-5,7,8-trimethyltocol. Chemical synthesis of α-tocopherol, by condensation of trimethyl hydroquinone with isophytol, yields *all-rac*-α-tocopherol, a mixture of four pairs of enantiomers. *RRR*-α-tocopherol and *all-rac*-α-tocopherol and their acetate and succinate esters are used commercially. The international unit (IU) of vitamin E activity (a measure of biological potency) has recently been redefined, 1 IU being equivalent to the biological activity of 1 mg *RRR*-α-tocopherol (formerly *all-rac*-α-tocopheryl acetate) (Lovell 1989 p.35). The biological potency of RRR-α-tocopherol is greater than that of *all-rac*-α-tocopherol, but the latter is usually more cost effective for use in animal feed supplements (Herting 1984).

1.2.2.2 Physical and chemical properties

The physical and chemical properties of tocopherols and tocotrienols were summarized by Kasparek (1980). Important in relation to the biological role of α-tocopherol are its solubility in oils, fats and fat solvents, and its oxidation reaction which yields a number of compounds including α-tocopheryl quinone (fig. 1.6) (Scott 1978).

Table 1.3. Eight naturally occurring compounds with vitamin E activity

Name
α-tocopherol
β-tocopherol
γ-tocopherol
δ-tocopherol
α-tocotrienol
β-tocotrienol
γ-tocotrienol
δ-tocotrienol

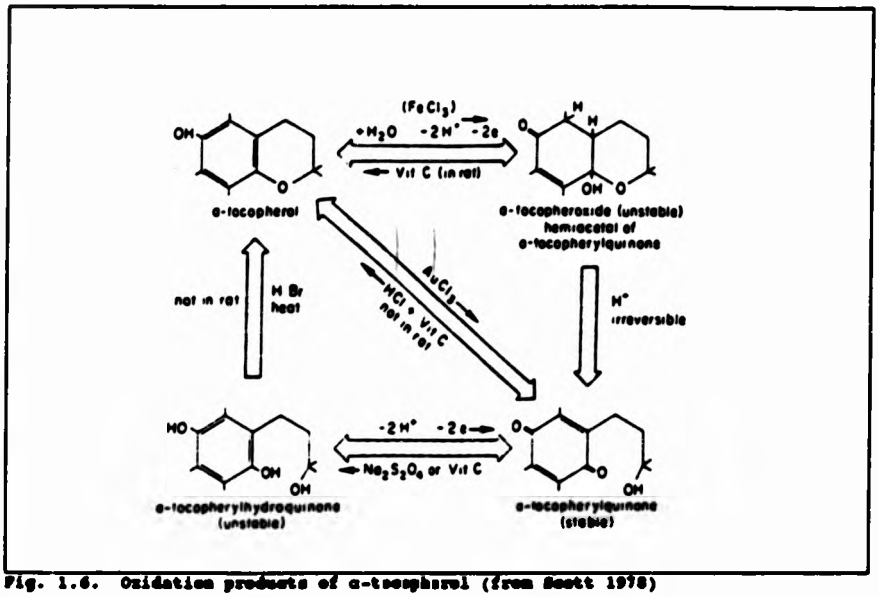

Fig. 1.6. Oxidation products of α-tocopherol (from Scott 1978)

1.2.2.3 Assay methods

Various methods for the assay of vitamin E in foods and tissues have been described by Desai (1980). The classical method to determine biological activity has been the foetal resorption test in female rats (Mason and Harris 1947). Various chromatographic procedures allow the qualitative identification

of compounds with vitamin E activity (Desai 1980). In studies in which vitamin E has been measured quantitatively in fish, the principal methods employed have been modifications of the colorimetric procedure of Emmerie and Engel (1938) and various high performance liquid chromatographic (HPLC) procedures (Hung *et al.* 1980a; Cowey *et al.* 1981; Buttriss and Diplock 1984) (see also section 2.2.1).

1.2.3 Vitamin E in fish tissues

There is a considerable body of literature on vitamin E concentrations in fish tissues as a result of nutritional analyses on fish and fish products for human and animal consumption. Jacquot (1961) and Higashi (1961) summarised early studies on the vitamin E content of fish tissues and later Love (1970; 1980) reviewed this subject. Smith and Nelson (1931) reported the presence of vitamin E in cod liver oil and Duboulos and Hedde (1942) demonstrated that other antioxidant substances besides tocopherols were also present in fish oils. Robeson and Baxter (1945) reported a concentration of 40 mg α-tocopherol 100 g^{-1} in liver oil from the shark, *Eugaleus galeus*. Kringstad and Folkvord (1949) used a modified Emmerie-Engel method to determine the vitamin E content of cod (*Gadus morhua*) roe and liver and found that liver concentrations were negligible, but that samples of fresh and canned roe contained between 5.25 and 7.70 mg 100 g^{-1} dry weight. Lange (1950) summarized the available information on the tocopherol content of food products and animal tissues including fish. Harris *et al.* (1950) reported that α-tocopherol was the predominant form in fish flesh. Brown (1953) measured vitamin E concentrations in the liver oil of various marine teleosts and gave the following figures: anglerfish 31.9, blue rayfish 25.3, cod 29.3, haddock 18.0, ling 27.2 and turbot 45.0 mg 100 g^{-1}. Mori *et al.* (1957) measured larger concentrations in liver oil (about 1 mg g^{-1}) than in oil from the pyloric caeca in various fish species and suggested that there was little variation in vitamin E content of liver oils from different species. These workers also noted high vitamin E activity in the gonad and its depletion during spawning. Pennock *et al.* (1962) determined α-tocopherol concentrations in heart, liver and muscle from various freshwater and marine teleost species and Nazir and Magar (1963,1964)

determined tocopherol concentrations in a mackerel, *Rastrelliger canagurta*, and a shark, *Carcharias ellioti*. Mega (1965) measured vitamin E concentrations in fish and 'shells' from the Sea of Japan and found high concentrations in the gonads, and low concentrations in organs high in oil. Dicks (1965) listed α-tocopherol concentrations in fish and fish products from a number of sources.

1.2.4 The biological role of vitamin E

At a biochemical level, vitamin E is believed to function as a free-radical scavenger, primarily in the termination of free-radical initiated chain reactions, and particularly (non-enzymic) autoxidation reactions involving polyunsaturated fatty acids in cell membranes (McCay and King 1980). Such events may be initiated during the course of normal metabolism (Fong *et al.* 1973), and indeed are necessary for the formation of certain biomolecules, such as eicosanoids (Rice and Kennedy 1988). Regulated antioxidant activity is therefore an important homeostatic mechanism. As a liposoluble antioxidant active *in vivo*, vitamin E interacts with other mechanisms of cellular defence against oxidative damage including enzymes containing copper, zinc and manganese (superoxide dismutases) and selenium (glutathione peroxidase) (fig. 1.7) (Bell and Cowey 1985). The high vitamin E activity of α-tocopherol may be explained solely on the basis of its suitability as an *in vivo* antioxidant, but other biochemical roles in addition to this antioxidant function have been proposed including chemically specific roles in nucleic acid and protein metabolism (Catignani 1980).

Diplock and Lucy (1973) proposed that α-tocopherol may play a physical as well as chemical role in the maintenance of membrane structure. This study showed that the C-4 and C-8 methyl groups of the phytol side chain of α-tocopherol matched with pockets provided by *cis* double bonds of arachidonic acid. Incorporation of α-tocopherol at this location would thus decrease membrane fluidity. Molenaar *et al.* (1972, 1980) reviewed information relating to the role of vitamin E in maintaining the structure and function of cellular and sub-cellular membranes and concluded that the effects of vitamin E deficiency could be largely mediated through impairment of this function.

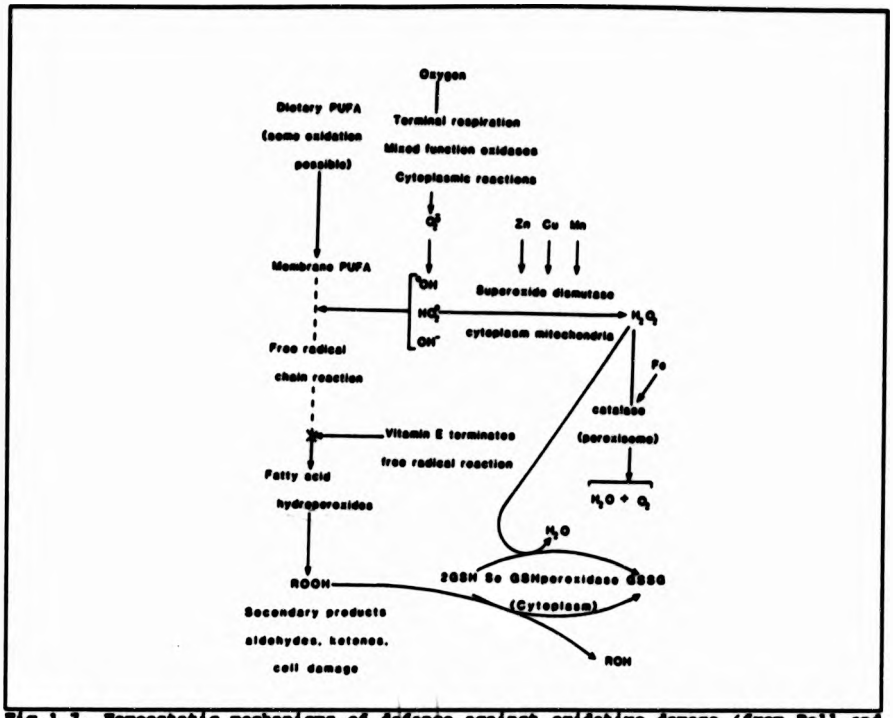

Fig.1.7. Homeostatic mechanisms of defence against oxidative damage (from Bell and Cowey 1985)

Specifically, the structure of membrane phospholipids, membrane fragility and permeability and the action of membrane-bound enzymes were all shown to be affected by vitamin E status. Molennaar *et al.* (1980) developed a hypothesis linking the biochemical and ultrastructural effects of vitamin E deficiency with microscopic and macroscopic effects of deficiency, based on alterations to basic membrane functions. At present these mechanisms are largely speculative. Nevertheless, as might be expected on the basis of this hypothesis, vitamin E deficiency has been shown experimentally to have a wide variety of manifestations. In fact it is probably responsible for the largest number of disorders due to any single vitamin deficiency (Herting 1984).

1.2.5 Vitamin E requirement and deficiency signs in fish

The variety of signs resulting from or related to vitamin E deficiency

in fish is summarized in table 1.4. These include structural and functional effects at biomolecular, cellular, tissue, and whole animal levels. A number of deficiency signs are consistently noted and some form a useful basis for tests of vitamin E status. Using these procedures, some agreement on minimum requirements has been reached for several species important in aquaculture.

1.2.5.1 Early experimental studies of the vitamin E requirements and effects of deficiency and excess in salmonids

Wolf (1951) devised a synthetic diet to study the effects of vitamin depletion on rainbow trout, *Oncorhynchus mykiss*. No effects on growth, mortality, erythrocyte count, behaviour or gross pathology were detected in fingerling fish fed the test diet, from which α-tocopherol was omitted, over a 25 week period. The test diet contained lipid sources which may have contained a significant amount of vitamin E, but no measurements of the dietary vitamin E concentration were made.

The α-tocopherol requirement of chinook salmon (*Oncorhynchus tshawytscha*) fingerlings was studied by Woodall *et al.* (1964) again using a synthetic laboratory diet. Basal diets containing 1% or 5% vitamin E-stripped herring oil were supplemented with 0, 10, 20, 40 and 80 mg *all-rac*-α-tocopherol 100 g^{-1} dry diet. In addition to these supplements, the stripped herring oil contributed 0.1 or 0.5 mg α-tocopherol 100 g^{-1} dry diet, but losses during preparation and storage were not quantified. The experimental fish weighed 0.45-0.47 g and had been reared on vitamin E-depleted diets since first-feeding. Duplicate groups were fed the test diets for a period of 24 weeks at a water temperature of 10°C. Tocopherol concentrations in fish tissues, measured using a modified Emmerie-Engel procedure, reflected the dietary vitamin E content. Gross signs of vitamin E deficiency in this species included exophthalmia and ascites, and lethargy. In addition, reduced weight gains and lower body lipid concentations were measured. A reduction in the mean size of erythrocytes was noted in groups fed diets containing both 1 and 5% herring oil without added α-tocopherol. Histological signs of deficiency included clubbed gill lamellae, epicarditis and ceroid deposition in the spleen and these were most pronounced in fish fed 5% herring oil without added

Table 1.4. Signs of vitamin E deficiency in fish

Deficiency sign	Atlantic salmon	Chinook salmon	Rainbow trout	Common carp	Channel catfish	Tilapia
mortality	+		+	+	+	+
inappetance					+	+
weight gain ↓	+	+	+	+	+	+
lethargy	+	+			+	
syncope	+					
exudative diathesis	+				+	
overt muscular dystrophy				+	+	
lordosis				+		
dermal depigmentation	+				+	+
skin haemorrhages						+
pale gills	+					
clubbed gills		+				
exophthalmia		+		+	+	
ascites	+	+		+	+	
yellow liver	+				+	
haematocrit ↓	+	+	+	+	+	
anisocytosis	+	+				
poikilocytosis	+	+				
renal glomerular sclerosis				+	+	
ceroid etc. pigment		+			+	+
atrophic haemopoietic tissue			+			+
exocrine pancreatic atrophy					+	
endocrine islet hypofunction				+		
hepatocyte hypertrophy				+		
skeletal myopathy	+		+	+	+	+
cardiac myopathy	+					+
epicarditis		+	+			
plasma PK activity ↑	+		+			
carcass protein	↑			↓		↓
carcass fat	↓	↓		↓		↓
susc. to haemolysis ↑	+		+		+	
microsomal peroxidn.↑	+		+		+	

vitamin E, less pronounced in fish fed 1% oil without vitamin E, and absent in groups fed even minimal α-tocopherol supplements. The effects of dietary lipid level on vitamin E nutrition are described in section 1.2.6.1. In a subsequent experiment, diets containing 2.5, 5.0, 10, 20 and 40 mg α-tocopherol 100 g^{-1} dry weight, plus 5% herring oil, were fed to fish as before. No differences in growth and no histological indications of α-tocopherol deficiency were detected among these groups. The α-tocopherol requirement of chinook salmon fingerlings was therefore estimated to be between 0.5 and 3 mg 100 g^{-1}, although in fish fed 2.5 and 5.0 mg 100 g^{-1} there was no significant accumulation of vitamin E in the tissues.

Whitmore (1965) described an anaemia of juvenile chinook salmon induced by the use of diets deficient in vitamin E. His material was collected during an earlier 22 week experimental study by Law *et al.* (1963). In this experiment, chinook salmon fingerlings were fed test diets, modified to provide various levels of rancidity, each with and without a vitamin E supplement (50 mg *RRR*-α-tocopheryl acetate 100 g^{-1} dry diet). Fish fed diets to which no vitamin E had been added showed marked reductions in blood haemoglobin concentration, haematocrit and percentage of lymphocytes, whilst the percentage of immature erythrocytes, total leucocyte count, and percentage of thrombocytes, granulocytes and monocytes were all above normal. These changes were apparent even in fish fed vitamin E-depleted diets with low TBA values and thus seemed to result from vitamin E deficiency rather than lipid rancidity. The combined effects of oxidized lipid and vitamin E deficiency are described in section 1.2.6.2.

Poston (1965) studied the effect of vitamin E depletion on fingerling brown trout (*Salmo trutta*). A modified formula of the diet described by Wolf (1951), in which the lipid fraction was treated to remove vitamin E, was prepared with and without the addition of vitamin E (approx. 16.5 mg 100 g^{-1} as *all-rac*-α-tocopheryl acetate) and fed to trout (mean weight 1.90 g) for 16 weeks at 11°C. After 10 weeks, microhaematocrits were significantly reduced in fish fed the vitamin E-deficient diet. Concomitantly, mortalities increased and weight gain was reduced. Stress tolerance was reduced in deficient fish, but there was no indication of histological change in any tissue examined. Dietary supplementation with 37 mg α-tocopheryl acetate 100 g^{-1} diet reduced

mortalities and increased haematocrits in deficient fish. The absence of histological change in contrast to Woodall *et al.* (1964) may have been due to species differences or differences in the vitamin E or fat content of the diet.

Poston and Livingston (1971) studied the effects of massive doses of dietary vitamin E on fingerling brook trout and described growth reduction and lowered haematocrits in fish fed a diet containing 500 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} diet for 20 weeks. There were also signs that this diet changed the liver lipid profile, giving rise to an increase in total lipids and a reduction in the linoleic acid fraction, but there was no increase in mortality.

1.2.5.2 Effects of vitamin E deficiency in Atlantic salmon

The effects of single and combined deficiencies of vitamin E and selenium in Atlantic salmon, *Salmo salar*, in freshwater were examined by Poston *et al.* (1976). Interactions between these nutrients are described in section 1.2.6.4. In the first four weeks of feeding, both separate and simultaneous deficiencies of vitamin E-selenium had no effect on weight gain, but caused increased mortalities. These were subsequently prevented by dietary supplementation with 50 mg *all-rac*-α-tocopheryl acetate plus 1 μg sodium selenite 100 g^{-1} dry diet. In a second experiment conducted at 14°C, salmon fry (0.9 g mean weight), previously reared for 12 weeks on a commercial diet, were transferred to one of four casein-based diets containing either 0 or 50 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} dry diet, together with either 0 or 1 μg sodium selenite 100 g^{-1}. Between 10 and 13 weeks from the start of the experiment fish fed the vitamin E-deficient diet, in the presence or absence of selenium, showed abnormal swimming behaviour and syncope (a transitory fainting) in response to handling or disturbance. Between 13 and 16 weeks a number of these fish died. The fish were examined after 16 weeks. No differences in weight gain were evident, but the following gross signs were described: pale gills, dermal depigmentation, ascites, yellow-orange liver and pyloric caeca, gall bladder distended with dark green bile and frothy, yellow, hindgut contents. Haematological examination showed extremely low haematocrits

with anisocytosis and poikilocytosis. Histological examination showed the presence of skeletal myopathy, apparent as enlargement of fibre bundles and invasion by giant cells, together with proliferation of connective tissue elements (Zenker's degeneration). Biochemical tests indicated that fish receiving the vitamin E supplement, with or without selenium, had increased carcass protein, reduced carcass fat and water, and increased plasma protein concentrations. Vitamin E supplementation, with or without selenium, also caused an inhibition of ascorbic acid-stimulated peroxidation in hepatic microsomes, an *in vitro* test of vitamin E status (Bieri and Anderson 1960).

Lall *et al.* (1987,1988) examined the role of vitamin E in nutrition and immune response in Atlantic salmon parr. Effects of vitamin E on immune function are described in section 1.2.5.4. A basal diet containing 0.55 mg 100 g^{-1} α-tocopherol, 5% herring oil and 5% tocopherol-stripped corn oil, or the basal diet supplemented with 1, 3, 6, 12 or 24 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} was fed for a period of 22 weeks. Growth rate was improved by supplementing the basal diet with 3 mg 100 g^{-1} vitamin E, but higher level supplements provided no additional benefit. Mortality was increased in fish fed the basal diet, with fish showing signs of lethargy, rapid opercular movements and erratic swimming behaviour prior to death. Tissue vitamin E concentrations reflected dietary levels and were highest in the liver and lowest in muscle. Fish fed the basal diet showed significantly lower haematocrits, increased erythrocyte fragility and histological evidence of myocardial and skeletal muscle degeneration.

Recently, Bell, McVicar, Mitchell and Cowey (unpublished) conducted two experiments to study the effects of vitamin E deficiency on Atlantic salmon parr (in freshwater) and post-smolts (in seawater) (G.Bell, personal communication 1987). In the first experiment, parr of 18-19 g were fed basal diets, containing 0.53 mg α-tocopherol and 0.0017 mg selenium 100 g^{-1} dry weight, with or without supplements of 8 mg α-tocopheryl acetate 100 g^{-1} and 0.09 mg selenium 100 g^{-1}, for a period of 12 weeks. In vitamin E-deficient groups, plasma tocopherol concentrations were reduced to 3 μg ml^{-1}. There was no difference in weight gain, but some mortality occurred among these fish. Haematocrit was reduced and susceptibility to haemolysis was increased. Plasma pyruvate kinase activity also increased indicating incipient muscle

degeneration, but in contrast to other studies on this species, myopathy was not observed histologically in any fish. In the second experiment, Atlantic salmon post-smolts were fed a fishmeal-based diet containing 0.4 mg 100 g^{-1} vitamin E, with or without a supplement of 8.5 mg *all-rac*-α-tocopheryl acetate 100 g^{-1}, for a period of 20 weeks. In fish fed the basal diet, α-tocopherol concentrations were reduced to 6 μg ml^{-1} in the plasma and 6 μg g^{-1} in the liver, haematocrits were reduced and erythrocyte susceptibility to haemolysis was increased. There was no effect on weight gain, but the level of mortality and other pathological changes were complicated by a secondary infection (G.Bell, personal communication 1987). No damage to the pancreas was present in any group, but about 60% developed exudative diathesis. Lesions were present in the heart and white skeletal muscle of all fish examined, reflected in elevated plasma PK activity in relation to fish fed the complete diet (C.Cowey, personal communication 1987). To date, there have been no attempts to closely define the vitamin E requirement of Atlantic salmon in seawater.

1.2.5.3. Studies on the vitamin E requirement of rainbow trout

Rainbow trout appear to be less susceptible to vitamin E deficiency than Atlantic salmon. Wolf (1951) found no effects of tocopherol depletion in this species (section 1.2.5.1) and Watanabe *et al.* (1973) noted that rainbow trout (5-8 g), fed a vitamin E-deficient diet for a period of 152 days, showed no alteration in triglyceride composition and no signs of deficiency except poor growth.

Hung *et al.* (1980*b*) studied the effects of dietary lipid oxidation on the vitamin E nutrition of rainbow trout fed practical-type (fishmeal-based) diets. Relationships between vitamin E and oxidised dietary lipid are described in section 1.2.6.2. Nine diets with three degrees of dietary lipid oxidation (POV=0.6, 2.6 and 5.0 mEq 100 g^{-1} oil) and three levels of vitamin E supplementation (3.3, 6.6 and 9.9 mg *all-rac*-α-tocopheryl acetate 100 g^{-1}) in addition to adventitious levels (<2.0 mg α-tocopherol 100 g^{-1}), were prepared. These were fed to trout (mean weight 1.5 g), held at 15°C, for a period of 24 weeks. Using an HPLC procedure without prior saponification (Hung *et al.* 1980*a*), mean liver α-tocopherol (free alcohol) concentrations in fish

fed the lowest level of vitamin E were determined to be 76 μg g^{-1}, and this was significantly increased by elevating the dietary vitamin E content. Determinations at 4-weekly intervals, of weight gain, food conversion ratio and mortality revealed no significant difference among groups on different diets; and carcass composition, haematocrit and plasma glutathione peroxidase activity measured at the completion of the experiment also showed no effects of treatment.

In a further study, Hung *et al.* (1981) re-examined the effects of oxidised fish oil together with α-tocopheryl acetate and ethoxyquin supplementation on the vitamin E nutrition of rainbow trout. The effects of dietary antioxidants on vitamin E nutrition are described in section 1.2.6.3. A factorial experiment was conducted using practical-type diets prepared with two degrees of oxidation of supplemental fish oil (7.5% in diet) (POV=0.5 and 12.0 mEq 100 g^{-1} oil, fresh and highly oxidized oil respectively), two levels of supplemental *all-rac*-α-tocopheryl acetate (0 and 3.3 mg 100 g^{-1}) and two levels of the antioxidant, ethoxyquin (0 and 12.5 mg 100 g^{-1},) in a practical-type diet. The basal diet contained ≤25 mg 100 g^{-1} adventitious vitamin E. The test diets were fed to trout (weight 2 g) held in freshwater at 15°C for a period of 24 weeks. In this study, minimum α-tocopherol (free alcohol) concentrations in fish fed fresh oil were 25 μg ml^{-1} in plasma and 180 μg g^{-1} in liver. Once again, there were no differences in weight gain, food conversion ratio, carcass composition or plasma glutathione peroxidase activities among the dietary groups. The results indicated that no vitamin E supplementation was needed to prevent vitamin E deficiency in rainbow trout fed this practical-type diet, which contained 7.5% fish oil (of which 20.7% comprised PUFA), together with 20% capelin fish meal (which undoubtedly supplied additional PUFA), since adventitious levels seemed to be adequate. However, the dependency of vitamin E requirement on the degree of oxidation of dietary lipid was demonstrated (section 1.2.6.2).

In an attempt to identify signs of vitamin E deficiency in this species, C.B.Cowey and colleagues used purified diets containing very low vitamin E levels. In these studies, in contrast to those of Hung *et al.* (1980*a*,*b*, 1981), a vitamin E assay procedure which included a saponification step prior to extraction was utilized. Cowey *et al.* (1981) examined the requirement for α-

tocopherol of trout fed diets with the minimum content of unsaturated fatty acids necessary to fulfill the essential fatty acid requirement (Castell *et al.* 1972). They fed a casein-based diet containing palmitic acid as the major lipid source and 1% linolenic acid (n-3) as the sole source of unsaturated fat, together with 0, 0.5, 1.0, 2.0, 3.0, 5.0, and 10.0 mg *all-rac*-α-tocopherol 100 g^{-1} dry diet. The adventitious vitamin E content of the basal diet was 0.6-0.66 μg 100 g^{-1}. Fish were held in freshwater at a constant 15°C and grew from 10 g to 51-56 g over the 16 week experimental period. Liver total lipid, liver and intestine phospholipid linoleic acid, liver and blood reduced glutathione concentrations, and activities of the antioxidant enzymes glutathione peroxidase, glutathione reductase, glutathione-S-transferase and manganese or cupro-zinc dependant superoxide dismutases were not affected by tocopherol deficiency. There was no difference in growth, food conversion efficiency or hepatosomatic index and tocopherol-deficient trout had no gross or sub-cellular pathologies (in the white muscle, liver or intestine). Erythrocyte fragility was significantly greater in fish fed the basal diet and haematocrits were also significantly reduced. Ascorbic acid-stimulated *in vitro* peroxidation in liver organelles indicated a tocopherol requirement of 2.0-3.0 mg 100 g^{-1} diet. Tocopherol concentrations in the liver of fish fed the unsupplemented diet were 7 μg g^{-1}. These were similar to those measured in Atlantic salmon exhibiting pathological signs of vitamin E deficiency (Poston *et al.* 1976; Bell, McVicar, Mitchell and Cowey, unpublished). The lack of pathological signs in this study was suggested to have been due to the effects of species, size or feeding history, or the level of dietary polyunsaturated fatty acid. The molar ratio of polyunsaturated fatty acids to tocopherol may provide a more appropriate indication of vitamin E status than tissue α-tocopherol concentrations *per se* (Evarts and Bieri 1974). In the livers of trout in this study, fed diets lacking or supplemented with α-tocopherol (10.0 mg 100 g^{-1}), these ratios were 980 and 170 respectively.

Higher levels of dietary polyunsaturated fatty acids were used in a second study (Cowey *et al.* 1983). This study utilized casein-based diets containing 10% fatty acids derived from marine fish oil, together with graded levels of vitamin E (2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 10.0 mg 100 g^{-1} as supplementary *all-rac*-α-tocopheryl acetate plus endogenous α-tocopherol).

Groups of trout were fed the experimental diets at a rate of 2% body weight day^{-1}, over a 16 week period, at a water temp of 15°C. During this time, mean fish weight increased from about 14 g to 102-112 g and liver vitamin E concentrations were reduced to 9 μg g^{-1}. In this study the PUFA:tocopherol ratio was 192 in fish fed vitamin E-adequate diets. Once again, there were no differences in weight gain and food conversion between treatments and no tissue pathologies were observed despite the higher level of lipid and its greater degree of unsaturation. Increased erythrocyte fragility was noted in fish fed the unsupplemented diet and ascorbic acid-stimulated *in vitro* peroxidation of liver microsomes reflected vitamin E intake, with little or no malondialdehyde being formed in the microsomes of fish fed diets containing 5 mg 100 g^{-1} or more. Thus the minimum requirement for rainbow trout fed diets containing 10% unoxidized lipid of which 25% PUFA was suggested to be 5 mg *all-rac*-α-tocopheryl acetate 100 g^{-1}.

Cowey *et al.* (1984) examined the effects of dietary vitamin E and oxidized oil concentrations on rainbow trout grown under natural, varying water temperatures. Once again the experimental diets were casein-based and contained no fish meal, they were considered to contain adequate adventitious selenium (0.01 mg 100 g^{-1}) and included 12% fish oil. A 2x2 factorial experiment was designed utilizing non-oxidized and moderately oxidized oils (POV 0.2 and 4.7 mEq 100 g^{-1} oil respectively) without or with supplemental *all-rac*-α-tocopheryl acetate (0 or 4 mg 100 g^{-1} diet). Measured dietary vitamin E concentrations were 39.52 mg 100 g^{-1} (unoxidized+vitamin E supplement), 1.84 mg 100 g^{-1} (unoxidized-vitamin E), 38.84 mg 100 g^{-1} (oxidized+vitamin E) and 0.74 mg 100 g^{-1} (oxidized-vitamin E). The fish, which had previously been reared on a vitamin E-adequate diet, were fed the experimental diets for a period of 14 weeks. During this time the water temperature fell from 12 to 6°C, the feeding rate was reduced from 7.5 to 3.0% body weight day^{-1} and the mean weight of the control group increased from 9.4 to 51.1 g. In contrast to previous studies, significant differences were discovered between treatments containing 4 mg α-tocopheryl acetate 100 g^{-1} diet and those that did not (ie. vitamin E replete and deficient). These differences applied to mortality, weight gain, haematocrit, erythrocyte fragility, liver and muscle α-tocopherol concentrations and *in vitro*

peroxidation in liver microsomes. Liver glutathione peroxidase activity and the proportions of fatty acids in the polar lipids of liver and muscle were not affected by the diets used. Severe degeneration of white skeletal muscle fibres occurred in trout in this study given diets lacking vitamin E. These changes consisted of an alteration in fibre size, and the fibres appeared to be rounded and partly swollen and partly shrunken. Sub-pericardial cellular infiltration was also noted, together with nuclear pyknosis and cytoplasmic vacuolation. In addition, there were signs of atrophy of haemopoietic tissue in the kidney. Vitamin E concentrations in the liver (6.4 $\mu g\ g^{-1}$) of these fish were similar to those measured in vitamin E-depleted fish with no indication of deficiency (Cowey *et al.* 1981; 1983), indicating that factors other than tissue vitamin E concentration were responsible for the expression of gross pathologies. Cowey *et al.* (1984) argued that since dietary selenium concentrations were higher than the minimum requirement and since glutathione peroxidase activities indicated that selenium status was adequate that such signs were not due to combined vitamin E-selenium deficiency. They suggested that the occurrence of mortality and myopathy and effects on weight gain may have been due to the lower water temperatures pertaining in this study. The hypothetical relationship between temperature and vitamin E requirement in poikilotherms is described in section 1.2.7.5. This interpretation, however, was not substantiated and other factors may explain the differences in experimental results between this and the earlier studies of Hung *et al.* (1980*b*; 1981) and Cowey *et al.* (1981, 1983). Firstly, high feeding rates used at low water temperatures in the 1984 study may have precipitated such signs (see section 3.1.3). Secondly, the faster flow rates (2 $l\ min^{-1}$ compared with 0.3 $l\ min^{-1}$) and slightly smaller fish size (7-9 g as opposed to 12 g) used by Cowey *et al.* (1984) would tend to increase the swimming speed (body length sec^{-1}) necessary to maintain position. Thus these fish may have suffered an exercise induced nutritional myopathy (see section 1.2.7.3).

1.2.5.4. Effects of vitamin E status on immune function in salmonids

In mice, the minimal daily α-tocopherol requirement for the prevention of erythrocyte haemolysis or peroxidation in hepatic microsomes *in vitro*, is

less than the optimum to maximise both antibody production and cell-mediated immune responses (Corwin and Gordon 1982). The vitamin E requirements necessary for the prevention of deficiency in fish may also be sub-optimal for resistance to infection. Blazer and Wolke (1984) fed rainbow trout (115 g) casein-based experimental diets containing 2.5% corn oil with or without 160 mg 100 g^{-1} supplementary α-tocopherol for a period of 12-17 weeks. Vitamin E depletion produced no lack of growth, no mortality and no gross or histopathological signs of deficiency. However, the specific immune response to sheep red blood cells and *Yersinia ruckeri*, T-cell (migration inhibition factor) and B-cell (plaque-forming cell) responses, haemagglutination, haemolysin, bacterial agglutination, serum protein, serum globulin, and the phagocytic index of peritoneal macrophages, were all impaired by α-tocopherol deficiency. In contrast, Lall *et al.* (1988) (see section 1.2.5.2) found no difference between groups of Atlantic salmon fed different levels of vitamin E, in resistance to *Aeromonas salmonicida* challenge, or in the humoral or complement response following vaccination with formalin-killed *A.salmonicida*. Hardie *et al.* (1990) also studied the effect of vitamin E on the immune response of Atlantic salmon. Salmon parr were maintained on casein-based diets containing 0.7, 8.6 or 80.0 mg vitamin E 100 g^{-1}, or a commercial feed containing 32.6 mg 100 g^{-1} vitamin E, for a period of 20 weeks at 14°C. During this time the fish grew from 26 to 121 g mean weight. No differences in weight gain, haematocrit, differential leucocyte count, serum protein or lysozyme concentrations were detected. Serum complement activity was reduced in fish fed 0.7 mg 100 g^{-1} and serum opsonization of *Aeromonas salmonicida* was increased in fish fed 80 mg 100 g^{-1} vitamin E. Mortality following challenge with *Aeromonas salmonicida* was significantly greater among fish fed the vitamin E-deficient diet than among those fed the commercial feed.

Furones *et al.* (1990) noted that rainbow trout fed diets supplemented with vitamin E showed the lowest mortality rate after experimental challenge with *Yersinia ruckeri*. Forster *et al.* (1988) studied the effect of diets containing oxidized herring oil, with or without vitamin E, on the growth and immunocompetence of juvenile coho salmon (*Oncorhynchus kisutch*). Semi-purified diets supplemented with unstabilized oil, oxidized and/or stabilized to different degrees, each with or without 100 mg *all-rac*-α-tocopheryl acetate

100 g^{-1}, were fed to coho salmon (*Oncorhynchus kisutch*). There was no significant difference in weight gain or food conversion efficiency between groups fed diets with and without supplemental α-tocopheryl acetate and no effects of diet on haematocrit, immunocompetence or resistance to challenge with *Vibrio* spp. were detected.

1.2.5.5. Experimental studies on the vitamin E nutrition of common carp (*Cyprinus carpio*)

Kinumaki *et al.* (1972) measured no deposition of α-tocopherol in carp fed diets containing 3 mg *all-rac*-α-tocopheryl acetate 100 g^{-1}, but supplementation in the range 10 to 100 mg 100 g^{-1} did lead to deposition and the rate of accumulation reflected the dietary vitamin E content.

The effects of vitamin E deficiency on carp were studied by Watanabe *et al.* (1970*a*). Fish weighing about 10 g were fed diets with and without supplemental *all-rac*-α-tocopherol (50 mg 100 g^{-1} dry diet). Ingredients of the basal diet, prepared to exclude endogenous α-tocopherol, included vitamin E-extracted casein and methyl esters (5% w/w) from soy-bean oil. Single groups of 50 fish, held at 20-23°C, were fed the experimental diets for a period of 17 weeks. Fish receiving the vitamin E-deficient diet showed lower weight gain and feed conversion efficiency. Apparent muscular dystrophy, characterised by loss of flesh on the back, developed within 90 days. Numbers of red and white blood cells decreased in the vitamin E-deficient group, the proportion of immature erythrocytes increased, and a change in the serum protein profile was noted. Histological signs of deficiency included a disturbance in the arrangement of white skeletal muscle fibres with atrophy and hyaline and granular degenerative change, sclerosis and necrosis of the renal corpuscles together with disappearance of the urinary tubules, some evidence of hypofunctional change in the endocrine pancreatic islets, enlargement of the pronephros due to an increase in number of lymphoid cells, hypertrophy and pyroninophilia of hepatocytes, and changes in the structure of the pituitary and cornea. The heart, gill, intestine, vertebrae, spleen, exocrine pancreatic tissue, thyroid gland, red muscle and gonad appeared normal and no evidence of ceroid was observed. Deficient fish also showed differences in proximate

composition having a high percentage moisture and low percentage fat and protein. In addition, their carcasses contained a higher proportion of oleic and a lower proportion of linoleic acid.

The α-tocopherol requirement of young carp was studied by Watanabe *et al.* (1970*b*) using the same basal diet as described above supplemented with 0, 10, 30, and 50 mg *all-rac*-α-tocopherol 100 g^{-1} diet. Groups of fish (mean weight about 1.5 g), held at 20-23°C, were fed the test diets for a period of 90 days. Once again, fish fed the vitamin E-deficient diet showed reduced weight gain, but diets containing more than 10 mg α-tocopherol 100 g^{-1} supported normal growth as indicated by weight gain, feed efficiency, and absence of histological lesions. Apparent muscular dystrophy appeared in fish fed the unsupplemented diet after about 50 days. Exophthalmia, ascites and lordosis, which were absent in the previous study using larger fish (Watanabe *et al.* 1970*a*), were observed in vitamin E-deficient young carp. Mortalities, in groups of fish fed the unsupplemented diet, were associated with lordosis and recorded from about 70 days. Some fish on vitamin E supplemented diets also showed lordosis, but to a lesser degree. Biochemical muscle analysis showed a high moisture and low protein content and a high TBA value. The α-tocopherol concentration in the hepatopancreas reflected the dietary content, and the concentration of ascorbic acid in the hepatopancreas showed a similar pattern. Histological examination showed similar signs to the previous study, but degeneration in the muscle and kidney was more severe. Ceroid, once again, was not found. A series of biochemical studies were conducted using material from this study. Watanabe *et al.* (1970*c*) analyzed the protein composition of the dystrophic muscle from vitamin E-deficient fish and found a reduction in the proportion of total, and myofibrillar, protein, and an increase in stromal protein. This was discovered to result from a loss of myosin and actomyosin from the myofibrillar protein fraction and was interpreted to suggest a disturbance to protein synthesis as a result of vitamin E deficiency. The lipid composition of vitamin E-deficient carp was described by Matsui *et al.* (1973) and Watanabe *et al.* (1973). Carp muscle lipids were found to show a triglyceride composition from C-43 to C-56. The dominant components in the fish fed vitamin E supplemented diets were C-50 to C-54, compared to C-43 to C-45 in the vitamin E-deficient fish. Furthermore, in the deficient carp,

linoleic acid was absent from the muscle lipid. Watanabe *et al.* (1977a) performed a further experiment using groups of carp fingerlings weighing about 30 g. These were fed either the basal diet as described above (except that soy-bean oil methyl esters were partially replaced by pollock liver oil methyl esters at a ratio of 3:2), or this diet supplemented with 70 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} diet, for a period of 90 days. Growth rate was reduced, but there was no mortality. Proximate analysis confirmed a high moisture and low protein content in the muscle and carcass. The fatty acid composition of various tissues showed a similar, though less pronounced, trend as before, with reductions in the percentage of linoleic acid in both polar lipids and triglycerides. There were no other marked differences in the composition of triglycerides and cholesteryl esters due to vitamin E deficiency, although muscle lipid C-54 and C-56 triglycerides and hepatopancreas lipid C-18 cholesteryl esters were reduced, the latter being replaced by C-16 and C-20 cholesteryl esters.

Watanabe and Takashima (1977) fed female adult carp (weight 76.5 to 115.5 g) basal diets, as described above, with or without *all-rac*-α-tocopheryl acetate (70 mg 100 g^{-1}) for a period of 17 months. Again, lower weight gain and apparent muscular dystrophy were recorded in fish fed the deficient diet. Gonad weight and gonadosomatic index were also significantly reduced. An increase in the percentage of water and a decrease in protein and lipid were noted in the ovary as in other tissues. A marked reduction in linoleic acid was observed in triglyceride and polar fractions in most of the deficient tissues. Once again there was an increase in low molecular weight triglycerides, and a decrease in the usually dominant C-52 to C-56 triglycerides. In the ovaries of deficient fish a reduction in polar lipids was noted and there were also decreases in 20:4(n-6), 20:5(n-3) and 22:6(n-3) and increases in 18:1(n-9) and 20:3(n-9) fatty acids. The pattern of fatty acid change due to vitamin E deficiency was similar to that seen in essential fatty acid deficiency (Castell *et al.* 1972; Watanabe *et al.* 1974, 1975). Similar histopathological findings were noted in vitamin E-deficient adult carp as in fingerlings, with degeneration of epaxial muscle, fewer, smaller insulin secretory cells in the endocrine pancreatic tissue, enlargement of the urinary tubules and changes in the pituitary. The ovaries of fish fed the

vitamin E-deficient diet were smaller and any oocytes were atretic and small. In control fish they were large and filled with yolk-granules and vesicles. Thus α-tocopherol deficiency in adult female carp, as in other animals, appeared to inhibit oocyte development.

1.2.5.6 Experimental studies of vitamin E nutrition in channel catfish

Dupree (1968) investigated the vitamin E requirement of channel catfish. Using tanks supplied with water free of natural food, 50 g fish (25-32 individuals) were fed diets containing 10% soybean protein, 10% casein or 10% fishmeal, each supplemented with 0, 1.0, 3.0, 5.0 or 20.0 mg of *RRR*-α-tocopheryl acetate 100 g^{-1}. The diets contained lard and cod liver oil as a lipid source, and were fed at a rate of 2-3% body weight day^{-1} for approximately 16 months. 100% mortality occurred in groups fed no α-tocopheryl acetate supplement. Before death, the fish became emaciated, lethargic and depigmented. They developed ascites and some showed slight exophthalmia. Maximum weight gain was achieved by the inclusion of 3 mg *RRR*-α-tocopheryl acetate 100 g^{-1} diet in casein and soybean based feeds, but only 1 mg 100 g^{-1} was required in the fish meal based diets, indicating that fish meal may have supplied up to 2 mg 100 g^{-1}. Without knowledge of the vitamin E content of the lipid components of the diet used in this study it was not possible to determine the absolute requirement of these fish for α-tocopheryl acetate.

Murai and Andrews (1974) studied interactions between α-tocopherol, oxidized menhaden oil and ethoxyquin in diets for fingerling channel catfish. The 3x3x2 factorial study included *all-rac*-α-tocopherol at 0, 2.5 and 10.0 mg 100 g^{-1} diet, 0, 1.0 and 10.0 g 100 g^{-1} oxidized oil (POV=6.0 mEq 100 g^{-1} oil), and 0 and 12.5 mg 100 g^{-1} ethoxyquin. Interactions between vitamin E and oxidized oil and dietary antioxidants are described in sections 1.2.6.2 and 1.2.6.3. Diets were casein based and contained 0.0068 mg 100 g^{-1} selenium, the lipid content was standardized to 10% using stripped lard, but losses of vitamin E during diet storage were not measured so absolute requirements could not be estimated. Experimental fish grew from an initial weight of about 14 g, to 143 g, they were held in well water at 26.5±0.5°C, and the diets were fed for a period of 16 weeks. High incidences of exudative diathesis,

depigmentation and mortality occurred in fish fed diets without oxidized oil, α-tocopherol or ethoxyquin, but no growth reduction, haematological changes, muscular dystrophy or fatty livers were observed in these fish. Based on the expression of deficiency signs in fish fed oxidized oil (section 1.2.6.2), Murai and Andrews suggested a requirement of 10.0 mg 100 g^{-1} *all-rac*-α-tocopherol, or 12.5 mg 100 g^{-1} ethoxyquin and 2.5 mg 100 g^{-1} α-tocopherol, in practical diets for catfish.

Lovell *et al.* (1984) studied the vitamin E requirement of channel catfish fingerlings fed diets containing a minimal level of polyunsaturated fatty acid. Casein-based diets containing 0.48% linoleic acid, 6% stripped lard and 0.04 mg 100 g^{-1} selenium, were supplemented with 0, 2.5, 7.5, 25.0 or 250.0 mg *all-rac*-α-tocopheryl acetate 100 g^{-1}. Fish grew from 5 to 45 g mean weight, at 28°C, over the 19 week experimental period. Feed was stored frozen and feeding rate was close to satiation, but no dietary vitamin E or malondialdehyde determinations were made. Growth rate was improved by supplementation of the basal diet with 2.5 mg α-tocopheryl acetate 100 g^{-1}, but no further increase was achieved with diets containing higher levels. Fish fed the basal diet were anorexic, lighter in colour, visibly thinner across the back, more easily excitable and had lower haematocrits, but failed to develop ascites or discoloration of internal organs. Histological signs in the α-tocopherol deficient fish included extreme atrophy and necrosis of the white (and to a lesser degree the red) muscle fibres, sclerotic glomeruli in the posterior kidney, and ceroid laden macrophages in the hepatic blood vessels. Fish fed 2.5 mg 100 g^{-1} α-tocopheryl acetate showed no gross pathological changes, but histological examination revealed necrosis of white muscle fibres and ceroid in liver blood vessels. No such changes were found in fish fed higher dietary levels. These results indicated that high levels of polyunsaturated fatty acids or oxidized lipids were not necessary for the production of myopathy in α-tocopherol deficient fish.

Wilson *et al.* (1984) also studied the vitamin E requirement of fingerling channel catfish using casein-based diets containing 1% cod liver oil plus 4% stripped lard and supplemented with 0 to 20.0 mg 100 g^{-1} *all-rac*-α-tocopheryl acetate. Diets contained adequate selenium, were stored frozen under nitrogen, and did not undergo any appreciable loss of α-tocopheryl

acetate. Catfish, initial weight 5-6 g, were fed the experimental diets, at 26.7±1.1°C, at a rate of 3% body weight day^{-1}. Two experiments were conducted, one for a period of 10 weeks at 25 fish tank^{-1}, and one for 16 weeks at 15 fish tank^{-1}. After this time stocking densities were reduced by 50% and the experiments continued for 14 weeks and 20 weeks respectively. No differences in growth rate or feed conversion efficiency were detected and no gross pathologies were apparent in fish fed the basal diets in either study. Erythrocyte haemolysis was detected in fish fed diets supplemented with 0 and 1.0 mg α-tocopheryl acetate 100 g^{-1}, but these fish showed no anaemia. In experiment 2, histological changes included multifocal haemosiderosis in the spleen and pancreatic tissue, and possibly reduction of pancreatic tissue. These were observed in fish fed unsupplemented diets, but not those receiving 2.0 mg vitamin E 100 g^{-1} or more. The minimum dietary vitamin E requirement, based on ascorbic acid-stimulated lipid peroxidation in hepatic microsomes collected after 16 weeks in experiment 2, was 5.0 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} dry diet.

Both Lovell *et al.* (1984) and Wilson *et al.* (1984) reported that the minimum vitamin E requirement suggested by Murai and Andrews (1974) was insufficient under practical conditions. Lovell *et al.* (1984) suggested that higher dietary levels of vitamin C used by Murai and Andrews may have had a sparing effect on vitamin E, thus reducing the apparent vitamin E requirement (section 1.2.6.5). In addition, Murai and Andrews (1974) used vitamin E as *all-rac*-α-tocopherol, whilst α-tocopheryl acetate was used in the other studies and under commercial conditions.

The absence of muscle degeneration as a result of simple vitamin E deficiency in the studies reported by Murai and Andrews (1974) and Wilson *et al.* (1984) is striking in comparison to the severe myopathy reported by Lovell *et al.* (1984). Its occurrence does not correlate with known vitamin E-lipid, or vitamin E-selenium relationships. Gatlin *et al.* (1986*b*) suggested that the higher stocking densities and lower dietary vitamin C concentrations used by Lovell *et al.* (1984) may have precipitated myopathy. To test these hypotheses, Gatlin *et al.* (1986*b*) studied the effects of stocking density and vitamin C status on vitamin E-adequate and vitamin E-deficient fingerling channel catfish. (see sections 1.2.7.7 and 1.2.6.5). Gatlin *et al.* (1986*b*)

also suggested that genetic (strain) differences may be responsible for such variability in the effects of deficiency (section 1.2.7.11). There are no clear differences in the level of forced exercise undergone by the fish used in these studies.

1.2.5.7 Experimental studies of vitamin E nutrition in tilapia

Lam (1985) fed *Oreochromis niloticus* fingerlings (4.5 g) semi-purified diets containing 6% stripped lard as a lipid source with five levels (0, 2.5, 5.0, 10.0 and 50.0 mg vitamin E 100 g^{-1}, for a period of 11 weeks. Deficiency signs included a lack of male sexual coloration, lesions in the trunk kidney and heart, and ceroid in the spleen and posterior kidney. Growth was improved in fish fed the diet supplemented with 2.5 mg 100 g^{-1} vitamin E compared to the unsupplemented group, but higher levels of addition did not improve weight gain. These results indicated a requirement for α-tocopherol by *O. niloticus* of 2.5 mg α-tocopheryl acetate 100 g^{-1} diet.

Satoh *et al.* (1987) also studied the vitamin E requirement of *O. niloticus* fingerlings. An experiment was conducted using casein-based diets and fish (initial weight 0.5 g) which had been reared on a tocopherol-free diet for the previous two weeks. The study lasted for 26 weeks and was carried out at 20°C. Graded levels of α-tocopheryl acetate (0, 5, 10, 30, 50 mg 100 g^{-1}) were added to a diet containing 5% lipid as fatty acid methyl esters. No overt deficiency signs were induced using diets containing 5% methyl esters, regardless of the level of dietary vitamin E, but fish receiving the basal diet showed a reduced hepatosomatic index and swollen, pale livers. These signs were prevented by the addition of more than 5 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} diet. Liver α-tocopherol concentrations were lowest in fish fed the basal diet and increased as the level of vitamin E in the diet increased. Liver total lipid concentrations increased in proportion to the dietary vitamin E level due to an increase in the triacylglyceride fraction. Fish fed the basal diet showed an increase in the proportions of 18:0 and 18:1(n-9) fatty acids and a reduction in 16:0 and 22:6(n-3) fatty acids in the liver polar lipids. Muscle proximate composition was also altered, with an increase in moisture content reflecting a reduction in protein and lipid.

Muscle thiobarbituric acid values were highest in fish fed the basal diet. Histological studies were not conducted. These results suggested a requirement of 5-10 mg α-tocopheryl acetate 100 g^{-1} in diets containing 5% lipid, but also indicated that higher dietary lipid levels increased the vitamin E requirement (see section 1.2.6.1).

1.2.5.8 Experimental studies of vitamin E nutrition in other fish species

Early experimental work on the effects of vitamin E deficiency in the guppy was described in section 2.1.1 (Cumings 1942). Yamakawa *et al.* (1975) studied the vitamin E requirement of the Japanese eel, *Anguilla japonica*. Deficiency signs included poor appetite, poor growth, haemorrhage and congestion in fins, and dermatitis. The minimum requirement of this species was estimated to be about 20.0 mg 100 g^{-1} diet (Lovell 1989).

1.2.6 Nutrient interrelationships

1.2.6.1 Vitamin E-lipid and vitamin E-polyunsaturated fatty acid interactions

In homeotherms, the requirement for vitamin E for the prevention of specific signs of deficiency is generally dependent upon the level of polyunsaturated fatty acids (PUFA) in the tissues (Dam 1962; Harris and Embree 1963; Green *et al.* 1967; Hayes *et al.* 1967; Green and Bunyan 1969; Alfin-Slater *et al.* 1972; Draper 1980*a*; Rice *et al.* 1985). This interaction can be envisaged to be a direct result of the increased risk of autoxidation in tissues containing larger amounts peroxidisable PUFA. In fish as in other species, the dietary PUFA content is reflected to some degree in the PUFA profile of the tissues (Bell *et al.* 1989); it therefore follows that increasing the dietary PUFA level or its degree of unsaturation, may increase the requirement for vitamin E. Such a relationship has not, however, been demonstrated for every vitamin E deficiency condition (Scott 1978). In addition to the effect on α-tocopherol requirement, dietary lipid levels may influence the efficiency of vitamin E digestion and absorption (section 1.2.9.5). Thirdly, peroxidation of dietary PUFA may cause the destruction of

α-tocopherol in feeds.

The results of Woodall *et al.* (1964) indicated that signs of vitamin E deficiency in chinook salmon were accentuated by the inclusion of larger quantities of herring oil, high in polyunsaturated fatty acids, in the diet. Furthermore, reductions in microhaematocrit, erythrocyte count and haemoglobin values, and an increase in the percentage of immature erythrocytes were observed only in fish fed diets containing 5% oil (highest level) without α-tocopherol. In contrast, the data of Cowey *et al.* (1981, 1983) suggested that increasing the dietary lipid (and PUFA) content failed to precipitate the expression of vitamin E deficiency in rainbow trout. In these studies both the total lipid content of the diet and the degree of unsaturation were increased. Watanabe *et al.* (1981*b*) discussed the relationship between dietary lipid levels and the α-tocopherol requirement of rainbow trout and described three experiments designed to examine this relationship. In the first, trout of average body weight 1 g were reared on a vitamin E-free diet for 20 days. Groups of these were then fed a partially-defined diet containing 15% pollock liver oil methyl esters and 0, 5, 10, 30 and 50 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} dry diet. The experiment continued for 12 weeks at 18-20°C during which the control group grew from 0.94 to 12.87 g. Fish fed the diet without vitamin E supplementation developed low appetite and reduced growth rate after 6 weeks of feeding, and began to show convulsions and mortalities after 7 weeks. The addition of 5 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} diet prevented the appearance of this syndrome, but larger supplements failed to improve growth or food conversion efficiency. In the second experiment, eight diets, four containing 5, 10, 15 and 20% methyl esters, 10, 5, 0 and 0% beef tallow and 5 mg 100 g^{-1} *all-rac*-α-tocopheryl acetate, and four with 15% methyl esters and 0, 5, 10 and 30 mg vitamin E 100 g^{-1}, were prepared. These were fed for 12 weeks at a water temperature of 16-22°C, during which the average weight of the control group increased from 1.27 to 10.43 g. Liver and carcass α-tocopherol concentrations were greatly reduced by increasing the degree of unsaturation of the dietary lipid. Weight gain was reduced as the percentage of PUFA in the diet increased but, although reduced feeding and growth were noted in fish fed the diets containing 15% methyl esters, convulsions and mortalities did not occur. Differences between this and the previous

experiment may have been due to the use of larger fish and possibly higher adventitious levels of dietary α-tocopherol. The results of this study indicated that, in a diet containing 15% lipid, 10 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} prevented deficiency signs. In a third experiment, conducted at 17-20°C, and using trout of mean weight 25.9-29.1 g, the effect of differences in the level of total lipid was examined in relation to the development of deficiency signs. Experimental diets containing 3, 5, 10 and 15% pollock liver oil plus 50 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} were fed to groups of fish for a period of 9 weeks. Liver lipid content was unchanged, liver and muscle α-tocopherol concentrations were higher in fish fed diets containing less than 10% fish oil, but liver contained more than muscle. These data support the hypothesis that increased dietary polyunsaturated lipid concentrations result in an increased vitamin E requirement and reduced tissue α-tocopherol concentrations.

Boggio *et al.* (1985) studied the effect of dietary α-tocopheryl acetate level together with lipid source, on the nutrition of rainbow trout. 30 g fish were fed herring meal-based diets containing either 8% herring oil (high PUFA) or 8% swine fat (low PUFA), and supplemented with 0, 5, 50 or 150 mg *all-rac*-α-tocopheryl acetate 100 g^{-1}, for a period of 124 days. At the end of this period, haematocrits were significantly lower in fish fed diets containing herring oil with 0 or 5 mg 100 g^{-1} α-tocopheryl acetate than in fish fed diets with swine fat and the same vitamin E supplements. This result demonstrated the effects of higher levels of polyunsaturated fatty acids on vitamin E nutrition.

The relationship between vitamin E nutrition and dietary polyunsaturated fatty acid content in carp fingerlings (weight 6.5 g) was studied by Watanabe *et al.* (1977*b*). Two experiments were conducted simultaneously over an 18 week period. In the first, partially characterized diets containing 5% methyl linoleate and 0, 10, 30, 50 and 100 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} were fed to groups of fish, and in the second, diets containing 10 mg α-tocopheryl acetate and 0, 2, 5, 10 and 15% methyl linoleate were used, together with a control containing 50 mg 100 g^{-1} vitamin E and 5% methyl esters. In experiment 1, weight gain was reduced and apparent muscular dystrophy was observed in the fish fed 0 and 10 mg 100 g^{-1} α-tocopheryl

acetate, but not in fish fed larger supplements. In experiment 2, fish fed diets containing 2% and 5% methyl linoleate, together with 10 mg 100 g^{-1} α-tocopheryl acetate, showed slightly lower growth than the control, but fish fed diets containing 10 and 15% methyl linoleate showed markedly lower growth and 35-40% developed apparent muscular dystrophy. This indicated that increasing the dietary polyunsaturated fatty acid content promoted the expression of signs of vitamin E deficiency. However, the preventive effect of higher levels of vitamin E was not demonstrated. In experiment 1, vitamin E concentrations in the muscle and hepatopancreas reflected dietary levels, and in experiment 2, tissue vitamin E concentrations were highest in the group fed 0% methyl linoleate. Malondialdehyde concentrations were highest in fish fed 0 mg vitamin E 100 g^{-1} and lowest in fish fed 0% methyl linoleate. Tissue lipid content and composition was broadly unaffected by either the α-tocopheryl acetate level, or the methyl linoleate content of the diets, however changes in the polyunsaturated fatty acid composition were again marked, particularly in the polar lipid fraction. Fish fed 0 mg α-tocopheryl acetate 100 g^{-1} showed lower proportions of (n-6) series fatty acids, higher proportions of 16:0, 16:1 and 18:1 fatty acids and reduced percentages of C-54 triglycerides. Fish fed the fat free diet also showed a reduction in (n-6) fatty acids and an increase in 16:1, 18:1 and 20:3 (n-9). A linear relationship between the proportion of C-54 triacylglyceride and the percentage dietary linoleate was noted in experiment 2.

Watanabe *et al.* (1981a) also studied the effect of the lipid content of the diet on the vitamin E requirement of carp fingerlings. Prior to the start of this study, fish had been reared for 2 weeks on a tocopherol-free diet containing 10% methyl palmitate. For the purpose of the experiment, the lipid source, methyl esters of pollock liver oil, was provided at levels of 0, 5, 10, 15, and 20% in partially-defined diets containing 5 mg *all-rac*-α-tocopheryl acetate. The control group was fed a diet containing 10% mixed lipid and 50 mg 100 g^{-1} vitamin E. 1.5 g carp were fed these experimental diets for 12 weeks at 18-22°C. Weight gain was lowest in fish given 0% lipid, probably due to essential fatty acid deficiency or the reduced energy content of the diets. Weight gain was greater in the group fed 5% methyl esters, but reduced in fish fed 10% and higher, and in fish fed 10% methyl esters and

above, apparent muscular dystrophy was observed. Once again fish showing muscular dystrophy were found to have high percentage moisture and low percentage protein compositions and increasing the level of dietary methyl esters above 5% resulted in a depletion of muscle and hepatopancreas α-tocopherol concentration. Changes in the fatty acid distribution were not recognised in this experiment perhaps since the pollock liver oil methyl esters were low in 18:2(n-6) fatty acids.

Satoh *et al.* (1987) fed 0.5 g *Oreochromis niloticus* diets containing 5, 10 or 15% fish oil methyl esters and 0 or 50 mg 100 g^{-1} α-tocopherol for a period of 9 weeks. Fish receiving diets containing 10% and 15% fish oil methyl esters without α-tocopherol supplementation began to show low appetite and reduced growth from the third week of feeding and showed poorer weight gain and feed conversion efficiency. Fish fed 15% lipid without vitamin E also showed 20% mortality during the course of the experiment. Supplementation with 50 mg 100 g^{-1} α-tocopheryl acetate improved these performance indices. Increasing the dietary lipid level from 5 to 15% reduced the concentration of α-tocopherol in the whole body. Increased moisture and TBA values and decreased protein and lipid content and muscle α-tocopherol concentrations were associated with higher dietary lipid levels and a lack of vitamin E supplementation.

Roem *et al.* (1990) studied the vitamin E requirement of the blue tilapia, *Oreochromis aureus*, in relation to the dietary lipid level. Semi-purified diets containing two levels of lipid (3% and 6% corn oil) and 0, 1.0, 2.0, 4.0 and 8.0 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} were fed to 2 g fish held at a temperature of 27°C, at 8-3% body weight per day, for 12 weeks. Fish fed the diets unsupplemented with vitamin E, at both lipid levels, exhibited reduced weight gain and food conversion efficiency and showed skin, snout, eye and fin haemorrhages, hyperaemic blood vessels on the head and operculum, anorexia, impaired erythropoiesis, erythrocyte disintegration, distended gall bladders, degeneration of cardiac and lateral skeletal muscle, ceroid, lipofuscin or melanin deposits in the liver and spleen, and lack of skin coloration. Muscle degeneration and liver pigment deposition was also noted in fish fed 6% lipid and 1.0 mg 100 g^{-1} α-tocopheryl acetate. The vitamin E requirement of this species was estimated to be 1.0 mg *all-rac*-α-tocopheryl

acetate 100 g^{-1} diet at 3% dietary lipid and 2.5 mg 100 g^{-1} at 6% lipid when diets contained 12.0 mg 100 g^{-1} butylated hydroxyanisoline (BHA). These authors suggested that vitamin E requirements be expressed as a function of dietary lipid level (ie. 0.3-0.4 mg α-tocopheryl acetate $percent^{-1}$ corn oil kg^{-1} diet for the blue tilapia).

1.2.6.2 Vitamin E-oxidized dietary lipid interactions

Whitmore (1965) found that, in chinook salmon fed vitamin E-deficient diets containing oxidized lipids, blood regeneration appeared to be delayed and impaired and signs of necrosis were observed in some erythrocytes. Early alterations were apparent within 4-6 weeks and the progressive changes suggested a disturbance to haemopoiesis. Fish fed rancid diets containing vitamin E developed slightly reduced haemoglobin and haematocrit values in comparison to fish fed vitamin E supplemented diets containing fresh oil. Therapeutic feeding of vitamin E to fish fed vitamin E-deficient, rancid diets, resulted in the blood indices returning to normal. This indicated that vitamin E can ameliorate the negative effects of rancid feed components.

Hung *et al.* (1980*b*) and Cowey *et al.* (1984) detected no effect of vitamin E-deficient diets containing 7.5% or 12% moderately oxidized oil (maximum POV about 5 mEq 100 g^{-1} oil) on weight gain, food conversion ratio and mortality or haematological and biochemical parameters in rainbow trout. However, Cowey *et al.* (1984) noted that fish fed oxidized oil in the absence of vitamin E showed more severe histological changes in the skeletal muscle, with areas of zonal necrosis, hyaline degeneration and increased eosinophilia. Hung *et al.* (1981) found that mortality was increased among rainbow trout fed diets containing 7.5% highly oxidized oil (POV=12 mEq 100 g^{-1} oil), and was reduced by supplementation of diets containing highly oxidized oil with *all-rac*-α-tocopheryl acetate. *In vitro* erythrocyte haemolysis was increased in fish fed highly oxidized oil, an effect which was also prevented by feeding α-tocopheryl acetate. Plasma and liver α-tocopherol concentrations were reduced in fish fed the highly oxidized oil. Moccia *et al.* (1984) described the histopathology and further details of the haematology and serum biochemistry of the fish used by Hung *et al.* (1981), together with a third

group fed extremely oxidized oil (POV=31.4 mEq 100 g^{-1} oil). Fish fed diets containing the highly or extremely oxidized oils without *RRR*-α-tocopheryl acetate supplements exhibited lower erythrocyte counts, haemoglobin concentrations and haematocrits. Gross pathological signs included splenic enlargement, liver discolouration, gall bladder distension and the presence of small amounts of viscous, yellow gut contents. In addition, fish from these groups had increased numbers of abnormally developing polychromatocytes, splenic haemosiderosis and hepatic focal necrosis and ceroidosis, but no muscular dystrophy. These observations suggested that mortalities in this study were due to the cumulative effect of liver dysfunction and anaemia. Supplementation with 3.3 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} prevented deficiency signs, but ethoxyquin supplementation exerted only partial protection in the absence of α-tocopheryl acetate. Řehulka (1990) found that reduction in erythrocyte count, haematocrit and haemoglobin in trout could be directly related to the degree of dietary lipid oxidation.

A histopathological study of carp fed α-tocopherol deficient diets with oxidized methyl linoleate was described by Miyazaki (1986). Experimental, casein-based, diets containing 10% fresh soybean oil (POV=0.48 mEq 100 g^{-1}) or methyl linoleate oxidized for 30, 40, 90 or 200 hrs (POV=67.5, 114.0, 160.0, 130.0 mEq 100 g^{-1}), were fed for a period of 5 months to fingerling carp (initial weight 1.g) held at 20°C. Diets containing soybean oil and 40 hr oxidized methyl linoleate were prepared with and without 25 mg α-tocopheryl acetate 100 g^{-1}. There was no significant difference in weight gain between groups fed soybean oil with and without α-tocopheryl acetate, but in groups fed diets containing oxidized oil with or without vitamin E, weight gain was significantly reduced at 5 months. Overt muscular dystrophy was observed most predominantly in fish fed the 30, 40 or 90 hr oxidized methyl linoleate without α-tocopheryl acetate. Histopathological examination of these fish showed mild to severe myopathy of the lateral white muscle. In milder cases there was focal atrophy and necrosis and splitting of fibres. In moderate cases, there was a more general degeneration and necrosis of fibres, some macrophage invasion was observed, and ceroid laden macrophages were noted in the hepatopancreas, spleen and kidney. In severe cases, most of the white muscle fibres were necrotic and atrophied, with separation of the sarcoplasm,

oedema and production of connective tissue. These lesions were noted on both sides of the lateral musculature. Larger numbers of ceroid laden macrophages were observed in the visceral organs. Hepatocytes showed atrophy and ceroid deposition and the B-cells of the pancreatic islets showed some degranulation. In contrast to fish fed unoxidized diets (Watanabe *et al.* 1970*a*,*b*), ceroid was observed even in the tissues of fish with no evidence of myopathy, including some from fish on vitamin E supplemented diets.

Murai and Andrews (1974) (section 1.2.5.6) found that channel catfish fed diets containing 10% oxidized menhaden oil (POV=6.0 mEq 100 g^{-1} oil) without supplemental α-tocopherol exhibited poor weight gain, feed conversion efficiency and survival, together with a high incidence of exudative diathesis, muscular dystrophy and loss of epidermal pigmentation. Exudative diathesis was manifested by exophthalmia, ascites and a swollen, inflamed vent. Affected fish showed increased susceptibility to stress. Fatty livers and anaemia (reduced haematocrit, increased numbers of polychromatocytes with rounded nuclei and increased numbers of haemolytic erythrocytes) were also noted, and histopathological changes in the muscle fibres (atrophy, hyaline or granular degeneration), kidney (hyaline droplet degeneration) and pancreatic tissue (atrophy, necrosis of acinar cells, degeneration of B-cells) were observed. Addition of α-tocopherol effectively prevented these signs.

Soliman (1982) and Soliman *et al.* (1983) described the pathological effects of feeding rancid lipid with and without vitamin E supplementation in diets for *Oreochromis niloticus* (Trewavas). Fish (initial weight 2.4-2.8 g) were fed defatted fishmeal-based diets for a period of 6 weeks at a rate of 5% body weight per day and at a temperature of 28°C. Diets contained 10% fresh (POV=4.28 mEq 100 g^{-1}) or oxidized (POV=144.6 mEq 100 g^{-1}) fish oil, both with and without 10 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} (2x2 factorial design). Specific growth rates and food conversion efficiencies were similar, but fish fed diets containing rancid lipid without vitamin E supplementation showed congestion of snout and fin vessels, lordosis, exophthalmia, cataract and orbital collapse, and oedema. These external signs appeared rapidly (within seven days) and 23% mortality occurred over the experimental period. Internally, darkening of the liver and distension of the bile duct were also observed. With the exception of lordosis, similar signs were noted in fish fed

the rancid lipid with vitamin E, but fish fed fresh lipid were clinically normal. Histologically, fish fed rancid lipid exhibited steatitis of all abdominal fat bearing tissues, loss of hepatic lipid and deposits of intracellular ceroid in the liver, spleen and kidney and choroid. A mild meningitis, subependymal oedema and a high frequency of pyknotic nuclei within cells of parenchymatous tissue were also recorded. Skin, heart and gills were unaffected, and skeletal muscle changes were minor being restricted to localized, bland sarcoplasmic degeneration, particularly in the red muscle around the lateral line and in the head. These signs were also present, but less marked, in fish fed diets supplemented with vitamin E.

Stéphan *et al.* (1989) studied the effects of oxidized fish oil and antioxidant deficiency in sea bass, *Dicentrarchus labrax*. Two experiments, one with 80 g and the other with 250 g fish were conducted using practical-type diets, one containing 7% oxidized fish oil and lacking supplemental vitamin E (adventitious concentration 0.04 mg 100 g^{-1}), choline and synthetic antioxidant. These were fed for a period of 5 months at a water temperature of 18°C. Fish fed the unsupplemented, oxidized oil diet show granular degeneration of muscle (particularly red muscle) fibres and increased plasma glutamic-oxaloacetic transaminase and creatine kinase activities and liver and muscle malondialdehyde concentrations. These changes were most marked in the groups of smaller fish which also had the best appetite and the best growth. A normochromic and normocytic anaemia and an increase in erythrocyte haemolysis were also observed. Sakaguchi and Hamaguchi (1979*a*,*b*) studied the effects of oxidized oil, with and without vitamin E and vitamin B2, in diets for cultured red sea bream (*Chrysophrys major*). In the first of these studies, diets containing moderately oxidized oil (POV=55 mEq 100g^{-1}) caused a reduction in hepatopancreatic fat, but this effect was not noted in fish fed severely oxidized oil (POV=155 mEq 100g^{-1}). Plasma triacylglyceride and cholesterol concentrations decreased in fish fed oxidized oil, particularly in fish fed diets containing severely oxidized oil and without vitamins E and B2. In the second study, oxidized oil was found to cause a decrease in hepatopancreatic glycogen and an increase in plasma glucose but the effect of vitamin E was not examined.

Several factors may be responsible for the pathological effects of

feeding oxidized oil. Forster *et al.* (1988) pointed out that oxidation may reduce the digestibility of the lipids, reduce the dietary concentrations of labile essential polyunsaturated fatty acids, vitamins and amino acids, and may diminish the activities of some digestive enzymes. Furthermore, oxidation products of lipids may be directly toxic if they are absorbed. Hung *et al.* (1980*b*) indicated that endogenous α-tocopherol concentrations in practical fish diets were significantly reduced by the addition of moderately oxidized oil and by storage for 24 weeks, but found that the concentrations of supplemental α-tocopheryl acetate were unaltered. Hung and Slinger (1980) reported that oxidized dietary lipid caused a reduction in the ascorbic acid content of feed and in liver ascorbate in fish fed these diets. Hung *et al.* (1981) showed that the percentage of polyunsaturated fatty acids in the total fatty acids were also significantly affected by the level of oxidation of the oil. Oxidized dietary lipids may reduce the protein efficiency ratio (Lohrey *et al.* 1978) and growth in fish (Ketola *et al.* 1989).

The practical significance of vitamin E-oxidized lipid interactions in aquaculture is demonstrated by the wide variety of clinical conditions involving diet rancidity in fish which respond to vitamin E treatment (section 1.2.8). However, a full explanation for the beneficial effect of vitamin E is difficult. Lipid hydroperoxide absorption may be enhanced by α-tocopherol. Takeuchi (1972) studied the absorption of lipid hydroperoxides in carp by comparing the uptake into various tissues of radio-labelled methyl linoleate (control) and radio-labelled methyl linoleate hydroperoxide (MLHP), administered via a tube into the gut. The carp absorbed little MLHP by 24 hours, but uptake was facilitated by vitamin E. In addition, α-tocopherol may prevent some toxic effects of lipid peroxides by the interruption of autoxidation reactions initiated by these compounds. Watanabe *et al.* (1970*b*) found that dietary α-tocopherol reduced the rate of loss of vitamin C and prevented an increase in the TBA value of the feed, but Hung *et al.* (1981) found that α-tocopheryl acetate supplements failed to prevent the loss of fatty acids or α-tocopherol from the diet. The acetate form has low antioxidant activity, but is stable as a feed supplement. Synthetic antioxidants are used commercially to protect labile dietary components against oxidative destruction.

1.2.6.3 Vitamin E-dietary antioxidant interactions

Several vitamin E deficiency diseases in homeotherms respond to dietary supplementation with synthetic antioxidants (Draper and Csallany 1958; Draper *et al.* 1958; Scott 1978). In such cases the possible *in vivo* activity of the antioxidant has been the subject of debate since these compounds also act to spare α-tocopherol from oxidation *in vitro*. Furthermore, since antioxidants lack specificity for the cell membrane they are eliminated from the tissues more rapidly than α-tocopherol. Antioxidants in commercial diets for fish inhibit lipid oxidation, conserve labile vitamins and other feed constituents, act to spare vitamin E and may have *in vivo* activity (Rumsey 1980). Ethoxyquin, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used in the preparation of fish feeds. Legal maximum dietary inclusion levels exist in some countries.

Hung *et al.* (1981) found that plasma and liver α-tocopherol concentrations in rainbow trout were unaffected by ethoxyquin and that no ethoxyquin supplementation was necessary in diets provided they contained fresh oil. Moccia *et al.* (1984) reported that ethoxyquin was less effective than α-tocopherol in preventing the pathological effects of oxidized oil, but did have some ameliorative effects. Murai and Andrews (1974) found that ethoxyquin supplementation prevented some, but not other, signs of vitamin E deficiency in channel catfish, and concluded that this antioxidant could partially replace α-tocopherol in feed. Similarly, Soliman (1982) recorded that BHT was also partially effective in the prevention of the pathological effects of feeding oxidized oil in *Oreochromis niloticus*.

1.2.6.4 Vitamin E-selenium interactions

As a component of glutathione peroxidase, selenium is involved in cellular defence against toxic products of peroxidation *in vivo* (Rotruck *et al.* 1973). Like vitamin E, selenium is therefore functional in the prevention of pathologies due to autoxidation of polyunsaturated fatty acids (Bell and Cowey 1985). In view of their related function it may be expected that the requirement for vitamin E may be dependent on selenium status and indeed,

selenium responsiveness has been demonstrated in several vitamin E deficiency conditions of homeotherms (Draper 1980a).

Poston *et al.* (1976) observed fragmented erythrocytes in Atlantic salmon deficient in both vitamin E and selenium. Combined vitamin E-selenium supplements increased liver, but not carcass, vitamin E concentrations above those achieved by vitamin E supplementation only. Histological lesions were most severe in combined vitamin E-selenium deficiency, but also present in groups singly deficient in either vitamin E or selenium. Selenium supplementation caused a large increase in plasma GPX activity, and this was enhanced in the presence of vitamin E, but vitamin E in the absence of selenium had no such effect.

Vitamin E-selenium interrelationships in rainbow trout were studied by Bell *et al.* (1985, 1986). Trout of mean weight 11 g, previously reared on commercial feeds, were fed one of four diets which were adequate or low in selenium or vitamin E or both (diet 1: 4.06 mg 100 g^{-1} vitamin E, 0.0869 mg 100 g^{-1} selenium; diet 2: 4.06 mg 100 g^{-1} vitamin E, 0.006 mg 100 g^{-1} selenium; diet 3: 0.214 mg 100 g^{-1} vitamin E, 0.0877 mg 100 g^{-1} selenium, and diet 4: 0.196 mg 100 g^{-1} vitamin E, 0.006 mg 100 g^{-1} selenium). Supplemental vitamin E was as *all-rac*-α-tocopheryl acetate, and supplemental selenium as sodium selenite, lipid was supplied as linoleic acid (1%) plus palmitic acid (9% in diet) and protected by the use of a dietary antioxidant. The experiment was conducted over a period of 40 weeks at a temperature of 15°C. Fish were fed at 2% body weight day^{-1} and the control group grew from 11.3 to 351.8 g over this period. Weight gain was reduced and exudative diathesis developed in animals fed the dually deficient diet, but no mortalities occurred and no histological signs of skeletal muscle degeneration were detected. Vitamin E deficiency alone caused no significant loss of growth and no exudative diathesis. A significant interaction between dietary vitamin E and selenium was detected on haematocrit and *in vitro* microsomal lipid peroxidation and, in plasma, dietary vitamin E had a significant effect on selenium concentration. Glutathione peroxidase activity was significantly reduced in the plasma and liver of trout fed selenium-deficient diets, but was unaffected by vitamin E intake. Plasma pyruvate kinase activity increased only in fish dually deficient in vitamin E-selenium, and may have indicated subclinical

muscle degeneration. Similarly, Bell, McVicar, Mitchell and Cowey (unpublished) found a synergistic effect of combined vitamin E and selenium deficiency on mortality, haematocrit reduction, increased susceptibility to haemolysis and plasma pyruvate kinase activity in Atlantic salmon parr (G.Bell; personal communication 1987).

Gatlin *et al.* (1986*a*) investigated vitamin E and selenium interrelationships in the nutrition of fingerling channel catfish. A 2x2 factorial experiment was conducted using a vitamin E-selenium deficient, casein-based diet (containing 0.25 mg α-tocopherol and 0.006 mg selenium 100 g^{-1}) alone, or supplemented with 5.0 mg *all-rac*-α-tocopheryl acetate 100 g^{-1}, 0.02 mg sodium selenite 100 g^{-1}, or both. The diet contained a high level of unsaturated fat (10% stripped corn oil). Triplicate groups of 80 g catfish (17 individuals) were fed the test diets at 3% body weight per day, at 27°C, for a period of 19 weeks. Some fish were removed and the experiment continued to 26 weeks. Combined deficiencies of vitamin E and selenium caused suppressed growth, reduced haematocrit, severe myopathy, exudative diathesis and death. Histologically, severe necrosis of skeletal muscle with mononuclear cell inflammatory infiltrate, and moderate to severe glomerulonephritis of the posterior kidney, were apparent. Singular deficiencies of either vitamin E or selenium did not consistently produce any of these deficiency signs. Vitamin E deficiency caused elevated ascorbic acid-stimulated peroxidation of hepatic microsomes, which was unaffected by selenium supplementation. Selenium deficiency caused reduced glutathione peroxidase activity and increased glutathione transferase activity in the liver, regardless of vitamin E supplementation.

1.2.6.5 Vitamin E-ascorbic acid interactions

Ascorbic acid can act as an *in vivo* antioxidant in non-lipid tissue compartments (Halver 1989) and may in some circumstances replace the functional requirement for vitamin E. Thus, Chen *et al.* (1980) demonstrated the sparing action of vitamin C on metabolism of vitamin E in vitamin E-deficient rats. In addition, Tappel (1968) and McCay (1985) presented evidence to suggest that ascorbic acid acts to regenerate vitamin E through reduction

of the deactivated α-tocopheroxyl radical to α-tocopherol.

Lovell *et al.* (1984) suggested that the presence or absence of myopathy due to vitamin E deficiency in channel catfish (Murai and Andrews 1974; Lovell *et al.* 1984; Wilson *et al.* 1984; Gatlin *et al.* 1986*a*) may be explained by differences in dietary ascorbic acid content. This interaction was studied by Gatlin *et al.* (1986*b*). A casein-based diet, deficient in vitamin E and ascorbic acid, was supplemented with 0 or 5.0 mg 100 g^{-1} *all-rac*-α-tocopheryl acetate and 0 or 6.0 mg vitamin C 100 g^{-1} (2x2 factorial design), and fed to groups of 15, 4.6 g catfish for 20 weeks. Fish fed diets without supplemental vitamin C had significantly reduced growth and feed efficiency regardless of vitamin E supplementation. No gross deficiency signs were noted in fish fed vitamin E-deficient diets with or without vitamin C supplementation. Vitamin E-deficient fish again showed increased microsomal lipid peroxidation, but this too was unaffected by vitamin C supplementation. Thus, vitamin C status did not appear to markedly influence the vitamin E nutrition of this species.

Poston and Combs (1979) examined the interrelationships between dietary requirements for selenium, vitamin E and vitamin C. A diet containing 0.008 mg selenium, 1 mg vitamin E and 5 mg vitamin C 100 g^{-1} was fed to groups of Atlantic salmon for a period of 26 weeks. Lethargy, anorexia, reduced muscle tone and increased mortality were induced. Affected fish lay on their side and made no attempt to swim when handled. Moribund fish had yellow-coloured livers and pyloric caeca. Normal haematocrit and the absence of scoliosis suggested that dietary levels of vitamins E and C were adequate, and the condition was attributed to selenium deficiency. Affected fish were fed repletion diets containing 0, 0.005, 0.010 or 0.015 mg selenium 100 g^{-1} plus supplements of either 8 mg vitamin E, 100 mg vitamin C or both 100 g^{-1} dry diet. Salmon fed no supplemental vitamin E or vitamin C grew slowly and showed high mortality, with the highest mortality in the diet without selenium supplementation. Single supplements of either vitamin E or vitamin C were equally effective in reducing mortality. This suggested that selenium requirements could be partially replaced by either vitamin E or ascorbic acid.

1.2.6.6 Vitamin E-Vitamin A interactions

Moore (1940) reported that vitamin E reduced the rate of vitamin A depletion from rat liver and high levels of dietary vitamin A have been found to increase vitamin E requirements (Pudelkiewicz *et al.* 1964) and reduce plasma vitamin E concentrations (Frigg and Brox 1984). Vitamin E may protect against vitamin A toxicity (Jenkins and Mitchell 1975), and may promote absorption of vitamin A. The mechanism of this relationship is uncertain, but Cawthorne *et al.* (1968) indicated that it was not simply a result of interactions between an antioxidant and an oxidizable substrate.

Poston (1968) studied the action of dietary tocopherol in a synthetic vitamin A test diet for brook trout, *Salvelinus fontinalis*. A 2x2 factorial experiment was designed utilizing high and low levels of vitamin A, each in combination with 20 or 125 mg *all-rac*-α-tocopheryl acetate 100 g^{-1} diet. High level vitamin E supplementation did not affect tissue vitamin A concentrations. Fish fed the higher levels of both vitamin A and vitamin E had higher haematocrits than fish fed single high-level supplements of either vitamin, and also a lower frequency of ventral subcutaneous discoloration. These results were interpreted to suggest that the lower level of vitamin E (20 mg 100 g^{-1}) was inadequate for the optimum formulation of a test diet for this species and that vitamins A and E may act synergistically in stimulating haemopoiesis. Sugii and Kinumaki (1968) found that vitamin E was distributed quite differently to vitamin A in the tissues of skipjack (*Katsuwonas pelamis*), carp and rainbow trout. Ushikusa *et al.* (1986), likewise, could find no correlation between tissue concentrations of vitamins E and A in carp. Watanabe *et al.* (1970*a*) noted that tissue vitamin A concentrations were slightly but not significantly lower in carp fed a vitamin E-deficient diet, and Watanabe *et al.* (1977*b*) found no significant difference in tissue vitamin A concentrations in carp fed different levels of vitamin E and polyunsaturated fatty acids.

1.2.6.7 Vitamin E-carotenoid interactions

Carotenoids may have an antioxidant function in fish (Tacon 1981) as in

other species (Burton and Ingold 1984). On this basis, some degree of inter-replacement between vitamin E and carotenoids may be expected, though none has yet been demonstrated in fish. However, Pozo *et al.* (1988) found that increasing the level of α-tocopherol in the diet of rainbow trout increased the deposition of canthaxanthin in the flesh, whilst Torrissen (1985) found that the quantity of astaxanthin laid down in the flesh of rainbow trout was not reduced even if α-tocopherol was excluded from the diet.

1.2.6.8 Vitamin E-iron interactions.

Iron may act as a pro-oxidant catalyzing the oxidation of polyunsaturated fatty acids and leading to the destruction of α-tocopherol in fish diets and tissues. The effects of iron toxicity are inhibited by vitamin E, presumably as a result of the prevention of oxidation (Scott 1978). Cumings (1942) used iron-treated diets to induce vitamin E deficiency in the guppy, and Desjardins *et al.* (1987) demonstrated that iron supplementation caused an increase in the malondialdehyde content of diets as a result of lipid oxidation and led to high mortality, poor growth, and the development of histological changes in the liver and kidney.

1.2.6.9 Vitamin E-sulphur amino acid interactions

Interactions between vitamin E and the sulphur-containing amino acids, methionine and cystine have been described in relation to the development and prevention of necrotic liver degeneration in rats (Schwarz and Foltz 1957) and skeletal myopathy in chicks (Scott 1970). The sparing effect of these amino acids on vitamin E requirement may be due to the reducing properties of sulphydryl forms of these nutrients (Draper 1980*a*). Interactions between vitamin E and sulphur amino acids seem not to have been demonstrated in fish.

1.2.6.10 Vitamin E-other nutrient interactions

In homeotherms, a wide variety of other nutrients have been proposed to interact with vitamin E, leading to or preventing the development of specific

deficiency syndromes, or resulting in variations in tissue α-tocopherol concentrations. These include vitamin B1 (D'Agostino 1952*a*), vitamin B6 (Young *et al.* 1955), vitamin B12 (Oski *et al.* 1966), nicotinic acid (D'Agostino 1952*b*), tryptophan (Bunce and Hess 1976), vitamin D2 (Selye *et al.* 1964), manganese (Lee *et al.* 1962), silver (Diplock *et al.* 1967), magnesium (Selisko 1961), zinc (Lü and Combs 1988), copper (Dove and Ewan 1981), mineral combinations (Van Vleet *et al.* 1981, Van Vleet 1982), sulphur dioxide (Scott 1973), choline (Wilson *et al.* 1973,1974), quinones (Spratt and Kratzing 1971), nitrite and ionophores (Tengerdy 1989), and fibre (DeLumen *et al.* 1982; Omaye and Chow 1984; Schaus *et al.* 1985; Kahlon *et al.* 1986). These interactions seem not to have been studied in fish.

1.2.7 Influence of physiological state and environment

1.2.7.1 Age and size

Cumings (1942) reported that the severity of signs of vitamin E deficiency in the guppy were related to the age of the fish when experiments were initiated. Watanabe *et al.* (1970*a*,*b*) described more severe signs of vitamin E deficiency in 1.5 g carp fry than in 10 g fingerlings (section 1.2.5.5). The difficulties of inducing vitamin E deficiency experimentally in large fish may be a result of the accumulation of greater tissue reserves which will also be dependent upon previous feeding history and vitamin E intake.

1.2.7.2 Growth rate

Vos *et al.* (1981) reported that myopathy as a result of vitamin E deficiency in ducklings could be prevented if vitamin E depletion was delayed until the animals reached two weeks of age. This coincided with a reduction in the growth rate of these animals which was demonstrated mathematically to result in a lesser degree of dilution of tissue vitamin E. It was therefore proposed that the rapid growth rate may be at least partly responsible for the increased susceptibility of young animals to vitamin E deficiency.

1.2.7.3 Exercise

Hulland (1985) distinguished between nutritional and exertional myopathies, yet several authors have reported the occurrence of exertional myopathies in animals with low tissue vitamin E concentrations (Christl 1971; Anderson *et al.* 1977) and therapeutic effects of vitamin E-selenium have been demonstrated upon these (Ullrey *et al.* 1985). It is speculated that the occurrence of muscle degeneration in vitamin E-deficient rainbow trout, as described by Cowey *et al.* (1984), may be explained by the increased level of exercise under the conditions of this study.

1.2.7.4 Maturation

Vitamin E is necessary for gonadal development in female carp (Watanabe and Takashima 1977). Accumulation of vitamin E in the ovary of carp was described by Kinumaki *et al.* (1972) even in deficient fish. Mézes (1986) recorded cyclical changes in the vitamin E content of the ovary, but not of plasma and liver, before and after ovulation in carp. Ushikusa *et al.* (1986) recorded that tissue tocopherol concentrations, generally, tended to increase during maturation in carp. On this basis it seems likely that the demand for vitamin E may be greater during periods of maturation.

1.2.7.5 Temperature

Cowey *et al.* (1984) suggested that the development of muscle degeneration as a result of vitamin E deficiency in rainbow trout may have been related to a reduction in water temperature. Low water temperatures lead to an increase in the proportion of polyunsaturated fatty acids in the lipid membranes of rainbow trout (Hazel 1979), and may therefore be expected to increase demand for vitamin E.

1.2.7.6 Salinity

Schulz *et al.* (1984) measured higher α-tocopherol concentrations in the

liver oils of marine fish than freshwater species. Atlantic salmon appear to be more susceptible to vitamin E deficiency in seawater than in freshwater (Bell, McVicar, Mitchell and Cowey, unpublished). This may reflect the fatty acid composition of fish in these different environments. Marine fish species have higher tissue concentrations of (n-3) fatty acids, whilst (n-6) fatty acids are more predominant in freshwater species (Cowey and Sargent 1972). Changes in salinity result in a shift in the polyunsaturated fatty acid composition of anadromous species (Lovern 1938; Lall and Bishop 1979; Sheridan *et al.* 1985; Halver 1989). Further consideration to this subject is given in section 3.3.1.

1.2.7.7 Stress

Stress factors have been implicated as the cause of changes in tissue vitamin E concentrations and the expression of signs of deficiency in various species. These are discussed in section 5.1.1. A possible relationship between the effects of stress on ascorbic acid and the role of ascorbic acid in regenerating active tocopherol has been proposed to link stress and vitamin E status (Putnam and Comben 1987). According to this hypothesis, marginal vitamin C deficiency caused by stress may reduce the availability of α-tocopherol and thus precipitate signs of vitamin E deficiency (Putnam and Comben 1987). Several studies have shown that stress alters ascorbic acid status in fish as in other animals (Baker 1967; Wedemeyer 1969; Thaxton and Pardue 1984; Thomas 1987). The relationship between ascorbic acid and vitamin E was described in section 1.2.6.5. To date there is no evidence that the two processes are linked and result in stress-induced signs of vitamin E deficiency.

1.2.7.8 Feeding rate

Reduced food intake or starvation may be expected to affect the vitamin E status of fish. Ackman and Cormier (1967) recorded a 50% reduction in α-tocopherol in the tissues of cod, *Gadus morhua*, starved for 6 months. High food intake may cause malabsorption in fish (Jobling 1986), giving rise to

nutritional imbalance. The possible effects of feeding rate on vitamin E status are considered in section 3.1.3.

1.2.7.9 Oxygen tension

Radi *et al.* (1988) found that more polyunsaturated fatty acids were present in the tissues of carp in conditions of hyperoxia and hypoxia, than in anoxia. The rate of lipid peroxidation and the activity of glutathione peroxidase increased and superoxide dismutase activity decreased as the oxygen tension was reduced. Vitamin E concentrations have not been measured in fish exposed to different oxygen concentrations, but vitamin E-deficient rats have been found to be more susceptible to increases or decreases in oxygen tension (Hove *et al.* 1945; Telford *et al.* 1954; Taylor 1956).

1.2.7.10 Toxins

Coffin and Combs (1981) demonstrated that the absorption of vitamin E was inhibited in chicks fed meal containing a mycotoxin and Verma and Tonk (1983) found that vitamin E concentrations in the tissues of the teleost, *Notopterus notopterus*, were affected by exposure to sublethal levels of mercury. Exposure to toxins may thus affect vitamin E status.

1.2.7.11 Genetic constitution

There are clear species differences in susceptibility to and effects of vitamin E deficiency. These may reflect inter-specific differences in PUFA concentrations and antioxidant activity (Gabryelak and Pérès 1986). Radi *et al.* (1987) found that tissue PUFA concentrations and activities of antioxidant enzymes were higher in herbivorous species. Gatlin *et al.* (1986*b*) suggested that strain differences may explain the inconsistency in experimental results of the effects of vitamin E deficiency in channel catfish. Genetic predispositions to antioxidant disorders are known to occur among homeotherms (Hoppe *et al.* 1989).

1.2.7.12 Infection

There is some evidence that infection may increase the vitamin E requirement or induce deficiency in mammals (McMurray and Rice 1982). Histological signs of vitamin E deficiency in fish suffering from microbial diseases were reported by Wood and Yasutake (1956), Ferguson and Rice (1980), Fjelstad and Heyeraas (1985), Snieszko *et al.* (1957) and Poppe *et al.* (1986).

1.2.8 Vitamin E-related clinical disease in fish

Nutritional fish diseases are difficult to define in absolute terms since simple deficiencies are rare and sufficient information to provide conclusive proof of their origin is often not available (Cowey and Roberts 1978). Nevertheless, several vitamin E-responsive conditions have been described in various fish species.

1.2.8.1 Salmonids

Lipoid liver degeneration

Lipoid liver degeneration (LLD) is characterised by the abnormal accumulation of lipid or ceroid pigment (Wood and Yasutake 1956) within hepatocytes. There is extreme anaemia and also some degeneration of splenic and renal haemopoietic tissue. The liver is swollen and bronzed, and the gills appear pale (Roberts and Bullock 1989; Roberts 1989). External indications include reduction in appetite, loss of equilibrium and some mortality. The condition was first described by Plehn (1909) and affects salmonids, mullets, channel catfish (*Ictalurus punctatus*), and several other species. The precise aetiology of LLD is not fully known, but it probably results from the use of rancid feed materials (Ghittino 1961; Woodall *et al.* 1964; Fowler and Banks 1969; Roald 1976; Smith 1979; Roald *et al.* 1981). Fowler and Banks (1969) indicated that damage to the liver and kidney in chinook salmon fed rancid diets, was prevented by the addition of vitamin E or a combination of vitamins E and C, but vitamin C alone was not completely effective. Roald (1976) demonstrated the therapeutic effect of α-tocopherol and ethoxyquin, and α-

tocopherol and selenium, on LLD in Atlantic salmon and Smith (1979) showed that LLD induced in rainbow trout by feeding a diet containing rancid fish meal was effectively prevented by the inclusion of *all-rac*-α-tocopheryl acetate plus ascorbic acid. Kawatsu (1960) described a dietary disease of rainbow trout in Japan, caused by feeding dried silk-worm pupae. The condition appeared to be similar to LLD in that affected fish showed anaemia together with enlarged, yellow-tinted livers, but the condition also led to heavy mortality. Kawatsu (1964) demonstrated that *all-rac*-α-tocopheryl acetate prevented anaemia associated with this condition, but did not report its effect on changes in the liver or on the mortality rate.

Nutritional myopathy

Kubota *et al.* (1981*b*) recorded a nutritional myopathy accompanying extensive ceroidosis in rainbow trout, caused by feeding rancid oil and corrected by increasing the α-tocopherol content of the diet. Besse *et al.* (1965) reported the occurrence of muscle degeneration in rainbow trout, which seemed to respond to dietary vitamin E supplementation. Skeletal myopathy in rainbow trout fry was also described by Carbery and Donnelly (1969). Affected fish had been fed a commercial diet which had been stored for some time. They exhibited increased mortality, pallor of the gills and other organs, and swelling, hyalinisation and disintegration of skeletal muscle fibres. Replacement of the diet with fresh liver resulted in the cessation of mortalities.

King (1975) described nutritional myopathy, together with steatitis, in rainbow trout fry in the United States and Venezuela. Bilateral hyaline degeneration of muscle fibres was apparent, particularly in the active caudal musculature. These conditions were successfully treated by dietary manipulation, and since they resembled vitamin E-selenium responsive diseases in cattle, cats and pigs, were assumed to have a common basis in vitamin E-selenium nutrition. Apparently similar conditions were noted in Atlantic salmon in Scotland and feral pickerel (*Esox* sp.) from Lake Cuyahoga, Ohio.

Pansteatitis

Roberts and Richards (1978) and Roberts *et al.* (1979) documented the

occurrence of pansteatitis, inflammatory infiltration of adipose tissue, in rainbow trout. Peri-pancreatic fat was most affected, but similar changes were also evident in lipoid tissue in the skin, heart and swimbladder. The main presenting signs were swimming aberrations, discoloration and high mortality after minimal stress. Swimbladder changes were considered pathognomic. The liver showed some inflammatory infiltration and also zones of necrosis, fatty degeneration and ceroid accumulation. Myopathy of the heart, skeletal and oesophageal muscles was also common. Pansteatitis in mink, cats, pigs and poultry is associated with feeding diets high in unsaturated fatty acids or certain types of trash fish or fish oils, and low in vitamin E, but in this study a change of diet failed to prevent mortalities. Baudin-Laurencin *et al.* (1985) described 'summer pathology' in marine farmed rainbow trout, and a similar condition has been reported in coho salmon (*Oncorhynchus kisutch*) (Aldrin *et al.* 1980). Peri-pancreatic leucocytic infiltration was similar to the description of pansteatitis (Roberts *et al.* 1979) and suggested a possible relationship with vitamin E nutrition. However, the origins of this condition are unknown.

Ferguson and Rice (1980) described an 'oedema syndrome' causing mortalities among post-spawned, farmed brown trout. Ascites, anaemia and myocardial degeneration together with low serum vitamin E titres (2.5 ppm), were suggestive of vitamin E deficiency. However, other features of the condition indicated a complex aetiology involving starvation and intercurrent infection.

Anaemia

Rainbow trout fry anaemia syndrome is a recently recognised condition of unknown origin which results in abnormal behaviour and mortality among rainbow trout fry. Typically, it affects more rapidly growing fish, perhaps indicating a nutritional aetiology (Scott 1989). Gross signs include ascites and gill pallor and histologically, bilateral muscle degeneration in red and white fibres (McCloughlin *et al.* 1990). Vitamin E supplementation or feed replacement has reduced the level of mortality in some cases (McCloughlin *et al.* 1990).

Pancreas disease

Pancreas disease (PD) was described in detail in section 1.1. The relationship between vitamin E nutrition and PD is discussed in chapter 4.

Hitra disease

'Hitra disease' has caused widespread and heavy mortalities among Atlantic salmon and rainbow trout in seawater farms in Norway since 1977. The condition has also been described in Scotland (Bruno *et al.* 1985,1986) and Faroe (Dalsgaard *et al.* 1988). It was initially ascribed an infectious origin (Egidius *et al.* 1981) and the causative agent, the bacterium, *Vibrio salmonicida* has since been identified (Holm *et al.* 1985, Egidius *et al.* 1986), Koch's postulates fulfilled (Egidius *et al.* 1981, 1984; Hjeltnes *et al.* 1987), successful vaccination achieved (Holm and Jørgensen 1987) and monoclonal antibodies towards the pathogen developed (Espelid *et al.* 1988). Various pathological features have been well described (Egidius *et al.* 1984; Soleim 1985; Bruno *et al.* 1986; Fjelstad and Heyeraas 1985; Poppe *et al.* 1985 *a,b*; Salte *et al.* 1987; Totland *et al.* 1988; Waagbø *et al.* 1988). Fjelstad and Heyeraas (1985) observed muscular and myocardial degeneration, together with hepatic ceroid accumulation, in affected fish, but were unable to culture bacteria. Poppe *et al.* (1985 *a,b*) suggested the condition may have a complex multifactorial origin, and both Fjelstad and Heyeraas (1985) and Poppe *et al.* (1986) pointed to similarities with vitamin E-selenium deficiency syndromes in other domesticated animals. Attempts to prevent the condition using parenteral vitamin E and selenium injection failed to demonstrate any significant effect (Salte *et al.* 1988). However, Hjeltnes *et al.* (1987) suggested that, since *Vibrio salmonicida* could not be isolated from every outbreak, the possibility that different aetiological factors may lead to similar clinical manifestations should be considered, implying that the term 'Hitra disease' may encompass more than one condition.

Cardiomyopathy syndrome

Also in Norway, a condition known as cardiomyopathy syndrome (CMS) (also called severe heart disease, acute heart failure or endomyocarditis) has been described (Amin and Trasti 1988; Ferguson *et al.* 1988, 1990). This condition

causes heavy mortality and involves severe disruption of the heart and skeletal muscle and primarily affects older fish. Ferguson *et al.* (1990) initially found no relationship between CMS and tissue vitamin E concentrations, but recent results suggest a correlation between the severity of cardiomyopathy and the degree of vitamin E depletion (T.Poppe, personal communication 1991).

Sudden death syndrome

This condition was described in section 1.1.2.2. Preliminary observations suggest that vitamin E concentrations are not markedly reduced in fish developing this condition (H.Rodger, W.Roy; unpublished observations), but a role for vitamin E in the pathogenesis of SDS is still considered plausible.

Histamine toxicosis

Teskeredžić *et al.* (1989) recorded mortality among farmed rainbow trout in Yugoslavia resulting from diets with high histamine and (thiobarbituric) acid values due to dietary protein and lipid decomposition respectively. In addition to its amelioration of the detrimental effects of rancid lipids, Watanabe *et al.* (1987) found that vitamin E supplementation reduced the prevalence of stomach erosion and improved weight gain in rainbow trout fed experimental diets containing histamine, although the histamine levels used in this study were 10-20 times those measured in diets on Yugoslavian farms. Brown fish meals are typically high in histamine. Miyazaki *et al.* (1985) described nutritional myopathy in carp resulting from the use of brown fish meals. The increasing use of this feedstuff in place of more expensive white fish meals may increase the frequency of problems associated with dietary histamine (Watanabe *et al.* 1987).

1.2.8.2 Common Carp

Sekoke disease

Sekoke disease of carp involves histological changes in the endocrine pancreatic tissue and hyperglycaemia together with muscular dystrophy, in fish

fed a diet containing quantities of dried silkworm pupae (Yokote 1970 *a,b,c*). Sugii and Kinumaki (1968) found that the tissue vitamin E distribution in affected carp was different to that in healthy fish. Miyazaki and Kubota (1981) demonstrated the corrective effect of vitamin E. Hashimoto *et al.* (1966) succeeded in inducing a similar condition experimentally by feeding a diet containing 10% oxidized saury oil and demonstrated that *all-rac*-α-tocopheryl acetate (50 mg kg^{-1}) was effective in preventing the condition. Watanabe and Hashimoto (1968) found muscle TBA value and histology to give an early indication of the onset of muscular dystrophy when diets contained 20% oxidized oil. Watanabe *et al.* (1966, 1967) reported that whilst vitamin E was effective, various synthetic antioxidants failed to prevent the development of experimentally induced muscular dystrophy, and Aoe *et al.* (1972) discovered that α-tocopherol had greater potency than either β-, γ- or δ-tocopherol in this respect. Similarly, Higashi *et al.* (1970) reported that muscular dystrophy in carp fed diets containing oxidized ethyl esters of fatty acids from pollock liver oil, was effectively prevented by the addition of *RRR*-α-tocopherol or *all-rac*-α-tocopherol, whereas *RRR*-non-α-tocopherols were less effective. The toxic factor responsible for the development of myopathy was found to be present in several fractions of the oxidized oil separated by column chromatography (Watanabe and Hashimoto 1968), and in both hydroperoxide and secondary decomposition products (Hata and Kaneda 1980). However, Watanabe *et al.* (1970*a*) commented on the similarities between Sekoke disease and experimental vitamin E deficiency in carp.

1.2.8.3 Channel catfish

Nutritional diseases have not been a serious problem for pond-cultured catfish, primarily because, with moderate stocking densities, natural pond organisms supplement the artificial feed and tend to correct any deficiency (Lovell 1975,1979). Unlike salmonids, farm-raised channel catfish are not reared on diets high in polyunsaturated fatty acids (Lovell *et al.* 1984).

Winter mortality syndrome

Wilson *et al.* (1984) noted the occurrence of a disease syndrome

involving anaemia, and other pathologies similar to those described for vitamin E deficiency. Jarboe *et al.* (1989) recorded the occurrence of 'winter mortality syndrome' in farmed channel catfish, causing losses of over 15% annual production in 1981/1982. The syndrome was characterized by the loss of epidermis from their backs and sides, often accompanied by external fungal infection, and 'sunken eyes'. These signs were regarded to be similar to those of vitamin E deficiency (as described by Murai and Andrews 1974), and a trial was undertaken under commercial conditions to ascertain if *all-rac*-α-tocopherol supplementation was effective in preventing the condition. The results of this trial were inconclusive since at no time were the clinical signs associated with the syndrome observed.

1.2.8.4 Yellowtail

Nutritional liver disease

Kimura (1963) examined blackened Japanese specimens of yellowtail (*Seriola quinqueradiata*), fed anchovies, and observed focal liver necrosis, kidney degeneration and anaemia. Nanba *et al.* (1973) described haematological observations on such fish but could detect no anaemia. Park (1978) recorded an apparently similar condition in cultured yellowtail in Korea, fed on frozen sand eel, and observed yellow-brown discolouration of the liver together with fatty degeneration. Kimura (1963) and Oka (1967) suggested feed rancidity to be the cause, but successful treatment with vitamin E was not recorded.

Nutritional myopathy

Sakaguchi and Hamaguchi (1969) studied the effect of oxidized oil and supplemental vitamin E on yellowtail. Groups of fish were fed for 93 days on artificial diets containing 8.5% either fresh (POV=11-13 mEq 100 g^{-1}) or oxidized oil (POV=480-500 mEq 100 g^{-1}) with or without added *all-rac*-α-tocopheryl acetate (60.0 mg 100 g^{-1} diet). Fish fed oxidized oil without added vitamin E showed reduced appetite and about 10% developed a leaning of the dorsal musculature similar to that reported in carp (Hashimoto *et al.* 1966). Such signs were not evident in fish fed oxidized oil plus vitamin E. A slightly higher TBA value was measured in the muscle of fish in the former

group, but there was no difference in the TBA value of the hepatopancreas. Hepatopancreatic glycogen content increased during the course of the experiment, except in fish fed oxidized oil, but transaminase activity (GOT and GPT) was higher in groups fed oxidized oil and was not reduced by vitamin E supplementation.

Kubota *et al.* (1980) documented a nutritional myopathy, manifested by emaciation and loss of muscle tone, in cultured yellowtail reared on a rancid fish diet. Histopathological examination of striated muscle showed degenerative changes, restricted to red muscle in mild cases and involving both red and white musculature in more advanced cases, together with ceroid accumulation in various organs.

1.2.8.5 Other fish species

Nutritional myopathy was recorded in puffer, *Fugu rubripes*, fry (Endo *et al.* 1979; Kubota *et al.* 1981*a*) again involving alterations in the striated muscle and the accumulation of ceroid in the visceral organs. Kubota *et al.* (1981*a*) demonstrated a preventive effect of α-tocopheryl acetate at a level of 250 mg 100 g^{-1} diet on the occurrence of this condition.

A clinical syndrome characterized by blindness, loss of pigmentation, erratic swimming behaviour and increased mortality has been described in breeding clownfish, *Amphiprion ocellaris*, fed a diet containing ingredients, high in polyunsaturated fatty acids, which had been subjected to freeze-thawing during preparation (Blazer and Wolke 1983). The condition was more prevalent at temperatures above 30°C, and 90% of the affected fish were female. Histopathological examination of affected fish revealed retinal degeneration, extensive ceroid deposits in visceral and macrophage accumulations, renal tubular calcium oxalate crystals and skeletal muscle necrosis (especially in the *adductor mandibule* and *levator operculi*). The condition was attributed to simultaneous vitamin E and A deficiency coupled with rancidity or peroxidation of dietary lipid and was corrected by reducing the water temperature, increasing the level of dietary vitamin E supplementation, and altering the diet preparation procedure. Ocular lesions in fish may be caused by vitamin A deficiency (Kitamura *et al.* 1967), but

have not previously been linked with vitamin E deficiency. However, Hayes (1974) has described retinal degeneration in monkeys resulting from single deficiencies of either vitamin E or vitamin A.

Huerkamp *et al.* (1988) described the occurrence of vitamin E deficiency in 6 month old, laboratory-raised goldfish, *Carassius auratus*, fed a shrimp-derived diet. Progressive fraying and deterioration of the tail and fins led to fungal infection and death. Histopathological examination revealed skeletal muscle degeneration in the muscles of the tail and caudal body wall compatible with vitamin E-selenium deficiency. Feed analysis indicated an inadequate vitamin E content and replacement of the diet cured the condition.

Pearse *et al.* (1974) recorded muscular dystrophy in cultured turbot, *Scophthalmus maximus*, fed trash fish. Although its origin was not precisely defined, the condition was putatively ascribed a nutritional aetiology since it responded to a vitamin and mineral supplement (Halver 1969) which included vitamin E. Nash *et al.* (1988) described an economically significant emaciative condition in snakeheads, *Channa striatus*, farmed in South-East Asia. The fish were reared on a trash fish diet and exhibited extensive striated myofibrillar and sarcoplasmic degeneration and necrosis, together with apparent hepatocellular lipid and ceroid accumulation. The origin of the condition was not determined, but similarities with vitamin E-selenium related myopathies were considered. Finally, intestinal steatosis in cultured sea bass, *Dicentrarchus labrax*, was reported by Deplano *et al.* (1989), but the cause of the condition was not defined.

1.2.9 Processes of vitamin E accumulation

1.2.9.1 Food sources

Wild fish are presumed to obtain vitamin E from their natural feeds in the form of *RRR*-α-tocopherol, but also as β, γ and δ-tocopherol. α-Tocopherol appears to be the main form present in fish tissues (Schulz *et al.* 1984; Ushikusa *et al.* 1987), although non-α-tocopherols were present particularly in the fatty tissues in carp (Ushikusa *et al.* 1986). Watanabe *et al.* (1981*c*) conducted feeding experiments to compare the assimilation of α, β, γ and δ-

tocopherol and to investigate the possible inter-conversion of these compounds. In the tissues, α-tocopherol predominated and its accumulation was high in relation to β, γ and δ-tocopherols. α-Tocopherol was accumulated in the liver and to a lesser degree in the muscle. There was no indication of any conversion of α-tocopherol to non-α-tocopherol, but the results suggested the possible conversion of δ to γ-tocopherol.

Intensively reared Atlantic salmon are fed fishmeal-based diets, commercially-prepared and stored under conditions of heat, light and moisture which may lead to destruction of any adventitious *RRR*-α-tocopherol. In addition to the effects of oxidized lipids and minerals, anti-vitamin E factors are present in certain feed materials of plant origin (eg. kidney and haricot beans, field pea, alfalfa, soybean and cottonseed) (Tacon and Jackson 1985; New 1987). Vitamin E supplements, usually in the form of *all-rac*-α-tocopheryl acetate, are added to the diet prior to pelleting. Current inclusion rates range from 10-40 mg 100 g^{-1} feed, compared to the minimum currently recommended for salmonids 3 mg 100 g^{-1} (NRC 1981) or 5 mg 100 g^{-1} (Cowey *et al.* 1983). Vitamin E in excess of the minimum requirement is incorporated to counteract losses during manufacture and storage (Scott 1973) and to provide a margin of safety between vitamin E supply and demand.

1.2.9.2 Digestion and absorption

Processes involved in the digestion and absorption of α-tocopherol in homeotherms have been reviewed by Gallo-Torres (1970, 1973, 1980) and Bjørneboe *et al.* (1990). Absorption efficiency is poor, generally between 20 and 40%, and dependent on the α-tocopherol content of the diet (Pudelkiewcz and Mary 1969).

The processes of digestion and absorption appear to be intimately related to lipid uptake, and pancreatic and biliary secretions play essential roles. Tocopheryl esters are believed to undergo hydrolysis in the gut lumen as a result of pancreatic esterase activity (Nakamura *et al.* 1975). Further esterase activity has been reported at the surface of enterocytes and intracellularly (Mathias *et al.* 1981). Hung *et al.* (1980*a*,*b*, 1981, 1982) demonstrated that *all-rac*-α-tocopheryl acetate is converted to α-tocopherol

during the processes of digestion and absorption in rainbow trout. In fish, dietary triglycerides are to a large extent hydrolysed into fatty acids and glycerol (Leger 1985). These processes are dependant on pancreatic lipases, together with pancreatic colipase and bile (bile salts, phospholipids and possibly alcohols) (Gallo-Torres 1970), and result in the formation of mixed micelles containing α-tocopherol. In fish, the absorption of lipids takes place mainly in the proximal intestine or in the pyloric caeca. Transfer of α-tocopherol to the enterocyte may occur by passive diffusion through the cell membrane (Gallo-Torres 1980). In mammals, the absorbed fatty acids undergo re-esterification to triacylglycerides which combine with specific phospholipids and apoproteins to form chylomicrons and very low-density lipoproteins (VLDL) (Leger 1985). Chylomicrons and high density lipoproteins (HDL) are responsible for the export of α-tocopherol from the gut (Bjørneboe *et al.* 1990).

1.2.9.3 Transport and metabolism

In mammals, the transportation of α-tocopherol, together with other lipids, from the gut occurs mainly via the lymphatic pathway, but in chickens there appears to be no compartmentalization between the portal blood vessels and lymphatics in relation to the absorption of lipids and liposoluble nutrients. In fish, there is little information on the relative importance of blood and lymph in lipid transport from the gut. Export of lipoproteins via the portal pathways was reported in carp, whilst in trout this did not appear to be a major route (Leger 1985).

α-Tocopherol in mammalian lymph and plasma is lipoprotein bound, although there is no evidence of any specific carrier molecule. Interactions between lipoproteins and cell receptors may facilitate tissue uptake of α-tocopherol and this process probably involves lipoprotein lipase activity. α-Tocopherol is taken up by all cells and incorporated into membranes of the mitochondria, microsomes, nucleus and plasma membrane. Pools of α-tocopherol exist in the liver, muscle and adipose tissue. Liver α-tocopherol is rapidly mobilized during depletion and secreted from the liver in association with VLDL, but mobilization from the muscle and adipose tissue is slow.

1.2.9.4 Tissue distribution

The question of mechanisms of uptake, transport and distribution of the synthetic *all-rac*-α-tocopheryl acetate in comparison to the naturally occurring form *RRR*-α-tocopherol was addressed by Hung *et al.* (1982) using orally administered radio-labelled vitamins. This study was conducted on rainbow trout, body weight 150-250 g and in water temperature 15°C. *RRR*-α-[5-methyl-^{3}H]tocopherol and *all-rac*-α-[3',4'^{14}C]tocopheryl acetate at a 3:10 ratio were administered *per os* to anaesthetized fish. These were allowed to recover then sacrificed after 2, 4, 8, 16, 32 and 64 hours and the distribution of the radioactivity in the plasma, liver, kidney, spleen, heart and skeletal muscle studied. Radioactivity in the plasma, liver, kidney, spleen and heart showed an exponential increase up to 32 hours, followed by a plateau or slight decline up to 64 hours. In skeletal muscle it increased exponentially up to 8 hours, then continued to increase linearly up to 64 hours. Comparison of plasma ^{3}H and ^{14}C radioactivity suggested that the uptake of *RRR*-α-tocopherol was 6 to 18 times greater than *all-rac*-α-tocopheryl acetate in the first 4 hours and 2 to 3 times greater between 8 and 64 hours. At the plateau, the amount of radioactivity (both ^{3}H and ^{14}C) was present in decreasing order in the liver > kidney > plasma > spleen > heart >> skeletal muscle. More than 87% of the ^{3}H and ^{14}C radioactivity was present as free α-tocopherol in the plasma and liver after 16 hours, and in the plasma, the radioactivity was bound primarily to plasma low density lipoproteins. These results support the hypothesis that the uptake, transport and distribution of *all-rac*-α-tocopheryl acetate after hydrolysis in the intestinal tract follows a similar pattern to *RRR*-α-tocopherol.

Cowey *et al.* (1981) administered *RRR*-α-[5-methyl-^{3}H]tocopherol to rainbow trout, weight 50-60 g. The radio-label was rapidly taken up by the plasma, adipose tissue and liver, but uptake into erythrocytes and white skeletal muscle was much slower. In most tissues radioactivity reached a plateau within three days, but in red muscle it increased over a 10-day period. The previous vitamin E intake had no effect on tissue distribution. In hepatocytes, α-tocopherol was concentrated in the mitochondria, with little in the cytosol. Ikeda and Taguchi (1966) measured significant vitamin E

concentrations in the fish heart. Ackman and Cormier (1967) found vitamin E to be more concentrated in the red than white muscle of cod (*Gadus morhua*). Sugii and Kinumaki (1968) found α-tocopherol to be evenly distributed in every organ, in skipjack tuna, rainbow trout and carp, although rainbow trout fed high levels of vitamin E accumulated it in the ovary. Ushikusa *et al.*(1987) found that α-tocopherol concentrations were high in the liver and adipose tissue of sea bream (*Pagrus major*). Bell, McVicar, Mitchell and Cowey (unpublished), measured 22 μg g^{-1}, 21 μg g^{-1} and 4 μg g^{-1}, in heart, red and white muscle of Atlantic salmon fed vitamin E-adequate diets and 3, 5 and 2 μg g^{-1} in the same tissues of fish fed a deficient diet. Cowey *et al.* (1981) measured concentrations of 7 μg g^{-1} in the liver, 0.7 μg g^{-1} in the muscle, 9.4 μg g^{-1} in the adipose tissue and 1.3 μg g^{-1} in the erythrocytes of rainbow trout fed an unsupplemented diet. Cowey *et al.* (1983) found that in vitamin E-deficient fish, tocopherol concentrations were highest in the brain and lowest in the skeletal muscle. Microsomes from the muscle of deficient trout were more susceptible to *in vitro* peroxidation than those from gill, heart or liver. Sugii and Kinumaki (1968) found that vitamin E-deficient fish showed preferential retention of vitamin E by some organs. Watanabe *et al.* (1970*a*) measured concentrations of 486, 14 and 41 μg g^{-1} in hepatopancreas, muscle and head kidney of 10 g carp, and these were reduced to 2.7, 2.8 and ~0 μg g^{-1} in vitamin E-deficient fish.

1.2.9.5 Factors affecting availability: intake, digestion, absorption, transport

Pomeranze and Lucarello (1953) reported that the efficiency of vitamin E absorption in mammals was dependent on the lipid intake, but Watanabe *et al.* (1977*b*) found no evidence that vitamin E absorption by carp was impaired by a fat free diet. In homeotherms, Akerib and Sterner (1971) found that vitamin E absorption was impaired by high levels of dietary polyunsaturated fatty acid and Baker *et al.* (1980) found that although fat stimulated the flow of bile and pancreatic juice, polyunsaturated fatty acids reduced the absorption and utilization of this nutrient.

1.2.9.6 Excretion and endogenous production

A large proportion of the dietary vitamin E is likely to be lost in the faeces since absorption efficiency is poor (Pudelkiewycz and Mary 1969). Other losses will occur in the bile and mucus. There is no evidence of any synthesis of vitamin E in fish (eg.by gut microflora), but α-tocopherol may be regenerated from the deactivated tocopheroxyl radical by the action of ascorbic acid (see section 1.2.5.6).

1.2.10 Uses of vitamin E in aquaculture

1.2.10.1 Broodstock nutrition

There is considerable interest in the development of diets for broodstock fish in order to optimize broodstock performance. In view of its essential function in reproduction, vitamin E has received particular attention in this respect. Watanabe (1985) reviewed the role of nutrition in broodstock performance and emphasized the importance of vitamin E. Takeuchi *et al.* (1981) found that in ayu (*Plecoglossus altivelis*) fed a diet low in vitamin E, the duration of spawning behaviour was reduced and one third of the total female broodstock fed this diet did not spawn. Among the offspring, survival rate to the eyed stage was poor as was hatchability, and the mortality of larvae from these eggs was high. The importance of vitamin E in reproduction has been confirmed in other species including carp (Watanabe and Takashima 1977), rainbow trout (Kinumaki *et al.* 1972; King 1984; King *et al.* 1983), and red sea bream (Watanabe *et al.* 1984). α-Tocopherol concentrations are high in the eggs and low in the tissues of broodstock after spawning. There is a rapid loss of egg vitamin E during development (Kinumaki *et al.* 1972). Cowey *et al.* (1985) presented a review of antioxidant systems in fish eggs. Alioshin (1987) found that joint administration of tocopherol and ascorbic acid prevented the toxic effects of dietary lipid peroxides on the ovary in rainbow trout, and observed that a dose of 0.04 mg α-tocopherol g^{-1} body weight day^{-1} stimulated mitotic division of the oogonia and their conversion into a meiotic state. Mézes and Vaddsz (1984) concluded that

injected α-tocopheryl acetate could be utilized by maturing female carp, Hsiao and Mak (1978*a*,*b*) found that vitamin E and human chorionic gonadotrophin increased the viability of sperm from farmed male ayu (*Plecoglossus altivelis*), and Juarez-Palacios and Palomo Martinez (1980) noted that vitamin E and luteinizing chorionic gonadotrophin induced spawning in female carp (*Ctenopharyngodon idella*).

1.2.10.2 Larval nutrition

The development of purified diets for nutritional studies on larval fish has been difficult, thus little is known about the effects of vitamin E on survival and development in fish larvae. However, Watanabe *et al.* (1983) found that enrichment of dietary *Artemia salina* with liposoluble vitamins improved survival of larval red sea bream.

1.2.10.3 Product quality

In intensive aquaculture it is important to consider the effects of diet on product quality. The effects of dietary vitamin E on the quality and storage stability of milk and meat were described by Marusich (1980). O'Keefe and Noble (1978) found that fillets of fingerling channel catfish fed various levels of vitamin E for a period of 97 days exhibited significant oxidative rancidity after frozen storage for 3 months if diets contained up to 5 mg *all-rac*-α-tocopheryl acetate 100 g^{-1}, and that subsequent refrigeration resulted in fat oxidation in fillets from fish fed less than 40 mg 100 g^{-1}. Hung and Slinger (1982) studied the effect of dietary vitamin E on muscle α-tocopherol concentrations in rainbow trout and its relationship to storage stability as assessed by changes in TBA value. The diets and fish used were as described by Hung *et al.* (1980*b*) (section 1.2.5.3). Only the highest levels of dietary vitamin E increased muscle α-tocopherol concentrations and there was no difference in storage stability between fish fed high and low levels of vitamin E for 24 weeks. Boggio *et al.* (1985) found that the dietary α-tocopheryl acetate level did not significantly affect the sensory attributes of trout fillets evaluated fresh or after prolonged frozen storage. However,

fish fed higher levels of vitamin E had lower malondialdehyde concentrations after frozen storage at -80°C for four months. Poso *et al.* (1988) found that dietary α-tocopherol seemed neither to protect canthaxanthin in trout fillets from fading during cold storage, nor the white muscle lipids from oxidative rancidity, though a little protection of dark muscle lipids was observed. Frigg *et al.* (1990) also found that flesh malondialdehyde concentrations in rainbow trout were influenced by the dietary vitamin E intake. In this study significant differences in the organoleptic criteria were detected.

1.2.10.4 Alleviation of stress

In mammals, vitamin E is believed to be involved in the mediation of cortisol production by the adrenal as a response to stress. In addition, effects of vitamin E on the pituitary may indirectly influence cortisol production (Kitabchi 1980). Vitamin E supplementation may help alleviate stress in fish, but currently there is no experimental evidence in favour of this hypothesis. Further relevant information is given in chapter 5.

1.2.10.5 Prevention of toxicity

Green and Bunyan (1969) and Menzel (1980) reviewed information on the protective effects of vitamin E against certain environmental pollutants. This protection may again be dependant on the antioxidant function of vitamin E, but has not been specifically investigated in fishes. Nevertheless, on the basis of mammalian studies, vitamin E supplementation of fish diets may increase resistance towards certain environmental toxins.

1.2.10.6 Resistance to disease

The beneficial effect of vitamin E supplementation on immune function and resistance to disease in rainbow trout was described by Blazer and Wolke (1984) (section 1.2.5.4). Although other experimental studies with Atlantic salmon show lesser responses, there is some justification for the belief that dietary vitamin E supplementation may increase resistance to infectious

disease. Further studies are required under practical conditions to quantify these effects.

1.2.11 Vitamin E nutrition of farmed Atlantic salmon

The effects of deficiency and data on the vitamin E requirement of Atlantic salmon were described in section 1.2.5.2. The majority of these studies were conducted on young fish in freshwater where the provision of 3 mg α-tocopheryl acetate 100 g^{-1} diet was considered to be adequate (Lall *et al.* 1988). Recently, muscle degeneration interpreted to be indicative of vitamin E deficiency has been observed in association with several recognised pathological conditions of Atlantic salmon farmed in seawater (section 1.2.8.1). The role of vitamin E in any of these conditions has still to be demonstrated, but these observations suggest that the vitamin E nutrition of fish at this stage in their life-cycle may be inadequate. The following study was conducted in order to examine the vitamin E nutrition of farmed salmon in seawater, particularly in relation to its role in the development of pancreas disease. The investigation consisted of field studies on healthy fish and fish suffering from pancreas disease, and experimental studies conducted to examine the effects of various husbandry practices on vitamin E status.

CHAPTER 2

GENERAL METHODS

2.1 SAMPLING PROCEDURE

2.1.1 Field studies

A routine sampling procedure was devised and used to collect plasma, liver and tissues for histology from fish farms in a uniform manner. Section 4.2 describes modifications to the standard procedure used at individual sites during studies of pancreas disease outbreaks. Before collecting any fish, samples of feed and details of the health and feeding history of the stock were collected. Tank or cage units were chosen after consultation with farm staff and, whenever possible, the behaviour, particularly the feeding response of the fish, was observed. Records were kept using a standardised data sheet.

Batches of fish were captured with a dip net and killed with a blow to the head. As soon as possible after capture, blood was collected from the caudal vein of each fish, using heparinized 1 ml, 2 ml or 5 ml disposable syringes fitted with 23 or 25 gauge needles. Blood collected in this way was transferred to marked 13.5 ml polystyrene tubes. Alternatively, for very small fish (ie. less than 30 g), blood was allowed to flow directly into pre-heparinized tubes after severing the tail.

Once blood had been collected from every fish, the condition and external appearance of each individual was noted and the weight and standard length recorded. This data was later used to calculate the condition factor (k) according to Fulton (1904) where:

$$k = 100 \times \frac{weight\,(g)}{length\,(cm)^3}$$

The body cavity was opened with a ventral incision and the sex and state of maturity determined whenever possible. The internal organs were examined, particularly for the presence of food in the gut and fat surrounding the pyloric caeca. The liver was carefully excised and placed in a marked container. Tissues for histology were collected as described in section 2.4. On return to the laboratory, blood was centrifuged at 2000 rpm for 10 min and the plasma transferred into marked tubes. Plasma, liver and feed samples were then frozen at -20°C.

2.1.2 Experimental studies

A more rigorous procedure was used to collect samples during experimental studies. In order to minimize the effects of sampling, efforts were made to avoid disturbing the fish prior to capture. Randomization procedures were used to determine the order in which tanks were sampled and once the tank lid was opened the fish were caught rapidly and blood collected within 15 min.

Batches of five or six fish as appropriate were captured by dip-net and immediately anaesthetized in 2-phenoxyethanol (1 ml l^{-1}) (Sehdev *et al.* 1963). Blood was drawn into heparinized syringes from the caudal vein and transferred to 13.5 ml polystyrene tubes kept on ice. Each fish was killed with a blow to the head, the condition and external appearance was observed and the weight and standard length recorded. The condition factor (k) was later calculated as described above. The body cavity was opened ventrally and the internal appearance, including sex and state of maturity, was noted. The liver was excised and placed in a marked plastic bag and, on some occasions, samples for histology were collected as described in section 2.4. Feed samples were also collected if required. Where appropriate, haematocrit and leucocrit measurements were made as described in section 2.3. The remaining blood was centrifuged at 2000 rpm for 10 min and the plasma transferred into labelled tubes. Plasma, liver and feed samples were then frozen at -20°C.

2.2 BIOCHEMICAL DETERMINATIONS

Analar grade chemicals were used in the following procedures, except where stated. Absorbance readings were made using a Pye Unicam SP500 Series 2 spectrophotometer.

2.2.1 Vitamin E determination using high performance liquid chromatography

α-Tocopherol determinations were made using a high performance liquid chromatography (HPLC) procedure devised by C.Cowey and G.Bell (G.Bell, personal communication), based on the published methods of Cowey *et al.* (1981)

and Hung *et al.* (1980a). Minor modifications were adopted for use with liver and feed.

2.2.1.1 Materials

a. 2% Pyrogallol in ethanol. Freshly made prior to each use by dissolving 1.0 g pyrogallol in 50 ml absolute ethanol

b. 60% Potassium hydroxide solution

c. Hexane (HPLC grade) with 0.00125% butylated hydroxytoluene (BHT)

d. Methanol (HPLC grade)

e. 95% HPLC Methanol

f. Vitamin E standard: 10 μg ml^{-1} α-tocopherol in methanol. Approximately 0.2 g *RRR*-α-tocopherol (Eastman Kodak, Rochester, New York) was weighed to 4 decimal places and dissolved in exactly 100 times (w/v) HPLC methanol. Serial dilutions were used to obtain 1.00 mg ml^{-1} stock and 10 μg ml^{-1} working solutions.

2.2.1.2 Procedure for the determination of α-tocopherol concentrations in fish plasma

Saponification and hexane extraction was carried out in stoppered, 15 ml, graduated test-tubes. In these tubes, 0.2 ml plasma was mixed with 4.0 ml 2% pyrogallol in ethanol. After heating for 5 min in a water bath at 70°C, the tubes were flushed with nitrogen and 1.0 ml 60% potassium hydroxide solution added. The reaction mixture was then heated for a further 20 mins at 70°C, shaking at 5 min intervals. Subsequently, the tubes were cooled on ice and their contents made up to 10 ml with distilled water. α-Tocopherol was extracted by shaking the reaction mixture with hexane-BHT. 4.0 ml hexane-BHT was added to each tube and the contents mixed vigorously for 1 min then allowed to stand. After phase separation, 3.0 ml of the upper layer was decanted into glass vials. Hexane was evaporated from these vials, under nitrogen, on a hot plate at 40°C. The dried residues were then redissolved in 1.0 ml absolute methanol and the resulting solutions stored in a refrigerator until assayed by HPLC.

Reverse phase, isocratic, adsorption HPLC was used to determine the α-tocopherol concentration in these solutions. Solvent flow was generated by a Gilson Model 302 HPLC pump and 95% methanol formed the mobile phase, flowing at a rate of 2.0 ml min^{-1}. Samples and standards were introduced through a Rheodyne Model 7128 valve fitted with a 0.10 ml injection loop. Separation took place in a 5 μm, octadecylsilane HPLC column (Spherisorb) (internal diameter 4.6 mm, length 25 cm). The absorbance of the effluent, at 293 nm, was monitored using a Gilson Model HM/HPLC Holochrome UV/VIS detector. Detector output was displayed on a Spectra-Physics SP4290 programmable integrator. A sample volume of 0.10 ml was passed from a microlitre syringe into the Rheodyne valve loop and injected onto the column. α-Tocopherol eluted after approximately 16 min and was detected as a labelled peak on the chart trace. Peak areas were calculated automatically by the integrator software. Output was calibrated by running 0.10 ml volumes of the 10 μg ml^{-1} α-tocopherol standard before and after each use.

2.2.1.3 Modifications for the determination of α-tocopherol concentrations in fish liver

Approximately 0.2 g liver was accurately weighed and homogenised with 4.0 ml 2% pyrogallol in ethanol using a Polytron homogeniser fitted with a 12 mm diameter aggregate. Subsequently, the procedure followed that for plasma.

2.2.1.4 Modifications for the determination of α-tocopheryl concentrations in fish feed

Pelleted fish feed was ground with a pestle and mortar and approximately 0.2 g weighed accurately into 15 ml test-tubes. 4.0 ml 2% pyrogallol in ethanol was then added to the tubes and the contents homogenised. Saponification and extraction was then as for plasma, except that the hexane and water phases were separated by gentle centrifugation (1 min at 100-200 rpm).

Table 2.1. Recovery of added α-tocopherol from plasma

Added α-tocopherol (μg ml^{-1})	Measured vitamin E (μg ml^{-1})
0	44
0	40
10	42
10	47
20	47
20	55
30	65
30	70
40	75
40	79
50	95
50	77

Regression equation: Measured=37.5+0.954 Added
Coefficient of variation=9.8%

2.2.1.5 Calculation

a. Since the peak area is proportional to the α-tocopherol concentration in the methanol solution:

$$W_u = \frac{A_u}{A_s} \times (C_s \times V_s \times V_u)$$

where

W_u is the unknown weight of α-tocopherol in the methanol solution (μg)

A_u is the peak area of the unknown α-tocopherol in methanol solution

A_s is the peak area of the α-tocopherol standard

C_s is the concentration of the α-tocopherol standard (10 μg ml^{-1})

V_s is the volume of α-tocopherol standard applied to the column (0.1 ml)

V_u is the volume of the unknown α-tocopherol in methanol solution applied to the column (0.1 ml)

b. Since only 3 ml of the total 4 ml volume of hexane was dried down:

where

W_u is the weight of α-tocopherol in the reaction mixture (μg)

Table 2.2. Recovery of added α-tocopheryl acetate from plasma

Added α-tocopheryl acetate (μg ml^{-1})	Measured vitamin E (μg ml^{-1})
0	37
0	35
10	53
10	45
20	52
20	51
30	62
30	70
40	70
40	83
50	80
50	95

Regression equation: Measured=35.8+1.01 Added

$$W_a = \frac{4}{3} \times W_c$$

c. Since this reaction mixture contains only a fraction of a millilitre of plasma or a fraction of a gram of liver or feed:

$$C_o = \frac{W_a}{V_o}$$

where

C_o is the α-tocopheryl concentration in the sample (μg ml^{-1} or μg g^{-1})

V_o is the volume of sample in the reaction mixture (ml or g)

2.2.1.6 Accuracy and precision

As a measure of the accuracy of the assay, vitamin E determinations were made on samples, from a plasma pool, to which known quantities of α-tocopherol had been added. Solutions were prepared containing 0, 10, 20, 30, 40 and 50 μg *RRR*-α-tocopherol 0.10 ml^{-1} HPLC methanol. In duplicate tubes, 0.10 ml aliquots of each solution were mixed with 0.90 ml plasma. These were assayed

as described above and the results plotted on a graph of added versus measured α-tocopherol. Regression analysis indicated that the fitted line had a gradient of 0.954, equivalent to an accuracy of 95.4% over this range of values. The precision within each assay, estimated as the coefficient of variation of individual points around this fitted line was 9.8% (table 2.1). Between assay precision was determined from measured values of α-tocopherol in pool plasma used for quality control. A coefficient of variation of 20% was calculated from 23 measurements made on quality control plasma between May 1989 and May 1990 (table 2.4). The assay showed a linear response over the range of concentrations measured and the minimum concentration distinguishable from zero in the solution applied to the column was 0.5 μg ml^{-1} (equivalent to about 7 μg ml^{-1} in plasma) (data not shown).

2.2.1.7 Specificity

One aspect of the assay's specificity, the effect of esterification of α-tocopherol on the accuracy of vitamin E determinations, was examined in order to determine the efficiency with which the procedure would measure α-tocopherol in tissues as the less readily available acetate form. Vitamin E assays were conducted on plasma samples to which known quantities of α-tocopheryl acetate had been added. Solutions were prepared containing 0, 10, 20, 30, 40 and 50 μg *all-rac*-α-tocopheryl acetate 0.1 ml^{-1} methanol, and 0.1 ml of each solution was mixed with 0.9 ml plasma. The results of vitamin E analyses on these samples are summarised in table 2.2. Regression analysis of added α-tocopheryl acetate versus measured vitamin E, yielded a gradient of 1.01, indicating that α-tocopherol in the acetate form was also measured with a high precision using this assay.

2.2.1.8 Effect of storage

Bunnell *et al.* (1965) and Bender (1978) found significant losses of vitamin E from foodstuffs during frozen storage, but Losowsky and Leonard (1967) found that plasma vitamin E concentrations in human plasma were unaffected by storage at -15°C for 2 months. In order to assess the effects

Table 2.3. α-Tocopherol concentrations measured during short-term freezer storage of plasma samples

Time (days)	Measured α-tocopherol concentration (μg ml^{-1})
0	107
0	84
1	94
1	94
3	102
3	91
7	104
7	86

Spearman's ρ = 0.000 (NS)

of frozen storage on α-tocopherol in salmon plasma, studies of both short- and long-term changes in vitamin E content were conducted. Vitamin E determinations were made on a pool of plasma after 0, 1, 3 and 7 days storage at -20°C. The measured values, given in table 2.3, showed no significant correlation with time and therefore indicate that short-term freezer storage had no effect on plasma α-tocopherol content. Similarly, vitamin E measurements made on quality control plasma stored at -20°C between May 1989 and May 1990, also showed no correlation with time, and indicate that no significant change in plasma α-tocopherol content occurred over this period (table 2.4).

2.2.2 Determination of plasma malondialdehyde concentration

Plasma malondialdehyde concentrations were estimated using a colorimetric method, based on the reaction with thiobarbituric acid, similar to that described by Wilbur *et al.* (1949).

2.2.2.1 Materials

a. Sodium phosphate buffer (10 mM; pH 7.0)

b. 20% Trichloroacetic acid

Table 2.4. α-Tocopherol concentrations measured in quality control plasma

Month	Plasma α-tocopherol concentration (μg ml^{-1})					
May 1989	47			54		
June	47	44	37	41	43	41
July	66	63	49	49		
August	45	65	48	44	42	
September	43					
October						
November	29		39		47	
December						
January 1990						
February						
March						
April	36					
May	33					

Spearman's ρ = -0.345 (NS)
Mean = 46±9 μg ml^{-1} Co-efficient of variation = 20%

c. 0.2% Butylated hydroxytoluene (BHT) in ethanol

d. 50 mM Thiobarbituric acid (TBA). Freshly made prior to use by dissolving 0.7205 g TBA in 100 ml distilled water.

2.2.2.2 Procedure for the determination of plasma malondialdehyde concentrations

Plasma was diluted 1 in 5 or 1 in 10 with phosphate buffer and 1.0 ml aliquots measured into 7 ml screw-cap test-tubes. To each unknown, and to a blank containing 1.0 ml phosphate buffer, 0.70 ml trichloroacetic acid was added. After shaking, 0.10 ml BHT in ethanol, followed by 1.20 ml TBA solution, was pipetted into each tube. The tubes were capped, mixed and placed in a water bath at 100°C for 10 min. They were then cooled on ice and centrifuged for 5 min at 2000 rpm to remove precipitate. The absorbance of the resulting supernatants, at 532 nm, was measured against the prepared blank using 1 cm glass cuvettes.

2.2.2.3 Calculation

a. The TBA-MDA adduct has a molar extinction coefficient of 1.56×10^{-12} μM^{-1} cm^{-1}, hence in a 1 cm cuvette :

$$C_u = \frac{Abs_u}{1.56 \times 10^{12}}$$

where

C_u is the concentration of TBA-MDA in the supernatant (μmol l^{-1})

Abs_u is the absorbance of the reaction mixture

b. Since only a fraction of a millilitre of plasma was included in the total volume of the reaction mixture:

$$C_o = C_u \times (\frac{V_t}{V_o})$$

where

C_o is the concentration of MDA in the plasma (μmol l^{-1})

V_t is the total volume of the reaction mixture (3.0 ml)

V_o is the volume of plasma in the reaction mixture (ml)

2.2.3 Determination of plasma pyruvate kinase activity

Plasma pyruvate kinase (PK) (E.C. 2.7.1.40) activities were measured using a test combination supplied by the Boehringer Corporation Ltd. This test is based on the method described by Beisenherz *et al.* (1953).

2.2.3.1 Materials

a. Triethanolamine buffer (0.16 mol l^{-1}; pH 7.5) containing 0.12 mol l^{-1} potassium chloride, 21 mmol l^{-1} magnesium (II) sulphate and 1.3 mmol l^{-1} ethylenediaminetetraacetate (EDTA)

b. Nicotinamide adenine dinucleotide phosphate / phosphoenol pyruvate (NADH/PEP). 6 mmol l^{-1} NADH and 32.5 mmol l^{-1} PEP in distilled water

c. Lactate dehydrogenase (LDH) (240 U ml^{-1})

d. Adenosine diphosphate (ADP) (0.1 mol l^{-1})

e. 0.9% sodium chloride solution (NaCl)

2.2.3.2 Procedure for the determination of plasma pyruvate kinase activities

In 7 ml test-tubes, 0.10 ml NADH/PEP was mixed with 2.5 ml buffer solution. 0.50 ml plasma, or a suitable dilution of plasma in 0.9% NaCl (1 in 2; 1 in 10; 1 in 100), followed by 0.05 ml LDH solution, were added. The contents of the tubes were mixed and, after an interval of 5 min, transferred to a cuvette in which the absorbance (Abs_1) was measured. After a further 10 min the absorbance (Abs_2) was measured, 0.1 ml ADP was added, and the mixture stirred. The absorbance was read immediately (Abs_3), and again after a further 10 min (Abs_4). Assays were conducted at room temperature. Readings were made in 1 cm silica cuvettes, at 340 nm, against an air blank.

2.2.3.3 Calculation

a. The change in absorbance (ΔAbs) was calculated as:

$$\Delta Abs=(Abs_1-Abs_2)-(Abs_3-Abs_4)$$

b. Pyruvate kinase activity in the test-tube was found by interpolation using tabulated values of ΔAbs and PK activity supplied together with the kit.

c. Finally, the activity in the plasma was calculated by multiplying by the original dilution factor. PK activity was expressed in milliunits (mU) ml^{-1}, where 1 mU is equivalent to 1 nmol substrate utilized min^{-1} at 25°C.

2.2.4 Determination of plasma creatine phosphokinase activity

Plasma creatine phosphokinase (CPK) (E.C. 2.7.3.2) activities were determined using a test combination supplied by Sigma Chemical Co. Ltd. This test is based on a modification of the method described by Hughes (1962).

2.2.4.1 Materials

a. TRIZMA buffer (pH 7.5) containing magnesium (II) sulphate

b. Sodium phosphocreatine, hydrate. 3 mg ml^{-1} in Tris buffer

c. ADP-glutathione. 0.1 mmol adenosine diphosphate (ADP) and reduced glutathione in 6 ml water

d. p-Hydroxymercuribenzoate (p-HMB) 0.05 mol l^{-1}

e. Alkali solution. 1.5 mol l^{-1} sodium hydroxide plus sodium carbonate

f. α-Naphthol. 20 mg ml^{-1} in alkali solution. Freshly prepared before use.

g. Diacetyl. 0.5 ml l^{-1}

h. Creatine standard. 0.4 mmol l^{-1} in distilled water.

2.2.4.2 Procedure for the determination of plasma creatine phosphokinase activities

Aliquots of 0.10 ml plasma diluted 1 in 10 or 1 in 100 in water, or 0.10 ml water (blank), were pipetted into 15 ml test tubes containing 0.50 ml sodium phosphocreatine. These were placed for several minutes in a water bath at 37°C before 0.2 ml ADP-glutathione solution were added. The tubes were then mixed and incubated for 30 min at 37°C. Mixing after each addition, 1.0 ml α-naphthol, 1.0 ml diacetyl solution and 7.0 ml water were pipetted into each tube. These tubes were returned to the water bath for 15-20 min, to allow colour development, then centrifuged for 5 min at 2000 rpm. The absorbance of the supernatants was read at 520 nm, against the water blank, using 1 cm glass cuvettes.

Serial dilutions of the creatine standard were made over the range 0.08 to 0.40 µmol l^{-1}. To 1.0 ml of each standard solution was added 1.0 ml α-naphthol, 1.0 ml diacetyl, and 7.0 ml water. The tubes were mixed and left for 15 min to allow colour development. Absorbance values were measured as above using the zero standard as blank.

2.2.4.3 Calculation

a. A standard curve was constructed to relate creatine concentration (proportional to CPK activity) to absorbance. CPK activity in the unknowns was calculated by interpolation on this standard curve.
b. The results were corrected by multiplying by the appropriate dilution factor and CPK activity expressed in international units (IU) ml^{-1}, where 1 IU is equivalent to 1 µmol creatine formed min^{-1} at 25°C.

2.2.5 Determination of plasma total protein concentration

Plasma protein concentrations were measured using a test combination supplied by the Boehringer Corporation Ltd. This test is based on the Biuret method as described by Weichselbaum (1946).

2.2.5.1 Materials

a. Biuret reagent comprising 0.1 N sodium hydroxide, 16 mmol l^{-1} sodium-potassium tartrate, 15 mmol l^{-1} potassium iodide and 6 mmol l^{-1} copper (II) sulphate
b. Reagent for blank comprising 0.1 N sodium hydroxide and 16 mmol l^{-1} potassium-sodium-tartrate
c. Protein standard. 60 mg ml^{-1}

2.2.5.2 Procedure for the determination of plasma protein concentrations

5.0 ml Biuret reagent were added to 0.10 ml protein standard, or 0.1 ml plasma, in 7 ml test-tubes. These were mixed and incubated for 30 min at 20-25°C. The absorbance of the standard and each unknown was measured against a reagent blank, at 546 nm, in 1 cm glass cuvettes.

2.2.5.3 Calculation

The plasma protein concentration was determined as follows:

$$C_u = \frac{Abs_u}{Abs_s} \times C_s$$

where

Abs_u is the absorbance of the unknown

Abs_s is the absorbance of the standard

C_u is the protein concentration in the plasma sample (mg ml^{-1})

C_s is the protein concentration in the standard (60 mg ml^{-1})

2.2.6 Plasma 'total' lipid determination

Plasma 'total' lipid concentrations were determined using a colorimetric procedure based on the sulpho-phosphovanillin reaction (Zöllner and Kirsch 1962).

2.2.6.1 Materials

a. Phosphovanillin. To 100 ml 0.6% solution of vanillin in distilled water were added 400 ml orthophosphoric acid. This was stored in a brown bottle at room temperature for up to four weeks.

b. Sulphuric acid, concentrated

c. 0.9% sodium chloride solution (NaCl)

d. 10 mg ml^{-1} cholesterol standard. Approximately 1 g cholesterol was weighed to 4 decimal places and dissolved in exactly 100 times (w/v) absolute ethanol.

2.2.6.2 Procedure for the determination of plasma 'total' lipid concentrations

Aliquots of 0.10 ml plasma, or a suitable dilution of plasma in 0.9% NaCl (1 in 2; 1 in 4), or 0.10 ml cholesterol standard, were pipetted into 7 ml boiling tubes. From a burette, 2.0 ml concentrated sulphuric acid were added to both unknowns and standard. After thorough mixing, the tubes were stoppered with cotton wool and heated for 10 min in a boiling water bath. They were then removed and cooled. Aliquots of 0.10 ml were taken into 12 ml

reaction tubes and a blank containing 0.10 ml sulphuric acid was also prepared. 5.0 ml phosphovanillin reagent were added to each tube including the acid blank. The tubes were then mixed and incubated for 15 min in a water bath at 37°C before being allowed to cool. The absorbance of unknowns and standard, relative to the acid blank, was measured at 540 nm in 1 cm glass cuvettes.

2.2.6.3 Calculation

a. The lipid concentration in the sample was determined as follows:

$$C_u = \frac{Abs_u}{Abs_s} \times C_s$$

where

Abs_u is the absorbance of the unknown

Abs_s is the absorbance of the standard

C_u is the concentration of lipid in the reaction mixture (mg ml^{-1})

C_s is the concentration of the standard (10 mg ml^{-1}).

b. The concentration of lipid in plasma was then calculated by correcting for any dilution factor:

$$C_o = C_u \times (10 \times V_o)$$

where

C_o is the lipid concentration in the plasma (mg ml^{-1})

V_o is the original volume of plasma (ml)

2.2.7 Determination of plasma cortisol concentration by radioimmunoassay

Plasma cortisol concentrations were determined by radioimmunoassay according to a modification of the published method of Pickering *et al.* (1987a).

2.2.7.1 Materials

a. BSA-saline. 1% bovine serum albumin (RIA grade from Sigma) and 0.9%

sodium chloride in distilled water

b. Dextran-coated charcoal. 0.5% activated charcoal (Norit PM5 from BDH), 0.1% dextran (grade C from BDH) and 0.9% sodium chloride in distilled water

c. Ethyl acetate. Aristar grade

d. Antiserum (anti-cortisol-3-(O-carboxymethyl)oxime HSA). Obtained from Steranti Research Ltd. in lyophilized form. Reconstituted with 2.0 ml BSA-saline and stored frozen in tubes containing 0.10 ml aliquots. For the assay the contents of one tube were diluted to 5.0 ml with BSA-saline.

e. Labelled hormone ([1,2,6,7-^{3}H]cortisol). Stock 1 supplied by Amersham International as a preparation with an activity of 9.25 MBq in 0.25 ml toluene-ethanol (9:1). Stock 2 was prepared by diluting 0.02 ml stock 1 with 2.0 ml absolute ethanol. For working, 0.10 ml of stock 2 were made up to 5.0 ml with BSA-saline (working solution contained approx. 22 200 dpm in 0.05 ml).

f. Cortisol standards. A 50 ng ml^{-1} stock solution was prepared in ethanol from cortisol supplied in pre-weighed vials by Sigma. Two working solutions were prepared : 1) 0.80 ml stock plus 9.2 ml ethyl acetate to give 4.0 ng ml^{-1} cortisol solution; 2) 1.00 ml 4.0 ng ml^{-1} cortisol solution plus 7.0 ml ethyl acetate to give 0.5 ng ml^{-1} cortisol solution.

g. Scintillation fluid. Optiphase HiSafe II (Pharmacia)

2.2.7.2 Procedure for the determination of cortisol concentration in plasma samples

A set of standards was prepared over the range 0-800 pg cortisol tube^{-1} from the 0.5 ng ml^{-1} and 4.0 ng ml^{-1} working solutions. The standards were made in duplicate, in 3.5 ml polypropylene test tubes, as shown in table 2.5. In 1.5 ml microcentrifuge tubes, 1.0 ml ethyl acetate was added to 0.2 ml plasma and the mixture shaken vigorously. After centrifugation for 5 min at 2500 rpm, appropriate volumes of supernatant (usually 0.2 ml) were transferred to 3.5 ml test tubes. Standards and unknowns were then evaporated to dryness in a

Table 2.5. Preparation of cortisol standards

Cortisol concentration pg tube^{-1}	ng ml^{-1}	Ethyl acetate (µl)	0.5 ng ml^{-1} standard (µl)	4.0 ng ml^{-1} standard (µl)
0	0	200	0	0
50	0.2	100	100	0
100	0.4	0	200	0
200	0.8	150	0	50
400	1.6	100	0	100
800	3.2	0	0	200

vacuum oven at 35°C. To each tube was added 0.10 ml BSA-saline and 0.05 ml labelled hormone. These were mixed and 0.10 ml antiserum added. The reaction solution was then mixed thoroughly and incubated at 4°C for at least four hours.

After incubation, the tubes were removed onto ice and 0.10 ml dextran-coated charcoal measured quickly into each. The tube contents were mixed and left to stand for exactly 5 min to allow the coated charcoal to adsorb the unbound cortisol. The charcoal was then removed from suspension by centrifugation at 1200 g for 5 min, at a temperature of 4°C. Aliquots of 0.20 ml of the supernatant were transferred to scintillation vials and mixed thoroughly with 5.0 ml scintillation fluid. A 'total counts' vial was also prepared, consisting of 0.05 ml labelled hormone in 5.0 ml scintillant. A Packard Tri-Carb 2000CA Scintillation counter was used to estimate the ^{3}H activity (disintegrations min^{-1} averaged over a period of 5 min) in standards, unknowns and total counts vials.

2.2.7.3 Calculation

a. Since counting was performed on only 0.20 ml of the total 0.35 ml supernatant available after separation:

$$D_a = D_u \times \left(\frac{0.35}{0.20}\right)$$

where

D_s are the dpm's measured in each vial

D_s are the dpm's in corresponding tube supernatants

b. Since D_s corresponds to the fraction of labelled hormone bound to antiserum, percentage binding can be calculated as follows:

$$\%B = \frac{D_s}{D_t} \times 100$$

where

$\%B$ is the percentage of labelled hormone binding to the antiserum

D_t is the total dpm in 0.05 ml tritiated cortisol solution

c. A standard curve was plotted as $\%B$ versus concentration of cortisol per tube for the set of standards. The cortisol concentration in each unknown tube was then calculated by interpolation on this standard curve.

d. The concentration of cortisol in the sample of plasma was then calculated as follows:

$$C_p = \frac{(\frac{C_s}{V_e})}{V_s}$$

where

C_p is the concentration of cortisol in plasma (ng ml^{-1})

C_s is the concentration of cortisol in the tube (ng ml^{-1})

V_e is the original volume of plasma (0.2 ml)

V_s is the volume of extract dried down (usually 0.2 ml)

2.2.8 Determination of total lipid and polyunsaturated fatty acid concentrations

Polyunsaturated fatty acid concentrations in plasma and liver samples were determined using a combination of high-performance thin-layer chromatography (HPTLC) and gas-liquid chromatography (GLC). These analyses were performed by G. Bell, University of Stirling, according to the methods published by Tocher and Harvie (1988) and Cowey *et al.* (1985). Total lipid concentrations were measured during the course of these analyses using the

method of Folch *et al.* (1957).

2.2.8.1 Materials

a. Chloroform:methanol (2:1 v/v) plus 0.05% butylated hydroxytoluene (BHT)

b. Potassium chloride. 0.88% in aqueous solution

c. Developer 1. Methyl acetate : isopropanol : chloroform : methanol : 0.25% aqueous potassium chloride. 25:25:25:10:9 by volume

d. Developer 2. Hexane:diethyl ether:acetic acid. 80:20:2 by volume

e. 3% copper acetate in 8% phosphoric acid

f. Lipid class standards. Sigma Ltd.

g. Sulphuric acid. 1% in anhydrous methanol

h. 2% Potassium hydrogen carbonate

i. Hexane:diethyl ether (1:1 by volume)

j. TLC solvent. Hexane:diethyl ether:acetic acid. 85:15:1.5 by volume

k. Dichloromethane containing 0.05% BHT

l. Fatty acid methyl ester standards

All solvents used were HPLC grade.

2.2.8.2 Procedure for the determination of total lipid and polyunsaturated fatty acid concentrations in fish tissues

Liver and plasma samples were homogenized with approximately 10 volumes 2:1 chloroform:methanol containing 0.05% BHT. The homogenate was filtered and the filtrate washed with 0.2 volumes 0.88% potassium chloride. After centrifugation, the lower chloroform layer was removed into pre-weighed tubes and the solvent evaporated under nitrogen. The tubes were dried by vacuum desiccation, for one hour, after which they were reweighed to quantify the lipid content. The lipid was redissolved in chloroform:methanol to a concentration of 50 mg ml^{-1}. The tubes were then purged with argon and stored at -20°C. The lipid class composition was determined with 1-2 µl of this solution as described by Tocher and Harvie (1988).

1-2 µl of the lipid extracts were spotted onto HPTLC silica gel 60

plates (10 x 10cm x 0.25mm; Merck, Darmstadt, FRG) which had been pre-run in diethyl ether and activated for 1 hour at 120°C. They were run for 6 cm using Developer 1 to separate polar lipids, dried and fully developed using Developer 2 to separate neutral lipids and cholesterol. The lipids were visualized by spraying with 3% copper acetate in 8% phosphoric acid and charring at 160°C for 20 min, then identified by comparison with pure standards. Lipid classes were quantified by densitometry using a Shimadzu CS-930 dual wavelength TLC scanner and DR-2 recording integrator.

Polyunsaturated fatty acid concentrations were determined using GLC as described by Cowey *et al.* (1985). 0.1 ml aliquots of the original lipid extracts were methylated using acid-catalyzed transmethylation as described by Christie (1982). 2 ml 1% sulphuric acid in anhydrous methanol were added to the lipid extract and the mixture heated overnight at 50°C. 1.0 ml 2% potassium hydrogen carbonate solution was added and the lipids were extracted by shaking twice with 5 ml aliquots of hexane:diethyl ether (1:1 v/v). After extraction, the methyl esters were purified by preparative thin-layer chromatography using silica gel 60 TLC plates (20 x 20 cm x 0.25 mm; Merck, Darmstadt, FRG) and hexane:diethyl ether:acetic acid (85:15:1.5) as the developing solvent. The methyl esters were redissolved in dichloromethane containing BHT, and analyzed using a Carlo Erba 4160 gas chromatograph (Fisons Ltd., Crawley, UK), fitted with a free fatty acid phase fused silica capillary column (internal diameter 0.22 mm, length 50 mm) (SGE Ltd., Milton Keynes, UK), and equipped for splitless injection. Hydrogen gas was used as the carrier and a thermal gradient from 150 to 220°C was applied. Individual methyl esters were identified by comparison with known standards and a well characterized fish oil, and also by reference to published data (Ackman 1980; Ackman and Eaton 1978; Bell *et al.* 1983). Fatty acid peaks were quantified using a Shimadzu CR 3-A recording integrator.

2.2.8.3 Calculation

a. The total lipid concentration (C_t) was calculated from the change in weight of the reweighed vial (ΔW_t) and the initial volume (V_o) of plasma or weight of tissue extracted.

$$C_t = \Delta M_t \div V_t$$

b. The percentage of total lipid as fatty acid (%*fa*) was determined from the results of the lipid class analysis as follows. Total lipids comprised different proportions of a number of lipid classes the percentages (%*a*) of which were determined by HPTLC. Each class was considered to contain a fixed percentage (%*b*) of fatty acid estimated on the basis of an average fatty acid molecular weight of 291.8. The percentage of PUFA within each lipid class (%*c*) in the sample was calculated as:

$$\%c = \%a \times (\%b \div 100)$$

c. The concentration of fatty acid (C_a) in the sample was:

$$C_a = C_t \times (\%fa \div 100)$$

d. From the results of GLC analyses, the percentage concentration of individual polyunsaturated fatty acids (%*x*) in the sample was determined, and the concentration of individual PUFAs (C_x) calculated as:

$$C_x = \%x \times C_a$$

These were converted into molarity or molality (C_y) by dividing by the molecular weight. C_y's were then summed to give an estimate of the total PUFA concentration in the sample (C_s).

e. The concentration of PUFA was expressed in μmol g^{-1} or ml^{-1} plasma.

f. An example calculation is shown in table 2.6.

g. PUFA:vitamin E molar ratios were calculated by dividing α-tocopherol concentration (μg ml^{-1} or g^{-1}) by 430.7 (the molecular weight of α-tocopherol), then dividing μmol PUFA by μmol vitamin E. PUFA:lipid ratios were expressed on a weight basis. PUFA concentrations (μmol ml^{-1} or g^{-1}) were multiplied by 291.8 (taken to be the average molecular weight of PUFA), divided by 1000 (to convert μg to mg), and then divided by the lipid concentration in mg ml^{-1}.

Table 2.6. Example of the calculation of polyunsaturated fatty acid concentrations in fish tissue

a) Total lipid concentration (C_t) = 32 mg g^{-1}

b) Class composition

Lipid class	percent of total (%a) from HPTLC	percent as fatty acid (%b)	percent of total as fatty acid (%c)
Sterol esters	14.8	41.6	6.2
Triacylglycerols	1.0	90.2	0.9
Free fatty acids	34.5	100	34.5
Cholesterol	16.6	0	0
Polar lipids	18.2	75.1	13.7
Total (%fa)			55.3

c) Total fatty acid concentration (C_a) = 32 x (55.3 ÷100) = 17.7 mg g^{-1}

d) Polyunsaturated fatty acid composition

PUFA	percent in sample (%x)	conc. in sample (C_x) (mg g^{-1})	Mol. wt.	Molality (C_y) (µg g^{-1})
18:2(n-6)	2.4	0.42	280	1.5
18:3(n-6)(n-3)	0.4	0.07	278	0.3
18:4(n-3)	0.2	0.04	276	0.1
20:2(n-6)	0.4	0.07	308	0.2
20:3(n-6)	-	-	306	-
20:4(n-6)(n-3)	2.0	0.35	304	1.2
20:5(n-3)	1.6	0.28	302	0.9
22:5(n-3)	0.5	0.09	328	0.3
22:6(n-3)	10.1	1.79	326	5.5
Total (Cu)				10.0

e) Thus the total polyunsaturated fatty acid concentration (C_u) in the liver sample was 10.0 µmol g^{-1}

2.3 HAEMATOLOGY

Blood samples were sometimes subjected to haematocrit and leucocrit determination. Those collected during the experimental studies described in section 5.2 were measured in batches of 10 or 12, so that blood did not stand for more than two hours.

Haematocrit (packed red cell volume) was determined as described by Hesser (1960). Freshly collected blood was drawn into heparinised

microhaematocrit tubes (length 7.5 mm, outside diameter 1.5 mm, volume 75 µl). The tips were sealed with plasticine and the tubes centrifuged for 4 min in a Hawksley haematocrit centrifuge. The haematocrit, expressed as a percentage of the total tube contents, was then measured directly using a microhaematocrit reader. Leucocrit (packed leucocyte-thrombocyte volume) was measured as described by McLeay and Gordon (1977). The centrifuged microhaematocrit tubes were viewed at x 35 magnification under a binocular microscope fitted with an ocular micrometer. The height of the buff-coloured layer overlying the packed red cells was measured and expressed as a percentage of the total tube contents.

2.4 HISTOLOGY

2.4.1 Sample collection and fixation

Tissues for histology were collected from freshly killed fish. The body cavity was opened as described in section 2.1 and the organs carefully removed. Tissues sampled included pancreas-caeca, heart, liver, kidney, gill spleen, oesophagus, stomach, hindgut (anterior and posterior), skin and red and white skeletal muscle. These were cut into small cubes and fixed in phosphate buffered 4% formaldehyde (table 2.7) for a minimum of 7 days.

Table 2.7. Phosphate buffered 4% formaldehyde fixative

sodium dihydrogen phosphate, monohydrate	4 g
disodium hydrogen orthophosphate, anhydrous	6 g
40% formaldehyde solution	100 ml
distilled water	900 ml

2.4.2 Processing and sectioning

Routine processing and staining of tissue samples for histology was carried out by M.Beveridge, L.Sinclair and S.Millar (University of Stirling). In addition a number of samples were prepared using facilities available at the Dunstaffnage Marine Laboratory. In both cases, the procedures used closely

followed those described by Bullock (1978). Fixed tissue was cut to size, placed into histological cassettes and loaded into baskets on a Reichert-Jung Histokinette 2000 automatic tissue processor. The tissues were dehydrated, cleared and impregnated with paraffin wax according to the schedule shown in table 2.8. They were then set into wax blocks, trimmed, and 5 µm sections cut using a rotary microtome. The sections were then transferred to a water bath at 50°C, floated onto microscope slides, and allowed to dry at 60°C for at least 1 hour.

Table 2.8. Schedule for processing tissues for histology

	Bath	Time (hrs)
1.	50% methylated spirits	1
2.	80% methylated spirits	2
3.	100% methylated spirits	2
4.	100% methylated spirits	2
5.	100% methylated spirits	2
6.	absolute alcohol	2
7.	absolute alcohol	2
8.	chloroform	2
9.	chloroform	2
10.	paraffin wax	1
11.	paraffin wax	2
12.	paraffin wax	2

2.4.3 Staining and observation

The sections were stained with haematoxylin and eosin, according to the schedule shown in table 2.9, and then mounted under coverslips with DPX mountant. The preparations were observed at x 40, x 100 and x 400 magnification using an Olympus CH compound microscope. Observations were recorded on a standard form. Photomicrographs were printed from transparencies taken on Kodachrome film, at x 50 and x 125 magnification, using a Leitz Ortholux II microscope and Orthomat 35 mm camera. Sections of pancreas-caeca were checked individually for evidence of exocrine pancreatic degeneration (EPD) as described by Munro *et al.* (1984). Heart and skeletal muscle sections

Table 2.9. Schedule for haematoxylin and eosin staining of tissue sections

	Bath	Time (mins)
1.	Xylene	5
2.	Alcohol I	2
3.	Methylated spirits	2
4.	Running Tap Water	rinse
5.	Haematoxylin	5
6.	Wash in Tap Water	rinse
7.	Acid Alcohol	rinse
8.	Wash in Tap Water	rinse
9.	Scott's Tap Water	2
Check staining microscopically at this stage		
10.	Wash well in Tap Water	rinse
11.	Eosin	5
12.	Quick wash in Tap Water	rinse
13.	Methylated spirits	2
14.	Alcohol II	2
15.	Alcohol III	2
16.	Xylene	5
17.	Xylene	5

were examined for signs of structural change as described by Cowey *et al.* (1984) and Ferguson *et al.* (1986*a*) (see section 1).

2.5 DATA ANALYSIS

Statistical procedures recommended by Sokal and Rohlf (1981) and Conover (1980) were used for data analyses.

2.5.1 Computer facilities

The analyses were performed using a DEC Vax 8650/VMS system, sited at the British Geological Survey, Nottingham, and operated by NERC Computer Services. Time series graphs and bar charts were generated using SAS/GRAPH (SAS Institute Inc.) from summary statistics produced using Genstat V

(Rothampsted Experimental Station). Genstat V was also used to carry out tests and transformations for normal distribution, analyses of variance, multiple comparisons, contrasts and Pearson's correlation tests. Minitab statistical software (Minitab Inc.) was used to perform Spearman's test for rank correlation, Kruskal-Wallis and Mann-Whitney U-tests, and also to calculate a number of parametric statistics using small data sets.

2.5.2 Descriptive statistics

Histograms and cumulative frequency plots were used to test for normal distribution. The raw data, square root+1 and log+1 transformations were used for these tests. Standard deviations of means are given in the text and tables, but standard errors were used in the preparation of the figures.

2.5.3 Analysis of variance

Analysis of variance was used to assign probability figures to differences between various groupings of normally distributed data. In field studies this procedure was primarily used to compare biochemical data at different times, although comparisons of farms, cages and sick and healthy individuals, males and females, and salmon and grilse were also carried out using single factor analyses of variance. In the experimental studies described in section 5 more complex models, involving a combination of treatment and blocking factors and nested and crossed designs, were analyzed. Further details of the models used are given in section 5.2. If necessary the data were subjected to square root+1 or log+1 transformation to improve the homogeneity of variance. Graphs of residuals against fitted values were used to indicate any appropriate transformation (Lane *et al.* 1987). Significance was tested at $p=0.05$ and $p=0.01$. Significant treatment-time interactions were further analyzed using the T (for equal sample sizes) and GT2 (for unequal sample sizes) methods of pairwise multiple comparison to indicate which times differed significantly at $p=0.05$. Contrasts were used to test for differences between sets of means. Both pairwise multiple comparisons and contrasts were calculated using Gabriel's approximate method (Gabriel 1978; Sokal and Rohlf

1981). Data sets which did not show normal distribution even after transformation, but for which similar tests were required, were analyzed using the Mann-Whitney U-test (if k=2) or the Kruskal-Wallis test (if k>2). These methods were used to compare treatments within each level of time for some experimental data, but were not sufficiently flexible to accommodate time-wise variation as in analysis of variance. Both were tested at p=0.05 and p=0.01. Non-parametric methods of multiple comparison were not used since the relevant data sets never reached the minimum number of observations (n>8) required for these tests.

2.5.4 Correlation and Regression analysis

Pearson's product-moment correlation coefficient was used to test for association between two sets of independent, normally distributed data, after transformation if necessary. Spearman's test for rank correlation was the non-parametric alternative if the data did not show bivariate normal distribution. The significance of Pearson's correlation coefficient was tested at p=0.05 and p=0.01, and Spearman's at p=0.05, p=0.01 and p=0.001. Spearman's test for rank correlation was also used to test for trends with time as suggested by Daniels (1950).

CHAPTER 3

STUDIES ON THE VITAMIN E STATUS OF HEALTHY, FARMED ATLANTIC SALMON

3.1 INTRODUCTION

Nutritional status may be defined as the adequacy or otherwise with which the supply of a nutrient in the diet meets the demand for that nutrient within the body or tissue. Historically, vitamin E status has been assessed using histological, biochemical and gross indicators of deficiency as described in section 1.2.5. Whilst these methods provide a useful indication of vitamin E activity, they are secondary and therefore suffer from poor sensitivity and uncertainty of cause. Accurate analytical procedures for the direct measurement of tissue α-tocopherol concentrations complement, but cannot yet replace, these criteria of vitamin E status. Knowledge of the normal range, and of the relative effects of the multitude of factors which may influence vitamin E status, is necessary before a judgement of the adequacy of vitamin E nutrition, or otherwise, can be made with confidence on the basis of measured tissue concentrations. Nevertheless, direct determination of vitamin E concentrations has allowed a greater understanding of the function of vitamin E and its role in disease processes (Farrell 1980).

3.1.1 Tissue vitamin E concentrations in healthy, farmed salmon

Tissue vitamin E concentrations during normal growth and development have not previously been monitored over an extended period for any fish species, yet this information is important in order to determine the normal range and perhaps to identify periods of vulnerability to deficiency. In order to obtain this basic information, vitamin E concentrations in plasma and liver of farmed Atlantic salmon, held under commercial production conditions, were monitored over a 20 month period, from the time of smoltification and transfer to seawater, through to maturation of grilse after one year in seawater.

3.1.2 Comparison of tissue vitamin E in farmed and wild salmon

Factors such as diet, environmental conditions, feeding regime and husbandry practices may be expected to influence vitamin E status (see section 1.2). Previous workers found evidence to suggest that farmed salmon have lower

tissue vitamin E concentrations than wild salmon (Poppe *et al.* 1985*c*). Since this suggests that the vitamin E nutrition of farmed stock is inadequate, a limited study of tissue vitamin E concentrations in wild-caught salmon was conducted to provide more data for comparison of farmed and wild fish.

3.1.3 Effect of food intake on tissue vitamin E concentrations

Feeding and growth rates may affect vitamin E-selenium status in other vertebrates (Vos *et al.* 1981; Peter *et al.* 1985). High feeding rates are also considered to influence the expression of overt pancreas disease although the mechanism of this action is unknown (section 1.1.3.6). No gross indications of vitamin E deficiency were detected in rainbow trout fed vitamin E-depleted diets at a rate of 2% body weight day^{-1} (Hung *et al.* 1981; Cowey *et al.* 1981, 1983). In contrast, Cowey *et al.* (1984) described severe muscle damage and other pathological signs of vitamin E deficiency in fish of a similar size, fed similar diets at between 7.5 and 3.0% body weight day^{-1}. In order to test the hypothesis that high feeding rates promote the expression of vitamin E deficiency, a study of the effect of feeding rate on plasma vitamin E concentrations was conducted. The processes which could mediate such a response are entirely speculative. Vos *et al.* (1981) inferred that the expression of nutritional myopathy in ducklings resulted from dilution of vitamin E in the tissues due to rapid growth, but the growth rate obtained in the study by Cowey *et al.* (1984) was lower than previously (Hung *et al.* 1981; Cowey *et al.* 1981, 1983). A further possibility in aquaculture is that impaired water quality, due to overfeeding, may precipitate a stress response leading to the expression of vitamin E deficiency. Relationships between vitamin E status and stress responses are examined in detail in chapter 5, but plasma cortisol concentrations in fish under different feeding regimes were also measured in the present study.

Ackman and Cormier (1967) found that α-tocopherol concentrations were reduced by about half in the muscle of cod, *Gadus morhua*, starved for 3 months, but there is no published information on vitamin E concentrations during starvation in farmed Atlantic salmon. Atlantic salmon become anorexic during maturation and also when suffering from pancreas disease. In addition,

on commercial salmon farms, healthy fish are often starved for a period prior to harvest. Since vitamin E status may influence the storage stability of fish flesh (section 1.2.10.3), this starvation period may affect product quality. In order to determine the effect of starvation on tissue vitamin E concentrations in one sea-winter salmon, vitamin E analyses were performed on tissues collected at weekly intervals during a period of 1 month starvation.

3.2 MATERIALS AND METHODS

3.2.1 Tissue vitamin E concentrations in healthy, farmed Atlantic salmon

3.2.1.1 Sample collection

A series of monthly samples was collected to monitor changes in tissue vitamin E concentrations in healthy farmed salmon under commercial production conditions. The study was conducted between February 1988 and September 1989 using fish from Stirling University's fish farm sites at Howietown and Oban. Sampling began when the stock was still held in freshwater and continued through the period of smoltification and growth in seawater, ending after the maturation of one sea-winter fish. Samples were collected at monthly intervals according to the procedure described in section 2.1.1. The dates, cages and number of fish sampled are given in table 3.1. An effort was made to collect fish from the same cage population, but this was not always possible. Material was collected for histology and examined for signs of pancreas disease by J.Turnbull (Howietown Fish Farm, Stirlingshire). All fish collected between February and May 1988 were classified as parr or smolt on the basis of external appearance (Hoar 1976) and a number of individuals collected between March and September 1989 were classified according to sex and state of maturity (ie. salmon or grilse) by internal examination.

3.2.1.2 Biochemical analysis

Vitamin E concentrations were measured in plasma, liver and feed samples as detailed in section 2.2.1. Lipid and polyunsaturated fatty acid

Table 3.1. Sampling schedule for study of tissue vitamin E concentrations in healthy, farmed salmon

Date	Cage(s)	No.fish
12.2.88	6	10
11.3.88	1B	10
12.4.88	1B	10
12.5.88	1B	11
13.6.88	1,3	10
12.7.88	1,3	11
16.8.88	1,3	10
12.9.88	1,3	10
12.10.88	1,3	10
11.11.88	1,3	8
13.12.88	1,3	8
16.1.89	1,3	8
9.2.89	1,3	8
13.3.89	1,3	6
12.4.89	2,3	6
10.5.89	1,2	6
14.6.89	1,2	6
12.7.89	3	4
11.8.89	3	4
11.9.89	3	4

concentrations in a number of plasma and liver samples were determined as described in section 2.2.8.

3.2.1.3 Statistical analysis

Spearman's rank correlations were used to determine the trend shown by each of the biochemical parameters with time. The data were transformed and tested for normal distribution and the means at each time compared using analysis of variance. Significant differences were further investigated using pairwise multiple comparisons and contrasts between selected sets of data.

Differences between parr and smolts, smolts in freshwater and seawater, males and females, and mature and immature one sea-winter fish were examined using the Mann-Whitney U-test. Correlation analyses between the various biochemical measurements, fish weight and condition factor were performed using Pearson's test and correlations with water temperature were made using Spearman's test.

3.2.2 Tissue vitamin E concentrations in mature, wild salmon

In order to gather comparative data from wild fish, blood and liver samples were collected from ten, sexually mature, wild-caught salmon held in tanks at Kinnaber on the River North Esk, near Montrose. These had been held without food for a period of six weeks prior to sampling on 21st December 1988. The fish were tagged and the age of each was known. The sex of each fish was determined at the time of sampling. Plasma and liver vitamin E concentrations were determined as described in section 2.2.1. The Mann-Whitney U-test was used to test for differences in tissue vitamin E concentrations between male and female fish and Spearman's test was used for correlation analysis.

3.2.3 The effects of ration size on plasma vitamin E and cortisol concentrations

In October and November 1987, an experiment was conducted to examine the effects of food intake on plasma vitamin E concentrations. The holding facilities comprised three $2m^3$ tanks (1400 l capacity), each supplied with seawater at a rate of 10 l min^{-1}. One hundred and five post-smolt salmon (mean weight 244±36 g) were distributed evenly between the three tanks (35 fish $tank^{-1}$). The fish were allowed to recover for 14 days during which time they were fed at a rate of 2% body weight day^{-1} using a diet, formulated to commercial standards, supplied by BP Nutrition Ltd. At the end of this recovery period a baseline sample of ten fish $tank^{-1}$ was collected. Groups of fish were then fed as follows:

1) control: fed at 2% body weight day^{-1}

2) low: fed at 1% body weight day^{-1}

3) high: fed at 3% body weight day^{-1}

Due to the limited number of tanks available, it was not possible to replicate each treatment. The experiment continued for 6 weeks. Ration size was calculated each day from predicted growth rates and the fish were fed three times daily. Daily ration was not adjusted for temperature which fell steadily from 12.0 to 9.5°C during the course of the experiment. Ten fish were sampled from each tank after three and six weeks as described in section 2.1.2. Plasma cortisol concentrations were measured in fish 1-5, and plasma vitamin E in fish 6-10 as described in section 2.2. Plasma cortisol concentrations between treatments, but within each time stratum, were compared using the Kruskal-Wallis test. Analysis of variance with treatment and time as factors was used to examine the plasma vitamin E data. Correlation analyses were conducted using Pearson's or Spearman's methods as appropriate.

3.2.4 The effect of starvation on tissue vitamin E concentrations

During April 1988, a group of one sea-winter salmon held in a cage in Dunstaffnage Bay was subjected to a period of up to 1 month without feeding in a study of the effects of starvation on weight gain and body composition (Okoro 1988). Plasma and liver samples collected from five fish at weekly intervals by N. Okoro (Stirling University) were assayed for vitamin E as described in section 2.2.1. Spearman's test for rank correlation was used to describe the trend with time, and the median values at each time were compared using the Kruskal-Wallis test. Tests for correlation between variates were also performed using Spearman's test.

3.3 RESULTS AND DISCUSSION

3.3.1 Tissue vitamin E concentrations in healthy, farmed Atlantic salmon

3.3.1.1 Results

In 1988, fish at the freshwater site underwent smoltification in March and April and were transferred to seawater in May 1988. Maturation of one sea-winter fish began in May 1989 and, in September, mature fish (grilse) were graded out of the study cages. No evidence of pancreas disease was found, either in histological samples or from gross examination of the fish, at any time during the course of the study.

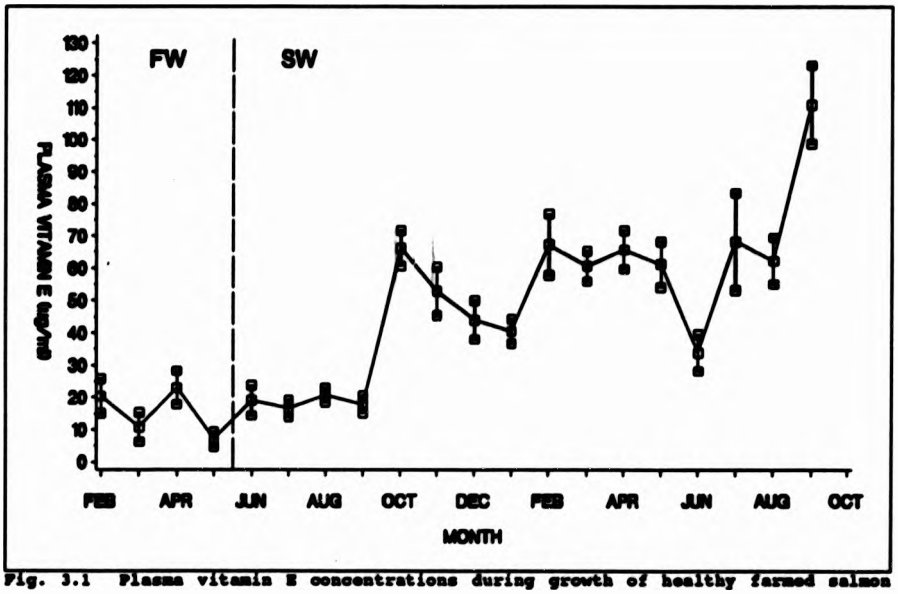

Fig. 3.1 Plasma vitamin E concentrations during growth of healthy farmed salmon (mean±se)

Mean monthly plasma vitamin E concentrations measured in these fish between February 1988 and September 1989 are shown in table 3.2 and illustrated in fig. 3.1. Between February and September 1988, mean plasma vitamin E concentrations ranged from 7±7 to 23±17 µg ml^{-1} (mean±standard deviation), but in October they rose sharply and between October 1988 and

Table 3.2. Plasma vitamin E concentrations in healthy, farmed salmon (mean±sd)

Month	n	Plasma vitamin E ($\mu g\ ml^{-1}$)
February 1988	10	20±17
March	8	11±13
April	10	23±17
May	8	7±7
June	10	19±15
July	11	17±9
August	10	21±7
September	10	18±8
October	10	66±17
November	8	53±21
December	8	44±17
January 1989	8	40±11
February	8	67±27
March	6	61±12
April	6	66±15
May	6	61±17
June	6	34±14
July	4	68±30
August	4	62±14
September	4	111±24

August 1989 they ranged between 34±14 and 68±30 $\mu g\ ml^{-1}$. At the final sample, in September 1989, concentrations of 111±24 $\mu g\ ml^{-1}$ were recorded. A significant tendency to increase was found during the course of the study ($p<0.001$ using Spearman's correlation test). The data were normalized using a square root transformation and analysis of variance showed significant differences among plasma vitamin E concentrations measured each month ($p<0.001$). The results of multiple pairwise comparisons of the means, using the GT2 method, are illustrated in fig.3.2. No significant differences could be detected among plasma vitamin E concentrations measured over the period February to September 1988. Means between October 1988 and September 1989 were also relatively homogenous although differences were detected between the

final sample, in September 1989, and samples from November and December 1988 and January and June 1989 ($p<0.05$). However, of 96 possible pairwise comparisons between means from the period February to September 1988 with means from the period October 1988 and September 1989, 82 comparisons were significantly different ($p<0.05$). A contrast between the data from February to September 1988 with the data from October 1988 to September 1989 showed that these were significantly different ($p<0.05$).

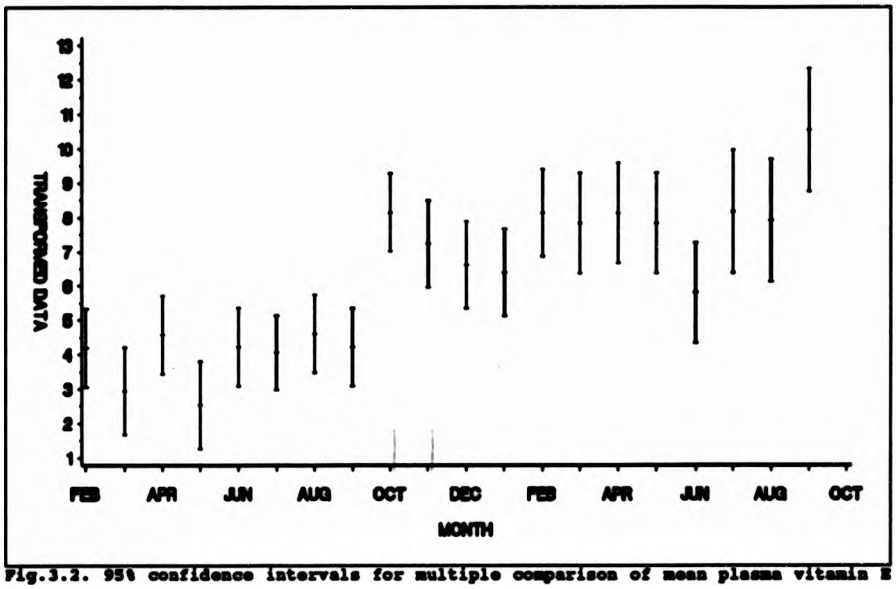

Fig.3.2. 95% confidence intervals for multiple comparison of mean plasma vitamin E concentrations. Overlapping confidence intervals mean no significant differences

Liver vitamin E concentrations measured in these fish are shown in table 3.3 and illustrated in fig. 3.3. In general, these were an order of magnitude higher than in plasma. Between February and June 1988, liver vitamin E concentrations ranged from 51±64 to 200±182 μg g^{-1} wet weight. In July, they increased sharply to 471±33 μg g^{-1} and remained at between 268±103 and 754±176 μg g^{-1} until September 1989. Liver vitamin E concentrations also showed a significant increase over the course of the study ($p<0.001$). The data was subjected to square root transformation and analysis of variance again showed significant differences between the means ($p<0.001$). Multiple comparisons, using the T-method, showed fewer significant differences than for plasma

Table 3.3. Liver vitamin E concentrations in healthy farmed salmon (mean±sd)

Month	n	Liver vitamin E ($\mu g\ g^{-1}$)
February 1988	3	200±182
March	3	51±64
April	3	86±33
May	3	92±49
June	3	164±110
July	3	471±33
August	3	469±139
September	3	318±134
October	3	499±159
November	3	268±103
December	3	580±88
January 1989	3	517±250
February	3	754±176
March	3	468±117
April	3	369±31
May	3	716±377
June	3	334±202
July	3	437±255
August	3	707±233
September	3	522±84

vitamin E (fig.3.4). No differences were found among samples collected between February and June 1988, and none were detected between July 1988 and September 1989, but 28 of 60 pairwise comparisons made between the former and latter sets of samples were significantly different ($p<0.05$). Even so, the confidence interval for a contrast between data from February to July 1988 and data from August 1988 to September 1989 was not significant. Pearson's test showed a significant overall correlation between plasma and liver vitamin E concentrations throughout the study ($p<0.01$).

Vitamin E concentrations in feed samples collected during the freshwater phase, between February and May 1988, averaged 21±6 mg 100 g^{-1} (n=6), whilst in samples collected during the seawater phase, in October and November, the

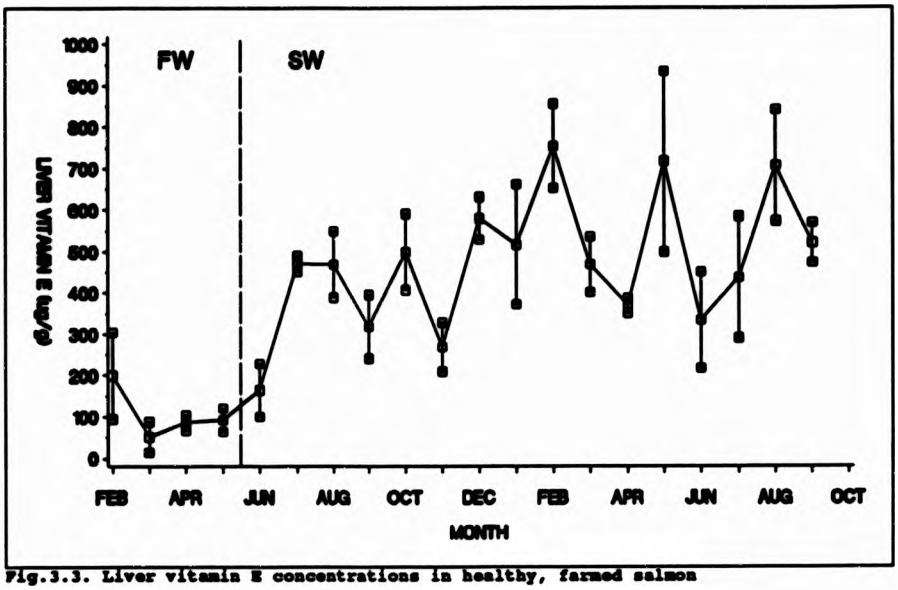

Fig.3.3. Liver vitamin E concentrations in healthy, farmed salmon

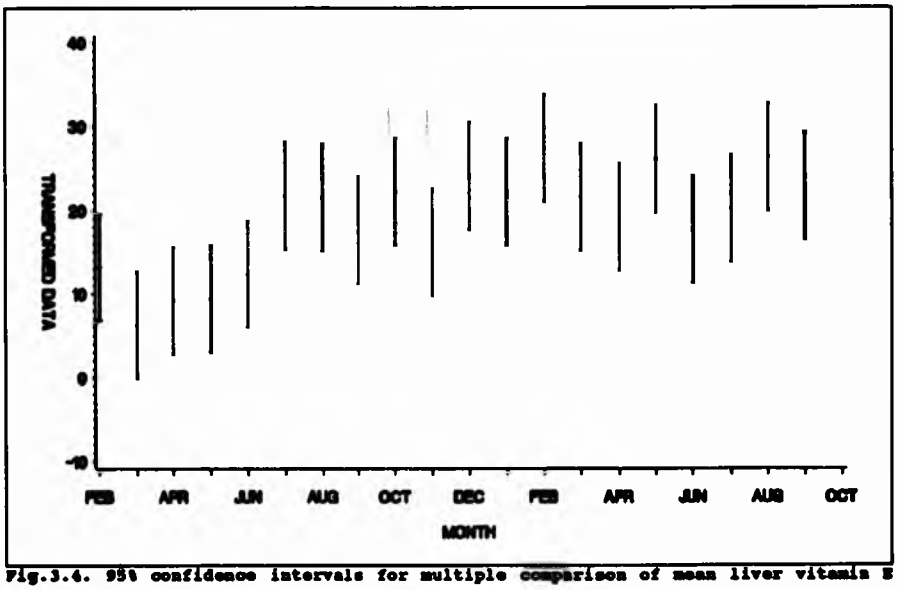

Fig.3.4. 95% confidence intervals for multiple comparison of mean liver vitamin E concentrations

figure was 16±0.3 mg 100 g^{-1} (n=5). The Mann-Whitney U-test indicated that these results were significantly different ($p<0.05$).

Table 3.4. Plasma lipid and polyunsaturated fatty acid concentrations in healthy farmed salmon (mean±sd)

Month	n	Plasma lipid (mg ml^{-1})	Plasma PUFA (μmol ml^{-1})
July 1988	3	12±1	7±1
August	3	8±1	5±1
September	5	6±4	4±3
October	3	11±3	8±2
November	5	17±6	12±5
December	3	4±2	4±2
January 1989	5	10±6	8±5
February	3	11±4	8±3
March	3	10±3	7±2
April	5	15±4	12±3
May	5	14±7	10±6
June	5	8±5	7±4
July	4	9±6	7±4
August	3	18±3	12±2
September	4	12±7	9±6

Plasma lipid and polyunsaturated fatty acid concentrations were only determined in fish collected between July 1988 and September 1989. These results are summarised in table 3.4. Plasma lipid concentrations ranged from 4±2 to 18±3 mg ml^{-1}, but showed no significant trend with time over this period (fig.3.5). The data was subjected to square root transformation and analysis of variance indicated that significant differences existed between the means ($p<0.05$). However, multiple comparisons, using the GT2 method, failed to show any significant differences between individual pairs of means at 95% confidence level. No clear groups appropriate for contrasts were recognised. A significant correlation between plasma lipid and plasma vitamin E concentrations was found ($p<0.01$).

Plasma polyunsaturated fatty acid concentrations are shown in fig. 3.6. These ranged from 3±1 to 12±5 μmol ml^{-1} between July 1988 and September 1989. They increased significantly with time ($p<0.05$), but closely reflected total lipid concentrations. A square root transformation was applied to the data and

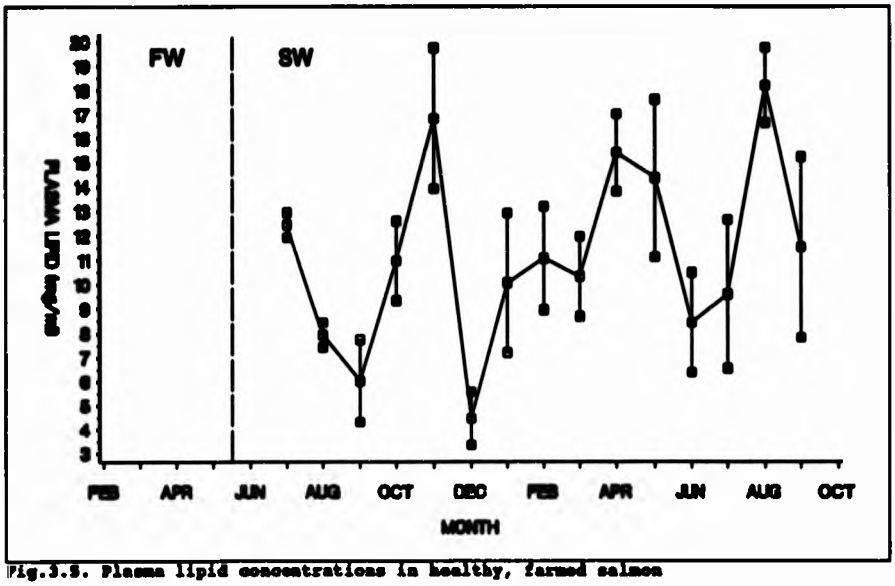

Fig.3.8. Plasma lipid concentrations in healthy, farmed salmon

significant differences were detected using analysis of variance ($p<0.05$). Once again, multiple comparisons failed to show any significant differences between individual pairs and no clear groupings suitable for contrasts were recognised. Plasma polyunsaturated fatty acid concentrations showed a significant correlation ($p<0.01$) with both plasma vitamin E and plasma lipid concentrations.

Plasma PUFA:lipid ratios (w/w) are shown in table 3.5 and illustrated in fig. 3.7. These ranged from 0.17±0.01 to 0.18±0.02 in July, August and September 1988, but then rose sharply, and, between October 1988 and September 1989, ranged between 0.20±0.01 and 0.24±0.02. A significant positive correlation with time was found using Spearman's test ($p<0.001$). Analysis of variance of the untransformed data showed that significant differences occurred among the means ($p<0.001$). Pairwise multiple comparisons, using the GT2 method (fig. 3.8), showed that significant differences lay between the July 1988 and October, January, June and July 1989 samples, the August 1988 and October, December, January, June and July 1989 samples and the September 1988 and January, June and July 1989 samples ($p<0.05$). However, the confidence interval for a contrast between the data from July, August and September 1988

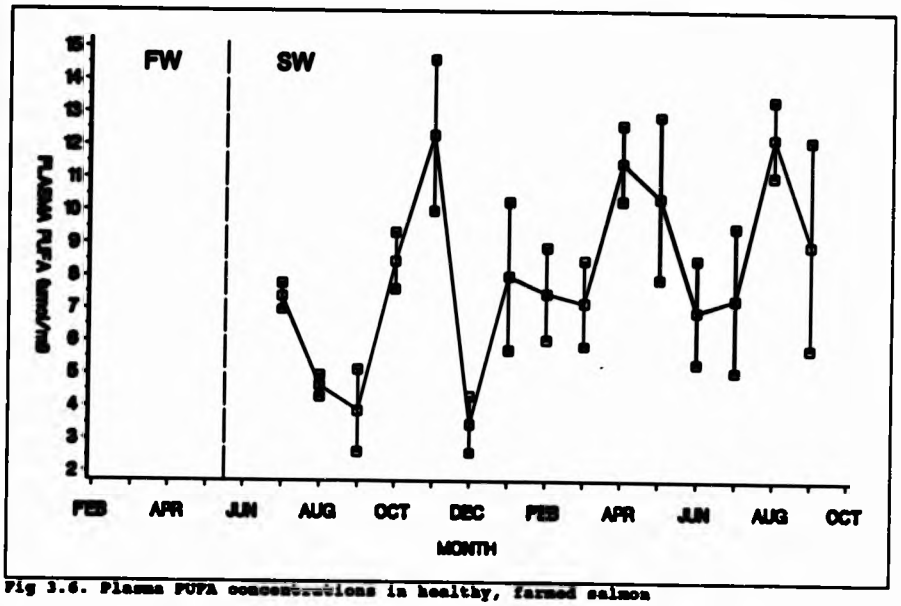

Fig 3.6. Plasma PUFA concentrations in healthy, farmed salmon

and the data from October 1988 to September 1989 was not significant. Pearson's test revealed a significant correlation between the plasma PUFA:lipid ratio and the plasma vitamin E concentration ($p<0.05$).

The molar ratios of PUFA:vitamin E in plasma are also shown in table 3.5 and illustrated in fig.3.9. These ranged between 174±196, in September 1988, and 33±19, in September 1989, and showed a significant, but irregular, downward trend during the course of the study ($p<0.01$). A log transformation was applied to the data, but no significant differences between the means each month were found using analysis of variance. A significant correlation was found between plasma PUFA:vitamin E ratios and plasma lipid concentrations ($p<0.01$), but not between plasma PUFA:vitamin E and plasma PUFA:lipid ratios.

Liver lipid and polyunsaturated fatty acid concentrations were measured in three fish from each month between February and July 1988 and between February and July 1989 (table 3.6). No samples were analysed from the period August 1988 to January 1989. Liver lipid concentrations are shown in fig. 3.10. These fell, from 75±27 mg g^{-1} (wet weight) in February to 24±2 mg g^{-1} in July 1988. Between February and July 1989 they remained low, at between 25±3 and 34±6 mg g^{-1}. Spearman's test showed that there was a significant negative

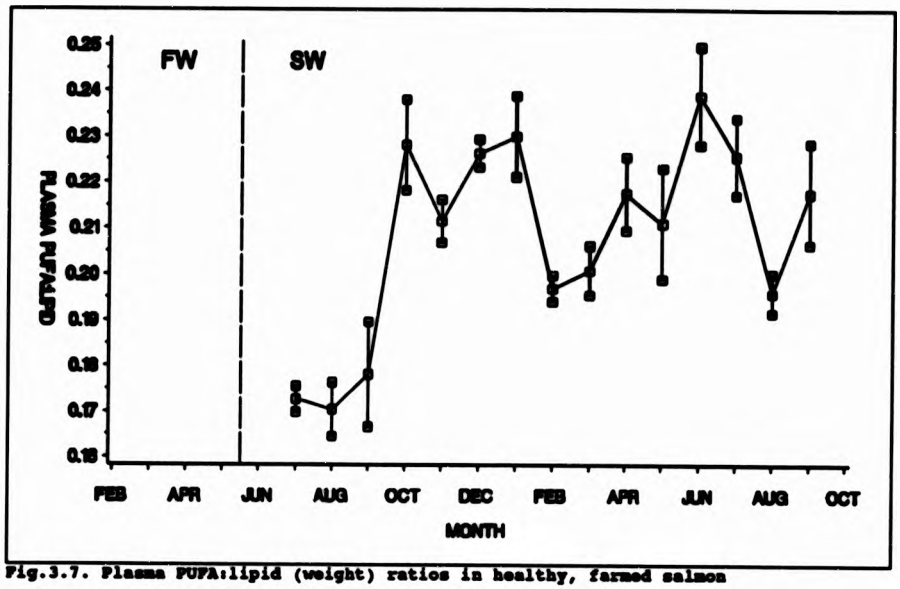

Fig.3.7. Plasma PUFA:lipid (weight) ratios in healthy, farmed salmon

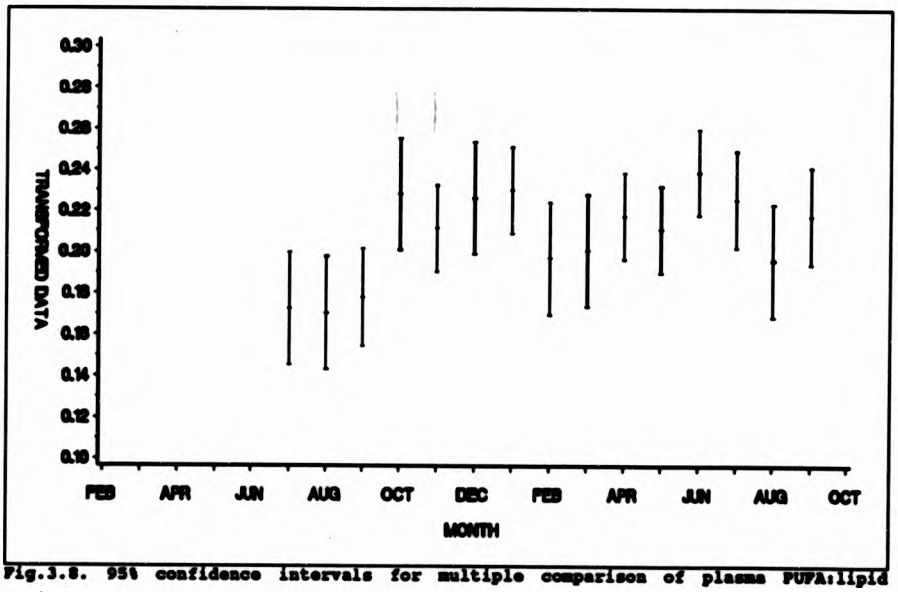

Fig.3.8. 95% confidence intervals for multiple comparison of plasma PUFA:lipid (weight) ratios.

relationship between liver lipid and time ($p<0.01$). The data were subjected to a log transformation and analysis of variance revealed significant

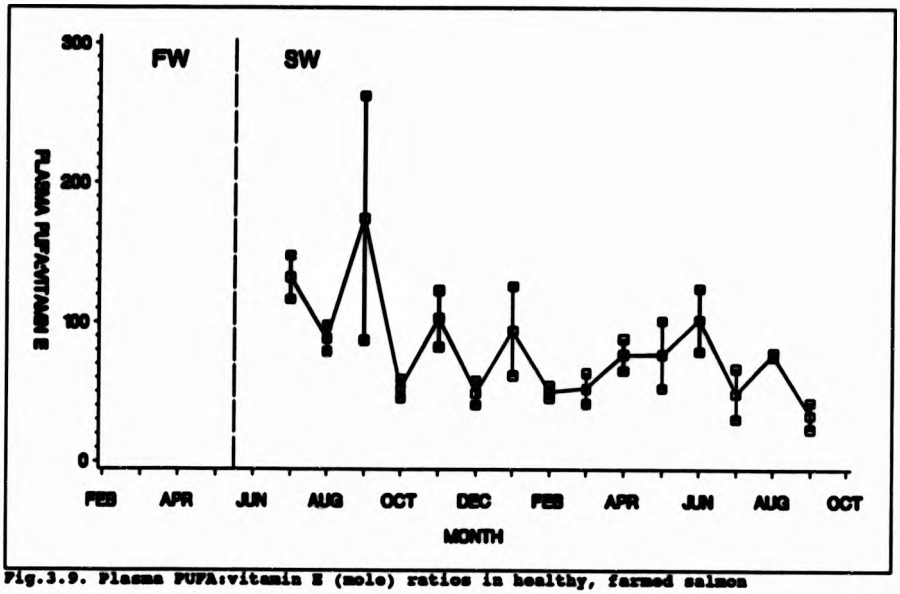

Fig.3.9. Plasma PUFA:vitamin E (mole) ratios in healthy, farmed salmon

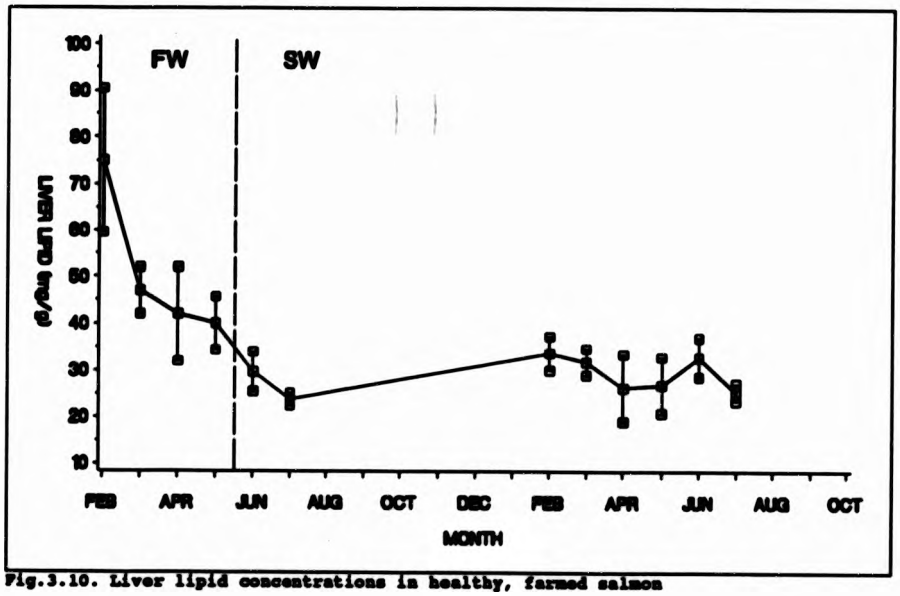

Fig.3.10. Liver lipid concentrations in healthy, farmed salmon

differences among the monthly means ($p<0.01$). Multiple comparisons, using the T-method, showed that liver lipid concentrations measured in February 1988

Table 3.5 Plasma PUFA:lipid and PUFA:vitamin E ratios in healthy farmed salmon (mean±sd)

Month	n	PUFA:lipid (wt.ratio)	n	PUFA:vitamin E (mole ratio)
July 1988	3	0.17±0.00	3	133±27
August	3	0.17±0.01	3	88±16
September	4	0.18±0.02	5	174±196
October	3	0.23±0.02	3	52±12
November	5	0.21±0.01	5	103±46
December	3	0.23±0.00	3	49±15
January 1989	5	0.23±0.02	5	94±72
February	3	0.20±0.00	3	50±7
March	3	0.20±0.01	3	52±19
April	5	0.22±0.02	5	77±25
May	5	0.21±0.03	5	77±54
June	5	0.24±0.02	5	102±51
July	4	0.23±0.02	4	49±36
August	3	0.20±0.01	3	77±2
September	4	0.22±0.02	4	33±19

were significantly different to those measured in July of the same year ($p<0.05$). No contrasts were performed on these data. Liver lipid concentrations showed no correlation with either liver vitamin E or plasma lipid concentrations.

Liver polyunsaturated fatty acid concentrations are illustrated in fig.3.11. These fell, from 22±5 μmol g^{-1} in February 1988 to 13±3 μmol g^{-1} in March, before rising to 19±5 μmol g^{-1} in June and 16±3 μmol g^{-1} in July. The following year they were higher and ranged from 21±9 to 28±2 μmol g^{-1} between February and July 1989. A significant increase with time was found using Spearman's test ($p<0.001$). The raw data was used for analysis of variance and significant differences ($p<0.05$) were detected between the means each month. However, no significant differences were detected during pairwise comparisons and a contrast between data from February to July 1988 and February to July 1989 was also not significant. No significant correlations were found between liver polyunsaturated fatty acid concentrations and liver lipid or vitamin E

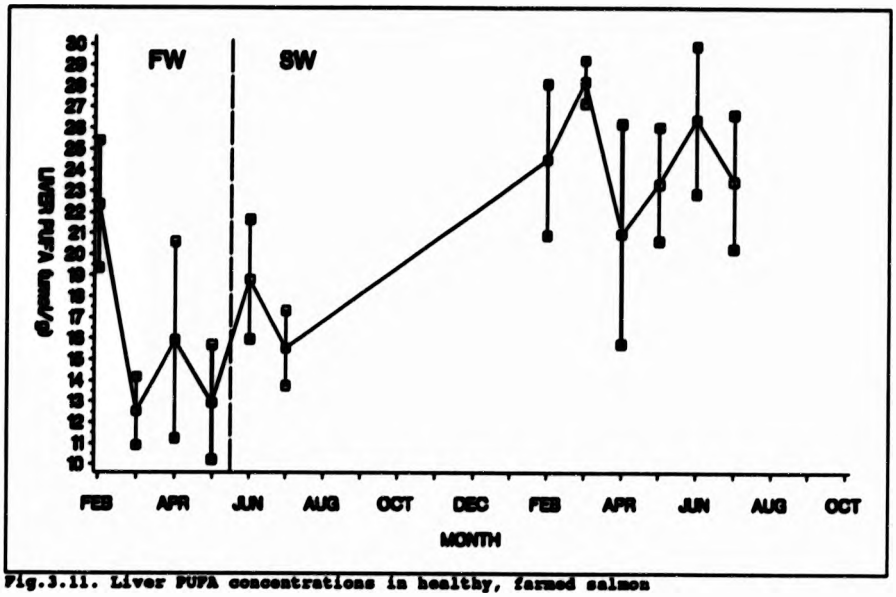

Fig.3.11. Liver PUFA concentrations in healthy, farmed salmon

or plasma polyunsaturated fatty acid concentrations.

Liver PUFA:lipid (weight) ratios are shown in table 3.7 and fig. 3.12. Between February and May 1988 this ratio ranged from 0.08±0.03 to 0.11±0.02. It rose sharply to 0.18±0.01 in June, after transfer to seawater, and between February and July 1989 rose from 0.22±0.08 to 0.27±0.03. Again a positive association with time was calculated ($p<0.001$). Analysis of variance of the untransformed data revealed significant differences among the monthly means ($p<0.01$). Multiple comparison, using the T-method, showed the following pairs to be significantly different: February 1988 and March, May and July 1989, March 1988 and March, April, May and July 1989, April 1988 and May 1989 and May 1988 and March, May and July 1989 ($p<0.05$) (fig.3.13). However, using a contrast, the means from the period February to May 1988 (in freshwater) were not significantly different to those measured between June 1988 and September 1989 (during the seawater phase). No significant correlation was found between liver PUFA:lipid and liver vitamin E concentrations.

Liver PUFA:vitamin E molar ratios are also shown in table 3.7 and in fig. 3.14. Both measurements were made for only 14 individuals. PUFA:vitamin E ratios fell, from 100±0 (n=1) in June to 12±0 (n=1) in July, then recovered,

Table 3.6 Liver lipid and polyunsaturated fatty acid concentrations in healthy farmed salmon (mean±sd)

Month	n	Liver lipid (mg g^{-1})	Liver PUFA (μmol g^{-1})
February 1988	3	75±27	22±5
March	3	47±9	12±3
April	3	42±17	16±8
May	3	40±10	12±5
June	3	30±7	19±5
July	3	24±2	16±3
August	0		
September	0		
October	0		
November	0		
December	0		
January 1989	0		
February	3	34±6	24±6
March	3	32±5	28±2
April	3	26±12	21±9
May	3	27±10	23±5
June	3	33±7	26±6
July	3	35±3	23±6

to 49±44, in September 1989. No overall trend with time was found and no significant differences were detected by analysis of variance of the log transformed data. No significant correlation was found between liver PUFA:vitamin E ratios and either liver lipid concentrations or liver PUFA:lipid ratios.

In February and March 1988, all fish collected were parr and showed characteristic parr markings. In April, all but one of the fish collected showed 'silvering' and brown fin fringes and were therefore judged to be smolts. In May, all fish collected were smolts. No significant differences in any of the biochemical parameters were detected between the samples from February/March and April/May, according to both Mann-Whitney U-tests and the multiple comparisons described above. Transfer to seawater took place in May, between the May and June samples. Again no significant differences were

Table 3.7 Liver PUFA:lipid and PUFA:vitamin E ratios in healthy farmed salmon (mean±sd)

Month	n	PUFA:lipid (wt. ratio)	n	PUFA:vitamin E (mole ratio)
February 1988	3	0.10±0.05	0	
March	3	0.08±0.03	0	
April	3	0.11±0.02	0	
May	3	0.09±0.02	0	
June	3	0.18±0.01	1	100±0
July	3	0.19±0.04	1	12±0
August	0		0	
September	0		0	
October	0		0	
November	0		0	
December	0		0	
January 1989	0		0	
February	3	0.22±0.08	1	17±0
March	3	0.26±0.02	2	31±3
April	3	0.24±0.07	2	19±9
May	3	0.31±0.21	2	13±5
June	3	0.24±0.05	3	49±40
July	3	0.27±0.03	2	49±44

detected in any of the biochemical parameters between these two months.

Data on sex and state of maturation was collected from a total of 24 fish captured between March and September 1989. From this data, no differences in plasma or liver vitamin E concentrations could be detected between males and females or between maturing and non-maturing salmon, although higher mean values were measured in male fish (table 3.8). Similarly, no differences between males and females, or mature or immature one sea-winter fish, were detected in plasma or liver polyunsaturated fatty acid or lipid concentrations, or in the PUFA:lipid or PUFA: vitamin E ratios, in either tissue.

Mean weights, lengths and condition factors for these fish are given in table 3.9. Weight gain is illustrated in fig. 3.15. The mean weight increased from 23±3 g in February 1988 to 2094±286 g in September 1989. Weight loss was

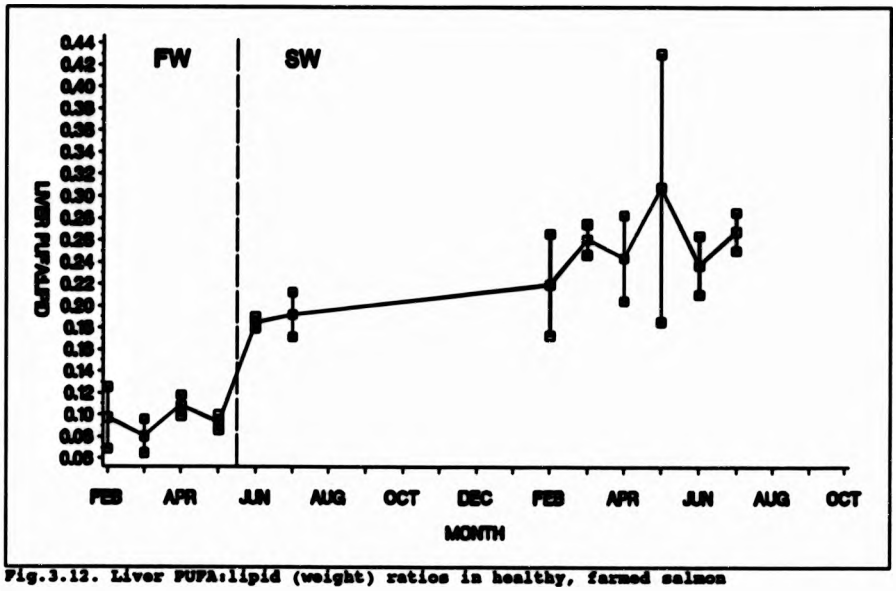

Fig.3.12. Liver PUFA:lipid (weight) ratios in healthy, farmed salmon

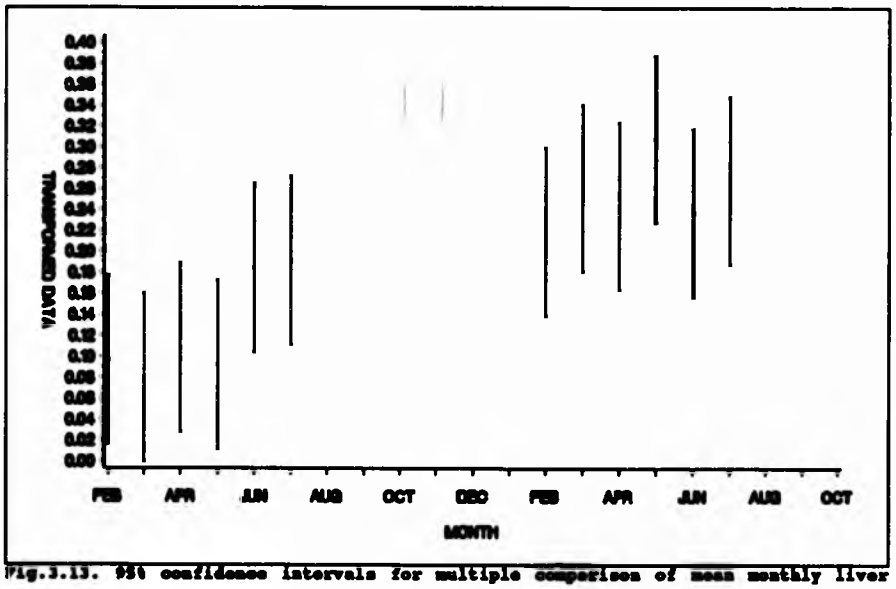

Fig.3.13. 95% confidence intervals for multiple comparison of mean monthly liver PUFA:lipid (weight) ratios

recorded between February and March and June and July 1989. Weight data was log transformed before correlation analysis. Using Pearson's test, plasma and

Table 3.8 Plasma and liver vitamin E concentrations in male and female farmed salmon (means±sd)

	n	Plasma vitamin E ($\mu g\ ml^{-1}$)	n	Liver vitamin E ($\mu g\ g^{-1}$)
Male	11	68±33	7	562±311
Female	12	60±27	7	530±204

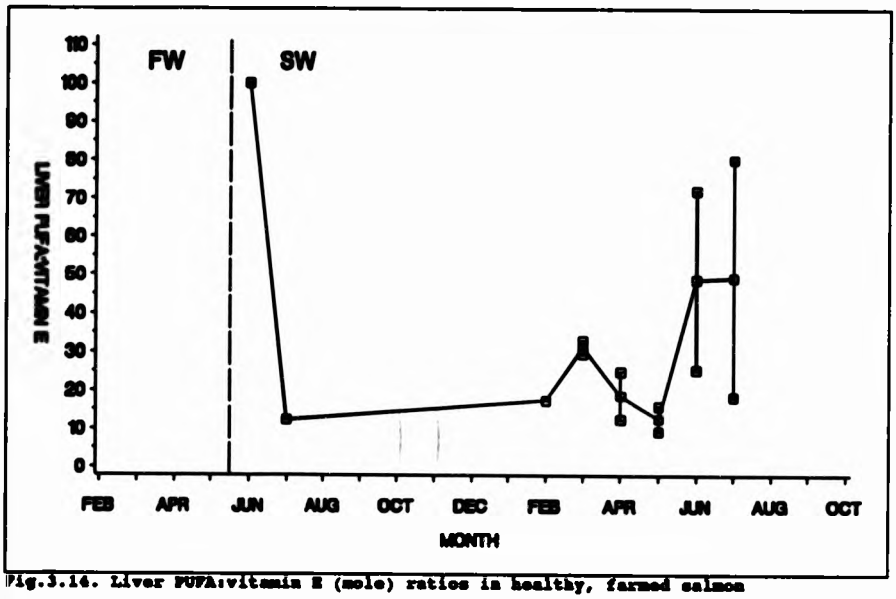

Fig.3.14. Liver PUFA:vitamin E (mole) ratios in healthy, farmed salmon

liver vitamin E and liver lipid and polyunsaturated fatty acid concentrations, plasma and liver PUFA:lipid ratios ($p<0.01$), and plasma polyunsaturated fatty acid concentrations ($p<0.05$), were found to be positively correlated with fish weight; the ratio of PUFA:vitamin E in plasma was negatively correlated ($p<0.05$), and plasma lipid and liver PUFA:vitamin E showed no correlation. Fish weights were significantly lower in parr collected in February/March than in smolts from April/May 1988 ($p<0.001$) and were also significantly lower in fish collected from freshwater in May than in those from seawater in June ($p<0.01$). No significant differences in fish weight were found between males and females or between salmon and grilse.

Condition factor increased from 1.28±0.15 to 1.39±0.09 between February

Table 3.9 Weight, length and condition factor of healthy farmed salmon (mean±sd)

Month	n	Weight (g)	Length (cm)	Condition factor
February 1988	9	23±3	12.1±0.8	1.28±0.15
March	10	23±2	12.1±0.3	1.32±0.05
April	10	26±7	12.2±1.3	1.39±0.09
May	11	43±6	14.8±0.8	1.32±0.06
June	10	57±10	16.7±0.9	1.23±0.05
July	11	88±24	19.4±1.8	1.19±0.06
August	10	189±31	25.1±1.4	1.19±0.07
September	10	297±44	27.8±1.3	1.38±0.08
October	10	498±71	31.7±1.6	1.56±0.11
November	8	649±98	34.4±1.9	1.59±0.06
December	8	916±153	38.7±1.3	1.58±0.05
January 1989	8	1005±131	41.0±1.9	1.45±0.06
February	8	1171±279	43.3±2.5	1.42±0.11
March	6	1098±333	42.4±3.1	1.40±0.15
April	6	1620±473	47.8±3.6	1.45±0.14
May	6	1622±628	47.5±5.4	1.45±0.21
June	6	1702±394	50.0±3.8	1.35±0.14
July	4	1354±448	47.2±4.0	1.25±0.10
August	4	1668±282	49.4±1.2	1.38±0.16
September	4	2094±286	51.5±5.7	1.66±0.69

and April 1988, and then fell, during the period of smoltification and seawater transfer, to 1.18±0.06 in July. During the late summer and autumn of 1988 it rose steadily, reaching 1.59±0.06 in November 1988. It decreased to 1.25±0.10 in July and then rose to 1.66±0.69 in September 1989. These changes are illustrated in fig. 3.16. Overall there was a significant improvement in condition factor with time ($p<0.001$). The raw data was suitable for parametric statistical analysis. Plasma vitamin E ($p<0.01$) and plasma lipid and polyunsaturated fatty acids ($p<0.05$) showed a positive correlation with condition factor, but no significant correlation was found between condition factor and liver vitamin E, lipid or polyunsaturated fatty acid concentrations or with plasma or liver PUFA:lipid or PUFA:vitamin E ratios. Condition factors

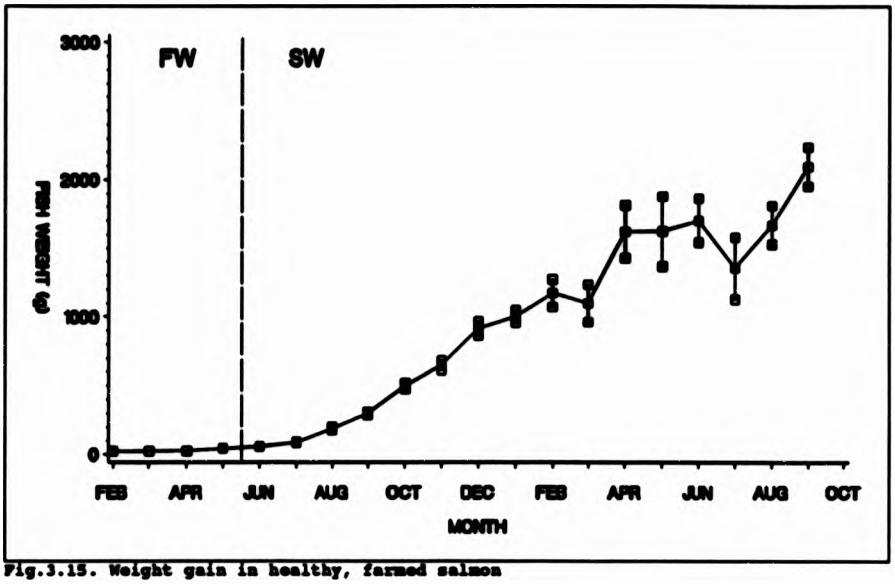

Fig.3.15. Weight gain in healthy, farmed salmon

showed no significant differences between parr from February/March and smolts from April/May, but in fish collected from freshwater in May they were significantly higher than in those collected from seawater in June ($p<0.01$). There were no significant differences in condition factor between male and female fish, but state of maturation did have significant effects, grilse having higher k-factors than salmon ($p<0.05$).

Typical monthly water temperatures during the study period are shown in fig. 3.17. These ranged between 4 and 14°C. Only liver lipid concentrations showed a significant correlation with water temperature ($p<0.05$). Higher liver lipid concentrations were associated with lower water temperatures.

3.3.1.2 Discussion

During the course of this study, plasma vitamin E concentrations ranged from 7 to 68 $\mu g\ ml^{-1}$ and liver concentrations from 51 to 754 $\mu g\ g^{-1}$. These cover a wide range, but both showed a tendency to increase with time and also a correlation with body weight. It therefore seems important that age and/or size should be considered when one is describing the normal range in healthy

fish. Although no further tests of vitamin E status were performed on these fish, the accumulation of stored α-tocopherol in the liver suggests that the dietary vitamin E content, at 16 mg 100 g^{-1}, was adequate in seawater.

Plasma vitamin E concentrations in the late parr stage lay between 7 and 23 μg ml^{-1} and liver concentrations ranged from 33 to 182 μg g^{-1}. During experimental studies, Bell, McVicar, Mitchell and Cowey (unpublished) measured concentrations of 19 μg ml^{-1} in plasma and 17 μg g^{-1} in liver of Atlantic salmon parr fed diets containing 85 mg kg^{-1} vitamin E (G.Bell, personal communication 1987). These plasma concentrations are similar, but liver concentrations are less than those measured here, probably since the dietary vitamin E content was lower in the earlier study. Hung *et al.* (1980a) showed that liver vitamin E concentrations in rainbow trout (*Oncorhynchus mykiss*) were correlated with dietary vitamin E over the range 4.3 to 76.9 mg 100 g^{-1}. Hardie *et al.* (1990) measured plasma vitamin E concentrations from 16 to 277 μg ml^{-1}, and liver vitamin E concentrations from 12 to 3097 μg g^{-1}, in Atlantic salmon parr fed diets containing between 0.7 and 80.0 mg vitamin E 100 g^{-1}. However, at very high dietary levels, tissue α-tocopherol concentrations would be expected to plateau as limits to the capacity of absorption are exceeded

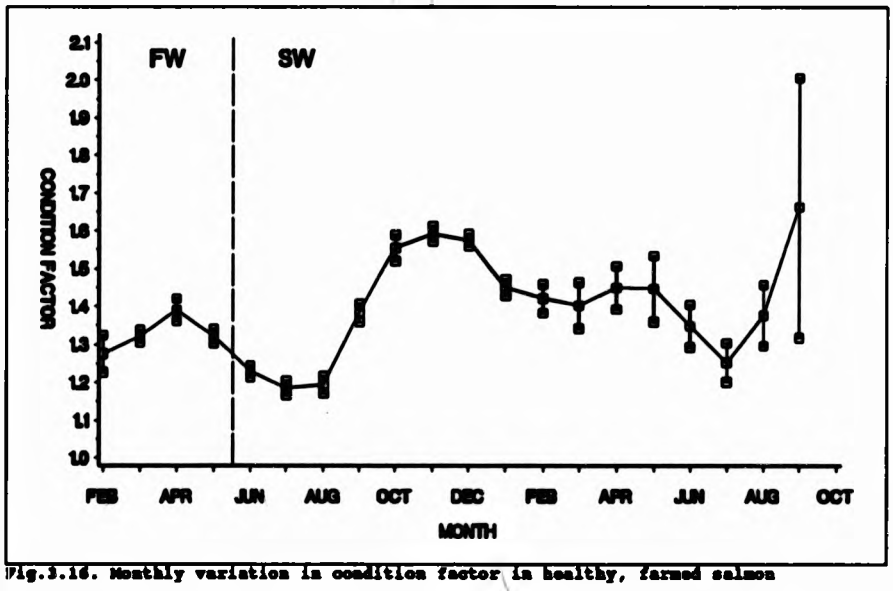

Fig.3.16. Monthly variation in condition factor in healthy, farmed salmon

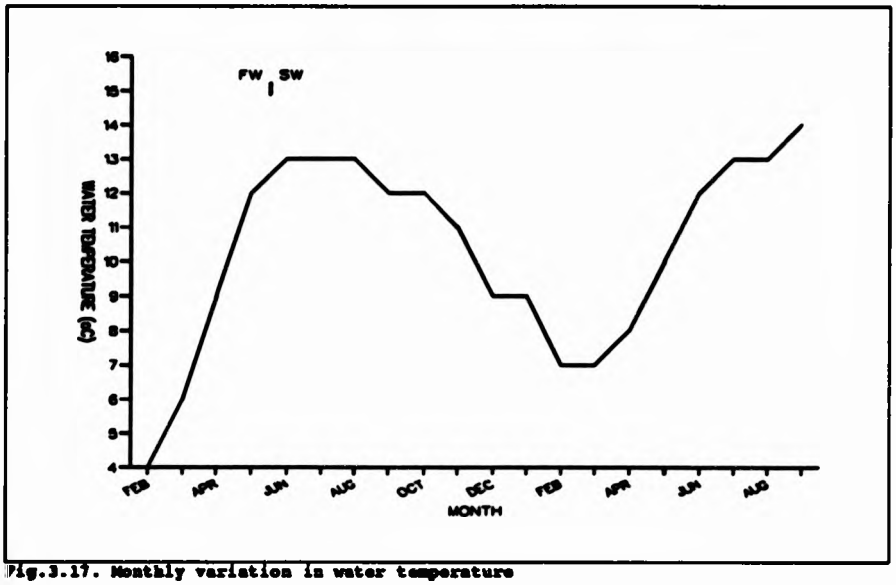

Fig.3.17. Monthly variation in water temperature (Pudelkiewicz and Mary 1969).

In the present study, liver vitamin E concentrations in post-smolts in seawater increased significantly between two and six weeks after transfer to seawater and between two and fifteen months post-transfer ranged from 268 to 754 μg g^{-1}. Plasma vitamin E concentrations remained at levels close to those found in freshwater until mid-October, 18 weeks post-transfer. At this time they increased and between October 1988 and August 1989 ranged from 34 to 68 μg ml^{-1}. As in common carp (Mézes 1986), there was no evidence of any cyclic seasonal variation. Bell *et al.* (1987*b*) measured α-tocopherol concentrations of 43±8 μg ml^{-1} in plasma and 316±66 μg g^{-1} in the liver of healthy, farmed salmon, aged one sea-year, fed a commercial diet the vitamin E content of which not stated. Ferguson *et al.* (1986*b*) found concentrations equivalent to 29 μg ml^{-1} in plasma and 281 μg g^{-1} in liver of post-smolts, mean weight approximately 900 g, fed a commercial diet with a measured vitamin E content of 9.8 mg 100 g^{-1}. Thus the results of the present study seem to be within a realistic normal range for fish fed diets containing approximately 20 mg vitamin E 100 g^{-1}.

Sharp increases, above the general rising trend, were found in α-

tocopherol concentrations in both the plasma and the liver during the first sea-summer. These give rise to two statistically distinct sub-groupings (see figs. 3.1 and 3.3). In liver there was a doubling of the vitamin E concentration between the first and second months in seawater, but in plasma a similar increase did not occur until between the fourth and fifth months in the sea. Since dietary vitamin E concentrations were slightly lower in seawater than in freshwater, these increases cannot be attributed to differences in the vitamin E content of the feed. An increase in feeding rate after transfer to seawater could lead to the accumulation of vitamin E in the tissues, but it is difficult to explain, on this basis, why these sharp increases occurred at different times in plasma and liver.

Plasma total lipid and polyunsaturated fatty acid concentrations were highly variable during the seawater phase. That this was largely a result of changes in the total lipid concentration is evident from the PUFA:lipid ratio which remained at between 0.17 and 0.24. Interestingly, the largest shift in the PUFA:lipid ratio occurred between September and October 1988, precisely the time of the jump in plasma vitamin E concentrations described above. A similar response was observed in the liver where the PUFA:lipid ratio rose sharply between May and June, just prior to the increase in liver vitamin E. Sheridan *et al.* (1985) found increases in the proportion of long-chain polyunsaturated fatty acids in the tissues of steelhead trout, *Oncorhynchus mykiss*, in anticipation of entry to seawater. Similar differences in the tissue fatty acid profile in the freshwater and marine stages of migratory fish species are well known (Lovern 1938; Lall and Bishop 1979; Halver 1989). Schulz *et al.* (1984) measured higher tocopherol concentrations in the liver and spleen of marine fish than in freshwater species and related this to differences in lipid composition. However, there appear to be no reports of both tissue PUFA:lipid ratios and vitamin E concentrations in migratory salmonids entering seawater, so further studies will be necessary to substantiate the apparent correlation described here.

Liver total lipid concentrations showed a rapid fall between February and July 1988, coinciding with the period of smoltification and seawater transfer. Sheridan *et al.* (1983) showed that in steelhead trout such changes resulted from triacylglyceride catabolism associated with the parr-smolt

transformation. In the present study, this coincided with a reduction in condition factor, typical of the metamorphosis between parr and smolt (Hoar 1976).

Plasma PUFA:vitamin E ratios ranged between 33 and 174 and liver PUFA:vitamin E between 12 and 100. These are well within the critical proportions suggested by Cowey *et al.* (1981) for rainbow trout in freshwater (section 1.2.5.3). The PUFA:vitamin E ratio in the liver was consistently lower than in the plasma, possibly reflecting hepatic storage of vitamin E. Highest ratios were recorded in both tissues soon after transfer to seawater. This suggests that the risk of vitamin E deficiency may be increased at this time.

3.3.2 Tissue vitamin E concentrations in mature, wild salmon

Individual tissue vitamin E concentrations together with the age and sex of each wild fish examined are given in table 3.10. The mean plasma vitamin E concentration was 63±20 μg ml^{-1}, and the mean liver concentration 84±54 μg g^{-1}. No significant differences in plasma vitamin E or liver vitamin E concentrations were detected between males and females, although the mean values were again higher in the male than the female fish (table 3.11). No significant correlation was detected between plasma and liver vitamin E concentrations in individual fish. Ushikusa *et al.* (1987) found that mature female carp had significantly higher gonad α-tocopherol concentrations than males. Gonad vitamin E concentrations were not measured in the present study.

Poppe *et al.* (1985*c*) measured serum vitamin E concentrations of 42±14 μg ml^{-1} in wild fish and 22±12 μg ml^{-1} in farmed fish weighing 0.4 to 4.6 kg, and found these to be significantly different. In the present study wild fish showed plasma concentrations of 63±20 μg ml^{-1}, whilst in farmed fish a comparable range was 34 to 68 μg ml^{-1}. Vitamin E concentrations measured in the tissues of wild salmon in the present study were therefore similar to the highest concentrations measured in farmed salmon aged 1 sea-winter and over. Since all wild fish examined were at least this age there is no evidence to support the suggestion by Poppe *et al.* (1985*c*) that farmed salmon have lower tissue vitamin E concentrations than their wild counterparts. However, in the

Table 3.10 Age, sex and tissue vitamin E concentrations in mature, wild salmon

Fish no.	Age	Sex	Plasma vitamin E ($\mu g\ ml^{-1}$)	Liver vitamin E ($\mu g\ g^{-1}$)
1	221	M	103	180
2	?21	M	65	112
3	221	F	41	102
4	?21	F	62	62
5	221	M	37	108
6	?21	M	68	6
7	221	F	56	90
8	?21	F	65	30
9	211	M	83	130
10	321	F	48	24

present study the wild fish had been held without food for six weeks, so may have undergone some loss of vitamin E since feeding in the sea.

Table 3.11 Plasma and liver vitamin E concentrations in male and female, mature, wild salmon (mean±sd)

	n	Plasma vitamin E ($\mu g\ ml^{-1}$)	Liver vitamin E ($\mu g\ g^{-1}$)
Male	5	71±24	107±63
Female	5	54±10	62±35

3.3.3 The effect of ration size on plasma vitamin E and cortisol concentrations

Mean plasma vitamin E concentrations in each experimental group at 0, 3 and 6 weeks are shown in table 3.12 and fig.3.18. These ranged between 24±19 $\mu g\ ml^{-1}$ at 3 weeks in fish fed the low ration and 76±21 $\mu g\ ml^{-1}$ at 6 weeks in fish fed the high ration. No transformation was necessary prior to analysis of variance and no differences could be detected between treatments. However, differences between groups of fish collected at different times were significant ($p<0.01$).

Table 3.12 Plasma vitamin E concentrations in fish fed different ration sizes (mean±sd)

Time (weeks)	Treatment	n	Plasma vitamin E ($\mu g\ ml^{-1}$)
0	1	5	56±19
	2	5	32±8
	3	5	52±21
3	1	5	35±21
	2	5	40±20
	3	5	24±19
6	1	5	51±22
	2	5	76±21
	3	5	57±27

Table 3.13. Plasma cortisol concentrations in fish fed different ration sizes (mean±sd)

Time (weeks)	Treatment	n	Plasma cortisol ($ng\ ml^{-1}$)
0	1	5	0.85±0.34
	2	5	0.84±0.27
	3	5	0.69±0.30
3	1	5	9.85±10.51
	2	5	2.88±4.61
	3	5	0.78±0.40
6	1	5	1.98±0.41
	2	5	0.61±0.10
	3	5	1.14±0.67

groups, but measured in different fish, are shown in table 3.13. Cortisol concentrations ranged between 0.61±0.10 and 2.88±4.61 ng ml^{-1}, except at 3 weeks in fish fed the medium ration where a mean of 9.85±10.51 was measured (fig.3.19). These data could not be normalised by any of the transformations described in section 2.5. Kruskal-Wallis tests showed significant differences in plasma cortisol concentrations between the treatments at both 3 and 6 weeks. Multiple comparisons were not performed, but plasma cortisol

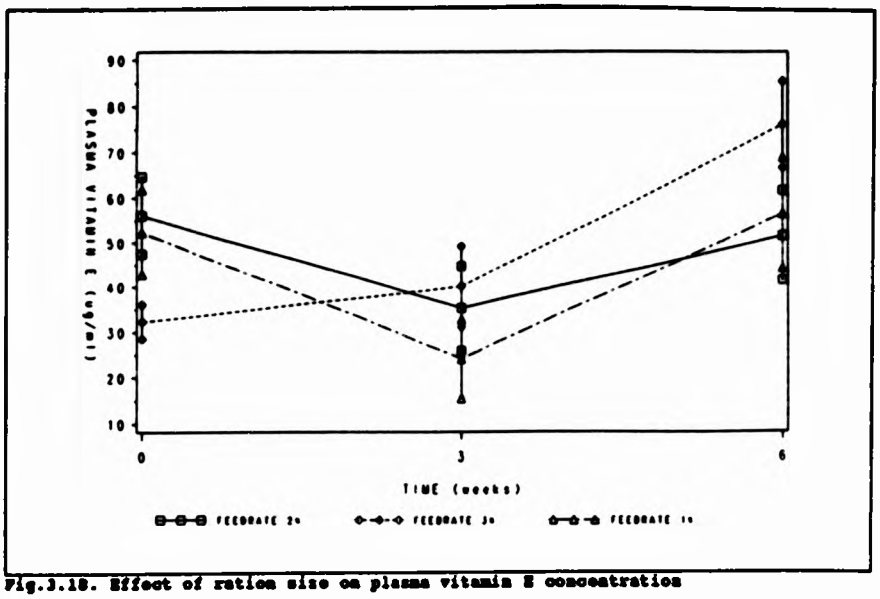

Fig.3.18. Effect of ration size on plasma vitamin E concentrations

concentrations in each of these time strata were highest in fish fed at 2% body weight. Mean plasma vitamin E and cortisol concentrations showed no significant correlation.

Weight and condition factor data are given in table 3.14. No transformation was necessary for any of these measurements prior to analysis of variance. No treatment or treatment-time differences were detected but significant changes with time in both weight ($p<0.001$) and condition factor ($p<0.01$) were found.

The results of correlation analyses showed no significant association between plasma vitamin E and weight or condition factor using Pearson's test, or between plasma cortisol and fish weight or condition factor using Spearman's test.

The different experimental feeding rates used in this study had no effect on plasma vitamin E concentrations which stayed within the normal range over the 6 week experimental period. Highest plasma cortisol concentrations were measured in fish fed the control diet (2% body weight day^{-1}). This may reflect social interactions among fish in this tank and illustrates the need for duplication of treatments. No significant correlation between vitamin E

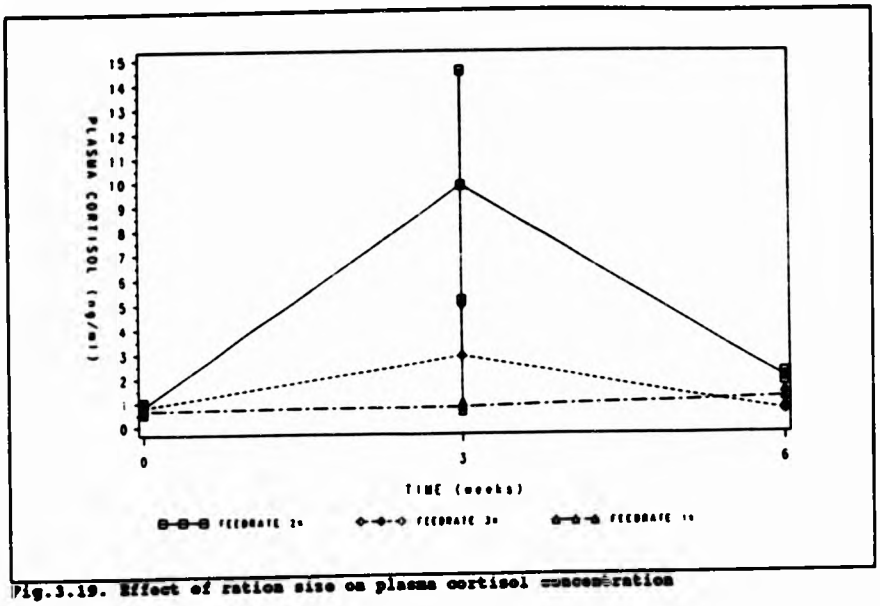

Fig.3.19. Effect of ration size on plasma cortisol concentration

and cortisol concentrations was detected. Austreng *et al.* (1987) noted that underfeeding may provoke a stress response as a result of aggression in relation to competition for food, whilst overfeeding may have similar effects due to impaired water quality.

Table 3.16. Weight and condition factor in one sea-winter salmon during a period of starvation (mean±sd)

Starvation time (weeks)	n	Weight (g)	Condition factor
1	5	3079±335	1.37±0.10
2	5	3053±414	1.43±0.09
3	5	3204±184	1.43±0.12
4	5	2762±257	1.45±0.13

3.3.4 The effect of starvation on tissue vitamin E concentrations

Mean plasma and liver vitamin E concentrations measured in one sea-winter fish after 1,2,3 and 4 weeks without food are shown in table 3.15

Table 3.14 Weight and condition factor of fish in feeding rate experiment (mean±sd)

Time (weeks)	Treatment	n	Weight (g)	Condition factor
0	1	10	288±37	1.38±0.09
	2	10	272±38	1.31±0.08
	3	10	295±38	1.31±0.09
3	1	10	320±61	1.39±0.07
	2	10	358±29	1.43±0.06
	3	10	346±50	1.35±0.06
6	1	10	428±78	1.38±0.10
	2	10	408±60	1.40±0.04
	3	10	384±74	1.38±0.05

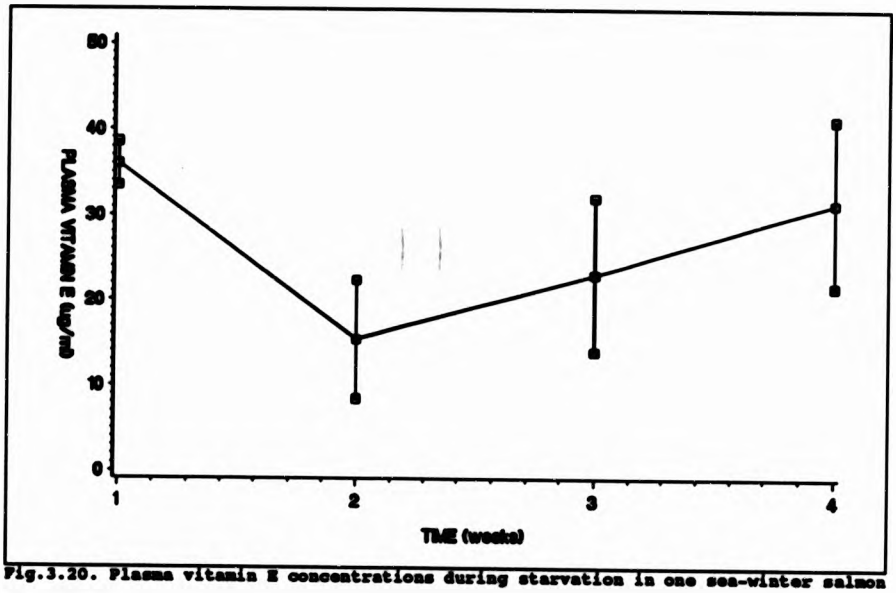

Fig.3.20. Plasma vitamin E concentrations during starvation in one sea-winter salmon

and figs. 3.20 and 3.21. Plasma vitamin E concentrations ranged from 15±16 to 36±6 µg ml^{-1}, and liver vitamin E concentrations from 147±30 to 263±216 µg g^{-1}. Neither showed any clear reduction during the course of the study. Spearman's rank correlation coefficients between both plasma and liver vitamin E concentrations and time were non-significant, and no differences between the

Table 3.15 Plasma and liver vitamin E concentrations in one sea-winter salmon during a period of starvation (measured)

Starvation time (weeks)	n	Plasma vitamin E ($\mu g\ ml^{-1}$)	Liver vitamin E ($\mu g\ g^{-1}$)
1	5	36±6	199±76
2	5	15±16	263±216
3	5	23±20	147±30
4	5	31±22	175±125

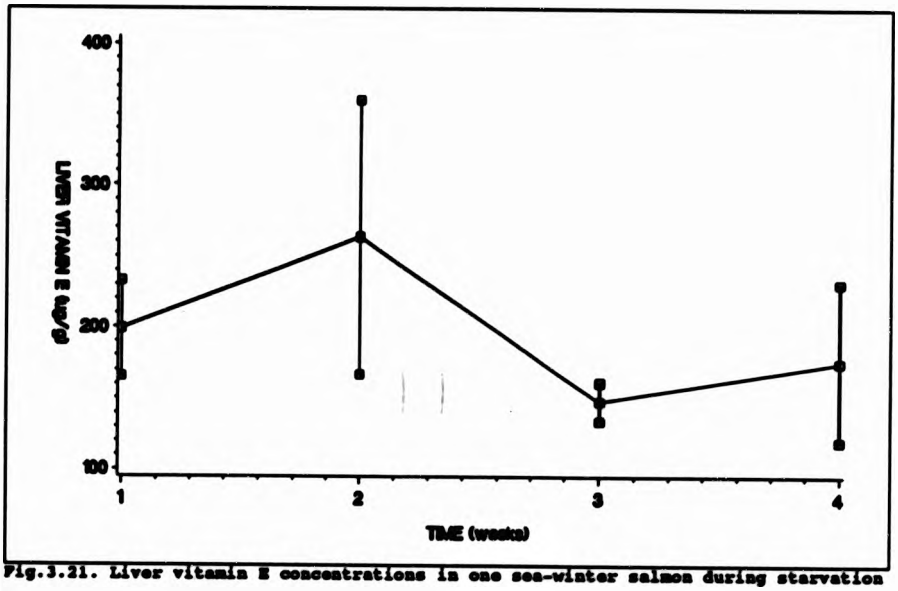

Fig.3.21. Liver vitamin E concentrations in one sea-winter salmon during starvation

median values at each time were detected using the Kruskal-Wallis test. No significant correlation was found between plasma and liver vitamin E concentrations in this study.

Weight and condition factor data are given in table 3.16. In this study, no significant correlations were found between plasma and liver vitamin E concentrations and either weight or condition factor using Spearman's test. In one sea-winter fish, starvation for up to four weeks had no significant effect on plasma or liver α-tocopherol concentrations which remained within the normal range for 1 sea-winter fish. Frigg *et al.* (1990) reported that the

oxidative stability of trout fillets was markedly affected by the α-tocopherol content of the flesh. Provided that muscle α-tocopherol concentrations behave similarly to plasma and liver concentrations, there is no reason to suppose that starvation for 1 month prior to harvest will impair the storage properties of the fish flesh as a result of vitamin E depletion.

CHAPTER 4

FIELD INVESTIGATIONS INTO THE ROLE OF VITAMIN E IN PANCREAS DISEASE OF FARMED ATLANTIC SALMON

4.1 INTRODUCTION

The primary purpose of the present study was to examine the role of vitamin E in PD. However, as is evident from the review presented in section 1.1, there are indications that exocrine pancreatic degeneration in PD may be caused by an infectious agent. Development of a transmission model, as described in section 1.1.4.3, may show to what extent this agent is involved in the clinical expression of PD. Successful transmission does not necessarily exclude vitamin E deficiency as a proximal cause of EPD, but experimental studies have so far failed to demonstrate pancreatic necrosis induced by vitamin E and/or any interrelated nutritional deficiency in Atlantic salmon (Poston *et al.* 1976; Bell *et al.* 1987*a*; Lall *et al.* 1988; Bell, McVicar, Mitchell and Cowey, unpublished). In contrast, muscle degeneration is commonly, though not exclusively, a feature of vitamin E deficiency in Atlantic salmon (section 1.2.5.2).

4.1.1 Vitamin E depletion in pancreas disease

A link between vitamin E nutrition and pancreas disease was first demonstrated by Ferguson *et al.* (1986*b*). These workers measured α-tocopherol concentrations in the plasma and liver of fish with clinical signs of PD and found them to be significantly lower than in apparently healthy fish. This observation was confirmed in a second case by Bell *et al.* (1987*b*) and extended to include white muscle. The first aim of the present study was to determine to what extent vitamin E depletion may be regarded as a consistent finding in PD. To this end, plasma samples were collected for vitamin E analysis from affected fish during 10 naturally occurring outbreaks of PD. In addition, for comparative purposes, vitamin E determinations were conducted on plasma samples from fish with experimentally-induced EPD.

The cause of vitamin E depletion in PD is not known. Analysis of feed from an affected site showed no evidence of unusually low levels of vitamin E or selenium (Ferguson *et al.* 1986*b*), and although fatty acid analysis showed that the diet contained 14 to 15% lipid, of which more than 50% was polyunsaturated (C18:2 and above), peroxide values were low. This suggested

that vitamin E depletion was not a result of dietary lipid oxidization. Liver fatty acid profiles were similar in affected and unaffected groups suggesting that high tissue concentrations of polyunsaturated fatty acids were not responsible for vitamin E depletion.

Bell *et al.* (1987*b*) suggested that vitamin E depletion could be explained by anorexia in the affected fish, but Ferguson *et al.* (1986*b*) rejected this explanation since a group of clinically normal, feeding animals, with histopathological signs of PD, had significantly lower mean tissue vitamin E concentrations than a healthy control group. In order to gather further information on the relationship between food intake, vitamin E and pancreas disease, records were kept of the presence or absence of food in the guts of individual fish collected during case studies.

Pancreas degeneration in chicks has been shown to impair vitamin E absorption (Thompson and Scott 1970) and pancreatic fibrocystis in man may also lead to reduced tissue α-tocopherol concentrations as a result of malabsorption (Gordon *et al.* 1958; Farrell *et al.* 1977). Similarly, exocrine pancreatic degeneration in PD may inhibit vitamin E absorption and lead to, or exacerbate existing, deficiency states. In the present study, this hypothesis was tested by comparing tissue vitamin E concentrations in fish with and without EPD, regardless of any overt PD. Thompson and Scott (1970) suggested that reduced pancreatic lipase production was responsible for impaired vitamin E uptake. However, Ferguson *et al.* (1986*b*) reported no change in liver lipids suggesting that lipid digestion was not impaired. In the present study, plasma lipid concentrations were measured so as to determine whether apparent changes in vitamin E simply reflected alterations in lipid status. In the light of these results, possible causes of vitamin E depletion were considered.

From preliminary studies, it became clear that in order to more fully understand the nutritional changes associated with PD it would be necessary to collect material during the early stages of disease. This was difficult when samples were collected following veterinary diagnosis since at that stage the outbreak was usually well-advanced. Consequently, a detailed study was conducted on a site with a history of PD, monthly samples being collected from the time of transfer to seawater through to recovery from an outbreak of PD.

This material was used to study the time course of changes in tissue vitamin E and plasma lipid concentrations in relation to the development of exocrine pancreatic degeneration and the onset of overt PD.

4.1.2 Effects of vitamin E depletion in PD

Ferguson *et al.* (1986*a*) described degeneration of the cardiac, skeletal and oesophageal muscles in association with PD, and pointed to a resemblance with nutritional, toxic and exertional myopathies occurring in other farmed species. McVicar and Munro (1989) regarded muscle involvement as a secondary condition. In order to gauge the prevalence of PD-associated myopathy, histological sections from the heart and skeletal muscle of fish collected from each outbreak were examined. Since muscle degeneration has been described in experimental studies of vitamin E deficiency in Atlantic salmon (Poston *et al.* 1976; Lall *et al.* 1988; Bell, McVicar, Mitchell and Cowey, unpublished) (section 1.2.5.2), the role of vitamin E in the development of myopathy associated with PD is of interest. T.Poppe (personal communication 1991) found evidence of a correlation between the degree of tissue vitamin E depletion and the severity of cardiomyopathy in farmed Atlantic salmon in Norway. However, Bell *et al.* (1987*b*) observed that the tissue vitamin E concentrations measured in fish with PD, whilst reduced, were higher than those induced in otherwise healthy salmonids without the development of myopathy due to vitamin E deficiency. Whilst it is difficult to test categorically whether or not muscle degeneration in PD occurs as a result of vitamin E depletion, an attempt was made in the present study to correlate the occurrence of cardiomyopathy with tissue vitamin E concentrations in natural PD outbreaks.

4.1.3 Treatment of pancreas disease using vitamin E

Previous attempts to prevent or treat PD by dietary supplementation with *all-rac*-α-tocopheryl acetate have proved ineffective in reducing the incidence or severity of PD in Scotland (McVicar and Munro 1989). However, a critical evaluation of the effects of this practice has not been carried out. In particular, it is uncertain whether or not vitamin E supplementation has any

effect on the incidence of PD-associated myopathy or even tissue α-tocopherol concentrations in PD. Therefore, data was gathered from field studies to give some indication of the effects of high-level dietary α-tocopheryl acetate supplementation in PD.

Pancreatic, mucosal and intracellular esterases are believed to be responsible for the conversion of dietary α-tocopheryl acetate to the biologically active α-tocopherol in the gut and gut epithelium (section 1.2.9.2). Insufficient esterase activity as a result of pancreas degeneration may reduce the rate of hydrolysis and perhaps lead to the uptake of vitamin E as α-tocopheryl acetate. Indeed, in mammals, Nakamura *et al.* (1975) found that α-tocopheryl esters could be absorbed intact, whilst Newmark *et al.* (1975) demonstrated that the rate of hydrolysis of intra-muscularly administered α-tocopheryl acetate limited the bioavailability of vitamin E. Unavailability of the acetate form could explain the inability of diets containing large supplements of α-tocopheryl acetate to prevent apparent vitamin E deficiency signs in fish with PD, and could also account for the presence of such signs in fish with significant amount of vitamin E in their tissues. α-Tocopheryl acetate is used as a vitamin E supplement in commercial salmon diets since it is more stable than α-tocopherol. However, α-tocopherol may be expected to be more effectively utilised, provided it can be delivered without substantial loss. Two experiments were conducted to study the effects of α-tocopherol on fish with PD. The first involved injection of affected fish with α-tocopherol and the second incorporation of high levels of α-tocopherol into the diet of fish with PD. The effects of these treatments on plasma vitamin E concentrations, plasma muscle enzyme activities and histopathology were examined.

In homeotherms, the absorption of lipid-soluble forms of vitamin E, in association with lipid uptake, is dependent on pancreatic lipase (section 1.2.9.2). Water-miscible vitamin E preparations may be absorbed through different mechanisms and may correct vitamin E depletion in PD. Schmandke and Schmidt (1965) found that α-tocopherol was absorbed twice as efficiently from aqueous as from oily solutions. Water-miscible vitamin E compounds have been shown to increase tissue vitamin E concentrations, and to reduce creatinuria, in infants with malabsorption due to pancreatic fibrocystis (Witowsky *et al.*

1962; Harries and Muller 1971). Such products are now commercially available for use in animal feeds (Papas *et al.* 1989).

4.2 MATERIALS AND METHODS

Between June 1987 and October 1990, twelve natural outbreaks of PD were studied. Ten of these occurred at farms in the west of Scotland and two in County Galway, Eire. Samples were taken in accordance with the procedure described in section 2.1.1, with some minor inconsistencies, particularly during 1987 (cases A-D) when the methodology was being developed. Further details are recorded below in chronological order.

4.2.1 Case A (Site 1; Ardnamurchan, Argyll, Scotland)

This farm was visited on 9th June, 31st July, 9th September and 8th October 1987. At the first visit, five grilse were sampled, together with a total of six post-smolts, three from each of cages 6 and 9. At the second visit, three fish were collected from cage 6 and four from cage 9, plus two, one sea-winter fish from a cage which had suffered from PD the previous year. At the third and fourth visits, five fish were collected from each of cages 6 and 9. Material for histological studies was taken at each visit, but fish weights were not determined. Blood samples were collected without the use of anticoagulant and were frozen whole. Serum samples were discoloured by severe haemolysis. Vitamin E determinations were made on serum only. Comparisons between feeding and non-feeding fish were performed using the Mann-Whitney U-test.

4.2.2 Case B (Site 2; South Uist, Scotland)

Farm 2 was visited on 22nd July, 18th August, 25th September and 12th November 1987. In addition, samples collected by a company employee on 20th July were also made available: these comprised tissues for histology, livers, and blood collected in tubes containing potassium-EDTA anticoagulant, from nine fish from cage D8 and two from cage E13. At the first visit, 10 fish from

cage D8 and five from cage D9 were collected and killed with an overdose of anaesthetic. At the second, five fish were collected from cage D6, six from D8 and seven from cage F2 on a second site (2a), located in a different sea-loch several miles south, and with no history of PD. At the third visit, six fish from D8, five from D10, four from D5 and one from D1 were taken, and at the final visit three fish from each of cages D8 and D10 were collected. Material for histology was taken at each visit and blood was collected without the use of anticoagulant and frozen whole. Fish weights were measured only on 18th August, 25th September and 12th November. Again, serum samples were discoloured by severe haemolysis. Vitamin E determinations were made on serum samples and also on a number of feed samples collected on August 18th. The Mann-Whitney U-test was used to compare serum vitamin E concentrations in fish with and without exocrine pancreatic degeneration, and in feeding and non-feeding animals. Spearman's rank correlation method was used to test for association between serum vitamin E concentrations and weight and condition factor data where available.

4.2.3 Case C (Site 3; Lorn, Argyll, Scotland).

This site was visited twice in 1987, on 16th September and 14th October, and on both dates five fish from each of cages 6 and 12 were collected. Material for histology was taken and unheparinized blood was collected and frozen whole. Fish weights were measured only on the second visit. Serum samples again suffered haemolysis. Vitamin E determinations were made on serum and groups of feeding and non-feeding fish were compared as described above. Spearman's rank correlation method was again used to test for a relationship between serum vitamin E concentrations and weight and condition factor.

4.2.4 Case D (Site 4; Lorn, Argyll, Scotland).

This site was visited only once in 1987, on 30th November. Six fish were collected from cage 5 and three from cage 6. Histological samples were collected and, again, unheparinized blood was frozen whole and serum was discoloured by haemolysis. Vitamin E determinations were made only on serum

samples. Statistical analyses were as for case C.

In 1988, 1989 and 1990 the uniform sampling practice described in section 2.1.1 was adhered to more strictly. Fish were normally brought ashore before blood collection and dissection.

4.2.5 Case E (Site 3; Lorn, Argyll, Scotland).

In 1988, a series of samples was collected from site 3 to show the time course of changes in tissue vitamin E concentrations during the development of PD, to study the relationship between plasma vitamin E and plasma lipid concentrations, and to examine in more detail the relationship between vitamin E depletion and pancreatic degeneration. Site 3 was selected for these studies because it had a previous history of PD uncomplicated by other diseases.

Sample collection began soon after the fish had been transferred to seawater and continued at monthly intervals until the fish developed gross signs of PD. The site was visited ten times between May 1988 and February 1989: the precise dates, cages and numbers of fish collected are shown in table 4.1. Plasma and liver vitamin E concentrations were measured according to the procedure described in section 2.2.1 and plasma lipid concentrations were measured as described in section 2.2.6. Histological samples were prepared and examined as described in section 2.4.

The statistical relationship with time for each set of biochemical data was examined using Spearman's rank correlation method. Tests for normal distribution were conducted, appropriate transformations for parametric analyses were selected and analyses of variance were carried out in order to test for differences between the means for each month. Subsequently, pair-wise multiple comparisons were made using the GT2 method. Comparisons between feeding and non-feeding fish, and individuals with and without pancreatic necrosis, were also made using analysis of variance. Pearson's method of correlation was used to test for association between pairs of variables. Spearman's correlation test and analysis of variance and multiple comparison procedures were used to compare different cages on the affected site and to compare affected and unaffected farms. Further details of these procedures are

Table 4.1. Sampling schedule for case study E

Date	Cage	No.fish
31.5.88	10	5
	13	5
1.7.88	10	5
	11	5
2.8.88	10	5
	13	5
1.9.88	10	5
	13	5
30.9.88	10	5
	13	5
31.10.88	1	5
	8	5
	11	5
1.12.88	8	5
	10	5
	11	5
	13	5
30.12.88	10	5
	13	5
31.1.89	10	5
	13	5
28.2.89	10	5
	11	5

given in section 4.3.5.6.

4.2.6 Case F (Site 5; Argyll, Scotland).

Samples from this site were collected by a company employee at fortnightly intervals between 23rd June and 9th December 1988. Pooled samples of liver, muscle and pancreas-caeca were collected from four groups of fish fed diets with and without a citric acid supplement, each under two different feeding regimes. No analyses were performed on these tissues since no effect

of treatment was observed, but some case details were recorded.

4.2.7 Case G (Site 6; Co.Galway, Ireland).

This farm was visited on 9th August 1988. Fish were collected from four cages: 2 and 5 on site 6a and 3 and 6 on site 6b. Five fish were sampled from each cage and tissues samples were subsequently processed for histology. Blood, liver and pancreas-caeca were not collected in this instance due to the distance of the site from the laboratory.

4.2.8 Case H (Site 7; Lorn, Argyll, Scotland).

This site was visited six times between August and October 1988. All fish were collected from cage A on the site. The dates and numbers of fish collected are given in table 4.2. Plasma samples from each visit were analyzed for vitamin E, lipid and malondialdehyde content as described in section 2.2. In addition, plasma pyruvate kinase activities were determined in a number of samples collected on the first and second visits only. Tissues were processed for histology as before. Spearman's test for rank correlation was used to examine relationships between plasma vitamin E, lipid and malondialdehyde concentrations, fish weight and condition factor, and time. The data were transformed and tested for normal distribution: Pearson's or Spearman's methods were used to test for association between variables and analysis of variance or the Mann-Whitney U-test was used to compare feeding and non-feeding fish. Comparisons between individuals with and without pancreatic necrosis were made using the Mann-Whitney test.

4.2.9 Case I (Site 8; Lorn, Argyll, Scotland).

This site was visited five times. Dates, cages and numbers of fish collected are shown in table 4.3. Tissues collected at each visit were processed for histology and plasma vitamin E concentrations measured in all fish. In addition, plasma pyruvate kinase activities were determined in samples collected at the second visit only. Statistical analyses were similar

Table 4.2. Sampling schedule for case study H

Date	No.fish
31.8.88	11
7.9.88	10
13.9.88	10
20.9.88	10
27.9.88	10
18.10.88	6

to those used in case H.

Table 4.3. Sampling schedule for case study I

Date	Cage	No.fish
14.10.88	8	5
	10	5
20.10.88	9	10
28.10.88	10	10
4.11.88	10	10
6.12.88	10	10

During the series of visits to this farm, a field experiment was conducted to examine the effect of injected vitamin E on the course of PD in individual fish. A preliminary experiment confirmed that it was feasible to administer vitamin E in molten cocoa-butter to healthy fish by parenteral injection. The results of this trial, shown in table 4.4, indicated that a dose of 1 ml, 0.5 g ml^{-1} *all-rac*-α-tocopherol in cocoa butter provided an increase in plasma vitamin E measurable after 1 week. Thus the method was applied to fish suffering from PD.

In October 1988, soon after PD was suspected on site 8, forty five fish were captured from an affected cage (cage 10). These were anaesthetized in 2-phenoxyethanol (0.5 ml l^{-1}) and individually marked with a Panjet and dye. Fifteen fish, selected at random, were given 1 ml *all-rac*-α-tocopherol (Sigma Chemical Co.) in molten cocoa-butter (0.5 g ml^{-1}) by intra-peritoneal

Table 4.4. Plasma vitamin E concentrations resulting from cocoa butter implants containing different quantities of vitamin E (mean±sd)

Implant vitamin E ($g\ ml^{-1}$)	n	Plasma vitamin E ($\mu g\ ml^{-1}$)
0	3	6±5
0.25	4	8±6
0.5	4	22±5

injection. Further groups of fifteen fish served as sham (cocoa-butter) injected and uninjected controls. These fish were kept separately in a small cage for 15 days and, at the end of this period, blood samples and tissues for histology were collected from each individual. Plasma CPK, PK, protein and vitamin E concentrations were determined as described in section 2.2. Material for histology was prepared according to the methods described in section 2.4. Statistical comparisons between the treatments were made using analysis of variance. Correlation analyses were made using Pearson's method.

4.2.10 Case J (Site 9; Isle of Mull, Argyll, Scotland).

Farm 9 was visited on 7th and 19th September 1989. Fish from this outbreak were used in a study to determine the effects of high dietary levels of a non-esterified vitamin E source (α-tocopherol) on the vitamin E status and course of PD in affected fish. 1 kg *all-rac*-α-tocopherol (Roche Ltd., Herts.) was stirred into 60 kg stabilized capelin oil and mixed onto 1 tonne feed to give a final concentration of 100 mg added α-tocopherol 100 g^{-1} diet. This diet was offered to fish in cage 10 for the 12 day period between visits to this site. Fish in cage 9 were fed a standard 'high energy' diet. At the first visit, seven fish were collected from cage 9 and six from cage 10, and at the second, seven were taken from each of cages 9 and 10. On each occasion tissues were collected for histology and biochemical analyses. Vitamin E concentrations in plasma and feed, and plasma PK activities, were determined as described in section 2.2. Statistical comparisons between treatments were made using the Mann-Whitney U-test. Spearman's method was used for correlation analysis.

A bench test was conducted by J. Roberts (BP Nutrition Ltd.) to determine the stability of α-tocopherol when incorporated into fish feed as described above. 5 kg salmon grower pellets containing 20 mg 100 g^{-1} vitamin E as α-tocopheryl acetate were coated with normal fish oil, and a further 5 kg were coated with fish oil containing α-tocopherol at a concentration sufficient to give an additional 50 mg vitamin E 100 g^{-1} diet. Vitamin E determinations were made on both batches at 1, 2, 3 and 5 weeks. Changes in the dietary vitamin E content with time were examined using Spearman's test.

4.2.11 Case K (Site 4; Lorn, Argyll, Scotland).

Material was collected at this site on 10th and 23rd August 1990. Fish from this outbreak were used in a trial to evaluate the use of a water-miscible vitamin E source to supply vitamin E to fish with PD. The vitamin E source (Rovimix E 20W) was provided by Roche Ltd., Herts and contained 20 mg g^{-1} *all-rac*-α-tocopheryl acetate in a polyethyleneglycol polypropyleneglycol ether matrix. This supplement was mixed onto BP Mainstream fish food at 62.5 g 25 kg^{-1} or 50 mg vitamin E 100 g^{-1} food. The pellets were then coated with gelatine to provide water stability and the diet was offered to fish suffering from PD for a period of 12 days. Eight and ten fish, respectively, were collected from a single cage (cage 1) at the beginning and end of this period. Tissue samples were processed for histology and plasma and liver vitamin E concentrations were measured as described in section 2.2.1. The Mann-Whitney U-test was used to compare tissue vitamin E concentrations before and after treatment.

4.2.12 Case L (Site 10; Co. Galway, Ireland).

A number of plasma and liver samples collected on 8th October 1990, from fish suffering heavy mortalities during a PD outbreak on a farm in Ireland, were sent by H. Rodger (University College, Galway) for vitamin E analysis. These samples were taken from a single cage but included fish in different stages of PD.

4.2.13 Tissue vitamin E concentrations in experimentally-induced PD

Plasma samples from fish which had been subjected to experimental challenge with PD, as described in section 1.1.4.3, were made available by R. Raynard (DAFS, Aberdeen). Groups of fish were given parenteral injections of kidney material from fish with and without PD. These fish were then used in an experiment to examine the effects of the corticosteroid analogue, dexamethasone, on susceptibility to pancreatic necrosis (Raynard 1989, unpublished).

The experimental treatments were as follows:

1) cocoa butter only
2) dexamethasone plus cocoa butter
3) dexamethasone plus cocoa butter/ PD challenged
4) PD challenged

These fish were held in tanks for a period of 6 weeks and were not fed during this time. Groups of 5 fish from treatments 1 and 2, and 10 fish from treatments 3 and 4 were sacrificed at weekly intervals. Histological samples were examined from all fish by A. McVicar (DAFS, Aberdeen), and classified as PD positive or negative according to the occurrence of exocrine pancreatic necrosis. Vitamin E determinations were made on a total of 57 plasma samples from treatments 1, 3 and 4, collected between two and six weeks after challenge. Analysis of variance was used to compare plasma vitamin E concentrations from fish with and without signs of pancreatic degeneration, and in PD challenged and sham challenged groups.

4.3 RESULTS AND DISCUSSION

4.3.1 Case A (Site 1)

4.3.1.1 Case history

Site 1 had suffered an outbreak of PD during 1986. In 1987, smolts were received on 23rd April and 19th-27th May. They were fed diets containing 20

to 40 mg 100 g^{-1} supplementary vitamin E as *all-rac*-α-tocopheryl acetate. During the four month period in which this site was visited the fish grew from 17±1 cm to 28±3 cm in length. At the time of the first visit (9.6.87), there were no signs of PD in the smolt stock and, although some fish were in poor condition, the majority were feeding. At the second visit (31.7.87), the stock was suffering severely from sea-lice and furunculosis, problems which may have been related to high stocking densities and excessive handling. Several fish examined showed mucous gut contents and distended gall-bladders which indicated lack of feeding (Talbot and Higgins 1982). In addition, many fish, including some with food in their guts, showed low levels of peri-pancreatic fat. One sea-winter fish from the 1986 PD outbreak were held on the same site and a number still appeared to be emaciated. In 1987, PD was diagnosed by staff at Stirling University on 11th August and the gross signs were evident in both cages studied at the time of the third visit (9.9.87). However, the pathology was reported to be complicated by a concurrent outbreak of furunculosis. Stocking densities had been reduced just prior to the occurrence of PD and company employees felt that handling may have precipitated this outbreak. At the time of the fourth visit (8.10.87), furunculosis had been eliminated, but PD persisted in both cages studied.

4.3.1.2 Histopathology

Histological samples from a total of eleven fish were examined. No evidence of exocrine pancreatic necrosis was observed in any of three smolts examined from the first visit and two from the second. Two one sea-winter fish examined at the second visit were found to have pockets of pancreatic acinar tissue within areas of fibrotic tissue. Two fish examined from the third visit showed exocrine pancreatic degeneration (EPD) and thus the diagnosis of PD was confirmed. No samples from the fourth visit were examined. No clear evidence of cardiac or skeletal myopathy was found in any fish from this outbreak, either before or during the period of overt disease.

4.3.1.3 Vitamin E

Vitamin E concentrations measured in serum samples from case A are summarised in table 4.5. In smolts from cages 6 and 9 these did not exceed 12±8 μg ml^{-1} at any time during the study. In addition, low concentrations (10±9 μg ml^{-1}) were also measured in grilse collected at the first visit. Low values, particularly in apparently healthy fish, may have been due to loss of α-tocopherol as a result of haemolysis. On the three dates for which comparative data is available, fish from cage 6 had lower mean vitamin E concentrations than fish from cage 9, but, in both, the highest concentrations were measured in samples from the final visit. Serum vitamin E concentrations appeared to be lower in the two fish with confirmed exocrine pancreatic degeneration (2±2 μg ml^{-1}) than in three healthy smolts for which comparative data was available (7±2 μg ml^{-1}), but these data were insufficient to warrant statistical analysis. No significant difference in serum vitamin E concentrations was detected between fish with and without food particles in their guts.

4.3.2 Case B (Site 2)

4.3.2.1 Case history

This site had suffered from PD in 1985 when the outbreak was the subject of the study described by Ferguson *et al.* (1986*b*). Broodstock were held on the site in the interim period. In 1987, smolts were received between 24th April and 5th May and were fed diets containing up to 40 mg 100 g^{-1} added α-tocopheryl acetate. Throughout the summer of 1987 the stock suffered from a chronic sea-louse infestation which necessitated regular treatment and which, in turn, may have precipitated PD. PD was first recognised in a single cage of smolts (cage D8) on 19th September and the diagnosis was later confirmed by staff at Stirling University. At the time of the outbreak the affected cage contained approximately 6000 fish at a density of less than 10 kg m^{-3}. These fish were 'Conon' strain and the cage population had previously shown the best growth of all the cages in the group. During the study period they grew in

Table 6.5. Serum vitamin E concentrations in case A (measured)

Date	Cage	n	Serum vitamin E ($\mu g\ ml^{-1}$)
9.6.87	3 (smolts)	2	2±4
	(grilse)	5	10±9
31.7.87	6	3	2±4
	9	4	6±3
9.9.87	6	5	3±6
	9	5	8±7
8.10.87	6	5	5±4
	9	5	12±8

length from 22±2 cm to 34±2 cm. At the first visit (22.7.87), these fish showed typical behavioral signs of PD (ie. hanging listlessly in the corners of the cage, refusing food) and, in addition, peri-pancreatic fat deposits were depleted. In contrast, fish from cage D9 appeared healthy. At the second visit (18.8.87), a second cage of smolts (cage D6) was beginning to show behaviour typical of PD. These were 'Pool' strain and also showed reduced levels of peri-pancreatic fat. Interesting husbandry differences were recorded between site 2 and the nearby site 2a which was operated by the same company. Fish on site 2a were fed almost entirely by hand and at a relatively low rate compared to other sites. The average growth rate was low and fat reserves were minimal, but disease problems were less often encountered and PD had never been found. At the time of the third visit (25.9.87), signs of recovery were noted in cage D8, but several other cages (including D5 and D10) showed signs of PD. At this stage, healthy fish weighed approximately 500 g, whereas fish with PD averaged about 300 g. Healthy fish were seen to have orange coloured livers through feeding on pigmented diets but, although fed the same diet, the livers of fish with PD were dark brown in colour. At the fourth visit (12.11.87), all the fish were feeding, but some were in poor condition and signs of haemorrhage between the pyloric caeca were observed in fish from both cages examined (D8 and D10). The outbreak was less severe than in previous years and farm staff attributed this to the regulation of feeding rates early in seawater phase.

4.3.2.2 Histopathology

Histological samples from a total of ten fish from this outbreak were studied. Examination of four individuals confirmed that a severe exocrine pancreatic necrosis was already apparent in fish collected on 20th and 22nd July from cage D8, but there was no evidence of similar pathology in one fish examined from the healthy cage D9 at this time. Cardiomyopathy, similar to that described in association with PD by Ferguson *et al.* (1986a), was present in fish from the affected cage D8 on 20th and 22nd July 1987, but not in the healthy fish from cage D9. On 18th August, the pancreatic tissue of one fish examined from cage D6 and one from site 2b were both found to be intact, but both fish were considered to show signs of cardiomyopathy. On 25th September, one fish from the previously affected cage D8 was examined and found to be apparently healthy, but fish from cages D5 and D10 showed pancreatic degeneration and the individual from cage D10 also showed cardiomyopathy. No sections from 12th November were examined. The presence of cardiomyopathy in this outbreak is significant since it demonstrates the occurrence of such pathology in fish fed relatively high levels of α-tocopheryl acetate (see below).

4.3.2.3 Vitamin E

Vitamin E concentrations in feed samples collected during this study were 37.2±1.6 mg 100 g^{-1} at site 2, and 22.0 mg 100 g^{-1} at site 2a. Serum vitamin E concentrations measured in fish from case B are shown in table 4.6. Mean concentrations of less than 5 μg ml^{-1} were found in fish from the affected cage D8 in all but the final sample when a concentration of 15±19 μg ml^{-1} was measured. Concentrations of 8±10 μg ml^{-1} and below were also measured in the other affected cages (D5, D6 and D10) during the course of the study. Higher levels, measured in cage D10 at the final sample (21±16 μg ml^{-1}), coincided with recovery in this cage. A concentration of 33 μg ml^{-1} was measured in a single fish collected from the healthy cage D1, but samples from other healthy cages E13, D9 and F2 on site 2a did not exceed 8±4 μg ml^{-1}. Again haemolysis of blood prior to removing serum may have resulted in the

Table 4.6. Serum vitamin E concentrations in case B (mounted)

Date	Cage	n	Serum vitamin E ($\mu g\ ml^{-1}$)
20.7.87	D8	9	4±6
	E13	2	8±4
22.7.87	D9	4	8±4
	D8	10	4±4
18.8.87	D6	5	6±9
	D8	6	2±4
	F2 (Site 2a)	7	7±7
25.9.87	D8	6	4±4
	D10	5	4±4
	D5	4	8±10
	D1	1	33±0
12.11.87	D8	3	15±19
	D10	3	21±16

loss of vitamin E from the samples. Statistical comparisons using the Mann-Whitney U-test indicated that there was no significant difference in serum vitamin E concentrations between fish with or without exocrine pancreatic necrosis or cardiomyopathy. However, the same test applied to feeding and non-feeding fish showed significant differences, serum vitamin E concentrations being higher in fish with food in their guts ($p<0.05$). In addition, significant positive correlations were found between serum vitamin E and both weight ($p<0.05$) and condition factor ($p<0.01$).

4.3.3 Case C (Site 3)

4.3.3.1 Case history

This site had suffered from PD in 1984 and 1985, but not 1986. No broodstock were present on the site, but one and two sea-winter fish were held nearby. In 1987, the fish were transferred to sea at a weight of approximately 40 g and fed to appetite (ie. above recommended feeding rates) during their

first summer in seawater. They were held at 6500 fish cage^{-1} at a density of less than 10 kg m^{-3}. They too had a recurrent problem with sea-lice which required approximately monthly treatment. PD was diagnosed by staff at Stirling University in August and at the time of the first visit (16.9.87) signs of PD were present in all 14 cages of smolts on the site. In samples collected from cage numbers 6 and 12, most fish had food in their guts, but little peri-pancreatic fat was observed even in fish still accepting food. Signs of haemorrhage were occasionally evident between the pyloric caeca in fish from both cages. Mean fish weights were approximately 140 g at the first visit and 315±143 g at the second. At the time of the second visit (14.10.87), food was present in the gut of all fish examined from cages 6 and 12 and all cages appeared to be showing signs of recovery. Even sick fish were observed to eat high energy feed if sprinkled in the corners.

4.3.3.2 Histopathology

Histological examination of two fish collected at the second visit confirmed the occurrence of EPD in both cages studied, but a third individual collected from one of the affected cages (cage 6) showed intact pancreatic tissue. No evidence of muscle degeneration was found in any of these fish.

4.3.3.3 Vitamin E

Serum vitamin E concentrations were again found to be low, although again perhaps partly as a result of haemolysis. On 16th September, mean concentrations of 6±8 μg ml^{-1} in cage 6 and 7±11 μg ml^{-1} in cage 12 were measured, and on 14th October slightly higher values, 10±10 μg ml^{-1} in cage 6 and 13±9 μg ml^{-1} in cage 12, were recorded. Serum vitamin E concentrations were lower in the two fish with exocrine pancreatic necrosis than in the one individual with an intact pancreas, but insufficient data was available to warrant statistical comparison. No significant difference was found between serum vitamin E concentrations in fish with and without food particles in their alimentary tracts, but a significant positive correlation was shown between serum vitamin E concentrations and both fish weight ($p<0.01$) and

condition factor ($p<0.001$).

4.3.4 Case D (Site 4)

4.3.4.1 Case history

This site had no previous history of PD. In 1987, it received smolts in mid May and these were fed on commercial diets containing 20 mg 100 g^{-1} added α-tocopheryl acetate. PD was first noticed early in November and confirmed later in the month by staff at Stirling University. No behaviourial signs of the condition were evident at the time of the visit to this farm, on 30th November 1987, but fish from the affected cage 5 weighed 518±352 g and many had little or no peri-pancreatic fat, whilst healthy fish from cage 6 weighed 593±108 g and showed large amounts of gut-associated fat. This outbreak occurred in triploid stock.

4.3.4.2 Histopathology

Patches of exocrine pancreatic degeneration were obvious in the histological samples from two fish collected from cage 5, but pancreatic acini were still visible in some sections. No sign of heart muscle involvement was seen in either fish.

4.3.4.3 Vitamin E

Serum vitamin E concentrations of 18±21 μg ml^{-1} were measured in six fish from the affected cage 5, and 27±0 μg ml^{-1} in two fish from the healthy cage 6. Haemolysis may again have resulted in some loss of α-tocopherol from the samples. In the two individuals in which pancreatic degeneration was confirmed, serum vitamin E concentrations of 0 and 3 μg ml^{-1} were measured respectively. No significant difference in serum vitamin E was found between feeding and non-feeding fish and rank correlation coefficients between vitamin E and fish weight and condition factor were both non-significant.

4.3.5 Case E (Site 3)

4.3.5.1 Case history

During the first five monthly visits to case E, no gross signs of PD were noticed and the fish appeared to be growing and feeding well. Overt PD was first observed on 31st October 1988 in four adjacent cages on a raft containing only smolts. The affected fish were already thin when PD was first noticed, but at this stage only about 200 fish in each cage exhibited the characteristic listless, hanging behaviour. Of the cages studied at this visit, only cage 1 showed signs of overt PD. Internal examination showed that fish from cage 1 had little peri-pancreatic fat, few had any trace of food in their guts, and the hindgut blood vessels were again prominent. The case was confirmed by staff at Stirling University towards the end of November, but some fish were also identified as suffering from 'late transfer syndrome'. Larger numbers of fish showed listless, hanging behaviour at the time of the visit on 1st December, and almost all cages showed signs of PD, but to varying degrees. Of the cages studied on this date, 8 and 10 had relatively few fish hanging at the surface, whilst 11 and 13 had large numbers of such individuals. Traces of food were present in the guts of all but one of the fish collected, however, a number of fish from each cage were emaciated and had no visible peri-pancreatic fat. Farm workers confirmed that they had noticed a recent improvement in feeding activity and suggested that recovery may have been assisted by continuing to offer small pellets rather than increasing feed size as planned. The colour of the plasma collected during this visit showed a correlation with the gross indications of PD: healthy fish had bright orange plasma whilst that of sick individuals was pale in colour. A further improvement in feeding response was recorded on 30th December, but many cages still contained large numbers of fish hanging close to the surface and cage to cage differences were still very obvious. In particular, three out of four fish collected from cage 13 were emaciated and had no peri-pancreatic fat, whilst three out of four from cage 10 were in good condition and had large fat reserves. Size-wise grading was carried out in January due to the large variation in fish weights within individual cages. On 31st January and

28th February, food was observed in the guts of all fish examined. Hanging behaviour was not noted in the populations regularly sampled, but the two worst affected cages contained many small fish thought unlikely to recover.

Table 4.7. Weight and condition factor of fish from case E (mean±sd)

Date	n	Weight (g)	Condition factor
31.5.88	10	47±11	1.28±0.08
1.7.88	10	84±28	1.23±0.09
2.8.88	10	176±23	1.26±0.07
1.9.88	10	315±46	1.47±0.07
30.9.88	10	468±95	1.74±0.19
31.10.88	15	420±138	1.32±0.17
1.12.88	12	516±184	1.36±0.13
30.12.88	8	515±325	1.34±0.17
31.1.89	7	721±262	1.33±0.09
28.2.89	6	767±465	1.35±0.15

4.3.5.2 Changes in weight and condition factor

During the course of the study, fish on this site grew from 47±11 to 767±262 g. Over this period weight was positively correlated with time ($p<0.001$). Mean weights each month are shown in table 4.7 and fig. 4.1. Between May and September, mean fish weight increased by a factor of 10 from 47±11 to 468±95 g, but in October a weight loss, to 420±138 g, was recorded. Between October and December, during the period of overt PD, the mean weight rose to only 515±325 g, but larger monthly increases to 721±262 g in January, and 767±262 g in February, were measured during recovery.

Changes in condition factor are also shown in table 4.7 and illustrated in fig. 4.2. Again a significant positive correlation with time was found ($p<0.05$). In May, after transfer to seawater, a condition factor of 1.28±0.08 was recorded. Similar values were found during June and July. Between July and September this figure increased from 1.26±0.07 to 1.74±0.19, but it then fell sharply, to 1.32±0.17 in October, coinciding with the first signs of overt PD. Between October and December, during the period of overt disease, condition factors ranged from 1.32±0.17 to 1.36±0.13. There was little evidence of any

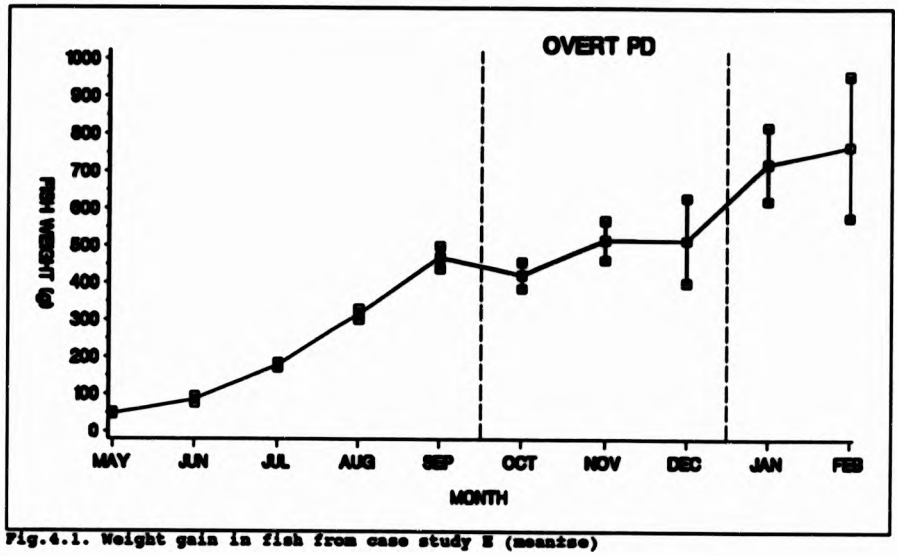

Fig.4.1. Weight gain in fish from case study E (meanise)

increase during January and February. Both weight and condition factor required to be log transformed for parametric statistical analysis.

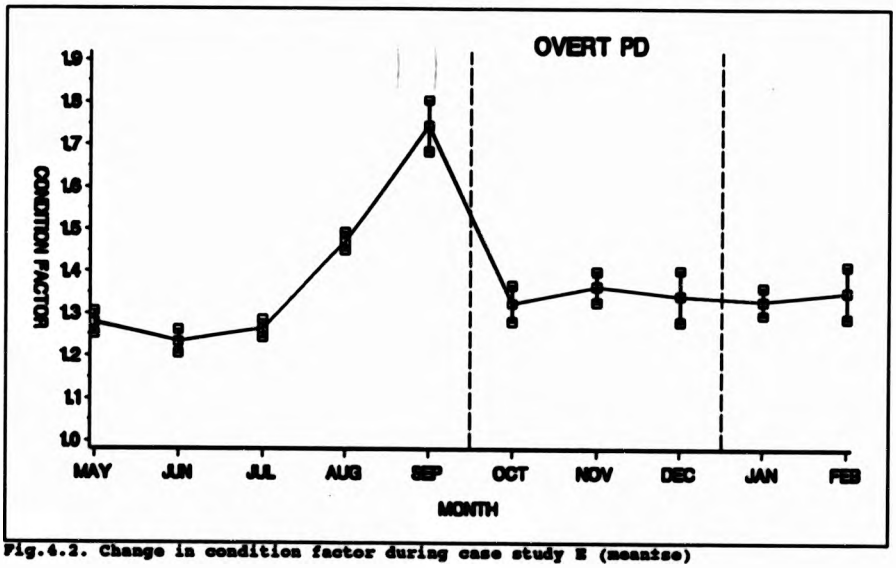

Fig.4.2. Change in condition factor during case study E (meanise)

Food particles were found in the intestinal tracts of eighty-five of the ninety-eight fish collected during the course of this study. The numbers and

proportions of feeding and non-feeding fish each month are shown in table 4.8 and fig 4.3. No difference in weight was found between fish which were or were not feeding, but a significant difference in condition factor was detected ($p<0.05$), feeding fish being in better condition.

Table 4.8. Numbers of non-feeding fish, and numbers of fish with exocrine pancreatic degeneration, from case E

Date	Total	No. feeding	Total	No. with EPD
31.5.88	10	8	10	0
1.7.88	10	10	10	0
2.8.88	10	10	10	0
1.9.88	10	10	10	0
30.9.88	10	8	6	4
31.10.88	15	11	15	11
1.12.88	12	11	12	7
30.12.88	8	7	8	6
31.1.89	7	7	7	5
28.2.89	6	6	6	4

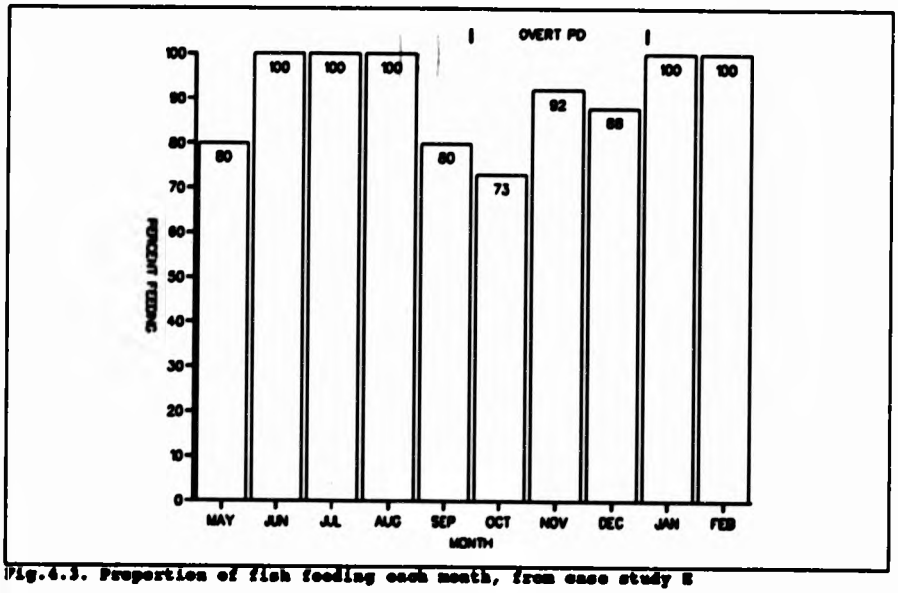

Fig.4.3. Proportion of fish feeding each month, from case study E

4.3.5.3 Histopathology

Histological material was examined from ninety-four fish collected during this series of samples. No evidence of exocrine pancreatic degeneration was observed in any of the forty fish collected between 31st May and 1st September. However, from material taken during the period 30th September to 28th February, it was clear that between 50 and 75% of fish examined each month did show a generalized pancreatic necrosis. These results are summarized in table 4.8 and in fig. 4.4. They support the suggestion of Munro *et al.* (1984) that exocrine pancreatic degeneration in PD develops rapidly. No evidence of cardiomyopathy was found in any of the histological material examined during the course of this study. Fish with pancreatic necrosis had significantly different mean weights to those without ($p<0.001$). Interestingly, fish with pancreatic degeneration were on average heavier. More rapidly growing fish have previously been suggested to be more vulnerable to PD (section 1.1.3.4). There was no difference in condition factor between these groups.

Of 36 individuals with EPD, only 9 had no food particles in their gut and 5 of these were collected in October when the first overt signs of PD were observed. Thus, in agreement with Munro *et al.* (1984), inappetance appears to be an early feature of the acute phase of the condition. Four fish, three of which were collected at the first sample, were not feeding, but were considered to have intact pancreatic tissue. Although such fish may have been affected to some degree, they did not show total exocrine pancreatic degeneration.

4.3.5.4 Vitamin E : time course study

Mean plasma vitamin E concentrations measured in fish collected each month during this study are shown in table 4.9 and illustrated in fig. 4.5. Plasma vitamin E concentrations increased from 20±29 $\mu g\ ml^{-1}$ in May 1988 to 65±23 $\mu g\ ml^{-1}$ in February 1989. Spearman's test indicated that over the course of the study they showed a significant positive correlation with time ($p<0.001$). In May and June, plasma vitamin E concentrations were 20±29 and

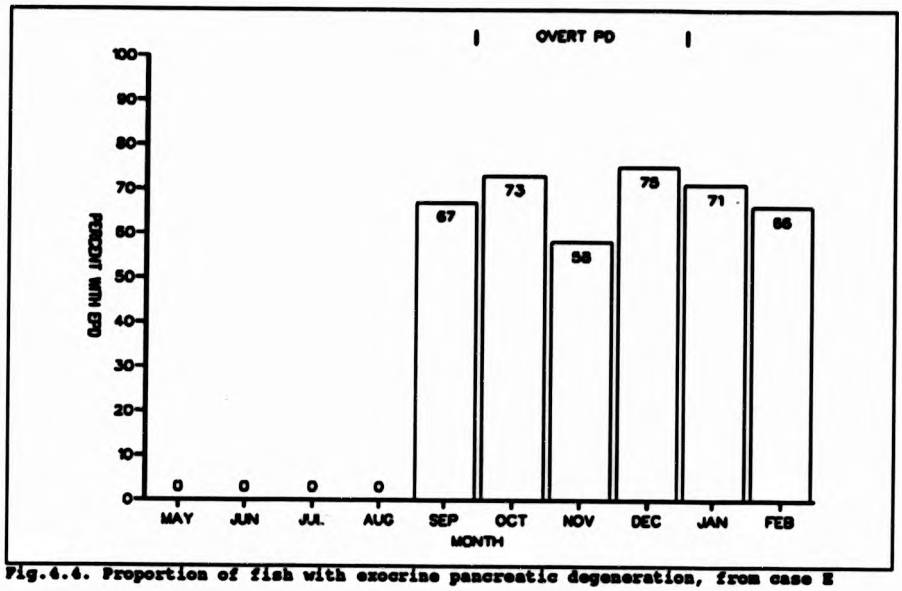

Fig.4.4. Proportion of fish with exocrine pancreatic degeneration, from case E

14±14 μg ml^{-1} respectively. In July they increased to 37±21 μg ml^{-1}, but between August and December they stayed relatively constant, ranging between 29±9 and 35±21 μg ml^{-1}. The largest increase, from 34±27 to 65±23 μg ml^{-1}, occurred between December and January, during recovery from PD. This was followed by a slight fall, to 52±29 μg ml^{-1}, in February. Analysis of variance of the square root transformed data confirmed that significant differences existed between the means at different times ($p<0.001$). Multiple pairwise comparisons, using the GT2 method, indicated that plasma vitamin E concentrations in May were significantly different from those in January, and that those in June differed from those in both January and February ($p<0.05$).

Liver vitamin E concentrations are also shown in table 4.9 and in fig. 4.6. Overall, liver vitamin E concentrations showed a reduction, from 284±128 μg g^{-1} in May to 180±118 μg g^{-1} in February. A significant negative relationship between liver vitamin E concentrations and time was found over the course of the study period ($p<0.001$). Mean liver vitamin E concentrations of 284±128 and 218±94 μg g^{-1} were recorded in May and June respectively. A maximum of 400±66 μg g^{-1} was recorded in July, but the mean fell sharply thereafter, reaching 118±155 μg g^{-1} in October. Subsequently, liver vitamin E

Table 4.9. Plasma and liver vitamin E concentrations in fish from case E (mean±sd)

Date	n	Plasma vitamin E ($\mu g\ ml^{-1}$)	n	Liver vitamin E ($\mu g\ g^{-1}$)
31.5.88	6	20±29	6	284±128
1.7.88	8	14±14	6	218±94
2.8.88	10	37±21	6	400±66
1.9.88	9	29±9	6	317±94
30.9.88	10	35±21	6	151±91
31.10.88	15	31±27	9	118±154
1.12.88	12	33±25	12	136±175
30.12.88	8	34±27	6	169±218
31.1.89	7	65±23	6	146±39
28.2.89	6	52±29	6	160±118

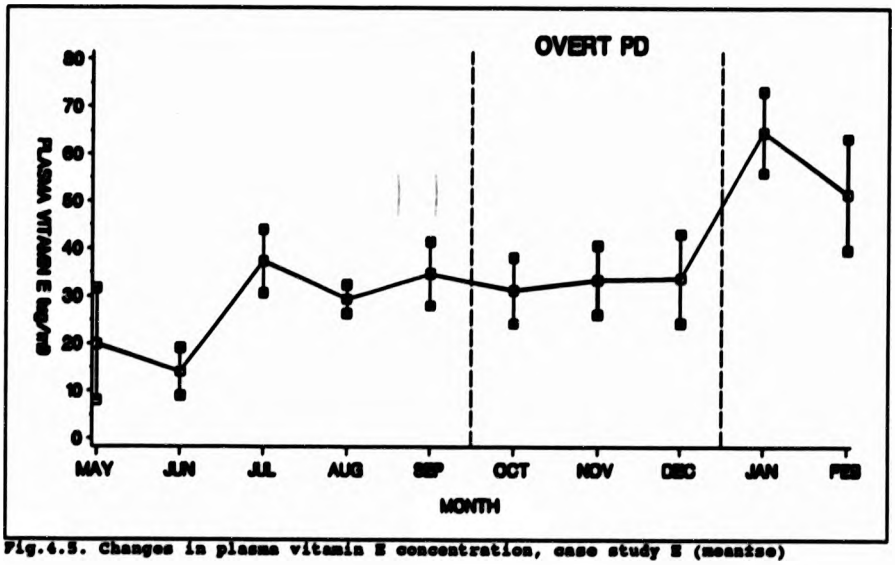

Fig.4.5. Changes in plasma vitamin E concentration, case study E (mean±sd)

concentrations remained low, increasing from 118±155 to only 169±218 $\mu g\ g^{-1}$ during the period of overt PD between October and December, and showing no signs of a sharp increase upon recovery. The data was subjected to square root transformation and analysis of variance showed that significant differences existed between the means at different times ($p<0.01$). The GT2 method of

multiple comparison indicated that significant differences occurred between liver vitamin E concentrations in July 1988 and those in October and November ($p<0.05$). Pearson's test for correlation revealed a significant positive relationship between plasma and liver vitamin E concentrations in this study ($p<0.01$). The timing of the onset of vitamin E depletion is difficult to determine. Multiple comparisons indicate that vitamin E depletion began sometime between July and October and examination of fig. 4.6 suggests that there was a steady loss of hepatic α-tocopherol from July (ie. prior to the onset of overt PD). However, this interpretation was not supported statistically since significant differences were not found between the July, August and September samples.

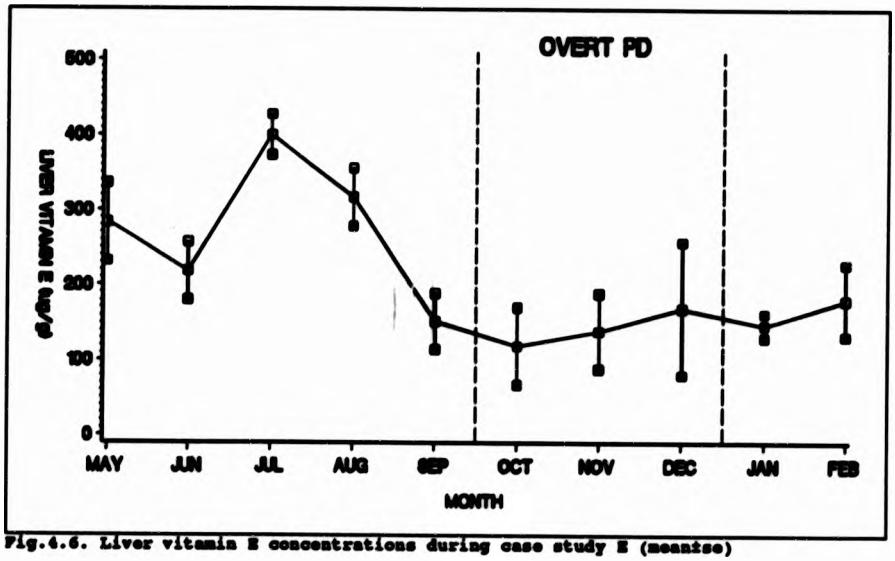

Fig.4.6. Liver vitamin E concentrations during case study E (meanise)

Pearson's correlation test also revealed a significant relationship between plasma vitamin E concentration and both fish weight and condition factor ($p<0.01$), but liver vitamin E concentration showed no association with either. No difference in plasma vitamin E concentration was found between feeding and non-feeding fish, but significant differences in liver vitamin E concentrations were detected ($p<0.05$), these being lower on average in non-feeding fish. No difference in plasma vitamin E concentration was detected between individuals with and without pancreatic acinar tissue, but once again

liver vitamin E concentrations were significantly different ($p<0.001$), being lower in fish with signs of pancreatic necrosis than in those without.

4.3.5.5 Plasma lipid concentrations

Plasma lipid concentrations increased, from 5±4 mg ml^{-1} in May to 20±6 mg ml^{-1} in February, and showed a positive correlation with time over this period ($p<0.001$). Between May and September, the mean concentration increased steadily from 5±4 to 18±6 mg ml^{-1}. It fell to 13±4 mg ml^{-1} in October and stayed close to this level during the period of overt PD, but increased during recovery, from 12±4 mg ml^{-1} in December to 20±3 mg ml^{-1} in January. These results are summarised in table 4.10 and illustrated in fig. 4.7. No transformation was necessary prior to analysis of variance which showed that significant differences existed between the mean plasma lipid concentrations at different times ($p<0.001$). Multiple pairwise comparisons showed differences between plasma lipid concentrations in May and those in August, September, November, January and February ($p<0.05$). Pearson's test for correlation showed a positive association between plasma lipid and vitamin E concentrations ($p<0.01$). Although plasma lipid concentrations appeared to show a more marked increase than vitamin E during the pre-acute phase, and a more obvious fall during the acute stage of the outbreak, both showed signs of recovery between December and January. A significant positive correlation was also detected between plasma lipid concentration and both fish weight and condition factor ($p<0.01$). No significant difference in mean plasma lipid concentration was found between groups of feeding and non-feeding fish or between individuals with and without exocrine pancreatic degeneration.

4.3.5.6 Cage differences

Of the two cages, 10 and 13, for which most data is available, gross signs of PD seemed more prevalent in cage 13. In histological samples, collected between September and February, exocrine pancreas degeneration was observed in 8 out of 16 fish (50%) from cage 10 and 11 out of 14 (79%) from cage 13. In order to investigate the nature of cage to cage differences in

Table 4.10 Plasma lipid concentrations in fish from case E (mean±sd)

Date	n	Plasma lipid (mg ml^{-1})
31.5.88	5	5±4
1.7.88	4	10±3
2.8.88	6	13±3
1.9.88	6	15±3
30.9.88	6	18±6
31.10.88	9	13±4
1.12.88	12	14±6
30.12.88	6	12±4
31.1.89	6	20±3
28.2.89	6	20±6

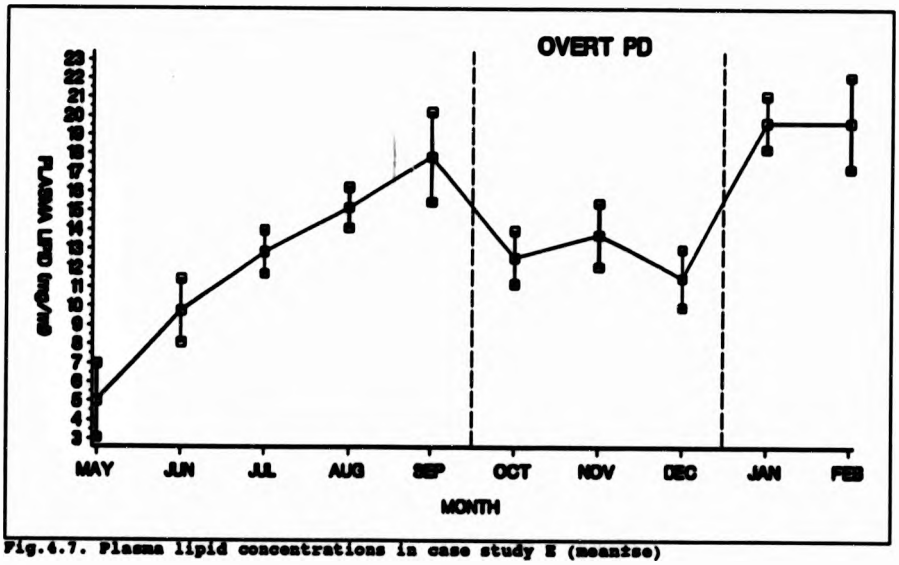

Fig.4.7. Plasma lipid concentrations in case study E (mean±se)

biochemical data, weight, and condition factor, further statistical analyses were conducted. Spearman's coefficients of rank correlation were calculated to describe the relationship, within each cage, of each variate with time. In addition, the data sets were subjected to a nested analysis of variance, with

cage and cage-time as treatment factors, in order to test for differences between cages overall and at each month. Tests for normal distribution showed that the transformations carried out above were also appropriate for the data restricted to cages 10 and 13. Significant cage-time effects were examined for differences between the cages at each month using the GT2 method of multiple comparison.

Plasma vitamin E concentrations measured in each cage at each time are shown in table 4.11 and fig. 4.8. In cage 10, plasma vitamin E concentrations increased from 0 $\mu g\ ml^{-1}$ in May to 60±36 $\mu g\ ml^{-1}$ in February. In cage 13, they stayed relatively constant between May and December 1988, ranging between 17±11 and 32±27 $\mu g\ ml^{-1}$, but increased to 57±17 $\mu g\ ml^{-1}$ in January. Spearman's test showed a significant positive correlation between plasma vitamin E concentration and time in cage 10 ($p<0.01$), but no correlation in cage 13. Using analysis of variance, significant differences were found between the means in different cages at different times ($p<0.01$), but multiple pairwise comparisons failed to detect any significant differences between the means in different cages in any particular month.

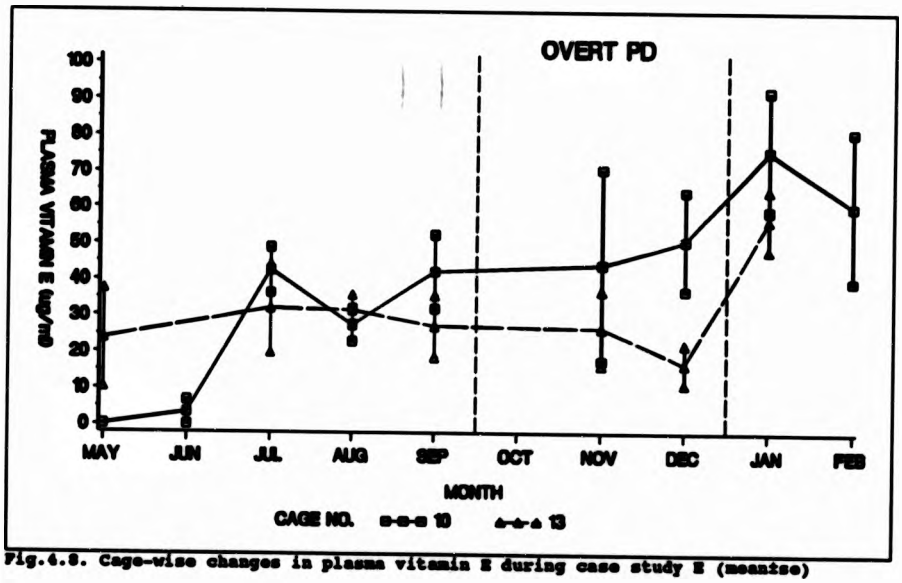

Fig.4.8. Cage-wise changes in plasma vitamin E during case study E (meanise)

Liver vitamin E concentrations in each of the two cages are shown in

Table 4.11. Plasma and liver vitamin E concentrations cage-wise from case E (mean±sd)

Date	Cage	n	Plasma vitamin E ($\mu g\ ml^{-1}$)	n	Liver vitamin E ($\mu g\ g^{-1}$)
31.5.88	10	1	0±0	3	219±112
	13	5	24±30	3	348±125
1.7.88	10	3	3±6	3	176±113
	13	0	-	0	-
2.8.88	10	5	43±14	3	399±35
	13	5	32±27	3	400±99
1.9.88	10	5	27±10	3	370±27
	13	4	32±8	3	264±113
30.9.88	10	5	42±23	3	116±63
	13	5	27±19	3	186±114
31.10.88	10	0	-	0	-
	13	0	-	0	-
1.12.88	10	3	44±46	3	229±322
	13	3	27±18	3	130±188
30.12.88	10	4	51±27	3	317±230
	13	4	17±11	3	21±15
31.1.89	10	3	76±28	3	167±41
	13	4	56±17	3	125±26
28.2.89	10	3	60±36	3	231±148
	13	0	-	0	-

table 4.11 and fig. 4.9. In cage 10, liver vitamin E concentrations varied greatly, ranging between 116±63 and 399±35 $\mu g\ g^{-1}$, but showed no clear trend during the course of the study. In cage 13, they rose from 348±125 $\mu g\ g^{-1}$ in May to 400±99 $\mu g\ g^{-1}$ in July, then fell to 21±15 $\mu g\ g^{-1}$ in December before recovering slightly to 125±26 $\mu g\ g^{-1}$ in January. A significant negative correlation between liver vitamin E concentrations and time was found in cage 13 ($p<0.01$), but in cage 10 there was no significant relationship. Analysis of variance indicated that significant differences existed between the means in each cage at each time($p=0.05$), but again no significant differences were found between the cages in any particular month.

Changes in plasma lipid concentrations in each cage are shown in table

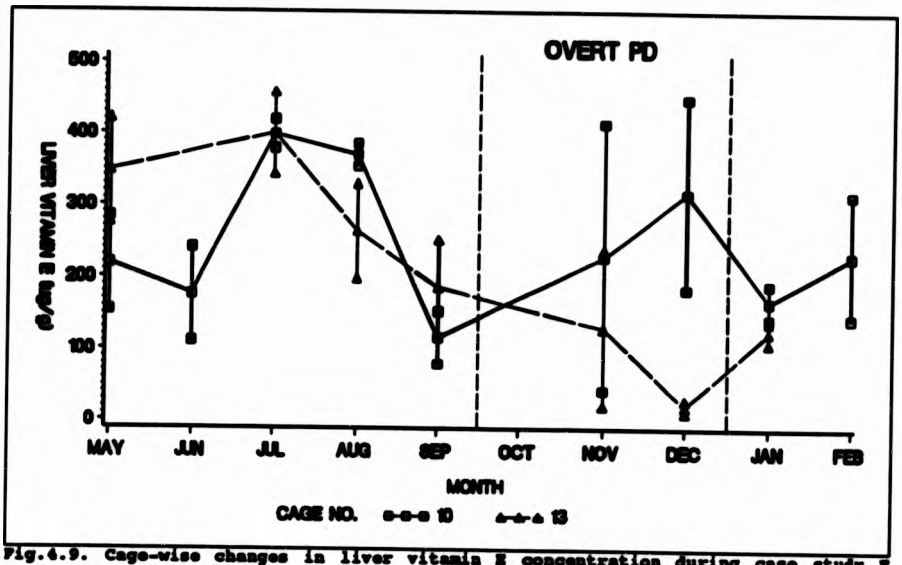

Fig.4.9. Cage-wise changes in liver vitamin E concentration during case study E (mean±se)

4.12 and fig. 4.10. In cage 10, plasma lipid concentrations rose from 3±3 mg ml^{-1} in May to 23±6 mg ml^{-1} in February. In cage 13 they rose from 8±6 mg ml^{-1} in May to a maximum of 18±4 mg ml^{-1} in September, fell to 11±3 mg ml^{-1} in November, and rose again to 23±6 mg ml^{-1} in February. Plasma lipid concentrations showed a significant positive correlation with time in cage 10 ($p<0.001$), but no correlation with time in cage 13. Significant differences were again found by analysis of variance ($p=0.01$), but no significant differences were detected between the means in any one month.

Fish weights and condition factors are shown in tables 4.13 and 4.14, and figs 4.11 and 4.12. In cage 10, fish weight increased from 45±11 g in May to 449±41 g in September, fell slightly to 419±244 g in November, then rose to 934±653 g in February. Over the first seven months of the study, a similar pattern was seen in cage 13. Here, the mean fish weight increased from 49±12 g in May to 504±179 g in November, but in December it fell to 287±111 g, then recovered to 637±83 g in January. The average condition factor of fish in both cages ranged from 1.23 to 1.30 between May and July, increased to more than 1.69 in September, fell sharply, to 1.28±0.22 in cage 10 in November and 1.21±0.14 in cage 13 in December, and stayed low for the remainder of the

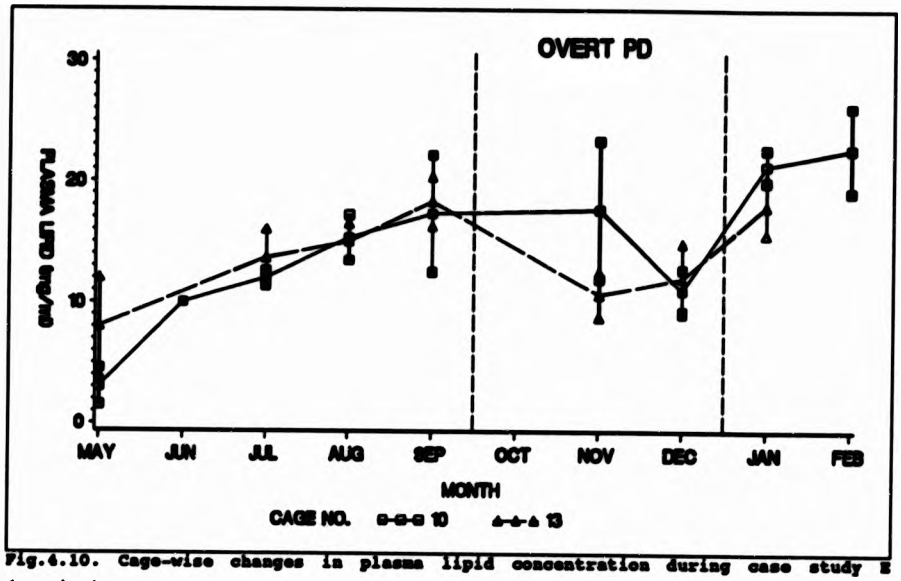

Fig.4.10. Cage-wise changes in plasma lipid concentration during case study E (means±se)

study period. A significant correlation between weight and time was found in both cages ($p<0.001$), but condition factor showed a significant relationship with time only in cage 10 ($p<0.01$). Analysis of variance showed significant cage-time effects in both weight and condition factor ($p<0.001$). Multiple comparisons showed that the mean weights were significantly different in December ($p<0.05$), being higher in cage 10 than cage 13, but no differences in condition factor were detected between the two cages in any one month.

4.3.5.7 Comparisons between farms with and without PD

The data collected between June and February from this site was compared with data, collected over the same time period, from the farm with no PD described in section 3.3.1. Statistical tests were as for the cage comparisons. Spearman's coefficients of rank correlation were calculated between the data from each farm and time. The data sets were then subjected to nested analysis of variance with farm and farm-time as the treatment factors and significant differences were further analysed using the GT2 method. Tests of normal distribution showed that the transformations used

Table 4.12. Plasma lipid concentrations cage-wise from case E (mean±sd)

Date	Cage	n	Plasma lipid (mg ml^{-1})
31.5.88	10	3	3±3
	13	2	8±6
1.7.88	10	1	10±0
	13	0	-
2.8.88	10	3	12±1
	13	3	14±4
1.9.88	10	3	15±3
	13	3	15±3
30.9.88	10	3	17±8
	13	3	18±4
31.10.88	10	0	-
	13	0	-
1.12.88	10	3	18±10
	13	3	11±3
30.12.88	10	3	11±3
	13	3	12±5
31.1.89	10	3	21±2
	13	3	18±4
28.2.89	10	3	23±6
	13	0	-

above were again suitable for the combined data sets.

Plasma vitamin E concentrations measured on each farm between June 1988 and February 1989 are shown in tables 3.2 and 4.9 and fig. 4.13. On the unaffected farm, plasma vitamin E concentrations ranged from 17±9 to 21±7 µg ml^{-1} between June and September, rose to 66±17 µg ml^{-1} in October, fell gradually to 41±11 µg ml^{-1} in January, then increased to 67±27 µg ml^{-1} in February. On the affected site, plasma vitamin E concentrations increased from 14±14 µg ml^{-1} in June to 37±21 µg ml^{-1} in July, then stayed relatively constant, ranging from 29±9 to 37±21 µg ml^{-1} between July and December. In January they increased to 65±23 µg ml^{-1} and in February they fell back to 52±29 µg ml^{-1}. Significant positive rank correlations between plasma vitamin

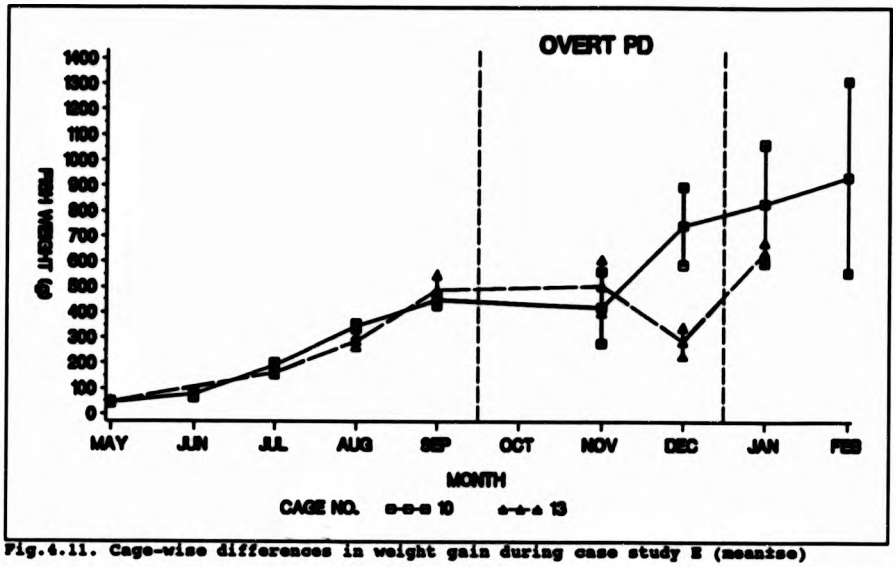

Fig.4.11. Cage-wise differences in weight gain during case study E (meanise)

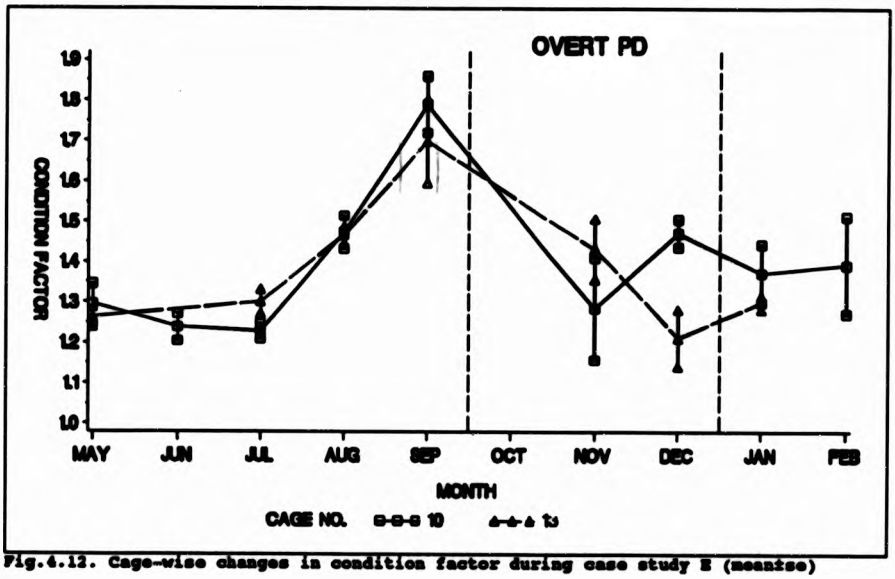

Fig.4.12. Cage-wise changes in condition factor during case study E (meanise)

E concentrations and time were detected on both the farm with PD ($p<0.01$) and the farm without ($p<0.001$). Analysis of variance showed that significant differences could be detected between the means on different sites at

Table 4.13. Weight and condition factor cage-wise from case E (seaated)

Date	Cage	n	Weight (g)	Condition factor
31.5.88	10	5	45±11	1.30±0.11
	13	5	49±12	1.26±0.05
1.7.88	10	5	76±27	1.24±0.08
	13	0	-	-
2.8.88	10	5	191±22	1.23±0.04
	13	5	162±14	1.30±0.07
1.9.88	10	5	344±24	1.47±0.09
	13	5	286±46	1.47±0.05
30.9.88	10	5	449±41	1.79±0.16
	13	5	488±134	1.70±0.23
31.10.88	10	0	-	-
	13	0	-	-
1.12.88	10	3	419±246	1.28±0.22
	13	3	504±179	1.43±0.13
30.12.88	10	4	743±309	1.47±0.07
	13	4	287±111	1.21±0.14
31.1.89	10	3	832±404	1.37±0.13
	13	4	637±83	1.30±0.03
28.2.89	10	3	934±653	1.39±0.21
	13	0	-	-

different times ($p<0.001$). Multiple pairwise comparisons showed that significant differences between the sites were found in October ($p<0.05$), with plasma vitamin E concentrations being higher on the unaffected site.

Liver vitamin E concentrations on each farm are shown in tables 3.3 and 4.9 and in fig. 4.14. On the unaffected farm, liver vitamin E concentrations rose from 164±111 μg g^{-1} in June to 471±33 μg g^{-1} in July and, although ranging as low as 268±103 μg g^{-1} in November, subsequently showed a general increase, reaching 754±176 μg g^{-1} in February. In contrast, on the affected site mean liver vitamin E concentrations increased from 218±94 to 400±66 μg g^{-1} between June and July, but fell to 151±91 μg g^{-1} in September and remained low, ranging from 118±155 to 180±118 μg g^{-1} between October and February. On both

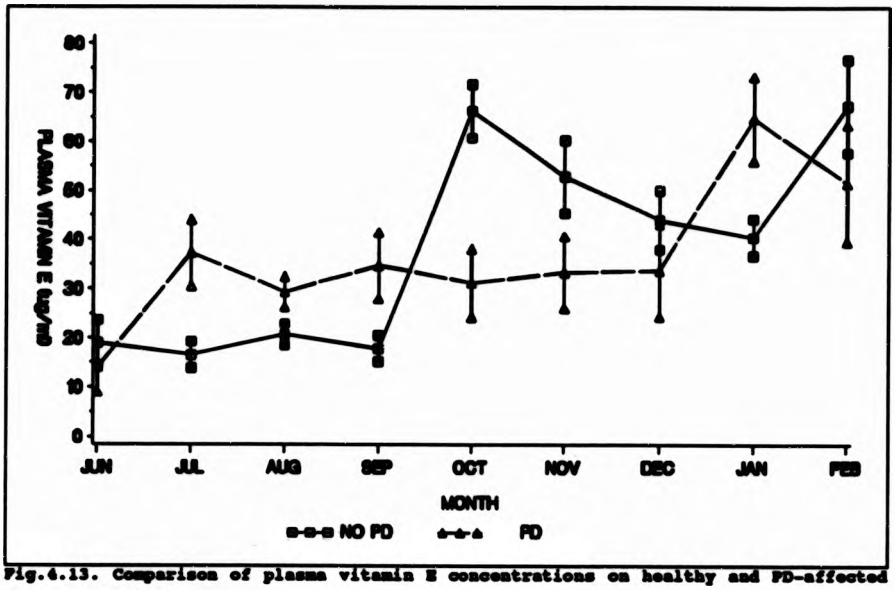

Fig.4.13. Comparison of plasma vitamin E concentrations on healthy and PD-affected farms (means±se)

sites a significant relationship between liver vitamin E concentration and time was detected ($p<0.01$), but whereas on the farm without PD this correlation was positive, on the farm with PD it was negative. Liver vitamin E concentrations were significantly different between sites at different times ($p<0.001$), and also differed significantly overall ($p<0.001$). Multiple comparisons showed significant differences between farms in the months of October, December and February when liver vitamin E concentrations were lower on the affected site ($p<0.05$). This coincided with the period of overt PD on the affected site. There were no significant differences prior to this time.

Plasma lipid concentrations are shown in tables 3.4 and 4.10 and in fig. 4.15. On the unaffected site, these fell from 12±1 mg ml^{-1} in July to 6±4 mg ml^{-1} in September, rose to 17±7 mg ml^{-1} in November, fell to 4±2 mg ml^{-1} in December, and rose once again to 11±4 mg ml^{-1} in February. On the affected site, plasma lipid concentrations rose from 10±3 mg ml^{-1} in June to 18±6 mg ml^{-1} in September, fell to 13±4 mg ml^{-1} in October and 12±4 mg ml^{-1} in December, then rose to 20±4 mg ml^{-1} in January and 20±6 mg ml^{-1} in February. No correlation between plasma lipid concentration and time was found on the site

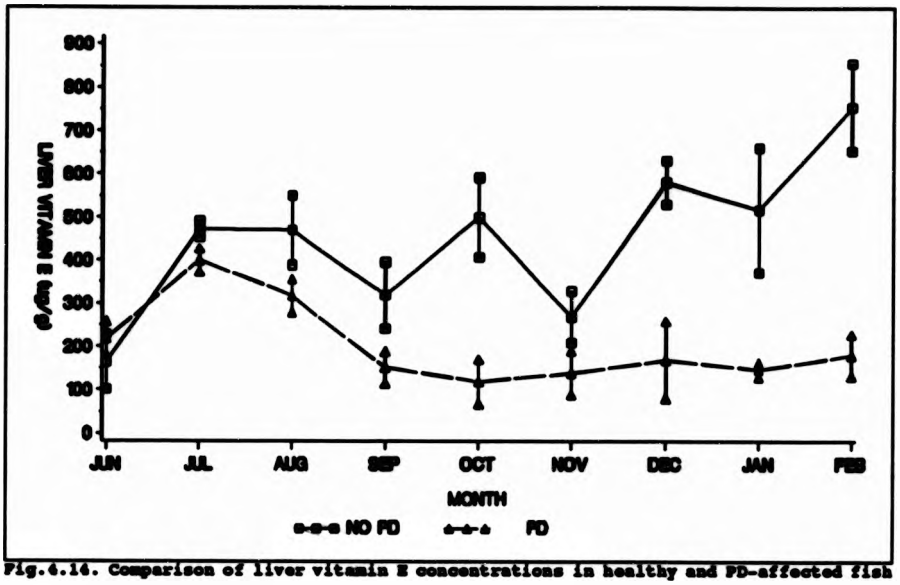

Fig.4.16. Comparison of liver vitamin E concentrations in healthy and PD-affected fish (mean±se)

without PD, but on the affected farm a positive correlation was detected ($p<0.05$). Mean plasma lipid concentrations differed significantly between sites and times ($p<0.001$), and also between sites overall ($p<0.001$). Multiple comparisons revealed significant differences in September and January ($p<0.05$) and in both cases plasma lipid concentrations were higher on the affected farm.

Weight and condition factor data for these farms are shown in tables 3.9 and 4.7, and in figs 4.16 and 4.17. On the unaffected farm, the mean fish weight increased from 57±8 g in June to 300±44 g in September and 1171±279 g in February, whilst on the affected site it increased from 84±28 g to 468±95 g between June and September, but then fell to 420±138 g in October, and increased to only 767±465 g in February. Similarly, on the unaffected farm, the mean condition factor increased from less than 1.25, in June, July and August, to 1.59±0.06 in November. It then fell gradually to 1.42±0.11 in February. On the affected farm it increased from 1.23±0.09 in June and 1.26±0.07 in July to 1.74±0.19 in September, fell sharply to 1.32±0.17 in October, and ranged from 1.32±0.17 to 1.37±0.13 between October and February.

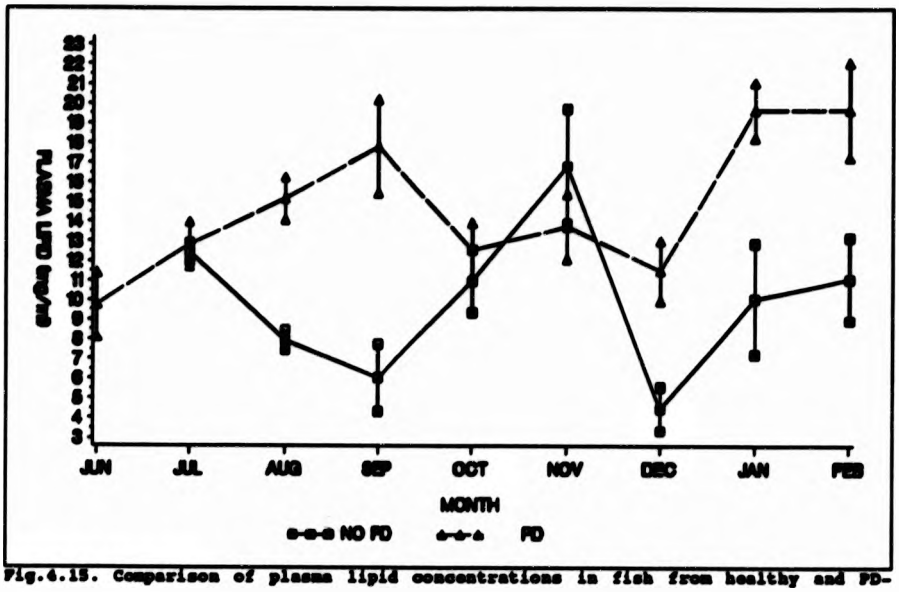

Fig.4.15. Comparison of plasma lipid concentrations in fish from healthy and PD-affected farms (means±se)

A significant relationship between fish weight and time was found on both sites ($p<0.001$), but whilst condition factor improved with time on the unaffected farm ($p<0.001$), no significant correlation was found on the affected site. Significant farm-time interactions were detected in both cases ($p<0.001$). Fish weights differed ($p<0.05$) in July, August and September prior to the PD outbreak when fish on the affected farm weighed more than on the unaffected site, and also in December and February when fish on the unaffected farm weighed more. Similarly, the mean condition factor of fish on the site developing PD was higher in August and September, but lower in October, November and December. This observation gives some support to the suggestion that fish in better condition may be more vulnerable to PD.

4.3.6 Case F (Site 5)

4.3.6.1 Case history

No visits were made to this farm site, but signs of PD were reported to

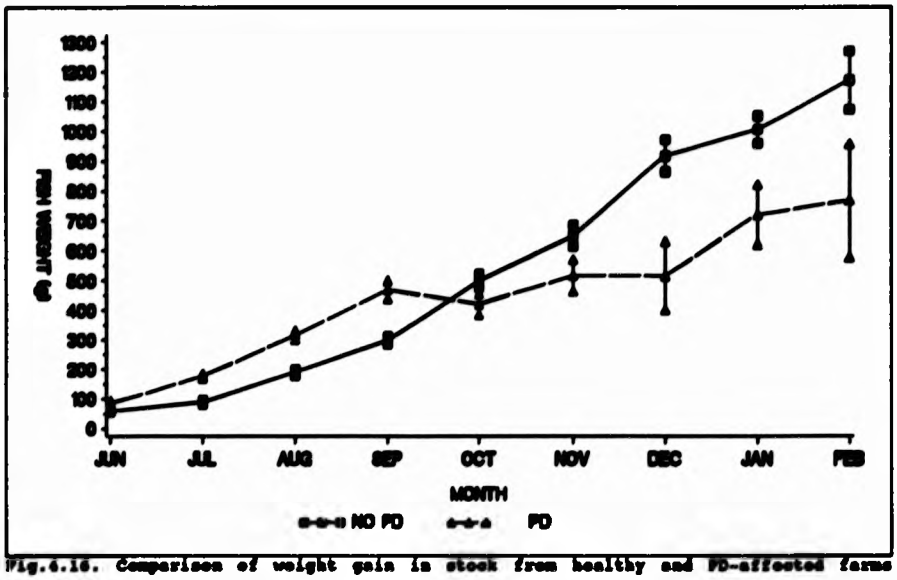

Fig.4.16. Comparison of weight gain in stock from healthy and PD-affected farms (means±se)

have occurred at the end of September 1988. The site had previously suffered annual outbreaks. Feeding rate was reduced immediately after signs were noticed and the stock showed signs of recovery, but the disease recurred two weeks later when normal feeding rates were resumed.

4.3.7 Case G (Site 6)

4.3.7.1 Case history

Annual outbreaks of PD had been recorded on this farm since 1985. In 1988, the first sign of PD was a reduction in feeding response in the smolt stock, noted on 28th May, soon after transfer to seawater. Routine histological monitoring was unsuccessful in detecting earlier evidence. This case was characterised by a high mortality rate in the affected population: deaths began to occur on 17th June and peaked in mid-July. At the time of the visit in August losses of 50% and 30% had occurred on sites 6a and 6b respectively; this amounted to a total of over 50 000 fish. Mortalities, of uncertain cause, but probably a result of secondary infection or the presence

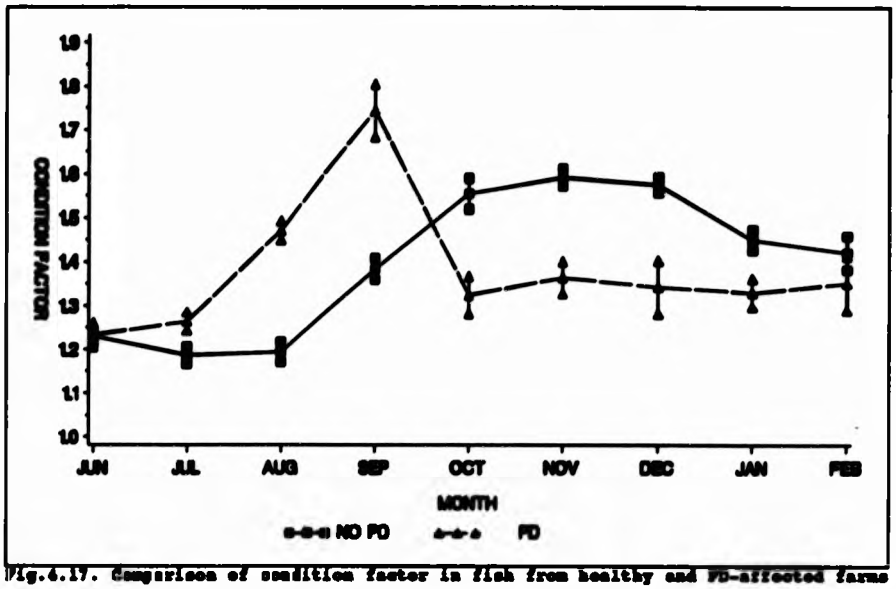

Fig.4.17. Comparison of condition factor in fish from healthy and PD-affected farms (means±se)

of severe heart lesions, occurred before emaciation became severe and were particularly heavy following the stress of delousing treatment. All cages of smolts were affected, but the timing and severity of disease and mortality showed cage to cage variation. Cages on the end of rafts appeared to be worst affected. Cages of fish on site 6a developed signs of disease in accordance with the sequence in which they were stocked during transfer to the sea site, but fish on site 6b all developed signs simultaneously and slightly later than on site 6a. Sick fish had white, gaping mouths and showed no regular swimming activity. Strong currents affected the site, but water flow through cages may have been reduced by the regular accumulation of large amounts of drifting seaweed. There were no signs of differences in susceptibility due to stock origin. The mean weight of fish collected was 79 g and the condition factor 0.97. Procedures for disease management included feeding to half the usual rate, using high energy diets and feed coated with pre-digested fish products to encourage feeding, and antibiotics to counteract secondary infection. However, these appeared to have little effect.

4.3.7.2 Histopathology

Histological sections were examined from a total of eleven fish from this outbreak. Exocrine pancreatic degeneration was recognised in eight individuals, three from cage 2 and one from cage 5 on site 6a, and two each from cages 3 and 6 on site 6b. Pancreas sections from the remaining three individuals were not sufficiently clear to recognise exocrine pancreatic tissue if it did occur. Severe cardiomyopathy was evident in two fish from cage 2 and one from cage 5 both on site 6a. The remaining eight heart preparations were too poor for clear recognition of such changes.

4.3.8 Case H (Site 7)

4.3.8.1 Case history

PD was diagnosed on site 7 at the end of August 1988. The outbreak was described as an acute case on the basis of histopathological observations including signs of haemorrhage in the peri-pancreatic fat. At the time of the first visit, on 31st August, fish collected from one affected cage were still in good condition, although their stomachs and hindguts contained no food. Peri-pancreatic fat was present but appeared pink. Smaller (7 m^2) cages of smolts appeared to be unaffected, but large numbers of fish of the same age and stock were lying at the surface, along the edges of three 15m^2 cages. All three cages had begun to show signs of PD together in mid August and, subsequently, delousing treatment had seemed to cause an increase in severity. The fish were fed Ewos feed with a very high concentration of vitamin E (200 mg 100 g^{-1} as α-tocopheryl acetate).

There was no previous history of PD on the farm, so it was interesting to note that growers on the same raft were apparently unaffected. Intercurrent furunculosis caused mortalities in anorexic PD fish; those that were feeding were treated with antibiotics. A similar picture was evident at the time of the second visit on 7th September, but by 13th September, only 3 weeks after the case was first diagnosed, some signs of recovery were noted. At this visit there were fewer fish at the surface and these appeared to be more active,

particularly in response to food. Post-mortem examination confirmed the presence of food in the guts of approximately half of the fish examined at this time and this proportion subsequently increased. By the 18th October all fish showed a vigorous feeding response with the exception of a few severely emaciated runts in the cage corners. However, fish weights were more variable than was normal for this time of year.

4.3.8.2 Fish weight and condition factor

Fish weights and condition factors are shown in table 4.14 and figs 4.18 and 4.19. Fish weights ranged from 136±37 to 155±43 g and showed little change during the first five visits, but increased to 283±95 by 18th October. Condition factors fell from 1.18±0.06 on the first to 0.79±0.09 on the fifth visit, but recovered to 1.33±0.09 at the final visit. Condition factor ($p<0.05$), but not fish weight, showed a significant negative correlation with time. Weight data was subjected to log transformation, no transformation was required for condition factor. Significant differences in condition factor, but not weight, were detected between feeding and non-feeding fish ($p<0.05$). Condition factor was higher in non-feeding fish.

Table 4.14. Weight and condition factor of fish from case H (mean±sd)

Date	n	Weight (g)	Condition factor
31.8.88	11	140±30	1.18±0.06
7.9.88	10	155±43	1.17±0.11
13.9.88	10	152±36	1.12±0.15
20.9.88	10	136±37	1.00±0.08
27.9.88	10	103±35	0.79±0.09
18.10.88	6	283±95	1.33±0.09

4.3.8.3 Histopathology

A total of ten fish were examined for evidence of pancreatic necrosis and muscle degeneration, two at each visit except from that of 20th September. On 31st August, 7th September and 13th September, both fish studied showed

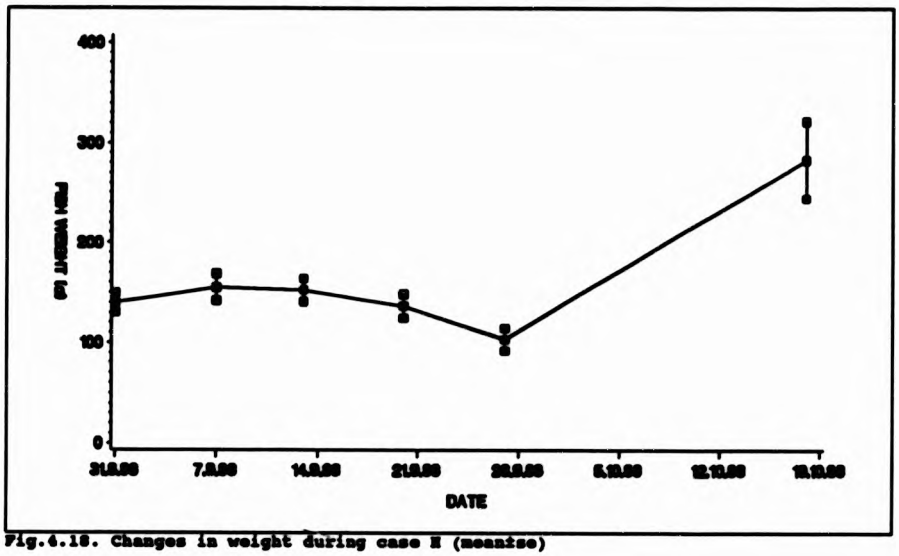

Fig.4.18. Changes in weight during case E (meanse)

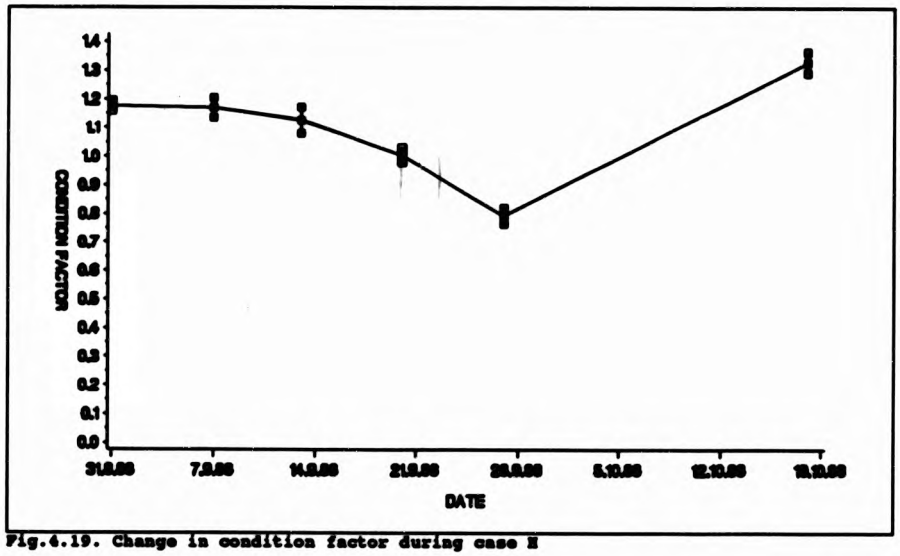

Fig.4.19. Change in condition factor during case E

exocrine pancreatic degeneration, on 27th September one fish was similarly affected and the other had intact pancreatic tissue, and on 18th October both appeared healthy. Heart muscle involvement was noted in all but one of the fish with pancreatic degeneration, but none of the fish with normal pancreatic

tissue. Significant differences in weight ($p<0.05$), but not condition factor, were detected between fish with and without pancreatic necrosis. The mean weight was lower in fish with EPD. In this outbreak, as in case B, muscle degeneration was described in association with exocrine pancreatic degeneration in fish fed high levels of α-tocopheryl acetate. Furthermore, as described below, these fish had very low tissue vitamin E concentrations.

4.3.8.4 Vitamin E

Mean plasma vitamin E concentrations at each visit are shown in table 4.15 and fig. 4.20. Plasma vitamin E concentrations were very low at the start of the study, averaging 3±2 μg ml^{-1} at the first visit and 0±1 μg ml^{-1} at the second. They rose to 10±14 μg ml^{-1} after three weeks and 7±9 μg ml^{-1} after four weeks, and on 18th October, seven weeks after the first visit, they reached 21±17 μg ml^{-1}. It is important to emphasize that such low concentrations were measured in fish previously fed a diet containing as much as 100 mg α-tocopheryl acetate 100 g^{-1}. This data had a skewed frequency distribution even after transformation, so non-parametric methods of statistical analysis were used. Spearman's test for rank correlation showed a significant increase in plasma vitamin E concentrations during the course of the study ($p<0.01$). Also using Spearman's test, a significant relationship was found between plasma vitamin E concentrations and weight ($p<0.05$), but not condition factor. No difference in plasma vitamin E concentrations was detected between feeding and non-feeding fish, using the Mann-Whitney U-test, but significant differences were detected between fish with and without pancreatic degeneration and cardiomyopathy ($p<0.05$). Higher mean vitamin E concentrations were measured in fish with no evidence of histological change.

4.3.8.5 Plasma lipid

Plasma lipid concentrations measured over the course of this study are presented in table 4.16 and fig. 4.21. These fell slightly from 10±4 mg ml^{-1} at the first visit to 7±5 mg ml^{-1} on the fifth visit, but by the final visit had increased to 15±4 mg ml^{-1}. No significant correlation with time was

detected using Spearman's test but, as in case E, a significant positive association between plasma lipid and plasma vitamin E concentrations was found

Table 4.15. Plasma vitamin E concentrations in fish from case H (measles)

Date	n	Plasma vitamin E ($\mu g\ ml^{-1}$)
31.8.88	11	3±2
7.9.88	8	0±1
13.9.88	10	6±9
20.9.88	10	10±14
27.9.88	10	7±9
18.10.88	6	20±17

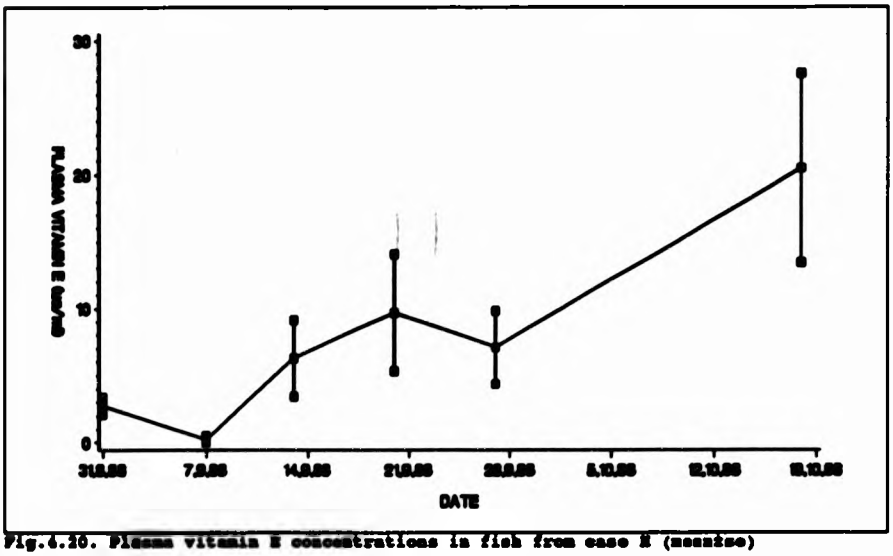

Fig.4.10. Plasma vitamin E concentrations in fish from case H (measles)

($p<0.001$). The data were subjected to square root transformation prior to parametric analyses. Pearson's test showed that plasma lipid concentrations were positively correlated with weight ($p<0.01$) and condition factor ($p<0.01$). As for vitamin E, analysis of variance showed no difference in plasma lipid concentrations between feeding and non-feeding fish. However, in contrast to vitamin E, the Mann-Whitney test detected no significant difference in plasma

lipid concentrations between individuals with and without pancreatic necrosis.

Table 4.16. Plasma lipid concentrations in fish from case H (mean±sd)

Date	n	Plasma lipid (mg ml^{-1})
31.8.88	10	10±4
7.9.88	7	8±4
13.9.88	10	7±3
20.9.88	10	8±6
27.9.88	10	6±5
18.10.88	6	15±4

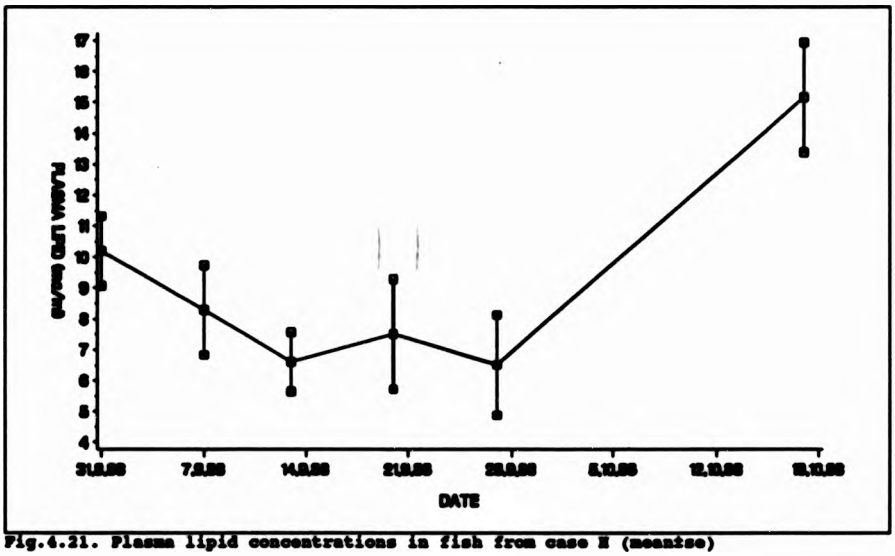

Fig.4.21. Plasma lipid concentrations in fish from case H (mean±se)

4.3.8.6 Plasma malondialdehyde

Table 4.17 and fig. 4.22 show plasma malondialdehyde concentrations measured during the course of this outbreak. These fell from a maximum of 53±39 μmol l^{-1} at the first visit, to 7±7 μmol l^{-1} at the fourth and 11±9 μmol l^{-1} at the fifth visits, but increased to 21±10 μmol l^{-1} at the final visit.

These are low in comparison to those measured in healthy experimental fish (section 5.3). A significant negative correlation with time ($p<0.001$), but no significant correlation with plasma vitamin E concentration was found using Spearman's test. A log transformation was applied to the data and a significant positive association was found between plasma malondialdehyde and lipid concentrations ($p<0.01$), and between plasma malondialdehyde concentration and both weight ($p<0.01$) and condition factor ($p<0.05$) using Pearson's test. Analysis of variance showed a significant difference in plasma malondialdehyde concentrations between feeding and non-feeding fish ($p<0.01$), the mean concentration being higher in non-feeding fish. Duthie *et al.* (1989) used plasma malondialdehyde concentrations as an index of the degree of tissue peroxidation. In the present study, there was no significant correlation between plasma vitamin E and malondialdehyde concentrations and there is no suggestion that plasma malondialdehyde concentrations are increased concommitantly with a reduction in plasma vitamin E during outbreaks of PD.

Table 4.17. Plasma malondialdehyde concentrations in fish from case E (mean±sd)

Date	n	Plasma malondialdehyde (μmol l^{-1})
31.8.88	11	53±39
7.9.88	10	44±46
13.9.88	10	19±5
20.9.88	10	7±7
27.9.88	10	11±9
18.10.88	6	21±10

4.3.8.7 Plasma pyruvate kinase

Plasma PK measurements made on plasma samples collected during the first two visits averaged 186±104 mU ml^{-1} at the first and 6±8 mU ml^{-1} at the second. The former is high, whilst the latter is within the normal range measured in healthy experimental fish (section 5.3). Bell *et al.* (1987*b*) demonstrated that plasma PK activity was increased in fish with PD. A significant positive rank correlation between plasma PK activity and vitamin E concentration ($p<0.05$)

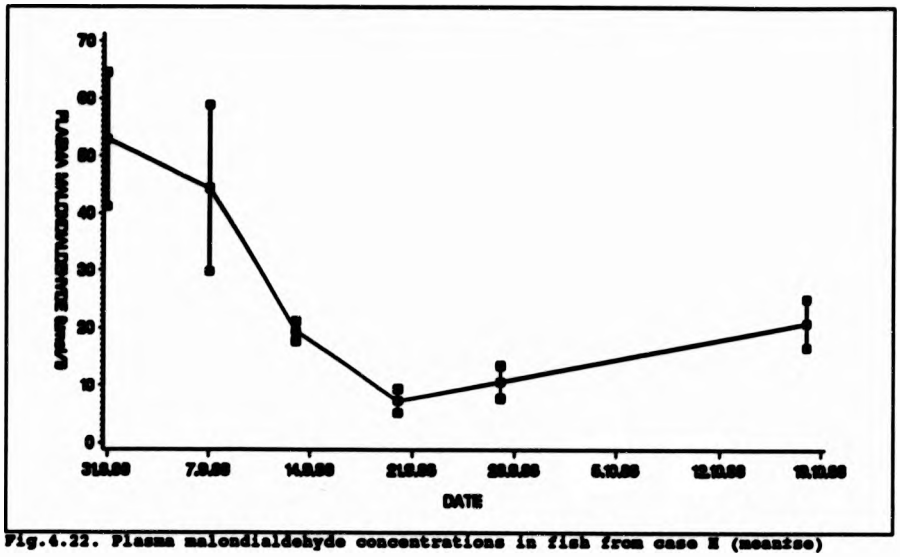

Fig.4.22. Plasma malondialdehyde concentrations in fish from case H (mean±se)

was detected using Spearman's test, but correlations between PK activity and plasma lipid and malondialdehyde concentrations, fish weight and condition factor, were not significant. Elevated plasma PK activity was regarded as an indicator of incipient muscle degeneration and was found to increase in vitamin E-selenium deficient rainbow trout (Bell *et al.* 1985, 1986).

4.3.9 Case I (Site 8)

4.3.9.1 Case history

On site 8, PD was suspected at the end of the first week in October 1988, after two cages of smolts on a raft of ten exhibited a poor feeding response. A third cage subsequently also showed signs of PD. This case showed the pattern of progression typical of a chronic outbreak of PD. At the first visit on 14th October, the affected fish were seen hanging in a group deep in one corner of the cage. The depth and position of these fish appeared to be related to wind strength and current direction. This hanging behaviour was noticed intermittently during the first visit, but thereafter appeared to be continuous except at meal times when the group was disturbed by active,

feeding fish in the same cage. PD was confirmed by staff at DAFS, Aberdeen on 4.11.88, and the report indicated that both early and late stages of PD were represented in the sample which had been collected in early October. This suggested that the condition had been present for some time before overt signs were noticed and that it developed intermittently or slowly in the population. In this respect it differed from case H. These smolts were stocked at 28 000 fish cage^{-1} in 15m^{2} galvanised steel cages. Two of the cages containing the affected stock had small mesh nets and the raft had recently been the target of a seal attack. As elsewhere, salmon lice posed a continual problem and delousing treatments were used regularly. The farm had suffered an outbreak of PD in 1987 at a different site two hundred metres south of the smolt raft. The affected population, now growers, had been moved to a site north of the smolt raft, but these fish showed no overt signs of PD in 1988. In the first samples of affected smolts, peri-pancreatic fat was present but pink, most fish showed no signs of food in the gut, and the hindgut blood vessels were often prominent. At the second visit (20.10.88), gut-associated adipose tissue was present in some fish, but not in others. The affected population was utilised in the experimental study described below. During the course of this experiment it was observed that sick fish appeared to have low resistance to handling and anaesthesia. On 28th October the affected fish appeared to be more active, but the presence in the sample of a large fish with early signs of PD suggested that the outbreak was continuing. The sample of 4th November showed no further improvement. By 6th December the fish in the corners of the cages were more active and difficult to catch, indicating the onset of recovery. This was supported by the observation of food in the guts of even the most emaciated fish. However, at that time three other cages showed a decrease in feeding response and began to exhibit the characteristic listless, hanging behaviour typical of PD.

4.3.9.2 Fish weight and condition factor

The weights and condition factors of fish collected during this study are shown in table 4.18. Over the course of the study, the mean weight ranged from 229±107 to 318±90 g and the condition factor from 1.12±0.09 to 1.24±0.07.

Neither fish weight nor condition factor showed any significant trend with time. A log transformation was applied to fish weight, but no transformation was necessary for condition factor. Neither showed any difference between feeding and non-feeding fish.

Table 4.18. Weight and condition factor of fish from case I (measured)

Date	n	Weight (g)	Condition factor
14.10.88	10	252±32	1.15±0.07
20.10.88	10	246±61	1.18±0.08
28.10.88	10	229±107	1.12±0.09
4.11.88	10	318±90	1.24±0.07
6.12.88	10	263±111	1.12±0.10

4.3.9.3 Histopathology

Sections prepared from 12 fish were examined to confirm the occurrence of exocrine pancreas degeneration. Examination of two heart sections from material collected on 20th October revealed evidence of severe cardiomyopathy in this outbreak.

4.3.9.4 Vitamin E

Plasma vitamin E concentrations measured in these fish are shown in table 4.19 and illustrated in fig. 4.23. They fell from 49±41 μg ml^{-1} on 14th October to 8±6 μg ml^{-1} on 28th October, then recovered slightly to 16±7 μg ml^{-1} on 4th November and 13±7 μg ml^{-1} on 6th December. Over the course of the study they showed a significant negative rank correlation with time ($p<0.01$). Plasma vitamin E data were subjected to a log transformation prior to parametric analyses. Analysis of variance showed that there was no significant difference in plasma vitamin E concentrations between feeding and non-feeding fish. Plasma vitamin E concentrations were significantly correlated with fish weight ($p<0.01$), but not with condition factor.

Table 4.19. Plasma vitamin E concentrations in fish from case I (meanise)

Date	n	Plasma vitamin E ($\mu g\ ml^{-1}$)
14.10.88	10	49±41
20.10.88	10	14±8
28.10.88	10	8±6
4.11.88	10	16±7
6.12.88	10	13±7

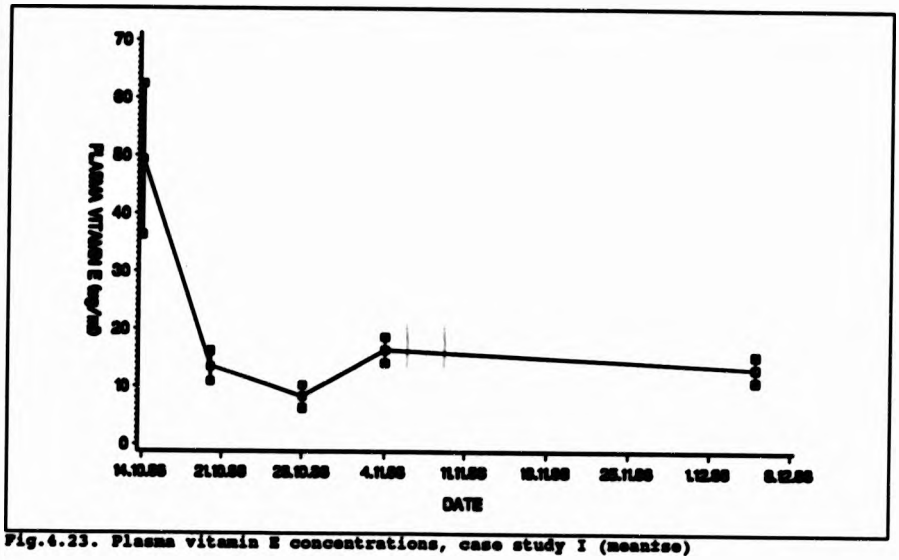

Fig.4.23. Plasma vitamin E concentrations, case study I (meanise)

4.3.9.5 Plasma pyruvate kinase

Plasma PK activity was determined in samples collected on 20th October only. A high mean value of 112±170 mU ml^{-1} was measured. There was no significant association between plasma PK activity and weight, but a significant negative correlation with condition factor was found. Thus plasma PK elevation in PD may be indicative of a general loss in muscle bulk as suggested by Bell *et al.* (1987*b*).

4.3.9.6 Effect of parenterally-administered vitamin E on PD

Table 4.20 and figs 4.24 and 4.25 show the results of plasma vitamin E and protein determinations on fish, from case I, used in the experimental study of the effects of parenterally-administered vitamin E on PD. After a treatment period of 15 days, plasma vitamin E concentrations were 21±7 μg ml^{-1} in the control group, 18±4 μg ml^{-1} in the sham injected group, and 24±15 μg ml^{-1} in the group given vitamin E in cocoa butter. The data were subjected to a log transformation, and analysis of variance indicated that no significant differences existed between the treatments, despite the results of a preliminary study conducted using healthy fish in which a similar dose produced a significant rise in plasma vitamin E concentrations. Plasma protein concentrations were 41±9, 44±11 and 46±10 mg ml^{-1}, respectively. No transformation was needed prior to analysis of variance and again no differences were found between the treatments. Consequently enzyme activities based on plasma volume, rather than protein concentration, were compared.

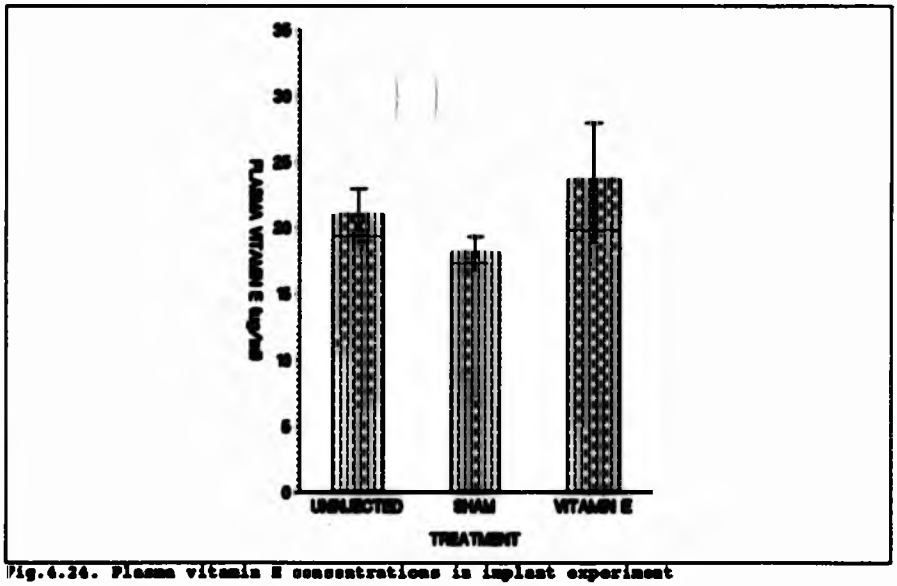

Fig.4.24. Plasma vitamin E concentrations in implant experiment

Plasma PK and CPK activities in each experimental group are given in table 4.21 and figs. 4.26 and 4.27. Plasma PK activity was 324±252 mU ml^{-1} in

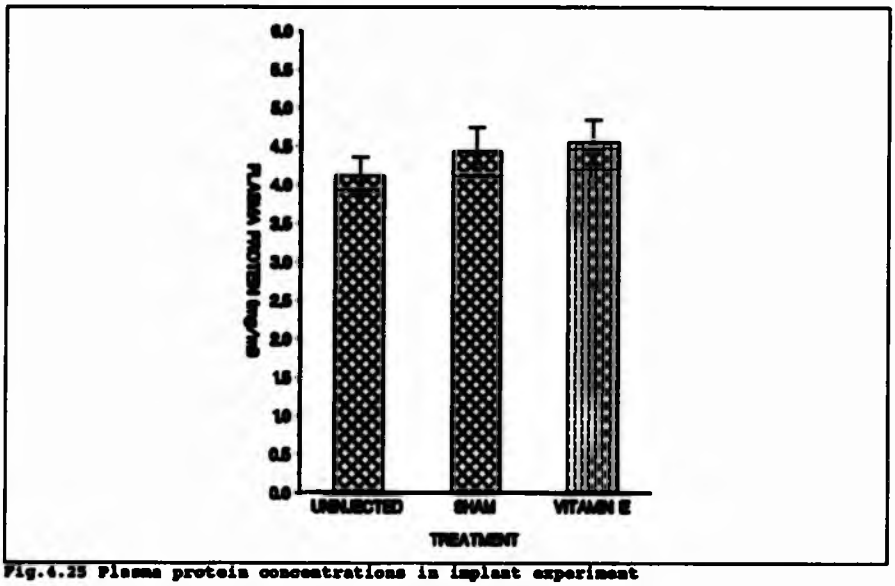

Fig.4.25 Plasma protein concentrations in implant experiment

the untreated group, 239±383 mU ml^{-1} in the sham treated, and only 92±62 mU ml^{-1} in the group treated with vitamin E. Plasma CPK activity showed a similar pattern, measuring 16±16 IU ml^{-1} in the uninjected group, 14±19 IU ml^{-1} in the sham, and 12±9 IU ml^{-1} in the vitamin E treated group. Both sets of data were log transformed. Analysis of variance showed significant treatment differences in PK ($p<0.05$), but not CPK activity. Multiple comparisons, using the GT2 method, failed to detect significant differences between any of the three pairs at 95% confidence level. Nevertheless, the data show that plasma PK activities were much lower in the vitamin E-treated group than either the sham-treated or uninjected control groups. Both PK and CPK activities were slightly lower in fish given cocoa-butter than in untreated fish. The vitamin E content of the cocoa butter implant was not quantified, but may have contained sufficient to cause a slight reduction in enzyme activity.

A significant positive correlation between plasma vitamin E concentrations and CPK activity was found in these samples ($p<0.05$), but no significant association was found between plasma PK activity and either vitamin E or CPK. Plasma protein concentrations showed a significant positive correlation with CPK activity ($p<0.01$), but no correlation with plasma vitamin

Table 4.20. Effect of vitamin E implantation on plasma chemistry in fish from case I (mean±sd)

Treatment	n	Plasma vitamin E ($\mu g\ ml^{-1}$)	n	Plasma protein ($mg\ ml^{-1}$)
Untreated	13	21±7	14	4.1±0.9
Sham	12	18±4	13	4.4±1.1
Vitamin E	13	24±15	12	4.6±1.0

Table 4.21. Effect of vitamin E implantation on plasma chemistry in fish from case I (mean±sd)

Treatment	n	Plasma CPK ($IU\ ml^{-1}$)	n	Plasma PK ($mU\ ml^{-1}$)
Untreated	15	16±16	15	324±252
Sham	15	14±19	14	239±383
Vitamin E	13	12±9	12	92±62

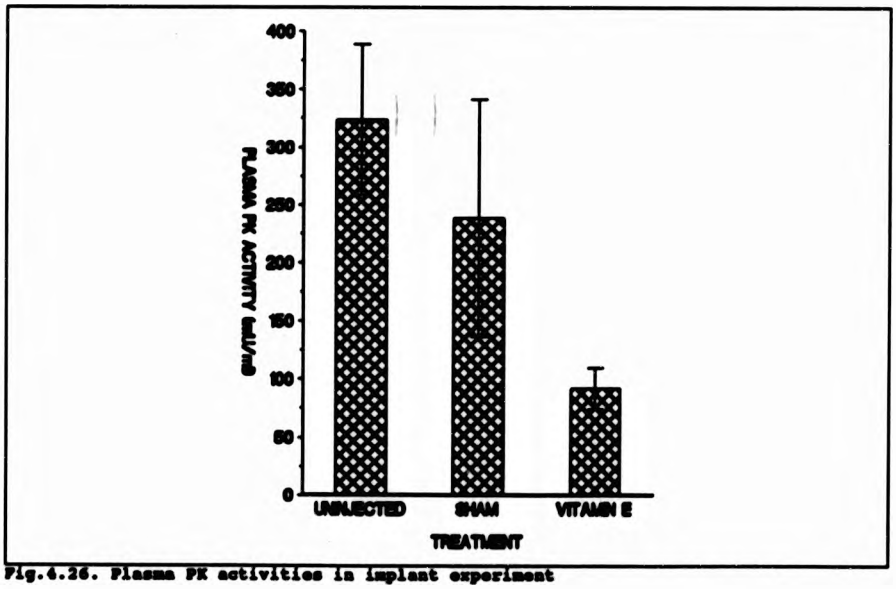

Fig.4.26. Plasma PK activities in implant experiment

E concentration or PK activity. Plasma vitamin E concentrations showed a significant positive correlation with weight ($p<0.05$), but not with condition

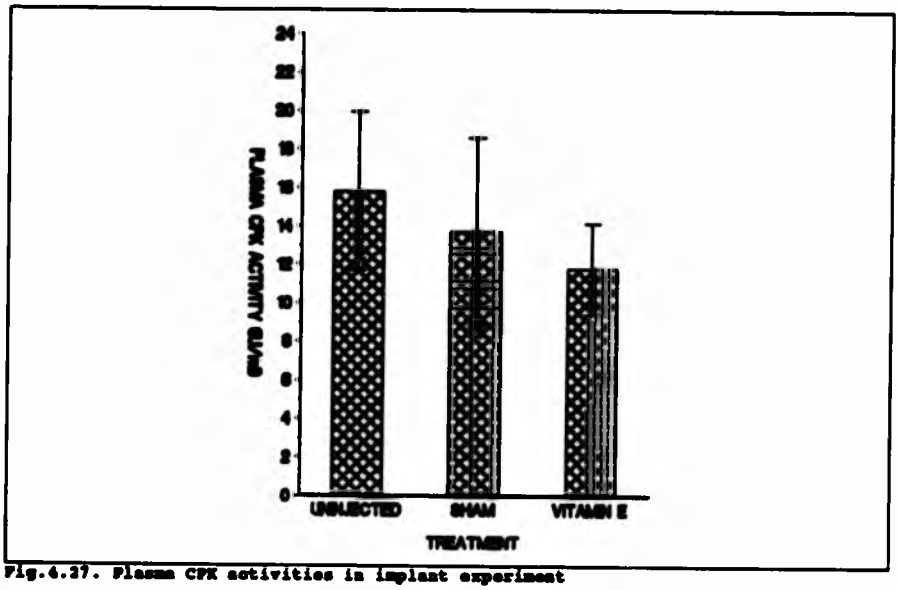

Fig.4.27. Plasma CPK activities in implant experiment

factor. Plasma protein concentrations were correlated with neither. Both plasma CPK and PK activities were positively correlated with weight ($p<0.01$ and $p<0.05$ respectively), but showed no association with condition factor. No exocrine pancreatic tissue was observed in sections from any of the forty-three fish remaining in this study at the completion of treatment. However, eleven of the forty-three fish were found to be feeding; six of these had been given no treatment, three had been sham-treated and two given vitamin E by injection.

Mézes and Vádasz (1984) successfully used vitamin E injection to increase tissue α-tocopherol concentrations in common carp, and Salte *et al.* (1988a) could detect no effect of vitamin E-selenium injection on survival in Atlantic salmon affected by Hitra disease, but showed that tissue concentrations of both these nutrients were increased by this treatment. In contrast to these, and the results of a preliminary study, no significant increase in plasma vitamin E was detected in fish with PD given *all-rac*-α-tocopherol by intra-peritoneal injection. This may be interpreted to indicate that the rate of vitamin E metabolism is increased in fish with PD, or that the dose used was insufficient to cause a significant rise. Nevertheless, this

treatment was effective in reducing plasma PK activity and therefore suggests that muscle degeneration in PD may respond to treatment with vitamin E.

4.3.10 Case J (Site 9)

4.3.10.1 Case history

This site had had a good record of growth in 1988 which was its first year of operation. At the time of the initial visit to this outbreak, fish in cage 9 were already exhibiting the characteristic hanging behaviour typical of PD, but most appeared to be feeding, and this was confirmed by the presence of food in the gut in all fish examined. In contrast, in cage 10 many fish were hanging listlessly, a poor feeding response was observed, and internal examination indicated that fish in this cage had little peri-pancreatic fat. At the second visit, both groups were feeding well and their guts contained food although gut-associated fat deposits were still depleted in some individuals. Muscle lesions were identified by pathologists, from Stirling University, in fish both with and without pancreatic lesions. The mean weight of the fish collected was 140±61 g and the condition factor 1.15±0.19.

4.3.10.2 The effect of a non-esterified dietary vitamin E source

Vitamin E concentrations in feed samples measured in duplicate were 124.4±6.8 mg 100 g^{-1} in the α-tocopherol supplemented diet and 18.5±2.1 mg 100 g^{-1} in the 'high energy' diet. In cage 10, fed the supplemented diet, plasma vitamin E concentrations at the beginning of the trial were found to be 5±3 μg ml^{-1}. After feeding the test diet for a period of 12 days they were unchanged at 5±6 μg ml^{-1}. In the same cage, plasma PK activities rose from 7±8 mU ml^{-1} on the first visit to 710±753 mU ml^{-1} at the end of the trial. The Mann-Whitney U-test indicated that plasma PK activities ($p<0.01$), but not vitamin E concentrations, showed significant differences before and after the study. For comparison, in cage 9 fed the 'high energy' diet, plasma vitamin E concentrations fell from 19±15 to 5±8 μg ml^{-1} between visits, and plasma PK activity showed little change, falling from 202±131 to 124±136 mU ml^{-1}. In

this cage, plasma vitamin E concentration ($p<0.05$), but not plasma PK activity, was significantly changed. There was no significant correlation between plasma vitamin E and pyruvate kinase activity during the course of this study, but both plasma vitamin E and PK showed a positive correlation with fish weight ($p<0.01$ in both cases), and also with condition factor ($p<0.05$ and $p<0.01$ respectively). Histological sections from all 27 fish were examined. Some exocrine tissue was observed in one fish from cage 10 from the second visit, but in the remaining 26, including the other six fish which were fed the test diet, there was no pancreatic acinar tissue. Slight to severe structural changes were noted in sections of heart from three fish including two which had received the α-tocopherol supplemented diet.

4.3.10.3 Stability of α-tocopherol in fish feed

The results of the bench study on the stability of α-tocopherol in fish feed are shown in table 4.21 and fig. 4.24. The vitamin E content of the diet coated with α-tocopherol in fish oil fell sharply from an initial concentration assumed to be 71.5 mg 100 g^{-1}, to 34.3 mg 100 g^{-1} after one week, and then more gradually, to 29.5 mg 100 g^{-1} after 5 weeks. In contrast, concentrations in the control diet, containing only the usual α-tocopheryl acetate supplement, were relatively constant and ranged from 21.5 to 22.7 mg 100 g^{-1}. There was a significant negative rank correlation between vitamin E concentration and time in the test diet ($p<0.01$), and a significant positive correlation ($p<0.05$) in the control diet.

Table 4.22. Stability of α-tocopherol in fish feed

Time (weeks)	0	1	2	3	4	5
Total vitamin E acetate + alcohol (mg kg^{-1})	715*	343	321	314		295
Basal vitamin E acetate only (mg kg^{-1})	215	218	215	221		227

*assumed

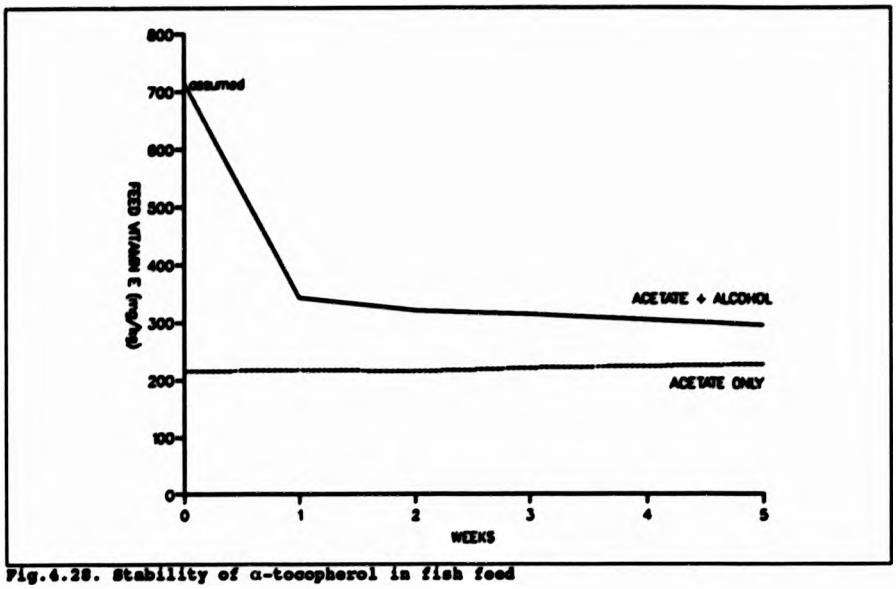

Fig.4.28. Stability of α-tocopherol in fish feed

4.3.11 Case K (Site 4)

4.3.11.1 Case history

An outbreak of PD at site 4 had been studied in 1987, but in 1988 and 1989 there was no sign of the condition despite extensive screening. In 1990, smolts were received in May and the first signs of PD were noticed during the first week of August, following an outbreak of furunculosis. At this time the fish were fed a diet containing 17.5 mg 100 g^{-1} added α-tocopheryl acetate. Fish collected had a mean weight of 112±36 g and an average condition factor of 1.10±0.10. All fish examined had little gut-associated fat. On the first visit all but one of the fish had no food in their guts, but on the second all but two were feeding.

4.3.11.2 Histopathology

Pancreas and muscle sections were examined from three fish collected at the first visit. Pancreatic acinar tissue was observed in two of these, but

all three were considered to show mild cardiac changes.

4.3.11.3 Experiment: the effect of a water-miscible vitamin E source

Plasma vitamin E concentrations on the first visit averaged 15±18 μg ml^{-1}. After a period of 12 days feeding a diet containing a water-miscible form of vitamin E these had risen to 33±27 μg ml^{-1}. However, over the same period, liver vitamin E concentrations fell from 496±364 to 361±352 μg ml^{-1}. Using the Mann-Whitney U-test, no significant differences in either plasma or liver vitamin E concentrations were found before and after the trial, and signs of cardiomyopathy were observed in fish fed the test diet on both occasions. However, the slight increase in plasma vitamin E concentrations observed following short-term supplementation with this water-miscible form suggests that this vitamin E source may be useful if administered over a longer period.

4.3.12 Case L (Site 10)

4.3.12.1 Case history

PD was recognised at site 10 early in September 1990 and progressed as usual for a period of about five weeks. At this time high mortalities began to be experienced in the affected cages. Mortalities occurred among fish in reasonably good condition. Microbiological studies revealed no evidence of an infectious agent, but histopathology showed the presence of cardiomyopathy, and also liver necrosis, in affected fish.

4.3.12.2 Vitamin E

Plasma vitamin E concentrations measured in moribund, listless, and apparently healthy groups of fish from this outbreak averaged 12±14, 15±6 and 40±17 μg ml^{-1} respectively. In the liver, group values were 246±196, 310±175 and 423±74 μg g^{-1}.

4.3.13 Plasma vitamin E concentrations in experimentally-induced PD

Two weeks after challenge, exocrine pancreatic degeneration was found in 65% of the fish injected with kidney material from fish with PD, but the exocrine tissue of all sham injected fish was intact. However, in subsequent weeks this proportion was reduced so that by six weeks post-challenge only 27% of fish challenged with active material showed EPD. No significant difference in susceptibility to EPD was found between group 3 fish treated with dexamethasone, and group 4 controls (R. Raynard, personal communication 1989).

Averaged over the whole experimental period, the mean plasma vitamin E concentration in fish challenged with material from fish with PD was 21±13 μg ml^{-1}, and in sham injected fish was 27±18 μg ml^{-1}. In fish found to have EPD, the mean plasma vitamin E concentration was 14±11 μg ml^{-1}, whilst in fish with intact pancreatic tissue, concentrations of 27±15 μg ml^{-1} were measured. The data was log transformed and analysis of variance revealed significant differences between fish with and without EPD ($p<0.01$), but not between fish subjected to active challenge, and sham-injected controls. This suggests that vitamin E depletion is associated with pancreatic degeneration rather than the challenge itself.

CHAPTER 5

EXPERIMENTAL STUDIES OF THE RELATIONSHIP BETWEEN VITAMIN E STATUS AND PHYSIOLOGICAL STRESS RESPONSES IN FARMED ATLANTIC SALMON

5.1 INTRODUCTION

5.1.1 The effect of stress on vitamin E status

Whilst factors inducing stress in fish may lead to immunosuppression and increased susceptibility to infectious disease, non-specific stressors also provoke a variety of metabolic effects, including changes in nutritional status (reviewed by Mazeaud *et al.* 1977 and Love 1980, chapter 4). Mitchell and Heffron (1982) described various forms of stress syndrome in pigs, which were characterized by muscle degeneration induced by routine husbandry procedures, such as transportation, high temperatures, exercise and fighting. Other stress-induced myopathies have been described in man (Thomson and Robinson 1980), birds (Henschel and Louw 1978), horses (Hammel and Raker 1972), zebra (Hofmeyer *et al.* 1973), antelope (Harthoorn *et al.* 1974; Gericke and Hofmeyr 1976) and deer (Pertz and Sundberg 1978; Ullrey *et al.* 1985). Duthie *et al.* (1988) presented evidence to indicate that the porcine stress syndrome may result from increased peroxidation of cell membrane lipids due to an antioxidant disorder, and Duthie and Arthur (1989) demonstrated that the condition will respond to dietary vitamin E supplementation. Dvoràk (1979) described investigations into the effects of stress on the vitamin E status of pigs, including studies which demonstrated a relationship between adrenocortical activity and plasma vitamin E concentrations. Dvoràk *et al.* (1976) showed that the development of myopathy due to experimental vitamin E deficiency was stimulated by the application of various stressors. Similarly, severe myopathy in vitamin E-selenium deficient yearling cattle can be precipitated by exercise, transportation, and other non-specific stressors (Allen *et al.* 1975; Anderson *et al.* 1977; Van Vleet *et al.* 1977; Koller and Exon 1986).

Inconsistencies in the results of experimental studies of vitamin E deficiency in rainbow trout (Hung *et al.* 1980*b*, 1981; Cowey *et al.* 1981, 1983, 1984) and channel catfish (Murai and Andrews 1974; Lovell *et al.* 1984; Wilson *et al.* 1984) may be partly explained by variations in husbandry procedures, such as stocking density and frequency of handling. The effect of stocking density on vitamin E status in channel catfish (*Ictalurus punctatus*) was

examined by Gatlin *et al.* (1986*b*). In this study, groups of fingerling catfish were held at four different stocking densities. Each of these groups was fed a vitamin E free basal diet, or a diet supplemented with 5.0 mg *all-rac*-α-tocopheryl acetate 100 g^{-1}, for a period of 19 weeks. Increased stocking density significantly reduced growth and feed conversion efficiency, but did not affect plasma cortisol concentrations. There was no significant difference in *in vitro* peroxidation in hepatic microsomes, or other signs of vitamin E deficiency, among groups of fish held at different stocking densities. However, microsomal peroxidation rates were significantly higher in fish fed the vitamin E depleted diet. Tissue vitamin E concentrations were not measured. Since species differences in stress-susceptibility are known to occur (Pickering 1981), similar studies with different fish species are desirable.

Factors inducing stress in fish have repeatedly been shown to precede the occurrence of overt PD (McVicar 1987). In searching for the cause of vitamin E depletion in fish with PD, the hypotheses that factors causing non-specific stress responses in farmed salmon resulted in tissue vitamin E depletion and/or in the expression of signs of vitamin E deficiency were developed. Studies aimed at testing these ideas are described in Experiments 1, 2 and 3 below.

5.1.2 Vitamin E status and stress-susceptibility

In aquaculture, manipulation of the diet may provide a means for the regulation of the effects of stress. Barton *et al.* (1988), working with juvenile chinook salmon, found that the dietary lipid composition affected the magnitude of some metabolic responses to handling and confinement. Mazik *et al.* (1987) found dietary vitamin C supplementation to improve tolerance of channel catfish to high ammonia and low dissolved oxygen concentrations. Recently, combined vitamin C and E dietary supplements have been suggested to reduce losses of farmed salmon following handling (J.Roberts, personal communication 1990). The effects of vitamin E supplementation on the cortisol response to handling and on responses to crowding were studied in Experiments 4 and 5.

In both sets of experiments, plasma and liver vitamin E concentrations were determined, and plasma malondialdehyde concentrations, haematocrit, plasma pyruvate kinase activities and muscle histology were variously used as indicators of vitamin E status. Plasma cortisol concentrations were used to quantify the response to acute stressors and leucocrit and growth rate were used as indices of the degree of chronic stress.

5.2 MATERIALS AND METHODS

These studies were conducted over a period of 18 months using Atlantic salmon obtained as S1 smolts from Howietown Fish Farm, Stirlingshire. The holding facilities used for the experiments are described in appendix 1. Fish were transferred from freshwater directly into the experimental tanks in May 1988. They were fed a commercial diet at a rate of 1-3% body weight per day according to body size and water temperature, and were fed twice daily Monday to Friday and once daily (half ration) at weekends. Records of feeding regime, mortalities, and water temperature were kept throughout the time the fish were resident in the experimental unit.

5.2.1 Experiment 1

In August 1988, a total of 1158 fish (mean weight 126 g) were pooled and counted at random, 193 per tank, into each of the six experimental tanks. They were then allowed to recover for 8 weeks during which time they were fed the usual commercial diet, containing 16.4 mg 100 g^{-1} vitamin E, at 2.5% body weight day^{-1}. These were used in an experiment to study the effects of short-term crowding and handling on tissue vitamin E concentrations.

On day 0, six fish were captured from each tank as a baseline sample. The following treatments were then allocated at random, two tanks per treatment:

1) unhandled control
2) crowding only
3) crowding and netting

The treatments were applied as follows. Fish in the control tanks were left undisturbed. Those subjected to crowding only were exposed to a lowering of the water level, to about 20 cm depth, and refilling over a period of about 15 min. Those subjected to crowding and handling were exposed to a reduction in water depth as above; they were then netted into a 2 m diameter, aerated tank and, after a period of approximately 10 min, returned to their original tank.

Six fish from each tank were sampled after 4 hrs, 1 day, 2 days, 3 days and 4 days as described in section 2.1.2. No food was offered for the duration of the experiment. The water temperature during this experiment ranged from 11.0 to 12.2°C. Plasma α-tocopherol, malondialdehyde and cortisol concentrations were measured as described in section 2.2. The resulting data sets were tested for normal distribution and transformed if necessary. Analysis of variance was used to examine the effects of treatment, time and fish number. For clarity, the analysis was restricted to data from either groups 1 and 2 or groups 1 and 3. A partially nested, partially crossed design was utilised, with tank as a blocking factor and treatment, time and fish number and their interactions as treatment factors in the analysis. The T-method of multiple pairwise comparison was used to further investigate significant treatment-time effects. Alternatively, if the data were not normally distributed, the non-parametric Mann-Whitney U-test was used to compare group means at each time. Tests for correlation between the various data sets were made using Pearson's or Spearman's tests as appropriate.

5.2.2 Experiment 2

Fish remaining from Experiment 1 were pooled and distributed at random, 155 fish per tank, into the six experimental tanks. They were held for five weeks to recover, and fed the same commercial diet at 1.5% body weight day^{-1}. An experiment was then conducted to examine the effect of chronic crowding on tissue vitamin E concentrations. Since crowding is known to affect food intake, an unfed control group was also used (Pickering 1981).

On day 0, a baseline sample of 6 fish per tank was collected. The following treatments were then applied to duplicate tanks, again assigned at

Table 5.1. Stocking densities in Experiment 2

Time	Stocking density (kg m^{-3})		
	Control	Unfed	Crowded
0 hr	7.3	7.5	20.6
3 day	6.8	7.3	16.2
1 week	7.0	5.7	19.3
2 weeks	7.5	5.7	18.1
3 weeks	7.5	5.5	17.6
4 weeks	6.7	4.9	17.5

random.

1) control
2) unfed
3) crowded

Stocking densities throughout the experiment are shown in table 5.1. Fish in groups 1 and 2 were held in tanks with a water depth of 80 cm, volume approximately 7 m^3, whilst group 3 fish were held in water depth 20 cm, giving a volume of 2.5 m^3 for the duration of the experiment. Fish subjected to treatments 1 and 3 were offered food at a rate of 1.25% body weight day^{-1}. Fish in group 2 were deprived of food for the duration of the experiment, but were disturbed at feeding time by lifting the tank lid. The diet used was the usual commercial diet with a measured vitamin E content of 16.4 mg 100 g^{-1}. During the study period the seawater temperature fell from 10.7 to 9.8°C.

Table 5.2. Vitamin E content of diets used in Experiment 3

Diet	n	Vitamin E content (mg kg^{-1})
Regular	6	160±37
Low Vitamin E	6	111±29

Six fish per tank were sampled at 3 days, 1 week, 2 weeks, 3 weeks and 4 weeks according to the procedure detailed in section 2.1.2. Plasma and liver vitamin E and plasma malondialdehyde concentrations were measured using the

methods given in section 2.2, and haematocrit and leucocrit were measured as described in section 2.3. The results were subjected to statistical analysis as above, and analyses of variance were again restricted to groups 1 and 2 and groups 1 and 3. In addition, tests for correlation with water temperature were performed using Spearman's test and data from the control group 1.

5.2.3 Experiment 3

The remaining fish were pooled and redistributed at random among the six tanks, 118 per tank. These were allowed to recover for three weeks before the third experiment began, and during this period were fed the commercial diet at 1.25 % body weight day^{-1}. These fish were used to study the effect of crowding on tissue vitamin E concentrations and indicators of muscle degeneration in fish fed a diet lacking supplementary vitamin E.

Table 5.3. Stocking densities in Experiment 3

Time (months)	Stocking density (kg m^{-3})		
	Regular diet Uncrowded	Low vitamin E Uncrowded	Low vitamin E Crowded
0	8.7	9.9	23.3
1	9.1	8.4	22.6
2	9.5	10.4	24.6
3	10.8	10.4	21.8
4	9.7	10.9	27.4
5	14.0	14.4	32.9
6	14.6	13.7	33.7

The low vitamin E diet was of a commercial-type supplied by BP Nutrition Ltd. with no supplementary vitamin E. The regular diet was to a standard formulation. The results of vitamin E determinations on the experimental diets are shown in table 5.2. On day 0, a baseline sample of five fish per tank was collected and the fish subjected to the following treatments assigned to duplicate tanks at random.

1) regular diet; uncrowded

2) low vitamin E diet; uncrowded

3) low vitamin E diet; crowded

A feeding rate of 1.25% body weight day^{-1} was employed except during the first month when 1.1% was used. Stocking densities throughout the experiment are shown in table 5.3. As in Experiment 2, uncrowded fish (groups 1 and 2) were held in water depth 80 cm, volume 7 m^3 and crowded fish (group 3) in water depth 20 cm, volume 2.5 m^3. The seawater temperature ranged between 8.0 and 14.0°C during the course of this study.

Five fish per tank were sampled, as described in section 2.1.2, every 4 weeks between 1 and 6 months after the experiment began. Plasma and liver vitamin E concentrations, plasma malondialdehyde and protein concentrations and plasma pyruvate kinase activities were measured as described in section 2.2. Samples for the determination of haematocrit and leucocrit were collected as described in section 2.3. Samples for histology were collected and processed as detailed in section 2.4. These results were statistically analyzed as described previously, although in this case the analyses of variance were restricted to groups 1 and 2, and 2 and 3. In addition, differences between males and females were tested using analysis of variance with sex as a treatment factor.

5.2.4 Experiment 4

Fish subjected to treatments 1 and 2 in Experiment 3 were utilised in a further experiment without re-randomization. These groups had been reared for six months on diets with and without supplementary vitamin E respectively. In Experiment 4, the effects of these treatments on responses to acute stress were examined.

Eighty-two fish in each of the four tanks continued to be fed the experimental diets for a five day period after the completion of Experiment 3. On day 0, a baseline sample of five fish per tank was collected and both groups were subjected to a short period of crowding and handling. The treatments here were as follows:

1) regular diet; crowding and netting

2) low vitamin E diet; crowding and netting

Five fish per tank were sampled at four hours, 1 day, 3 days and 5 days as described in section 2.1.2. The fish were not fed for the duration of this experiment. Plasma vitamin E, malondialdehyde and cortisol concentrations were determined as described in section 2.2. Haematocrit and leucocrit were measured as described in section 2.3. The results were compared using analysis of variance and correlation tests as described above, except that during analysis of variance of the biochemical data only treatment and time were employed as treatment factors. In the case of non-normally distributed data, the Mann-Whitney U-test was again used the to compare treatment means at each time. Differences due to sex or state of maturity were tested using a single-factor analysis of variance.

5.2.5 Experiment 5

The remaining fish (384 in total) were pooled and distributed at random, 64 per tank, into the six experimental tanks. They were left to recover for three weeks and fed at a rate of 0.5% body weight day^{-1} on the usual commercial diet. Subsequently, they were used to examine the effects of graded dietary vitamin E supplements on responses to chronic crowding stress.

Table 5.4. Preparation of diets used in Experiment 5

Treatment	Vitamin E supplement		Oil (kg)	Feed (kg)	n	Measured dietary vitamin E (mg kg^{-1})
	(g)	(mg kg^{-1})				
1	0	0	1.5	25	2	51±1
2	2.5	50	1.5	25	4	107±20
3	10	200	1.5	25	4	217±12

The basal diets were again of commercial type, supplied by BP Nutrition without supplementary vitamin E or carotenoid pigment, and were not pre-coated with fish oil. Three experimental diets were prepared to contain 0, 5.0 and

20.0 mg added vitamin E 100 g^{-1} diet as shown in table 5.4. Rovimix E50 adsorbate (Roche Ltd., Herts.) was used as the vitamin E source. This preparation consists of *all-rac*-α-tocopheryl acetate adsorbed onto silicic acid and contains 500 mg kg^{-1} α-tocopheryl acetate. The appropriate quantity of Rovimix E50 adsorbate was mixed into fish oil using a Hobart food mixer, and then coated onto the feed, at 6% oil by weight, using a cement mixer. The measured vitamin E contents of each of the experimental diets are also shown in table 5.4.

Table 5.5. Stocking densities in Experiment 5

Time (weeks)	Stocking density (kg m^{-3})		
	Crowded 0 mg kg^{-1}	Crowded 50 mg kg^{-1}	Crowded 200 mg kg^{-1}
0	21.3	24.1	28.4
1	23.8	25.6	20.3
3	23.1	22.2	20.6
6	16.0	17.3	19.2
9	15.8	20.3	17.3
12	17.7	20.8	17.1

On day 0, five fish per tank were collected as a baseline sample. The following treatments were then allocated at random to duplicate tanks:

1) crowded; 0 mg 100 g^{-1} added vitamin E
2) crowded; 5.0 mg 100 g^{-1}
3) crowded; 20.0 mg 100 g^{-1}

All three groups of fish were held in water depth 20 cm, volume 2.5 m, for the duration of the experiment. Stocking densities are shown in table 5.5. The diets were fed at a rate of 0.4% body weight day^{-1}. Water temperature during this experiment fell from 11.4 to 8.9°C.

Five fish per tank were sampled at 1 week, 3 weeks, 6 weeks, 9 weeks and 12 weeks as described in section 2.1.2. Plasma and liver vitamin E and plasma malondialdehyde concentrations were determined as described in section 2.2, haematocrit and leucocrit were measured as in section 2.3, and tissues were processed for histology as detailed in section 2.4. Statistical analysis was

as before, except that all three treatment groups were compared in one analysis of variance, and tests for correlation with water temperature were performed using entire data sets rather than any control group as previously.

5.3 RESULTS AND DISCUSSION

5.3.1 Experiment 1

Table 5.6. Plasma α-tocopherol concentrations in Experiment 1 (mean±sd)

Time	Treatment	n	Plasma α-tocopherol ($\mu g\ ml^{-1}$)
0 hrs	Unhandled	6	113±54
	Crowded	6	65±35
	Netted	6	107±61
4 hrs	Unhandled	6	54±14
	Crowded	6	66±25
	Netted	6	79±24
1 day	Unhandled	6	58±14
	Crowded	6	66±17
	Netted	6	41±17
2 days	Unhandled	6	48±15
	Crowded	6	36±13
	Netted	6	49±16
3 days	Unhandled	6	45±14
	Crowded	6	25±18
	Netted	6	33±15
4 days	Unhandled	6	51±16
	Crowded	6	47±28
	Netted	6	53±18

Mean plasma α-tocopherol concentrations for each treatment at each time in Experiment 1 are shown in table 5.6. Figs 5.1 and 5.2 show plasma α-tocopherol concentrations in groups of fish subjected to crowding and crowding and handling in relation to controls. In the unhandled control group 1, plasma vitamin E concentrations fell from a maximum of 113±54 $\mu g\ ml^{-1}$ at 0 hrs, to 54±14 $\mu g\ ml^{-1}$ at 4 hrs and thereafter stayed fairly constant, falling to only

51±16 μg ml^{-1} at 4 days. Lower initial levels were measured in group 2 which was subjected to short-term crowding only. In this group, plasma vitamin E concentrations of 65±35, 66±25 and 66±17 μg ml^{-1} were measured at 0 hrs, 4 hrs and 1 day respectively. These then fell slightly and between 2 and 4 days ranged between 25±18 and 47±28 μg ml^{-1}. In group 3, subjected to both crowding and handling, plasma concentrations of 107±61 μg ml^{-1} were measured at 0 hrs. These fell to 79±24 μg ml^{-1} at 4 hrs and 41±17 μg ml^{-1} at 1 day, and between days 2 and 4 ranged from 33±15 to 53±18 μg ml^{-1}. The data from both groups 1 and 2 and 1 and 3 were subjected to square root transformation prior to analysis of variance. Significant effects of time ($p<0.001$) were found within the data from groups 1 and 2 and 1 and 3 and, in addition, treatment-time-number interactions ($p<0.01$) were detected in the comparison of groups 1 and 3.

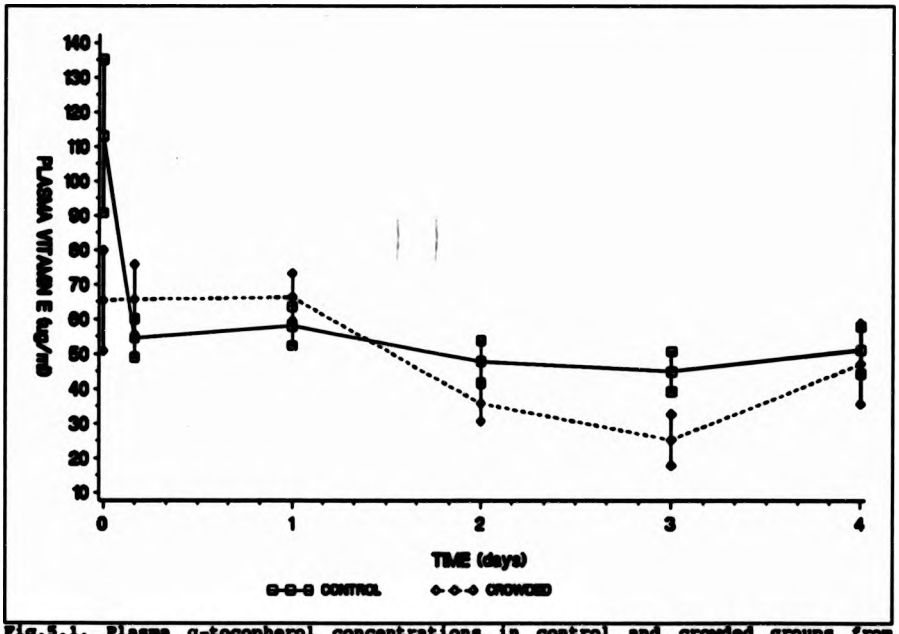

Fig.5.1. Plasma α-tocopherol concentrations in control and crowded groups from Experiment 1 (mean±se)

Plasma malondialdehyde concentrations are summarized in table 5.7. In group 1, they rose from 2±2 μmol l^{-1} at 0 hrs, to a maximum of 17±13 μmol l^{-1} after 4 days. In the crowded group 2, they ranged more widely, but also showed

Table 5.7. Plasma malondialdehyde concentrations in Experiment 1 (mean±sd)

Time	Treatment	n	Plasma malondialdehyde (μmol l^{-1})
0 hrs	Unhandled	6	2±1
	Crowded	5	6±2
	Netted	6	4±2
4 hrs	Unhandled	5	4±2
	Crowded	6	4±2
	Netted	5	4±2
1 day	Unhandled	6	13±5
	Crowded	6	1±0
	Netted	6	13±5
2 days	Unhandled	6	6±2
	Crowded	5	3±1
	Netted	6	2±1
3 days	Unhandled	6	7±3
	Crowded	6	14±6
	Netted	6	10±4
4 days	Unhandled	6	13±5
	Crowded	6	10±4
	Netted	6	4±2

an overall increase, from 8±6 μmol l^{-1} at 0 hrs to 9±3 at 2 and 27±14 μmol l^{-1} at 3 days, and to 22±10 μmol l^{-1} at 4 days. In group 3, they increased from 6±4 μmol l^{-1} at 0 hrs to 22±10 at 3, and 14±4 μmol l^{-1} at 4, days. These data were subjected to square root transformation before analysis of variance and, in comparisons of both groups 1 and 2 and 1 and 3, significant effects of time ($p<0.001$), but no treatment effects were found. Pearson's test showed that plasma vitamin E and malondialdehyde concentrations were negatively correlated ($p<0.01$).

Plasma cortisol concentrations are given in table 5.8 and illustrated in figs. 5.3 and 5.4. Throughout the study, mean cortisol concentrations remained below 5 ng ml^{-1} in all groups. In the control group, they measured 2.4±2.2 ng ml^{-1} at 0 hrs and 2.6±2.8 ng ml^{-1} at 4 hrs, fell to 0.3±0.2 ng ml^{-1} at 1 day, and rose to 1.4±2.7 after 4 days. In the crowded group they

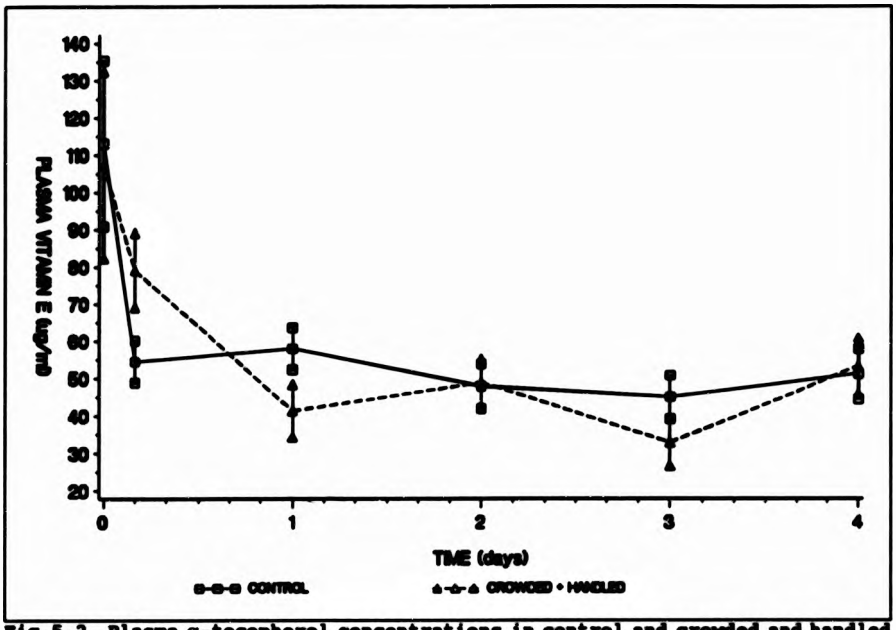

Fig.5.2. Plasma α-tocopherol concentrations in control and crowded and handled groups from Experiment 1 (mean±se)

decreased from 3.0±3.8 ng ml^{-1} at 0 hrs to 0.6±0.6 at 4 hrs and, between 1 and 4 days, ranged from 0±0.1 to 1.5±1.8 ng ml^{-1}. In group 3, subjected to crowding and handling, they rose from a 0 hr mean of 3.1±2.7 to 4.8±2.3 ng ml^{-1} at 4 hrs, then fell to below 0.1 ng ml^{-1} on days 2 and 3 and 0.7±0.5 ng ml^{-1} on day 4. These data could not be normalized by either square root or log transformation and were therefore analyzed using non-parametric methods. Mann-Whitney U-tests carried out on the data from groups 1 and 2 and 1 and 3 at each time revealed significant differences only between groups 1 and 3 at 1 day ($p<0.05$). Spearman's correlation test showed a significant positive association between plasma cortisol and vitamin E concentrations and a significant negative association between plasma cortisol and malondialdehyde concentrations ($p<0.001$).

Weight and condition factor data are given in table 5.9. Mean fish weights in each treatment group at each time, ranged from 204±48 to 260±32 g and condition factors varied between 1.34±0.07 and 1.72±0.12. The untransformed weight data was used for analyses of variance. In both analyses,

Table 5.8. Plasma cortisol concentrations in Experiment 1 (mean±sd)

Time	Treatment	n	Plasma cortisol (ng ml^{-1})
0 hrs	Unhandled	6	2.4±2.2
	Crowded	6	3.0±3.8
	Netted	6	3.1±2.7
4 hrs	Unhandled	6	2.6±2.8
	Crowded	6	0.6±0.6
	Netted	6	4.8±2.3
1 day	Unhandled	6	0.3±0.2
	Crowded	6	0.2±0.2
	Netted	6	1.3±1.0
2 days	Unhandled	6	0.9±1.3
	Crowded	6	1.5±1.8
	Netted	6	0.0±0.0
3 days	Unhandled	6	0.7±1.2
	Crowded	6	0.0±0.1
	Netted	6	0.0±0.1
4 days	Unhandled	6	1.4±2.7
	Crowded	6	0.7±1.4
	Netted	6	0.7±0.5

significant effects of weight ($p<0.05$), but no treatment effects were detected. Similarly condition factor, again untransformed, showed significant time-wise differences ($p<0.001$) when the data was restricted to treatments 1 and 2 and 1 and 3. Treatment-time interactions were also detected ($p<0.05$) within the data restricted to groups 1 and 3, but pairwise multiple comparisons failed to detect any significant differences between these treatments at any time. Using Pearson's test, significant positive correlation coefficients were calculated between plasma vitamin E concentrations and fish weight ($p<0.01$) and condition factor ($p<0.001$), and significant negative associations were found between plasma malondialdehyde concentrations and weight ($p<0.05$) and condition factor ($p<0.001$). Spearman's test showed that plasma cortisol concentrations were also positively correlated with weight ($p=0.05$) and condition factor ($p<0.05$).

Table 5.9. Weight and condition factor of fish from Experiment 1 (mean±sd)

Time	Treatment	n	Weight (g)	Condition factor
0 hrs	Unhandled	12	239±59	1.63±0.13
	Crowded	12	249±45	1.61±0.21
	Netted	12	236±43	1.72±0.12
4 hrs	Unhandled	12	260±32	1.54±0.08
	Crowded	12	258±54	1.50±0.12
	Netted	12	248±48	1.52±0.10
1 day	Unhandled	12	252±51	1.52±0.07
	Crowded	12	221±30	1.50±0.05
	Netted	12	224±56	1.43±0.08
2 days	Unhandled	12	241±50	1.46±0.06
	Crowded	12	229±52	1.44±0.11
	Netted	12	231±41	1.44±0.05
3 days	Unhandled	12	238±39	1.44±0.09
	Crowded	12	213±44	1.42±0.10
	Netted	12	225±37	1.41±0.11
4 days	Unhandled	12	208±53	1.35±0.09
	Crowded	12	222±42	1.43±0.10
	Netted	12	204±48	1.34±0.07

In Experiment 1, cortisol concentrations of between 0 and 3 ng ml^{-1} were measured in unhandled and crowded groups of fish. These seem to be within the normal resting range for Atlantic salmon (Pickering *et al.* 1987*b*). A transient plasma cortisol elevation was noted at four hours post-treatment in fish subjected to crowding and handling, but normal levels were recovered by 1 day. Plasma cortisol elevation is a well recognised response to acute stress (Donaldson 1981) and was used in the present study to give some indication of the severity of the experimental procedures. In this respect it is interesting that the crowding procedure, whilst clearly causing increased activity in the fish, did not lead to any detectable cortisol elevation.

No changes in plasma α-tocopherol concentrations as a result of either crowding or crowding and handling were discernible in the current study. In addition, higher cortisol concentrations were not associated with lower

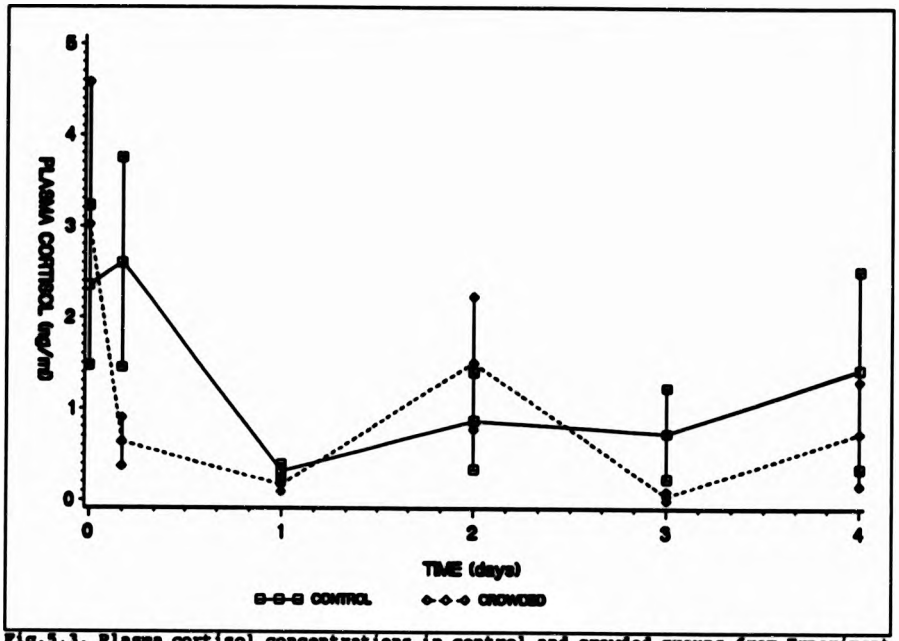

Fig.5.3. Plasma cortisol concentrations in control and crowded groups from Experiment 1 (meanise)

vitamin E concentrations, rather a positive correlation was detected. Dvorák (1979) found that stress in pigs caused either an increase or a decrease in plasma vitamin E concentrations depending upon its duration. In the present study, changes in plasma vitamin E concentrations, in all groups during the course of the experiment, reflected differences in the weight and condition factor of fish sampled.

Hayashi and Takagi (1977) reported high thiobarbituric acid values in tissue lipids of *Sardinops melanostica* affected by the severe stress of gill-net capture. These may have resulted from increased oxygen consumption and tissue lipid peroxidation. In the present study, there was no increase in the degree of peroxidation, as assessed by plasma malondialdehyde concentrations, following either acute stress. However, plasma vitamin E and malondialdehyde concentrations showed a significant negative correlation, suggesting that malondialdehyde production is suppressed in fish with higher tissue vitamin E concentrations. Muscle thiobarbituric acid values were found by Watanabe *et al.* (1970*b*) to be elevated in vitamin E-deficient carp.

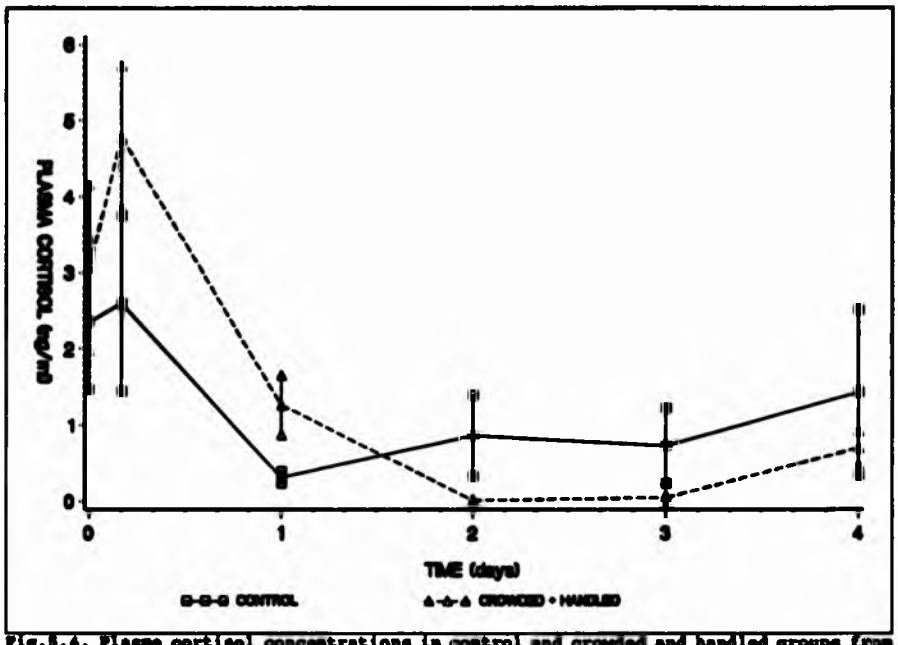

Fig.5.4. Plasma cortisol concentrations in control and crowded and handled groups from Experiment 1 (means±se)

5.3.2 Experiment 2

Plasma α-tocopherol concentrations measured in samples from Experiment 2 are summarized in table 5.10. The data from groups 1 and 2 are compared in fig. 5.5 and from groups 1 and 3 in fig. 5.6. In the control group 1, plasma vitamin E concentrations fell from 53±14 µg ml^{-1} on day 0, to 46±9 µg ml^{-1} at 1 week, then rose to 58±8 µg ml^{-1} at 4 weeks. In the unfed group 2, they fell from 51±12 µg ml^{-1} on day 0 to 36±22 µg ml^{-1} at 1 week, rose to 51±19 µg ml^{-1} at 2 weeks, then dropped to 17±12 µg ml^{-1} at 3, and 16±8 at 4 weeks. In group 3, subjected to chronic crowding, they fell from 50±6 µg ml^{-1} on day 0, to a minimum of 38±15 µg ml^{-1} at 2 weeks, then increased to 46±5 µg ml^{-1} after 4 weeks. These were lower than controls throughout the experiment. No transformation of the data, prior to analysis of variance, was necessary for either treatment comparison. Significant treatment ($p<0.05$) and treatment-time ($p<0.01$) effects were detected by analysis of variance of the data from treatments 1 and 2. Pairwise multiple comparisons showed that plasma vitamin

Table 5.10. Plasma α-tocopherol concentrations in Experiment 2 (mean±sd)

Time	Treatment	n	Plasma vitamin E ($\mu g\ ml^{-1}$)
0 days	Control	6	53±14
	Unfed	6	51±12
	Crowded	6	50±6
3 days	Control	6	48±22
	Unfed	6	45±14
	Crowded	6	43±18
1 week	Control	6	46±9
	Unfed	6	36±22
	Crowded	6	40±14
2 weeks	Control	6	49±9
	Unfed	6	51±19
	Crowded	6	38±15
3 weeks	Control	6	56±23
	Unfed	6	17±12
	Crowded	6	46±16
4 weeks	Control	6	58±8
	Unfed	6	16±8
	Crowded	6	46±5

E concentrations were significantly lower in the unfed group than in controls at 3 and 4 weeks ($p<0.05$). No significant treatment effects were detected between groups 1 and 3 and no time effects were found in either comparison.

Liver vitamin E concentrations are shown in table 5.11 and figs. 5.7 and 5.8. In the control group these fell gradually, from 355±183 $\mu g\ g^{-1}$ on day 0 to 231±91 $\mu g\ g^{-1}$ at 3 weeks, then increased to 453±131 $\mu g\ g^{-1}$ at 4 weeks. In the unfed group they fell more steeply, from 354±67 $\mu g\ g^{-1}$ on day 0, to 57±36 $\mu g\ g^{-1}$ at 3 weeks and 98±40 $\mu g\ g^{-1}$ at 4 weeks. In the crowded group they also showed an initial reduction, from 346±153 $\mu g\ g^{-1}$ on day 0 to 210±131 at 1 week, but then recovered to 409±144 $\mu g\ g^{-1}$ at 3 weeks and 330±119 $\mu g\ g^{-1}$ at 4 weeks. Again no transformation of the data was necessary prior to analysis of variance for either treatment comparison. Significant treatment-time interactions ($p<0.01$), and effects of time ($p<0.01$), were detected by analysis

Table 5.11. Liver α-tocopherol concentrations in Experiment 2 (measured)

Time	Treatment	n	Liver α-tocopherol ($\mu g\ g^{-1}$)
0 days	Control	6	355±183
	Unfed	6	354±67
	Crowded	6	346±153
3 days	Control	6	332±89
	Unfed	6	326±118
	Crowded	6	328±114
1 week	Control	6	294±89
	Unfed	6	240±178
	Crowded	6	210±131
2 weeks	Control	6	340±129
	Unfed	6	237±123
	Crowded	6	243±100
3 weeks	Control	6	231±91
	Unfed	6	57±36
	Crowded	6	409±144
4 weeks	Control	6	453±131
	Unfed	6	108±98
	Crowded	6	330±119

of variance of the data from treatments 1 and 2. Pairwise multiple comparisons showed that the means in the control and unfed groups of fish differed significantly at 4 weeks ($p<0.05$). No significant effects were detected by analysis of variance using data from groups 1 and 3. Pearson's test showed a positive correlation between plasma and liver vitamin E concentrations in this study ($p<0.01$).

The results of plasma malondialdehyde determinations are shown in table 5.12. In group 1, plasma malondialdehyde concentrations of 30±9 $\mu mol\ l^{-1}$ were measured on day 0; these fell to 11±6 $\mu mol\ l^{-1}$ at 2 weeks, then increased to 18±9 $\mu mol\ l^{-1}$ by 4 weeks. In groups 2 and 3 a similar pattern was observed. In the unfed fish, they fell from 23±4 $\mu mol\ l^{-1}$ on day 0, to 1±2 $\mu mol\ l^{-1}$ at 2 weeks, then rose to 18±7 $\mu mol\ l^{-1}$ at 3 and 11±3 at 4 weeks, whilst in the crowded group they fell from 23±13 $\mu mol\ l^{-1}$ on day 0, to 6±5 $\mu mol\ l^{-1}$ at 2

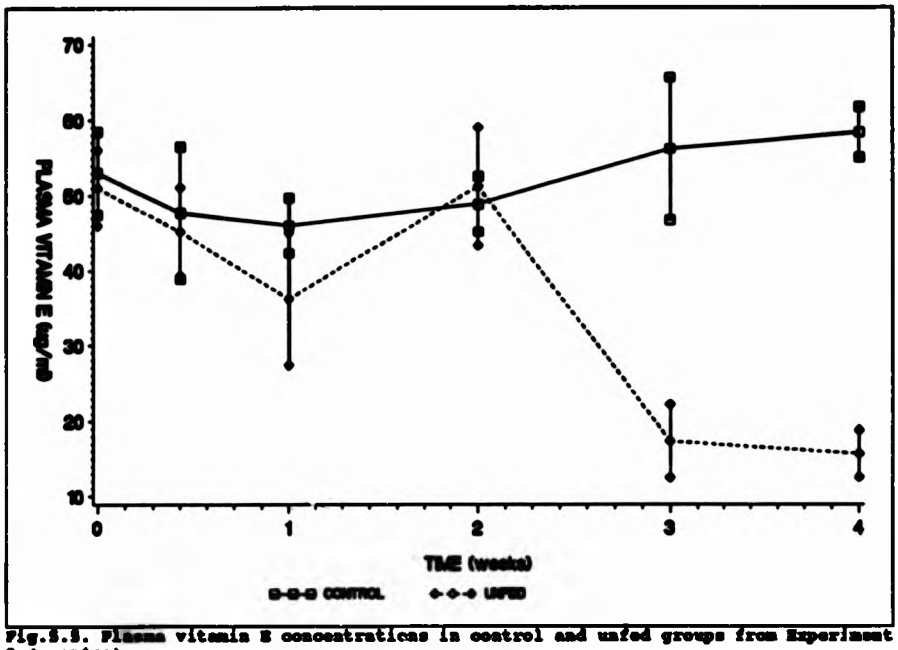

Fig.5.8. Plasma vitamin E concentrations in control and unfed groups from Experiment 2 (means±se)

weeks, then increased to 18±9 μmol l^{-1} at 4 weeks. Square root transformations were used prior to statistical analysis. In both cases analysis of variance revealed significant changes with time ($p<0.001$), but no treatment or treatment-time effects. In addition, significant effects of fish number were detected ($p<0.05$) in the comparison of groups 1 and 2. The untransformed data was used for correlation analyses. In this study no significant correlation between plasma vitamin E and malondialdehyde concentrations was found.

Haematocrit and leucocrit data are given in table 5.13. Changes in haematocrit in the experimental groups are shown in figs 5.9 and 5.10. In the control group, haematocrits fell from 42±3% on day 0 to 39±3% at 3 days and 38±2% after 1 week, then recovered to 42±3% at 3 weeks and 41±5% at 4 weeks. In the unfed group, they fell from 40±2% on day 0 to 39±4% on day 3, increased to 43±7% at 1 and 43±3% at 3 weeks, then dropped to 37±4% at 4 weeks. In the crowded group, they measured 41±3% on day 0 and 41±2% on day 3, rose to 42±5% at 1 week, fell to 37±4% at 3 weeks, and then increased to 40±4% at week 4. Analysis of variance of the untransformed data from groups 1 and 2 and 1 and

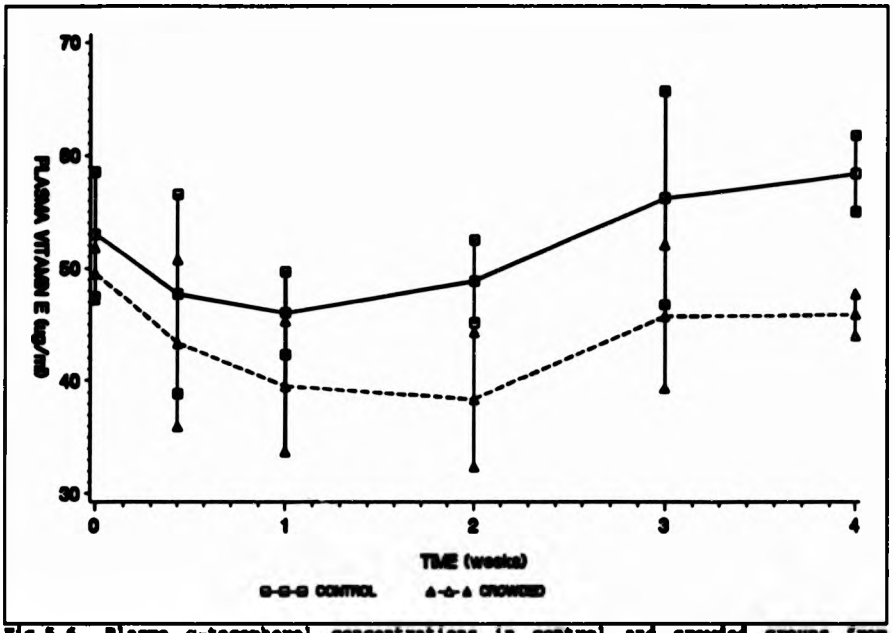

Fig.5.6. Plasma α-tocopherol concentrations in control and crowded groups from Experiment 1 (mean±se)

3 revealed significant effects of fish number ($p<0.001$), with higher haematocrits being measured in fish bled later rather than sooner. Significant effects of time were detected in the comparison between groups 1 and 2, but not between groups 1 and 3. Significant treatment-time interactions were found ($p<0.001$) in both cases, but multiple comparisons detected significant differences only between treatments 1 and 3 at 3 weeks ($p<0.05$). There was no correlation between haematocrits and either plasma or liver vitamin E concentrations, but a significant positive association was detected between haematocrits and plasma malondialdehyde concentrations ($p<0.01$).

Leucocrit measurements are shown in figs 5.11 and 5.12. In the control group, leucocrits of 1.19±0.23%, 1.21±0.19% and 1.19±0.20% were recorded at day 0, 1 day and 1 week respectively, but these fell sharply and between 2 and 4 weeks ranged from 1.02±0.09 to 1.03±0.14%. In the unfed group they fell irregularly, from a maximum of 1.15±0.11% on day 0 to a minimum of 1.03±0.15% at 4 weeks, and in the crowded group they showed an initial fall, from 1.14±0.15% on day 0 to 1.05±0.22% at 1 week, but then recovered to 1.19±0.06%

Table 5.12. Plasma malondialdehyde concentrations in Experiment 2 (meantsd)

Time	Treatment	n	Plasma malondialdehyde (μmol l^{-1})
0 days	Control	6	30±9
	Unfed	6	23±4
	Crowded	6	23±13
3 days	Control	6	15±4
	Unfed	6	12±10
	Crowded	6	12±6
1 week	Control	6	11±6
	Unfed	6	8±10
	Crowded	6	14±6
2 weeks	Control	6	6±8
	Unfed	6	1±2
	Crowded	6	6±5
3 weeks	Control	6	17±3
	Unfed	6	18±7
	Crowded	6	8±6
4 weeks	Control	6	18±9
	Unfed	6	11±3
	Crowded	6	18±9

on week 4. Again there was no need for transformation prior to analysis of variance for either treatment pair. Significant effects of time ($p<0.01$) were detected by analysis of variance of the data from groups 1 and 2. Analysis of variance of the data from groups 1 and 3 revealed significant treatment-time interactions ($p<0.05$), but no significant differences were detected between the means at any time using multiple pairwise comparisons. There was no significant correlation between leucocrits and haematocrits, nor between leucocrits and plasma or liver vitamin E or plasma malondialdehyde concentrations.

Weight and condition factor data are given in table 5.14. Condition factors in groups 1 and 2 are illustrated in fig.5.13, and in groups 1 and 3 in fig. 5.14. During the course of this study, fish in the control group grew from 343±85 g on day 0, to 392±88 g at 4 weeks, fish in the unfed group lost

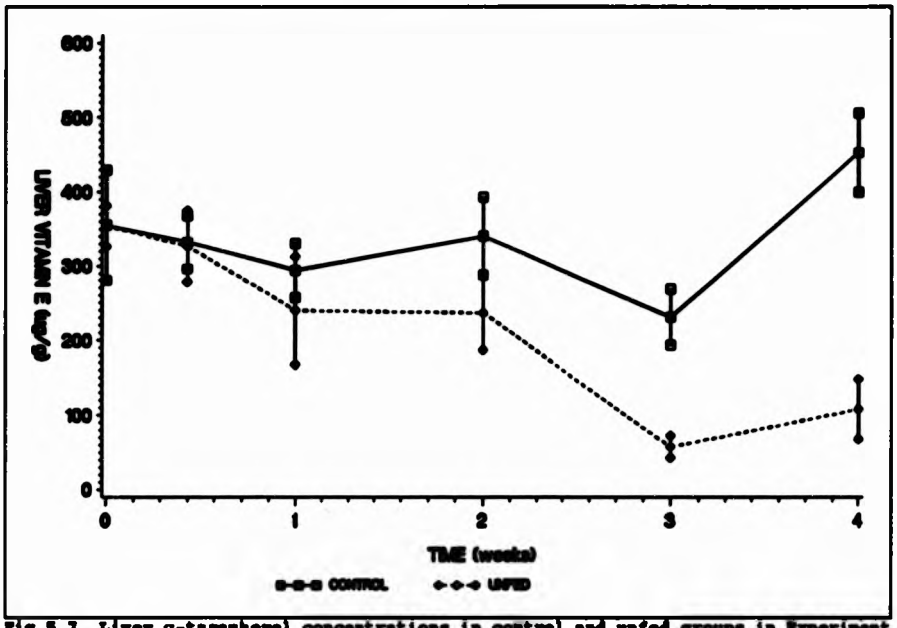

Fig.5.7. Liver α-tocopherol concentrations in control and unfed groups in Experiment 2 (meanise)

weight, from 357±42 g at day 0, to 293±63 g at 4 weeks and, in the crowded group, mean sample weights stayed fairly constant, the fish growing from 358±42 g on day 0, to only 380±108 g at 4 weeks. Condition factor decreased in all groups, but to different degrees. In the control group, it fell from 1.54±0.06 at day 0, to 1.40±0.08 at 4 weeks, in the unfed group it showed the largest drop, from 1.47±0.07 to 1.27±0.04 over the same period, and in the crowded group it fell from 1.46±0.07 to 1.38±0.08. No transformation of weight or condition factor was necessary prior to analysis of variance. Significant treatment-time interactions ($p<0.05$) were found by analysis of variance of the weight data from groups 1 and 2, but multiple comparisons detected no pairwise differences at any time. Comparison of treatments 1 and 3 showed only significant effects of time ($p<0.05$). Analysis of variance of condition factor data from groups 1 and 2 showed significant time ($p<0.001$) and treatment-time ($p<0.001$) effects, as well as overall treatment differences ($p<0.05$). Multiple pairwise comparisons showed that condition factors in the unfed group differed significantly from those of the controls at 1,2,3 and 4 weeks ($p<0.05$).

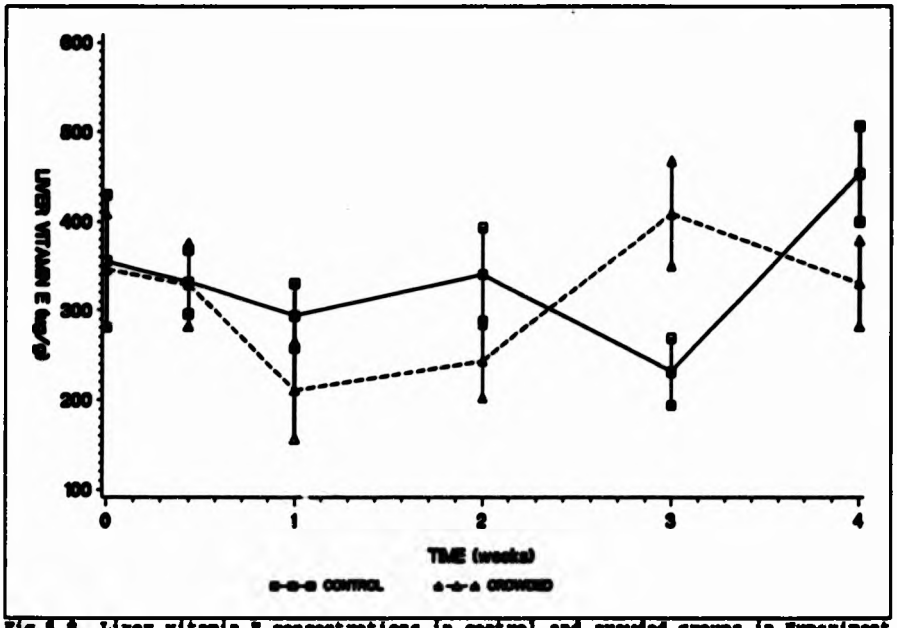

Fig.5.8. Liver vitamin E concentrations in control and crowded groups in Experiment 2 (meanise)

Condition factors in groups 1 and 3 showed significant time ($p<0.001$) and overall treatment differences ($p<0.05$), but no treatment-time effects.

Pearson's test showed that fish weight was significantly correlated with none of the above biochemical and haematological parameters. Condition factor showed a positive correlation with plasma and liver vitamin E and plasma malondialdehyde concentrations ($p<0.01$), but not with either haematocrit or leucocrit.

A significant correlation was detected between water temperature and leucocrit in group 1 during the course of this experiment ($p<0.01$), but none of the other biochemical or haematological parameters showed any relationship with water temperature.

Plasma cortisol concentrations are unsuitable for use as indicators of chronic stress since they may show compensation (Thomas 1990). McLeay and Gordon (1977) used leucocrit to detect a stress response in coho salmon exposed to kraft-mill effluent for long periods. In the present study, this test was used in an attempt to quantify the degree of chronic stress induced

Table 5.13. Haematocrit and leucocrit measurements in Experiment 2 (means±sd)

Time	Treatment	n	Haematocrit (%)	Leucocrit (%)
0 days	Control	12	42±3	1.19±0.23
	Unfed	12	40±2	1.15±0.11
	Crowded	12	41±3	1.34±0.15
3 days	Control	12	39±3	1.21±0.19
	Unfed	12	39±4	1.07±0.15
	Crowded	12	41±2	1.10±0.19
1 week	Control	12	38±2	1.19±0.20
	Unfed	12	43±7	1.14±0.19
	Crowded	12	42±5	1.05±0.22
2 weeks	Control	12	39±3	1.02±0.09
	Unfed	12	42±7	1.05±0.14
	Crowded	12	39±4	1.07±0.24
3 weeks	Control	12	42±3	1.03±0.14
	Unfed	12	43±3	1.15±0.28
	Crowded	12	37±4	1.14±0.15
4 weeks	Control	12	41±5	1.02±0.13
	Unfed	12	37±4	1.03±0.15
	Crowded	12	40±4	1.19±0.20

by crowding. The pattern of leucocrit suppression followed by recovery in fish subjected to this procedure is typical of the leucocyte response to chronic stress observed, for example, by Pickford *et al.* (1971) (Pickering 1981). However, no statistically significant difference was detected between crowded and control groups in the present study. The control group showed a sharp reduction in leucocrit between weeks 1 and 2 when the water temperature also fell sharply. Weight gain also provided some indication of the degree of chronic stress. Mean weights were higher in the control group, particularly at weeks 2 and 3, whilst crowded fish showed little weight gain during the course of the study.

Dvorák *et al.* (1976) found that long-term stimulation of the hypothalamo-pituitary-adrenal axis resulted in reduced tissue α-tocopherol concentrations and the expression of signs of vitamin E deficiency in pigs.

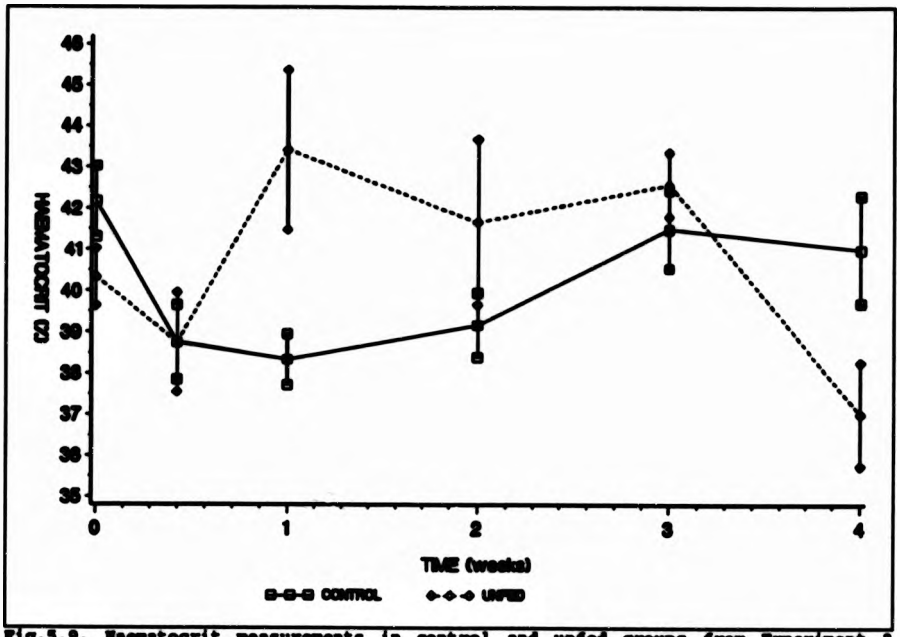

Fig.5.9. Haematocrit measurements in control and unfed groups from Experiment 2 (means±se)

In the present study, plasma vitamin E concentrations showed a gradual depletion in crowded fish when compared to controls, but these differences were not statistically significant. Liver vitamin E concentrations showed a similar response except at 3 weeks when unexpectedly high concentrations were measured in crowded fish and unusually low concentration in controls. Longer periods of crowding may lead to the development of statistically significant differences in tissue vitamin E concentrations. In Experiment 2, plasma malondialdehyde concentrations showed no effect of treatment and no correlation with plasma vitamin E concentration.

Haematocrits showed an initial increase in the crowded group, perhaps in response to increased oxygen demand, but were then reduced, though not to levels associated with vitamin E deficiency. Haematocrits showed no correlation with vitamin E concentration. Fish number effects on haematocrit probably reflect the release of erythrocytes from storage in the spleen. Splenic contraction, in response to catecholamines, allows a rapid increase in numbers of circulating erythrocytes (Yamamoto *et al.* 1980).

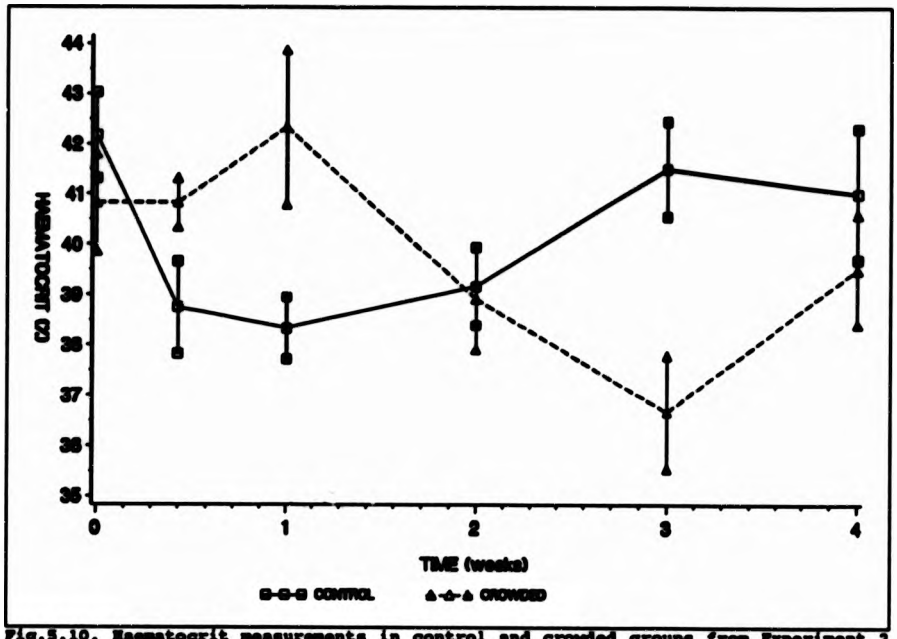

Fig.5.10. Haematocrit measurements in control and crowded groups from Experiment 2 (means±se)

Starvation of experimental fish caused irregular changes in plasma vitamin E concentrations between weeks 0 and 2, and a sharp drop at weeks 3 and 4 when these were significantly lower than in controls. Similarly in the liver, a slight drop occurred after 2 weeks, but at 3 and 4 weeks there was a marked fall. These results are in contrast to those obtained with one sea-winter fish in sea-cages, described in section 3.3.4. Differences in the effects of starvation between these studies may be due to the smaller size of the tank-held fish, or may reflect their higher initial tissue α-tocopherol concentrations. In mammals, the liver contains rapidly and slowly mobilised pools of α-tocopherol (Gallo-Torres 1980). In this experiment, the loss of vitamin E due to starvation may be due to mobilization of the readily-available excess α-tocopherol in the tissues.

Haematocrit increased and leucocrit showed no change as a result of starvation. Love (1980) noted that the short-term effects of starvation on the cellular components of blood are highly variable.

Table 5.14. Weight and condition factor in Experiment 2 (measured)

Time	Treatment	n	Weight (g)	Condition factor
0 days	Control	12	343±85	1.54±0.06
	Unfed	12	357±68	1.47±0.07
	Crowded	12	358±42	1.46±0.07
3 days	Control	12	336±87	1.40±0.07
	Unfed	12	363±102	1.42±0.04
	Crowded	12	294±96	1.36±0.07
1 week	Control	12	357±54	1.47±0.06
	Unfed	12	294±61	1.37±0.06
	Crowded	12	365±103	1.41±0.07
2 weeks	Control	12	403±99	1.41±0.05
	Unfed	12	308±71	1.29±0.05
	Crowded	12	359±90	1.37±0.06
3 weeks	Control	12	422±71	1.44±0.09
	Unfed	12	313±74	1.27±0.05
	Crowded	12	364±94	1.40±0.05
4 weeks	Control	12	392±88	1.40±0.08
	Unfed	12	293±63	1.27±0.04
	Crowded	12	380±108	1.38±0.08

5.3.3 Experiment 3

Plasma α-tocopherol concentrations measured in samples from Experiment 3 are summarized in table 5.15. Differences between groups 1 and 2 are illustrated in fig 5.15 and between groups 2 and 3 in fig. 5.16. In group 1, fed the normal diet and held at low stocking density, plasma vitamin E concentrations increased from 40±6 μg ml^{-1} on day 0, to 66±14 μg ml^{-1} at 2 months, fell to 42±25 μg ml^{-1} at 4 months, and increased to 58±24 μg ml^{-1} at 6 months. In group 2, fed the low vitamin E diet and held at low stocking density, concentrations of 39±12 and 33±9 μg ml^{-1} were measured on day 0 and at 1 month, these increased to 70±16 μg ml^{-1} at 4 months, and then fell to 42±15 μg ml^{-1} at 6 months. In group 3 fish, fed the low vitamin E diet and held in crowded conditions, plasma vitamin E concentrations fell from 41±13

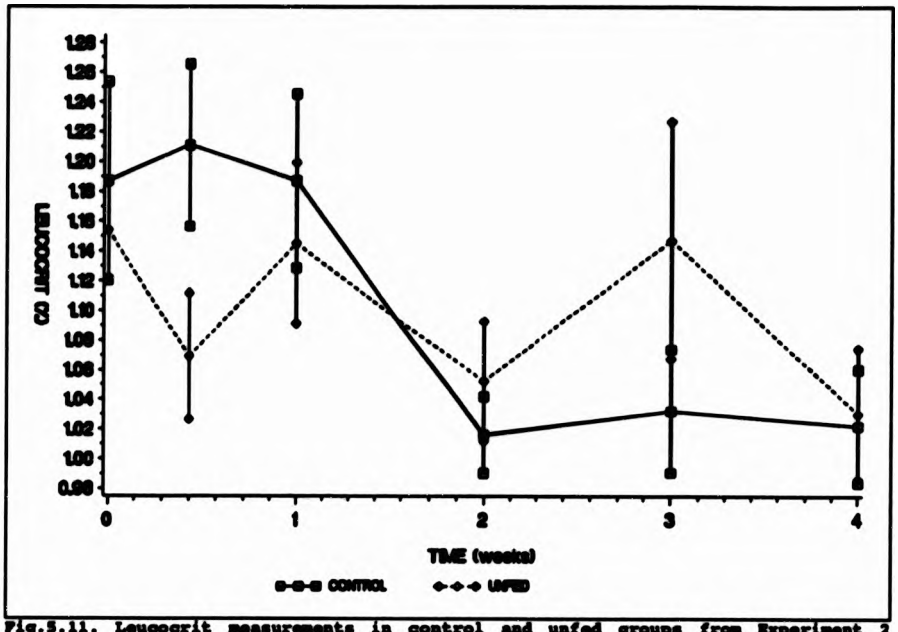

Fig.5.11. Leucocrit measurements in control and unfed groups from Experiment 2 (means±se)

$\mu g\ ml^{-1}$ on day 0 to 26±11 $\mu g\ ml^{-1}$ at 1 month, and stayed low, measuring 28±8 $\mu g\ ml^{-1}$ at 2 and 33±16 $\mu g\ ml^{-1}$ at 3 months. At 4 months an increase to 65±46 $\mu g\ ml^{-1}$ was recorded, but a further drop occurred, to 37±14 $\mu g\ ml^{-1}$ at 5 and 44±18 $\mu g\ ml^{-1}$ at 6 months. Data from groups 1 and 2 were subjected to analysis of variance without transformation. Significant treatment-time interactions ($p<0.05$) were detected, and time effects were also found ($p<0.01$). However, multiple pairwise comparisons of the treatment-time means detected no treatment differences at any particular time. Data from groups 2 and 3 were subjected to square root transformation and in this case analysis of variance showed significant effects only of time ($p<0.01$). The complete data were subjected to square root transformation before further analysis.

Liver α-tocopherol concentrations are shown in table 5.16 and group comparisons illustrated in figs. 5.17 and 5.18. In group 1 these showed an overall fall during the course of the experiment, dropping from 242±92 $\mu g\ g^{-1}$ on day 0 to a minimum of 144±104 $\mu g\ g^{-1}$ at 6 months, although ranging as high as 316±118 $\mu g\ g^{-1}$ at 3 months. In group 2, they fell steadily from 188±54 to

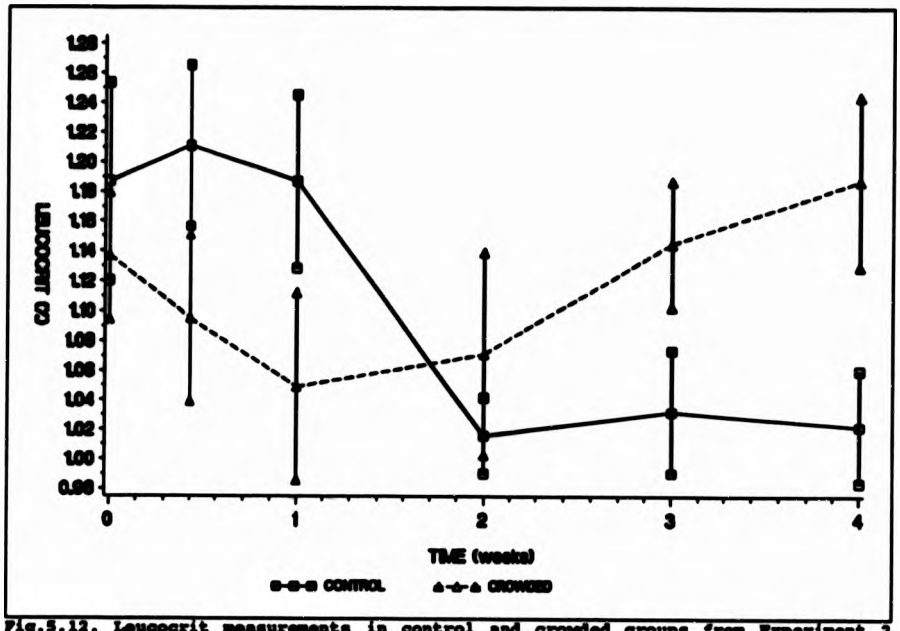

Fig.5.12. Haematocrit measurements in control and crowded groups from Experiment 2 (mean±se)

72±30 μg g^{-1} between 0 and 6 months, and in group 3 they fell more steeply during the first 8 weeks of the experiment, from 211±44 μg g^{-1} at day 0 to 88±42 μg g^{-1} at 1 and 70±20 at 2 months, then ranged from 80±47 to 115±22 μg g^{-1} between 3 and 6 months. This data was subjected to square root transformation before statistical analysis. Analysis of variance of the data from groups 1 and 2 showed significant effects of time ($p<0.01$) and overall effects of treatment ($p<0.05$), lower concentrations being measured in the livers of fish fed the unsupplemented diet. The data from groups 2 and 3 showed significant effects only of time ($p<0.001$). Pearson's test showed a significant correlation between plasma and liver vitamin E concentrations in this study ($p<0.01$).

Plasma malondialdehyde concentrations are given in table 5.17. In group 1, these rose from 17±9 μmol l^{-1} on day 0, to 29±5 μmol l^{-1} at 2 months, fell sharply to 11±5 μmol l^{-1} at 3 months, then increased to 24±9 and 23±8 μmol l^{-1} at 5 and 6 months respectively. In group 2, they dropped from 24±14 μmol l^{-1} on day 0 to 20±7 μmol l^{-1} at 2 months. Low concentrations (7±6 μmol l^{-1}) were

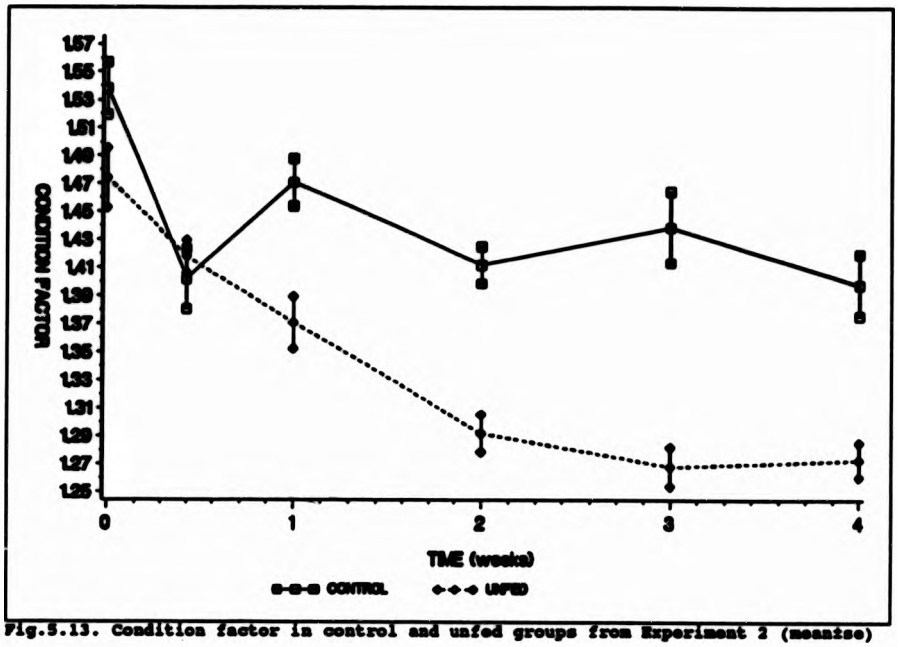

Fig.5.13. Condition factor in control and unfed groups from Experiment 2 (mean±se)

again measured at 3 months, they increased to 17±8 μmol l^{-1} at 4 months, then fell to 10±3 μmol l^{-1} at 6 months. In group 3, they were relatively low and stable, ranging from 11±6 μmol l^{-1} on day 0 to 17±11 μmol l^{-1} at 6 months. Statistical analyses were performed using the untransformed data. Analysis of variance of the data from groups 1 and 2 showed significant effects only of time ($p<0.001$). Significant treatment-time interactions ($p<0.05$) and fish number effects ($p<0.01$) were found in groups 2 and 3. Multiple comparisons could detect no pairwise differences between the treatment means at any time. The number effects indicated a tendency for higher malondialdehyde concentrations to be measured in fish sampled later. Pearson's test showed that there was no significant correlation between plasma vitamin E and malondialdehyde concentrations in this study.

Plasma protein concentrations are shown in table 5.18. In group 1, plasma protein concentrations ranged from 4.8±3.0 to 7.5±2.7 mg ml^{-1}, between 0 and 5 months, increasing to 10.3±6.1 mg ml^{-1} at 6 months. In group 2, they ranged between 4.7±1.9 and 7.5±2.0 mg ml^{-1}, and in group 3 they fell from 7.3±1.6 mg ml^{-1} on day 0, to a minimum of 3.7±1.2 mg ml^{-1} at 1 month, then

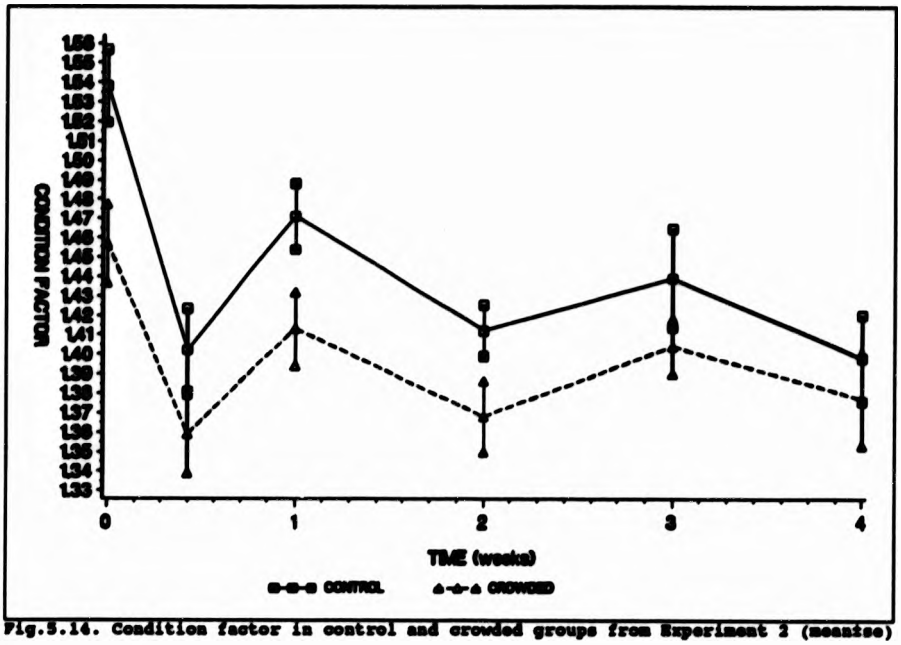

Fig.5.14. Condition factor in control and crowded groups from Experiment 2 (means±se)

gradually recovered to 5.5±0.5 mg ml^{-1} at 5 and 8.5±4.8 mg ml^{-1} at 6 months. These data were log transformed for statistical analysis. Analysis of variance of the data from groups 1 and 2 showed significant effects only of time ($p<0.001$), but in the data from groups 2 and 3, time ($p<0.001$), number ($p<0.05$) and treatment-time effects ($p<0.05$) were detected. There was a tendency for plasma protein concentrations to increase with fish number, and multiple comparisons revealed significant treatment differences at 1 month ($p<0.05$). Pearson's correlation test showed that plasma protein concentrations were positively correlated with both plasma and liver vitamin E concentrations, and with plasma malondialdehyde concentrations ($p<0.01$ in each case).

Plasma pyruvate kinase activities are shown in table 5.19. These were relatively low and close to the limits of detection of the assay procedure. In view of the treatment effects on plasma protein described above, PK activities were expressed per mg protein. In group 1, plasma PK activities ranged from 0±0 to 2.1±3.6 mU mg^{-1}. In group 2, a similar range was found except at 1 month when activities of 5.0±6.0 mU mg^{-1} were measured, and group

Table 5.15. Plasma α-tocopherol concentrations in Experiment 3 (mean±sd)

Time (months)	Treatment	n	Plasma α-tocopherol ($\mu g\ ml^{-1}$)
0	Regular Uncrowded	6	40±6
	Low vitamin E Uncrowded	6	39±12
	Low vitamin E Crowded	6	41±13
1	Regular Uncrowded	6	44±22
	Low vitamin E Uncrowded	6	33±9
	Low vitamin E Crowded	6	26±11
2	Regular Uncrowded	6	66±14
	Low vitamin E Uncrowded	6	56±8
	Low vitamin E Crowded	6	28±8
3	Regular Uncrowded	6	60±20
	Low vitamin E Uncrowded	6	64±17
	Low vitamin E Crowded	6	33±16
4	Regular Uncrowded	6	42±25
	Low vitamin E Uncrowded	6	70±16
	Low vitamin E Crowded	6	65±46
5	Regular Uncrowded	6	61±13
	Low vitamin E Uncrowded	6	48±13
	Low vitamin E Crowded	6	37±14
6	Regular Uncrowded	6	58±24
	Low vitamin E Uncrowded	6	42±15
	Low vitamin E Crowded	6	44±18

Table 5.16. Liver α-tocopherol concentrations in Experiment 3 (mean±sd)

Time (months)	Treatment	n	Liver α-tocopherol ($\mu g\ g^{-1}$)
0	Regular Uncrowded	6	242±92
	Low vitamin E Uncrowded	6	188±94
	Low vitamin E Crowded	6	211±44
1	Regular Uncrowded	6	227±79
	Low vitamin E Uncrowded	6	146±36
	Low vitamin E Crowded	6	88±42
2	Regular Uncrowded	6	284±123
	Low vitamin E Uncrowded	6	155±25
	Low vitamin E Crowded	6	70±20
3	Regular Uncrowded	6	316±118
	Low vitamin E Uncrowded	6	101±37
	Low vitamin E Crowded	6	83±42
4	Regular Uncrowded	6	175±80
	Low vitamin E Uncrowded	6	147±65
	Low vitamin E Crowded	6	115±22
5	Regular Uncrowded	6	225±33
	Low vitamin E Uncrowded	6	117±48
	Low vitamin E Crowded	6	80±47
6	Regular Uncrowded	6	144±104
	Low vitamin E Uncrowded	6	72±30
	Low vitamin E Crowded	6	87±39

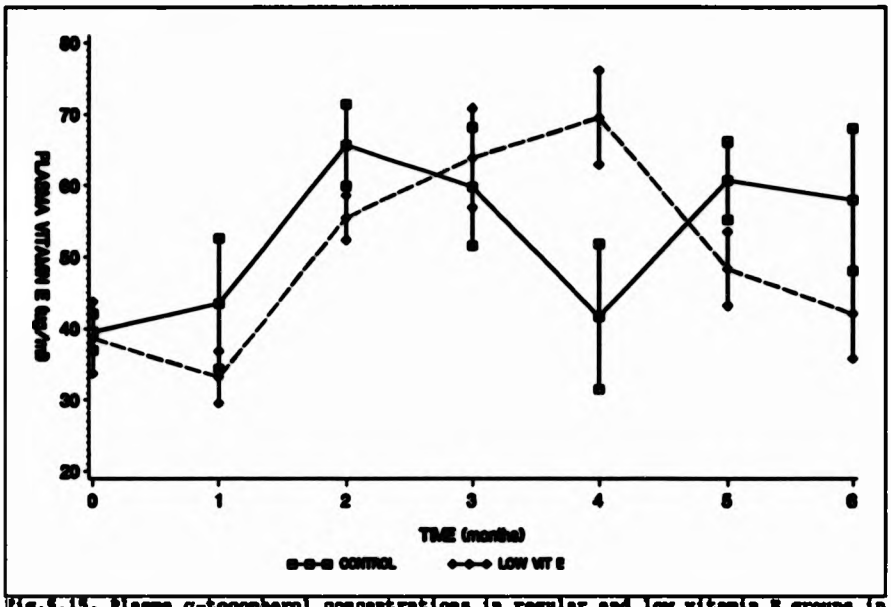

Fig.5.15. Plasma α-tocopherol concentrations in regular and low vitamin E groups in Experiment 3 (means±se)

3 showed a similar pattern to group 2, with activites as high as 8.5±10.1 mU mg^{-1} measured at 1 month. Neither square root nor log transformations created normally distributed data. The Mann-Whitney U-test showed no differences between the three groups at any time, but Spearman's test indicated that plasma PK activities were negatively correlated with plasma vitamin E concentrations ($p<0.01$).

Haematocrit and leucocrit measurements are summarized in table 5.20. Haematocrits increased in all treatment groups during the course of the experiment. In group 1, they rose from 42±4% at day 0 to 44±4% at 6 months, but ranged as high as 47±4% at 4 months. In group 2, they rose steadily, from 41±4 to 46±5% between 0 and 6 months, and in group 3 they were more variable, rising from 40±4% on day 0 to 46±4% at 6 months, but ranging as low as 38±6% at 1 month and as high as 49±5% at 4 months. No data transformation was necessary prior to statistical analysis. Analyses of variance revealed effects of time ($p<0.01$ in the comparison of groups 1 and 2, and $p<0.001$ in the comparison of groups 2 and 3), and fish number effects ($p<0.001$ in both cases). Lower haematocrits were again measured in fish sampled sooner after

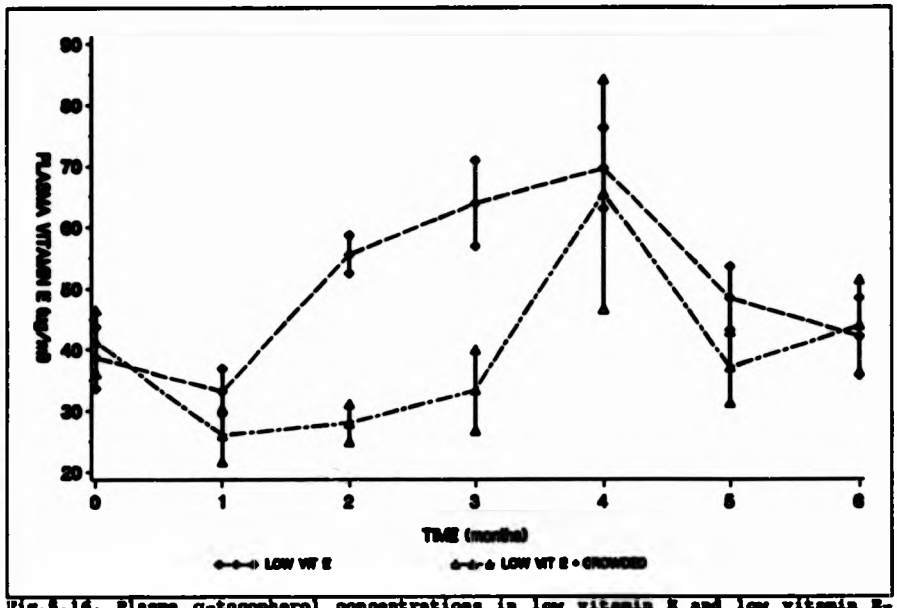

Fig.8.16. Plasma α-tocopherol concentrations in low vitamin E and low vitamin E-crowded groups in Experiment 3 (means±se)

capture.

In group 1, leucocrits ranged between 1.41±0.25% on day 0 and 0.99±0.20% at 2 months; in group 2 they fell from 1.17±0.36 to 1.00±0.19% between 0 and 5 months, then rose to 1.48±0.17% at 6 months, and in group 3 they increased from 1.20±0.19 to 1.39±0.24%, but dipped to 1.00±0.14% at 3 months. These data were log transformed. Analysis of variance of data from groups 1 and 2 showed significant effects of time ($p<0.001$) and number ($p<0.05$), and both treatment-time and treatment-number interactions ($p<0.01$). However, multiple pairwise comparisons showed no treatment differences at any time. No further analysis was applied to the number or treatment-number means. Analysis of variance of the data from groups 2 and 3 showed significant effects only of time ($p<0.001$). There was no significant correlation between haematocrits and leucocrits, but positive correlations were found between haematocrits and plasma vitamin E ($p<0.01$), plasma malondialdehyde ($p<0.05$) and plasma protein ($p<0.01$) concentrations, and a negative correlation was detected between haematocrits and plasma PK activities ($p<0.01$). Leucoorits showed a positive correlation only with plasma vitamin E concentrations ($p<0.05$).

Table 5.17. Plasma malondialdehyde concentrations in Experiment 3 (mean±sd)

Time (months)	Treatment	n	Plasma malondialdehyde (μmol l^{-1})
0	Regular Uncrowded	6	17±9
	Low vitamin E Uncrowded	6	24±14
	Low vitamin E Crowded	6	12±6
1	Regular Uncrowded	6	27±7
	Low vitamin E Uncrowded	6	22±8
	Low vitamin E Crowded	6	12±8
2	Regular Uncrowded	6	29±5
	Low vitamin E Uncrowded	6	20±7
	Low vitamin E Crowded	6	17±7
3	Regular Uncrowded	6	11±5
	Low vitamin E Uncrowded	6	7±6
	Low vitamin E Crowded	6	12±7
4	Regular Uncrowded	6	22±10
	Low vitamin E Uncrowded	6	17±8
	Low vitamin E Crowded	6	16±12
5	Regular Uncrowded	6	24±9
	Low vitamin E Uncrowded	6	17±6
	Low vitamin E Crowded	6	16±6
6	Regular Uncrowded	6	23±8
	Low vitamin E Uncrowded	6	10±3
	Low vitamin E Crowded	6	17±11

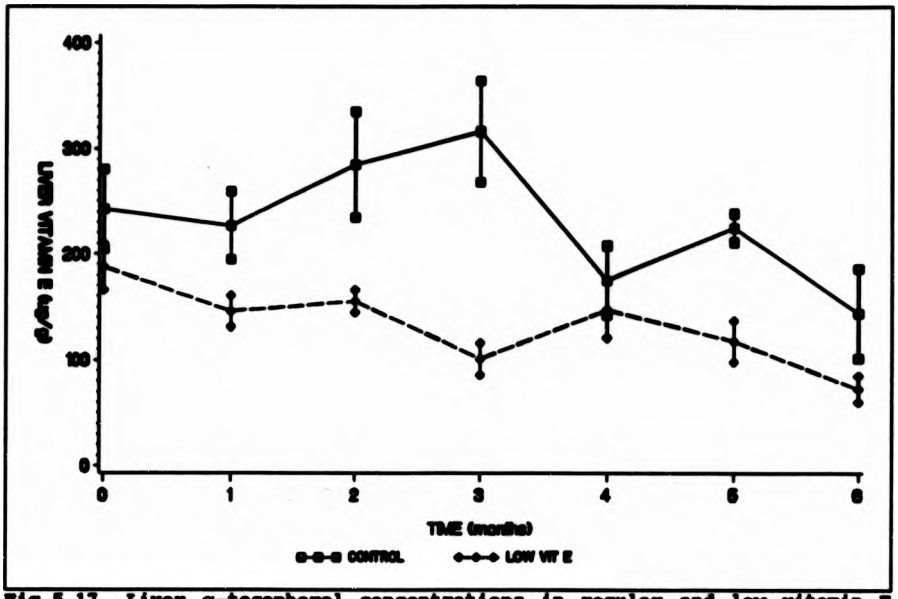

Fig.5.17. Liver α-tocopherol concentrations in regular and low vitamin E groups in Experiment 3 (mean±se)

Details of the weight and condition factor of fish collected during this experiment are shown in table 5.21. Fish in group 1 grew from 521±124 g on day 0, to 1173±381 g after 6 months. Group 2 showed a similar weight gain, from 592±147 g to 1100±283 g, but group 3 performed less well, growing from 516±199 to only 999±176 g over the 6 month period. The data was subjected to log transformation before statistical analysis. Analysis of variance of group 1 and 2 data revealed significant effects of time ($p<0.001$) and fish number ($p<0.05$). Within the data from groups 2 and 3 there were significant effects of time ($p<0.001$) and also overall treatment effects ($p<0.05$), fish subjected to crowding weighing less on average than those held at low stocking densities. Condition factor varied greatly in group 1, increasing from 1.32±0.04 at day 0, to 1.47±0.30 at 1 month, falling to 1.30±0.31 at 4 months, then increasing to 1.45±0.12 at 6 months. In groups 2 and 3 it was less variable. In group 2 it rose from 1.36±0.05 to just 1.39±0.12 between 0 and 1 month, fell to 1.33±0.11 at 3 months and increased to 1.49±0.10 at 6 months. In group 3, it fell from 1.36±0.10 at day 0, to 1.30±0.04 at 1 month, then increased to 1.48±0.10 at 6 months. The untransformed data was used for

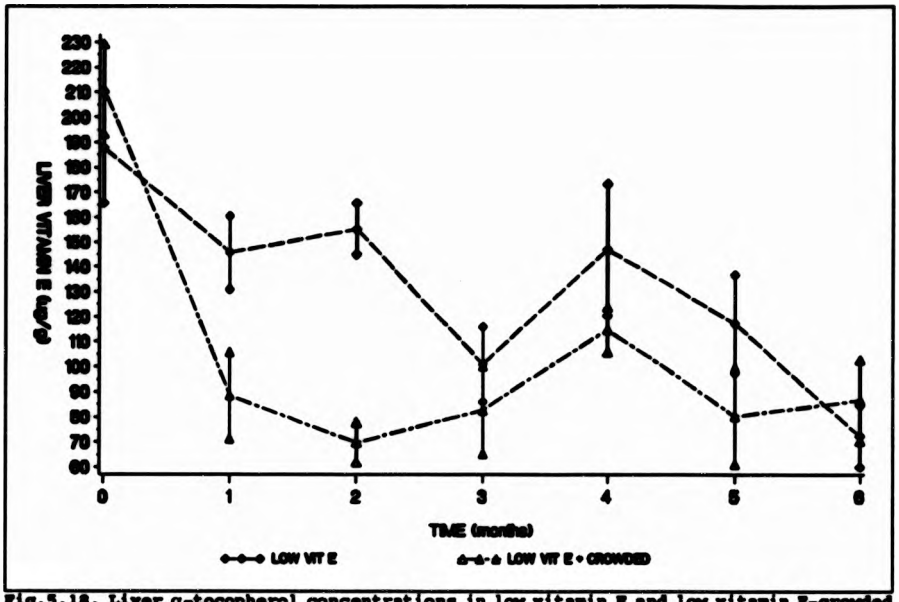

Fig.5.18. Liver α-tocopherol concentrations in low vitamin E and low vitamin E-crowded groups in Experiment 3 (means±se)

statistical analysis. Significant effects of time ($p<0.001$) but no treatment effects were detected within the data from both groups 1 and 2 and groups 2 and 3. Fish weight showed positive correlations with plasma vitamin E ($p<0.01$) and protein ($p<0.01$) concentrations and haematocrits ($p<0.01$), and a negative correlation with plasma PK activity ($p<0.05$). Condition factor showed positive associations with plasma vitamin E ($p<0.05$) and protein ($p<0.01$) concentrations and haematocrits ($p<0.01$), and a negative association with plasma PK activity ($p<0.05$).

Sex was determined in 168 individuals collected from all but the day 0 sample: 79 males and 89 females were counted. Analysis of variance showed that there were significant differences in haematocrit ($p<0.05$), weight ($p<0.001$) and condition factor ($p<0.01$) between males and females. The means in each case were greater in males than in females. In addition, liver vitamin E concentrations showed significant differences between the sexes ($p<0.05$), but these were higher in females than in males.

A significant negative correlation ($p<0.05$) was found between water

Table 5.18. Plasma protein concentrations in Experiment 3 (mean±sd)

Time (months)	Treatment	n	Plasma protein (mg ml^{-1})
0	Regular Uncrowded	6	6.3±1.2
	Low vitamin E Uncrowded	6	7.5±2.0
	Low vitamin E Crowded	6	7.3±1.6
1	Regular Uncrowded	6	6.2±0.4
	Low vitamin E Uncrowded	6	6.0±1.1
	Low vitamin E Crowded	6	3.7±1.2
2	Regular Uncrowded	6	7.5±2.7
	Low vitamin E Uncrowded	6	5.7±1.0
	Low vitamin E Crowded	6	4.5±0.5
3	Regular Uncrowded	6	4.8±3.0
	Low vitamin E Uncrowded	6	4.7±1.9
	Low vitamin E Crowded	6	4.8±1.2
4	Regular Uncrowded	6	6.7±2.3
	Low vitamin E Uncrowded	6	6.5±1.0
	Low vitamin E Crowded	6	5.8±1.2
5	Regular Uncrowded	6	7.0±1.7
	Low vitamin E Uncrowded	6	5.8±1.0
	Low vitamin E Crowded	6	5.5±0.5
6	Regular Uncrowded	6	10.3±6.1
	Low vitamin E Uncrowded	6	6.3±1.0
	Low vitamin E Crowded	6	8.5±4.8

Table 5.19. Plasma pyruvate kinase activities in Experiment 3 (meanted)

Time (months)	Treatment	n	Plasma PK (mU mg protein^{-1})
0	Regular Uncrowded	5	0.29±0.64
	Low vitamin E Uncrowded	6	0.78±0.89
	Low vitamin E Crowded	6	1.29±1.06
1	Regular Uncrowded	6	0.58±1.43
	Low vitamin E Uncrowded	6	5.00±5.99
	Low vitamin E Crowded	6	8.47±10.07
2	Regular Uncrowded	6	0±0
	Low vitamin E Uncrowded	6	0±0
	Low vitamin E Crowded	6	0.70±1.72
3	Regular Uncrowded	6	0±0
	Low vitamin E Uncrowded	6	2.20±2.88
	Low vitamin E Crowded	6	0±0
4	Regular Uncrowded	6	2.14±3.61
	Low vitamin E Uncrowded	6	0.83±1.33
	Low vitamin E Crowded	6	1.70±2.41
5	Regular Uncrowded	6	1.03±2.53
	Low vitamin E Uncrowded	6	0.57±0.90
	Low vitamin E Crowded	6	0.33±0.82
6	Regular Uncrowded	6	0.48±0.74
	Low vitamin E Uncrowded	6	1.22±1.65
	Low vitamin E Crowded	6	0±0

Table 5.20. Haematocrit and leucocrit measurements in Experiment 3 (mean±sd)

Time (months)	Treatment	n	Haematocrit (%)	Leucocrit (%)
0	Regular Uncrowded	10	42±4	1.41±0.25
	Low vitamin E Uncrowded	10	41±4	1.17±0.36
	Low vitamin E Crowded	10	40±4	1.20±0.19
1	Regular Uncrowded	10	45±3	1.10±0.16
	Low vitamin E Uncrowded	10	42±4	1.07±0.17
	Low vitamin E Crowded	10	38±6	1.09±0.26
2	Regular Uncrowded	10	44±3	0.99±0.20
	Low vitamin E Uncrowded	10	44±4	1.07±0.21
	Low vitamin E Crowded	10	41±5	1.15±0.21
3	Regular Uncrowded	10	42±4	1.03±0.16
	Low vitamin E Uncrowded	10	43±5	0.98±0.14
	Low vitamin E Crowded	10	42±5	1.04±0.21
4	Regular Uncrowded	10	47±4	1.29±0.29
	Low vitamin E Uncrowded	10	45±2	1.14±0.21
	Low vitamin E Crowded	10	49±5	1.29±0.27
5	Regular Uncrowded	10	44±5	1.08±0.16
	Low vitamin E Uncrowded	10	46±5	1.00±0.19
	Low vitamin E Crowded	10	45±4	1.15±0.23
6	Regular Uncrowded	10	44±4	1.18±0.20
	Low vitamin E Uncrowded	10	46±5	1.48±0.17
	Low vitamin E Crowded	10	46±4	1.39±0.24

Table 5.21. Weight and condition factor in Experiment 3 (mean±sd)

Time (months)	Treatment	n	Weight (g)	Condition factor
0	Regular Uncrowded	10	521±124	1.32±0.04
	Low vitamin E Uncrowded	10	592±147	1.36±0.05
	Low vitamin E Crowded	10	516±199	1.36±0.10
1	Regular Uncrowded	10	572±132	1.47±0.30
	Low vitamin E Uncrowded	10	527±129	1.39±0.12
	Low vitamin E Crowded	10	523±78	1.30±0.04
2	Regular Uncrowded	10	625±134	1.39±0.09
	Low vitamin E Uncrowded	10	679±141	1.33±0.09
	Low vitamin E Crowded	10	594±228	1.30±0.07
3	Regular Uncrowded	10	742±219	1.36±0.17
	Low vitamin E Uncrowded	10	712±225	1.33±0.11
	Low vitamin E Crowded	10	552±150	1.31±0.09
4	Regular Uncrowded	10	702±238	1.30±0.31
	Low vitamin E Uncrowded	10	786±206	1.44±0.18
	Low vitamin E Crowded	10	731±187	1.43±0.14
5	Regular Uncrowded	10	1065±285	1.46±0.11
	Low vitamin E Uncrowded	10	1099±257	1.50±0.17
	Low vitamin E Crowded	10	923±219	1.49±0.10
6	Regular Uncrowded	10	1173±381	1.45±0.12
	Low vitamin E Uncrowded	10	1100±283	1.49±0.10
	Low vitamin E Crowded	10	999±176	1.48±0.10

temperatures and liver vitamin E concentrations, but none of the other biochemical and haematological parameters were correlated with water temperature.

Histological preparations of heart, skeletal muscle and pancreas were examined from two fish per tank at 3 months, and 2, 3 or 4 fish per tank at 6 months. At 3 months there was no indication of any histopathological change in any of the samples, but at 6 months mild changes in the cardiac muscle were observed in 9 of the 20 samples examined. These changes comprised an increase in cellularity due to leucocyte infiltration of the tissue, and shrinkage of muscle fibres. In addition, yellow, fatty deposits within the epicardium were common. These changes were mild in comparison to those described in section 4, and were observed in fish from all tanks and treatments.

Plasma vitamin E concentrations in fish fed a diet without vitamin E supplementation were no different to those in fish fed diets containing supplementary α-tocopheryl acetate. However, liver vitamin E concentrations were significantly lower. In contrast, Hardie *et al.* (1990) and Hung and Slinger (1982) found that both liver and plasma vitamin E concentrations reflected dietary vitamin E concentrations. In the present study, diets were not formulated to be vitamin E deficient, but were rather more marginal with respect to vitamin E than those used commercially. In fish fed the low vitamin E diet, plasma vitamin E concentrations showed an initial rise and then decreased, whilst liver concentrations fell throughout the course of the experiment. Thus there were indications of a preferential loss from the liver, whilst plasma levels were maintained.

There was no consistent evidence of vitamin E deficiency (as described in section 1.2.5) in fish fed the unsupplemented diet. The dietary vitamin E content had no effect on plasma malondialdehyde concentrations. Plasma malondialdehyde concentrations were in fact slightly (though not significantly) lower in fish fed the low vitamin E diet, and overall they showed no significant correlation with plasma vitamin E concentrations. Haematocrits also showed no significant treatment effects, indicating no marked reduction as a result of vitamin E deficiency (section 1.2.5). Similarly, no differences in leucocrit were detected and, histologically, there was no sign of treatment-related muscle or pancreas pathology. Plasma

pyruvate kinase activity in the fish fed low vitamin E diets showed a slight increase at 1 month but recovered to very low levels by 2 months. Storage of plasma samples may have resulted in loss of activity, and the assay temperature may have been sub-optimal, but no differences were detected between the treatments. Fish fed the vitamin E-supplemented and unsupplemented diets also showed no differences in weight gain or condition factor.

Liver vitamin E concentrations were found to be significantly higher in maturing, female fish. This may indicate that vitamin E is accumulated in the liver prior to deposition in the developing ovary. In addition, a significant negative correlation with water temperature was detected. This may reflect a higher vitamin E demand at lower water temperatures (section 1.2.7.5).

Among groups of fish fed the unsupplemented diet, plasma and liver vitamin E concentrations were generally lower in those subjected to crowding than in the uncrowded groups but, as in Experiment 2, these differences were not statistically significant, despite the long duration of Experiment 3. Since starvation was demonstrated to result in a marked reduction in both plasma and liver α-tocopherol concentrations, the effects of crowding on food intake may explain any slight effect of chronic stress on tissue vitamin E concentrations. Again there was no evidence of increased plasma malondialdehyde concentrations as a result of vitamin E deficiency. This suggests that crowded fish were not more prone to lipid peroxidation *in vivo*. In the crowded group, haematocrits were lower at 1 and 2 months but then increased above those of the uncrowded-unsupplemented group. This pattern was similar to the measured changes in plasma vitamin E concentration but there is no other evidence that haematocrit can be directly related to plasma vitamin E concentrations. There was no sign of any histological change in crowded fish fed the unsupplemented diet, but plasma pyruvate kinase activity showed a small peak at 1 month which was slightly higher than that seen in the uncrowded group. Fish weight and condition factor were reduced to a lesser degree in fish fed the unsupplemented diet, suggesting that vitamin E supplementation may be of some benefit in fish under chronic stress. This hypothesis was further examined in Experiment 5. However, in agreement with Gatlin *et al.* (1986*b*), there was little evidence from the present study to indicate that stressful husbandry conditions can precipitate vitamin E

deficiency in fish.

Table 5.22. Plasma α-tocopherol concentrations in Experiment 4 (mean±sd)

Time	Treatment	n	Plasma α-tocopherol ($\mu g\ ml^{-1}$)
0 hrs	Regular Netted	6	48±24
	Low vitamin E Netted	6	56±26
4 hrs	Regular Netted	6	34±21
	Low vitamin E Netted	6	27±23
1 day	Regular Netted	6	37±21
	Low vitamin E Netted	6	38±19
3 days	Regular Netted	6	63±29
	Low vitamin E Netted	6	27±17
5 days	Regular Netted	6	31±17
	Low vitamin E Netted	6	18±8

5.3.4 Experiment 4

Plasma α-tocopherol concentrations measured in Experiment 4 are shown in table 5.22. In group 1, reared on the vitamin E-supplemented diet prior to handling, plasma α-tocopherol concentrations dropped from 48±24 $\mu g\ ml^{-1}$ to 34±21 $\mu g\ ml^{-1}$ at 4 hrs and 37±21 at 1 day, increased to 63±29 $\mu g\ ml^{-1}$ at 3 days, and fell once more to 31±17 $\mu g\ ml^{-1}$ at 5 days. In group 2, fed the low vitamin E diet prior to handling, they fell from 56±26 $\mu g\ ml^{-1}$ at 0 hrs to 27±23 $\mu g\ ml^{-1}$ at 4hrs, recovered to 38±19 $\mu g\ ml^{-1}$ at 1 day and fell again to 18±8 $\mu g\ ml^{-1}$ at 5 days. A log transformation was applied to the data before statistical analysis. Significant effects of time only ($p<0.05$), were found during the course of the study.

Table 5.23. Plasma malondialdehyde concentrations in Experiment 4 (meantsd)

Time	Treatment	n	Plasma malondialdehyde (μmol l^{-1})
0 hrs	Regular Netted	6	19±9
	Low vitamin E Netted	6	21±10
4 hrs	Regular Netted	6	18±9
	Low vitamin E Netted	6	24±14
1 day	Regular Netted	6	11±5
	Low vitamin E Netted	6	14±5
3 days	Regular Netted	6	12±2
	Low vitamin E Netted	6	10±3
5 days	Regular Netted	6	14±9
	Low vitamin E Netted	6	6±5

Plasma malondialdehyde concentrations measured in these fish are summarized in table 5.23. In group 1, plasma malondialdehyde concentrations fell from 19±9 μmol l^{-1} at 0 hrs, to 11±5 μmol l^{-1} at 1 day, then increased slightly, to 14±9 μmol l^{-1} at 5 days. In group 2 they showed a similar response, falling from 21±10 and 24±14 μmol l^{-1} at 0 and 4 hrs, to 6±5 μmol l^{-1} at 5 days. A square root transformation was applied to the data and, once again, significant effects of time ($p<0.001$), but no significant treatment effects, were noted. Pearson's test showed a significant positive correlation between plasma vitamin E and malondialdehyde concentrations ($p<0.05$).

Plasma cortisol concentrations are shown in table 5.24 and fig. 5.19. In both groups these showed an increase at 4 hrs returning to basal levels by 1 day. In group 1, plasma cortisol concentrations rose from 1.7±1.4 ng ml^{-1} at 0 hrs to 8.0±2.7 ng ml^{-1} at 4 hrs then dropped back, ranging from 0.4±0.4 to 0.7±0.3 ng ml^{-1}, between days 1 and 5. In group 2 the initial increase was

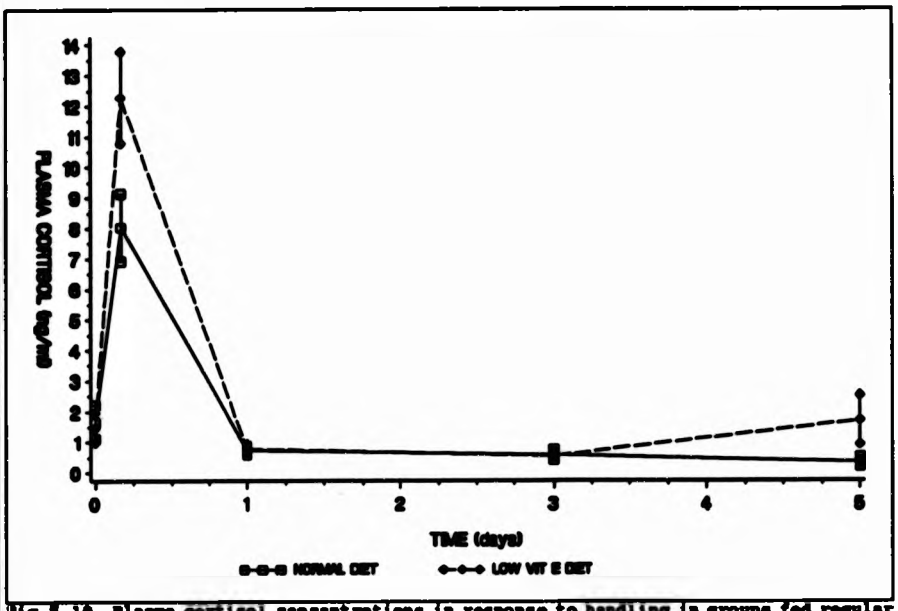

Fig.5.19. Plasma cortisol concentrations in response to handling in groups fed regular and low vitamin E diets (mean±se).

larger, from 1.5±1.2 ng ml^{-1} at 0 hrs to 12.3±3.7 ng ml^{-1} at 4 hrs, but between 1 and 5 days they recovered to between 0.6±0.3 and 1.7±2.0 ng ml^{-1}. These data were not normally distributed even after transformation, so non-parametric methods of analysis were used. No differences between the treatments were detected at any time using the Mann-Whitney U-test. Spearman's test showed a significant rank correlation between plasma cortisol and malondialdehyde ($p<0.05$), but not between plasma cortisol and vitamin E concentrations.

Haematocrit and leucocrit measurements are summarized in table 5.25. In group 1, haematocrits of between 44±6 and 46±4% were measured throughout the study, except at 3 days when values as low as 42±5% were found. In group 2, they showed a slight initial drop from 46±3 to 44±6%, but then rose to 46±6% on day 5. Analysis of variance of the untransformed data showed significant fish number effects ($p<0.001$), but no treatment or time effects.

In group 1, leucocrits fell, sharply, from 1.32±0.32% at 0 hrs to 0.89±0.16% at 4 hrs, recovered to 1.15±0.27% after 1 day, and fell slightly to 0.95±0.30 and 1.02±0.22% at 3 and 5 days. In group 2 the initial fall was

Table 5.26. Plasma cortisol concentrations in Experiment 4 (mean±sd)

Time	Treatment	n	Plasma cortisol (ng ml^{-1})
0 hrs	Regular Netted	6	1.7±1.4
	Low vitamin E Netted	6	1.5±1.2
4 hrs	Regular Netted	6	8.0±2.7
	Low vitamin E Netted	6	12.3±3.7
1 day	Regular Netted	6	0.7±0.3
	Low vitamin E Netted	6	0.8±0.4
3 days	Regular Netted	6	0.6±0.4
	Low vitamin E Netted	6	0.6±0.3
5 days	Regular Netted	6	0.4±0.4
	Low vitamin E Netted	6	1.7±2.0

less marked, from 1.40±0.31 to 1.07±0.07% at 4 hrs, but thereafter the response was similar to group 1, ranging from 1.00±0.31 to 1.20±0.39%, between 1 and 5 days. A square root transformation was applied to the data and significant effects of time ($p<0.001$), but not of treatment, were detected. There was no significant association between haematocrit and leucocrit and no significant correlations between either haematological parameter and any of the biochemical data described above.

Weight and condition factor are given in table 5.26. In Experiment 4, sample weights ranged from 935±304 to 1225±324 g. Analysis of variance of square root transformed weight data revealed significant treatment-time effects ($p<0.05$) and treatment-time-number effects ($p<0.05$). Multiple comparison of the treatment-time means showed no differences between the treatments at any time. Treatment-time-number interactions were not examined. Condition factor appeared to be consistently higher in group 1 than in group

Table 5.25. Haematocrit and leucocrit measurements in Experiment 4 (meanted)

Time	Treatment	n	Haematocrit (%)	Leucocrit (%)
0 hrs	Regular Netted	10	45±6	1.32±0.32
	Low vitamin E Netted	10	46±3	1.40±0.31
4 hrs	Regular Netted	10	44±6	0.89±0.16
	Low vitamin E Netted	10	44±6	1.07±0.07
1 day	Regular Netted	10	46±4	1.15±0.27
	Low vitamin E Netted	10	45±4	1.20±0.39
3 days	Regular Netted	10	42±5	0.95±0.30
	Low vitamin E Netted	10	46±4	1.00±0.31
5 days	Regular Netted	10	46±4	1.02±0.22
	Low vitamin E Netted	10	46±6	1.17±0.30

2. In group 1 a maximum of 1.48±0.09 was measured at 0 hrs and a minimum of 1.42±0.10 at 5 days, whilst in group 2 the range was between 1.38±0.08 and 1.44±0.14. However, analysis of variance of the raw data detected no significant overall treatment effect. There was a significant positive correlation between fish weight and haematocrit ($p<0.01$) and between condition factor and plasma vitamin E concentration ($p<0.05$), but no other correlations were found.

The sex and state of maturity of 99 fish was determined: the numbers of males to females was 52:47, and of salmon to grilse was 43:56. 36 males and 20 females were sexually mature. Significant differences between the sexes in weight ($p<0.01$) and haematocrit ($p=0.001$) were found. In both cases these were greater in males than in females. Differences in weight ($p<0.001$) and condition factor ($p<0.01$) were found between mature and non-mature fish, and here grilse had higher averages than salmon. In addition, plasma

Table 5.26. Weight and condition factor in Experiment 4 (mean±sd)

Time	Treatment	n	Weight (g)	Condition factor
0 hrs	Regular Netted	10	1011±204	1.48±0.09
	Low vitamin E Netted	10	1094±356	1.44±0.14
4 hrs	Regular Netted	10	935±304	1.43±0.12
	Low vitamin E Netted	10	1051±323	1.42±0.10
1 day	Regular Netted	10	1165±285	1.46±0.08
	Low vitamin E Netted	10	935±162	1.39±0.12
3 days	Regular Netted	10	974±264	1.42±0.10
	Low vitamin E Netted	10	1070±159	1.38±0.08
5 days	Regular Netted	10	970±194	1.42±0.10
	Low vitamin E Netted	10	1225±324	1.43±0.08

malondialdehyde concentrations were found to be significantly greater in salmon than in grilse ($p<0.05$).

As in Experiment 1, handling and crowding had no effect on plasma vitamin E or malondialdehyde concentrations. Plasma cortisol concentrations rose to 8 ng ml^{-1} in the vitamin E supplemented group and 12 ng ml^{-1} in fish fed the unsupplemented diet. These increases are greater than those measured in Experiment 1, despite the similarities of the treatment, but again cortisol concentrations recovered baseline levels within one day. No differences in the effects of acute stress on haematocrit and leucocrit, in relation to vitamin E status, were found in this study. There was thus no evidence to indicate that vitamin E status affected the magnitude of any response to crowding and handling.

Table 5.27. Plasma α-tocopherol concentrations in Experiment 5 (mean±sd)

Time (weeks)	Treatment	n	Plasma α-tocopherol ($\mu g\ ml^{-1}$)
0	Crowded 0 mg kg^{-1}	6	41±15
	Crowded 50 mg kg^{-1}	6	35±15
	Crowded 200 mg kg^{-1}	6	44±31
1	Crowded 0 mg kg^{-1}	6	40±20
	Crowded 50 mg kg^{-1}	6	40±14
	Crowded 200 mg kg^{-1}	6	43±23
3	Crowded 0 mg kg^{-1}	6	45±26
	Crowded 50 mg kg^{-1}	6	41±19
	Crowded 200 mg kg^{-1}	6	35±14
6	Crowded 0 mg kg^{-1}	6	31±13
	Crowded 50 mg kg^{-1}	6	32±13
	Crowded 200 mg kg^{-1}	6	40±17
9	Crowded 0 mg kg^{-1}	6	32±15
	Crowded 50 mg kg^{-1}	6	42±29
	Crowded 200 mg kg^{-1}	6	28±23
12	Crowded 0 mg kg^{-1}	6	21±12
	Crowded 50 mg kg^{-1}	6	22±28
	Crowded 200 mg kg^{-1}	6	23±19

5.3.5 Experiment 5

Plasma vitamin E concentrations measured in Experiment 5 are given in table 5.27. These fell in all groups during the course of the study. In group 1, fed no added vitamin E, plasma concentrations fell from 41±15 μg ml^{-1} on day 0, to a minimum of 21±12 μg ml^{-1} at 12 weeks, but reached 45±26 μg ml^{-1} at 3 weeks. In group 2, fed the 5 mg 100 g^{-1} supplement, plasma vitamin E concentrations fell from 35±15 to 22±28 μg ml^{-1} at 12 weeks, but reached 42±29 μg ml^{-1} at 9 weeks. In group 3, fed the 20 mg 100 g^{-1} vitamin E supplement, they fell from 44±39 μg ml^{-1} on day 0, to 23±19 μg ml^{-1} at 12 weeks. The untransformed data was subjected to analysis of variance, but no significant effects of treatment or time were detected.

Liver vitamin E concentrations in these fish are given in table 5.28. In group 1, they fell from 147±110 μg g^{-1} on day 0, to 93±82 μg g^{-1} at 1 week, and 83±37 μg g^{-1} at 12 weeks. In group 2, they rose from 74±70 μg g^{-1} on day 0 to 177±94 μg g^{-1} at 12 weeks. In group 3 they were more variable. In this group they fell from 108±81 μg g^{-1} on day 0, to 86±36 μg g^{-1} at 1 week, rose to 118±82 μg g^{-1} at 6 weeks, fell again to 92±43 μg g^{-1} at 9 weeks and then rose to 175±97 μg g^{-1} at 12 weeks. The data were subjected to log transformation before statistical analysis. Analysis of variance revealed no significant effects of treatment or time, but plasma and liver vitamin E concentrations were positively correlated ($p<0.01$).

Plasma malondialdehyde concentrations are shown in table 5.29. Fish in all groups showed an increase in plasma malondialdehyde concentrations between 0 and 3 weeks followed by a return to low levels between 6 and 12 weeks. In group 1, plasma malondialdehyde concentrations increased from 0±0 μmol l^{-1} on day 0, to 4.8±4.6 μmol l^{-1} at 3 weeks, then fell to 0.2±0.4 μmol l^{-1} at 9 weeks. In group 2, they increased from 1.3±1.8 to 5.6±3.7 μmol l^{-1} between 0 and 3 weeks, then fell 1.0±2.4 μmol l^{-1} at 6 weeks. In group 3, they increased from 0±0 to 8.0±2.9 μmol l^{-1} at 3 weeks, dropped to 0±0 μmol l^{-1} again at 6 weeks, and reached 2.2±1.8 μmol l^{-1} at 12 weeks. None of the usual transformations yielded normally distributed data, so non-parametric tests were utilised. The Kruskal-Wallis test detected no treatment differences at any time, and Spearman's test showed no correlation between plasma

Table 5.28. Liver α-tocopherol concentrations in Experiment 5 (mean±sd)

Time (weeks)	Treatment	n	Liver α-tocopherol (μg g^{-1})
0	Crowded 0 mg kg^{-1}	6	147±110
	Crowded 50 mg kg^{-1}	6	74±70
	Crowded 200 mg kg^{-1}	6	108±81
1	Crowded 0 mg kg^{-1}	6	93±82
	Crowded 50 mg kg^{-1}	6	89±43
	Crowded 200 mg kg^{-1}	6	86±36
3	Crowded 0 mg kg^{-1}	6	90±54
	Crowded 50 mg kg^{-1}	6	84±58
	Crowded 200 mg kg^{-1}	6	89±47
6	Crowded 0 mg kg^{-1}	6	103±45
	Crowded 50 mg kg^{-1}	6	108±43
	Crowded 200 mg kg^{-1}	6	118±82
9	Crowded 0 mg kg^{-1}	6	89±19
	Crowded 50 mg kg^{-1}	6	121±75
	Crowded 200 mg kg^{-1}	6	92±43
12	Crowded 0 mg kg^{-1}	6	83±37
	Crowded 50 mg kg^{-1}	6	177±94
	Crowded 200 mg kg^{-1}	6	175±197

Table 5.29. Plasma malondialdehyde concentrations in Experiment 5 (mean±sd)

Time (weeks)	Treatment	n	Plasma malondialdehyde ($\mu mol\ l^{-1}$)
0	Crowded 0 mg kg^{-1}	6	0±0
	Crowded 50 mg kg^{-1}	6	0±0
	Crowded 200 mg kg^{-1}	6	0±0
1	Crowded 0 mg kg^{-1}	6	1±2
	Crowded 50 mg kg^{-1}	6	1±2
	Crowded 200 mg kg^{-1}	6	2±1
3	Crowded 0 mg kg^{-1}	6	5±5
	Crowded 50 mg kg^{-1}	6	6±4
	Crowded 200 mg kg^{-1}	6	8±3
6	Crowded 0 mg kg^{-1}	6	2±2
	Crowded 50 mg kg^{-1}	6	1±2
	Crowded 200 mg kg^{-1}	6	0±0
9	Crowded 0 mg kg^{-1}	6	0±0
	Crowded 50 mg kg^{-1}	6	1±2
	Crowded 200 mg kg^{-1}	6	2±4
12	Crowded 0 mg kg^{-1}	6	2±2
	Crowded 50 mg kg^{-1}	6	1±1
	Crowded 200 mg kg^{-1}	6	2±2

Table 5.30. Haematocrit and leucocrit measurements in Experiment 5 (measured)

Time (weeks)	Treatment	n	Haematocrit (%)	Leucocrit (%)
0	Crowded 0 mg kg^{-1}	10	47±7	0.72±0.19
	Crowded 50 mg kg^{-1}	10	42±6	0.88±0.16
	Crowded 200 mg kg^{-1}	10	45±7	0.88±0.17
1	Crowded 0 mg kg^{-1}	10	45±4	0.83±0.16
	Crowded 50 mg kg^{-1}	10	44±4	1.00±0.21
	Crowded 200 mg kg^{-1}	10	44±7	0.76±0.17
3	Crowded 0 mg kg^{-1}	10	46±9	0.85±0.20
	Crowded 50 mg kg^{-1}	10	47±7	1.00±0.55
	Crowded 200 mg kg^{-1}	10	49±9	0.80±0.23
6	Crowded 0 mg kg^{-1}	10	46±9	0.75±0.20
	Crowded 50 mg kg^{-1}	10	44±6	0.67±0.15
	Crowded 200 mg kg^{-1}	10	50±9	0.69±0.26
9	Crowded 0 mg kg^{-1}	10	46±6	0.75±0.24
	Crowded 50 mg kg^{-1}	10	48±8	0.88±0.18
	Crowded 200 mg kg^{-1}	10	46±7	0.69±0.22
12	Crowded 0 mg kg^{-1}	10	48±8	0.76±0.23
	Crowded 50 mg kg^{-1}	10	48±5	0.64±0.17
	Crowded 200 mg kg^{-1}	10	48±10	0.68±0.19

malondialdehyde and plasma vitamin E concentrations. Haematocrit and leucocrit measurements from this experiment are shown in table 5.30. In group 1 they stayed fairly constant, falling from 47±7% on day 0 to 45±4% at 1 week, then rising to 48±8% at 12 weeks. In group 2, they rose irregularly from 42±6% on day 0 to 48±5% at 12 weeks, and in group 3 they increased from 45±4 on day 0 and 44±4% at 1 week, to 49±9 at 3 and 50±9 at 6 weeks, fell to 46±7% at 9 weeks and rose again to 48±10% at 12 weeks. The data was subjected to square root transformation. Analysis of variance revealed only significant effects of fish number ($p<0.001$). Haematocrit was positively correlated with liver vitamin E ($p<0.01$) and plasma malondialdehyde concentrations ($p<0.05$ by Spearman's test), but not with plasma vitamin E concentration.

In group 1, leucocrits rose from 0.71±0.19 on day 0, to 0.83±0.16 at 1 week, and 0.85±0.20 at 3 weeks, then fell to 0.75±0.20 at 6, 0.75±0.24 at 9 and 0.76±0.23% at 12 weeks. In group 2, they were initially higher, rising from 0.88±0.16 to 1.00±0.21 at 1 week and 1.00±0.55 at 3 weeks, but then fell to 0.67±0.15 at 6, 0.88±0.18 at 9 and 0.64±0.17% at 12 weeks. In group 3 they showed a slight fall over the course of the experiment, from 0.88±0.17 on day 0, to 0.68±0.19% at 12 weeks. The data was log transformed before statistical analysis. Analysis of variance showed overall effects of treatment ($p<0.05$) and of time ($p<0.01$), but no significant treatment-time interaction. Multiple comparisons indicated that the treatment means in groups 2 and 3 were significantly different ($p<0.05$), being greater in group 2. A significant negative correlation was found between haematocrit and leucocrit ($p<0.01$). Leucocrit was positively correlated with plasma vitamin E and negatively correlated with liver vitamin E concentrations ($p<0.01$ in each test).

Weight and condition factor data are summarized in table 5.31. Fish weights did not show the expected regular increase during the course of this study. A net increase from 941±213 to 1286±721 g and 1064±520 to 1511±748 g between day 0 and 12 weeks was found in groups 1 and 2 respectively, but in group 3 there was a slight net decrease from 1258±443 g to 1246±572 g over this period. The data were log transformed prior to statistical analysis. Analysis of variance showed no significant treatment or time effects. Condition factors too were variable. In group 1, they rose from 1.31±0.15 on day 0, to 1.38±0.13 at 3 weeks, then fell to 1.26±0.19 at 9, and 1.29±0.27 at

Table 5.31. Weight and condition factor in Experiment 5 (meantsd)

Time (weeks)	Treatment	n	Weight (g)	Condition factor
0	Crowded 0 mg kg^{-1}	10	941±213	1.31±0.15
	Crowded 50 mg kg^{-1}	10	1064±520	1.39±0.13
	Crowded 200 mg kg^{-1}	10	1258±443	1.36±0.15
1	Crowded 0 mg kg^{-1}	10	1140±226	1.36±0.16
	Crowded 50 mg kg^{-1}	10	1228±540	1.36±0.09
	Crowded 200 mg kg^{-1}	10	976±151	1.35±0.14
3	Crowded 0 mg kg^{-1}	10	1211±432	1.38±0.13
	Crowded 50 mg kg^{-1}	10	1164±388	1.34±0.14
	Crowded 200 mg kg^{-1}	10	1077±361	1.30±0.17
6	Crowded 0 mg kg^{-1}	10	923±297	1.31±0.19
	Crowded 50 mg kg^{-1}	10	1002±431	1.22±0.13
	Crowded 200 mg kg^{-1}	10	1111±212	1.26±0.15
9	Crowded 0 mg kg^{-1}	10	1014±336	1.26±0.19
	Crowded 50 mg kg^{-1}	10	1306±356	1.34±0.15
	Crowded 200 mg kg^{-1}	10	1114±315	1.32±0.15
12	Crowded 0 mg kg^{-1}	10	1286±721	1.29±0.27
	Crowded 50 mg kg^{-1}	10	1511±748	1.45±0.28
	Crowded 200 mg kg^{-1}	10	1246±572	1.29±0.12

12, weeks. In group 2, they were reduced from 1.39±0.13 on day 0, to 1.22±0.13 at 6 weeks, then increased to 1.45±0.28 at 12 weeks. In group 3, they dropped from 1.36±0.15 to 1.26±0.15 between 0 and 6 weeks, then rose to 1.32±0.15 at 9, and 1.29±0.12 at 12, weeks. Analysis of variance of the square root transformed data again showed no significant treatment or time effects. Pearson's test showed positive correlations between both weight and condition factor and plasma vitamin E concentration and leucocrit ($p<0.01$), but no correlation with liver vitamin E concentration. Spearman's test showed no correlation between plasma malondialdehyde concentrations and either weight or condition factor.

Sex and state of maturity were determined in all 180 fish collected during this study. Of these 88 were male and 92 female. 80 (91%) of the males and 55 (60%) of the females were sexually mature. Analysis of variance indicated that condition factor ($p<0.001$; F>M), weight ($p<0.05$; F>M), haematocrit ($p<0.001$; M>F), leucocrit ($p<0.001$; F>M) and liver vitamin E concentration ($p<0.001$; M>F) differed significantly between the sexes. Differences between grilse and salmon were found in weight ($p<0.001$; S>G), condition factor ($p<0.001$; S>G), haematocrit ($p<0.05$; G>S), leucocrit ($p<0.001$; S>G) and plasma and liver vitamin E concentration ($p<0.001$; S>G in both cases).

Water temperature was positively correlated with plasma vitamin E concentration and leucocrit ($p<0.01$) and negatively correlated with liver vitamin E concentration ($p<0.05$). No correlation with water temperature was found for plasma malondialdehyde concentration or haematocrit.

Histological sections from three fish per tank were examined from the 12 week sample. No pathological signs were noted consistently, although fat-laden vacuoles were occassionally noted within hearts and one fish showed an unusually high degree of leucocyte infiltration of the peri-pancreatic fat.

No effects of treatment were detected within any of the parameters measured in Experiment 5. In particular, there was no difference in growth rate among fish fed any level of vitamin E, despite the suggestion in Experiment 3 that vitamin E supplementation may improve weight gain. As a result of the onset of maturity, a large proportion of fish sampled during Experiment 5 were found not to be feeding. Nevertheless, liver α-tocopherol

concentrations showed an increase during the course of the study in all groups. However, this experiment must be repeated using immature fish to confirm the absence of any treatment effect.

CHAPTER 6

GENERAL DISCUSSION

6.1 PANCREAS DISEASE

6.1.1 Case histories

Pathological and epizootiological findings in PD were similar to those described previously (section 1.1) and the studies presented in chapter 4 meet the need for documented case histories to illustrate the more general descriptions provided by Munro *et al.* (1984) and McVicar (1987). Three gross clinical features were consistently found. Anorexia characterised all of these outbreaks, and listless, hanging behaviour and loss of weight and condition were observed in the majority of the cases. The case studies included examples of acute and chronic outbreaks of PD and provided serial data from one outbreak. Stress factors were commonly identified as precipitating overt PD and possible routes for transmission of an infectious agent were noted in some, but not all, cases. However, case details were not collected on any fixed basis and there is a need for more objective epizootiological studies in order to identify features predisposing farmed salmon to PD.

6.1.2 Histopathology

Each case of PD was confirmed by the recognition of total exocrine pancreatic necrosis (section 1.2.1.2), in association with gross clinical signs, within the affected population. At present this is the most accurate criterion on which to base a diagnosis of PD. However, since these changes are believed to be secondary to some causative factor, they cannot be regarded to provide a definitive diagnosis. Differences between individuals in the degree of exocrine pancreatic necrosis, even within a single cage, were noted in all cases. Nevertheless, as demonstrated in case E, pancreatic degeneration appeared to occur synchronously (between monthly visits) in all affected fish. This is in agreement with the results of the pathological study described by Munro *et al.* (1984). Cage to cage differences in the proportion of affected fish were noted in case E and these reflected differences in the prevalence of overt PD. Ferguson *et al.* (1986*b*) found that gross signs were not always associated with histological indications of PD. This is supported in the

present study by the finding that fish with exocrine pancreatic degeneration often showed evidence of feeding activity. (ie. their guts contained food).

6.2 VITAMIN E NUTRITION OF ATLANTIC SALMON

The primary purpose of the present study was to determine the role (if any) of vitamin E in the development of PD. The stimulus for this was the observation, by Ferguson *et al.* (1986*a*,*b*), of signs indicative of vitamin E deficiency in farmed salmon suffering from PD. However, the occurrence of similar signs in other recognized disease conditions of Atlantic salmon in seawater, suggested that the vitamin E nutrition of this species, at this stage in its life-cycle, was inadequate. Thus it was necessary to consider, in more general terms, the vitamin E nutrition of Atlantic salmon farmed in seawater.

6.2.1 Tissue α-tocopherol concentrations and vitamin E deficiency

A normal range for plasma and liver vitamin E concentrations in farmed Atlantic salmon was suggested in section 3.3.1. In the present study there was no evidence to indicate that this range was lower than in wild salmon, but further studies are required both to verify the data presented from healthy, farmed fish and to conduct a more detailed comparison between farmed and wild salmon.

In field and experimental studies, a correlation between tissue vitamin E concentration and condition factor was frequently found. This suggests that tissue vitamin E concentrations reflect the overall plane of nutrition of the fish and may therefore be useful as a index of general health and well-being. In support of this, the present study also demonstrated that starvation caused a reduction in tissue vitamin E concentrations. Ferguson and Rice (1980) measured low tissue vitamin E concentrations in brown trout in poor condition.

Conversely, the use of tissue α-tocopherol measurements as specific indicators of vitamin E deficiency is restricted by the limitations described in section 3.1. In particular, there is a scarcity of information on which tissue concentrations should be considered adequate and which deficient. In

addition, since vitamin E status is believed to be affected to a large degree by interacting nutritional, environmental and husbandry-related factors, it would be difficult to determine if particular animals were vitamin E-deficient solely on the basis of measured tissue vitamin E concentrations. Much more work is required to determine the relative importance of these interacting factors on the vitamin E status of salmon.

In vivo microsomal lipid peroxidation and erythrocyte haemolysis tests provide functional indications of vitamin E status and have been used to determine minimum α-tocopherol requirements in experimental studies on salmonids (Poston *et al.* 1976; Cowey *et al.* 1981, 1983, 1984). In addition, well-defined pathological changes, such as muscle degeneration and anaemia, have been widely recognised as indicators of vitamin E deficiency in fish (section 1.2.6). In order to confirm the effects of vitamin E deficiency on Atlantic salmon in seawater, it is necessary to repeat the studies of Bell, McVicar, Mitchell and Cowey (unpublished) (section 1.2.5.2) and these could be extended to examine factors, particularly level of exercise, which may affect the development of myopathy in vitamin E deficiency. It is also necessary, when relating the results of experimental studies of vitamin E deficiency to the situation on farms, to have data available on normal ranges for biochemical and histological parameters in healthy fish. Consequently, it would be worthwhile to randomly screen farmed salmon for signs similar to those observed in experimental vitamin E deficiency.

In the studies presented in section 5.3, there was no evidence that non-specific stress factors had any significant effect on tissue vitamin E concentrations or on the expression of pathological signs of deficiency in fish fed unsupplemented diets. Tissue malondialdehyde concentrations bore no consistent relationship to vitamin E concentrations. Sensitive biochemical indicators of vitamin E status were not examined in the present study, but Gatlin *et al.* (1986*b*) found no difference in the degree of *in vivo* microsomal lipid peroxidation between stressed and unstressed channel catfish. Starvation resulted in vitamin E depletion in one study (section 5.3.2), but not in another (section 3.3.4). Despite this inconsistency, the results of the former demonstrate that changes in food intake may affect vitamin E status. However, under experimental conditions (section 3.3.3), no differences in plasma

vitamin E concentrations were detected in fish fed at rates of between 1% and 3% body weight day^{-1}.

6.2.2 Level of dietary supplementation

There was no evidence from the study presented in section 3.3.1 to suggest that current levels of vitamin E supplementation are inadequate for healthy, farmed Atlantic salmon. Salmon in seawater, fed diets containing approximately 20 mg *all-rac*-α-tocopheryl acetate 100 g^{-1}, showed an accumulation of vitamin E in the liver and plasma and a progressive reduction in the PUFA:vitamin E ratio in these tissues. Although more sensitive tests were not performed, no histological evidence of vitamin E deficiency was noted. However, it is important to be aware that vitamin E requirements may vary during the course of development.

Interestingly, it appears that transfer to seawater may result in an increased requirement for vitamin E associated with an increase in the proportion of polyunsaturated fatty acids in the tissues. This suggestion requires experimental confirmation, but, in this light, vitamin E supplementation of diets for smolts prior to and after transfer to seawater may be advised.

Changes in vitamin E requirements may also be expected as a result of differences in growth rate, season, sex and state of maturity. In some fish species vitamin E treatment has enhanced reproductive performance. Further examination of the vitamin E requirement of salmon broodstock in relation to reproduction may be particularly rewarding, but there is also a case for the re-examination of vitamin E requirements in Atlantic salmon at other stages in their life history and in particular during growth in seawater.

There is little evidence that vitamin E supplementation increases resistance to disease in Atlantic salmon (section 1.2.5.5), and in the present study, responses to stress were unaffected by the level of dietary vitamin E supplementation. However, further studies are required to confirm that high-level vitamin E supplementation produces no economic advantage in terms of survival and performance of Atlantic salmon under farm conditions. Studies on the effects of dietary vitamin E supplementation on product quality of farmed

Atlantic salmon may also be of commercial value.

From the literature review presented in section 1.2, it is clear that nutrient interactions should be considered in relation to vitamin E requirements when formulating new diets. In particular, factors which increase tissue polyunsaturated fatty acid concentrations (such as lipid quality and quantity), or the degree of dietary lipid oxidation (pro-oxidants), may increase vitamin E requirements. On the other hand, increases in available selenium or vitamin C (for example by the use of new, stable forms of ascorbic acid) may act to spare vitamin E. Further studies on vitamin E-nutrient interactions in relation to the formulation of practical diets may be useful in order to optimize the vitamin E nutrition of farmed salmon.

6.2.3 Form of vitamin E supplement

Alternative forms of vitamin E supplement are increasingly becoming available for use in animal feeds (Papas *et al.* 1989). These include both 'natural' (*RRR*-) and 'synthetic' (*all-rac*-) products, as the free-alcohol or a variety of esters, and in lipid-soluble, water-miscible and water-soluble forms. Commercially produced lipid-soluble forms include mixed tocopherols (*RRR*-α, β, γ and δ-tocopherols), *all-rac*- and *RRR*-α-tocopherol and *all-rac*- and *RRR*-α-tocopheryl acetate and succinate. Water-miscible forms include α-tocopheryl acetate (*all-rac* or *RRR*-) dispersed within a polyethylene glycol or gelatin matrix. A water-soluble form, *RRR*-α-tocopheryl polyethylene glycol 1000 succinate, has recently become available. Synthetic (*all-rac*) α-tocopheryl acetate is most often used as the vitamin E supplement in salmon diets, but natural forms may have a higher specific activity (Scott and Desai 1964). Wild fish are expected to receive most vitamin E as the naturally occurring *RRR*-α-tocopherol, whereas farmed fish are fed diets supplemented with *all-rac*-α-tocopheryl acetate. Hidiroglou *et al.* (1988*a*) showed that sheep fed *RRR*-α-tocopherol developed higher tissue vitamin E concentrations than those fed larger amounts of *all-rac*-α-tocopherol to provide the same biological activity, and Hidiroglou *et al.* (1988*b*) found that natural forms of either the alcohol or acetate form increased plasma α-tocopherol concentrations in cattle at a faster rate than the synthetic forms.

In the present study, no advantage in the use of *all-rac*-α-tocopherol as opposed to α-tocopheryl acetate was found, despite the hypothesis that the acetate form may be absorbed in an unavailable form. As demonstrated in section 4.3.10.3., the free alcohol suffered considerable loss due to oxidation in the feed whilst the acetate ester was stable. Lipid-soluble forms show poor absorption efficiency in mammals, whilst water-miscible forms are absorbed more effectively (Schmandke and Schmidt 1965). In the present study, a water-miscible form was shown to increase tissue vitamin E concentrations in fish, but further studies are required to quantify its efficacy. The water-soluble *RRR*-α-tocopheryl polyethylene glycol 1000 succinate, has recently been found to be absorbed efficiently by large zoo ungulates (elephants and black rhinoceros), whereas fat-soluble or water-miscible *RRR*- and *all-rac*- forms showed poor absorption (Papas *et al.* 1989). In rainbow trout, Hung *et al.* (1982) found that the rate of uptake of natural (*RRR*-) α-tocopherol was greater than that of *all-rac*-α-tocopheryl acetate, but the experimental technique did not allow quantification of the absorption efficiency. Experimental comparisons of the relative efficency of the various forms may indicate economic alternatives for use in fish feeds.

6.3 THE ROLE OF VITAMIN E IN PANCREAS DISEASE

6.3.1 Plasma vitamin E concentrations in natural outbreaks of PD

Ferguson *et al.* (1986*b*) recorded vitamin E concentrations of 2 and 6 μg ml^{-1} in plasma, and 21 and 65 μg g^{-1} in liver, of Atlantic salmon showing overt and sub-clinical signs of PD respectively, during one outbreak of PD in the Western Isles. Bell *et al.* (1987*b*) gave values of 17 μg ml^{-1} in plasma, 119 μg g^{-1} in liver and 6 μg g^{-1} in white muscle in fish in the acute phase of an outbreak in the Shetland Islands. In the present study, the minimum mean plasma concentration ranged from 0 (case H) to 29 μg ml^{-1} (case E) (table 6.1). Low values recorded from the 1987 case studies (cases A to D) must be treated with caution since blood samples suffered severe haemolysis which may have reduced the vitamin E content. Nevertheless, this range, which encompasses the data of Ferguson *et al.* and Bell *et al.*, is lower than the

Table 6.1. Minimum mean plasma vitamin E concentrations, together with the presence or absence of muscle degeneration, summarized from each case study

Case no.	Plasma vitamin E ($\mu g\ ml^{-1}$)	Muscle involvement
A	2	N
B	2	Y
C	6	N
D	18	N
E	31	N
H	0	Y
I	8	Y
J	5	Y
K	15	Y
L	12	Y

suggested normal range of 34 to 68 $\mu g\ ml^{-1}$ described in section 3.3.1. Vitamin E depletion may therefore be regarded to be a consistent feature of PD.

The selection of groups of fish for unbiased statistical comparison was difficult. Comparisons of healthy and affected farm sites revealed differences in plasma and liver vitamin E concentrations as well as in plasma lipid concentrations, fish weight and condition factor (section 4.3.5.7). However, the use of fish from different sites introduces the possibility of site-wise variability independent of the occurrence of PD. Comparisons of cage populations during case study E suggested that plasma and liver vitamin E concentrations reflected the level of overt PD within a cage, although these differences were not statistically significant (section 4.3.5.6). Since apparently healthy cage populations on an affected site may show sub-clinical indications of PD (Ferguson *et al.* 1986*b*), these are also not ideal as controls. Individual classification on the basis of histopathology is time-consuming, but at present the most accurate method of comparing affected and unaffected fish during the acute and post-acute phases. In case E, this method demonstrated that liver vitamin E concentrations were significantly lower in fish with EPD, but no significant difference in plasma vitamin E concentrations was detected. In the future, rapid diagnostic tests may allow useful comparisons to be made during the earlier stages of PD.

Larger numbers of fish were examined in the present study than previously, and samples were collected on several dates. Serial sampling during the course of the acute case H suggested that plasma vitamin E concentrations were reduced early in the outbreak, but in the chronic case I a gradual reduction during the course of the outbreak was noted. In case E, plasma vitamin E concentrations were not reduced during the course of the outbreak, but failed to show the increase found in healthy fish. Liver vitamin E concentrations in fish with PD were measured only in case E. These reached a minimum of 118 $\mu g\ g^{-1}$ compared to 21 and 65 $\mu g\ g^{-1}$, and 119 $\mu g\ g^{-1}$, in studies described by Ferguson *et al.* (1986*b*) and Bell *et al.* (1987*b*). Fewer fish showed gross signs of PD in case E and the outbreak was therefore judged less severe than in other cases studied. This is reflected in the tissue vitamin E concentrations which were reduced to a lesser degree than in other cases (table 4.23). Serial changes in the liver revealed a gradual diminution of vitamin E during the course of case E. Interestingly, this appeared to begin between July and August, prior to the onset of overt PD in September/October. Early changes in tissue vitamin E concentrations have been observed previously in fish developing PD (R.Palmer, personal communication 1988). However, in the present study, differences between the means in July and August were not demonstrated statistically.

Fish from case E failed to show the sharp increase in plasma α-tocopherol concentration which, in healthy fish, was associated with a rise in the proportion of lipid as polyunsaturated fatty acid. Plasma polyunsaturated fatty acid concentrations were not measured in samples from case E, but total lipid concentrations were generally higher than those found in healthy fish, which suggests that the PUFA:vitamin E ratio may have been increased substantially in these fish. Further biochemical analysis would be necessary to confirm that plasma PUFA concentrations were not reduced during case E, but Ferguson *et al.* (1986*b*) found no difference in tissue PUFA profiles between PD-affected and unaffected fish. In case E, although an initial rise in liver vitamin E concentrations was evident for two months after transfer, α-tocopherol concentrations subsequently fell. Depletion of hepatic α-tocopherol reserves suggests that the vitamin E supply is inadequate to meet demand. Since vitamin E depletion is a consistent feature of pancreas

disease it is important to consider possible explanations for this effect.

In the present study, both healthy and affected farmed fish were fed similar diets containing approximately 20 mg 100 g^{-1} α-tocopherol. A simple inadequacy of dietary vitamin E is therefore unlikely to be the cause of vitamin E depletion in fish with PD. Ferguson *et al.* (1986*b*) also considered that measured vitamin E and selenium concentrations in the diets of fish with PD were adequate and, in addition, they noted that there was no evidence of lipid oxidation in the diets of affected fish. Since farmed salmon are successfully reared on commercial diets ostensibly the same as those fed to salmon developing PD, there is no evidence to suggest that any aspect of diet formulation or preparation is responsible for vitamin E depletion in PD.

Although effects of starvation on vitamin E concentrations were demonstrated in section 5.3.2, all fish in case E were known to be feeding in July and August and the majority of fish were appetant throughout the period of overt PD. It therefore seems unlikely that anorexia is the sole cause of vitamin E depletion in PD, although it may play a large part during the acute phase of disease. A reduction in food intake is also unlikely to be the main cause of vitamin E depletion given the high condition factors and the general increase in plasma lipid concentrations measured in these fish prior to the development of overt PD.

In case E, pancreatic necrosis was first observed only when liver vitamin E concentrations were already reduced. However, it is difficult to ascertain whether or not vitamin E depletion preceded the onset of structural change in the pancreas. Since plasma lipid concentrations showed no sign of any decrease prior to the overt phase of PD there was no suggestion that a general lipid malabsorption was a feature of pre-acute PD. However, malabsorption due to pancreatic insufficiency probably contributed to vitamin E depletion during the acute phase. The possibility of a specific malabsorption of vitamin E seems unlikely, given the close relationship between vitamin E and lipid uptake described in section 1.2.9.2.

Alternatively, an increased metabolic demand for α-tocopherol may result from the actions of various environmental and husbandry related factors (section 1.2.7). The possibility that non-specific stress responses lead to vitamin E depletion was considered in section 5, but there was no support for

the hypothesis that these are directly responsible for vitamin E depletion in PD. There is some evidence that infection may increase the vitamin E requirement or induce deficiency in mammals (McMurray and Rice 1982). Histological signs of vitamin E deficiency in fish suffering from microbial diseases were reported by Wood and Yasutake (1956), Ferguson and Rice (1980), Fjelstad and Heyeraas (1985), Sniesko *et al.* (1957) and Poppe *et al.* (1986). In the present study, significant differences in plasma vitamin E concentrations were observed in fish with experimentally-induced exocrine pancreatic degeneration, but differences between challenged and unchallenged groups overall were not significant. These differences developed despite the fact that these fish were not fed, and were therefore not caused by malabsorption or differences in food intake. Vitamin E depletion may occur as a direct result of the process of degeneration of the pancreatic tissue. Reactive oxygen metabolites are involved in leucocyte responses to tissue damage (Badwey and Karnovsky 1980) and loss of vitamin E in PD may be due to the production of these free-radicals during the processes of tissue repair. Chardavoyne *et al.* (1989) indicated that similar metabolites are involved in the pathogenesis of acute pancreatitis in mammals. It would be interesting to examine the effects of pancreatic degeneration from other causes on tissue vitamin E concentrations in salmon. In this respect it may be significant that muscle degeneration has been found in association with clinical outbreaks of IPN (Sniesko *et al.* 1957).

6.3.2 Effects of depletion

If depletion is severe or prolonged, or exacerbated by elevated tissue PUFA concentrations, pathological signs of vitamin E deficiency may be induced in fish with PD. Bell *et al.* (1987*b*) found that tissue vitamin E concentrations in PD were not reduced to a level comparable to those produced experimentally which resulted in the development of deficiency signs in Atlantic salmon. In contrast, the results summarised in table 6.1 demonstrate that, in some cases of PD, plasma vitamin E concentrations may fall to very low levels (less than 5 $\mu g\ ml^{-1}$ in some outbreaks). These are well within the range at which muscle degeneration has occurred in experimental studies of

vitamin E deficiency in salmon (section 1.1.5.2). Furthermore, as stated previously, it is difficult to be sure at what level tissue vitamin E concentrations in Atlantic salmon farmed in seawater may be considered deficient.

Although it remains possible that vitamin E deficiency is a feature of PD, it has yet to be demonstrated that muscle degeneration, in association with PD, is a result of vitamin E depletion. In support, mean minimum plasma α-tocopherol concentrations were found to be lower in cases H and I, which exhibited muscle degeneration, than in case E which did not. However, there was no general correlation between the occurrence of muscle degeneration and the degree of vitamin E depletion in each outbreak. This observation is hardly surprising given that differences in the course and severity of PD among individual fish may be reflected in greater variability of tissue vitamin E concentrations in an affected population. Comparisons between individual fish, with and without muscle degeneration, may provide more convincing data, but in the present series of studies such comparisons were not conducted. Even if a correlation between vitamin E depletion and the occurrence (and severity) of muscle degeneration were to be demonstrated, it is important to note that this would not necessarily imply any causal relationship.

The hypothesis that muscle degeneration in PD is due to vitamin E deficiency would be further supported by the observation of other vitamin E deficiency signs in fish with PD. Whilst some signs of PD resemble those of vitamin E deficiency (table 6.2), other specific haematological or histological signs of vitamin E deficiency have not been described in PD-affected fish. In particular, no evidence of anaemia has been shown consistently in PD, but further haematological studies could be justified. Tissue malondialdehyde concentrations were increased as a result of *in vivo* lipid peroxidation in vitamin E deficient carp (Watanabe *et al.* 1970*b*) and red sea bream (*Chrysophrys major*) (Murata and Yamauchi 1989), but there was no significant relationship between plasma vitamin E and malondialdehyde concentrations in case H in the present study. However, malondialdehyde concentrations were initially higher than in vitamin E replete healthy, experimental fish (section 4.3.8.6). Plasma PK activities measured in cases H and I were also elevated in relation to samples collected from healthy fish

Table 6.2. Clinical signs common to vitamin E deficiency and pancreas disease

weight loss
inappetance
cachexia
poor stress tolerance
epicarditis
elevated plasma PK
cardiac and skeletal myopathy

during experimental studies. The degree of elevation in cases H and I was similar to that described by Bell *et al.* (1987*b*) in fish with PD, but less than that described in vitamin E-selenium deficient salmonids (Bell *et al.* 1985, 1986; section 1.2.5.2).

Another possible consequence of vitamin E depletion in PD relates to its role in susceptibility to infection. Raynard *et al.* (1990) induced vitamin E depletion by feeding low vitamin E diets to experimental salmon and then subjected these fish to challenge with kidney material from fish with PD. Vitamin E depleted fish showed increased susceptibility to exocrine pancreatic degeneration as a result of challenge. However, the practical significance of this finding is uncertain since it is not clear whether vitamin E depletion precedes or succeeds pancreatic degeneration in PD.

If vitamin E depletion precedes pancreatic necrosis, then the association between vitamin E depletion and pancreatic degeneration as a result of infection in PD may be understood as an increased susceptibility to infection, but the process of vitamin E depletion remains a mystery. The suggested early vitamin E depletion, together with the apparent occurrence of signs of vitamin E deficiency in other diseases of salmon support this interpretation. Alternatively, if vitamin E depletion is considered to be a consequence of infection, then the processes of vitamin E depletion can be explained, but any pathological consequences must be viewed as secondary and the occurrence of signs of vitamin E deficiency in association with other conditions must be considered to be unrelated and not indicative of a general problem in the vitamin E nutrition of farmed salmon. This interpretation is

supported by the lack of evidence of vitamin E depletion in healthy salmon (section 3.3.1), the absence of a statistically significant reduction in tissue vitamin E concentrations prior to acute PD (section 4.3.5.4) and the success in inducing exocrine pancreatic degeneration (albeit to a lesser degree) in vitamin E-adequate fish (Raynard *et al.* 1990).

6.3.3 Effects of vitamin E supplements on PD

In case H, there were no signs that high-level vitamin E supplementation was successful in elevating tissue vitamin E concentrations or in preventing vitamin E depletion. This may be a result of failure to feed or to absorb α-tocopheryl acetate from the diet. However, Pudelkiewicz and Mary (1969) showed that, in chicks fed graded levels of α-tocopheryl acetate, plasma vitamin E concentrations plateaued at levels above 66.7 mg 100 g^{-1} diet. This suggests that there are limitations to the uptake of large quantities of dietary vitamin E even in healthy animals. Histological examination confirmed the presence of muscle degeneration, perhaps as a result of vitamin E deficiency, in fish with PD fed diets containing high levels of α-tocopheryl acetate (cases B and H). The beneficial effects of dietary vitamin E supplements on various vitamin E-responsive myopathies of fish were described in section 1.2.8, and failure to prevent myopathy associated with PD may be attributed to the unavailability of the vitamin E supplement for the reasons suggested above, rather than to any lack of response to vitamin E. Indeed, the reduction in plasma PK activity in PD-affected fish treated with vitamin E by injection suggests that the myopathy will in fact respond to vitamin E, although there was no histological evidence of any improvement. Mitowsky *et al.* (1962) found that injected vitamin E was effective in preventing creatinuria, indicative of muscle degeneration, in children with cystic fibrosis.

Fish suffering a natural outbreak of PD fed a diet containing 100 mg α-tocopherol 100 g^{-1} showed no elevation of plasma vitamin E and no reduction in plasma PK activity. The majority of fish collected were actively feeding and, although there was considerable loss, elevated α-tocopherol levels were still measured in the feed after 5 weeks storage. It is possible that any absorbed α-tocopherol was rapidly metabolised as described above, but, given

the severe degree of pancreatic atrophy, it seems more likely that impaired digestion and absorption prevented the uptake of significant amounts of the supplement. Nevertheless, the study demonstrated that the use of α-tocopheryl acetate as a feed supplement, in place of the biologically active α-tocopherol, was not responsible for the failure to elevate tissue vitamin E concentrations in fish with PD.

The absorption of α-tocopherol and α-tocopheryl acetate are believed to be closely linked to the absorption of lipids and dependent on pancreatic and biliary secretions (section 1.2.9.2). Thus water-miscible vitamin E supplements may be more effectively absorbed in fish with PD. In section 4.3.11.3, plasma vitamin E concentrations in fish fed a water-miscible form of α-tocopheryl acetate were found to double over the 12 day experimental period. Although this increase was not statistically significant, and a slight (although again not significant) depletion was recorded in the liver, these results provide some indication that water-miscible forms of vitamin E may circumvent malabsorption of vitamin E during outbreaks of PD. However, further studies are required to verify this conclusion and to study the effects of this and other vitamin E compounds on myopathy in PD.

APPENDIX

EXPERIMENTAL TANK FACILITY

A.1 INTRODUCTION

A tank system was constructed for the purpose of conducting the experimental work described in section 5. This system was designed to hold up to 500 kg Atlantic salmon in six three-metre diameter fibreglass tanks and was sited at the Dunstaffnage Marine Laboratory (fig. A.1. inset). Due to limitations on the flow available from an existing pump, and the difficulty of disposing of a large volume of effluent via a soakaway, the system was built to recirculate up to 50% of the water flowing through the tanks.

A.2 DESIGN

A.2.1 General description

Filtered seawater was taken from a bay at a point 250 m to the north-west of the tank site, using a shore-based pump. Water was pumped into a small header tank and from there fed by gravity into the stock tanks. The rate of water flow through each tank was approximately 80 l min^{-1}. Tank effluent passed into a concrete channel and then to a large fibreglass settling tank where solids were removed. A submersible pump in one end of the settling tank pumped about half of the incoming water into an array of biofilters where ammonia was removed. The treated water then rejoined the main supply to the stock tanks. Waste water flowed from the settling tank into a large, gravel-filled pit or 'soakaway' from where it percolated into the surrounding ground. Stock tanks and biofilters were supplied with air, and an alarm system monitored the action of both pumps. A diagrammatic representation of the system is shown in fig. A.1.

A.2.2 Design and construction

The main seawater pump was a positive displacement Mono Merlin, model no. SAE 12M1R5/M1, with a maximum flow capacity of 317 l min^{-1}. It was powered by a 4KW 3-phase electric motor connected via a V-belt coupling to drive the pump at 720 rpm. The rotor and coupling rod were constructed of type 316

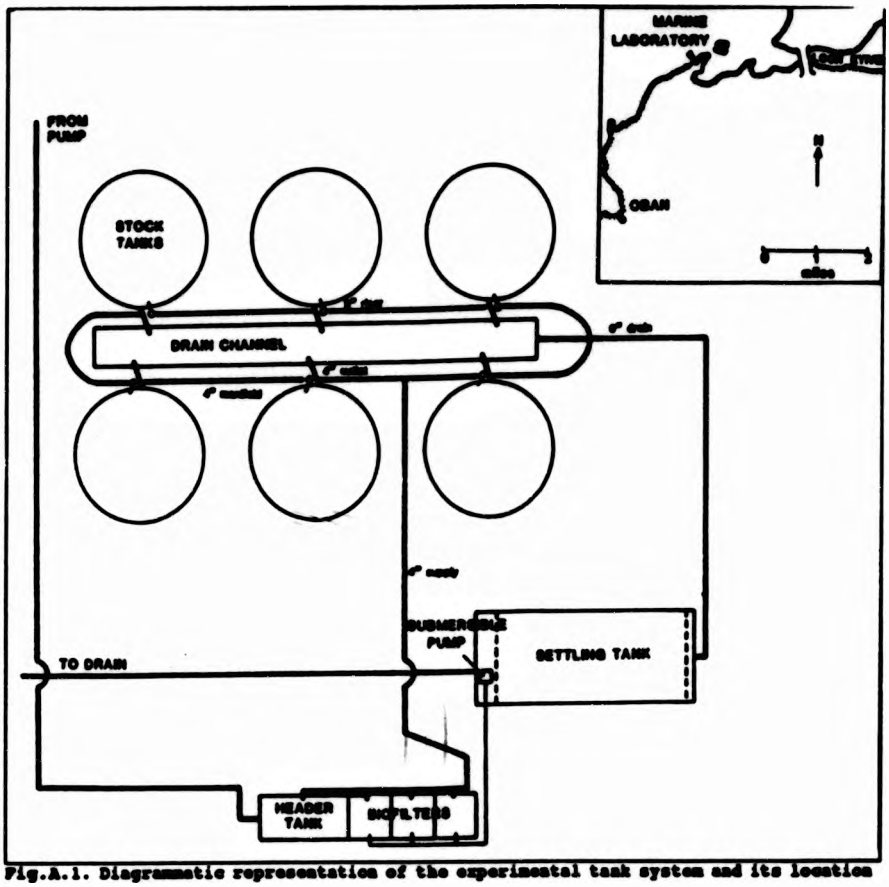

Fig.A.1. Diagrammatic representation of the experimental tank system and its location

stainless steel and the stator was of industrial grade nitrile rubber. This pump was manufactured by Mono pumps Ltd. Manchester, and supplied by Flowplant Ltd., Edinburgh.

The main pipeline was constructed of 90 mm diameter medium density polyethylene (MDPE) pipe. It ran a distance of approximately 250 m and was laid in a trench for most of its length. The intake was protected with two sub-sand filters dug into the seabed below the extreme low water mark (SWS Filtration Ltd., Hartburn, Northumberland) (Cansdale 1982).

Calculation 1. Expected flow from beach pump.

The positive displacement pump had a maximum flow of 317 l min^{-1} at 720 rpm, equivalent to 5.278 l s^{-1}. The main pipeline had a length of 250 m, and a diameter of 90 mm. From tabulated figures for MDPE pipework (Uponor Ltd. 1987), water flowing through a 90 mm diameter pipeline at a rate of 5.3 l s^{-1} induces a hydraulic gradient of 0.022. Over a distance of 250 m this corresponds to head of water (*hf*) equivalent to:

$$hf = 0.022 \times 250 = 5.5 \text{ m}$$

In addition, due to the elevation of the header tank above the point from which water was drawn, there was an actual head of 9.5 m against which the pump had to operate. Thus the total head (*H*) was:

$$H = 9.5 + 5.5 = 15\text{m or } 1.5 \text{ bar}$$

According to the manufacturers specifications, our pump could provide a flow of 300 l min^{-1} against 1.5 bar. However, there were also minor head losses, in particular due to the sub-sand filters, which were difficult to calculate. In practice a flow rate of 200-250 l min^{-1} was measured at the inlet to the header tank, depending on the state of the tide.

Filter, header and stock tanks were of one-piece, glass-reinforced plastic (GRP) supplied by PPS Glassfibre Ltd., Inverurie, Aberdeen. The settling tank was of GRP coated plywood and supplied by Cruachan Marine Ltd., Taynuilt, Argyll. All were supplied with lids. The header tank measured 2m x 1m x 1m deep. It was sited on scaffolding at a height of 2.5 m above ground level. The outlet pipe was three inches in diameter and fitted with a valve to regulate flow and thus maintain a constant head of water.

The main supply pipe was constructed of four-inch unplasticized polyvinylchloride (uPVC) and received water from header and filter tanks before descending to ground level. This pipe supplied a four inch diameter ring main. Three inch diameter risers fed water from the ring main into the stock tanks. These risers were fitted with valves to control the flow of water

into each tank.

Calculation 2. Pipe diameter of main supply to stock tanks.

A flow rate of 500 l min^{-1} or 8.3 l s^{-1}, was required through the main supply pipe (see below). The head available between the water surface in the header tank and the inlets to the stock tanks was 2 m. The length of pipe required between the header/filter array and the stock tanks was estimated to be 20 m, thus the hydraulic gradient (h) was calculated as:

$$h = 2 \div 20 = 0.1$$

From tabulated values relating to uPVC pipes (British Plastics Federation 1980), the minimum pipe diameter allowing a flow of 8.3 l s^{-1} against a hydraulic gradient of 0.1 was found to be three inches. In addition minor head losses due to elbows, valves, etc. were not quantified, but had to be allowed for. A combination of four and three inch pipe proved satisfactory in practice.

The stock tanks were circular, three metres in diameter and 1.25 m deep, and held a maximum of 8.0 m^3 water. The tank inlets were three inch diameter spargers and the outlets, protected by a flat screen, exited centrally through the base of each tank. The outlet pipes were of four inch diameter uPVC, feeding into three inch diameter 'swinging-arm' type stand pipes which controlled the water level within the stock tanks.

Calculation 3. Tank volume requirements.

A maximum stock biomass of 500 kg was selected as the basis for the design since fish weights of up to 2.5 kg per individual were expected and adequate numbers were required for experimentation. The total tank volume (V) required to hold 500 kg of fish at the relatively low density of 10 kg m^{-3} is:

$$V = 500 \div 10 = 50 \text{ m}^3$$

This volume could be met:

a) using thirteen 2m diameter tanks (individual volume=3.9 m^3)

b) using six 3m tanks (volume=8.8 m^3), or

c) using four 4m tanks (15.7 m^3).

Six 3m tanks were selected as allowing the required stock biomass and density, whilst giving adequate replication of treatments.

Calculation 4. Water flow requirements.

The total seawater requirement was calculated under the assumption that 1 l min^{-1} provides an adequate supply of oxygen to support 1 kg salmonid fish (Huguenin and Colt 1989). Thus the total requirement (Q) was simply:

$$Q = 500 \text{ kg} \times 1 \text{ l min}^{-1} \text{ kg}^{-1} = 500 \text{ l min}^{-1}$$

Water flowed from the stock tanks into a concrete channel which measured 0.7 m x 10.0 m x 0.5 m deep and carried water via a six inch drain into the settling tank. The dimensions and characteristics of this tank are shown in fig. A.2.

Calculation 5. Settling tank volume.

Muir (1981) reported that, in jar settling tests, up to 90% of solids from trout culture were removed from the water column within 15 mins, thus the volume of the settlement tank was calculated to give a residence time of this duration. At a flow rate of 500 l min^{-1}, or 0.5 m^3 min^{-1}, the volume (V) required to give a 15 min residence time is:

$$V = 15 \times 0.5 = 7.5 \text{ m}^3$$

To increase sedimentation efficiency the settlement area was maximised and the tank fitted with removeable baffles to increase upward water movement.

The second baffle also provided a pumping chamber containing relatively clean water. A submersible Grindex Minex (type 170720) centrifugal pump was

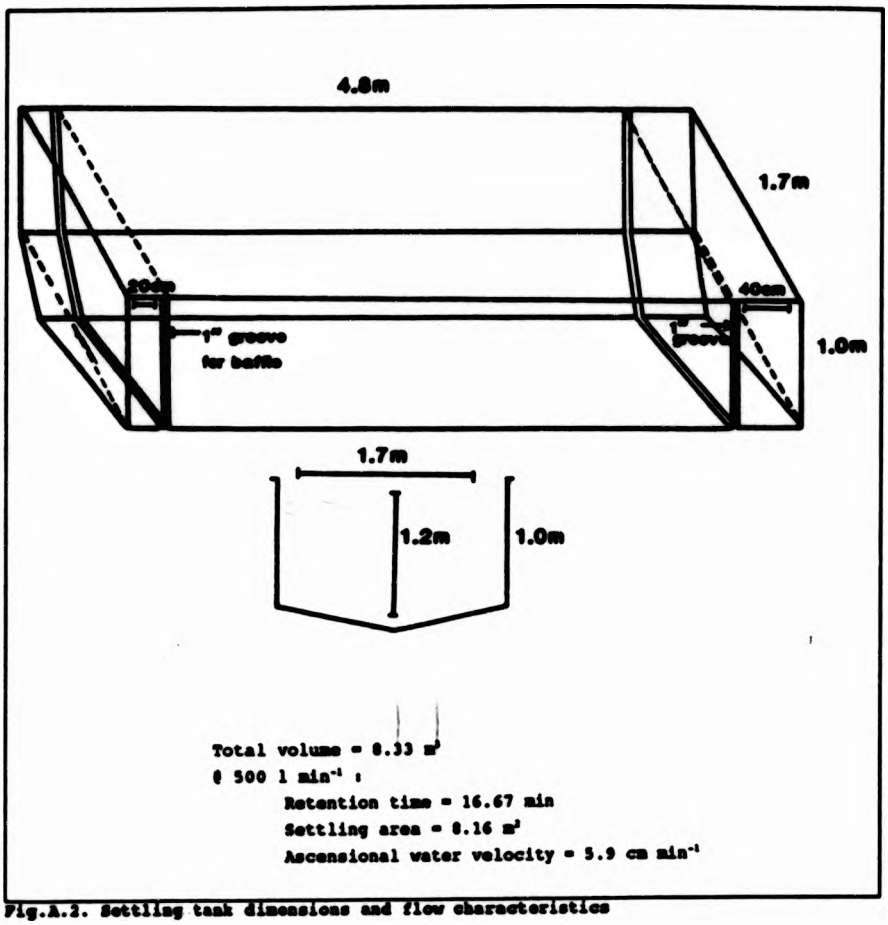

Fig.A.2. Settling tank dimensions and flow characteristics

situated within this chamber. This pump was supplied by Ritchie, MacKenzie and Co. Ltd., Glasgow, powered by a 1.1KW 3-phase electrical motor, and operated at 2800 rpm with a maximum capacity of 675 l min^{-1}. It was fitted with zinc anodes to protect against corrosion. Approximately 9 m of two inch diameter pipework carried water to the three biofilters which were situated on scaffolding together with the header tank at a height of 2.5 m above ground level.

Calculation 6. Flow requirement from submersible pump.

A flow of 250 l min^{-1} or 4.2 l s^{-1} was required, through a pipe two inches in diameter and 9 m in length, to the array of biofilters. According to tables, the hydraulic gradient induced by water flowing at 4.2 l s^{-1} through a two inch pipe is 0.07. Thus the head (*hf*) due to frictional losses within the pipe was:

$$hf = 0.07 \times 9 = 0.63 \text{ m}$$

In addition there was a vertical distance of 2.5+1+1 m, giving a total head (*H*) of:

$$H = 0.63 + 4.5 = 5.13 \text{ m or approximately } 0.5 \text{ bar}$$

Thus the pump was selected to give a flow of 250 l min^{-1} at 0.5 bar.

The biofilters measured 1m x 1m x 1m deep and contained mesh bags filled with biofilter medium. Two inch diameter outlets linked back into the main supply. Both inlet and outlet pipes were fitted with valves so that it was possible to isolate each tank. Additional pipework was installed to enable backflushing.

Calculation 7. Biofilter volume.

The expected production of ammonia (NH_3-N) from salmonid fish can be estimated as 0.0365 x weight of feed supplied (Liao 1970). For 500 kg stock biomass, fed at 2% body weight day^{-1}, the total ammonia production (*Np*) was calculated to be:

$$Np = 0.0365 \times 10 \text{ kg} = 0.365 \text{ kg } NH_3\text{-N per day}$$

The biofilter medium used (Filterpak CP50; Mass Transfer Ltd., Kendal), had a specific area of approximately 200 m^2 m^{-3} and an NH_3-N removal efficiency of about 1 g m^{-2} day^{-1}. Thus the volume (*V*) required to remove 0.365 kg NH_3-N per

day was:

$$V = 365 \div (200 \times 1) = 1.825\ m^3$$

In practice, three $1m^3$ filter tanks were installed to allow cleaning and reconditioning of the filters when necessary.

Overflows from the header and filter tanks were constructed of four inch diameter pipe and led into the settling tank. A six inch diameter drain led a distance of about 100 m from the settling tank to the 'soakaway'. This consisted of a pit about three metres wide and 20 m long, excavated to a depth of about four metres and filled with cobbles. Waste water entering the soakaway drained below ground level into the earth.

Aeration was supplied by a BVC blower (type YP3 100) driven by a 0.75 KW, 3-phase electric motor running at 10000 rpm. This unit was supplied by D.D.Lamson PLC, Gosport, Hants. It had a capacity of 300 $ft^3\ min^{-1}$ and was connected to a two inch uPVC ring main. Dome diffusers, one in each biofilter and two in each stock tank, were linked to the ring main by 3/4 inch Sircoflex hose and uPVC pipe. The 3/4 inch pipes were fitted with valves to regulate air flow.

Calculation 8. Blower power.

The estimated oxygen requirement for 500 kg Atlantic salmon, assuming a maximum active metabolic rate of 1 g $O_2\ kg^{-1}\ hr^{-1}$, is 500g $O_2\ hr^{-1}$ (Liao 1971). A typical transfer efficiency (oxygen out/power in) for a blower-diffuser system is 1.22 kg-O_2 per KW-hr (Huguenin and Colt 1989). Assuming an overall blower efficiency of 67%, the minimum power rating (W) was calculated as:

$$W = (1/67) \times (0.5) \div 1.22 = 0.61KW \qquad \text{(Huguenin and Colt 1989)}$$

The tank area was fenced and a shed provided for working and storage, and for installation of the electrical supply. The three-phase supply was taken, via a busbar connection, from the mains supply in the laboratory building to a distribution board located in the shed. From here, connections

were made by underground cabling to the beach pump, the submersible pump and the blower. In addition, a single phase supply was provided for compound and shed lighting, power points and the alarm system. Each circuit was wired through a main circuit breaker, and additional protection was given by the installation of residual current circuit breakers as appropriate. In case of power failure, the main laboratory generator, previously wired into the buiding, provided an emergency supply.

The alarm system was designed and built by D. Milroy, University of Stirling. The main pump was monitored using a pair of float switches, situated in the header tank, which were activated in the event that water fell below a certain level in this tank. The submersible pump was monitored by a reed-type vertical float switch situated in a blind end of the two inch biofilter supply as described by Ross and Muir (1987). Both sets of switches were linked into an alarm panel which emitted visual and audible alarms.

A.3 PERFORMANCE

A.3.1 Fish growth and survival

Approximately twelve hundred Atlantic salmon smolts were transferred from freshwater directly into the experimental system in May 1988. These were reared in the system over a period of about two years, during which time they were subjected to the experimental studies described in section 5.

The fish grew from a mean weight of approximately 40 g in May 1988 to 1145 g in February 1990, equivalent to a specific growth rate (SGR) averaging 0.532% day^{-1}. Food conversion efficiency (FCE) was calculated to be 51% over this 21 month period. Both SGR and FCE are lower than would be expected on commercial farms (Austreng *et al.* 1987), probably partly due to the effects of experimental procedures, although the nature of the holding facilities and feeding practices may also have had some effect.

A high percentage of grilse were found among fish held for over one year in the experimental system. In summer 1989, approximately 75% of the fish in the experimental tanks were sexually mature, whilst the same genetic stock held in seawater cages over the same period showed a much lower proportion of

grilse. The cause of such a high maturation rate is unknown, but other small seawater tank systems have suffered similarly. Increased availability of feed on the bottom of the tank may be responsible since a high food intake promotes maturation (Thorpe 1989).

No serious disease problems were experienced during the experimental period. Sea lice were notably absent from all fish in the tank system and few unexplained mortalities occurred. The loss of about 30 fish soon after transfer into the system was attributed to damage during transfer. A similar number failed to begin feeding after transfer and starved after 3 or 4 months. In addition, after the completion of the experimental work, 36 mature fish died from a suspected fungal infection.

A.3.2 Water quality

A number of water quality parameters were measured at intervals during the time fish were kept within the system. Water temperature was measured each day using a mercury thermometer suspended in a stock tank. The temperature ranged from 7.5°C in February 1989 and January 1990, to 14°C in August and September 1988 and 1989. Further results are shown in fig. A.3.

Water salinity was measured at approximately monthly intervals during the first year of operation using a temperature-salinity probe (Valeport Ltd., Dartmouth, Devon). These results are shown in table A.1. The salinity ranged from a minimum of 29.1 ppt to a maximum of 33.6 ppt. Similar fluctuations occur locally in Loch Linnhe from where the water was drawn and it seems likely that these are brought about by changes in the freshwater input due to rainfall, and the episodic release of low salinity water from Loch Etive (Milne 1972).

Water oxygen content was monitored at approximately monthly intervals during the first year of operation using the Winkler method described by Strickland and Parsons (1972). The results of these analyses are shown in table A.2. Concentrations of 0.714 and 0.683 mg-at O_2 l^{-1} were measured in the header tank during the first two months of operation. These correspond to 125 and 127% saturation respectively. Supersaturation of water with oxygen and nitrogen can occur as a result of leakage in the suction line to a pump

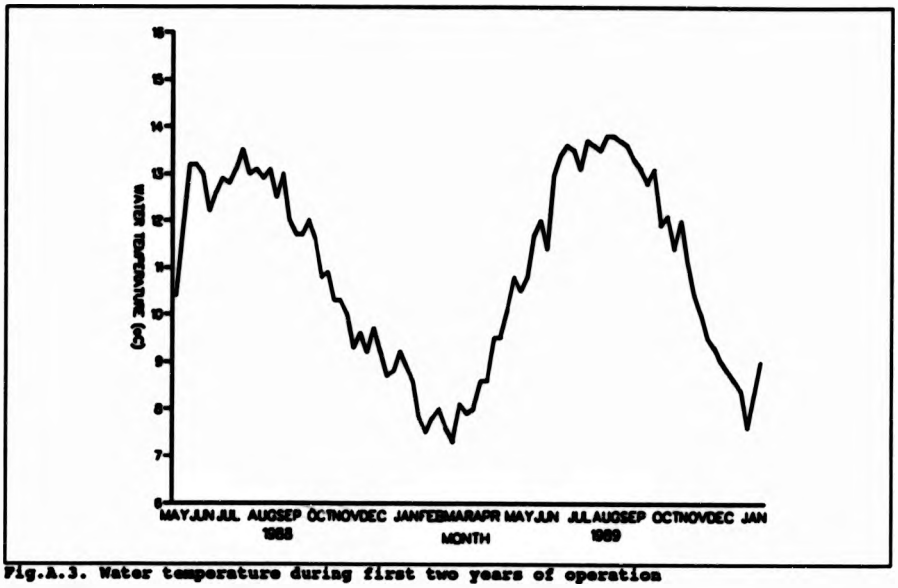

Fig.A.3. Water temperature during first two years of operation

Table A.1. Salinity measurements in experimental tanks

Date	Salinity (ppt)
25.5.88	32.4
29.6.88	-
30.8.88	31.8
29.9.88	29.1
29.10.88	31.9
30.11.88	32.6
8.12.88	-
10.2.89	29.5
19.4.89	31.4
8.12.89	33.6

causing air entrainment and dissolution under pressure (Kils 1977), and it seems likely that this mechanism was responsible for such high concentrations in the header tank soon after start-up. These were reduced to 0.375 mg-at l^{-1} (equivalent to 66% saturation) in the third month of operation and subsequently stayed low, presumably since any leakage had ceased. In the stock

tanks the effects of these fluctuations were minimized by the use of aeration and oxygen concentrations, ranging from 0.445 mg-at l^{-1} (81%) in August 1988 to 0.575 mg-at l^{-1} (100%) in December 1989, were less variable. These are above the minimum concentrations for salmonids recommended by Wickins (1981). No signs of gas-bubble disease (D'Aoust 1989) were observed in any fish held in the system.

Table A.2. Seawater oxygen content in the experimental system

Date	Header		Stock tanks	
	mg-at l^{-1}	%	mg-at l^{-1}	%
25.5.88	0.714	125	0.579	100
29.6.88	0.683	127	0.561	104
30.8.88	0.365	66	0.445	81
29.9.88	0.375	64	0.465	82
29.10.88	0.362	-	0.510	90
30.11.88	-	-	0.519	90
8.12.88	0.371	-	0.541	-
10.2.89	0.471	78	0.620	103
19.4.89	0.461	78	0.532	90
8.12.89	0.423	74	0.575	100

The suspended solid content of water samples was measured regularly during the first four months of operation and occassionaly thereafter, using the method described by Strickland and Parsons (1972). These data are shown in table A.3. In the header tank, total suspended solids were low on each occassion and averaged 0.019 g l^{-1}, indicating effective operation of the sub-sand filters. The proportion of organic to inorganic matter was approximately equal, at about 0.009 g l^{-1} of each. In the stock tanks total suspended solids averaged 0.021 g l^{-1}, of which 0.010 g l^{-1} were organic and 0.011 g l^{-1} inorganic. Such concentrations are slightly higher than the 0.015 g l^{-1} recommended by Wickins (1981).

pH was measured on two occassions at several points within the system, and ranged from 7.52 to 7.71. This is within the normal range for seawater (Strickland and Parsons 1972).

Table A.3. Seawater suspended solids content in experimental system

Date	Header ($g\ l^{-1}$)			Stock tanks ($g\ l^{-1}$)		
	Total	Organic	Inorg.	Total	Organic	Inorg.
30.5.88	0.200	0.015	0.005	0.023	0.010	0.012
30.6.88	0.200	0.009	0.010	0.020	0.010	0.011
28.8.88	0.018	0.010	0.008	0.019	0.009	0.010
29.9.88	0.017	0.006	0.011	0.021	0.011	0.010
6.12.88	-	-	-	0.020	0.008	0.012
19.4.89	-	-	-	0.021	0.008	0.013
8.12.89	0.018	0.007	0.011	0.022	0.011	0.012

Total ammonia-ammonium (NH_3-NH_4^+) concentrations were measured on seven occassions using a Hach test kit (Hach Co., Namur, Belgium). These showed little variation and were close to the lower limit of detection of the kit. In the header tank NH_3-NH_4^+ concentrations were always less than 0.1 mg l^{-1}, but in the stock tanks they were usually slightly higher at 0.2 or 0.3 mg l^{-1}. These are within safe limits for salmonids at the pH and temperature ranges described above (Westers and Pratt 1977; Wickins 1981). Water samples taken at the inlet and outlet of the biofilters in September 1988 were assayed for NH_3-NH_4^+ by M.Philips, University of Stirling using the indo-phenol blue method modified for seawater (Solárzano 1969). These averaged 228 and 178 µg l^{-1} respectively and indicate that the biofilters removed only about 22% of the total. However, at higher NH_3-NH_4^+ concentrations the efficiency would be expected to improve (Kaiser and Wheaton 1983).

A.3.3 Maintenance and running costs

Besides feeding the fish twice each day, cleaning and maintenance was required on a regular basis. The stock tanks and drainage channel were cleaned and sludge pumped from the settling tank once each week. The biofilters were cleaned every three months on average, and the header tank siphoned once every six months. Over the two year period, problems with biofouling were significant only in the concrete channel which supported growth of green and

brown algae, and in the fish tanks which became coated in brown algae after several months. No problems were experienced with the sub-sand filters after initial difficulties with installation, but the main pump required servicing and replacement of moving parts twice each year. The submersible pump was provided with a backup, but it was only necessary to use this on one occassion over the two year period when the first pump required servicing. Similarly the blower required servicing only once over the two year period.

REFERENCES

Ackman, R.G. (1980) Fish lipids, part 1. *In* Advances in Fish Science and Technology (ed. J.J. Connell) Fishing News Books Ltd., Farnham, Surrey, UK. pp.86-103.

Ackman, R.G. and Cormier, M.G. (1967) α-Tocopherol in some Atlantic fish and shellfish with particular reference to live holding without food. *J. Fish. Res. Bd. Canada. 24*, 357-373.

Ackman, R.G. and Eaton, C.A. (1978) Some contemporary applications of open-tubular gas-liquid chromatography in analysis of methyl esters of longer-chain fatty acids. Fette Seifen Anstr. Mittel *80*, 21-37.

Akerib, M. and Sterner, W. (1971) Inhibition of vitamin E absorption by a lipid fraction. Internat. J. Vit. Nutr. Res. *41*, 42-43.

Aldrin, J.F., Boeuf, G., Mauviot, J.C., Messager, J.L. and Mével, M. (1980) Observations sur les 'mortalitiés d'été' dans un élevage de saumons coho; etudé de certains aspects métaboliques par analyse en compostantes principales. Ann. Inst. Michel Pacha. *12*, 13-27.

Alfin-Slater, R.B., Shimma, Y., Hansen, H., Wells, P., Aftergood, L. and Melnick D. (1972) Dietary fat composition and tocopherol requirement: III. Quantitative studies on the relationship between dietary linoleate and vitamin E. J. Am. Oil Chem. Soc. *49*, 395-402.

Alioshin, S.A. (1987) On the possibility of using α-tocopherol and ascorbic acid to protect the reproductive system of trout from toxic food peroxides. Vest. Leningrad Univ. (Biol.) *2*, 3-8 (In Russian).

Allen, W.M., Bradley, R., Berrett, S., Parr, W.H., Swannack, K., Barton, C.R.Q. and MacPhee, A. (1975) Degenerative myopathy with myoglobinuria in yearling cattle. Br. vet. J. *131*, 292-308.

Amin, A.B. and Trasti, J. (1988) Endomyocarditis in Atlantic salmon in Norwegian seafarms. A case report. Bull. Eur. Ass. Fish Pathol. *8*, 70-71.

Anderson, P.H., Bradley, R. and Berrett, S. (1977) The sequence of myodegeneration in nutritional myopathy of the older calf. Br. vet. J. *133*, 160-165.

Anon. (1988) Pancreas disease still an enigma. Bull. Aquacult. Assoc. Can. *88*, 2.

Aoe, H., Abe, I., Saito, T., Fukawa, H. and Koyama, H. (1972) Preventive effects of tocols on muscular dystrophy of young carp. Bull. Jap. Soc. Sci. Fish. *38*, 845-851.

Austreng, E., Storebakken, T. and Åsgård, T. (1987) Growth rate estimates for cultured Atlantic salmon and rainbow trout. Aquaculture *60*, 157-160.

Badwey, J.A. and Karnovsky, M.L. (1980) Active oxygen species and the function of phagocytic leukocytes. Ann. Rev. Biochem. *49*, 695-726.

Baker, E.M. (1967) Vitamin C requirements in stress. Am. J. Clin. Nutr. *20*, 583-590.

Baker, H., Frank, O., DeAngelis, B. and Feingold, S. (1980) Plasma tocopherol in man at various times after ingesting free or acetylated tocopherol. Nutr. Rep. Int. *21*, 531.

Barton, B.A., Schreck, C.B. and Fowler, L.G. (1988) Fasting and diet content affect stress-induced changes in plasma glucose and cortisol in juvenile chinook salmon. Prog. Fish-Cult. *50*, 16-22.

Baudin-Laurencin, F., Aldrin, J.F., Messager, J.L. and Tixerant, G. (1985) "Summer pathology" in marine cultured rainbow trout. *In* Fish and Shellfish Pathology (ed. A.E. Ellis). Academic Press, London. pp.211-221.

Beisenherz, G., Boltze, H.J., Bücher, T., Czok, R., Garbade, K.H., Meyer-Arendt, E. and Pfleiderer, G. (1953) Diphosphofructose aldolase, phosphoglyceraldehyde dehydrogenase, lactic acid dehydrogenase and pyruvate kinase from rabbit muscle in one process. Z. Naturforsch. *8(b)*, 555-577.

Bell, J.G. and Cowey, C.B. (1985) Roles of vitamin E and selenium in the prevention of pathologies related to fatty acid oxidation in salmonids. *In* Nutrition and Feeding in Fish (eds C.B. Cowey, A.M. Mackie, and J.G. Bell) Academic Press, London. pp.333-347.

Bell, J.G. and Cowey, C.B. (1989) Digestibility and bioavailability of dietary selenium from fishmeal, selenite, selenomethionine and selenocystine in Atlantic salmon (*Salmo salar*). Aquaculture *81*, 61-68.

Bell, J.G., Cowey, C.B., Adron, J.W. and Shanks, A.M. (1985) Some effects of vitamin E and selenium deprivation on tissue enzyme levels and indices of tissue peroxidation in rainbow trout (*Salmo gairdneri*). Br. J. Nutr. *53*, 149-157.

Bell, J.G. Pirie, B.J.S., Adron, J.W. and Cowey, C.B. (1986) Some effects of selenium deficiency on glutathione peroxidase (E.C. 1.11.1.9) activity and tissue pathology in rainbow trout (*Salmo gairdneri*). Br. J. Nutr. *55*, 305-311.

Bell, J.G., Cowey, C.B., Adron, J.W. and Pirie, B.J.S. (1987*a*) Some effects of selenium deficiency on enzyme activities and indices of tissue peroxidation in Atlantic salmon parr (*Salmo salar*). Aquaculture *65*, 43-54.

Bell, J.G., McVicar, A.H. and Cowey, C.B. (1987*b*) Pyruvate kinase isozymes in farmed Atlantic salmon (*Salmo salar*): pyruvate kinase and antioxidant parameters in pancreas disease. Aquaculture *66*, 33-41.

Bell, J.G., Youngson, A., Mitchell, A.I. and Cowey, C.B. (1989) The effect of enhanced intake of linoleic acid on the fatty acid composition of tissue polar lipids of post-smolt Atlantic salmon (*Salmo salar*). Lipids *24*, 240-242.

Bell, M.V., Simpson, C.M.F. and Sargent, J.R. (1983) (n-3) and (n-6) polyunsaturated fatty acids in the phosphoglycerides of salt-secreting epithelia from two marine fish species. Lipids *18*, 720-726.

Bencosme, S.A. and Lechago, J.L. (1971) Functional anatomy of the pancreas. *In* The Exocrine Pancreas (eds I.T. Beck and D.G. Sinclair). J. and A. Churchill, London. pp.2-26.

Bender, A.E. (1978) Food processing and nutrition. Academic Press, London.

Besse, P., Levaditis, J.C., Guillon, J.C. and de Kinkelin, P. (1965) Occurrence of viral diseases in the rainbow trout hatcheries in France. First histopathological results. Ann. N.Y. Acad. Sci. *126*, 543-546.

Bieri, J.G. and Anderson, A.A. (1960) Peroxidation of lipids in tissue homogenates as related to vitamin E. Arch. Biochem. Biophys. *90*, 105-110.

Bjerneboe, A., Bjerneboe, G-E. Aa. and Drevon C.A. (1990) Absorption, transport and distribution of vitamin E. J. Nutr. *120*, 233-242.

Blazer, V.S. and Wolke, R.E. (1983) Ceroid deposition, retinal degeneration and renal calcium oxalate crystals in cultured clownfish, *Amphiprion ocellaris*. J. Fish Dis. *6*, 365-376.

Blazer, V.S. and Wolke, R.E. (1984) The effects of α-tocopherol on the immune response and non-specific resistance factors of rainbow trout (*Salmo gairdneri* Richardson). Aquaculture *37*, 1-9.

Boggio, S.M., Hardy, R.W., Babbitt, J.K. and Brannon, E.L. (1985) The influence of dietary lipid source and alpha-tocopherol acetate level on product quality of rainbow trout (*Salmo gairdneri*). Aquaculture *51*, 13-24.

British Plastics Federation (1980) Flow chart for uPVC pipes. Publ. No 104/10 BPF, London.

Brinn, J.E., Jr. (1973) The pancreatic islets of bony fishes. Amer. Zool. *13*, 653-665.

Brown, F. (1953) Occurrence of vitamin E in cod and other fish liver oils. Nature *171*, 790-791.

Bruno, D.W., Hastings, T.S., Ellis, A.E. and Wootten, R. (1985) Outbreak of cold-water vibriosis in Atlantic salmon in Scotland. Bull. Eur. Ass. Fish Pathol. *5*, 62-63.

Bruno, D.W., Hastings, T.S. and Ellis, A.E. (1986) Histopathology, bacteriology and experimental transmission of cold-water vibriosis in Atlantic salmon, *Salmo salar*. Dis. aquat. Org. *1*, 163-168.

Bullock, A.M. (1978) Laboratory methods. *In* Fish Pathology (ed. R.J. Roberts) Ballière-Tindall, London. pp.235-267.

Bunce, G.E. and Hess, J.L. (1976) Lenticular opacities in young rats as a consequence of maternal diets low in tryptophan and/or vitamin E. J. Nutr. *106*, 222-229.

Bunnell, R.H., Keating, J., Quaresimo, A. and Parman, G.K. (1965) Alpha-tocopherol content of foods. Am. J. Clin. Nutr. *17*, 1.

Burton, G.W. and Ingold, K.U. (1984) β-Carotene: an unusual type of lipid antioxidant. Science *224*, 569-573.

Buttriss, J.L. and Diplock, A.T. (1984) High-performance liquid chromatography methods for vitamin E in tissues. Meth. Enzymol. *105*, 131-138.

Cansdale, G. (1981) Seawater abstraction. *In* Aquarium Systems (ed. A.D. Hawkins) Academic Press, London. pp.47-62.

Carbery, J.T. and Donnelly, W.J.C. (1969) Skeletal myopathy in rainbow trout fry. Vet. Rec. *85*, 586-587.

Castell, J.D., Sinnhuber, R.O., Wales, J.H. and Lee, D.J. (1972) Essential fatty acids in the diet of rainbow trout (*Salmo gairdneri*): growth, feed conversion and some gross deficiency symptoms. J. Nutr. *102*, 77-86.

Catignani, G.L. (1980) Biochemical function. 2. Role in nucleic acid and protein metabolism *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York. pp.318-332.

Cawthorne, M.A., Bunyan, J., Diplock, A.T., Murrell, E.A. and Green, J. (1968) On the relationship between vitamin A and vitamin E in the rat. Br. J. Nutr. *22*, 133-143.

Chardavoyne, R., Asher, A., Bank, S., Stein, T.A. and Wise, L. (1989) Role of reactive oxygen metabolites in early cardiopulmonary changes of acute hemorrhagic pancreatitis. Dig. Dis. Sci. *34*, 1581-1584.

Chen, L.H. and Lin C.T. (1980) Some enzymatic changes associated with pathological changes in rats with long-term vitamin E dificiency. Nutr. Rep. Int. *21*, 387-395.

Chen, L.H., Lee, M.S., Hsing, W.F. and Chen S.H. (1980) Effect of vitamin C on tissue antioxidant status of vitamin E deficient rats. Internat. J. Vit. Nutr. Res. *50*, 156-162.

Chen, L.H., Chow, C.K. and Thacker, R.R. (1983) Plasma pyruvic kinase isozyme electrophoretic pattern of vitamin E-deficient rats. Nutr. Rep. Int. *27*, 207-212.

Christiansen, D.C. and Klungsøyr, L. (1987) Metabolic utilization of nutrients and the effects of insulin in fish. Comp. Biochem. Physiol. *88B*, 701-711.

Christie, W.W. (1982) Lipid Analysis. 2nd edn. Pergamon, Oxford.

Christl, H. (1971) Dtsch. Tier. Wch. *78*, 204-207 (cited by Rice *et al.* 1985).

Coffin, J.L. and Combs, G.F. (1981) Impaired vitamin E status of chickens fed T-2 toxin. Poultry Sci. *60*, 385-392.

Conover, W.J. (1980) Practical nonparametric statistics. 2nd edn. J. Wiley, New York. 493 pp.

Corwin, L.M. and Gordon R.K. (1982) Vitamin E and immune regulation. Ann. N.Y. Acad. Sci. *393*, 437-451.

Cowey, C.B. and Roberts, R.J. (1978) Nutritional pathology of teleosts. *In* Fish Pathology (ed. R.J. Roberts) Ballière-Tindall, London. pp.216-226.

Cowey, C.B. and Sargent, J.R. (1972) Fish nutrition. Adv. Mar. Biol. *10*, 383-492.

Cowey, C.B., Adron, J.W., Walton, M.J., Murray, J., Youngson A. and Knox, D. (1981) Tissue distribution, uptake, and requirement for α-tocopherol of rainbow trout (*Salmo gairdneri*) fed diets with a minimal content of unsaturated fatty acids. J. Nutr. *111*, 1556-1567.

Cowey, C.B., Adron, J.W. and Youngson, A. (1983) The vitamin E requirement of rainbow trout (*Salmo gairdneri*) given diets containing polyunsaturated fatty acids derived from fish oil. Aquaculture *30*, 85-93.

Cowey, C.B., Degener, E., Tacon, A.G.J., Youngson, A. and Bell, J.G. (1984) The effect of vitamin E and oxidized fish oil on the nutrition of rainbow trout (*Salmo gairdneri*) grown at natural, varying water temperatures. Br. J. Nutr. *51*, 443-451.

Cowey, C.B., Bell, J.G., Knox, D., Fraser, A. and Youngson, A. (1985) Lipids and lipid antioxidant systems in developing eggs of salmon (*Salmo salar*). Lipids *20*, 567-572.

Crampton, V. and Jackson, A. (1981) Profit in salmon: large or small? Harvest size is the critical factor. Fish Farmer *4*, 24-26.

Cumings, H.W. (1942) The effects of vitamin E deficiency in the guppy, *Lebistes reticulatus*. Anat. Rec. *84*, 499-500 (Abstract).

D'Agostino, L. (1952a) Influenza dell'α-tocoferolo sulla eliminazione urinaria di aneurina. Bull. Soc. Ital. Biol. Sper. *28*, 157-159.

D'Agostino, L. (1952*b*) α-Tocoferolo ed eliminazione urinaria di acido nicotinico. Bull. Soc. Ital. Biol. Sper. *28*, 159-161.

Dalsgaard, I., Jürgens, O. and Mortensen, A. (1988) *Vibrio salmonicida* isolated from farmed Atlantic salmon in the Faroe Islands. Bull. Eur. Ass. Fish Pathol. *8*, 53-54.

Dam, H. (1962) Interrelations between vitamin E and polyunsaturated fatty acids in animals. Vit. Horm. *20*, 527-540.

Daniels, H.E. (1950) Rank correlation and population models. *J.* Roy. Stat. Soc. (B) *12*, 171-181.

D'Aoust, B.G. (1989) Gas bubble disease - an appraisal. Fish Farmer *12*, 34-37.

DeLumen, B.O., Lubin, B., Chiu, D., Reyes, F.S. and Omaye, S.T. (1982). Bioavailability of vitamin E in rats fed diets containing pectin. Nutr. Res. *2*, 73.

Deplano, M., Connes, R., Diaz, J.P. and Paris, J. (1989) Intestinal steatosis in the farm-reared sea bass, *Dicentrarchus labrax*. Dis. Aquat. Org. *6*, 121-130.

Desai, I.D. (1980) Assay methods. *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York. pp.67-98.

Desjardins, L.M., Hicks, B.D. and Hilton, J.W. (1987) Iron catalyzed oxidation of trout diets and its effect on the growth and physiological response of rainbow trout. Fish Physiol. Biochem. *3*, 173-182.

Dicks, M.W. (1965) Tocopherol content of fish and fish products. Univ. Wyoming Agric. Exp. Sta. Bull. *435*. pp. 154-156.

Diplock, A.T. and Lucy, J.A. (1973) The biochemical modes of action of vitamin E and selenium. FEBS Lett. *29*, 205-210.

Diplock, A.T., Green, J., Bunyan, J., McHale, D. and Muthy, I.R. (1967) Vitamin E and stress. 3. The metabolism of *D*-α-tocopherol in the rat under dietary stress with silver. Br. J. Nutr. *21*, 115-125.

Donaldson, L.R. (1943) Histological studies of the pancreas and associated tissues of wild and experimentally fed young chinook salmon. Am. J. Physiol. *138*, 560-564.

Donaldson, E.M. (1981) The pituitary-interrenal axis as an indicator of stress in fish. *In* Stress and Fish (ed. A.D. Pickering) Academic Press, London. pp.11-47.

Dove, C.R. and Ewan, R.C. (1981) The effect of copper, iron or zinc addition on the vitamin E status of growing pigs and the natural levels in food. J. Anim. Sci. *65*, Suppl.1., 134 (Abstract).

Draper, H.H. (1980*a*) Nutrient interrelationships. *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York. pp.272-288.

Draper, H.H. (1980*b*) Role of vitamin E in plants, microbes, invertebrates and fish. *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York. pp.391-395.

Draper, H.H. and Csallany, A.S. (1958) Action of *N, N'*-diphenyl-p-phenylene-diamine in tocopherol deficiency diseases. Proc. Soc. Exp. Biol. Med. *99*, 739-742.

Draper, H.H., Goodyear, S., Borbee, K.D. and Connor Johnson, B. (1958) A study of the nutritional role of antioxidants in the diet of the rat. Br. J. Nutr. *12*, 89-97.

Dubick, M.A., Yu, G.S.M. and Majumdor, A.P.N. (1989) Morphological and biochemical changes in the pancreas of the copper-deficient female rat. J. Nutr. *119*, 1165-1172.

Duboulos, P. and Nedde, M.F. (1942) La présence des substances antioxygéniquès dans les tissues des animaux. Trav. membranes soc. chim. biol. *24*, 1137-1144.

Dupree, H.K. (1968) Vitamin E requirement of channel catfish. Progress in Sport Fishery Research, Division of Fishery Research, Bureau of Sport Fisheries and Wildlife. *77*, 220-223.

Duthie, G.G. and Arthur, J.R. (1989) The antioxidant abnormality in the stress-susceptible pig: effect of vitamin E supplementation. Ann. N.Y. Acad. Sci. *570*, 322-334.

Duthie, G., Arthur, J. and Hoppe, P. (1988) Porcine stress syndrome, free radicals and vitamin E. *In* Oxygen Radicals in Biology and Medicine (eds K.A. Taylor, J.F. Ward and C. von Sonntag) Proceedings of the 4th International Conference on Oxygen Radicals, San Diego, Plenum Press. pp. 605-609.

Duthie, G.G., Arthur, J.R., Nicol, F. and Walker, M. (1989) Increased indices of lipid peroxidation in stress susceptible pigs and effects of vitamin E. Res. Vet. Sci. *46*, 226-230.

Dvorák, M. (1979) Interrelation between adrenocortical activity and vitamin E level with regard to the development of myopathy in pigs. Acta Agric. Scand. Suppl.*21*.

Dvorák, M., Cerná, J., Majman, L., Raszyk, J., Toulová, M. and Urbanová, J. (1976) The role of stress in the pathogenesis of nutritional muscular dystrophy in pigs. Zentbl. Vet. Med. A*23*, 384-393.

Egidius, E., Andersen, K., Clausen, E. and Raa, J. (1981) Cold-water vibriosis or 'Hitra disease' in Norwegian salmonid farming. J. Fish Dis. *4*, 353-354.

Egidius, E., Soleim, S. and Andersen, K. (1984) Further observations on cold-water vibriosis or Hitra disease. Bull. Eur. Ass. Fish Pathol. *4*, 50-51.

Egidius, E., Wiik, R., Andersen, K., Hoff, K.A. and Hjeltnes, B. (1986) *Vibrio salmonicida* sp. nov., a new fish pathogen. Int. J. Syst. Bacteriol. *36*, 518-520.

Emmerie, A. and Engel C. (1938) Colorimetric determination of *d,l*-α-tocopherol (vitamin E). Nature *142*, 873.

Endo, M., Miyazaki, T., Kubota, S.S., Oobayashi, M., and Matumoto, M. (1979) Studies on nutritional myopathy syndrome in cultured fishes - II. Nutritional myopathy of puffer fry. Fish Pathol. *13*, 183-187.

Espelid, S., Holm, K.O., Hjelmeland, K. and Jørgensen, T. (1988). Monoclonal antibodies against *Vibrio salmonicida* : the causative agent of coldwater vibriosis ('Hitra disease') in Atlantic salmon, *Salmo salar* L. J. Fish Dis. *11*, 207-214.

Evans, H.M. and Bishop, K.S. (1922) On the existence of a hitherto unrecognized dietary factor essential for reproduction. Science *56*, 650-651.

Evans, H.M., Emerson, O.H. and Emerson, G.A. (1936) The isolation from wheat germ oil of an alcohol, α-tocopherol, having the properties of vitamin E. J. Biol. Chem. *113*, 319-332.

Evarts, R.P. and Bieri, J.G. (1974) Ratios of polyunsaturated fatty acids to α-tocopherol in tissues of rats fed corn or soybean oils. Lipids *9*, 860-864.

Farrell, A.P. (1987) Coronary flow in a perfused rainbow trout heart. J. Exp. Biol. *129*, 107-123.

Farrell, A.P., Saunders, R.L., Freeman, H.C. and Mommsen, T.P. (1986) Arteriosclerosis in Atlantic salmon: effects of dietary cholesterol and maturation. Arteriosclerosis *6*, 453-461.

Farrell, A.P., Hammons, A.M., Graham, M.S. and Tibbits, G.F. (1988) Cardiac growth in rainbow trout, *Salmo gairdneri*. Can. J. Zool. *66*, 2368-2373.

Farrell, P.M. (1980) Deficiency states, pharmacological effects and nutrient requirements. *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York. pp.520-620.

Farrell, P.M., Bieri, J.G., Fratantoni, J.F., Wood, R.E. and Di Sant'Agnese, P.A. (1977) The occurrence and effects of human vitamin E deficiency. A study in patients with cystic fibrosis. J. Clin. Invest. *60*, 233-241.

Ferguson, H.W. and Rice, D.A. (1980) Post-spawning mortalities in brown trout, *Salmo* trutta L. J. Fish Dis. *3*, 153-160.

Ferguson, H.W., Roberts, R.J., Richards, R.H., Collins, R.O. and Rice, D.A. (1986*a*) Severe degenerative cardiomyopathy associated with pancreas disease in Atlantic salmon, *Salmo salar* L. J. Fish Dis. *9*, 95-98.

Ferguson, H.W., Rice, D.A. and Lynas, J.K. (1986*b*) Clinical pathology of myodegeneration (pancreas disease) in Atlantic salmon (*Salmo salar*). Vet. Rec. *119*, 297-299.

Ferguson, H.W., Poppe, T. and Speare, D. (1988) Severe heart disease in Norwegian Atlantic salmon. Abstract from International Fish and Health Conference, p.142, Fish Health Section, Vancouver, BC, Canada.

Ferguson, H.W., Poppe, T. and Speare, D.J. (1990) Cardiomyopathy in farmed Norwegian salmon. Dis. Aquat. Org. *8*, 225-231.

Fernholz, E. (1938) Constitution of alpha-tocopherol. J. Am. Chem. Soc. *60*, 700-705.

Fjolstad, M. and Heyeraas, A.L. (1985) Muscular and myocardial degeneration in cultured Atlantic salmon, *Salmo salar* L., suffering from 'Hitra disease'. J. Fish Dis. *8*, 367-372.

Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. *226*, 497-509.

Fong, K.-L., McCay, P.B., Poyer, J.L., Keele, B.B. and Misra, H. (1973) Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl free radicals produced during flavin enzyme activity. J. Biol. Chem. *248*, 7792-7797.

Forster, I., Higgs, D.A., Bell, G.R., Dosanjh, B.S. and March, B.E. (1988) Effect of diets containing herring oil oxidized to different degrees on growth and immunocompetence of juvenile coho salmon (*Oncorhynchus kisutch*). Can. J. Fish. Aquat. Sci. *45*, 2187-2194.

Fowler, L.G. and Banks, J.L. (1969) Tests of vitamin supplements and formula changes in the Abernathy salmon diet, 1966-67. Tech. Pap. Bur. Sport Fish. Wildl., Washington *26*, 1-19.

Frigg, M. and Bros, J. (1984) Relationships between vitamin A and vitamin E in the chick. Internat. J. Vit. Nutr. Res. *54*, 125-134.

Frigg, M., Prabucki, A.L. and Ruhdel, E.U. (1990) Effect of dietary vitamin E on oxidative stability of trout fillets. Aquaculture *84*, 145-158.

Fulton, T.W. (1904) The rate of growth of fishes. Fisheries Board of Scotland Annual Report *22* (3), 141-241, Edinburgh.

Furones, D., Alderman, D.J., Fletcher, T.C. and White, A. (1990) Influence of dietary vitamin E on the susceptibility of rainbow trout (*Onchorynchus mykiss*) to infection with *Yersinia ruckeri*. Poster presented at Bacterial diseases of fish conference, June 26-29 1990, Stirling, Scotland, Abs. p.43.

Gabriel, K.R. (1978) A simple method of multiple comparison of means. J. Amer. Stat. Assoc. *73*, 724-729.

Gabryelak, T. and Pérès, G. (1986) Comparative antioxidant enzyme and lipid peroxidation study in erythrocytes and liver of some freshwater fish. Acta Biol. Hung. *37*, 219-224.

Gallo-Torres, H.E. (1970) Obligatory role of bile for the intestinal absorption of vitamin E. Lipids *5*, 379-384.

Gallo-Torres, H.E. (1973) Studies on the intestinal lymphatic absorption, tissue distribution and storage of vitamin E. Acta Agric. Scand. Suppl. *19*, 97-104.

Gallo-Torres, H.E. (1980) Absorption *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York. pp.170-192.

Gatlin, D.M. III, Poe, W.E. and Wilson, R.P. (1986*a*) Effects of singular and combined dietary deficiencies of selenium and vitamin E on fingerling channel catfish (*Ictalurus punctatus*). J. Nutr. *116*, 1061-1067.

Gatlin, D.M. III, Poe, W.E. and Wilson, R.P. (1986*b*) Effects of stocking density and vitamin C status on vitamin E-adequate and vitamin E-deficient fingerling channel catfish. Aquaculture *56*, 187-195.

Gericke, M.D. and Hofmeyer, J.M. (1976) Aetiology and treatment of capture stress and myopathy in springbok. S. Afr. J. Sci. *72*, 28.

Ghittino, P. (1961) Eziologia, patogenesi e tentativi di transmissione della "degenerazione lipoidea epatica" nella trota iridea (*Salmo gairdneri*). Veterinaria Italiana *23*, 3-21 (In Italian).

Gordon, H.H., Nitowsky, H.M., Tildon, J.T. and Leving, S. (1958) Studies of tocopherol deficiency in infants and children V. An interim summary. Pediatrics *21*, 673-681.

Green, J. and Bunyan J. (1969) Vitamin E and the biological antioxidant theory. Nutr. Abstr. Review *39*, 321-345.

Green, J., Diplock, A.T., Bunyan, J. McHale, D. and Muthy, I.R. (1967) Vitamin E and stress. I. Dietary unsaturated fatty acid stress and the metabolism of α-tocopherol in the rat. Br. J. Nutr. *21*, 69-101.

Gries, C.L. and Scott, M.L. (1972) Pathology of selenium deficiency in the chick. J. Nutr. *102*, 1287-1296.

Halver, J.E. (1969) Vitamin requirements. *In* Fish in Research (eds O.W. Neuhaus and J.E. Halver) Academic Press, London. pp.209-232.

Halver, J.E. (1989) Fish Nutrition, 2nd edn. Academic Press. London.

Hammel, E.P. and Raker, C.W. (1972) Exertional myopathy *In* Equine Medicine and Surgery. Am. Vet. Publ., Illinois. pp.587-591.

Hardie, L.J., Fletcher, T.C. and Secombes, C.J. (1990) The effect of vitamin E on the immune response of the Atlantic salmon (*Salmo salar* L.). Aquaculture *87*, 1-13.

Harries, J.T. and Muller, D.P.R. (1971) Absorption of different doses of fat soluble and water miscible preparations of vitamin E in children with cystic fibrosis. Arch. Dis. Childh. *46*, 341-344.

Harris, P.L. and Embree, N.D. (1963) Quantitative consideration of the effect of polyunsaturated fatty acid content of the diet upon the requirements for vitamin E. Am. J. Clin. Nutr. *13*, 385-392.

Harris, P. L., Quaife, M.L. and Swanson, W.J. (1950) Vitamin E content of foods. J. Nutr. *40*, 367-381.

Harthoorn, A.M., van der Walt, K. and Young, E. (1974) Possible therapy for capture myopathy in captured wild animals. Nature *247*, 577.

Hashimoto, Y., Okaichi, T., Watanabe, T., Furukawa, A. and Umezu, T. (1966) Muscle dystrophy of carp due to oxidized oil and the preventive effect of vitamin E. Bull. Jap. Soc. Sci. Fish. *32*, 64-69.

Hata, K. and Kaneda, T. (1980) Effect of autoxidized oil on carp. Bull. Jap. Soc. Sci. Fish. *46*, 997-1000.

Hayashi, K. and Takagi, T. (1977) On the fatty acid composition of fish affected by excessive stress. Bull. Jap. Soc. Sci. Fish. *43*, 1189-1194.

Hayes, K.C. (1974) Retinal degeneration in monkeys induced by deficiencies of vitamin E and A. Investigative Ophthalmology *13*, 499-510.

Hayes, K.C., Neilsen, S.W. and Rousseau, J.E., Jr. (1967) Vitamin E deficiency and fat stress in the dog. J. Nutr. *99*, 196-209.

Hazel, J.R. (1979) Influence of thermal acclimation on membrane lipid composition of rainbow trout liver. Am. J. Physiol. *5*, R91-R101.

Henschel, J.R. and Louw, G.N. (1978) Capture stress, metabolic acidosis and hyperthermia in birds. S. Afr. J. Sci. *74*, 305-306.

Herting, D.C. (1984) Vitamin E. *In* Kirk-Othmer Encyclopedia of Chemical Technology 3rd edn. (Wiley, New York) *24*, 214-227.

Hess, W.N. (1935) Reduction of the islets of Langerhans in the pancreas of fish by means of diet, overeating and lack of exercise. J. Exp. Zool. *70*, 187-195.

Hesser, E.F. (1960) Methods for routine fish hematology. Prog. Fish Cult. October issue, 164-171.

Hidiroglou, N., McDowell, L. and Pastrama, R. (1988*a*) Bioavailability of various vitamin E compounds in sheep. Internat. J. Vit. Nutr. Res. *58*, 189-197.

Hidiroglou, N., Laflamme, L. and McDowell, L. (1988*b*) Blood plasma and tissue concentration of vitamin E in beef cattle as influenced by supplementation of various tocopherol compounds. J. Anim. Sci. *66*, 3227-3234.

Higashi, H. (1961) Vitamins in fish - with special reference to edible parts. Ch 13 *In* Fish as Food, Vol. 1 (ed. G. Borgstrom) Academic Press, London. pp. 411-486.

Higashi, H., Nakamizo, S., Ozaki, H. and Terada, K. (1970) Vitamins (Kyoto) *42*, 243-247.

Hilton, J.W., Hodson, P.V. and Slinger, S.J. (1980) The requirement and toxicity of selenium in rainbow trout (*Salmo gairdneri*). J. Nutr. *110*, 2527-2535.

Hjeltnes, B., Andersen, K., Ellingsen, H.-M. and Egidius, E. (1987) Experimental studies on the pathogenicity of a *Vibrio* sp. isolated from Atlantic salmon, *Salmo salar* L., suffering from Hitra disease. J. Fish Dis. *10*, 21-27.

Hoar, W.S. (1976) Smolt transformation: evolution, behaviour, and physiology. J. Fish. Res. Board Can. *33*, 1234-1252.

Hodson, P.V., Hilton, J.W. and Slinger, S.J. (1986) Accumulation of waterborne selenium by rainbow trout (*Salmo gairdneri*), eggs, fry and juveniles. Fish Physiol. Biochem. *1*, 187-196.

Hofmeyer, J.M., Louw, G.N. and du Preez, J.S. (1973) Incipient capture myopathy as revealed by blood chemistry of chased zebras. Madoqua Ser. 1 *7*, 45-50.

Holm, K.O. and Jørgensen, T. (1987) A successful vaccination of Atlantic salmon, *Salmo salar* L., against 'Hitra disease' or coldwater vibriosis. J. Fish Dis. *10*, 85-90.

Holm, K.O., Strøm, E., Stensvåg, K., Raa, J. and Jørgensen, T. (1985) Characteristics of a *Vibrio* sp. associated with the "Hitra disease" of Atlantic salmon in Norwegian fish farms. Fish Path. *20*, 125-129.

Hoppe, P.P., Duthie, G.G., Arthur, J.R., Schoner, F.J. and Wiesche, H. (1989) Vitamin E and vitamin C supplementation and stress-susceptible pigs: effects of halothane and pharmacologically induced muscle contractions. Livestock Production Science *22*, 341-350.

Houlihan, D.F., Agnisola, C., Lyndon, A.R., Gray, C. and Hamilton, N.M. (1988) Protein synthesis in a fish heart: responses to increased power output. J. exp. Biol. *137*, 565-587.

Hove, E.L., Hickman, K and Harris, P.L. (1945). Effect of tocopherol and of fat on the resistance of rats to anoxic anoxia. Arch. Biochem. *8*, 395.

Hsiao, S.M. and Mak, W.C. (1978*a*) Artificial fertilization and incubation of fertilized eggs of pond reared ayu. China Fish. Mon. *305*, 2-10.

Hsiao, S.M. and Mak, W.C. (1978*b*) Artificial fertilization of ayu (*Plecoglossus altivelis*) feeding mainly on benthic algae. China Fish. Mon. *306*, 8-12.

Huerkamp, M.J., Ringler, D.H. and Chrisp, C.E. (1988) Vitamin E deficiency in goldfish fed a shellfish derived diet. Lab. Anim. Sci. *38*, 178-182.

Hughes, B.P. (1962) A method for the estimation of serum creatine kinase and its use in comparing creatine kinase and aldolase activity in normal and pathological sera. Clin. Chim. Acta *7*, 579.

Huguenin, J.E. and Colt, J. (1989) Design and operating guide for aquaculture seawater systems. Developments in Aquaculture and Fisheries Science V.20. Elsevier, Amsterdam. 264pp.

Hulland, T.J. (1985) Muscles and Tendons *In* Pathology of Domestic Animals *1*, 3rd edn. (ed. K.V.F. Jubb, P.C. Kennedy and N. Palmer) Academic Press, London. pp.139-199.

Hung, S.S.O. and Slinger, S.J. (1980) Effect of oxidized fish oil on the ascorbic acid nutrition of rainbow trout (*Salmo gairdneri*). Internat. J. Vit. Nutr. Res. *50*, 393-400.

Hung, S.S.O. and Slinger, S.J. (1982) Effect of dietary vitamin E on rainbow trout (*Salmo gairdneri*): muscle α-tocopherol and storage stability. Internat. J. Vit. Nutr. Res. *52*, 120-125.

Hung, S.S.O., Cho, C.Y. and Slinger, S.J. (1980*a*) High performance liquid chromatographic determination of α-tocopherol in fish liver. J. Assoc. Off. Anal. Chem. *63*, 889-893.

Hung, S.S.O., Cho, C.Y. and Slinger, S.J. (1980*b*) Measurement of oxidation in fish oil and its effect on vitamin E nutrition of rainbow trout (*Salmo gairdneri*). Can. J. Fish. Aquat. Sci. *37*, 1248-1253.

Hung, S.S.O., Cho, C.Y. and Slinger, S.J. (1981) Effect of oxidized fish oil, *DL*-α-tocopheryl acetate and ethoxyquin supplementation on the vitamin E nutrition of rainbow trout (*Salmo gairdneri*) fed practical diets. J. Nutr. *111*, 648-657.

Hung, S.S.O., Moon, T.W., Hilton, J.W. and Slinger, S.J. (1982) Uptake, transport and distribution of *DL*-α-tocopheryl acetate compared to α-tocopherol in rainbow trout (*Salmo gairdneri*). J. Nutr. *112*, 1590-1599.

Hyldgaard-Jensen, J. (1977) Nutritional deficiencies and blood enzymes with special emphasis on vitamin E and selenium. Annual Meeting of Vitaminology, Hoffman-La Roche and Cie, Paris, Feb 10, 1977.

Ikeda, S. and Taguchi, T. (1966) Improved assay method and levels of vitamin E in fish tissues. Bull. Jap. Soc. Sci. Fish. *32*(4), 346-351.

IUPAC-IUB Commission on Biochemical Nomenclature (1974) Nomenclature of tocopherols and related compounds. Recommendations (1973). Eur. J. Biochem. *46*, 217-219.

Jacquot, R. (1961) Organic constituents of fish and other aquatic animal foods. *In* Fish as Food (ed. G. Borgstrom) Academic Press, London. pp.145-209

Jarboe, H.H., Robinette, H.R. and Bowser, P.R. (1989) Effect of vitamin E supplementation of channel catfish production feeds. Prog. Fish-Cult. *51*, 91-94.

Jenkins, M.Y. and Mitchell, G.V. (1975) Influence of excess vitamin E on vitamin A toxicity in rats. J. Nutr. *105*,1600-1606.

Jobling, M. (1986) Gastrointestinal overload - a problem with formulated feeds. Aquaculture *51*, 257-263.

Juarez-Palacios, J.R. and Palomo Martinez, G.G. (1980) Resultados preliminares del empleo de gonadotropina carionica (HGC-LH) para lograr el desove de la carpa herbivora, *Ctenopharynodon idellus* (Cuv. et Val.). *In* Memorias del 2. simposio latinoamericana de acuacultura, Mexico City, 13th November 1978. pp.1089-1105

Kahlon, T.S., Chow, F.I., Hoefer, J.L. and Betschart, A.A. (1986) Bioavailability of vitamins A and E as influenced by wheatbran and bran particle size. Cereal Chem. *63*, 490.

Kaiser, G.E. and Wheaton, F.W. (1983) Nitrification filters for aquatic culture systems: state of the art. J. World Maricul. Soc. *14*, 302-324.

Kasparek, S. (1980) Chemistry of tocopherols and tocotrienols. *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York. pp.7-66

Kawatsu, H. (1960) On the dietary disease of rainbow trout (*Salmo irideus* Gibbons) caused by feeding of dried silk-worm pupae - I. On the relationship between the disease and growth. Bull. Freshw. Fish. Res. Lab. *10*, 41-52 (In Japanese).

Kawatsu, H. (1964) On the dietary disease of rainbow trout (*Salmo irideus* Gibbons) caused by feeding of dried silk-worm pupae - III. Preventive effects of vitamin B complex and vitamin E on anaemia caused by the dried silk-worm pupae. Bull. Freshw. Fish. Res. Lab. *14*, 1-9 (In Japanese).

Kent, M.L. and Elston, R.A. (1987) Pancreas disease in pen-reared Atlantic salmon in North America. Bull. Eur. Ass. Fish Pathol. *7* (2) 29-31.

Ketola, H.G., Smith, C.E. and Kindschi, G.A. (1989) Influence of diet and oxidative rancidity on fry of Atlantic and coho salmon. Aquaculture *79*, 417-423.

Kils, U. (1977) The salinity effects on aeration in mariculture. Meeresforsch. *25*, 201-206.

Kimura, M. (1963) Studies on diseases of fish under culture - I. Blackened specimens of the yellow tail, *Seriola quinqueradiata* T. and S. Bull. Jap. Soc. Sci. Fish. *29* (10), 905-910 (In Japanese).

King, I.B. (1984) Influence of vitamin E in reproduction in rainbow trout (*Salmo gairdneri*). Publ. Wash. Sea Grant WSH-TH-85-3.

King, I.B., Hardy, R.W. and Halver, J.E. (1983) The effect of dietary vitamin E on the distribution of α-tocopherol in rainbow trout (*Salmo gairdneri*) during ovarian maturation. *In* Salmonid Reproduction: An International Symposium. 31st October-2nd November 1983, Bellevue, Washington. Publ. Wash. Sea Grant. pp.24-25

King, J.M. (1975) Nutritional myopathy in fish. *In* Pathology of Fishes (eds W.E. Ribelin and G.E. Migaki) Univ. of Wisconsin Press. pp.787-792.

Kinumaki, T. Sugii, K., Iida, H. and Takahashi, T. (1972) Addition of fat soluble vitamins to the feeding stuffs for parent rainbow trout with particular reference on the vitamin levels of eggs and fry. Bull. Tokai Reg. Res. Lab. *71*, 133-160.

Kitabchi, A.E. (1980) Hormonal status in vitamin E deficiency. *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York. pp.348-371.

Kitamura, S., Suwa, T., Ohara, S. and Nakagawa, K. (1967) Studies on vitamin requirements of rainbow trout - II. The deficiency symptoms of fourteen kinds of vitamin. Bull. Jap. Soc. Sci. Fish. *33* (12), 1120-1125.

Koller, L.D. and Exon, J.H. (1986) The two faces of selenium - deficiency and toxicity - are similar in animals and man. Can. J. Vet. Res. *50*, 297-306.

Kringstad, H. and Folkvord, S. (1949) The nutritive value of cod roe and cod liver. J. Nutr. *38*, 489-502.

Kubota, S.S., Funahasi, N., Endo, M. and Miyazaki, T. (1980) Studies on nutritional myopathy syndrome in cultured fishes - I. Nutritional myopathy in cultured yellowtail. Fish Pathol. *15*, 75-80.

Kubota, S.S., Endo, M., Miyazaki, T. and Ohbayashi, T. (1981a) Studies on the nutritional myopathy syndrome in cultured fishes - III. A prophylaxis effect of vitamin E on nutritional myopathy in cultured puffers. Fish Pathol. *16*, 9-13 (In Japanese).

Kubota, S.S., Miyazaki, T., Tsuda, S. and Kasuya, K. (1981*b*) Studies on nutritional myopathy syndrome in cultured fishes - IV. Nutritional myopathy accompanying extensive ceroidosis in rainbow trout. Bull. Fac. Fish. Mie Univ. *8*, 107-115 (In Japanese).

Lall, S.P. and Bishop, F.J. (1979) Studies of the nutrient requirements of rainbow trout, *Salmo gairdneri*, grown in sea water and fresh water. *In* Advances in Aquaculture (eds T.V.R. Pillay and W.A. Dill) F.A.O., Rome. pp.580-584.

Lall, S.P., Olivier, G., Hines, J.A. and Ferguson, H.W. (1987) The role of vitamin E in Atlantic salmon (*Salmo salar*) nutrition and immune response. Poster presented at the International symposium on feeding and nutrition in fish, Bergen, 23-27 August 1987. Abstract p.42.

Lall, S.P., Olivier, G., Hines, J.A. and Ferguson, H.W. (1988) The role of vitamin E in nutrition and immune response of Atlantic salmon (*Salmo salar*). *In* Bull. Aquacult. Assoc. Canada *88*, 76-78.

Lam, V. (1985) Dietary requirement for α-tocopherol by *Tilapia nilotica*. M.S. thesis, Auburn Univ., Auburn, Alabama. 66pp.

Lane, P., Galwey, N. and Alvey, N. (1987) Genstat 5: An Introduction. Oxford Science Publications, Claredon Press, Oxford. 163 pp.

Lang, H. (editor) (1981) Creatine Kinase Isoenzymes. Pathophysiology and Clinical Application. Springer-Verlag, Berlin. 317 pp.

Lange, W. (1950) Cholesterol, phytosterol and tocopherol content of food products and animal tissues. J. Am. Oil Chem. Soc. *27*, 414-422.

Law, D.K., Westgate, J.W., McKee, T.B. and Sinnhuber, R.O. (1963) Effects of vitamin E and certain antioxidants on the toxicity of autoxidised fats fed to chinook salmon. Unpublished manuscript, Oregon Fish Commission.

Lee, Y.C.P., Kuha, K.T., Visscher, M.B. and King, J.T. (1962) Role of manganese and vitamin E deficiency in mouse paralysis. Am. J. Physiol. *203*, 1103-1108.

Leger, C. (1985) Digestion, absorption and transport of lipids. *In* Nutrition and Feeding in Fish (eds C.B. Cowey, A.M. Mackie, and J.G. Bell) Academic Press, London. pp. 299-331

Liao, P.B. (1971) Water requirements of salmonids. Prog. Fish-Cult. *33*, 210-215.

Lohrey, E.E., Hughes, I.R. and Gray, I.K. (1978) Effect of dietary lipid oxidation on measurement of protein efficiency ratios. J. Assoc. Off. Anal. Chem. *61*, 104-110.

Longnecker, D.S. (1982) Pathology and pathogenesis of diseases of the pancreas. Am. J. Physiol. *107*, 103-121.

Losowsky, M.S. and Leonard, P.J. (1967) Evidence of vitamin E deficiency with malabsorption or alcoholism and the effects of therapy. Gut *8*, 539-543.

Love, R.M. (1970) The Chemical Biology of Fishes. Academic Press, London. 547 pp.

Love, R.M. (1980) The Chemical Biology of Fishes, vol.2 (Advances 1968-77). Academic Press, London. 943 pp.

Lovell, R.T. (1975) Nutritional deficiencies in intensively cultured catfish. *In* Pathology of Fishes (eds W.E. Ribelin and G.E. Migaki) Univ. of Wisconsin Press. pp. 721-729.

Lovell, R.T. (1979) Nutritional diseases in channel catfish. *In* Advances in Aquaculture (eds T.V.R. Pillay and T.A. Dill) F.A.O., Rome. pp.605-609

Lovell, T. (1989) Nutrition and feeding of fish. Van Nostrand Reinhold, New York.

Lovell, R.T., Miyazaki, T. and Rabegnator, S. (1984) Requirement for α-tocopherol by channel catfish fed diets low in polyunsaturated triglycerides. J. Nutr. *114*, 894-901.

Lovern, J.A. (1938) Fat metabolism in fishes. Factors influencing the composition of the depot fat of fishes. Biochem. J. *32*, 1214-1224.

Lü, J. and Combs, G.F. Jr. (1988) Excess dietary zinc decreases tissue α-tocopherol in chicks. J. Nutr. *118*, 1349-1359.

Machlin, L.J., Gabriel, E., Spiegel, H.E., Horn, L.R., Brin, M. and Nelson, J. (1978) Plasma activity of pyruvate kinase and glutamic oxalacetic transaminase as indices of myopathy in the vitamin E-deficient rat. J. Nutr. *108*, 1963-1968.

Marusich, W.L. (1980) Vitamin E as an *in vivo* lipid stabilizer and its effect on flavor and storage properties of milk and meat. *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York pp.445-472.

Mason, K.E. and Harris, P.L. (1947) Bioassay of vitamin E. *In* Biological Symposia *12*, (ed. J. Cattell *et al.*) 459-483.

Mathias, P.M., Harries, J.T., Peters, T.J. and Muller, D.P.R. (1981) Studies on the *in vivo* absorption of micellar solutions of tocopherol and tocopheryl acetate in the rat: demonstration and partial characterization of a mucosal esterase localized to the endoplasmic reticulum of the enterocyte. J. Lipid Res. *22*, 829-837.

Matsui, M., Watanabe, T. and Ikekawa, N. (1973) Effect of α-tocopherol deficiency on carp III. Analytical method for triglyceride composition of fish lipid. Bull. Jap. Soc. Sci. Fish. *39*, 367-373.

Mazeaud, M.M., Mazeaud, F. and Donaldson, E.M. (1977) Primary and secondary effects of stress in fish: some new data with a general review. Trans. Amer. Fish. Soc. *106*, 201-212.

Mazik, P.M., Brandt, T.M. and Tomasso, J.R. (1987) Effects of dietary vitamin C on growth, caudal fin development, and tolerance of aquaculture-related stressors in channel catfish. Prog. Fish-Cult. *49*, 13-16.

McCay, P.B. (1985) Vitamin E: interactions with free-radicals and ascorbate. Ann. Rev. Nutr. *5*, 323-340.

McCay, P.B. and King, M.M. (1980) Biochemical function 1. Vitamin E: its role as a biologic free radical scavenger and its relationship to the microsomal mixed function oxidase system. *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York pp. 289-317.

McLeay, D.L. and Gordon, M.R. (1977) Leucocrit: a simple haematological technique for measuring acute stress in salmonid fish including stressful concentrations of pulpmill effluent. J. Fish. Res. Board. Can. *34*, 2164-2175.

McLoughlin, M., Kennedy, S. and Kennedy, G. (1990) Rainbow trout fry mortality, field experiences and possible causes. Paper presented at the Scottish Fish Farming Conference, Edinburgh 13-15th February 1990.

McMurray, C.H. and Rice, D.A. (1982) Vitamin E and selenium deficiency diseases. Irish Vet. J. *36*, 57-67.

McVicar, A. (1986) A spreading threat to salmon. Fish Farmer *9* (4), 18-19.

McVicar, A.H. (1987) Pancreas disease of farmed Atlantic salmon, *Salmo salar*, in Scotland: epidemiology and early pathology. Aquaculture *67*, 71-78.

McVicar, A.H. (1990) Infection as a primary cause of pancreas disease in farmed Atlantic salmon. Bull. Eur. Ass. Fish Pathol. *10*, 84-87.

McVicar, A.H. and Munro, A.L.S. (1987) Pancreas disease in farmed Atlantic salmon: evidence for an infectious aetiology. ICES Mariculture committee CM 1987/ F:6. 6 pp.

McVicar, A.H. and Munro, A.L.S. (1988) Further results in the study of the aetiology of pancreas disease of farmed Atlantic salmon. ICES Mariculture committee CM 1988/ F:35.

McVicar, A.H. and Munro, A.L.S. (1989) Pancreas disease in farmed Atlantic salmon: evidence for an infectious aetiology. *In* Aquaculture: a review of recent experience. O.E.C.D., Paris. pp.70-81

Mega, A. (1965) Studies of vitamin E in marine products (part 2). Determination of vitamin E content of fishes and shells in Nippon Sea. Rep. Hokkaido Inst. Publ. Health *15*, 93-97 (In Japanese).

Menzel, D.B. (1980) Protection against environmental toxicants. *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York. pp.474-494.

Mézes, M. (1986) Investigations on vitamin E content and lipid peroxidation in blood and tissues of common carp (*Cyprinus carpio* L.). Aquacult. Hung. *5*, 71-78.

Mézes, M. and Vadász, G. (1984) Effect of injected vitamin E on lipid peroxide and vitamin E content of certain tissues of carp. Aquacult. Hung. *4*, 51-54.

Milne, P.H. (1972) Hydrography of Scottish west coast sea lochs. Mar. Res. J. H.M.S.O., Edinburgh.

Mitchell, G. and Heffron, J.J.A. (1982) Porcine stress syndromes. Adv. Food Res. *28*, 167-230.

Miyazaki, T. (1986) A histopathological study on the carp fed α-tocopherol deficient diets including oxidized methyl linoleate. Fish Pathol. *21*, 73-79.

Miyazaki, T. and Kubota, S. (1981) Studies on nutritional myopathy syndrome in cultured fishes V. Sekokeosis in carp fed on rancid pupae of silkworm. Bull. Fac. Fish. Mie Univ. *8*, 117-128.

Miyazaki, T., Tsuda, A. and Kubota, S.S. (1985) Studies on the nutritional myopathy syndrome in cultured fishes 9. Histopathology of carp fed brown meal. Bull. Fac. Fish. Mie Univ. *12*, 131-137 (In Japanese).

Moccia, R.D., Hung, S.S.O., Slinger, J. and Ferguson, H.W. (1984) Effect of oxidized fish oil, vitamin E and ethoxyquin on the histopathology and haematology of rainbow trout, *Salmo gairdneri* Richardson. J. Fish Dis. *7*, 269-282.

Molenaar, I., Vos, J. and Hommes, F.A. (1972) Effect of vitamin E deficiency on cellular membranes. Vitam. Horm. *30*, 45-82.

Molenaar, I., Hulstaert, C.E. and Hardonk, M.J. (1980) Role in function and ultrastructure of cellular membranes. *In* Vitamin E: A Comprehensive Treatise (ed. L.J. Machlin) Marcel Dekker Inc., New York. pp. 373-389.

Moore, T. (1940) The effect of vitamin E deficiency on the vitamin A reserves of the rat. Biochem. J. *34*, 1321-1328.

Mori, T., Naito, K. and Mejika, K. (1957) Vitamin E in fish products II. Vitamin E contents in fish and shellfish. Lecture presented at 49th meeting Comm. Research Fat soluble vitamins. Vitamins (Kyoto) *13*, 537 (summary)

Morton, R.A. (1968) The history of vitamin research. Int. S. Vit. Res. *38*, 5-44.

Muir, J.F. (1982) Recirculated water systems in aquaculture. *In* Recent Advances in Aquaculture (eds J.F. Muir and R.J. Roberts) Croom-Helm, London. pp. 357-446.

Munro, A.L.S. (1988) Infectious pancreatic necrosis (IPN). D.A.F.S. Aquaculture series no. 2. 4 pp.

Munro, A.L.S., Ellis, A.E., McVicar, A.H., McLay, H.A. and Needham, E.A. (1984) An exocrine pancreas disease of farmed Atlantic salmon in Scotland. Helgol. Meersunters. *37*, 571-586.

Murai, T. and Andrews, J.W. (1974) Interactions of dietary α-tocopherol, oxidized menhaden oil and ethoxyquin on channel catfish (*Ictalurus punctatus*). J. Nutr. *104*, 1416-1431.

Murata, M. and Yamauchi, K. (1989) Relationship between the 2-thiobarbituric acid values of some tissues from cultured red sea bream and its dietary α-tocopherol levels. Nippon Suisan Gakkaishi *55*, 1435-1439.

Nanba, K., Nakagawa, H., Murachi, S., Kayama, M. and Nakamura, T. (1973) Blood properties of cultured yellowtail, *Seriola quiqueradiata* Temminck and Schlegel 1. Effect of disease caused by anchovy, *Engraulis japonica* Houthyn, as diet. J. Fac. Fish. Anim. Husb., Hiroshima Univ. *12*, 89-99.

Nakamura, T., Aoyama, Y., Fujita, T. and Katsui, G. (1975) Studies on tocopherol derivatives V. Intestinal absorption of several *d,l*-3,4-^{3}H-2-α-tocopheryl esters in the rat. Lipids *10*, 627-633.

Nash, G., Roberts, R.J., Chinabut, S., Aneerat, S. and Limsuwan, C. (1988) Emaciation of pond-cultured snakehead, *Channa striatus* (Fowler). J. Fish Dis. *11*, 215-224.

Nazir, D.J. and Magar, N.G. (1963) Chemical composition of mackerel (*Rastrelliger canagurta*) and changes in the nutritive value during storage. Indian J. Technol. *1*, 247-249.

Nazir, D.J. and Magar, N.G. (1964) Determination of ubiquinone and tocopherol in some tissues of shark (*Carcharias ellioti* Day). Biochem. J. *90*, 268-270.

New, M.B. (1987) Feed and feeding of fish and shrimp: a manual on the preparation and presentation of compound feeds for shrimp and fish in aquaculture. ADCP/REP/87/29 U.N.D.P., F.A.O., Rome 129 pp.

Newmark, H.L., Pool, W., Bauernfeind, J.C. and De Ritter, E. (1975) Biopharmaceutic factors in parenteral administration of vitamin E. J. Pharmaceut. Sci. *64*, 655-657.

Nitowsky, H.D., Tildon, J.T., Levin, S. and Gordon, H.H. (1962) Studies of tocopherol deficiency in infants and children VII. The effect of tocopherol on urinary, plasma, and muscle creatine. Am. J. Clin. Nutr. *10*, 368-378.

NRC (National Research Council) (1981) Nutrient requirements of coldwater fishes. National Academy Press, Washington D.C.

O'Connell, C.P. (1976) Histological criteria for diagnosing the starving condition in early post-yolk sac larvae of the Northern anchovy, *Engraulis mordax* Girard. J.exp. mar. Biol. Ecol. *25*, 285-312.

Oka, M. (1967) Fish Pathology *1* (2) 1-19 (In Japanese) (cited by Wanba *et al.* 1973).

O'Keefe, T.M. and Noble, R.L. (1978) Storage stability of channel catfish (*Ictalurus punctatus*) in relation to dietary level of α-tocopherol. J Fish. Res. Board Can. *35*, 457-460.

Okoro, N. (1988) Evaluation of weight loss in Atlantic salmon (*Salmo salar*) during starvation prior to smoking. M.Sc. thesis, Univ. of Stirling, Stirling, Scotland 58 pp.

Omaye, S.T. and Chow, F.I. (1984) Effect of hard red spring wheat bran on the bioavailability of lipid-soluble vitamins and growth in rats fed for 56 days. J. Food Sci. *49*, 504.

Oski, F.A., Myerson, R., Barness, L.A. and Williams, W.J. (1966) A possible interrelationship between vitamin E and vitamin B12. Am. J. Clin. Nutr. *18*, 307.

Palmer, R. (1988) Development of pancreas disease in Ireland. Paper presented at the Irish salmon farming conference, Clifden, 5-6th February 1988.

Papas, A.M., Cambre, R.C. and Citino, S.B. (1989) Vitamin E: considerations in practical animal feeding and case studies with elephants and rhinoceros. Paper presented at the 8th Dr. Scholl conference on the nutrition of captive wild animals, Chicago, Illinois, 8-9th December 1989.

Park, S.-I. (1978) Nutritional liver disease of cultured yellowtail, *Seriola quinqueradiata*, caused by feed deficiency. Bull. Kor. Fish. Soc. *11*, 1-4.

Pearse, L., MacQueen, A. and Roberts, R.J. (1974) Muscular dystrophy in cultured turbot (*Scophthalmus maximus* L.). Vet. Rec. *94*, 435-437.

Pennock, J.F., Morton, R.A., Lawson, D.E.M. and Laidman, D.L. (1962) Quinones and related compounds in fish tissues. Biochem. J. *84*, 637-640.

Pertz, L. and Sundberg, D.P. (1978) Malignant hyperthermia induced by atropine and xylazine in a fallow deer. J. Am. Vet. Med. Assoc. *173*, 124.

Peter, D.W., Hunter, R.A., Whanger, P.D. and Buscall, D.J. (1985) Feed intake as a factor influencing selenium status in young sheep. *In* T r a c e Elements in Man and Animals (eds C.F. Mills, I. Bremner and J.K. Chesters) Commonwealth Agricultural Bureaux, Slough. pp. 487-490.

Pickering, A.D. (editor) (1981) Stress and Fish. Academic Press, London.

Pickering, A.D., Pottinger, T.G. and Sumpter J.P. (1987*a*) On the use of dexamethasone to block the pituitary-interrenal axis in the brown trout, *Salmo trutta* L. Gen. Comp. Endocrinol. *65*, 346-353.

Pickering, A.D., Griffiths, R. and Pottinger, T.G. (1987*b*) A comparison of the effects of overhead cover on the growth, survival and haematology of juvenile Atlantic salmon, *Salmo salar* L., brown trout, *Salmo trutta* L, and rainbow trout, *Salmo gairdneri* Richardson. Aquaculture *66*, 109-124.

Pickford, G.E., Srivistava, A.K., Slicher, A.M. and Pang, P.K.T. (1971) The stress response in the abundance of circulating leucocytes in the killifish, *Fundulus heteroclitus*, I. The cold shock sequence and the effects of hypophysectomy. *J. Exp. Zool. 117*, 89-96.

Plehn, M. (1909) Über die leber der salmoniden. Allgemeine Fischwirtsschaftszeitung *24*, 525-527.

Poirier, A., Baudin-Laurencin, F., Bodennec, G. and Quentel, C. (1986) Experimental poisoning of the rainbow trout (*Salmo gairdneri* Richardson) by engine diesel oil: mortalities, haematological changes, histology. Aquaculture *55*, 115-137 (In French).

Pomeranze, J. and Lucarello, R.J. (1953) Tocopherol response curves and fat absorption. J. Lab. Clin. Med. *42*, 700-704.

Poppe, T.T. (1987) Exocrine pancrease disease og hemoragisk syndrom hos laks. Beslektede lidelser. Frisk Fisk 1987, 10-11 (In Norwegian).

Poppe, T., Freslie, A., Koppang, N. and Håstein, T. (1985*a*) Muscular dystrophy with exudative haemorrhagic diathesis in farmed Atlantic salmon - "Nitra disease". Norsk Veterin. *97*, 159-165.

Poppe, T.T., Håstein, T. and Salte, R. (1985*b*) "Nitra disease" (Haemorrhagic syndrome) in Norwegian salmon farming: present status. *In* Fish and Shellfish Pathology (ed. A.E. Ellis) Academic Press, London. pp.223-229

Poppe, T., Håstein, T., Freslie, A., Norheim, G. and Heum, M. (1985*c*) Vitamin E, selenium, copper and zinc in Atlantic salmon (*Salmo salar* L.): comparative studies of wild and farmed fish. Bull. Eur. Ass. Fish Pathol. *5*, 28-29.

Poppe, T.T., Håstein, T., Freslie, A., Koppang, N. and Norheim, G. (1986) Nutritional aspects of Haemorrhagic syndrome ('Nitra disease') in farmed Atlantic salmon, *Salmo salar*. Dis. aquat. Org. *1*, 155-162.

Poppe, T., Rimstad, E. and Hyllseth, B. (1989) Pancreas disease in Atlantic salmon (*Salmo salar*) postsmolts infected with infectious pancreatic necrosis virus (IPNV). Bull. Eur. Ass. Fish Pathol. *9*, 83-85.

Poston, H.A. (1965) Effect of dietary vitamin E on microhaematocrit, mortality, and growth of immature brown trout. Fish. Res. Bull. N.Y. *28*, 1-10.

Poston, H.A. (1968) The role of dietary tocopherol and cod-liver oil in a synthetic vitamin A test diet for brook trout. Fish. Res. Bull. N.Y. *31*, 39-43.

Poston, H.A. and Livingston, D.L. (1971) Effects of massive doses of dietary vitamin E on fingerling brook trout. Fish. Res. Bull. N.Y. *33*, 9-12.

Poston, H.A. and Combs, G.F. Jr. (1979) Interrelationships between requirements for dietary selenium, vitamin E and *L*-ascorbic acid by Atlantic salmon (*Salmo salar*) fed a semi-purified diet. Fish Health News *8* (4), vi-vii.

Poston, H.A., Combs, G.F. Jr. and Leibovitz, L. (1976) Vitamin E and selenium interrelations in the diet of Atlantic salmon (*Salmo salar*): gross, histological and biochemical deficiency signs. J. Nutr. *106*, 892-904.

Pozo, R., Lavéty, J. and Love, R.M. (1988) The role of dietary α-tocopherol (vitamin E) in stabilising the canthaxanthin and lipids of rainbow trout muscle. Aquaculture *73*, 165-175.

Pudelkiewicz, W.J. and Mary, N. (1969) Some relationships between plasma, liver and excreta tocopherol in chicks fed graded levels of alpha-tocopheryl acetate. J. Nutr. *97*, 303-306.

Pudelkiewicz, W.J., Webster, L. and Matterson, L.D. (1964). Effects of high levels o dietary vitamin A acetate on tissue tocopherol and some related analytical observations. J.Nutr. *84*, 113-117.

Putnam, M.E. and Comben, N. (1987) Vitamin E. Vet. Rec. *121*, 541-545.

Radi, A.A.R., Matkovics, B. and Csengeri, I. (1987) Comparative studies of the phospholipid fatty acids and the antioxidant enzyme activities in fish with different feeding habits. Comp. Biochem. Physiol. *87B*, 49-54.

Radi, A.A.R., Matkovics, B. and Csengeri, I. (1988) Effects of various oxygen concentrations on antioxidant enzymes and the quantity of tissue phospholipid fatty acids in the carp. Acta Biol. Hung. *39*, 109-119.

Raynard, R.S., McVicar, A.H., Bell, J.G., Youngson, A., Knox, D. and Fraser, C.O. (1991) Nutritional aspects of pancreas disease of Atlantic salmon: the effects of dietary vitamin E and polyunsaturated fatty acids. Comp. Biochem. Physiol. *98A*, 125-131.

Řehulka, J. (1990) Effect of hydrolytically changed and oxidised fat in dry pellets on the health of rainbow trout, *Oncorhynchus mykiss* (Richardson). Aquacult. Fish. Mgmt. *21*, 419-434.

Rice, D.A. and Kennedy, S. (1988) Vitamin E: function and effects of deficiency. Br. vet. J. *144*, 482-496.

Rice, D.A., McMurray, C.H. and Kennedy, S. (1985) Polyunsaturated fatty acid induced nutritional degenerative myopathy in selenium-deficient steers. *In* Trace elements in man and animals (eds C.F. Mills, I. Bremner and J.K. Chesters) Commonwealth Agricultural Bureaux, Slough. pp. 232-237.

Roald, S.O. (1976) An outbreak of lipoid liver degeneration (LLD) in Atlantic salmon (*Salmo salar*) in a fish farm, and attempts to cure the disease. Nord. Vet.-Med. *28*, 243-249 (In Norwegian).

Roald, S.O., Armstrong, D. and Landsverk, T. (1981) Histochemical, fluorescent and electron microscopical appearance of hepatocellular ceroidosis in the Atlantic salmon, *Salmo salar* L. J. Fish Dis. *4*, 1-14.

Roberts, R.J. (editor) (1989) Fish Pathology 2nd edn. Ballière-Tindall, London.

Roberts, R.J. and Bullock, A.M. (1989) Nutritional Pathology. *In* Fish Nutrition 2nd edn. (ed. J.E. Halver) Academic Press, London. pp.423-8

Roberts, R.J. and Richards, R.H. (1978) Pansteatitis in farmed rainbow trout (*Salmo gairdneri* Rich.). Vet. Rec. *103*, 492-493.

Roberts, R.J., Richards, R.H. and Bullock, A.M. (1979) Pansteatitis in rainbow trout, *Salmo gairdneri* Richardson: a clinical and histopathological study. J. Fish. Dis. *2*, 85-92.

Robertson, O.H., Wexler, B.C. and Muller, B.F. (1961) Degenerative changes in the cardiovascular system of spawning Pacific salmon, *Oncorhynchus tshawytscha*. Circulation Research *9*, 826-834.

Robeson, C.B. and Baxter, J.G. (1945) Chemistry and physiology of the vitamins. Interscience, New York.

Roem, A.J., Kohler, C.C. and Stickney, R.R. (1990) Vitamin E requirements of the blue tilapia, *Oreochromis aureus* (Steindachner), in relation to dietary lipid level. Aquaculture *87*, 155-164.

Ross, L.G. and Muir, J.F. (1987) A simple water flow sensor for electronic alarm systems. Aquacult. Eng. *6*, 75-77.

Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G. and Hoekstra, W.G. (1973) Selenium: biochemical role as a component of glutathione peroxidase. Science *179*, 588-590.

Roy, B. (1990) Careful feeding may solve pancreas problem. Fish farmer *13* (5), 52-53.

Rumsey, G.L. (1980) Antioxidants in compounded feeds. *In* Fish feed technology ADCP/REP/80/11, F.A.O., Rome. pp.177-182.

Sakaguchi, M. and Hamaguchi, A. (1969) Influence of oxidized oil and vitamin E on the culture of yellowtail. Bull. Jap. Soc. Sci. Fish. *35*, 1207-1214.

Sakaguchi, M. and Hamaguchi, A. (1979*a*) Physiological studies on cultured red sea bream - II. Effect of oxidized oil on the chemical constituents in plasma, hepatopancreas and muscle. Bull. Jap. Soc. Sci. Fish. *45*, 449-453 (In Japanese).

Sakaguchi, M. and Hamaguchi, A. (1979*b*) Physiological studies on cultured red sea bream - III. Digestibility and changes in chemical constituents in plasma and hepatopancreas after feeding of oxidized oil. Bull. Jap. Soc. Sci. Fish. *45*, 545-548 (In Japanese).

Salte, R., Hafstad, P. and Åsgård, T. (1987) Disseminated intravascular coagulation in "Hitra disease" (Hemorrhagic syndrome) in farmed Atlantic salmon. Vet. Pathol. *24*, 378-385.

Salte, R., Åsgård, T. and Liestøl, K. (1988) Vitamin E and selenium prophylaxis against "Hitra disease" in farmed Atlantic salmon - a survival study. Aquaculture *75*, 45-55.

Satoh, S., Takeuchi, T. and Watanabe, T. (1987) Requirement of tilapia for α-tocopherol. Bull. Jap. Soc. Sci. Fish. *53*, 119-124.

Schaus, E.E., DeLumen, B.O., Chow, F.I., Reyes, P. and Omaye, S.T. (1985) Bioavailability in rats fed different levels of pectin. J. Nutr. *115*, 263.

Schmandke, H. and Schmidt, G. (1965) Absorption of tocopherol from oily and aqueous solutions. Int. Z. Vitaminforsch *39*, 128-130 (In German).

Schulz, H., Mueller, K. and Feldheim, W. (1984) Studies of tocopherol content in liver and spleen of some freshwater and marine fish species. Helgol. Meeresunters. *38*, 75-81.

Schwarz, K. and Foltz, C.M. (1957) Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. J. Am. Chem. Soc. *79*, 3292-3293.

Scott, M.L. (1970) Nutritional and metabolic interrelationships involving vitamin E, selenium and cystine in the chicken. Internat. J. Vit. Res. *40*, 334-343.

Scott, M.L. (1973) Effect of processing on the availability and nutritional value of vitamins. *In* Effects of processing on the nutritional value of feeds. Nat. Acad. Sci., Washington D.C. pp. 119-130.

Scott, M.L. (1978) Vitamin E. *In* The Fat-soluble Vitamins (Handbook of lipid research vol. 2) (ed. H.F. DeLuca) Plenum Press, New York. pp.133-210.

Scott, M.L. and Desai, I.D. (1964) The relative anti-muscular dystrophy activity of the *d*- and *l*- epimers of α-tocopherol and of other tocopherols in the chick. J. Nutr. *83*, 39-43.

Scott, P. (1989) Clues to the rainbow fry anaemia syndrome. Fish farmer *12* (6), 17.

Sehdev, H.S., McBride, J.R. and Fagerlund, U.H.M. (1963) 2-Phenoxyethanol as a general anaesthetic for sockeye salmon. J. Fish. Res. Bd. Canada *20*, 1435-1440.

Selisko, O. (1961) Magnesium - ein vitamin E synergist? Die Naturwissenschaften *48*, 556 (In German).

Selye, H., Tuchweber, B. and Gabbiana, G. (1964) Further studies on anacalciphylaxis. J. Am. Geriat. Soc. *12*, 207-214.

Sheridan, M.A., Allen, W.V. and Kersletter, T.H. (1983) Seasonal variations in the lipid composition of the steelhead trout, *Salmo gairdneri* Richardson, associated with the parr-smolt transformation. J. Fish Biol. *23*, 125-134.

Sheridan, M.A., Allen, W.V. and Kersletter, T.H. (1985) Changes in the fatty acid composition of steelhead trout, *Salmo gairdneri* Richardson, associated with the parr-smolt transformation. Comp. Biochem. Physiol. *80B*, 671-676.

Skjaerlund, J.M. (1989) Exocrine pancreatic degeneration during vitamin E and selenium deficiency in rats. Nutr. Rep. Int. *40*, 151-159.

Smart, I.J., Embury, D.H., Barr, D.A., Sinclair, A.J., Karunajeewa, H., Ewing, I., Reece, R.L., Forsyth, W.M. and Hooper, P.T. (1985) Absence of a role for selenium deficiency in the runting syndrome of broiler chickens in Australia. Avian Diseases *29*, 1201-1211.

Smith, A.O. and Nelson, V.E. (1931) Cod liver oil for reproduction and lactation. Proc. Soc. Exp. Biol. Med. *28*, 393.

Smith, C.E. (1979) The prevention of liver lipoid degeneration (ceroidosis) and microcytic anaemia in rainbow trout, *Salmo gairdneri* Richardson, fed rancid diets: a preliminary report. J. Fish. Dis. *2*, 429-437.

Snieszko, S.F., Wood, E.M. and Yasutake, W.T. (1957) Infectious pancreatic necrosis in trout. A.M.A. Arch. Pathol. *63*, 229-233.

Sokal, R.R. and Rohlf, F.J. (1981) Biometry 2nd edn. W.H. Freeman and Co., San Francisco. 859 pp.

Solangi, M.A. and Overstreet, R.M. (1982) Histopathological changes in two estuarine fishes, *Menidia beryllina* (Cope) and *Trinectes maculatus* (Bloch and Schneider), exposed to crude oil and its water soluble fractions. J. Fish Dis. *5*, 13-35.

Solárzano, L. (1969) Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr. *14*, 799-801.

Soleim, S. (1985) Kidney histology of Atlantic salmon (*Salmo salar*) suffering from cold-water vibriosis or Hitra disease. Bull. Eur. Ass. Fish Pathol. *5*, 88-91.

Soliman, A.K. (1982) The effects of oxidation of dietary lipid combined with alpha-tocopherol acetate and butylated hydroxy toluene supplementation on the tilapia *Oreochromis niloticus* (Trewavas). M.Sc. thesis, University of Stirling. 57 pp.

Soliman, A.K., Roberts, R.J. and Jauncey,K. (1983) The pathological effects of feeding rancid lipid in diets for *Oreochromis niloticus* (Trewavas). *In* Proc. Int. Symp. on Tilapia in Aquaculture; Nazereth, Israel. 8th-13th May 1983. (Compiled by L.Fishelson and Z.Yaron). Tel Aviv University Press, Israel. pp.193-199.

Spratt, M. G. and Kratzing, C.C. (1971) The effect of dietary lipids and α-tocopherol on RES activity in choline deficiency: RES and fatty livers. Res. J. Reticuloendothelial Soc. *10*, 319-329.

Stéphan, G., Messager, J.L., Quentel, C. and Baudin-Laurencin, F. (1989) Effects of dietary oxidized fish oil and antioxidant deficiency on sea bass *Dicentrarchus labrax* L. Poster presented at the 4th International Conference of the European Association of Fish Pathologists, Santiago de Compostela, Spain, 24-28 September.

Strickland, J.D.H. and Parsons, T.R. (1972) A practical handbook of seawater analysis. Bull. Fish. Res. Board Can. *167*.

Sugii, K and Kinumaki, T. (1968) Distribution of vitamin E in a few species of fish. Bull. Jap. Soc. Sci. Fish. *34*, 420-428 (In Japanese).

Sure, B. (1924) Dietary requirements for reproduction. II. The existence of a specific vitamin for reproduction. J. Biol. Chem. *58*, 693-709.

Tacon, A.G.J. (1981) Speculative review of possible carotenoid function in fish. Prog. Fish-Cult. *43*, 205-208.

Tacon, A.G.J. and Jackson, A.J. (1985) Utilisation of conventional and unconventional protein sources in practical fish feeds. *In* Nutrition and Feeding in Fish (eds Cowey, C.B., Mackie, A.M. and Bell, J.G.) Academic Press, London. pp.119-145.

Takeuchi, M. (1972) Effect of vitamin E and ethoxyquin on absorption of lipid hydroperoxide in carp. Bull. Jap. Soc. Sci. Fish. *38*, 155-159.

Takeuchi, M., Ishii, S. and Ogiso, T. (1981) Effect of dietary vitamin E on growth, vitamin E distribution, and mortalities of the fertilized eggs and fry in ayu, *Plecoglossus altivelis*. Bull. Tokai Reg. Fish. Res. Lab. *104*, 111-122.

Talbot, C. and Higgins, P.J. (1982) Observations on the gall bladder of juvenile Atlantic salmon, *Salmo salar* L., in relation to feeding. J. Fish Biol. *21*, 663-669.

Tappel, A.L. (1968) Will antioxidant nutrients slow aging processes? Geriatrics *23*, 97-105.

Taylor, D.W. (1956) The effects of vitamin E and of methylene blue on the manifestations of oxygen poisoning in the rat. J.Physiol. *131*, 200-206.

Telford, I.R., Wiswell, O.B. and Smith E.L. (1954) Tocopherol prophylaxis in multiple exposure to hypoxia. Proc. Soc. Exp. Biol. Med. *87*, 162-164.

Tengerdy, R.T. (1989) Vitamin E, immune response and disease resistance. *In* Vitamin E: Chemistry and Health Implications (eds A.T. Diplock, L.J. Machlin, L. Packer, and W.A. Pryor) Ann. N.Y. Acad. Sci. *570*, 335-344.

Teskeredžić, Z., Teskeredžić, E. and Malnar, L. (1989) High mortality of rainbow trout (*Salmo gairdneri*) in Yugoslavian fish farms caused by inadequate feed quality. Aquaculture *79*, 391-395.

Thaxton, J.P. and Pardue, S.L. (1984) Ascorbic acid and physiological stress. In Ascorbic Acid in Domestic Animals (eds I.Wegger, F.J.Taqwerker and J.Moustgaard). Royal Danish Agricultural Society, Copenhagen. pp.25-31.

Thomas, P. (1987) Influence of some environmental variables on the ascorbic acid status of striped mullet *Mugil cephalus* Linn., tissues. III. Effects of exposure to oil. J. Fish Biol. *30*, 485-494.

Thomas, P. (1990) Molecular and biochemical responses of fish to stressors and their potential use in environmental monitoring. Amer. Fish. Soc. Symp. *8*, 9-28.

Thompson, J.N. and Scott, M.L. (1970) Impaired lipid and vitamin E absorption related to atrophy of the pancreas in selenium-deficient chicks. J. Nutr. *100*, 797-809.

Thomson, C.D. and Robinson, M.F. (1980) Selenium in human health and disease with emphasis on those aspects peculiar to New Zealand. Am. J. Clin. Nutr. *33*, 303-323.

Thorpe, J. (1989) A technique for reducing grilse ratios and costs. Paper presented at the Scottish Fish Farming Conference, Edinburgh, 14th-16th February 1989.

Thorpe, J.E., Talbot, C., Miles, M.S., Rawlings, C. and Keay, D.S. (1990) Food consumption in 24 hours by Atlantic salmon in a sea cage. Aquaculture *90*, 41-47.

Tocher, D.R. and Harvie, D.G. (1988) Fatty acid composition of the major phosphoglycerides from fish neural tissues; (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (*Salmo gairdneri*) and cod (*Gadus morhua*) brains and retinas. Fish Physiol. Biochem. *5*, 229-239.

Torrissen. O.J. (1985) Pigmentation of salmonids: factors affecting carotenoid deposition in rainbow trout (*Salmo gairdneri*) Aquaculture *46*, 133-142.

Totland, G.K., Kryvi, H., Jodestøl, K.A., Christiansen, E.N., Tangerås, A. and Slinde, E. (1987) Growth and composition of the swimming muscle of adult Atlantic salmon (*Salmo salar* L.) during long-term sustained swimming. Aquaculture *66*, 299-313.

Totland, G.K., Nylund, A. and Holm, K.O. (1988) An ultrastructural study of morphological changes in Atlantic salmon, *Salmo salar*, L. during the development of cold water vibriosis. J. Fish Dis. *11*, 1-13.

Ullrey, D.E., Schmitt, S.M., Cooley, T.M., Ku, P.K. and Whetter, P.A. (1985) Selenium, vitamin E and capture myopathy in white-tailed deer. *In* Trace elements in man and animals (eds C.F. Mills, I.Bremner and J.K. Chesters). Commonwealth Agricultural Bureaux, Slough. pp.113-115.

Uponor Ltd. (1987) Upoten blue MDPE pipes and fittings. Uponar Ltd, Durem, England. p.23.

Ushikusa, T., Marumaya, T., Kanematsu, H., Niiya, I. and Matsumoto, T. (1986) Seasonal variation of tocopherols in different tissues of carp and their relationships to vitamin A and D. Nippon Eiyo, Shokuryo Gakkaishi *39*, 423-426 (In Japanese).

Ushikusa, T., Marumaya, T. Kanematsu, H. and Niiya, I. (1987) Tocopherol in different tissues of cultured sea bream. Seikatsu Eisei *31*, 27-30 (In Japanese).

Van Vleet, J.F. (1982) Amounts of eight combined elements required to induce selenium-vitamin E deficiency in ducklings and protection by supplements of selenium and vitamin E. Am. J. Vet. Res. *43*, 1049-1055.

Van Vleet, J.F., Crawley, R.R. and Anstutz, H.E. (1977) Myodegeneration associated with selenium-vitamin E deficiency in a pregnant heifer. J. Am. Vet. Med. Assoc. *171*, 443-445.

Van Vleet, J.F., Boon, G.D. and Ferrans, W. (1981) Induction of lesions of selenium-vitamin E deficiency in ducklings fed silver, copper, cobalt, tellerium, cadmium, or zinc: protection by selenium or vitamin E supplements. Am. J. Vet. Res. *42*, 1206-1217.

Verma, S.R. and Tonk, I.P. (1983) Effect of sublethal concentrations of mercury on the composition of liver muscles and ovary of *Notopterus notopterus*. Water Air Soi. Pollu. *20*, 287-292.

Vos, J., Hulstaart, C.E. and Molenaar, I. (1981) Nutritional myopathy in ducklings: a growth rate-dependant symptom of 'tissue peroxidosis' due to a net nutritional shortage of vitamin E plus selenium in skeletal muscle. Ann. Nutr. Metab. *25*, 299-306.

Waagbø, R., Sandnes, K. Espelid S., and Lie, Ø. (1988) Haematological and biochemical analyses of Atlantic salmon, *Salmo salar* L., suffering from coldwater vibriosos ('Hitra disease'). J. Fish Dis. *11*, 417-423.

Watanabe, T. (1985) Importance of the study of broodstock nutrition for further development of aquaculture *In* Nutrition and Feeding in Fish (eds C.B. Cowey, A.M. Mackie, and J.G. Bell) Academic Press, London. pp.395-414.

Watanabe, T. and Hashimoto, Y. (1968) Toxic compounds of oxidised saury oil inducing muscular dystrophy in carp. Bull. Jap. Soc. Sci. Fish. *34*, 1131-1140.

Watanabe, T. and Takashima, F. (1977) Effect of α-tocopherol deficiency on carp. VI. Deficiency symptoms and changes of fatty acid and triglyceride distributions in adult carp. Bull. Jap. Soc. Sci. Fish. *43*, 819-830.

Watanabe, T., Matsumura, Y. and Hashimoto, Y. (1966). Effect of natural and synthetic antioxidants on the incidence of muscle dystrophy of carp induced by oxidized saury oil. Bull. Jap. Soc. Sci. Fish. *32*, 887-891.

Watanabe, T. Tsuchiya, T. and Hashimoto, Y. (1967) Effect of DPPD and ethoxyquin on the muscular dystrophy of carp induced by oxidised saury oil. Bull. Jap. Soc. Sci. Fish. *33*, 843-847.

Watanabe, T., Takashima, F., Ogino, C. and Hibiya, T. (1970*a*) Effects of α-tocopherol deficiency on carp. Bull. Jap. Soc. Sci. Fish. *36*, 623-630.

Watanabe, T., Takashima, F., Ogino, C. and Hibiya, T. (1970*b*) Requirement of young carp for α-tocopherol. Bull. Jap. Soc. Sci. Fish. *36*, 972-976.

Watanabe, T. Takashima, F., Ogino, C. and Hibiya, T. (1970*c*) Effects of α-tocopherol deficiency on carp - II. Protein composition of the dystrophic muscle. Bull. Jap. Soc. Sci. Fish. *36*, 1231-1234.

Watanabe, T. Matsui, M., Takashima, F. and Ikekawa, N. (1973) Effect of α-tocopherol deficiency on carp - IV. The composition of triglycerides. Bull. Jap. Soc. Sci. Fish. *39*, 375-382.

Watanabe, T., Kobayashi, I. Utsue, O. and Ogino, C. (1974) Effect of dietary methyl linoleate on fatty acid composition of lipids in rainbow trout. Bull. Jap. Soc. Sci. Fish. *40*, 387-392.

Watanabe, T., Utsue, O., Kobayashi, I. and Ogino, C. (1975) Effect of dietary methyl linoleate and linolenate on growth of carp - I. Bull. Jap. Soc. Sci. Fish. *41*, 257-262.

Watanabe, T. Matsui, M., Kawabata, T. and Ogino, C. (1977*a*) Effect of α-tocopherol deficiency on carp - V. The compositions of triglycerides and cholesteryl esters in lipids of young carp. Bull. Jap. Soc. Sci. Fish. *43*, 813-817.

Watanabe, T., Takeuchi, T., Matsui, M., Ogino, C. and Kawabata, T. (1977*b*) Effect of α-tocopherol deficiency on carp - VII. The relationship between dietary levels of linoleate and α-tocopherol requirement. Bull. Jap. Soc. Sci. Fish. *43*, 935-946.

Watanabe, T., Takeuchi, T. and Wada, M. (1981*a*) Dietary lipid levels and α-tocopherol requirement of carp. Bull. Jap. Soc. Sci. Fish. *47*, 1585-1590.

Watanabe, T., Takeuchi, T., Wada, M. and Uehara, R. (1981*b*) The relationship between dietary lipid levels and α-tocopherol requirement of rainbow trout. Bull. Jap. Soc. Sci. Fish. *47*, 1463-1471.

Watanabe, T., Wada, M. Takeuchi, T. and Arai, S. (1981*c*) Absence of interconversion of tocopherols in rainbow trout. Bull. Jap. Soc. Sci. Fish. *47*, 1455-1462.

Watanabe, T., Tanniya, T., Oka, A., Hirata, M. Kitajuma, C. and Fujita, S. (1983) Improvement of dietary value of live foods for fish larvae by feeding them on ω3 highly unsaturated fatty acids and fat-soluble vitamins. Bull. Jap. Soc. Sci. Fish. *49*, 471-479.

Watanabe, T., Arakawa, T., Kitajima, C. and Fujita, S. (1984) Effect of nutritional quality of broodstock diets on reproduction of red sea bream. Bull. Jap. Soc. Sci. Fish. *50*, 495-501.

Watanabe, T., Takeychi, T., Satoh, S., Toyama, K. and Okuzumi, M. (1987) Effect of dietary histidine or histamine on growth and development of stomach erosion in rainbow trout. Nippon Suisan Gakkaishi *53*, 1207-1214.

Wedemeyer, G. (1969) Stress-induced ascorbic acid depletion and cortisol production in two salmonid fishes. Comp. Biochem. Physiol. *29*, 1247-1251.

Weichselbaum, T.E. (1946) An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. Amer. J. clin. Path. (Tech. Sect.) *10*, 40-49

Westers, H. and Pratt, K.M. (1977) Rational design of hatcheries for intensive salmonid culture, based on metabolic characteristics. Prog. Fish-Cult. *39*, 157-165.

Whitacre, M.E., Combs, G.E. Jr., Combs, S.B. and Parker, R.S. (1987) Influence of dietary vitamin E on nutritional pancreatic atrophy in selenium deficient chicks. J. Nutr. *117*, 460-467.

Whitmore, C.M. (1965) A microcytic anemia of juvenile chinook salmon resulting from diets deficient in vitamin E. Fish Commission of Oregon, Portland, Oregon. Contribution no. 29.

Wickins, J.F. (1981) Water quality requirements for intensive aquaculture: a review. Proc. World Symp on Aquaculture in Heated Effluents and Recirculation systems. Stavanger 28-30 May, 1980. (ed. K. Tiews) Heineman, Berlin. pp.17-37.

Wilbur, K.M., Bernheim, F., and Shapiro, O.W. (1949) The thiobarbituric acid reagent as a test for the oxidation of unsaturated fatty acids by various agents. Arch. Biochem. *24*, 305-313.

Wilson, R.B., Kula, N.S., Newberne, P.M. and Conner, M.W. (1973) Vascular damage and lipid peroxidation in choline-deficient rats. Exp. Biol. Pathol. *18*, 357-368.

Wilson, R.B., Newberne, P.M. and Kula, N.S. (1974) Protection by antioxidants against arterial sclerosis of chronic choline deficiency. Exp. Biol. Pathol. *21*, 118-122.

Wilson. R.P., Bowser, P.R. and Poe, W.E. (1984) Dietary vitamin E requirement of fingerling catfish. J. Nutr. *114*, 2053-2058.

Wolf, L.E. (1951) Diet experiments with trout. Prog. Fish-Cult. *13*, 17-24.

Wood, E.M. and Yasutake, B.A. (1956) Ceroid in fish. Am. J. Path. *32*, 591-603.

Woodall, A.N., Ashley, L.M., Halver, J.E., Olcott, H.S. and Van der Veen, J. (1964) Nutrition of salmonoid fishes. XIII. The α-tocopherol requirement of chinook salmon. J. Nutr. *84*, 125-135.

Yamakawa, T.S., Arai, S., Shimina, Y. and Watanabe, T. (1975) Vitamin E requirement for Japanese eel. Vitamins (Kyoto) *49*, 62.

Yamamoto, K.I., Itasawa, Y. and Kobayashi, H. (1980) Supply of erythrocytes into the circulating blood from the spleen of exercised fish. Comp. Biochem. Physiol. *65A*, 5-11.

Yokote, M. (1970*a*) Sekoke disease, spontaneous diabetes in carp, *Cyprinus carpio*, found in fish farms I. Pathological study. Bull. Freshw. Fish. Res. Lab. *20*, 39-72.

Yokote, M. (1970*b*) Sekoke disease, spontaneous diabetes in carp found in fish farms II. Some metabolic aspects. Bull. Jap. soc. Sci. Fish. *36*, 1214-1218.

Yokote, M. (1970*c*) Sekoke disease, spontaneous diabetes in carp found in fish farms III. Response to mammalian insulin. Bull. Jap. Soc. Sci. Fish. *36*, 1219-1223.

Young, J.M. Jr., Dinning, J.S. and Day, P.L. (1955) Metabolic interrelationships of vitamins E and B6. Proc. Soc. Exp. Biol. Med. *89*, 216-217.

Zöllner, N. and Kirsch, K. (1962) Über die quantitative Bestimmung von Lipoiden (micromethode) mittels der vielen naturlichen Lipoiden (allen bekaaten Plasmalipoiden) gemeinsamen sulpho-phosphovanillin-reaktion. Z. ges. exp. Med., Bd. *135*, S, 545-561 (In German).