STUDIES ON POLYUNSATURATED FATTY ACID NUTRITION IN THE LARVAE OF A MARINE FISH - THE HERRING, *Clupea harengus* L.

Thesis presented for the degree of
Doctor of Philosophy
of the University of Stirling

Clive Fox BSc. (Hons.) Liverpool .

NERC Unit of Aquatic Biochemistry
University of Stirling
Stirling, Scotland

Declaration

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree.
The work of which it is a record, except where specifically acknowledged, has been carried out by myself and all sources of information have been specifically acknowledged by means of reference.

Olive Fosc

Abstract

Despite increasing interest in the culture of cold-water marine fish, a satisfactory inert feed for the larvae of these species has not been developed. As a consequence, the majority of information on the nutritional requirements of the larval stages has been inferred from studies on juvenile or adult fish or derived from manipulations of the nutrient contents of live feeds. Although the latter technique has highlighted the importance of long-chain (n-3)polyunsaturated fatty acids (PUFAs), it is insufficiently precise to define the requirements for individual fatty acids.

In this study, the (n-3)PUFA nutrition of the larvae of the herring, *Clupea harengus* L. was studied. Firstly, the ability of the larvae to elongate and desaturate the fatty acids contained in two strains of brine shrimp, *Artemia*, was considered. Secondly, the feasibility of rearing herring larvae on an inert, microencapsulated diet was considered. Although it was concluded that long-term rearing trials using this method were not viable at the time, larvae weaned onto the inert feed, from live feed, achieved positive growth. Finally, a novel method for incorporating radiolabelled fatty acids, as metabolic tracers, into herring larvae was developed. The viability of using this system for studying the ability of larvae to elongate and desaturate the fatty acids 18:3(n-3) and 20:5(n-3) was demonstrated.

The results from all the trials indicated that a high rate of elongation and desaturation of 18:3(n-3) does not occur in herring larvae. Although some ability to elongate 20:5(n-3) to 22:5(n-3) was demonstrated, significant desaturation of this fatty acid to 22:6(n-3) did not occur. It is suggested that the rate of bioconversion of 20:5(n-3) to 22:6(n-3) is too low to be of nutritional significance.

Dedication

This work is dedicated to my parents who have provided much encouragement, support and financial assistance and to Duncan Seaton who died on July 25, 1990 after serving at DAFS for 40 years.

Acknowledgements

The author wishes to express his gratitude to Prof J. Sargent and to Dr J. Gamble for their supervision of this project.

To Frippak Feeds (Ltd.) and to Niel McDonald for providing encapsulated diets.

To the Danish Fisheries Research Institute, Charlottenland, for the provision of Baltic herring eggs and to the Scottish Marine Biology Association (SMBA) Laboratory, Oban, for provision of egg incubation facilities.

Thanks also:-

To Dr P. Tytler for his special interest in the latter part of this project and to Mr L. Brown for attempting to teach me the art of histology.

To all my collegues at the NERC Unit of Aquatic Biochemistry for their advice, encouragement and occassional remonstrations (especially to Dougie 'Where's my chair?' Tocher for entertainment value).

To Mr P. Hodgson for starting off my interest in computing.

To all at the Department of Agriculture and Fisheries for Scotland (DAFS) who became involved in this work, especially to those stationed at the Marine Research Unit, Poolewe, Wester Ross and to Duncan Seaton for the late nights spent fertilizing herring eggs.

Finally special thanks to Mel for all her support and for spending her holidays on the west coast of Scotland counting fish larvae.

This work was carried out under NERC Grant No. GT4/86/ALS/35.

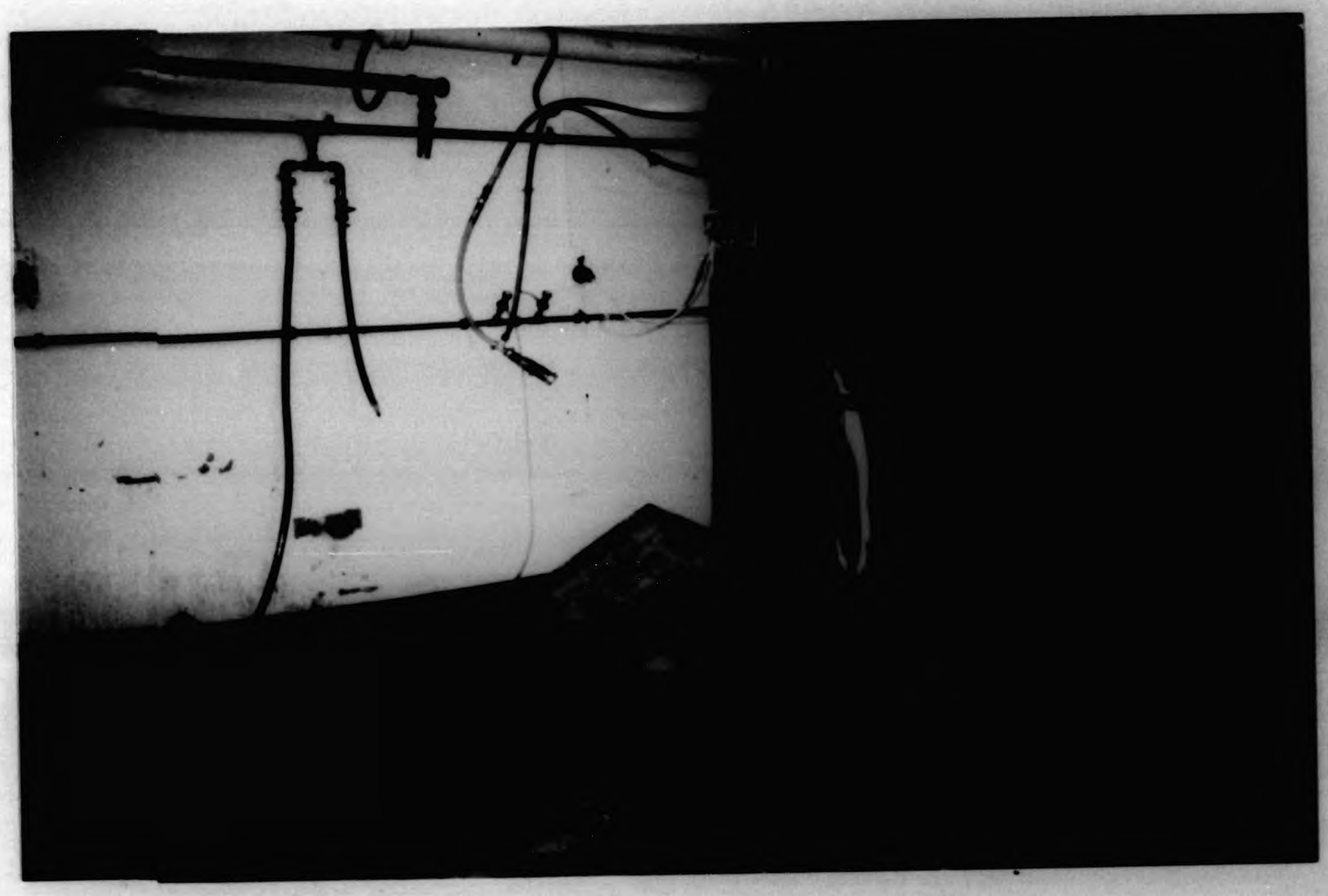


Plate I **Just when you thought you had everything under control, explosion damage in the aquarium at Stirling University, March 16, 1989.**

Contents

> **Studies in progress in several laboratories are particularly concerned with the nutritional requirements of cold-water marine fish larvae. This aspect is not yet, however, fully understood and is probably one of the main constraints in commercial mass rearing of marine fish fry.**
>
> **Tilseth (1990)**

Chapter 1 Introduction

This section outlines the nutrition of fish in relation to aquaculture and to the natural environment. Lipid nutrition in fish larvae, juveniles and adults is considered in more detail and the aims of this study are set out.

1.1 Nutrition in aquatic ecosystems

All heterotrophs depend upon the catabolism of organic compounds for their survival. For terrestrial ecosystems these compounds have traditionally been viewed as being ultimately derived from photosynthetic plants. By extension, in the aquatic environment a food chain from phytoplankton, via zooplankton to higher trophic levels was envisaged. However, there are several differences between terrestrial and aquatic ecosystems of direct relevance to nutrition. In aquatic ecosystems organisms often alter their feeding habits and niche with age. An example of this is the Atlantic menhaden (*Brevoortia tyrannus*) which feeds mainly on zooplankton in the larval stage but becomes almost exclusively herbivorous after metamorphosis. This change is associated with a switch from particulate to filter feeding (June & Carlson 1971). Unlike terrestrial organisms, those in aquatic ecosystems live suspended in a fluid medium containing dissolved nutrients which they may utilize (Stephens & Manahan 1984). Recent emphasis on nutrient cycling in the microbial loop

means that it is now necessary to envisage pelagic ecosystems as dynamic communities with potential interactions amongst all the components (Sherr & Sherr 1988). However, the contribution which bacteria and ciliates make to the nutrition of macro-zooplankton is currently unclear.

Because of the complexity of the natural ecosystem, the nutritional needs of aquatic animals can only be quantitatively defined in simplified experimental systems. The majority of our knowledge of fish nutrition has come from studies on species which are important in aquaculture. As aquaculture has become more intensive it has become increasingly reliant on artificially formulated feeds as replacements for, or supplements to, naturally produced food in ponds and cages. In order to produce diets which satisfy the physiological requirements of the cultured species, a considerable amount of work on fish nutrition has been carried out in recent years.

1.2 Fish nutrition

This subject has been recently reviewed by Steffens (1989), Halver (1989), Cowey *et al.* (1985) and Millikin (1982).

Fish, like all animals, require dietary inputs of protein, lipid, carbohydrate, minerals and vitamins. Although nutrients may be present in the diet they are not always available to the organism, they must be in a form which can be digested and absorbed.

Nutrients may be catabolized for energy or used as structural components. Materials may also be stored for mobilization at a later date. This may involve their conversion into more stable storage compounds.

The quantitative and possibly qualitative dietary requirements of fish relate to age (Dabrowski 1986) and environmental salinity. Zeitoun *et al.* (1974, 1973) found that the dietary protein requirement of rainbow

trout (*Salmo gairdneri*) fingerlings was influenced by salinity but the protein requirement of coho salmon (*Oncorhyncus kisutch*) smolts was not.

1.2.1 Digestion

This subject has been reviewed by Steffens (1989), Ash (1985) and Kapoor *et al.* (1975).

The oesophagus tends to be short and in most fishes incorporates the opening to the swim bladder. Species such as catfish possess a stomach but in the cyprinids this is absent. In those fish possessing stomachs, the form of this organ is highly variable. Certain species possess pyloric caeca which are thought to increase the residence time of food and the surface area for absorption of nutrients. The length and configuration of the intestine varies with species but generally the bile and pancreatic ducts open into the initial portion. There is no clearly defined rectum, closure of the intestine being effected by an anal sphincter.

Stomachless fish do not excrete hydrochloric acid or pepsinogen and protein digestion is accomplished at neutral to alkaline pH by tryptic-like enzymes. However, in fish possessing stomachs, acidic reactions do occur. Some lipolase and carbohydrase activity has also been detected in fish stomachs. A range of proteolytic enzymes is produced by the intestinal mucosa and the pancreas but the proteolytic activity in the intestine and pyloric caeca of young fish tends to be lower than that in the stomach.

Bile produced by the liver is of prime importance for emulsifying dietary lipid and facilitating its digestion by lipase enzymes. In most fish the majority of lipid digestion appears to occur in the intestine. The

digestion, absorption and fate of dietary lipid has been reviewed by Leger (1985).

A wide range of carbohydrases, produced mainly by the intestine and pancreas, have been detected in a variety of fish species.

The ontogeny of digestion in fish larvae is of interest since, at first feeding, the alimentary canal is structurally and functionally less complex than that of the adults (Govoni *et al.* 1986). In larval turbot, *Scophthalmus maximus*, Cousin *et al.* (1987) detected non-specific protease, amylase, esterase, aminopeptidase and acid and alkaline phosphatase activities in the guts at the time of first feeding. However, lipase activity was not noted until day 15 post-hatch. The ontogeny of lipase secretion has received little attention compared to that of the proteases.

Despite several studies it is still unclear whether exogenous proteases derived from live prey are important for digestion in larval fish (Hjelmeland *et al.* 1989, Lauff & Hofer 1984).

1.2.2 Energy

Fish convert feed efficiently into body tissue i.e. they have a low energy requirement per unit weight gain compared with mammals and birds. This is because they are poikilothermic, their bodies are supported by water and they excrete about 85% of their waste nitrogen as ammonia. Excretion of this low molecular weight compound, via a concentration gradient across the gills, occurs at little energetic cost. The metabolic rate of fish (relative to unit mass) generally decreases with weight. Thus the daily energy consumption (g^{-1} tissue) during routine metabolism at a specific temperature will be greater for a juvenile as opposed to an adult specimen (Steffens 1989).

Lipids, proteins and carbohydrates can all be used to supply energy. Since protein is economically the most expensive component of artificial diets, most formulated feeds for aquaculture attempt to supply the energy requirement in the form of carbohydrate or lipid. This is termed protein sparing (Reinitz *et al.* 1978).

The growth rate of fish is affected by the balance of nutrients obtained by the fish after the energetic costs of metabolic maintenance have been met. Growth rate will also be affected by water temperature, rearing conditions and the genetic potential of the fish. The use of terrestrial plant materials may incorporate antinutritional factors into artificial diets for fish. Protease inhibitors occur in soybean, rapeseed and rice whilst the toxin gossypol occurs in cottonseed oil (Tacon & Jackson 1985).

1.2.3 Amino acids and proteins

Proteins are, as a rule, degraded to their constituent amino acids in the digestive tract prior to absorption although there is evidence that pinocytosis of intact proteins followed by intracellular digestion can occur in young fish (Watanabe 1982b). The amino acids are transported to the body cells via the portal circulation. Amino acid storage does not take place to any great extent and so the acids are used as the building blocks for protein anabolism or are catabolized for energy. Protein anabolism requires an adequate supply of energy and only if this condition is met is growth possible.

Compared with other vertebrates, fish are characterized by a high dietary protein requirement. In aquaculture it is necessary to define the minimum dietary protein level consistent with a high growth rate and

good feed utilization by the fish. The minimum protein requirement of young chinook salmon, *Onchorhyncus tshawytscha* at 8°C water temperature is about 40% of the diet (by weight). The protein requirement is affected by water temperature, the amino acid spectrum of the protein, the energy content of the feed and the physiological state of the fish (Steffens 1989).

Studies using defined diets and radiolabelled acetate have shown that a wide range of fish species require a group of ten amino acids as essential dietary components (Cowey *et al.* 1970). These include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine whilst the remaining non-essential amino acids can be synthesised *de novo*. Additional techniques employed to study the amino acid requirements of fish have included measurements of the free amino acid pool in serum or muscle and quantification of the oxidation/deposition ratio for a universally ^{14}C-labelled amino acid. Problems exist in translating data derived from the above methods into practical diets since intact proteins often have a greater dietary value than is indicated by their amino acid spectrum. This may be partly due to overestimation of the amino acid requirements using crystalline acids which may be prone to leaching from diets.

Protein is the major organic component of fish comprising 65-75% of the dry weight. As well as being the main structural component of muscle, proteins fulfil enzymic roles. Since the properties of a protein are determined by its amino acid composition, essential amino acid deficiency will have a highly deleterious effect.

1.2.4 Carbohydrates

Carbohydrates are mainly catabolized for energy. In the wild, carnivorous fish receive only a minor portion of their diet as carbohydrate whilst herbivorous fish receive a greater portion, although much of this will be cellulose. Carbohydrates are ecomically cheap and often have a protein sparing effect but the degree to which fish can utilize them varies with species. Furuichi & Yone (1980) showed that the maximum levels of dextrin which could be incorporated into artificial diets were 10% for yellowtail (*Seriola quinqueradiata*), 20% for red sea bream (*Chrysophrys major*) and 30% for common carp (*Cyprinus carpio*). They suggested that fish which were naturally herbivorous or omnivorous could utilize carbohydrate more efficiently than those which were carnivorous.

The digestibility of carbohydrates is also related to their molecular complexity. Thus efficiency of uptake is normally in the order monosaccharides > disaccharides > polysaccharides (Millikin 1982).

Fibre is defined as the quantity of non-dissolved, ash-free residue after a diet has been extracted with dilute alkali and dilute mineral acid. The term includes a complex of polysaccharides including cellulose, lignin, pentosans and chitin. Fibre does not appear to be of direct nutritional importance for rainbow trout (Davies 1985) but a possible role in maintaining gut muscle tone remains to be evaluated. There is some evidence that several fish species possess chitin digesting enzymes in their guts but these are probably of bacterial origin (Clark *et al.* 1988, MacDonald *et al.* 1986, Goodrich & Morita 1977). Excessive fibre may reduce digestive efficiency by decreasing the gut residence time of food and possibly by chelating metal ions which serve as cofactors for digestive

enzymes. In practical formulated diets, excessive fibre may also reduce water quality (Leary & Lovell 1975).

1.2.5 Vitamins

Vitamins are low molecular weight compounds of widely differing composition which are essential to life, but which cannot as a rule be synthesised by higher animals. Although they are generally required only in trace amounts, failure to supply these compounds in the diet results in a wide range of pathological disorders. Eleven water-soluble vitamins and four fat-soluble vitamins (A, D, E and K) have been shown to be required by salmon and rainbow trout. Hypervitaminosis of the water-soluble vitamins is unlikely as any surplus is readily excreted but hypervitaminosis of the fat-soluble vitamins can occur. Since vitamin E is often included as an antioxidant in fish feed, excessive accumulation of this compound in the fish can be a potential problem.

Vitamins perform a wide range of metabolic functions. In several cases they act with, or are precursors of, coenzymes and interactions with mineral ions are also common.

1.2.6 Minerals

A variety of inorganic substances are required by aquatic organisms. Unlike terrestrial animals, fish can absorb some minerals from their environment. Since many minerals are required only in trace amounts, it has frequently been impossible to demonstrate deficiency syndromes. It is currently thought that 29 of the 90 naturally occurring elements are essential for life. Disregarding the macro-elements (C, H, N, S, Ca, Mg, P, Na, K and Cl), fifteen trace elements are considered essential

in animals. Amongst these, deficiency syndromes linked to physiological roles have been demonstrated for Cr, Co, Cu, I, Fe, Mn, Mo, Se, Zn and F. In ultra-clean environments, deficiency syndromes for Si, Ni, V, and As have been demonstrated but their physiological function is unclear. Although most of these elements have been detected in fish, the essentiality of only a few, namely Ca, P, Mg, Fe, Cu, Mn, Zn, I and Se have been investigated.

The macro-elements listed above are found in the major structural components of fish tissues whilst trace elements are frequently found as coenzymes. Interactions between minerals and other dietary components have been frequently demonstrated in terrestrial animals. Certain minerals have protective roles e.g. selenium will bind toxic elements such as mercury and thus reduce their bioavailability. Selenium also appears to act synergistically with vitamin E.

Finally it must be noted that many minerals exert toxic effects when present at excessive concentrations or in certain states e.g. organic and inorganic mercury. The ability of fish to excrete or detoxify these minerals varies with species and age.

1.3 Lipids

Lipids have received a large amount of attention recently since many dietary problems in aquaculture are related to this group of nutrients.

The lipids are probably the most intensively studied of the major biochemical components of aquatic organisms. This is due to the high levels of oils found in many aquatic organisms and their high (n-3) polyunsaturated fatty acid[1] (PUFA) content. Interest in (n-3)PUFAs has

[1] Fatty acids containing two or more unsaturated bonds

recently intensified due to their implication in human nutrition (Neuringer *et al.* 1988, Carroll 1986). Since this study was concerned with PUFA nutrition in the larvae of a marine fish, a fuller introduction to the lipids is provided in the following sections.

A summary of lipid structure, nomenclature and the pathways for fatty acid elongation and desaturation in animals is provided at Appendix I.

1.3.1 Lipid nutrition in fish

Several recent reviews of this subject are available (Sargent *et al.* 1989, Henderson & Tocher 1987, Watanabe 1982a, Castell 1979, Cowey & Sargent 1977) and only the major points are reiterated below.

As outlined above for carbohydrates, lipids have a sparing effect on proteins in artificial diets for fish. However, unlike carbohydrates, lipids are also essential components for growth. In commercial diets fat is normally included at between 10–15% (dry weight). There are considerable technical difficulties in preparing diets with higher lipid contents. Several studies have examined the optimum level of lipid in fish diets (see review by Millikin (1982).

The spectrum of fatty acids supplied in the diet is relatively unimportant from the point of view of energy provision. However, the specific fatty acids supplied are very important due to their structural and metabolic roles.

1.3.2 Freshwater ecosystems

In freshwater ecosystems fatty acids of the (n-6) series are more abundant than in marine ecosystems. Terrestrial plant seeds contain significant levels of 18:2(n-6) whilst in freshwater algae (n-6)PUFAs are found in higher levels than in marine algae. Considerable amounts of (n-3)PUFAs also occur with 18:3(n-3) usually being more abundant than 20:5(n-3) and 22:6(n-3). The thylakoid membranes of the chloroplasts of terrestrial plants are usually dominated by 18:3(n-3).

The fatty acids of aquatic insects have received attention due to their importance in freshwater food-webs. Of 58 genera of aquatic insects examined, all contained 18:2(n-6) and 18:3(n-3) with 20:4(n-6) and 20:5(n-3) present at up to 7% and 25% of total fatty acids respectively. Terrestrial insects and adults with aquatic larvae had lipids dominated by 18:2(n-6) and 18:3(n-3) (Hanson *et al.* 1985).

The fatty acids of freshwater fish have been reviewed by Henderson & Tocher (1987). The lipids of freshwater fish contain higher levels of 18:2(n-6), 20:4(n-6) and 18:3(n-3) than marine species. This appears to reflect the greater abundance of these fatty acids in the freshwater food-web and the terrestrial influence. The (n-3)/(n-6)PUFA ratio tends to be lower in freshwater fish but it must be emphasised that they still usually contain much more (n-3) than (n-6)PUFA.

1.3.3 Marine ecosystems

In contrast to freshwater ecosystems, pelagic food webs are characterized by high levels of (n-3)PUFAs derived ultimately from phytoplankton. Most information on phytoplanktonic fatty acids has been derived from laboratory-grown cultures. Although the lipid classes and fatty acids synthesised vary with environmental conditions and culture age, diatoms and dinoflagellates tend to be rich in 20:5(n-3) and 22:6(n-3) whilst Chlorophycae often have high levels of 18:4(n-3). In contrast, benthic macroalgae contain higher levels of (n-6)PUFA which is reflected in elevated levels of 20:4(n-6) in the polar lipids of benthic and inshore invertebrates (Sargent & Whittle 1981).

In general, C16 and C18(n-3) fatty acids found in phytoplankton are not very abundant in zooplankton. There is some evidence that these fatty acids can be converted by zooplankton to 20:5(n-3) and 22:6(n-3) (Morris *et al.* 1973). The extent to which zooplankton selectively retain or elongate and desaturate dietary fatty acids probably depends on species and environmental conditions.

Mature copepods, which form the major part of the diet of adult herring (Last 1989), contain wax esters consisting of fatty alcohols synthesised *de novo* esterified to fatty acids, largely of dietary origin (Sargent & Falk-Petersen 1988). The dietary fatty alcohols tend to be converted to the corresponding fatty acids by wax ester ingesting fish. As with the fatty acids derived directly from the zooplankton diet, these may then be stored as triacylglycerol, catabolized for energy or be incorporated into cell structure in the form of phospholipids.

The fate of dietary fatty acids in herbivorous fish such as post-metamorphosis menhaden has been less well studied.

The fatty acids of marine fish are characterized by very high (n-3)/(n-6)PUFA ratios. Large amounts of 22:6(n-3) and 20:5(n-3) are found but 22:5(n-3), 20:4(n-3) and 18:3(n-3) can occur, albeit at much lower levels. The saturate 16:0 is usually abundant with reduced levels of 18:0 occurring. Amongst the monoenes, 18:1 is usually the most abundant with less 16:1. The high levels of 22:1(n-11) and 20:1(n-9) found in the triacylglycerol of herring and similar zooplankton-consuming fish are derived from fatty alcohols found in wax ester rich copepods (Sargent *et al.* 1981).

1.3.4 The modification of dietary fatty acids by fish

Fish, like other vertebrates, are able to synthesise fatty acids *de novo* from glucose, pyruvate and acetate. The rates of lipogenesis are determined by the quantitative and qualitative balance of lipid, protein and carbohydrate in the diet as well as by environmental and species-specific factors (Greene & Selivonchick 1987, Wakil *et al.* 1983, Bloch & Vance 1977). The products of fatty acid synthesis are the saturated fatty acids, 14:0, 18:0 and especially 16:0. These products may be acted on by delta-9 desaturase to form the corresponding monoenes. Relatively large amounts of 18:1(n-9) may be synthesised *de novo* from 18:0 by this means. However, since fish, like other vertebrates do not possess the desaturases necessary to inter-convert (n-9), (n-6) and (n-3) series fatty acids, 18:2(n-6) and 18:3(n-3) must be supplied in the diet (see Appendix I).

1.3.5 Essential fatty acids in freshwater fish

When rats were reared on a fat free diet, pathological symptoms resulted (Burr & Burr 1929). Addition of oils containing 18:2(n-6) to the diet reversed these symptoms (Burr & Burr 1930). It is now well accepted that 18:2(n-6) is an essential nutrient in mammalian nutrition but an essential role for 18:3(n-3) has been hard to demonstrate till recently (Lamptey & Walker 1976). It is still unclear what the optimum (n-3)/(n-6) ratio is for human nutrition.

It is now known that it is the longer-chain, more unsaturated fatty acids which are necessary for normal metabolism. Most mammals are capable of elongating and desaturating 18C fatty acids to form these and so only 18:2(n-6) and 18:3(n-3) are essential in the diet. However, members of the cat family have a limited elongation/desaturation ability and must be supplied with pre-formed long-chain PUFAs (Rivers *et al.* 1975).

Early workers assumed that a similar situation to that in rats existed in fish. When rainbow trout were reared on diets deficient in PUFAs, the result was poor growth and pathological symptoms including fin erosion, heart myopathy and shock syndrome (Castell *et al.* 1972a). However, it was the addition of 1% 18:3(n-3) to the diet (by weight), rather than the addition of 18:2(n-6), which was most effective in preventing these symptoms. The reversal of the deficiency symptoms was correlated with changes in tissue fatty acids and histology (Castell *et al.* 1972b & 1972c). In fish reared on diets deficient in essential fatty acids, the level of 22:6(n-3) in the phospholipids decreased whilst 20:3(n-9), synthesised *de novo* from 18:1(n-9), accumulated. It was suggested that the ratio of these two fatty acids would provide a valid indicator of

essential fatty acid deficiency in trout. The presence of 18:3(n-3) in the diet inhibited the elongation and desaturation of 18:1(n-9). Since then, several studies using purified diets and radiolabelled fatty acids have shown that rainbow trout are capable of synthesising 22:6(n-3) and 20:5(n-3) from dietary 18:3(n-3) (Yamada *et al.* 1980, Kanazawa *et al.* 1979, Owen *et al.* 1975, Watanabe *et al.* 1974a).

Yu & Sinnhuber (1972) reared rainbow trout on a semi-purified diet supplemented with 18:3(n-3) or 22:6(n-3). Growth was best on the diet containing 1% additional 18:3(n-3) and was almost comparable on the diet containing 1% 22:6(n-3). However, Takeuchi & Watanabe (1976) found that addition of 0.5% or 1% of either 22:6(n-3) or a purified cuttlefish oil with a high long-chain (n-3)PUFA content to a defined diet improved growth, survival and feed conversion to a greater extent than adding 1% 18:3(n-3). Inclusion of the cuttlefish oil at 2% suppressed performance. It was noted that the best performance was on a diet containing 3% soybean oil + 2% pollack liver oil. Takeuchi & Watanabe (1977), investigating the active components in pollock liver oil, found that either 0.5% 20:5(n-3) or 0.5% 22:6(n-3) had a more powerful growth enhancing effect than 0.5% 18:3(n-3). The best gain however, was on a diet containing 0.25% of both 20:5(n-3) and 22:6(n-3). Unfortunately, in this trial they did not employ a pollock liver oil control diet.

Thus although freshwater fish, in general, can elongate and desaturate dietary 18:3(n-3), it is the longer chain (n-3)PUFAs which appear to exert the growth promoting effect. Supplying these fatty acids pre-formed in the diet is as, or probably more beneficial than supplying their precursor, 18:3(n-3).

Nutritional studies have shown that fatty acids of the (n-6) series appear to be required in low amounts or not at all by rainbow trout (Yu & Sinnhuber 1975). In trout fed 5% 18:2(n-6), reduced growth and increased mortality resulted (Yu & Sinnhuber 1976, 1975).

The majority of studies on the essential fatty acid requirements of freshwater fish have employed rainbow trout. However, it has been recently demonstrated that, although fingerling white fish *Coregonus lavaretus maraena* could convert dietary 18:3(n-3) to 22:6(n-3), supplementing the diet with 1% long-chain (n-3)PUFA was more effective in improving growth and feed efficiency and in reducing histological abnormalities than supplementation with 2% 18:3(n-3). This species is thus similar to rainbow trout in this respect (Watanabe *et al.* 1989a, Throngrod *et al.* 1989). Csengeri *et al.* (1979) studied the response of common carp to diets containing various levels of fatty acids and concluded that although the fish could elongate and desaturate 18:2(n-6) and 18:3(n-3), there was an upper dietary limit above which a greater rate of conversion did not occur. Farkas *et al.* (1977) working with the same species showed that dietary 18:2(n-6) was converted to 20:4(n-6) and 22:5(n-6) when the diet contained low levels of 18:3(n-3). Despite this, 20:3(n-9) also accumulated indicating that the anabolism of 20:4(n-6) and 22:5(n-6) was insufficient to counter the effects of the (n-3)PUFAs lost. Although growth data was not given, the implication was that carp, like trout require dietary (n-3) fatty acids.

A requirement by freshwater fish (n-3) fatty acids is by no means universal. Studies on the eel (*Anguilla japonica*) indicated that they require both (n-3) and (n-6) fatty acids (Takeuchi *et al.* 1980) whilst *Tilapia nilotica* (an omnivore) and *Tilapia zillii* (a euryhaline herbivore)

and appear to need (n-6) rather than (n-3) fatty acids (Takeuchi *et al.* 1983, Kanazawa *et al.* 1980). Some confusion appears to surround the requirements of the channel catfish, *Ictalurus punctatus* and more study is required (Satoh *et al.* 1989, Stickney & Hardy 1989). These differences between species may correlate with a number of factors such as geographical location, water temperature, feeding habits etc. More species will need to be examined under comparable experimental conditions before firm conclusions can be drawn.

1.3.6 Essential fatty acids in marine fish

Due to the high levels of (n-3) fatty acids found in marine fish, it seems probable that (n-3) fatty acids rather than (n-6) fatty acids will exert a greater growth promoting effect on marine fish. Bautista & De La Cruz (1988) demonstrated that 1% 18:3(n-3) improved the growth of fingerling milkfish (*Chanos chanos*) to a greater extent than 1% 18:2(n-6). In fish fed diets supplemented with 18:3(n-3), tissue levels of 20:5(n-3) were higher than in control fish but levels of 22:6(n-3) were reduced. The effect of including longer chain (n-3)PUFAs was not investigated.

Although the ability of rainbow trout to convert 18:2(n-6) and 18:3(n-3) to longer chain PUFAs has been confirmed many times, this ability is not found in all fish species. After rearing plaice (*Pleuronectes platessa*) on diets supplemented with 0.4% 18:3(n-3) or 18:2(n-6), Owen *et al.* (1972) found that triacylglycerides in the liver contained high levels of saturated and monounsaturated fatty acids but that the level of C20 and C22PUFAs had decreased. Owen *et al.* (1975) showed that when turbot were fed radiolabelled 18:1(n-9), 18:2(n-6) or 18:3(n-3) only small amounts were converted into longer, more highly unsaturated fatty acids. Cowey

et al. (1976a) demonstrated that the growth rate of juvenile turbot was not substantially improved by dietary supplementation with 18:2(n-6) or 18:3(n-3). However, the addition of cod-liver oil [high in long-chain (n-3) PUFAs] did bring about improved growth. Furthermore, the tissues of fish fed 18:2(n-6) and 18:3(n-3) contained elevated levels of 20:2(n-6) and 20:3(n-3) respectively. This suggested that chain elongation but not desaturation was occurring. Pathological changes in fish fed on diets low in (n-3)PUFA were recorded (Cowey *et al.* 1976b).

Studies using semi-purified diets have shown that the addition of a mixture of 20:5(n-3) and 22:6(n-3) stimulated growth in red sea bream whereas addition of 18:3(n-3) produced no improvement over the basal corn oil diet (Yone & Fujii 1975). This again indicated that it was the long-chain (n-3)PUFAs which exert a biological effect.

It has been postulated that in general, marine fish, unlike freshwater fish, have lost much of the reduced ability to convert 18:2(n-6) and 18:3(n-3) to longer chain, more highly unsaturated PUFAs. This may reflect the greater abundance of long-chain (n-3)PUFAs in the marine food-web. Brackish water fish appear to have an intermediate ability whilst the situation for species which move between fresh and seawater is unclear. Studies using radiolabelled fatty acids have confirmed the above trend for Japanese eel, European eel (*Anguilla anguilla*), red sea bream, rockfish (*Sebastiscus marmoratus*), globefish (*Fug rubipes*), black sea bream (*Mylio macrocephalus*), opaleye (*Girella nigricans*) and stripped mullet (*Mugil cephalus*) (Kissil 1987, Yamada 1980, Kanazawa *et al.* 1979).

1.3.7 Requirement for dietary 22:6(n-3) by fish

Little work has been carried out on the ability of fish to convert 20:5(n-3) to 22:6(n-3) since the oils commonly used in dietary studies contain both fatty acids. Using specially prepared oils, Bell *et al.* (1985a) showed that turbot fed on diets with high 20:5(n-3)/22:6(n-3) ratios exhibited poor survival and reduced tissue levels of 22:6(n-3). This suggested that turbot require 22:6(n-3) as well as 20:5(n-3) in their diet.

The growth and survival of juvenile striped jack, *Pseudocaranx dentex* was improved slightly by the addition of 1.8% 20:5(n-3) to the diet, but was improved considerably by the addition of 1.8% 22:6(n-3). Supplementation with 0.9% of each fatty acid produced an intermediate improvement. From body composition data, it is likely that 22:6(n-3) would not be suitable as the sole dietary PUFA over a longer rearing period. A certain level of both 22:6(n-3) and 20:5(n-3) is probably required in the diet (Watanabe *et al.* 1989b).

1.3.8 Retro-conversion of 22:6(n-3) to 20:5(n-3) by fish

The ability of fish to retro-convert 22:6(n-3) to 20:5(n-3) has been considered in only a few studies. No evidence for significant production of 20:5(n-3) from dietary 22:6(n-3) was found in juvenile striped jack (Watanabe *et al.* 1989) or in rainbow trout (Yu & Sinnhuber 1972). Similarly, Tocher *et al.* (1989) found no evidence for efficient retro-conversion of 22:6(n-3) by turbot fin or rainbow trout gonad cells in tissue culture.

1.3.9 Tissue culture studies

The use of tissue culture to investigate the bioconversion of fatty acids by fish is a relatively new development although the cell lines have been established for some time (up to 30 years). Tocher *et al.* (1989) investigated the effects of supplementing the foetal calf serum media used to culture turbot fin and rainbow trout gonad cells with different fish oil concentrates and fatty acids. When 18:3(n-3) was added, the trout cells converted a high percentage of it to 20:5(n-3) but only a small increase in 22:6(n-3) was observed. In contrast, the turbot cells converted a high percentage of it to 18:4(n-3) but only a very small amount to 20:4(n-3) and 20:5(n-3). No increase in 22:6(n-3) was noted. This implied that delta-6 desaturase was active but C18-C20 elongase activity was limited (see Appendix I for definitions). Turbot cells did show some ability to elongate and desaturate supplemented 20:5(n-3) to 22:5(n-3) and 22:6(n-3), but the addition of 22:6(n-3) itself to the media produced the greatest increase in concentration of this fatty acid in the cells. This confirmed the pattern predicted from the earlier rearing trials on juvenile trout and turbot except for the low delta-4 desaturase activity noted in the trout gonad cell cultures.

Despite these results, doubts exist over the integrity of the cell lines after several decades of culturing, especially as mammalian serum is used in the media. The cultured cells have lower (n-3)PUFA levels than are found in wild fish and increased levels of 20:3(n-9) or 18:2(n-9) (rainbow trout gonad and turbot fin respectively) which may indicate some degree of essential fatty acid deficiency (Tocher *et al.* 1988). The effect of these long-term distortions of the natural fatty acid patterns on the desaturase and elongation enzyme activities of these cells has not been established, although cell growth and division does not appear to be

affected. It must be noted that the cultivation temperature of 22°C is higher than that which would be experienced by these tissues in the wild.

1.4 The structural and metabolic roles of fatty acids

In order to understand why certain substances are essential to an organism, it is necessary to explain their roles. For the PUFAs it is further necessary to explain why only molecules of specific n- series (see Appendix I) and chain lengths appear to be capable of fulfilling these functions. Within the teleosts, the subject has been reviewed by Sargent *et al.* (1989) and Bell *et al.* (1986).

1.4.1 The role of fatty acids in cell membranes

The most obvious function of fatty acids are as major components of cell membranes. Fish biomembranes contain between 25% and 80% lipid, predominantly as phospholipid with varying amounts of cholesterol (Henderson & Tocher 1987). Early studies on the role of fatty acids concentrated on the physical properties of purified lipids. Thus, it is frequently stated that PUFAs, because of their low phase-transition temperature, maintain the fluidity of biomembranes. However, this does not explain why highly unsaturated fatty acids are essential since all PUFAs are fluid at temperatures well below those encountered in biological systems [e.g. 18:3(n-3) -10°C]. It is worth noting that the melting point of 22:6(n-3) at -44.4°C is actually higher than that of 20:5(n-3) at -54.4°C. Furthermore, human retina is highly enriched in 22:6(n-3) but the physiological temperature is 37°C. It must be borne in mind that membrane fluidity is the result of complex interactions between lipids, proteins and cholesterol and consideration of the physical

properties of purified components may be misleading. Fish, being poikilotherms, are especially useful models for studying the changes which occur in biomembranes with environmental temperature (Hazel 1984).

Lipids may fulfil multiple functions in biomembranes e.g. many of the proteins present have enzymic properties which may be influenced by lipids (Sandermann 1978).

The use of natural biomembranes such as ghost erythrocytes and models such as liposomes (Gier 1988) along with computer modelling (Applegate & Glomset 1986, Chapman 1982) is yielding valuable information but the precise roles of specific fatty acids and lipid classes in biomembranes remain unclear.

1.4.2 The role of (n-3)PUFAs in neural tissue

High levels of (n-3)PUFAs are present in mammalian retina, cerebral cortex, testis and sperm (Neuringer *et al.* 1988). The levels of (n-3)PUFAs in these tissues was highly conserved when rats were fed diets containing linoleic acid as the only lipid source (Tinoco *et al.* 1971). However, the fatty acid composition of neural tissue in foetal rats can be altered by feeding the pregnant dams essential fatty acid-deficient diets (Samulski & Walker 1982). There is evidence that this reduced the visual acuity, and possibly learning ability of the progeny (Lamptey & Walker 1976). Dietary (n-3)PUFA deficiency has been linked with visual impairment In rhesus monkeys (Neuringer *et al.* 1984). This research has serious implications for human infant nutrition, especially for infants fed on formulated feeds that currently lack long-chain (n-3)PUFAs.

1.4.3 The role of 22:6(n-3) in fish

The roles of 22:6(n-3) in mammals have been reviewed by Salem *et al.* (1986). In both freshwater and marine fish, elevated levels of 22:6(n-3) are found in the brain and especially in the retina (Tocher & Harvie 1988). The recent discovery of relatively high levels of di-22:6(n-3) molecular species in phosphatidylethanolamine and phosphatidylserine from trout brain, and also in phosphatidylcholine from trout retina, emphasises the importance of this fatty acid (Bell & Tocher 1989). As in terrestrial animals (n-3)PUFAs concentrated in neural tissues are highly conserved under starvation conditions (Bell & Tocher 1989, Tocher & Harvie 1988, Lamptey & Walker 1976). As yet the precise role of 22:6(n-3) in these tissues is unknown. Bell *et al.* (1985a) reported that mortalities in turbot fed on a diet high in 20:5(n-3) could be reduced by restoring the dietary 20:5(n-3)/22:6(n-3) ratio to 2.2. It is unclear from the paper whether this also reduced the level of gill pathologies described in section 1.4.5.

1.4.4 The role of 20:5(n-3) in fish

Kanazawa *et al.* (1982) showed that radiolabelled 20:5(n-3) was preferentially incorporated into the gall bladder, swim bladder and alimentary tract 24 h after injection into red sea bream. Moderately high levels were recovered from the liver and gills. Red sea bream reared on diets deficient in (n-3)PUFA frequently fail to show swim bladder inflation suggesting 20:5(n-3) is important for the development of this organ.

Apart from the study above there are few reports indicating a specific role for this fatty acid.

1.4.3 The role of 22:6(n-3) in fish

The roles of 22:6(n-3) in mammals have been reviewed by Salem *et al.* (1986). In both freshwater and marine fish, elevated levels of 22:6(n-3) are found in the brain and especially in the retina (Tocher & Harvie 1988). The recent discovery of relatively high levels of di-22:6(n-3) molecular species in phosphatidylethanolamine and phosphatidylserine from trout brain, and also in phosphatidylcholine from trout retina, emphasises the importance of this fatty acid (Bell & Tocher 1989). As in terrestrial animals (n-3)PUFAs concentrated in neural tissues are highly conserved under starvation conditions (Bell & Tocher 1989, Tocher & Harvie 1988, Lamptey & Walker 1976). As yet the precise role of 22:6(n-3) in these tissues is unknown. Bell *et al.* (1985a) reported that mortalities in turbot fed on a diet high in 20:5(n-3) could be reduced by restoring the dietary 20:5(n-3)/22:6(n-3) ratio to 2.2. It is unclear from the paper whether this also reduced the level of gill pathologies described in section 1.4.5.

1.4.4 The role of 20:5(n-3) in fish

Kanazawa *et al.* (1982) showed that radiolabelled 20:5(n-3) was preferentially incorporated into the gall bladder, swim bladder and alimentary tract 24 h after injection into red sea bream. Moderately high levels were recovered from the liver and gills. Red sea bream reared on diets deficient in (n-3)PUFA frequently fail to show swim bladder inflation suggesting 20:5(n-3) is important for the development of this organ.

Apart from the study above there are few reports indicating a specific role for this fatty acid.

1.4.5 The role of (n-6) fatty acids in fish

20:4(n-6) has been recorded at high levels in phosphatidylinositol from turbot gill tissue (Bell *et al.* 1985b), the salt-secreting epithelia of dogfish (*Scyliorhinus canicula*) and cod (Bell *et al.* 1983), and the ripe roes of several fish species (Tocher & Sargent 1984). In the gills of seawater eels, the rate of salt secretion has been shown to be related to hormone mediated changes in phosphatidylinositol turnover (Girrard *et al.* 1977). This is similar to the mammalian situation where phosphatidylinositol plays a key role in the transduction of hormone signals across the biomembrane.

Since phosphatidylinositol occurs in minor amounts in total fish lipid, it is likely that 20:4(n-6) is an essential fatty acid but is required only in trace amounts. Bell *et al.* (1985a) found gross pathologies in the gill filaments of turbot reared on special diets totally deficient in (n-6)PUFA. Only fish fed a diet containing fish oil [2.5% (n-3)PUFA and 0.23% (n-6)PUFA] showed good growth and survival. Florida pompano (*Trachinotus carolinus*) developed similar gill pathologies and atrophied operculae when reared on a lipid deficient diet, whereas fish fed on diets containing menhaden oil grew normally (Williams *et al.* 1985). Although Williams *et al.* attributed this to (n-3)PUFAs in the menhaden oil, the result could have been due to (n-6)PUFAs also in the oil. It may be noted that all fish oils contain (n-6)PUFA, albeit in small amounts and many compounds routinely used as constituents of experimental diets, such as starch and casein, unless highly purified also contain traces of (n-6)PUFAs.

Since marine fish generally contain very low levels of (n-6) fatty acids (in total lipid) their dietary requirements for PUFAs of this series

have received little attention. However, the possible roles for (n-6)PUFA outlined above imply that this area should receive more attention (Henderson *et al.* 1985).

1.4.6 The formation of oxygenated eicosanoids

The term eicosanoids covers any C20 fatty acid including the prostaglandins, thromboxanes and leukotrienes. These are cyclopentanoic acids derived by oxygenation of C20 PUFAs, especially 20:4(n-6). They are hormone-like substances which act near the site of synthesis and are effective at extremely low concentrations. In mammalian systems these compounds have been implicated in a wide range of physiological functions (Needleman *et al.* 1986). Cyclo-oxygenase from rainbow trout thrombocytes will act upon 20:5(n-3) to produce thromboxin (Henderson & Tocher 1987). However, the available evidence suggests that, despite the preponderance of (n-3)PUFA in tissue lipids, 20:4(n-6) is the preferred substrate for the cyclo-oxygenase pathways in fish. This has been demonstrated in turbot gill tissue by Henderson *et al.* (1985). The conversion of 20:5(n-3) to a hydroxylated derivative, which may have similar effects to prostaglandins, probably occurs in trout gills (German *et al.* 1983). In teleosts, spawning and related behaviour are affected by prostaglandins derived from 20:4(n-6), whilst gill homogenates appear to be very active for *in vitro* prostaglandin synthesis suggesting a role for the eicosanoids in the control of osmoregulation (Henderson *et al.* 1985).

1.5 Lipid nutrition in fish larvae

Most of the current knowledge of the fatty acid requirements of marine fish has been derived from studies on juveniles and adults. This is because larval marine fish are generally unwilling to ingest semi-purified or purified diets. However, it has been shown that the quantitative and possibly qualitative nutrient requirements of fish larvae can be different from those of the juveniles and adults (Izquierdo *et al.* 1989, Dabrowski 1986).

Cultivation of freshwater fish e.g. trout, carp and salmon has been established for some time (although salmon are reared at sea, they have freshwater larval and juvenile stages). The rearing of cold-water marine species has not developed so rapidly but interest in farming cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*) and wolf fish (*Anarhichas lupus*) is growing (Tilseth 1990, Kirk 1987). This is due to declining wild stocks, increasing cost of wild-caught fish and the benefits to human health of consuming food high in (n-3)PUFAs (Simopolous *et al.* 1986). With many of the marine fish species cultured experimentally, major problems in larval rearing have been encountered. These difficulties are frequently nutritional in origin (Tilseth 1990).

From a research view, nutritional deficiencies are liable to be manifested more rapidly in the fast growing larval stage; the larvae generally have a simple body structure, frequently with little pigmentation, making them suitable subjects for manipulative experimental work. Finally, there is considerable debate over the relative importance of starvation and predation in controlling the recruitment of fish to adult stocks in the wild (Purcell & Grover 1990, Houde 1987, May 1974). Nutritional experiments can contribute to this debate by defining the nutrient requirments of larvae in quantitative and qualitative terms.

A number of techniques have been used to study lipid nutrition in fish larvae. Several studies have examined the changes which occur in under starvation conditions. This has been done for the larvae of herring, *Clupea harengus* (Tocher *et al.* 1985a, Tocher *et al.* 1985b), cod (Fraser *et al.* 1988), gilthead sea bream (*Sparus aurata*) (Koven *et al.* 1989) and red sea bream (Tandler *et al.* 1989). These studies indicated that, under starvation conditions, levels of 20:5(n-3) and especially 22:6(n-3) were conserved for as long as possible at the expense of shorter chain fatty acids and those of the (n-9) and (n-6) series. These trends were taken to indicate the essential nature of the (n-3)PUFAs.

The lipid requirements of marine fish larvae have also been investigated using live food chains, artificial diets and radiotracers. The degrees of success and the information derived from these techniques is discussed in the introductions to chapters 3, 4 and 5 respectively.

1.6 Aims of this study

The aims of this study were to develop a method for investigating the essentiality of (n-3)PUFAs in the diet of a cold-water marine fish larva. Ideally, the method would allow both long-term rearing on a defined diet and the incorporation of radiolabelled tracers.

Herring were chosen for study since the larvae are relatively large (compared with cod or turbot, Russell 1976), the protocol for egg incubation and larval rearing is established (Blaxter 1968a), the eggs were obtainable and studies on their lipid nutrition in mesocosms had been previously undertaken (Fraser 1986).

The hypothesis investigated was that herring larvae require a dietary source of 22:6(n-3) and possibly 20:5(n-3) for normal development due to a limited ability to elongate/desaturate 18:3(n-3) to 20:5(n-3) and 20:5(n-3) to 22:6(n-3).

Chapter 2 Materials and methods

This section details materials and methods used throughout the ensuing chapters. Specialized techniques used for specific sections of work are described in the relevant chapters.

2.1 Source of herring larvae

Successful artificial spawning of the clupeoids, Atlantic menhaden and northern anchovy (*Engraulis mordax*), has been achieved using injections of pituitary hormone (Hettler 1981, Leong 1971) but has not been attempted with herring (Blaxter & Hunter 1982). The supply of larvae for research is thus dependant on the capture of mature male and female fish by trawling at known spawning sites. This was carried out at the times and areas detailed in subsequent chapters. Ripe fish were readily identified by the release of eggs and sperm when gentle pressure was applied to the abdominal wall. Selected fish were killed and the ovaries and testes excised and stored in chilled, clean glass jars (Blaxter 1968a). Fertilization was carried out in port within 10 h. The eggs were spread onto glass plates in a shallow tray of clean seawater. They were then fertilized for 15 min using sperm which had been obtained by squeezing the excised testes through a 250 µm mesh. The plates with attached eggs were then washed thoroughly with clean seawater ånd assembled into stacks for transport to the laboratory.

Incubation was carried out as detailed in subsequent chapters. The use of antibiotics was not found to be necessary for successful hatching.

2.2 Rearing of the rotifer, *Brachionus plicatilis*

The rotifer is used as a live feed for herring larvae, especially in the early stages of their growth (Blaxter & Hunter 1982). Rotifers had been obtained from a stock maintained at the DAFS Marine Research Unit, Poolewe. They were cultured at 27°C in 2 µm filtered seawater. For the spring 1987 and 1988 experiments the rotifers were fed on activated baker's yeast (Allinson, Queens Mills, Aire St, Castleford, Yorkshire) at a rate of 2 g x 10^6 rotifers day^{-1} and supplemented with algae [*Nannochloropsis occulata* Hubberd (CCAP 849\1)].

For the autumn 1988 and spring 1989 experiments, rotifers were fed algae alone. Ten litres of rotifer culture were filtered daily through two 68 µm meshes, the rotifers returned and the culture volume restored with fresh algal suspension.

Daily counts were made on 1 ml subsamples. For feeding to larvae the relevant volume of culture was passed through two 68 µm mesh nets. The entrapped rotifers were then washed, resuspended in clean seawater and distributed to the fish.

2.3 Algal culture

Prior to the autumn 1988 experiments, *Nannochloropsis occulata* was cultured in 5 l glass carboys. Aquarium seawater filtered to 2 µm and enriched with Guillard's F/2 medium (Thompson *et al.* 1988) was inoculated with algae from a sterile starter culture and aerated with 0.22 µm filtered air. Illumination was provided by a bank of fluorescent tubes. The algae were ready to use in 2 weeks.

From autumn 1988 onwards, larger volumes of algae were reared in 80 l plastic bags. These were managed in a similar manner to the carboy

cultures except that the water was bubbled daily with CO_2 until the pH was below 7.0.

Sterile stock cultures were maintained as outlined by McVey (1983).

2.4 Hatching of the brine shrimp, *Artemia*

The use of the brine shrimp for rearing fish larvae has been reviewed by Léger *et al.* (1986). Herring larvae may be reared for several weeks on rotifers but after this time the rotifers are too small to promote a good growth rate in the fish larvae. The rotifers may be replaced with naturally caught zooplankton, filtered to the required size range or with nauplii of the brine shrimp (Blaxter & Hunter 1982). Great Salt Lake cysts were supplied by Artemia Systems (Ghent, Belgium) and San Francisco Bay cysts by San Francisco Bay Brand Inc. (Newark, California, U.S.A.). The cysts were hatched in 0.22 µm filtered seawater for 24 hours at 27°C. After this time the aeration was turned off and the shrimps attracted to a light at the base of the hopper. Unhatched cysts and debris floated to the water surface. Shrimps were caught by draining the culture through a 150 µm mesh followed by washing and resuspension in clean seawater. Numbers were determined by rapidly heating a sample to kill the shrimps and counting subsamples. Fresh hatches were set up daily.

2.4.1 Discussion of *Artemia* hatching techniques

Reasonable hatching rates of the cysts were obtained with the system described above. It is possible to increase the hatching success by dissolution of the cyst walls with hypochlorite (Bruggeman *et al.* 1979). This process has the added advantage of eliminating the need to separate nauplii from the cyst cases and ensures that the nauplii are sterile

(Sorgeloos *et al.* 1983). In addition, unhatched, decapsulated *Artemia* have proved acceptable when fed directly to the larvae of milkfish (De los Santos *et al.* 1980) and the African catfish, *Clarias gariepinus* (Verreth *et al.* 1987). Decapsulated *Artemia* have however been reported to be poorly digested by the larvae of sole (*Solea solea*) leading to a low growth rate (Léger *et al.* 1986). Cyst decapsulation was not employed in the present work.

2.5 Sampling of larvae from rearing trials

A variety of techniques were used and are described in the relevant chapters.

2.6 Determination of standard lengths and dry weights of larvae

Larvae were removed from tank samples and anaesthetized in a seawater bath containing 0.05% (w/v) MS-222 (Sandoz). The standard lengths of individual larvae were measured directly under low power magnification on a calibrated slide (Graticules Ltd., Tonbridge, England) to ± 0.1 mm. The development stage was recorded as defined by Doyle (1977).

The larva was then transferred, via three washes of distilled water, onto numbered Teflon discs. The larvae were freeze dried overnight and subsequently weighed using a Perkin-Elmer microbalance (model AD-27) to ± 0.001 mg.

2.7 Extraction of lipid for biochemical analyses

2.7.1 Method of lipid extraction

Lipids were extracted from frozen, solvent-stored or freeze-dried material by Folch-Lees extraction as modified by Ways & Hanahan and described by Christie (Christie 1982, Ways & Hanahan 1964, Folch *et al.* 1957).

All solvents were HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Scotland). Butylated hydroxytoluene (BHT) was obtained from the Sigma Chemical Co. (Poole, Dorset, England) whilst other chemicals were supplied by BDH Chemicals Ltd. (Poole, Dorset, England) and were of 'Analar' grade.

Samples were suspended in 10 ml of methanol and homogenized on ice for 1 min using a Teflon-in-glass homogenizer (TriR model K43). The material was then homogenized for a further 2 min after the addition of 20 ml of chloroform. Solutions were filtered through pre-washed Whatman No 1 filter papers (Whatman, England) and the residues homogenized in 10 ml of chloroform:methanol (2:1 by vol) for 1 min. These were also filtered and 10 ml of 0.88% KCl (aq) added to the combined filtrates. After mixing, phase separation was obtained by centrifuging for 1 min at 2000 rpm. The upper phases were discarded and the organic phases dried under rotary vacuum. The lipids were transferred in chloroform:methanol (2:1 by vol) into pre-weighed storage vials (Pierce Ltd., Cambridge, England), the solvent removed under oxygen-free nitrogen stream and the samples desiccated overnight under vacuum. The lipid yields were determined gravimetrically.

Extracted lipids were stored prior to analysis in chloroform:methanol (2:1 by vol) under oxygen-free nitrogen at -20°C.

Antioxidants were not included prior to lipid class determination by Iatroscan as the antioxidant may interfere with the analysis.

2.7.2 Discussion of the lipid extraction methodology used

The Folch-Lees was originally developed for the extraction of lipids from brain tissue. Although the method has become the standard technique for extraction of lipid from a wide variety of material, it is not always suitable. Gangliosides and a fraction of glycolipids will be lost to the aqueous phase whilst lipoproteins may not be extracted. The procedure may also fail to extract lipids containing very long-chain fatty acids (Rézanka 1989). This method does not extract lipids efficiently from plant tissue but is generally 95-99% efficient for animal tissue samples of less than 1 g wet weight (Christie 1982). The method is suitable for use with herring larvae (Fraser 1986) providing precautions are taken to prevent the action of the active lipase enzymes present (see Chapter 4).

2.8 Thin-layer chromatography of lipid samples

2.8.1 Methods used for thin-layer chromatography

Chromatography was carried out on 20 x 20 cm thin-layer chromatography (TLC) or on 10 x 10 cm high performance thin-layer chromatography (HPTLC) pre-prepared silica Gel G plates (E. Merck, Darmstadlt, W. Germany). Plates were cleaned prior to use by developing fully in one dimension in chloroform:methanol (2:1 by vol). After solvent removal under vacuum, a 1 cm band of silica was scraped from the upper edge of the plate. The plate was then rotated through 90°, activated for 15 min at 110°C and samples spotted 1 cm from the lower edge.

Neutral lipids were separated in one dimension using hexane:ether:acetic acid (40:9:1 by vol) as described by Christie (1982).

Polar lipids were separated in one dimension using either propanol:chloroform:methanol:0.25% KCl (aq) (25:25:25:10:9 by vol) as described by Vitiello & Zanetta (1978) or chloroform:ethanol:water: triethylamine (30:35:6:35 by vol) as described by Leray & Pelletier (1987).

Separation of polar and neutral lipid classes from a single sample was achieved by developing the plates half-way in polar solvent. After drying under vacuum, the plate was developed fully in the neutral solvent system.

Separated classes were visualized under ultra-violet light after spraying with 2',7'-dichlorofluoroscein (0.1% w/v in 95% methanol containing 0.1% w/v BHT). When a permanent record was required and further analysis on the lipid classes was not planned, plates were charred for 20 min at 160°C after spraying with 3% cupric acetate in 8% orthophosphoric acid (Christie 1982).

2.8.2 Discussion of thin layer chromatography methods used

The separation of the neutral lipid classes in herring larvae presented no difficulties and the solvent system described above is recommended.

The separation of polar classes was considerably more difficult. The system proposed by Leray & Pelletier provided good separation of all the polar classes found in herring larvae. Unfortunately it was difficult to completely remove the solvent from the plates. The binder was affected and this led to cracking of the silica when the classes were detected by charring. In addition, residual solvent appeared to interfere with

subsequent neutral solvent separation in the two stage, one dimensional total separation of classes procedure. For these reasons the solvent system described by Vitiello & Zanetta is recommended. Good separation of phosphatidylethanolamine, cardiolipin, phosphatidylcholine and lysophosphatidylcholine was obtained. However, phosphatidylinositol and phosphatidylserine tended to run together whilst sphingomyelin occasionally failed to resolve from phosphatidylcholine. The solvent was however easily removed from the plate under vacuum and subsequent separation of the neutral lipids was successful.

2.9 Quantification of lipid classes

The quantification of lipid classes was carried out using the Iatroscan thin-layer chromatography coupled with flame-ionization detection system.

2.9.1 Method of operation of the Iatroscan TLC-FID system

The system was run as described by Fraser *et al.* (1985) with some modifications. An Iatroscan TH10 Mark II analyser (Iatron Laboratories, Tokyo, Japan) was linked to a Hewlett Packard 3390A recording integrator. The flame ionization detector was operated with an air flow of 2000 ml min^{-1} from an air pump. Hydrogen pressure was 0.71 kg cm^{-2}.

SII chromarods were stored in a water-saturated atmosphere between analyses and activated by passing them through the flame-ionization detector prior to use. Lipid samples were dissolved in chloroform:methanol (2:1 by vol) at 60 mg ml^{-1}. Each rod was spotted with 60 µg of lipid dispensed from a Hamilton microsyringe (Hamilton, Bonadue, Switzerland). Rods for neutral lipid class separation were developed in a

lined chromatography tank containing 1,2 dichloroethane:chloroform:acetic acid (92:8:0.1 by vol). Polar lipid classes were separated on a separate set of rods using chloroform:methanol:water (70:35:3.5 by vol). The chromatography tanks were suspended in a water bath at 25°C. When the solvent front had moved 10 cm from the origin, the rods were removed and dried at 100°C for 2.5 min. The rods were scanned after being allowed to stand at room temperature for a further 10 min.

Calibration curves were constructed from a composite standard containing cholesteryl oleate, triacylglycerol, cholesterol, oleic acid, phosphatidylcholine and phosphatidylethanolamine. All standards were obtained from the Sigma Chemical Co. Ltd. except for triacylglycerol which was purified from crude fish oil (Marinol). Standards were checked for purity by thin-layer chromatography prior to use.

The percentage class distribution was computed using both the polar and neutral separations. Estimation of the total amount of polar lipid from the neutral separation leads to overestimation of the phospholipids, due to the presence of non-lipid material at the origin. The relative amounts of total neutral lipid and phospholipids were thus calculated from the polar separations. Results were analysed using software developed by C. Fox and P. Hodgson (see Appendix II).

2.9.2 Results

Neutral lipid classes were well separated by the solvent system used but the polar lipid classes often failed to separate in the manner described by Fraser *et al.* (1985). Phosphatidylinositol and phosphatidylserine frequently ran together. Sphingomyelin also tended to

run under phosphatidylcholine. Analysis by HPTLC had indicated that a small amount of cardiolipin was present in samples of herring lipid. This class could not be resolved on the Iatroscan system.

The calibration curves used for spring 1989 samples are presented in Figs 2-1 and 2-2.

2.9.3 Discussion of the Iatroscan TLC-FID system

The results from the comparison of lipid class quantification using the Iatroscan system and using gravimetric-colorimetric methods by Fraser *et al.* (1985) suggested that the Iatroscan system under quantified phosphatidylethanolamine and slightly over quantified phosphatidylcholine. The calibration curves shown in Figs 2-1 and 2-2 confirmed this. It was shown that there was up to 30% error in the quantification of the two major polar lipids in herring larvae if standards containing only phosphatidylcholine were used. Ideally composite standards should be prepared containing all the polar classes to be analysed. Since this is not practical it was assumed that phosphatidylinositol, phosphatidylserine, sphingomyelin and lysophosphatidylcholine had the same detector response as phosphatidylethanolamine.

Studies have indicated that the degree of unsaturation and the chain lengths of the fatty acids in triacylglycerol can affect the separation characteristics of this class using the Iatroscan system (Oshima *et al.* 1987, Kramer *et al.* 1986). This in turn may affect peak integration. Although no significant difference in detector response was noted when comparing tripalmitin with triacylglycerol purified from the

natural fish oil, Marinol (Fraser *et al.* 1985), this potential error was minimized in this study by using a triacylglycerol derived from Marinol for the construction of the calibration curves.

Although the Iatroscan system allows rapid quantification of samples, it does incorporate several disadvantages. Relatively large amounts of lipid were needed (120 µg sample^{-1}) whereas complete analysis of a sample containing 20–30 µg can be carried out by HPTLC. The separation of polar lipid classes with the Iatroscan system was difficult and several classes failed to resolve. The lipid samples are destroyed by the flame-ionization detector as opposed to HPTLC where a permanent record is produced, which can be rescanned if needed. Finally, there appear to remain problems with the calibration of the Iatroscan system. It is probably for these reasons that this method has not lived up to its early promise (Ackman 1981) and in our laboratory recent work has returned to using HPTLC combined with densitometry (Olsen & Henderson 1989).

2.10 Gas-liquid chromatography (GLC)

This technique is now so well established as to require little discussion. Lipid samples were converted to methyl esters by acid catalysed transmethylation as described by Christie (1982). Samples of dry lipid were dissolved in 1 ml of toluene and sealed under oxygen-free nitrogen after the addition of 2 ml of 1% sulphuric acid in methanol. Catalysis was carried out overnight at 50°C. The reaction was terminated, after cooling by the addition of 2 ml of distilled water. The solution was washed with 5 ml hexane:ether (1:1 by vol) containing 0.01% BHT (w/v) and, after centrifuging for 1 min at 2000 rpm, the upper

organic phase was removed to a clean tube. The lower phase was then re-extracted using 5 ml hexane and the organic phases combined. The pooled organic phases were washed with 4 ml of 2% $KHCO_3$ (aq w/v) to remove 2',7'-dichlorofluoroscein. The methyl esters were concentrated by solvent evaporation under oxygen-free nitrogen stream and purified using NH_2 Sep-pak columns (Analytichem Int., Harbor City, California, U.S.A.). The crude methyl esters were applied to the column dissolved in 1 ml of hexane. Elution was with 4 ml of hexane and the columns were reactivated by washing with 4 ml of 2% acetic acid in diethyl ether followed by 8 ml hexane. Following concentration under oxygen-free nitrogen stream, methyl esters were stored at an estimated 3 mg ml^{-1} (assuming fatty acids comprise 60% of the original lipid) in hexane containing 0.01% BHT (w/v) at -20°C.

Methyl esters of specific lipid classes were prepared by direct transmethylation on silica after separation of total lipid by TLC. All classes except wax esters and sterol esters can be treated in this way (Christie 1972).

Methyl esters were separated using a Packard 436 gas chromatograph equipped with a CP wax 51 column (50 m by 0.32 mm). Gas pressures were 150 KPa for hydrogen and 200 KPa for nitrogen and air. The injection temperature was 100°C and the initial oven temperature 50°C. This was increased after 0.1 min to reach 235°C after 15 min and the remainder of the run was isothermal. Runs were terminated after 45 min. Data was collected using a Shimadzu C-R3A integrator. Peaks were identified by comparison with known standards and the results computed using software developed by C. Fox (see Appendix III).

Samples of methyl esters after autumn 1987 were prepared with 23:0 fatty acid as an internal standard. This fatty acid is not found naturally in herring larvae. The 23:0 fatty acid was added at a level of 10% (of the lipid sample by weight) prior to the addition of the transmethylating reagents. The advantage of including an internal standard is that quantification of the absolute amounts of fatty acids in the lipid sample is possible. Non-fatty acid material (which contributes to the lipid mass but is not converted to methyl esters) includes cholesterol and backbone molecules such as glycerol. The computations involved are more fully explained in Appendix III.

2.11 Separation of radioactively labelled methyl esters

Radioactively labelled methyl esters were analysed by argentation TLC (Christie 1982) or by packed column GLC (Olsen *et al.* 1990). The former technique separates the esters in terms of the numbers of double bonds in the fatty acids whilst the latter technique separates the sample by individual fatty acids.

2.11.1 Argentation TLC

All argentation TLC work was carried out under subdued lighting. 20 x 20 cm TLC plates were precleaned as described in section 2.8.1. Each plate was then sprayed with silver nitrate (2 g dissolved in 20 ml acetonitrile) then dried at 110°C for 30 min.

Radiolabelled samples were loaded alongside unlabelled standards (20 mg ml^{-1} Marinol:linseed oil:corn oil methyl esters mixed in the proportions 12:1:1 by vol). The plates were developed to 6 cm from the origin using toluene:ethyl acetate (9:1 by vol). After drying under vacuum

for 15 min, the plates were fully developed using ethyl ether:acetic acid (9:1 by vol).

Areas of radioactivity were identified by autoradiography and their locations compared against the unlabelled standard. The distribution of radioactivity was determined by scraping each sample track in 0.5 cm bands. The fractions were collected into scintillation vials and 10 ml of Optifluor (Canberra-Packard Instruments, Berks, U.K.) added. Levels of radioactivity were then assessed using a TriCarb 2000CA liquid scintillation counter (Canberra-Packard Instruments). The presence of silver nitrate in the samples leads to a high quench which was corrected for by the construction of quench calibration curves.

2.11.2 Packed column GLC

Radioactive fatty acid methyl esters were mixed with 20 µl of unlabelled standard (described above). Samples (5 µl) were separated using a Pye Unicam 104 gas chromatograph equipped with a 2 m x 4 mm column packed with 10% CP-Wax 51 coated on chromosorb 100-120 (Chrompack) using nitrogen as the carrier gas. The oven temperature remained at 220°C for 15 min following sample injection and then rose at 3°C min^{-1} to reach 250°C, the remainder of the run being isothermal. The column effluent was split prior to flame ionization detection allowing 90% to be trapped on Gilson Pipetman safety filters saturated with Optifluor scintillant (Packard). The radioactivity of filters containing fractions corresponding to identifiable components were determined by liquid scintillation spectrophotometry. Results were corrected for background counts using samples collected from the gas chromatograph over known time periods whilst running the unlabelled standard methyl ester mix.

2.11.3 Discussion

Although packed column GLC yields more information, argentation TLC is a more rapid technique. However, the separation of fatty acids with more than four double bonds tends to be poor. It was found that carry over of radioactivity between peaks tended to occur with the Pye 104. Thus neither of the techniques used to separate radiolabelled fatty acids was ideal.

Fig 2-1

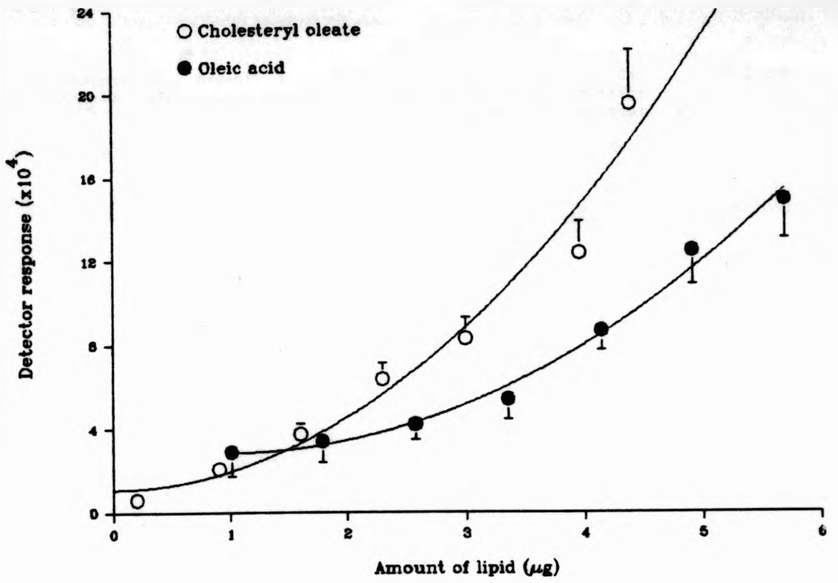
Calibration curves for cholesteryl oleate and oleic acid relating the weight of lipid to the peak area of the ionization detector response for the Iatroscan system

Each point is the mean of ten separate rods; unless otherwise shown by error bars, the standard deviation of the mean is less than the symbol size for each point

2nd order polynomial equations fitted to data

Cholesteryl oleate $y = 1.0996 - 0.0207x + 0.8703x^2$, $r^2 = 0.9889$

Oleic acid $y = 3.3739 - 1.0993x + 0.5658x^2$, $r^2 = 0.9633$



Calibration curves for cholesterol, triglyceride and phosphatidylethanolamine relating the weight of lipid to the peak area of the ionization detector response for the Iatroscan system

Each point is the mean of ten separate rods; unless otherwise shown by error bars, the standard deviation of the mean is less than the symbol size for each point

2nd order polynomial equations fitted to data

Cholesterol $y = 2.6752 + 1.5505x + 0.4237x^2$, $r^2 = 0.9903$

Triacylglycerol $y = 4.2574 - 0.5861x + 0.3259x^2$, $r^2 = 0.9831$

Phosphatidylethanolamine $y = 1.1252 + 0.6231x + 0.1221x^2$, $r^2 = 0.9955$

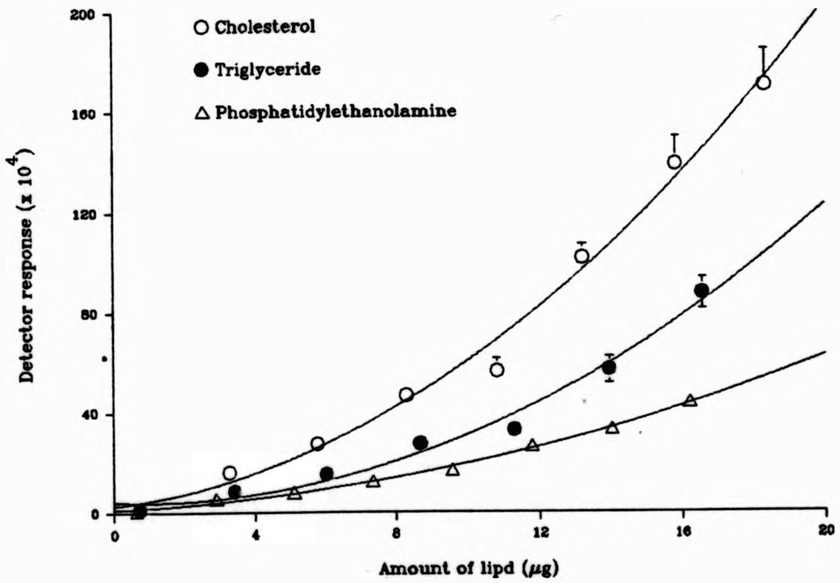


Fig 2-2 Calibration curves for total neutral classes and phosphatidylcholine relating the weight of lipid to the peak area of the ionization detector response for the Iatroscan system

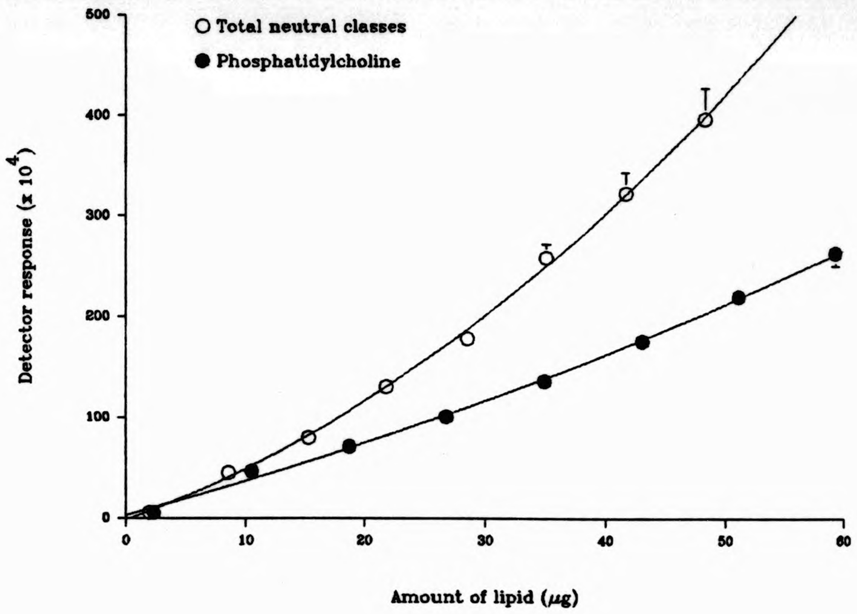
Each point is the mean of ten separate rods; unless otherwise shown by error bars, the standard deviation of the mean is less than the symbol size for each point

2^{nd} order polynomial equations fitted to data

Total neutrals $y_2 = -1.5752 + 4.1826x + 0.08523x^2$
$r^2 = 0.9872$

Total neutrals are defined as the peak area of the neutral classes from the polar solvent separation and include triacylglycerol, oleic acid, cholesterol and cholesteryl oleate

Phosphatidylcholine $y = 3.1384 + 3.1667x + 0.02018x^2$
$r^2 = 0.9980$



Chapter 3 The rearing of herring larvae on San Francisco Bay and Great Salt Lake *Artemia* nauplii

3.1 Introduction

Considerable information has been gained about the fatty acid requirements of marine fish larvae by using live feeds. One approach has been the rearing of fish larvae in large mesocosms to generate semi-natural food webs. To examine the transfer and modification of lipids through the system, samples of organisms from each trophic level are examined. However, the complete system is only under partial experimental control.

A second approach is laboratory rearing of fish larvae using live feeds which have had their fatty acid spectra modified.

3.1.1 Nutritional data for fish larvae from mesocosm studies

Mesocosms were developed in order to study planktonic ecosystems more conveniently than could be done in the open sea (Gamble *et al.* 1977). These workers demonstrated that the physical conditions within the mesocosm were somewhat different from those in the surrounding water due to the removal of horizontal mixing. Despite this, a self-sustaining phytoplankton/zooplankton community could be maintained over several months. The patterns of circulation and temperature within mesocosms were investigated further by Steele *et al.* (1977). Large-scale mesocosms have proved useful for studies on nutrients, species succession, pollution and trophic interactions (Gamble & Davies 1982). In terms of fish larvae, mesocosms have been used for studies on growth, survival, predation and feeding (Gamble & Houde 1984, Gamble *et al.* 1981, Øiestad & Moksness 1981, Schnack 1981). The results showed that given sufficient food and control

of potential predators, large numbers of marine fish larvae could be reared in these systems.

Gatten *et al.* (1983) took samples of zooplankton and herring larvae from 300 m^3 enclosures and showed that when the fish larvae were at the first feeding stage, total lipid in the larvae fell rapidly but levels of 18:4(n-3), 20:5(n-3) and 22:6(n-3) increased in the triacylglycerol fraction. It was suggested that this initial pattern was characteristic of phytoplankton fatty acids. Calanoid type fatty acids [20:1(n-9) and 22:1(n-11)] were only detected after metamorphosis at day 60. These results suggested that mesocosms could be used to follow marker fatty acids in the food web. Fraser *et al.* (1987) reared batches of herring larvae in mesocosms in which the level of zooplankton was maintained at between 6-8 nauplii l^{-1} or 30-40 nauplii l^{-1}. Analyses of lipids from phytoplankton, zooplankton and fish larvae demonstrated that the fatty acids of the triacylglycerol reserves tended to reflect those of the prey organisms available, whilst those of polar lipids were more conserved. Fatty acids characteristic of phytoplankton such as 18:4(n-3) could be traced through the food web. However, if data from mesocosm studies is to be interpreted correctly, the ability of the organisms at each trophic level to modify dietary fatty acids needs to be known. In order to achieve this, laboratory based studies are required.

3.1.2 Data from laboratory based nutritional studies using live diets

Several studies have used laboratory based food chains to examine the transfer of specific fatty acids between trophic levels. Normally a simple system is used with only one organism at each trophic level. The use of a monocultured algal strain as the primary producer gives a considerable degree of control over the initial fatty acid input into the

food chain. Although the fatty acids synthesised by microalgae can vary with culture conditions and growth phase (Ackman & Tocher 1968), the fatty acid spectra of different species are sufficiently constant for them to be used in nutritional studies (Scott & Middleton 1979). Using such a system, Kayama *et al.* (1963) was able to demonstrate that the brine shrimp was able to elongate and desaturate 18:3 to 20:5 fatty acid and that the guppy was probably able to convert 20:5 to 22:6 fatty acid.

The rotifer and the brine shrimp are widely used for fish rearing and research has concentrated on these two organisms e.g. studies on their nutritional value for commercially important species (Watanabe *et al.* 1983). The fatty acid composition of the rotifer is readily affected by that of the algae on which it is reared (Ben-Amotz *et al.* 1987, Watanabe *et al.* 1978). However, the rotifer is too small to support herring larval growth beyond the first few weeks and the larger brine shrimp must then be used (Blaxter & Hunter 1982). It is possible to increase the levels of certain nutrients in *Artemia* to some extent by rearing them on microalgae (Watanabe *et al.* 1980, Hinchcliffe & Riley 1972), various yeast strains grown in enriched media (Dendrinos & Thorpe 1987), lipid emulsions (Watanabe *et al.* 1982) or microcapsules (Walford & Lam 1987, Sakamoto *et al.* 1982). However, levels of short-chain fatty acids are more easily increased than those of polyunsaturates (Dendrinos & Thorpe 1987, Hinchcliffe & Riley 1972). Many experiments on the enrichment of brine shrimps have considered fatty acid content in percentage terms only but Tzoumas (1988) clearly demonstrated the dangers in this approach. Whilst feeding Brazilian strain *Artemia* with microcapsules containing 20:5(n-3) and 22:6(n-3) led to a percentage increase in 20:5(n-3) in the brine shrimp lipid, there was actually a decrease in absolute terms caused by a rapid reduction in total lipid over the incubation period. The content of

22:6(n-3) could not be increased and this fatty acid appears to be catabolized by the brine shrimps (Tzoumas 1988, Ballaer *et al.* 1985). An additional problem is that for enrichment, the incubation time must be prolonged for at least 24 h beyond instar I nauplii production, since the *Artemia* do not feed until after this stage (Ballaer *et al.* 1985).

Whilst this degree of control over the fatty acid content of live fish diets has proved sufficient for aquaculture purposes, where the prime concern is with reducing larval mortality, it is frequently inadequate for nutritional research.

Nevertheless, several studies have been carried out on rearing turbot using different algal strains to influence the fatty acid composition of rotifers. Howell (1979) demonstrated that growth of turbot larvae was poor when fed rotifers reared on the microalgae, *Dunaliella tertiolecta*. This was correlated with a lack of long-chain PUFAs in this algal strain. The conclusions were supported by subsequent work (Scott & Middleton 1979) and appeared to reinforce the results of studies using juveniles of this species i.e. that they have a limited ability to elongate and desaturate short-chain to long-chain PUFAs and thus require long-chain PUFAs in their diet (Owen *et al.* 1975). The results from a trial in which turbot larvae were reared on rotifers, San Francisco Bay *Artemia*, and the copepod *Eurytemora affinis*, demonstrated that growth and survival was best on the copepod (Witt *et al.* 1984). In terms of fatty acids, the copepods possessed more 20:5(n-3) and 22:6(n-3) than the other diets.

The growth of herring, plaice and turbot larvae reared on rotifers grown on baker's yeast and enriched with either yeast, *Isochrysis galbana* Park or *Nannochloropsis occulata* Hibberd has been considered

by Minkoff (1987). Analysis of the rotifers showed that yeast-enriched *Brachionus* had no 18:3(n-3) or 20:5(n-3) and trace amounts of 22:6(n-3). The *Nannochloropsis* enriched rotifers had 11-14% 20:5(n-3), whilst rotifers enriched with *Isochrysis* had 2-4% 20:5(n-3). Herring larvae reared for one month responded similarly to the algal-enriched diets, showing good growth and survival compared to those reared on yeast-enriched rotifers. However, in consequence of the large amount of man-power required to culture the rotifers and algae, the total numbers of larvae reared were small. This meant that lipid analysis of the larvae was not possible (Minkoff pers. comm.) and the growth responses were assumed to be due to the differences in fatty acid profiles of the diets. It was concluded that herring larvae were able to convert 20:5(n-3) to 22:6(n-3) but could not convert 18:3(n-3) to 20:5(n-3)..

Recently, Izquierdo *et al.* (1989) succeeded in defining the requirement of red sea bream larvae for (n-3)PUFAs using rotifers enriched with lipid emulsions. However, although the overall (n-3)PUFA content could be manipulated, precise control of the amounts of individual fatty acids in the rotifers was not possible.

3.1.3 Aim of this rearing trial

The objective of the trial described in this chapter was to raise herring larvae on two strains of *Artemia*, namely, San Francisco Bay strain, frequently classified by aquaculturists as a marine type containing relatively high 20:5(n-3) and some 22:6(n-3) (Watanabe *et al.* 1983), and Great Salt Lake strain, a freshwater type containing lower levels of 20:5(n-3) and minimal 22:6(n-3) (Ballaer *et al.* 1985). It had been planned to rear a third stock of larvae on a Chinese strain known to be highly

deficient in 20:5(n-3) and 22:6(n-3). Unfortunately the supply of cysts was destroyed in a fire at the Sea Fish Industry Laboratory, Ardtoe.

Labour was cut to a minimum by rejecting the option of enriching the *Artemia* nauplii as described above (methods which in any case have dubious benefits). This allowed the rearing of large numbers of fish larvae, sufficient to provide material for detailed lipid analyses as well as morphometric measurements.

The aims of the trial were to evaluate changes in the lipids of larvae reared on two diets with known fatty acid profiles in order to confirm that herring larvae were unable to convert 18:3(n-3) to 20:5(n-3) and to investigate whether they could elongate and desaturate 20:5(n-3) to 22:6(n-3).

3.2 Materials and methods

The procedures used to culture *Nannochloropsis occulata*, rotifers and *Artemia* are described in sections 2.2 - 2.4.

3.2.1 Supply of herring larvae

Fertile male and female herring were caught on Ballantrae Bank (55°8'N 004°20'W) off the Firth of Clyde by the R.V. Calanus on March 14, 1988. Gonads were excised and eggs fertilized as detailed in section 2.1. Stacks of eggs were transferred in pails of clean seawater to the DAFS Marine Research Unit, Poolewe. The stacks were placed in a 235 l black, plastic tub containing seawater filtered to 2µm. The incubation was carried out at 5°C in a constant temperature room. Each day 50% of the water in

the tank was siphoned out and replaced with fresh, filtered seawater which a small electric pump circulated over the eggs. The upper plate of each stack was checked daily to ensure that embryonic development was proceeding normally. Once the embryos had fully pigmented eyes, the water circulating pumps were removed and the daily plate check suspended to avoid inducing a premature hatch. During the three days prior to hatching the temperature was gradually raised to 8°C.

3.2.2 Tank layout

Six 235 l black PVC, circular, flat-bottomed tanks were set up in a constant temperature room as shown in Fig 3-1. Each tank was fitted with a barrel outlet covered initially with 250 µm mesh and when the herring larvae were larger with 400 µm mesh.

3.2.3 Water supply

Seawater from Loch Ewe was pumped into a header tank. It was then passed through 2 µm filters (Auto-Klean KS10N) before being fed to the tanks. Water input rates were adjusted to give a gentle trickle from the tank outlets. The flow was sufficient to exchange the water in the tanks at least once every 24 h. Inflow was via a debubbler (Fig 3-2) which removed small air bubbles generated by the drop in water pressure across the control valve. The location of the water inlet was designed to give an upward flow of water to aid flotation and clearance of diets from the tanks.

3.2.4 Lighting

Illumination was provided by a double bank of 40 W fluorescent (daylight type) tubes set 1 m above the tanks (Fig 3-2). The illumination levels had been previously recorded as described in section 4.2.5.

3.2.5 Temperature

The temperature of incoming seawater was not under control but the trial was run in a constant temperature room set to 8°C. Water temperatures in each tank were recorded every morning, afternoon and evening.

3.2.6 Tank cleaning

The flow of seawater through the tanks helped to flush away unused food. However, material did settle on the tank bases and had to be removed daily. Debris was collected into a pail by siphon and allowed to stand. Any live larvae were dipped out with a small beaker and returned to the rearing tank.

3.2.7 Experimental design

The stock of larvae were fed with rotifers enriched with *Nannochloropsis occulata* from 5 days post-hatch to 10 days post-hatch at a rate of 1000 particles l^{-1} given twice daily. At 11 days post-hatch the larvae were redistributed into six 235 l black plastic tubs (see section 3.2.2) to give approximately 5000 larvae $tank^{-1}$. Over the next three days

the rotifers were replaced with *Artemia* nauplii until by 14 days post-hatch only brine shrimps were being fed.

Tanks 1, 3 and 5 were fed on newly hatched San Francisco Bay *Artemia* (SFB) and tanks 2, 4 and 6 on newly hatched Great Salt Lake *Artemia* (GSL). The numbers of brine shrimps in each tank prior to feeding were assessed by taking four subsample counts from four cores of water drawn from each tank. Sufficient feed was added to restore the prey concentration to 2000 l^{-1}.

Larvae were analysed for length and weight at 10, 15, 25, 35 and 45 days post-hatch. Samples were also collected at these times for lipid analysis.

Mortality assessments were not carried out as previous work had failed to provide a satisfactory protocol for doing this in tanks of the size used in this study.

3.2.8 Sampling of larvae and *Artemia*

Larvae were sampled by attracting them to a bench light mounted on the edge of the tank and dipping them out with a beaker. The standard lengths and dry weights of the larvae were determined as described in section 2.6. Whilst larval lengths were being recorded, an estimate of the number of *Artemia* in the guts was made. A record was also kept of those larvae showing signs of feeding (orange staining or distention of the gut wall). The guts of those fish containing prey were gently squeezed with forceps to encourage the egestion of prey items prior to washing of the larva for dry weight determination.

Larvae for biochemical analysis were counted into a dish of seawater using a wide-bore pipette. They were washed onto a 200 µm mesh and rinsed with distilled water. Larvae were then transferred into 7.5 ml of chloroform:methanol (2:1 by vol) and stored at -20°C in Teflon capped glass bottles (Pierce U.K. Ltd., Cambridge, England).

Newly hatched brine shrimps were collected on a 250 µm mesh, washed with distilled water, freeze dried and stored at -20°C prior to analysis.

The dry weight of nauplii was estimated by counting out samples of 100 *Artemia* which had been rinsed in distilled water onto pre-weighed filter papers. The nauplii were then freeze dried and the weight determined by difference.

3.2.9 Biochemical analyses

Analyses of lipid content, lipid classes and fatty acids were carried out. The lipid content and class analyses were performed on duplicate samples from each tank at each sample point. The lipid remaining after these assays was pooled by diet and used for fatty acid analysis after the addition of 23:0 fatty acid as an internal standard. These methods are fully described in sections 2.7 - 2.10.

3.2.10 Statistical analysis of morphometric and biochemical data

The standard length and dry weight data were initially analysed to yield the median and upper and lower quartiles for individual tanks and for tanks pooled by diet.

The data sets for individual tanks were then analysed for goodness of fit to a normal distribution using the Chi-squared test (Zar 1984). At each sample point, data from several tanks did not fit the normal distribution acceptably ($p<0.05$). A variety of transformations were attempted but failed to remove the differences. The experiment was designed to allow comparison of the data by analysis of variance (ANOVA). However, this technique assumes normal distribution and homogeneity of variance. Although ANOVA is relatively robust to distortions in the above assumptions, the data in this case was analysed using non-parametric methods (Conover 1980).

Length and weight data were considered separately. For each sample point i.e. 15, 25, 35 and 45 days post-hatch a Kruskall-Wallis test was carried out between data from tanks making up each treatment. This test was designed to show if intra-treatment differences existed. Data for day 10 post-hatch are from the single stock tank.

Data sets were then pooled by treatment and tested for inter-treatment differences using Mann-Whitney tests. The Mann-Whitney test is frequently more powerful than the two-sample t test for nonparametric distributions or where one or both samples contain unusually high or low values (Conover 1980).

Lipid data, when in percentage terms, were arcsin transformed prior to the calculation of summary statistics.

3.3 Results

3.3.1 Light levels, tank design and layout

The measurements of light levels carried out in a previous experiment for the tank system used in this study are given in section 4.3.1. At no time did the water temperature in the tanks deviate from 8°C by more than 1°C.

3.3.2 Feeding incidence, length and weight data

The results for the numbers of prey in the guts of herring larvae are given in Table III,i. There was considerable variation with some larvae containing a large number of *Artemia*, whilst others contained none. The standard deviations are thus high. The mean values often differed widely from tank to tank within the treatment group. There was no clear relationship between the numbers of *Artemia* nauplii larva^{-1} and age but the percentage of larvae showing signs of feeding increased with age. The mean data for pooled samples indicated that the numbers of SFB and GSL *Artemia* in the guts of larvae were similar.

Morphometric measurements expressed as medians and quartiles for each tank and pooled by treatment are given in Tables III,ii and III,iii. Unpooled data are expressed graphically in Fig 3-3. Tanks receiving the same treatment showed similar trends. For larvae fed SFB *Artemia*, standard length increased in a linear fashion with time. However, increases in length for larvae fed GSL *Artemia* decreased after 25-35 days post-hatch (tanks 4 and 6) or became negative (tank 2). The dry weights of larvae fed SFB *Artemia* continued to increase with age up to termination

of the experiment, whilst the rate of increase for GSL fed larvae showed signs of declining after 25 (tank 6) or 35 days post-hatch.

Pooled length and weight data are represented graphically in Fig 3-4. Over the experimental period, median standard length increased from 10.4 to 16.0 and 15.2 mm, whilst median dry weight increased from 0.134 to 0.612 and 0.439 mg (for larvae fed SFB and GSL *Artemia* respectively).

3.3.3 Statistical analysis of morphometric data

The results of Kruskall-Wallis tests performed between tanks receiving the same diet are presented in Table III,iv. For larvae fed SFB *Artemia* there were no significant differences between the data at each sample point for dry weight, but intra-treatment differences did exist for larvae fed GSL *Artemia* for both standard length and dry weight. Thus, further analyses of morphometric data pooled by treatment must be regarded cautiously.

The results of the Mann-Whitney tests carried out on pooled data are presented in Table III,v. Inter-treatment differences were not significant at 15 and 25 days post-hatch but became highly significant ($p<0.01$) for both standard length and dry weight after this time. The probability levels indicated that the two diets had markedly different effects on length and weight increase which were greater than the intra-treatment differences detected.

3.3.4 Lipid class analyses

The results for lipid class analyses on samples from each tank are given in Tables III,vi to III,xi. In each table, the lipid class distribution expressed as percentage of total lipid is given, followed by the triacylglycerol/cholesterol ratio. The lipid content expressed in absolute and relative terms is then quoted. Lipid class data and lipid contents were then combined to give the absolute amount of each lipid class present in the larvae. The data pooled by treatment are given in Tables III,xii and III,xiii. The absolute lipid contents are given for triacylglycerol and for total polar lipids.

Both intra-treatment and inter-treatment differences in lipid class distributions were small. In all samples free fatty acids, monoacylglycerols and diacylglycerols were present in trace amounts only, indicating that sample degradation prior to analysis had not occurred. Sterol/wax esters comprised between 1.6 and 3.9% of total lipid and did not exhibit any trend with age.

For larvae fed SFB *Artemia*, the triacylglycerol content increased from 18.9 to 25.8% of total lipid (between 10 and 45 days post-hatch) which was equal to a maximum of 17.3 µg larva^{-1} at day 45. Larvae fed GSL *Artemia* showed a slightly lower increase reaching 21.8% or 14.5 µg larva^{-1} at day 45. Levels of cholesterol remained relatively constant with age comprising between 7.6 and 12.0% of total lipid.

Fig 3-4 illustrates the changes in triacylglycerol/cholesterol ratios which occurred with time. The pattern for both sets of larvae was similar but the decline after day 25 post-hatch was less steep for larvae fed SFB *Artemia*.

The majority of polar lipid was made up of two classes, phosphatidylcholine and phosphatidylethanolamine. In larvae from both dietary groups phosphatidylcholine declined from 37.2% to around 30% of total lipid, whilst the percentage of phosphatidylethanolamine increased with age.

Phosphatidylserine and phosphatidylinositol were not resolved from each other in any of the analyses and failed to resolve from phosphatidylethanolamine in most samples. Where these classes were resolved they comprised about 3.0% of total lipid. Sphingomyelin comprised between 2.0 and 4.6% of total lipid, lysophosphatidylcholine was not detected and cardiolipin could not be resolved from phosphatidylcholine using the Iatroscan system.

The lipid content of larvae (relative to dry weight) fell from 23.3% to 10.9% and 15.1% during the study (for larvae fed SFB and GSL *Artemia* respectively, Tables III,xii and III,xiii). Fig 3-5 illustrates the changes in absolute lipid with age in larvae pooled by diet. Both sets of larvae exhibited a decrease between days 10 and 15 and then showed increases in absolute lipid content. The initial increase was greatest for larvae fed SFB *Artemia* but the rate declined after day 35. Both groups of larvae had similar lipid contents at 45 days post-hatch (67.0 and 66.3 µg for larvae fed SFB and GSL *Artemia* respectively).

Relative to total lipid (Tables III,xii and III,xiii), total polar classes showed a slight decrease with age. The relative triacylglycerol content peaked at 25 and 35 days post-hatch (for larvae fed SFB and GSL *Artemia* respectively) and by the end of the trial comprised 25.8% of total lipid for SFB fed larvae but 21.8% for GSL fed larvae. The changes in absolute amounts of total polar lipid and triacylglycerol are shown graphically in Fig 3-5.

3.3.5 Fatty acid analyses

The results of fatty acid analyses performed on samples of SFB and GSL *Artemia* nauplii are given in Tables III,xiv and III,xv. Total lipid contained 67.8 and 73.1% as fatty acids (SFB and GSL respectively). The predominant fatty acids in SFB nauplii were 18:1(n-9) at 12.3%, 18:3(n-3) at 10.0%, 18:1(n-7) at 8.6% and approximately 8% of 16:0 and 16:1. The polyunsaturate, 20:5(n-3) was present at 5.3%. There was a trace of 22:5(n-3) but no 22:6(n-3). The fatty acid spectra of GSL nauplii was similar but with substantially more 18:3(n-3) at 19.1%, less 16:1 at 2.9% and a reduced 20:5(n-3) content (2.0%). The increased level of 18:3(n-3) in GSL *Artemia* gave them a higher (n-3)/(n-6) fatty acid ratio than for SFB *Artemia*.

The results for dry weights of *Artemia* nauplii (Table III,xvi) were similar to those quoted by Vanhaecke & Sorgeloos (1980). The percentage lipid data were combined with the estimates of nauplius dry weight to give the absolute fatty acid contents of nauplii (also in Table III,xvi). Great Salt Lake *Artemia* were slightly larger and approximately twice as heavy as SFB nauplii. In absolute terms, each GSL nauplius contained substantially more 18:1(n-9) and 18:3(n-3), slightly more 18:4(n-3) and slightly less 20:5(n-3). The absolute levels of 20:4(n-6) were greater in SFB nauplii (2.8 pg nauplius^{-1} as opposed to 0.8 pg nauplius^{-1}). In total each GSL nauplius contained 206.1 pg of fatty acids as opposed to 93.6 pg for SFB nauplii.

The results of fatty acid analyses on samples of herring larvae from each tank are given in percentage terms in Tables III,xvii to III,xxv. These results were converted to absolute amounts (µg larva^{-1}) and are

presented in Tables III,xxvi and III,xxvii. The fatty acid content of the two feeds are also given for comparison. At the termination of the trial, larvae fed SFB *Artemia* contained 38.26 µg fatty acid larva^{-1} whilst GSL fed larvae contained 37 µg larva^{-1}. The changes in amounts of various (n-3) fatty acids with age are shown graphically in Fig 3-5. The fatty acids of the herring larvae tended towards the pattern shown by the feed with time. Over the experimental period there was a rapid decline in the amount of 22:6(n-3) present, from 4.87 µg larva^{-1} to 2.41 and 2.25 µg larva^{-1} (in SFB and GSL fed fish respectively). The amount of 20:5(n-3) present, after an initial fall, increased more rapidly in larvae fed SFB *Artemia* reaching 4.42 µg larva^{-1} at 45 days post-hatch. In contrast the average content for larvae fed GSL *Artemia* at the same age was 2.78 µg. At the termination of the study, larvae fed GSL *Artemia* contained greater amounts of 18:3(n-3) than larvae fed SFB *Artemia* (5.77 µg larva^{-1} compared to 3.22 µg larva^{-1} respectively). The elevated content of 18:3(n-3) in larvae fed GSL *Artemia*, resulted in a higher (n-3)/(n-6) fatty acid ratio in these fish at 45 days post-hatch.

There was little change in the concentration of 18:3(n-6) in the herring larvae but a slight accumulation of 18:2(n-6). This was greater in larvae fed GSL *Artemia* reflecting the higher level of this fatty acid in the brine shrimps of this strain.

3.4 Discussion

3.4.1 Growth as measured by standard length and dry weight

The results showed that herring larvae grew better on SFB than on GSL *Artemia*. Poor growth may be attributed to a variety of factors e.g.

low ingestion rate, poor digestibility, nutritional inadequacy or a detrimental factor in the feed.

The ingestion rate may be affected by such factors as the size (Eggers 1977) and type of zooplankton (Checkley 1982), their pigmentation (Dendrinos *et al.* 1984) or differences in chemical feeding stimulants (Mearns 1986). Differences in ingestion rate due to the larger size of GSL *Artemia* nauplii (width 200 ± 20 as compared with 171 ± 14 µm) would be likely to be most pronounced in the early stages of the trial. However, from days 15 to 25 the increase in dry weight was comparable between the two treatments and it was only at a later age that differences became apparent. It was noted that GSL nauplii tended to be paler than SFB nauplii but the results of the larval gut content analyses failed to show that GSL *Artemia* were ingested at a lower rate than SFB *Artemia*. An attempt was made to measure the prey consumption of the larvae directly, by counting the numbers of *Artemia* removed from a beaker of known volume containing a known number of herring larvae over unit time (as in Houde & Schekter 1980). Replicate tests showed that the variation in results was very high. The rates measured varied between 6.9 to 13.9 and 2.5 to 11.1 nauplii larva^{-1} h^{-1} (for SFB and GSL nauplii respectively) for 30 day old herring larvae. The digestibility of the diets was not assessed.

3.4.2 Changes in the lipid content and class distribution of herring larvae

The lipid content and class distribution of larvae were assayed as additional parameters in assessing their growth on the two strains of *Artemia*. Fraser *et al.* (1987) provided data on changes in Clyde herring larvae reared at high (30 - 40 calanoid nauplii l^{-1}) and low (6 - 8 calanoid nauplii l^{-1}) food densities. Between 10 and 47 days post-hatch, Fraser *et al.* found that percentage triacylglycerol was correlated with food density,

sterols increased in under-nourished larvae but were relatively constant in well-nourished larvae, phosphatidylcholine decreased by approximately 10% at both feeding levels whilst phosphatidylethanolamine tended to increase and the levels of other polar lipids remained relatively constant.

In this trial, triacylglycerol increased at a comparable rate, as a percentage of total lipid, compared with well-nourished larvae from the work of Fraser *et al.* It has been suggested that the triacylglycerol/cholesterol ratio may be used as a nutritional condition index (Fraser 1989, Fraser *et al.* 1987). This concept is discussed more fully in the next chapter. The changes in triacylglycerol/cholesterol ratio from this study exhibited a different pattern to those recorded for enclosure reared larvae (Fraser 1989). The ratio declined steadily after 25 days post-hatch indicating that the both *Artemia* strains were nutritionally inadequate. In terms of absolute triacylglycerol content and triacylglycerol/cholesterol ratio, larvae reared on SFB *Artemia* performed better than those reared on GSL *Artemia*.

The most significant change in lipid classes which occurred was the decrease in phosphatidylcholine and concomitant increase in phosphatidylethanolamine resulting in almost equal proportions of these classes at 45 days post-hatch. This is in contrast to previous reports where at hatching, phosphatidylethanolamine only comprised 7.2% and although the class did increase in percentage terms with age, the levels never reached those of phosphatidylcholine (Fraser *et al.* 1987). It is suggested that the difference between these results was due to the different calibration method for the Iatroscan system used. Fraser *et al.* assumed that the detector response of phosphatidylethanolamine was the same as for phosphatidylcholine and constructed calibration curves for

phospholipids based only on this class (Fraser *et al.* 1985). The calibration for this study contained both classes and it was shown that the response of phosphatidylcholine was considerably greater than that of phosphatidylethanolamine (see section 2.9) thus leading to the under estimation of the latter class in previous work.

Marine fish roe contains high levels of phosphatidylcholine e.g. cod 45.6%, haddock 45.8%, saithe 49.6% (Tocher & Sargent 1984). A decline in phosphatidylcholine levels upon fertilisation of Atlantic halibut eggs has been reported, but the level then increased with development reaching 52% of total lipid by stage III (Falk-Petersen *et al.* 1989, Falk-Petersen *et al.* 1986). It has been suggested that the high levels of phosphatidylcholine act as a store for (n-3)PUFAs. It was postulated that the fatty acids were destined for biomembrane formation and so there was some advantage in storing them in the form of polar as opposed to neutral lipid. During larval development, selective catabolism of phosphatidylcholine has been recorded in herring (Tocher *et al.* 1985b) and cod (Fraser *et al.* 1988). In starving cod larvae about 33% of the 22:6(n-3) liberated by phosphatidylcholine catabolism was not oxidized but retained in the neutral lipids. This was in contrast to liberated 20:5(n-3) for which selective retention could not be demonstrated. This probably reflects the metabolic importance of 22:6(n-3) with shorter chain fatty acids being preferentially oxidized for energy (Tocher *et al.* 1985a).

The primary reason for high phosphatidylcholine levels in fish eggs, which decline with development is unclear. Possible explanations include the provision of inorganic phosphate for intermediary metabolism (Tocher & Sargent 1984), the provision of choline for conversion to betaine which can act as an intracellular osmolyte (Falk-Petersen *et al.* 1989), or to facilitate the transport of neutral lipids from the yolk sac to the developing embryo (Fraser *et al.* 1988).

Reports on the dietary importance of phosphatidylcholine for marine fish larvae organisms are currently fragmentary. The phosphatidylcholine component of lecithin has been implicated as an essential nutrient for larval ayu *Plecoglossus altivelis*, red seabream and knifejaw, *Oplegnathus fasciatus* (Kanazawa *et al.* 1983a, Kanazawa *et al.* 1983b). In view of the apparent importance of phosphatidylcholine in the development of fish embryos and larvae and its possible metabolic functions, more research is needed in the area of phospholipid nutrition.

3.4.3 The fatty acid composition of *Artemia*

As described in the review by Léger *et al.* (1986), *Artemia* nauplii are almost ubiquitously used in aquaculture for rearing fish larvae during the period when they require live feeds but are too large to take rotifers.

In this trial the feed level was kept at 2000 *Artemia* l^{-1}. One problem with feeding relatively high numbers of prey is that since only a fraction are consumed by the larvae, the biochemical composition of the remaining *Artemia* can change with starvation. For unfed brine shrimps, Claus *et al.* (1979) reported changes in fatty acid profile whilst Benijts *et al.* (1976) found that, although total lipid declined, the fatty acid profile was virtually unaltered. The fatty acid data presented in Tables III,xiv and III,xv are for newly hatched nauplii and thus there is no guarantee that the nauplii ingested by the fish larvae had exactly this composition.

It is well known that brine shrimps from different localities vary considerably in their biochemical composition (Léger *et al.* 1986). Several studies have found that SFB *Artemia* are superior to GSL *Artemia* for

growing marine fish larvae (Sorgeloos 1980). Feeding with the latter strain led to high mortality in sea bass larvae (Ballaer *et al.* 1985). These results were attributed to high pesticide residues but in earlier rearing trials with the prawn *Palaemon serratus*, Wickins (1972) concluded that nutritional inadequacy and not contamination was to blame.

It is commonly believed in aquaculture that SFB *Artemia* provide a good source of the fatty acids currently regarded as essential for rearing fish larvae. However, the fatty acid profile can vary considerably from year to year and even from batch to batch (Watanabe *et al.* 1982). In addition Léger *et al.* (1986) state that it is primarily the levels of 20:5(n-3) which determine the suitability of the strain for marine fish. The polyunsaturate 22:6(n-3) appears to be regarded as 'semi-essential' and the levels needed by fish larvae have not been well defined.

The results from this trial showed that there were only small differences in the relative fatty acid contents of the SFB and GSL nauplii used. The San Francisco Bay *Artemia* did possess slightly more 20:5(n-3) but neither strain possessed 22:6(n-3). As explained in Chapter 1, little work has been carried out on the requirement of fish larvae for 20:4(n-6). In view of the concentration of this fatty acid in phosphatidylinositol (Tocher & Sargent 1984) and its possible metabolic role as a prostaglandin precursor (Henderson *et al.* 1985), it should be present in the diet at a nominal level. Although there is no recommendation for the amounts of 20:4(n-6) required in the diet of fish larvae, this fatty acid was present in both strains of *Artemia* used in this trial.

The lipid content and fatty acid profiles for a typical North Sea copepod, *Calanus finmarchicus* were presented by Kattner & Krause (1987). The lipid content as a percentage of dry weight was between 8.4 (copepodid I) and 24.2% for adult females. The content of 20:5(n-3)

expressed as percentage total fatty acids was never less than 13.0% (copepodid I) and that of 22:6(n-3) never less than 9.9% (adult male). Levels of 18:3(n-3) were between 1.2 and 2.3%. The proportion of the lipid composed of wax esters increased from an initial levels of 20.1 and 41.0% to between 70.0% (adult female) and 91.5% (adult male). The fatty alcohol moiety of these wax esters was dominated by 22:1(n-11) and 20:1(n-9). The favoured food of young herring larvae are copepod nauplii whilst older larvae take copepodite stages (Bainbridge & Forsyth 1971). Assuming the lipid profile of the nauplii is similar to that of the copepodid stages, this composition may be taken as fairly typical of the diet of herring larvae feeding in the waters around the British Isles.

Compared to these levels, considerably lower amounts of long-chain PUFAs are supplied to larvae reared on *Artemia*, even when the brine shrimps are enriched (Walford & Lam 1987). Naturally collected *Acartia* contained high levels of (n-3)PUFAs (Watanabe *et al.* 1983) and Witt *et al.* (1984) reported that turbot survived and grew better when fed cultured copepods (*Eurytemora affinis*) as compared with SFB *Artemia*, a result which appeared to correlate with higher PUFA levels in the copepods. The low levels of PUFAs and in particular the lack of 22:6(n-3) in *Artemia*, although allowing survival and growth of marine fish larvae, may be cause sub-lethal detrimental effects. In the opinion of the author, it should be considerably easier to maintain high PUFA levels in cultured copepods as opposed to forcing the levels in *Artemia* unnaturally high. In view of the increasing cost of *Artemia* cysts, the uncertain quality of batches and their possible nutritional inadequacy every effort should now be directed towards the successful culture of copepods for marine fish larval rearing.

Other biochemical parameters such as the amino acid profile of the two *Artemia* strains used in this trial were not assessed. Although no

differences in amino acid profile between SFB and GSL *Artemia* were detected by Landau & Riehm (1985), results from this trial cannot be definitely attributed to the relatively small differences in fatty acid profiles found in the brine shrimps. In order to assess whether these differences exerted an effect on the composition of the larvae, the fatty acid profiles of the fish were also monitored throughout the trial.

3.4.4 Changes in the fatty acids of herring larvae

The results of rearing turbot larvae on live diets (Scott & Middleton 1979) have tended to confirm the hypothesis, derived from studies on juvenile and adult fish, that they possess some ability to elongate, but less ability to desaturate 18:3(n-3), 18:2(n-6) or 18:1(n-9) to longer chain PUFAs (Cowey *et al.* 1976a, Owen *et al.* 1975).

In this trial, the accumulation of 18:3(n-3) coupled with the reduction in 22:6(n-3) and 22:5(n-3) showed that little or no elongation and desaturation of 18:3(n-3) had occurred. The accumulation of 18:3(n-3) was greater for GSL fed larvae reflecting the higher amounts of this fatty acid in the *Artemia*. Since 20:5(n-3) was present in both *Artemia* strains, it cannot be definitely concluded that elongation and desaturation to this fatty acid had not occurred but the rate of conversion was certainly too low to maintain the levels of (n-3)PUFAs present in the larvae at 10 days post-hatch.

A slight accumulation of 18:4(n-3) was noted, this being greater in larvae fed GSL *Artemia*. This suggests that the delta-6 desaturase enzyme is partially active in herring larvae.

22:6(n-3) was totally absent from the *Artemia* and the decline in this fatty acid, accompanied by some accumulation of 20:5(n-3) indicated that there was negligible conversion of dietary 20:5(n-3) to 22:6(n-3) by the

larvae. The results thus confirmed that herring larvae behave in a similar way to turbot in terms of their limited ability for (n-3)fatty acid bioconversion.

The changes occurring in the polar and neutral fractions were not examined. Several studies (Fraser *et al.* 1987, Tocher *et al.* 1985) have indicated that long-chain PUFAs are preferentially retained in the polar lipids and depleted in the neutral lipids under starvation or poor food supply conditions. This is due to the fact that the polar lipids primarily form the biomembranes and it is more critical that their biochemical integrity is preserved. Koven *et al.* (1989) showed that when larval gilthead seabream were reared on PUFA deficient rotifers, a strategy for the conservation of (n-3) fatty acids occurred, but that under positive growth this was insufficient to maintain the 'correct' fatty acid spectra of the polar lipids. Although the fatty acid spectra of the polar and neutral lipids was not determined in this trial, it is likely that a similar situation occurred. Thus, positive growth could be maintained, even on GSL *Artemia*. However, after an initial period of high growth, the rate of increase declined. For larvae fed SFB *Artemia*, the levels of 20:5(n-3) in this strain may have been sufficient to maintain positive growth over a longer period of time, or the growth rate of these larvae might also have declined if the trial had been continued.

It is possible that accumulation of 20:5(n-3) can to some extent ameliorate the effects of losses of 22:6(n-3) from tissues. It is known that in rats a depletion of 22:6(n-3) from brain tissue results in an accumulation of 22:5(n-6) from the diet or 20:3(n-9) synthesised *de novo* from 18:1(n-9) (Salem *et al.* 1986). The accumulation of 20:5(n-3) from SFB *Artemia* by fish larvae in this trial demonstrated that a dietary source of 22:6(n-3) is not essential for growth under the conditions of this trial. It

is the opinion of the author however, that 22:6(n-3) deprivation is likely to have a detrimental effect on the health of the larvae. These results explain the findings of Minkoff (1987), described in section 3.1.2, and his erroneous conclusion that herring larvae could elongate and desaturate 20:5(n-3).

3.5 Conclusions

Growth of herring larvae as measured by increases in standard length, dry weight and lipid content was better when the larvae were reared on SFB as opposed to GSL *Artemia* nauplii.

The reason for these differences could not be unequivocally defined since many biochemical factors of the two diets were not measured. There were however differences in the (n-3)PUFA content of the two *Artemia* strains which may explain the results.

The fatty acid spectra of total lipid from larvae closely resembled that of their relative diets by the end of the trial. There was no evidence for efficient elongation and desaturation of 18:3(n-3) or of 20:5(n-3) to longer chain PUFAs by the larvae.

The following chapters detail experiments carried out to assess directly the elongation and desaturation ability for (n-3) fatty acids by herring larvae.

Table III,i The gut contents of herring larvae reared on San Francisco Bay and Great Salt Lake *Artemia*

The numbers of *Artemia* in the guts of larvae were recorded during length measurement.
The percentage of larvae showing signs of feeding, including those with positive prey items in their guts is also given.
Tank data are based on 30 larvae, pooled data on 90 larvae.

Larvae fed San Francisco Bay *Artemia*

	Age (days post-hatch)			
	15 mean ± sd	25 mean ± sd	35 mean ± sd	45 mean ± sd
Tank 1				
Artemia larva^{-1}	1.1 ± 2.0	2.5 ± 2.5	0.9 ± 1.4	0.9 ± 1.4
Percentage feeding	47.6	76.7	90.0	100.0
Tank 3				
Artemia larva^{-1}	1.5 ± 2.2	0.8 ± 1.2	1.3 ± 2.1	2.1 ± 2.7
Percentage feeding	36.6	83.3	100.0	100.0
Tank 5				
Artemia larva^{-1}	3.6 ± 1.9	1.4 ± 1.8	1.1 ± 1.6	1.6 ± 1.8
Percentage feeding	46.7	73.3	90.0	93.3
Pooled				
Artemia larva^{-1}	2.1 ± 2.1	1.7 ± 2.0	1.1 ± 1.7	1.5 ± 2.1
Percentage feeding	42.2	80.0	93.3	97.8

Larvae fed Great Salt Lake *Artemia*

	Age (days post-hatch)			
	15 mean ± sd	25 mean ± sd	35 mean ± sd	45 mean ± sd
Tank 2				
Artemia larva^{-1}	0.7 ± 1.6	1.2 ± 1.5	1.5 ± 2.5	1.3 ± 1.8
Percentage feeding	40.0	53.3	90.0	86.7
Tank 4				
Artemia larva^{-1}	4.4 ± 3.1	1.7 ± 1.6	0.6 ± 1.7	4.6 ± 5.6
Percentage feeding	90.0	90.0	80.0	100.0
Tank 6				
Artemia larva^{-1}	4.2 ± 2.8	1.5 ± 2.0	1.2 ± 1.9	1.0 ± 1.5
Percentage feeding	36.7	96.7	70.0	90.0
Pooled				
Artemia larva^{-1}	2.3 ± 3.0	1.5 ± 1.7	1.1 ± 2.1	2.3 ± 3.8
Percentage feeding	63.3	80.0	80.0	92.2

Table III,ii Standard lengths and dry weights for herring larvae reared on San Francisco Bay *Artemia*

Data are expressed as medians (med) and lower (q1) and upper quartiles (q2).
The number of larvae in the sample is also given (n).
Age is given in days post-hatch.
Data for 10 days post-hatch is derived from a single stock tank.

Tank 1

Age	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
10	30	10.4	10.0	10.5	30	0.134	0.128	0.144
15	30	11.5	11.0	11.8	30	0.173	0.145	0.190
25	30	12.4	11.1	13.5	22	0.276	0.204	0.362
35	30	14.7	13.8	15.4	30	0.402	0.329	0.511
45	30	15.6	14.6	17.0	30	0.596	0.377	0.676

Tank 3

Age	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
10	30	10.4	10.0	10.5	30	0.134	0.128	0.144
15	30	11.3	10.8	12.2	30	0.154	0.141	0.192
25	30	13.7	12.3	14.4	25	0.314	0.236	0.405
35	30	14.5	13.4	15.5	26	0.359	0.292	0.515
45	30	16.2	14.7	17.0	30	0.623	0.414	0.674

Tank 5

Age	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
10	30	10.4	10.0	10.5	30	0.134	0.128	0.144
15	30	11.8	11.0	12.2	30	0.170	0.146	0.194
25	30	13.6	11.3	14.4	28	0.317	0.150	0.379
35	30	14.4	13.8	15.7	30	0.351	0.293	0.465
45	30	16.7	14.8	17.8	30	0.607	0.377	0.810

San Francisco Bay *Artemia* fed larvae - pooled data

Age	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
10	30	10.4	10.0	10.5	30	0.134	0.128	0.144
15	90	11.4	11.0	11.9	90	0.168	0.144	0.194
25	90	13.1	11.5	14.2	75	0.295	0.216	0.381
35	90	14.5	13.7	15.5	86	0.376	0.303	0.515
45	90	16.0	14.7	17.1	90	0.612	0.382	0.684

Table III,iii Standard lengths and dry weights for herring larvae reared on Great Salt Lake *Artemia*

Notes as for Table III,ii

Tank 2

Age	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
10	30	10.4	10.0	10.5	30	0.134	0.128	0.144
15	30	11.3	11.0	11.5	29	0.157	0.138	0.175
25	30	13.6	11.3	14.4	27	0.317	0.150	0.379
35	30	16.0	14.9	17.4	30	0.529	0.376	0.770
45	30	14.6	14.0	15.7	30	0.393	0.322	0.462

Tank 4

Age	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
10	30	10.4	10.0	10.5	30	0.134	0.128	0.144
15	30	11.5	11.0	12.2	29	0.170	0.146	0.207
25	30	13.7	12.9	14.2	30	0.301	0.192	0.367
35	30	15.2	14.5	15.8	30	0.463	0.415	0.528
45	30	16.0	15.0	17.2	29	0.522	0.402	0.688

Tank 6

Age	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
10	30	10.4	10.0	10.5	30	0.134	0.128	0.144
15	30	11.5	11.1	12.1	30	0.175	0.151	0.193
25	30	14.0	13.0	14.7	28	0.348	0.252	0.399
35	30	14.5	13.8	15.6	30	0.380	0.316	0.491
45	30	15.0	14.1	16.6	30	0.416	0.342	0.589

Great Salt Lake *Artemia* fed larvae - pooled data

Age	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
10	30	10.4	10.0	10.5	30	0.134	0.128	0.144
15	90	11.4	11.0	11.9	88	0.164	0.143	0.192
25	90	13.5	12.4	14.5	85	0.288	0.196	0.381
35	90	15.2	14.4	16.4	90	0.453	0.363	0.603
45	90	15.2	14.1	16.4	89	0.439	0.343	0.576

Table III,iv Intra-treatment differences tested by Kruskall-Wallis tests

Measurements for standard lengths and dry weights of larvae within treatments were compared at each sample point using a Kruskall-Wallis test.
The null hypothesis was that the differences between treatments were not significant.
NS indicates acceptance of the null hypothesis. The null hypothesis was rejected at a probability of 0.05, values below this become increasingly significant.
Age is given in days post-hatch.

Age	*Artemia* strain	Tanks	Probability Length data	Probability Weight data
15	San Francisco Bay	(1,3,5)	NS	NS
	Great Salt Lake	(2,4,6)	NS	NS
25	San Francisco Bay	(1,3,5)	0.0340	NS
	Great Salt Lake	(2,4,6)	0.0144	0.0285
35	San Francisco Bay	(1,3,5)	NS	NS
	Great Salt Lake	(2,4,6)	0.0112	0.0198
45	San Francisco Bay	(1,3,5)	NS	NS
	Great Salt Lake	(2,4,6)	0.0319	0.0200

Table III,v Inter-treatment differences tested by Mann-Whitney tests between larvae pooled by treatment

Measurements for standard lengths and dry weights of larvae pooled by treatment were compared at each sample point using a Mann-Whitney test.
The null hypothesis was that the differences between treatments were not significant.
NS indicates acceptance of the null hypothesis. The null hypothesis was rejected at a probability of 0.01, values below this become increasingly significant.
Age is given in days post-hatch.

Age	Tanks compared	Probability Length data	Probability Weight data
15	1,3,5 against 2,4,6	NS	NS
25	1,3,5 against 2,4,6	NS	NS
35	1,3,5 against 2,4,6	0.00276	0.00342
45	1,3,5 against 2,4,6	0.00921	0.00221

Table III,vi Lipid content and class analyses from herring larvae reared on San Francisco Bay *Artemia*

Tank 1

The results given below are the means from duplicate analyses computed after arcsin transformation of the data, standard deviations were <0.5% unless shown.
Data for 10 days post-hatch is derived from a single stock tank.
T = trace, detected by HPTLC but below the resolution of Iatroscan analysis, class comprises <1.0% of total lipid.
FR = Failure to resolve lipid class.
- = Lipid class was not detected.

	Age (days post-hatch)				
Lipid class (%)	10	15	25	35	45
Sterol/Wax esters	2.5	3.2	2.0	1.9	2.2
Triacylglycerol	18.9	15.0	22.7	29.4	25.0
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	11.7	13.4	8.5	8.7	10.4
Phosphatidylethanolamine	23.2	26.1	28.1	26.2	29.9
Phosphatidylserine/inositol	3.2	4.1	1.9	FR	FR
Phosphatidylcholine	37.2	33.7	32.9	30.5	28.9
Sphingomyelin	3.3	4.5	3.9	3.3	3.6
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	33.1	31.6	33.2	40.0	37.6
Total polar classes	66.9	68.4	66.8	60.0	62.4
Triacylglycerol/Cholesterol	1.6	1.1	2.7	3.4	2.4
Larval dry weight (mg larva^{-1})	0.134	0.173	0.276	0.402	0.596
Lipid (µg larva^{-1})	31.2	26.7	26.4	55.8	61.9
Lipid (% larval dry weight)	23.3	15.4	9.6	13.9	10.4

The lipid class contents of the larvae in absolute terms were computed by combining the above data.

	Age (days post-hatch)				
Lipid class (µg larva^{-1})	10	15	25	35	45
Sterol/Wax esters	0.8	0.9	0.5	1.1	1.4
Triacylglycerol	5.9	4.0	6.0	16.4	15.5
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	3.7	3.6	2.2	4.9	6.4
Phosphatidylethanolamine	7.2	7.0	7.4	14.6	18.5
Phosphatidylserine/inositol	1.0	1.0	0.5	FR	FR
Phosphatidylcholine	11.6	9.0	8.8	17.0	17.9
Sphingomyelin	1.0	1.2	1.0	1.8	2.2
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	10.4	8.5	8.7	22.4	23.3
Total polar classes	20.8	18.2	17.7	33.4	38.6

Table III,vii Lipid content and class analyses from herring larvae reared on San Francisco Bay *Artemia*

Tank 3

The results given below are the means from duplicate analyses computed after arcsin transformation of the data, standard deviations were <0.5% unless shown.
Data for 10 days post-hatch is derived from a single stock tank.
T = trace, detected by HPTLC but below the resolution of Iatroscan analysis, class comprises <1.0% of total lipid.
FR = Failure to resolve lipid class.
- = Lipid class was not detected.

	Age (days post-hatch)				
Lipid class (%)	10	15	25	35	45
Sterol/Wax esters	2.5	4.3	1.9	1.8	3.1
Triacylglycerol	18.9	15.4	24.6	25.6	26.9
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	11.7	12.5	7.4	8.3	9.4
Phosphatidylethanolamine	23.2	25.0	23.4	25.7	26.3
Phosphatidylserine/inositol	3.2	2.3	5.7	FR	FR
Phosphatidylcholine	37.2	36.5	32.7	34.9	32.5
Sphingomyelin	3.3	4.0	4.3	3.7	1.8
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	33.1	32.2	33.9	35.6	39.4
Total polar classes	66.9	67.8	66.1	64.4	60.6
Triacylglycerol/Cholesterol	1.6	1.2	4.4	3.1	2.9
Larval dry weight (mg larva^{-1})	0.134	0.154	0.314	0.359	0.623
Lipid (µg larva^{-1})	31.2	24.7	38.4	63.0	65.5
Lipid (% larval dry weight)	23.3	16.0	12.2	17.5	10.5

The lipid class contents of the larvae in absolute terms were computed by combining the above data.

	Age (days post-hatch)				
Lipid class (µg larva^{-1})	10	15	25	35	45
Sterol/Wax esters	0.8	1.1	0.7	1.1	2.0
Triacylglycerol	5.9	3.8	9.4	16.1	17.6
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	3.7	3.1	2.8	5.2	6.2
Phosphatidylethanolamine	7.2	6.2	9.0	16.2	17.2
Phosphatidylserine/inositol	1.0	0.5	2.2	FR	FR
Phosphatidylcholine	11.6	9.0	12.6	22.1	21.3
Sphingomyelin	1.0	1.0	1.7	2.3	1.2
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	10.4	8.0	12.9	22.4	25.8
Total polar classes	20.8	16.7	25.5	40.6	39.7

Table III,viii Lipid content and class analyses from herring larvae reared on San Francisco Bay *Artemia*

Tank 5

The results given below are the means from duplicate analyses computed after arcsin transformation of the data, standard deviations were <0.5% unless shown.
Data for 10 days post-hatch are derived from a single stock tank.
T = trace, detected by HPTLC but below the resolution of Iatroscan analysis, class comprises <1.0% of total lipid.
FR = Failure to resolve lipid class.
- = Lipid class was not detected.

	Age (days post-hatch)				
Lipid class (%)	10	15	25	35	45
Sterol/Wax esters	2.5	3.7	2.1	1.3	1.3
Triacylglycerol	18.9	16.7	32.2	27.7	25.6
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	11.7	11.3	6.9	7.7	8.5
Phosphatidylethanolamine	23.2	28.3	25.6	24.4	32.2
Phosphatidylserine/inositol	3.2	2.6	FR	FR	FR
Phosphatidylcholine	37.2	33.9	30.3	36.3	30.0
Sphingomyelin	3.3	3.5	2.9	2.6	2.4
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	33.1	31.7	41.2	36.7	35.4
Total polar classes	66.9	68.3	58.9	63.3	64.6
Triacylglycerol/Cholesterol	1.6	1.5	4.7	3.6	3.0
Larval dry weight (mg larva^{-1})	0.134	0.170	0.317	0.351	0.607
Lipid (µg larva^{-1})	31.2	25.6	40.5	64.1	73.7
Lipid (% larval dry weight)	23.3	15.1	12.8	18.3	12.1

The lipid class contents of the larvae in absolute terms were computed by combining the above data.

	Age (days post-hatch)				
Lipid class (µg larva^{-1})	10	15	25	35	45
Sterol/Wax esters	0.8	0.9	0.9	0.8	1.0
Triacylglycerol	5.9	4.3	13.0	17.8	18.9
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	3.7	2.9	2.8	4.9	6.3
Phosphatidylethanolamine	7.2	7.2	10.4	15.6	23.7
Phosphatidylserine/inositol	1.0	0.7	FR	FR	FR
Phosphatidylcholine	11.6	8.7	12.3	23.3	22.1
Sphingomyelin	1.0	0.9	1.1	1.7	1.7
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	10.4	8.1	16.7	23.5	26.2
Total polar classes	20.8	17.5	23.8	40.6	47.5

Table III,ix Lipid content and class analyses from herring larvae reared on Great Salt Lake *Artemia*

Tank 2

The results given below are the means from duplicate analyses computed after arcsin transformation of the data, standard deviations were <0.5% unless shown.
Data for 10 days post-hatch are derived from a single stock tank.
T = trace, detected by HPTLC but below the resolution of Iatroscan analysis, class comprises <1.0% of total lipid.
FR = Failure to resolve lipid class.
- = Lipid class was not detected.

	Age (days post-hatch)				
Lipid class (%)	10	15	25	35	45
Sterol/Wax esters	2.5	3.8	2.3	1.5	1.8
Triacylglycerol	18.9	18.3	22.9	26.8	19.7
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	11.7	12.5	8.2	11.9	11.4
Phosphatidylethanolamine	23.2	25.7	30.6	26.7	32.3
Phosphatidylserine/inositol	3.2	1.0	0.6	FR	FR
Phosphatidylcholine	37.2	33.6	31.2	30.2	31.7
Sphingomyelin	3.3	5.1	4.2	2.9	3.1
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	33.1	34.6	33.4	40.2	32.9
Total polar classes	66.9	65.4	66.6	59.8	67.1
Triacylglycerol/Cholesterol	1.6	1.5	2.8	2.3	1.7
Larval dry weight (mg larva^{-1})	0.134	0.157	0.317	0.529	0.393
Lipid (µg larva^{-1})	31.2	26.4	42.0	45.0	52.5
Lipid (% larval dry weight)	23.3	16.8	13.2	8.5	13.4

The lipid class contents of the larvae in absolute terms were computed by combining the above data.

	Age (days post-hatch)				
Lipid class (µg larva^{-1})	10	15	25	35	45
Sterol/Wax esters	0.8	1.0	1.0	0.7	0.9
Triacylglycerol	5.9	4.8	9.6	12.1	10.3
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	3.7	3.3	3.4	5.4	6.0
Phosphatidylethanolamine	7.2	6.8	12.9	12.0	17.0
Phosphatidylserine/inositol	1.0	0.3	0.3	FR	FR
Phosphatidylcholine	11.6	8.9	13.1	13.6	16.7
Sphingomyelin	1.0	1.3	1.7	1.3	1.6
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	10.4	9.1	14.0	18.2	17.2
Total polar classes	20.8	17.3	28.0	26.9	35.3

Table III,x Lipid content and class analyses from herring larvae reared on Great Salt Lake *Artemia*

Tank 4

The results given below are the means from duplicate analyses computed after arcsin transformation of the data, standard deviations were <0.5% unless shown.
Data for 10 days post-hatch are derived from a single stock tank.
T = trace, detected by HPTLC but below the resolution of Iatroscan analysis, class comprises <1.0% of total lipid.
FR = Failure to resolve lipid class.
- = Lipid class was not detected.

	Age (days post-hatch)				
Lipid class (%)	10	15	25	35	45
Sterol/Wax esters	2.5	3.8	2.6	1.8	1.2
Triacylglycerol	18.9	19.0	27.4	25.0	23.3
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	11.7	11.6	7.7	8.6	10.3
Phosphatidylethanolamine	23.2	25.9	25.9	26.9	32.0
Phosphatidylserine/inositol	3.2	2.5	3.2	FR	FR
Phosphatidylcholine	37.2	33.2	30.8	33.9	30.8
Sphingomyelin	3.3	4.0	3.0	3.8	2.4
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	33.1	34.3	37.7	35.4	34.8
Total polar classes	66.9	65.7	62.3	65.6	65.2
Triacylglycerol/Cholesterol	1.6	1.6	3.6	2.9	2.3
Larval dry weight (mg larva^{-1})	0.134	0.170	0.301	0.463	0.522
Lipid (µg larva^{-1})	31.2	23.9	45.3	62.9	86.4
Lipid (% larval dry weight)	23.3	14.1	15.0	13.6	16.6

The lipid class contents of the larvae in absolute terms were computed by combining the above data.

	Age (days post-hatch)				
Lipid class (µg larva^{-1})	10	15	25	35	45
Sterol/Wax esters	0.8	0.9	1.2	1.1	1.0
Triacylglycerol	5.9	4.5	12.4	15.7	20.1
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	3.7	2.8	3.5	5.4	8.9
Phosphatidylethanolamine	7.2	6.2	11.7	16.9	27.6
Phosphatidylserine/inositol	1.0	0.6	1.4	FR	FR
Phosphatidylcholine	11.6	7.9	14.0	21.4	26.7
Sphingomyelin	1.0	1.0	1.1	2.4	2.1
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	10.4	8.2	17.1	22.2	30.0
Total polar classes	20.8	15.7	28.2	40.7	56.4

Table III,xi Lipid content and class analyses from herring larvae reared on Great Salt Lake *Artemia*

Tank 6

The results given below are the means from duplicate analyses computed after arcsin transformation of the data, standard deviations were <0.5% unless shown.
Data for 10 days post-hatch are derived from a single stock tank.
T = trace, detected by HPTLC but below the resolution of Iatroscan analysis, class comprises <1.0% of total lipid.
FR = Failure to resolve lipid class.
- = Lipid class was not detected.

	Age (days post-hatch)				
Lipid class (%)	10	15	25	35	45
Sterol/Wax esters	2.5	4.2	1.9	1.4	1.6
Triacylglycerol	18.9	18.6	30.0	24.8	22.2
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	11.7	11.9	9.3	9.5	11.1
Phosphatidylethanolamine	23.2	27.7	26.6	27.5	32.8
Phosphatidylserine/inositol	3.2	FR	FR	FR	FR
Phosphatidylcholine	37.2	32.9	30.6	34.7	30.0
Sphingomyelin	3.3	4.7	2.2	2.1	2.3
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	33.1	34.7	41.2	35.7	34.9
Total polar classes	66.9	65.3	58.8	64.3	65.1
Triacylglycerol/Cholesterol	1.6	1.6	3.2	2.6	2.0
Larval dry weight (mg larva^{-1})	0.134	0.175	0.348	0.380	0.416
Lipid (µg larva^{-1})	31.2	23.0	36.9	47.6	53.2
Lipid (% larval dry weight)	23.3	13.1	10.6	12.5	12.8

The lipid class contents of the larvae in absolute terms were computed by combining the above data.

	Age (days post-hatch)				
Lipid class (µg larva^{-1})	10	15	25	35	45
Sterol/Wax esters	0.8	1.0	0.7	0.7	0.9
Triacylglycerol	5.9	4.3	11.1	11.8	11.8
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	3.7	2.7	3.4	4.5	5.9
Phosphatidylethanolamine	7.2	6.4	9.7	13.1	17.4
Phosphatidylserine/inositol	1.0	FR	FR	FR	FR
Phosphatidylcholine	11.6	7.6	11.2	16.5	16.0
Sphingomyelin	1.0	1.0	0.8	1.0	1.2
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	10.4	8.0	15.2	17.0	18.6
Total polar classes	20.8	15.0	21.7	30.6	34.6

Table III,xii Lipid content and class analyses from pooled samples of herring larvae reared on San Francisco Bay *Artemia*

The results given below are given as the means of the previous analyses pooled by diet. The means were computed after arcsin transformation of the data.
Standard deviations were <0.5% unless shown.
Data for 10 days post-hatch are derived from a single stock tank.
T = trace, detected by HPTLC but below the resolution of Iatroscan analysis, class comprises <1.0% of total lipid.
FR = Failure to resolve lipid class.
- = Lipid class was not detected.

Lipid class (%)	Age (days post-hatch) 10	15	25	35	45
Sterol/Wax esters	2.5	3.8	2.0	1.7	2.1
Triacylglycerol	18.9	15.7	26.4	27.5	25.8
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	11.7	12.3	7.6	8.2	9.4
Phosphatidylethanolamine	23.2	26.4	27.0	25.4	29.4
Phosphatidylserine/inositol	3.2	3.0	FR	FR	FR
Phosphatidylcholine	37.2	34.8	33.3	33.9	30.7
Sphingomyelin	3.3	4.0	3.7	3.3	2.6
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	33.1	31.8	36.0	37.4	37.3
Total polar classes	66.9	68.2	64.0	62.6	62.7
Triacylglycerol/Cholesterol mean	1.6	1.3	3.9	3.4	2.8
± std dev	-	0.2	1.1	0.3	0.3
Larval dry weight (mg larva^{-1}) median	0.134	0.168	0.295	0.376	0.612
Lipid (µg larva^{-1}) mean	31.2	25.6	38.7	61.0	67.0
± std dev	0.6	1.0	3.2	4.5	6.7
Lipid (% larval dry weight) lipid/median	23.3	15.2	13.1	16.2	10.9

Lipid class (µg larva^{-1})	Age (days post-hatch) 10	15	25	35	45
Triacylglycerol mean	5.9	4.0	10.2	16.8	17.3
± std dev	0.1	0.2	0.8	1.2	1.7
Total polar classes mean	20.9	17.5	24.8	38.2	42.0
± std dev	0.4	0.7	2.0	2.8	4.2

Table III,xiii Lipid content and class analyses from pooled samples of herring larvae reared on Great Salt Lake *Artemia*

Notes as for Table III,xii.

Lipid class (%)	Age (days post-hatch) 10	15	25	35	45
Sterol/Wax esters	2.5	3.9	2.4	1.6	1.7
Triacylglycerol	18.9	18.6	26.8	25.5	21.8
Free fatty acids	T	T	T	T	T
Diacylglycerols	T	T	T	T	T
Monoacylglycerols	T	T	T	T	T
Cholesterol	11.7	12.0	8.5	10.0	11.0
Phosphatidylethanolamine	23.2	27.7	28.3	27.0	32.6
Phosphatidylserine/inositol	3.2	FR	FR	FR	FR
Phosphatidylcholine	37.2	33.2	30.8	33.0	30.9
Sphingomyelin	3.3	4.6	3.2	2.9	2.0
Lysophosphatidylcholine	-	-	-	-	-
Total neutral classes	33.1	34.5	37.7	37.1	34.5
Total polar classes	66.9	65.5	62.3	62.9	65.5
Triacylglycerol/Cholesterol mean	1.6	1.6	3.2	2.6	2.0
± std dev	-	0.1	0.4	0.3	0.3
Larval dry weight (mg larva^{-1}) median	0.134	0.164	0.288	0.453	0.439
Lipid (µg larva^{-1}) mean	31.2	24.4	41.4	51.8	66.3
± std dev	0.6	2.5	4.9	9.0	19.3
Lipid (% larval dry weight) lipid/median	23.3	14.9	14.4	11.4	15.1

Lipid class (µg larva^{-1})	Age (days post-hatch) 10	15	25	35	45
Triacylglycerol mean	5.9	4.5	11.1	13.2	14.5
± std dev	0.1	0.5	1.3	2.3	4.2
Total polar classes mean	20.9	16.0	25.8	32.6	43.4
± std dev	0.4	1.6	3.1	5.7	12.6

Table III,xiv Fatty acid spectra of San Francisco Bay *Artemia* nauplii

The results of analyses of three samples of newly hatched nauplii collected at intervals during the rearing trial are given.
Samples were analysed with 23:0 internal standard as described in the text.
The means and standard deviations were computed after arcsin transformation of percentage data.
T = Trace, fatty acid was detected but constituded <0.1% of the total lipid.
- = Fatty acid was not detected.

Percentage composition of total lipid

Fatty acid	Samples 1	2	3	Mean ± sd
14:0	1.1	0.7	0.7	0.8 ± 0.01
15:0	0.4	0.3	0.3	0.3 ± 0.02
16:0	8.9	7.8	7.5	8.1 ± 0.02
16:1	7.6	8.7	9.0	8.4 ± 0.02
16:2	0.8	0.7	0.5	0.7 ± 0.01
16:3	1.3	1.3	0.6	1.0 ± 0.05
16:4	0.1	-	-	T
18:0	2.8	3.1	3.0	3.0 ± 0.02
18:1(n-9)	13.2	12.2	11.4	12.3 ± 0.02
18:1(n-7)	8.1	8.6	9.1	8.6 ± 0.01
18:2(n-6)	4.9	3.4	3.6	3.9 ± 0.04
18:3(n-6)	0.2	0.4	0.4	0.3 ± 0.01
18:3(n-3)	10.7	9.3	9.9	10.0 ± 0.01
18:4(n-3)	1.6	1.1	1.2	1.3 ± 0.01
20:0	-	-	0.1	T
20:1	0.3	0.4	0.4	0.4 ± 0
20:2(n-6)	0.1	T	0.1	T
20:3(n-6)	0.2	0.2	0.2	0.2 ± 0
20:4(n-6)	2.0	2.0	1.9	2.0 ± 0
20:4(n-3)	0.3	0.3	0.3	0.3 ± 0
20:5(n-3)	5.2	5.5	5.1	5.3 ± 0
22:0	-	-	-	-
22:1	-	0.2	0.2	0.1 ± 0.1
22:5(n-3)	-	0.1	0.2	0.1 ± 0.1
22:6(n-3)	-	-	-	-
24:1	-	-	-	-
Total unknowns	1.3	0.6	0.4	0.7 ± 0.07
Total sats	13.3	12.0	12.0	12.4 ± 0.01
Total monoenes	29.2	30.0	29.8	29.7 ± 0
Total (n-3)	17.9	16.4	16.6	17.0 ± 0.01
Total (n-6)	7.4	6.1	6.2	6.6 ± 0.02
(n-3)/(n-6)	3.7	2.7	2.7	2.6 ± 0.03
Fatty acids	71.1	67.1	66.1	67.8 ± 0.08
Non fatty acids	28.9	32.9	33.9	32.2 ± 0.08

Table III,xv Fatty acid spectra of Great Salt Lake *Artemia* nauplii

Notes as for Table III,xiv.

Percentage composition of total lipid

Fatty acid	Samples 1	2	3	Mean ± sd
14:0	0.8	0.7	0.6	0.7 ± 0
15:0	0.2	0.1	0.1	0.1 ± 0.01
16:0	9.7	7.9	9.0	8.9 ± 0.03
16:1	3.5	3.1	2.2	2.9 ± 0.04
16:2	0.5	0.5	0.5	0.5 ± 0
16:3	0.5	1.0	0.7	0.7 ± 0.02
16:4	1.2	-	-	0.1 ± 0.4
18:0	3.4	3.1	3.6	3.4 ± 0.01
18:1(n-9)	16.5	12.8	15.5	14.9 ± 0.07
18:1(n-7)	6.2	6.0	5.4	5.9 ± 0.01
18:2(n-6)	5.1	3.9	5.5	4.8 ± 0.04
18:3(n-6)	0.2	0.2	0.3	0.2 ± 0
18:3(n-3)	21.3	17.3	18.7	19.1 ± 0.07
18:4(n-3)	2.6	2.2	3.2	2.7 ± 0.02
20:0	0.1	0.1	0.1	0.1 ± 0
20:1	0.4	0.4	0.4	0.4 ± 0
20:2(n-6)	0.1	0.1	0.2	0.1 ± 0.01
20:3(n-6)	-	-	-	-
20:4(n-6)	0.3	0.3	0.4	0.3 ± 0
20:4(n-3)	0.3	0.3	0.4	0.3 ± 0
20:5(n-3)	2.1	1.8	2.2	2.0 ± 0.01
22:0	-	-	-	-
22:1	0.2	0.2	0.2	0.2 ± 0
22:5(n-3)	0.1	-	0.1	T
22:6(n-3)	-	-	-	-
24:1	-	-	-	-
Total unknowns	3.5	4.1	7.0	4.8 ± 0.2
Total sats	14.2	11.9	13.6	13.2 ± 0.03
Total monoenes	41.2	34.4	23.5	32.8 ± 0.9
Total (n-3)	26.4	21.6	24.6	24.3 ± 0.08
Total (n-6)	5.4	4.5	6.4	5.4 ± 0.04
(n-3)/(n-6)	4.9	4.8	3.8	4.5 ± 0.02
Fatty acids	78.8	66.1	76.3	73.1 ± 0.6
Non fatty acids	21.2	33.9	23.7	26.9 ± 0.6

Table III,xvi Size, weight, lipid and fatty acid content of newly hatched *Artemia* nauplii

Lengths and weights were determined as given in the text. Means and standard deviations of percentages were computed after arcsin transformation.

	San Francisco Bay *Artemia* mean ± sd	n	Great Salt Lake *Artemia* mean ± sd	n
Body length (µm)	555 ± 71	30	635 ± 117	30
Body width (µm)	171 ± 14	30	200 ± 20	30
Dry weight (µg)	1.02 ± 0.37	9	2.04 ± 0.26	9
Lipid (% dry weight)	13.5 ± 0.003	3	13.8 ± 0.3	3
Lipid (µg nauplius^{-1})	0.138 ± 0.05	3	0.282 ± 0.04	3
Fatty acids (pg nauplius^{-1})				
14:0	1.1 ± 0.4		2.0 ± 0	
15:0	0.4 ± 0.2		0.3 ± 0.3	
16:0	11.2 ± 4.1		25.1 ± 0.4	
16:1	11.6 ± 4.2		8.2 ± 0.2	
16:2	1.0 ± 0.4		1.4 ± 0	
16:3	1.4 ± 0.5		2.0 ± 0.1	
16:4	T		0.3 ± 1.1	
18:0	4.1 ± 1.5		9.6 ± 0.1	
18:1(n-9)	17.0 ± 6.2		42.0 ± 0.8	
18:1(n-7)	11.9 ± 4.3		16.6 ± 0.3	
18:2(n-6)	5.4 ± 2.0		13.5 ± 0.3	
18:3(n-6)	0.4 ± 0.2		0.6 ± 0	
18:3(n-3)	13.8 ± 5.0		53.9 ± 1.0	
18:4(n-3)	1.8 ± 0.7		7.6 ± 0.2	
20:0	T		0.3 ± 0	
20:1	0.6 ± 0.2		1.1 ± 0	
20:2(n-6)	T		0.3 ± 0	
20:3(n-6)	0.3 ± 0.1		-	
20:4(n-6)	2.8 ± 1.0		0.8 ± 0.2	
20:4(n-3)	0.4 ± 0.3		0.8 ± 0	
20:5(n-3)	7.3 ± 2.7		5.6 ± 0.1	
22:0	-		-	
22:1	0.1 ± 0.1		0.6 ± 0	
22:5(n-3)	0.1 ± 0.1		T	
22:6(n-3)	-		-	
24:1	-		-	
Total unknowns	1.1 ± 0		13.5 ± 0.8	
Total sats	17.1 ± 6.2		37.2 ± 0.6	
Total monenes	41.0 ± 14.9		92.5 ± 3.8	
Total (n-3).	23.4 ± 8.5		68.5 ± 1.2	
Total (n-6)	8.9 ± 3.3		15.2 ± 0.3	
(n-3)/(n-6)	2.6 ± 0.03		4.5 ± 0.02	
Fatty acids	93.6 ± 34.0		206.1 ± 4.6	
Non fatty acids	44.4 ± 16.2		75.9 ± 2.7	

Table III,xvii Fatty acid spectra of total lipid in herring larvae from single stock tank, aged 10 days post-hatch

The results of analyses of samples from three stock tanks are given.
Samples were analysed with 23:0 internal standard as described in the text.
The means and standard deviations were computed after arcsin transformation of percentage data.
T = Trace, fatty acid was detected but constituded <0.1% of the total lipid.
- = Fatty acid was not detected.

Percentage composition of total lipid

Fatty acid	Tanks 1	2	3	Mean ± sd
14:0	1.1	1.1	1.1	1.1 ± 0
15:0	0.2	0.2	0.2	0.2 ± 0
16:0	12.6	11.8	10.8	11.9 ± 0.02
16:1	3.2	3.1	2.3	2.9 ± 0.002
16:2	0.7	0.2	0.3	0.4 ± 0.04
16:3	0.2	0.2	0.2	0.2 ± 0
16:4	T	0.1	0.2	0.1 ± 0.05
18:0	2.3	2.1	2.0	2.1 ± 0.003
18:1(n-9)	6.8	7.0	6.0	6.6 ± 0.01
18:1(n-7)	2.9	2.8	2.4	2.7 ± 0.007
18:2(n-6)	0.6	0.7	0.7	0.7 ± 0.001
18:3(n-6)	-	0.2	0.1	0.1 ± 0.05
18:3(n-3)	0.4	0.3	0.4	0.4 ± 0.002
18:4(n-3)	0.6	T	0.3	0.2 ± 0.2
20:0	-	-	-	-
20:1	0.5	0.7	0.6	0.6 ± 0.004
20:2(n-6)	T	T	T	T
20:3(n-6)	-	-	-	-
20:4(n-6)	0.9	0.7	0.7	0.8 ± 0.004
20:4(n-3)	0.3	0.2	0.3	0.3 ± 0.003
20:5(n-3)	6.8	7.0	6.2	6.7 ± 0.007
22:0	-	-	-	-
22:1	0.2	0.2	0.2	0.2 ± 0
22:5(n-3)	0.2	0.8	0.6	0.7 ± 0.004
22:6(n-3)	17.6	15.8	13.5	15.6 ± 0.08
24:1	0.2	0.3	T	0.1 ± 0.1
Total unknowns	0.5	0.7	0.8	0.7 ± 0.009
Total sats	16.1	15.1	14.0	15.3 ± 0.02
Total monoenes	13.8	14.0	11.4	13.0 ± 0.05
Total (n-3)	26.3	24.2	21.3	23.9 ± 0.02
Total (n-6)	1.5	1.7	1.5	1.6 ± 0.002
(n-3)/(n-6)	18.0	14.3	14.6	14.9 ± 0.08
Fatty acids	59.2	56.2	49.6	55.2 ± 0.2
Non fatty acids	40.8	43.8	50.4	44.8 ± 0.2

Table III,xviii Fatty acid spectra of total lipid in herring larvae aged 15 days post-hatch and reared on San Francisco Bay *Artemia*

Samples were analysed with 23:0 internal standard as described in the text.
The means and standard deviations were computed after arcsin transformation of percentage data.
T = Trace, fatty acid was detected but constituded <0.1% of the total lipid.
- = Fatty acid not detected.

Percentage composition of total lipid

Fatty acid	Tanks 1	3	5	Mean ± sd
14:0	0.8	0.6	0.5	0.6 ± 0.009
15:0	0.2	0.2	0.2	0.2 ± 0
16:0	11.5	10.3	10.1	10.6 ± 0.01
16:1	2.2	2.8	2.4	2.5 ± 0.01
16:2	0.3	0.6	0.3	0.4 ± 0.02
16:3	0.3	0.2	0.4	0.3 ± 0.009
16:4	0.2	0.1	0.2	0.2 ± 0.006
18:0	2.7	2.5	2.6	2.6 ± 0.001
18:1(n-9)	7.1	6.7	6.7	6.8 ± 0.002
18:1(n-7)	3.1	3.1	3.2	3.1 ± 0.0002
18:2(n-6)	0.8	1.0	0.9	0.9 ± 0.003
18:3(n-6)	0.1	T	0.1	T
18:3(n-3)	0.8	0.9	1.1	0.9 ± 0.006
18:4(n-3)	0.2	0.4	0.2	0.3 ± 0.01
20:0	T	T	T	T
20:1	0.5	0.4	0.4	0.4 ± 0.002
20:2(n-6)	T	T	0.1	T
20:3(n-6)	T	-	-	T
20:4(n-6)	1.1	1.0	1.0	1.0 ± 0.0008
20:4(n-3)	0.3	0.3	0.2	0.3 ± 0.003
20:5(n-3)	7.4	5.8	5.5	6.2 ± 0.04
22:0	-	-	-	-
22:1	0.1	0.3	0.1	0.2 ± 0.02
22:5(n-3)	0.6	0.6	0.5	0.6 ± 0.002
22:6(n-3)	15.3	12.8	12.4	13.5 ± 0.03
24:1	T	T	T	T
Total unknowns	1.2	1.3	0.9	1.1 ± 0.01
Total sats	15.2	13.7	13.4	14.0 ± 0.02
Total monoenes	13.1	13.3	12.8	13.0 ± 0.001
Total (n-3)	24.6	20.8	20.0	21.8 ± 0.09
Total (n-6)	1.9	2.0	2.1	1.9 ± 0.001
(n-3)/(n-6)	12.6	10.4	9.4	11.5 ± 0.07
Fatty acids	56.9	51.9	50.1	52.7 ± 0.1
Non fatty acids	43.1	48.1	49.9	47.3 ± 0.1

Table III,xix Fatty acid spectra of total lipid in herring larvae aged 15 days post-hatch and reared on Great Salt Lake *Artemia*

Notes as for Table III,xviii.

Percentage composition of total lipid

Fatty acid	Tanks 2	4	6	Mean ± sd
14:0	0.7	0.6	0.7	0.7 ± 0.001
15:0	0.2	0.2	0.2	0.2 ± 0
16:0	10.3	9.7	10.0	10.0 ± 0.003
16:1	2.4	3.3	2.6	2.8 ± 0.02
16:2	0.2	0.2	0.3	0.2 ± 0.003
16:3	0.4	0.4	0.3	0.4 ± 0.002
16:4	0.2	0.2	0.2	0.2 ± 0
18:0	2.6	2.6	2.5	2.6 ± 0.0003
18:1(n-9)	7.0	7.3	6.7	7.0 ± 0.004
18:1(n-7)	3.3	3.6	3.2	3.4 ± 0.003
18:2(n-6)	1.0	1.1	1.0	1.0 ± 0.0008
18:3(n-6)	0.1	0.1	0.1	0.1 ± 0
18:3(n-3)	1.5	1.9	1.2	1.5 ± 0.02
18:4(n-3)	0.3	0.3	0.2	0.3 ± 0.003
20:0	T	T	T	T
20:1	0.4	0.4	0.4	0.4 ± 0
20:2(n-6)	T	0.1	T	T
20:3(n-6)	0.1	T	-	T
20:4(n-6)	1.1	1.2	1.0	1.1 ± 0.002
20:4(n-3)	0.3	0.2	0.3	0.3 ± 0.003
20:5(n-3)	5.8	5.3	5.4	5.5 ± 0.003
22:0	0.1	T	T	T
22:1	0.2	0.1	0.2	0.2 ± 0.006
22:5(n-3)	0.5	0.5	0.5	0.5 ± 0
22:6(n-3)	12.8	10.9	11.8	11.8 ± 0.02
24:1	-	-	-	-
Total unknowns	0.6	0.5	1.2	0.7 ± 0.04
Total sats	13.9	13.1	13.4	13.5 ± 0.004
Total monoenes	13.2	14.6	13.0	13.8 ± 0.02
Total (n-3)	21.1	19.1	19.4	19.9 ± 0.02
Total (n-6)	2.3	2.5	2.0	2.2 ± 0.007
(n-3)/(n-6)	9.1	7.8	9.5	9.0 ± 0.03
Fatty acids	52.0	50.7	49.9	50.9 ± 0.01
Non fatty acids	48.0	49.3	50.1	49.1 ± 0.01

Table III,xx Fatty acid spectra of total lipid in herring larvae aged 25 days post-hatch and reared on San Francisco Bay *Artemia*

Samples were analysed with 23:0 internal standard as described in the text.
The means and standard deviations were computed after arcsin transformation of percentage data.
T = Trace, fatty acid was detected but constituded <0.1% of the total lipid.
- = Fatty acid not detected.

Percentage composition of total lipid

Fatty acid	Tanks 1	3	5	Mean ± sd
14:0	0.4	0.4	0.4	0.4 ± 0
15:0	0.2	0.2	0.2	0.2 ± 0
16:0	8.2	7.4	6.5	7.3 ± 0.03
16:1	5.2	4.8	3.2	4.4 ± 0.07
16:2	0.4	0.5	0.4	0.4 ± 0.002
16:3	0.6	0.5	0.7	0.6 ± 0.004
16:4	T	0.1	0.1	T
18:0	3.1	2.5	2.7	2.8 ± 0.009
18:1(n-9)	9.4	7.7	8.5	8.5 ± 0.02
18:1(n-7)	6.5	5.1	4.6	5.4 ± 0.05
18:2(n-6)	2.8	2.0	2.0	2.3 ± 0.02
18:3(n-6)	0.2	0.2	0.2	0.2 ± 0
18:3(n-3)	4.9	4.5	4.4	4.6 ± 0.004
18:4(n-3)	0.8	0.7	0.7	0.7 ± 0.001
20:0	T	T	0.1	T
20:1	0.3	0.4	0.3	0.3 ± 0.003
20:2(n-6)	0.1	T	0.1	T
20:3(n-6)	0.1	0.1	0.1	0.1 ± 0
20:4(n-6)	2.1	2.0	1.9	2.0 ± 0.001
20:4(n-3)	0.2	0.3	0.2	0.2 ± 0.003
20:5(n-3)	5.7	5.8	4.9	5.5 ± 0.01
22:0	-	-	-	-
22:1	0.2	0.3	0.1	0.2 ± 0.01
22:5(n-3)	0.3	0.3	0.3	0.3 ± 0
22:6(n-3)	6.6	6.6	5.7	6.3 ± 0.01
24:1	T	T	0.1	T
Total unknowns	0.4	1.0	0.4	0.6 ± 0.05
Total sats	12.0	10.5	9.7	10.7 ± 0.03
Total monoenes	21.7	18.3	16.8	18.8 ± 0.1
Total (n-3)	18.5	18.2	16.2	17.6 ± 0.03
Total (n-6)	5.3	4.3	4.2	4.6 ± 0.02
(n-3)/(n-6)	3.5	4.2	3.8	3.8 ± 0.009
Fatty acids	58.9	53.4	48.4	53.3 ± 0.3
Non fatty acids	41.1	46.6	51.6	46.7 ± 0.3

Table III,xxi Fatty acid spectra of total lipid in herring larvae aged 25 days post-hatch and reared on Great Salt Lake *Artemia*

Notes as for Table III,xx.

Percentage composition of total lipid

Fatty acid	Tanks 2	4	6	Mean ± sd
14:0	0.6	0.5	0.5	0.5 ± 0.002
15:0	0.1	0.1	0.1	0.1 ± 0
16:0	8.1	7.4	8.0	7.8 ± 0.005
16:1	3.3	3.3	2.6	3.1 ± 0.01
16:2	0.3	0.4	0.5	0.4 ± 0.006
16:3	0.7	0.6	0.6	0.6 ± 0.001
16:4	T	T	T	T
18:0	3.1	2.8	3.1	3.0 ± 0.003
18:1(n-9)	11.0	9.7	10.3	10.3 ± 0.01
18:1(n-7)	5.1	4.6	4.3	4.7 ± 0.009
18:2(n-6)	3.2	3.3	3.8	3.4 ± 0.008
18:3(n-6)	0.1	0.1	0.1	0.1 ± 0
18:3(n-3)	8.0	7.0	7.3	7.4 ± 0.01
18:4(n-3)	1.4	1.4	1.3	1.4 ± 0.0006
20:0	T	T	0.1	T
20:1	0.3	0.3	0.3	0.3 ± 0
20:2(n-6)	0.1	0.1	0.1	0.1 ± 0
20:3(n-6)	T	T	T	T
20:4(n-6)	1.2	1.5	1.5	1.4 ± 0.006
20:4(n-3)	0.3	0.3	0.3	0.3 ± 0
20:5(n-3)	4.3	4.1	4.3	4.2 ± 0.0009
22:0	T	T	T	T
22:1	0.2	T	0.2	0.1 ± 0.07
22:5(n-3)	0.3	0.3	0.3	0.3 ± 0
22:6(n-3)	6.4	5.8	6.7	6.3 ± 0.009
24:1	0.1	0.1	0.2	0.1 ± 0.006
Total unknowns	0.8	0.2	0.5	0.5 ± 0.05
Total sats	12.0	10.8	11.8	11.4 ± 0.01
Total monoenes	20.0	17.9	17.8	18.6 ± 0.03
Total (n-3)	20.6	18.9	20.1	19.9 ± 0.01
Total (n-6)	4.6	5.1	5.5	5.0 ± 0.01
(n-3)/(n-6)	4.5	3.7	3.6	4.0 ± 0.02
Fatty acids	59.0	54.0	56.9	56.4 ± 0.06
Non fatty acids	41.0	46.0	43.1	43.6 ± 0.06

Table III,xxii Fatty acid spectra of total lipid in herring larvae aged 35 days post-hatch and reared on San Francisco Bay *Artemia*

Samples were analysed with 23:0 internal standard as described in the text.
The means and standard deviations were computed after arcsin transformation of percentage data.
T = Trace, fatty acid was detected but constituded <0.1% of the total lipid.
- = Fatty acid not detected.

Percentage composition of total lipid

Fatty acid	Tanks 1	3	5	Mean ± sd
14:0	0.4	0.3	0.4	0.4 ± 0.002
15:0	0.1	0.1	0.1	0.1 ± 0
16:0	7.0	6.5	7.0	6.8 ± 0.003
16:1	3.9	3.6	4.1	3.9 ± 0.004
16:2	0.5	0.6	0.7	0.6 ± 0.004
16:3	0.5	0.4	0.5	0.5 ± 0.002
16:4	T	T	T	T
18:0	3.0	2.7	3.0	2.9 ± 0.003
18:1(n-9)	9.1	8.5	9.8	9.1 ± 0.01
18:1(n-7)	6.1	6.0	6.2	6.1 ± 0.0004
18:2(n-6)	2.9	2.5	3.2	2.9 ± 0.01
18:3(n-6)	0.2	0.1	0.3	0.2 ± 0.01
18:3(n-3)	4.7	4.4	4.7	4.6 ± 0.002
18:4(n-3)	0.7	0.6	0.7	0.7 ± 0.001
20:0	0.1	T	0.1	T
20:1	0.2	0.2	0.3	0.2 ± 0.03
20:2(n-6)	0.1	0.1	0.1	0.1 ± 0
20:3(n-6)	0.1	0.1	0.1	0.1 ± 0
20:4(n-6)	2.7	2.5	2.8	2.7 ± 0.002
20:4(n-3)	0.2	0.2	0.2	0.2 ± 0
20:5(n-3)	5.8	5.6	6.1	5.8 ± 0.003
22:0	0.2	0.2	T	0.1 ± 0.1
22:1	0.1	T	T	T
22:5(n-3)	0.3	0.3	0.3	0.3 ± 0
22:6(n-3)	4.6	4.0	4.2	4.3 ± 0.006
24:1	T	T	T	T
Total unknowns	0.4	0.9	0.5	0.6 ± 0.03
Total sats	10.7	9.8	10.6	10.3 ± 0.007
Total monoenes	19.4	18.2	20.4	19.3 ± 0.02
Total (n-3)	16.4	15.1	16.3	15.9 ± 0.01
Total (n-6)	6.1	5.2	6.5	6.0 ± 0.02
(n-3)/(n-6)	2.7	2.9	2.5	2.7 ± 0.004
Fatty acids	53.9	50.2	55.5	53.2 ± 0.07
Non fatty acids	46.1	49.8	44.5	46.8 ± 0.07

Table III,xxiii Fatty acid spectra of total lipid in herring larvae aged 35 days post-hatch and reared on Great Salt Lake *Artemia*

Notes as for Table III,xxii.

Percentage composition of total lipid

Fatty acid	Tanks 2	4	6	Mean ± sd
14:0	0.7	0.4	0.4	0.5 ± 0.01
15:0	0.1	0.2	0.1	0.1 ± 0.006
16:0	8.4	7.3	7.8	7.8 ± 0.01
16:1	2.7	2.0	2.1	2.3 ± 0.02
16:2	0.4	0.4	0.3	0.4 ± 0.002
16:3	0.8	0.3	0.7	0.6 ± 0.03
16:4	T	T	0.1	T
18:0	3.1	2.9	3.2	3.1 ± 0.002
18:1(n-9)	11.1	11.2	10.9	11.1 ± 0.0006
18:1(n-7)	5.0	4.1	4.3	4.5 ± 0.01
18:2(n-6)	3.6	3.1	3.2	3.3 ± 0.005
18:3(n-6)	0.1	0.2	0.2	0.2 ± 0.006
18:3(n-3)	8.9	8.3	7.7	8.3 ± 0.01
18:4(n-3)	1.8	1.4	1.2	1.5 ± 0.02
20:0	T	T	0.1	T
20:1	0.2	0.3	0.3	0.3 ± 0.003
20:2(n-6)	0.1	0.1	0.1	0.1 ± 0
20:3(n-6)	T	0.1	0.1	T
20:4(n-6)	1.4	1.1	1.3	1.3 ± 0.005
20:4(n-3)	0.3	0.3	0.3	0.3 ± 0
20:5(n-3)	4.1	4.0	4.4	4.2 ± 0.003
22:0	0.1	T	0.2	0.1 ± 0.1
22:1	0.1	T	T	T
22:5(n-3)	0.3	0.3	0.3	0.3 ± 0
22:6(n-3)	5.2	4.4	5.2	4.9 ± 0.01
24:1	T	0.1	0.1	T
Total unknowns	0.4	0.2	0.5	0.4 ± 0.02
Total sats	12.5	10.9	11.8	11.6 ± 0.02
Total monoenes	19.1	17.6	17.8	18.2 ± 0.01
Total (n-3)	20.7	18.7	19.1	19.5 ± 0.02
Total (n-6)	5.2	4.6	4.9	4.9 ± 0.005
(n-3)/(n-6)	3.9	4.1	3.9	4.0 ± 0.0009
Fatty acids	59.1	52.7	55.2	55.6 ± 0.1
Non fatty acids	40.9	47.3	44.8	44.4 ± 0.1

Table III,xxiv Fatty acid spectra of total lipid in herring larvae aged 45 days post-hatch and reared on San Francisco Bay *Artemia*

Samples were analysed with 23:0 internal standard as described in the text.
The means and standard deviations were computed after arcsin transformation of percentage data.
T = Trace, fatty acid was detected but constituded <0.1% of the total lipid.
- = Fatty acid not detected.

Percentage composition of total lipid

Fatty acid	Tanks 1	3	5	Mean ± sd
14:0	0.2	0.4	0.4	0.3 ± 0.01
15:0	0.1	0.3	0.2	0.2 ± 0.01
16:0	7.3	7.7	7.6	7.5 ± 0.001
16:1	3.7	4.0	4.2	4.0 ± 0.004
16:2	0.4	0.5	0.6	0.5 ± 0.005
16:3	0.9	0.5	1.0	0.8 ± 0.02
16:4	0.1	T	T	T
18:0	3.1	3.5	3.4	3.3 ± 0.003
18:1(n-9)	9.1	10.8	10.6	10.2 ± 0.02
18:1(n-7)	7.0	6.4	6.9	6.8 ± 0.004
18:2(n-6)	2.4	2.6	3.1	2.7 ± 0.01
18:3(n-6)	0.3	0.3	0.3	0.3 ± 0
18:3(n-3)	4.5	5.0	5.0	4.8 ± 0.005
18:4(n-3)	0.5	0.7	0.8	0.7 ± 0.009
20:0	0.1	0.1	0.1	0.1 ± 0
20:1	0.2	0.2	0.2	0.2 ± 0
20:2(n-6)	0.1	0.1	0.1	0.1 ± 0
20:3(n-6)	0.1	0.2	0.2	0.2 ± 0.006
20:4(n-6)	2.9	3.4	3.2	3.2 ± 0.005
20:4(n-3)	0.2	0.3	0.2	0.2 ± 0.003
20:5(n-3)	5.9	7.0	6.9	6.6 ± 0.02
22:0	0.2	T	0.2	0.1 ± 0.1
22:1	T	0.1	T	T
22:5(n-3)	0.3	0.4	0.4	0.4 ± 0.002
22:6(n-3)	3.0	4.1	3.7	3.6 ± 0.02
24:1	T	0.1	0.1	T
Total unknowns	0.2	0.2	0.6	0.3 ± 0.03
Total sats	10.9	11.9	11.9	11.5 ± 0.008
Total monoenes	20.0	21.6	22.0	21.2 ± 0.02
Total (n-3)	14.4	17.5	17.0	16.3 ± 0.05
Total (n-6)	5.7	6.6	6.9	6.5 ± 0.02
(n-3)/(n-6)	2.5	2.7	2.5	2.5 ± 0.001
Fatty acids	52.5	58.8	59.9	57.1 ± 0.2
Non fatty acids	47.5	41.2	40.1	42.9 ± 0.2

Table III,xxv Fatty acid spectra of total lipid in herring larvae aged 45 days post-hatch and reared on Great Salt Lake *Artemia*

Notes as for Table II,xxiv.

Percentage composition of total lipid

Fatty acid	Tanks 2	4	6	Mean ± sd
14:0	0.3	0.3	0.3	0.3 ± 0
15:0	0.1	0.1	0.1	0.1 ± 0
16:0	8.4	8.3	8.1	8.3 ± 0.0008
16:1	1.7	1.8	1.7	1.7 ± 0.0005
16:2	0.4	0.4	0.5	0.4 ± 0.002
16:3	0.6	0.6	0.6	0.6 ± 0
16:4	T	T	T	T
18:0	3.1	3.1	3.1	3.1 ± 0
18:1(n-9)	11.1	12.8	10.5	11.5 ± 0.03
18:1(n-7)	4.5	5.1	4.3	4.6 ± 0.01
18:2(n-6)	3.6	4.5	3.2	3.7 ± 0.03
18:3(n-6)	0.3	0.5	0.2	0.3 ± 0.02
18:3(n-3)	8.9	9.7	7.5	8.7 ± 0.04
18:4(n-3)	1.8	2.8	1.2	1.9 ± 0.09
20:0	T	0.1	0.1	T
20:1	0.2	0.3	0.2	0.2 ± 0.003
20:2(n-6)	0.2	0.2	0.1	0.2 ± 0.006
20:3(n-6)	T	0.1	0.1	T
20:4(n-6)	1.1	1.0	1.2	1.1 ± 0.002
20:4(n-3)	0.3	0.4	0.3	0.3 ± 0.002
20:5(n-3)	4.1	4.2	4.2	4.2 ± 0.0002
22:0	0.1	0.2	0.2	0.2 ± 0.006
22:1	0.1	T	T	T
22:5(n-3)	0.3	0.3	0.3	0.3 ± 0
22:6(n-3)	3.3	2.9	4.1	3.4 ± 0.03
24:1	T	T	T	T
Total unknowns	0.7	0.6	0.9	0.7 ± 0.008
Total sats	12.0	12.1	11.9	12.0 ± 0.0002
Total monoenes	17.6	19.9	16.7	18.0 ± 0.05
Total (n-3)	18.7	20.2	17.7	18.8 ± 0.03
Total (n-6)	5.2	6.3	4.8	5.3 ± 0.03
(n-3)/(n-6)	3.6	3.2	3.7	3.5 ± 0.005
Fatty acids	55.2	60.1	53.1	55.8 ± 0.1
Non fatty acids	44.8	39.9	46.9	44.2 ± 0.1

Table III,xxvi Fatty acid content of pooled samples of herring larvae reared on San Francisco Bay *Artemia* and of San Francisco Bay *Artemia* nauplii

The mean lipid and fatty acid contents of the larvae were calculated by multiplying the lipid content by the percentage fatty acid content given in Tables III,xvi to III,xxiv.
Standard deviations are not given.

		Age (days post-hatch)				
	San Fran Bay *Artemia*	10	15	25	35	45
Lipid content (µg nauplius/larva^{-1})	0.138	31.2	25.6	38.7	61.0	67.0
Fatty acid (µg nauplius/larva^{-1})						
14:0	0.0011	0.34	0.15	0.15	0.24	0.20
15:0	0.0004	0.06	0.05	0.08	0.06	0.13
16:0	0.0112	3.71	2.71	2.83	4.14	5.02
16:1	0.0116	0.90	0.64	1.70	2.38	2.68
16:2	0.0010	0.12	0.10	0.15	0.37	0.34
16:3	0.0014	0.06	0.08	0.23	0.31	0.54
16:4	T	0.03	0.05	T	T	T
18:0	0.0041	0.66	0.67	1.08	1.77	2.21
18:1(n-9)	0.0170	2.06	1.74	3.29	5.55	6.83
18:1(n-7)	0.0119	0.84	0.79	2.09	3.72	4.56
18:2(n-6)	0.0054	0.22	0.23	0.89	1.77	1.81
18:3(n-6)	0.0004	0.03	T	0.08	0.12	0.20
18:3(n-3)	0.0138	0.12	0.23	1.78	2.81	3.22
18:4(n-3)	0.0018	0.06	0.08	0.27	0.43	0.47
20:0	T	-	T	T	T	0.07
20:1	0.0006	0.19	0.10	0.12	0.12	0.13
20:2(n-6)	T	T	T	T	0.06	0.07
20:3(n-6)	0.0003	-	T	0.04	0.06	0.13
20:4(n-6)	0.0028	0.25	0.26	0.77	1.65	2.14
20:4(n-3)	0.0004	0.09	0.08	0.08	0.12	0.13
20:5(n-3)	0.0073	2.09	1.59	2.13	3.54	4.42
22:0	-	-	-	-	0.06	0.07
22:1	0.0001	0.06	0.05	0.08	T	T
22:5(n-3)	0.0001	0.22	0.15	0.12	0.18	0.27
22:6(n-3)	-	4.87	3.46	2.44	2.62	2.41
24:1	-	0.03	T	T	T	T
Total unknowns	0.0001	0.21	0.28	0.23	0.37	0.20
Total sats	0.0171	4.77	3.58	4.14	6.28	7.71
Total monoenes	0.0410	4.06	3.33	7.28	11.77	14.20
Total (n-3)	0.0235	7.46	5.58	6.81	9.70	10.92
Total (n-6)	0.0091	0.50	0.49	1.78	3.67	4.36
(n-3)/(n-6)	2.58	14.92	11.39	3.83	2.64	2.50
Fatty acids	0.0936	17.22	13.49	20.63	32.45	38.26
Non fatty acids	0.0444	13.98	12.11	18.07	28.55	28.74

Table III,xxvii Fatty acid content of pooled samples of herring larvae reared on Great Salt Lake *Artemia* and of Great Salt Lake *Artemia* nauplii

Notes as for Table III,xxvi.

		Age (days post-hatch)				
	Great Salt Lake *Artemia*	10	15	25	35	45
Lipid content (µg nauplius/larva^{-1})	0.282	31.2	24.4	41.4	51.8	66.3
Fatty acid (µg nauplius/larva^{-1})						
14:0	0.0020	0.34	0.17	0.21	0.26	0.20
15:0	0.0003	0.06	0.05	0.04	0.05	0.07
16:0	0.0251	3.71	2.44	3.23	4.04	5.50
16:1	0.0082	0.90	0.68	1.28	1.19	1.13
16:2	0.0014	0.12	0.05	0.17	0.21	0.27
16:3	0.0020	0.06	0.10	0.25	0.31	0.40
16:4	0.0003	0.03	0.05	T	T	T
18:0	0.0096	0.66	0.63	1.24	1.61	2.06
18:1(n-9)	0.0420	2.06	1.71	4.26	5.75	7.62
18:1(n-7)	0.0166	0.84	0.83	1.95	2.33	3.05
18:2(n-6)	0.0134	0.22	0.24	1.41	1.71	2.45
18:3(n-6)	0.0006	0.03	0.02	0.04	0.10	0.20
18:3(n-3)	0.0539	0.12	0.37	3.06	4.30	5.77
18:4(n-3)	0.0076	0.06	0.07	0.58	0.78	1.26
20:0	0.0003	-	T	T	T	T
20:1	0.0011	0.19	0.10	0.12	0.16	0.13
20:2(n-6)	0.0003	T	T	0.04	0.05	0.13
20:3(n-6)	-	-	T	T	T	T
20:4(n-6)	0.0008	0.25	0.27	0.58	0.67	0.73
20:4(n-3)	0.0008	0.09	0.07	0.12	0.16	0.20
20:5(n-3)	0.0056	2.09	1.34	1.74	2.18	2.78
22:0	-	-	T	T	0.05	0.13
22:1	0.0006	0.06	0.05	0.04	T	T
22:5(n-3)	T	0.22	0.12	0.12	0.16	0.20
22:6(n-3)	-	4.87	2.89	2.61	2.54	2.25
24:1	-	0.03	-	0.04	T	T
Total unknowns	0.0135	0.21	0.17	0.21	0.21	0.46
Total sats	0.0372	4.77	3.29	4.72	6.01	7.96
Total monoenes	0.0925	4.06	3.17	7.70	9.43	11.93
Total (n-3)	0.0685	7.46	4.73	8.23	10.01	12.46
Total (n-6)	0.0152	0.50	0.54	2.07	2.54	3.51
(n-3)/(n-6)	4.51	14.92	8.76	3.98	3.94	3.55
Fatty acids	0.2061	17.22	12.42	23.35	28.80	37.00
Non fatty acids	0.0759	13.98	11.98	18.05	23.00	29.30

Fig 3-1 Tank layout in the constant temperature room at the DAFS Marine Research Unit, Firemore Bay, Poolewe

Numbers shown indicate the tank numbers used in this study

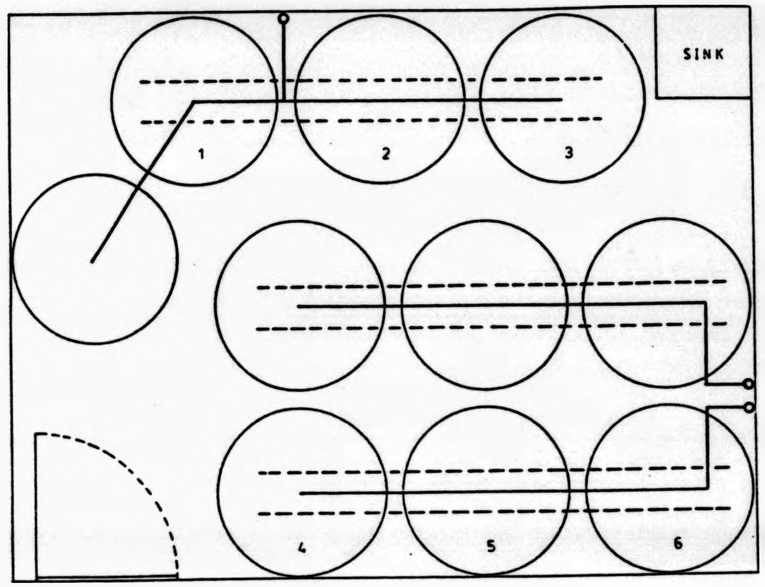


Fig 3-2 . Cross section through tanks, lights and water system

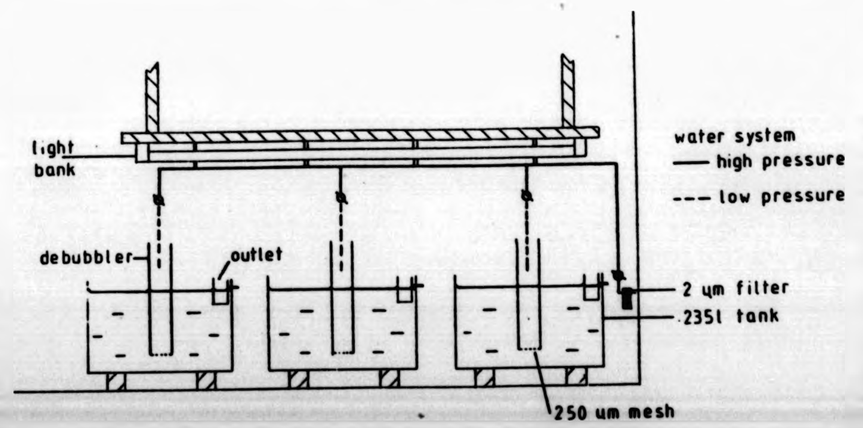
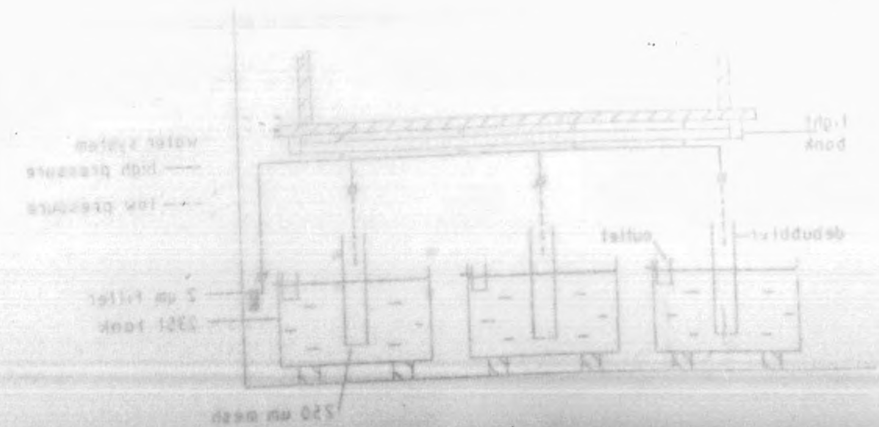
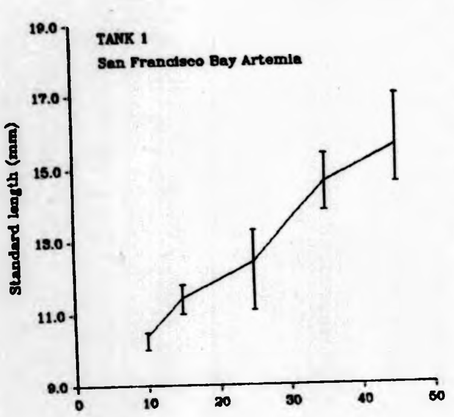
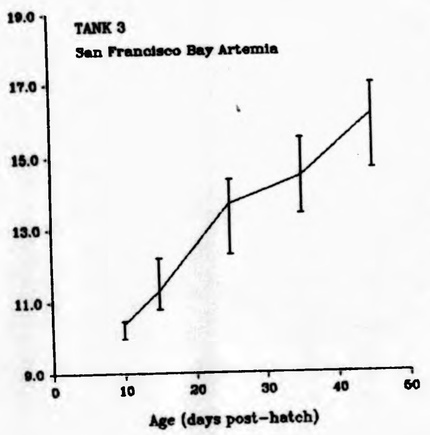


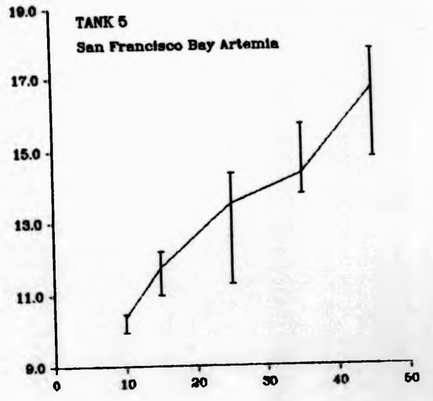
Fig 3-3 **Plots of standard lengths and dry weights against age for herring larvae reared on San Francisco Bay or Great Salt Lake *Artemia*, by tank**

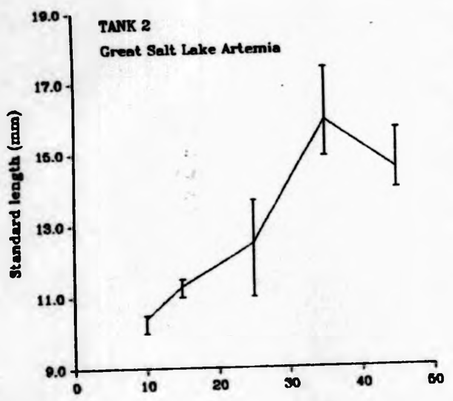
Medians joined by continuous plot
Vertical bars indicate the upper and lower quartiles
The number of individuals in each sample was 30 unless indicated by numbers on the vertical bars

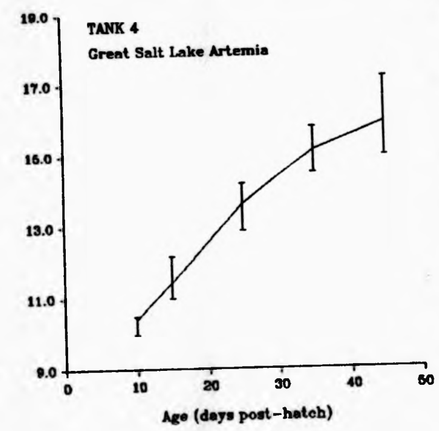


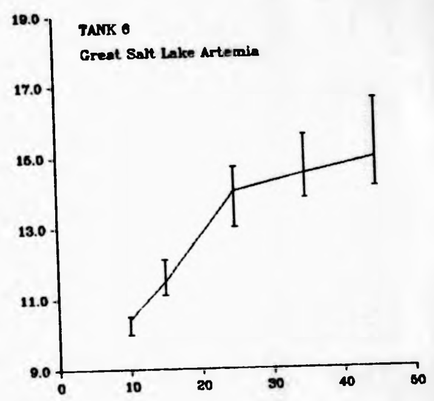

TANK 1
San Francisco Bay Artemia
Standard length (mm)
19.0
17.0
15.0
13.0
11.0
9.0
0
10
20
30
40
50

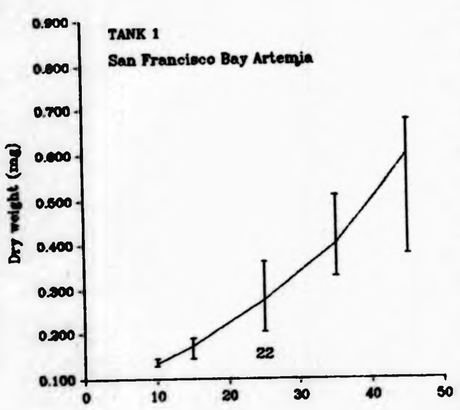

TANK 3
San Francisco Bay Artemia
19.0
17.0
15.0
13.0
11.0
9.0
0
10
20
30
40
50
Age (days post-hatch)

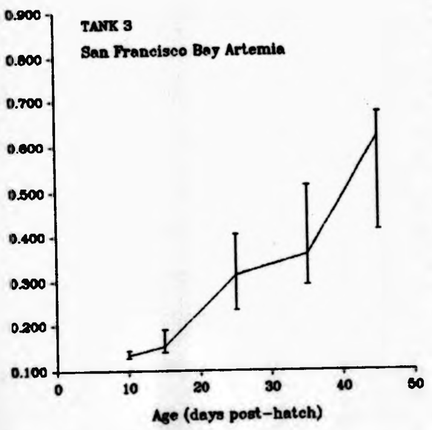

TANK 5
San Francisco Bay Artemia
19.0
17.0
15.0
13.0
11.0
9.0
0
10
20
30
40
50

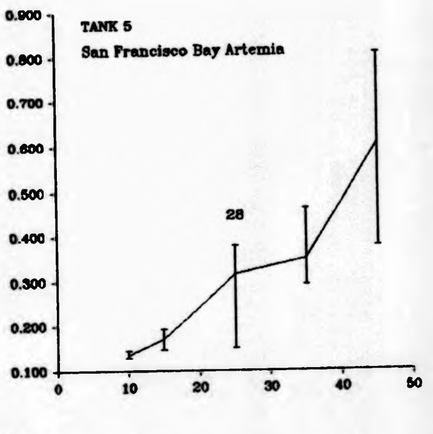

TANK 2
Great Salt Lake Artemia
Standard length (mm)
19.0
17.0
15.0
13.0
11.0
9.0
0
10
20
30
40
50

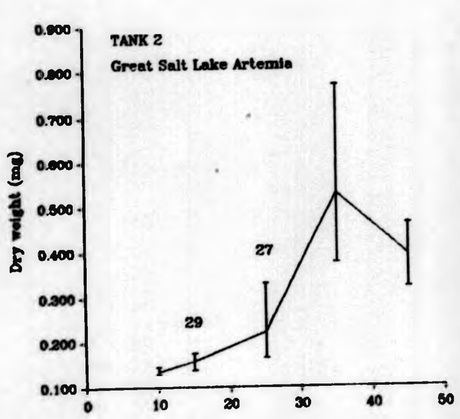

TANK 4
Great Salt Lake Artemia
19.0
17.0
15.0
13.0
11.0
9.0
0
10
20
30
40
50
Age (days post-hatch)

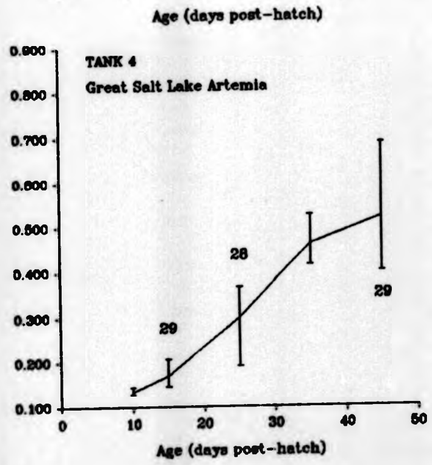

TANK 6
Great Salt Lake Artemia
19.0
17.0
15.0
13.0
11.0
9.0
0
10
20
30
40
50


TANK 1
San Francisco Bay Artemia
Dry weight (mg)
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
22
0
10
20
30
40
50


TANK 3
San Francisco Bay Artemia
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0
10
20
30
40
50
Age (days post-hatch)


TANK 5
San Francisco Bay Artemia
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
28
0
10
20
30
40
50


TANK 2
Great Salt Lake Artemia
Dry weight (mg)
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
29
27
0
10
20
30
40
50


TANK 4
Great Salt Lake Artemia
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
29
28
29
0
10
20
30
40
50
Age (days post-hatch)

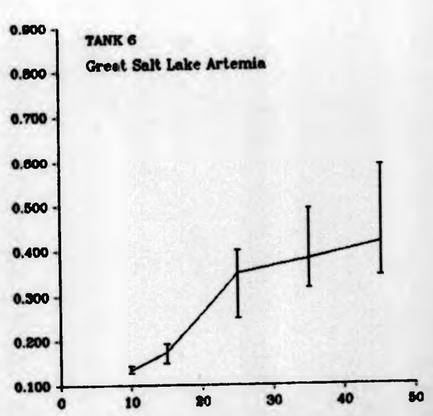
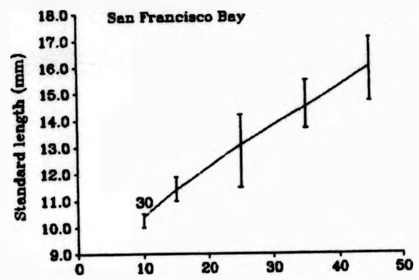
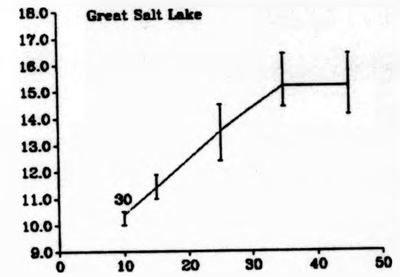

TANK 6
Great Salt Lake Artemia
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0
10
20
30
40
50

Fig 3-4 Plots of standard lengths of herring larvae against age, samples pooled by feed type

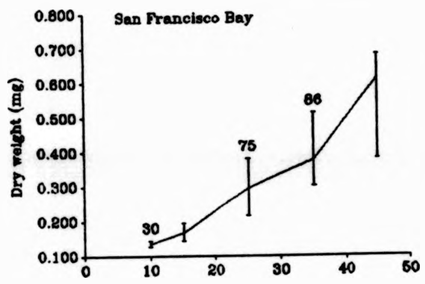
Medians joined by continuous plot
Vertical bars indicate upper and lower quartiles
The number of individuals in each sample was 90 unless indicated by numbers on the vertical bars

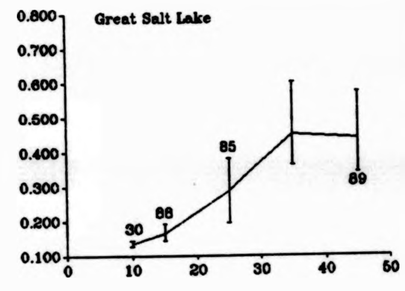




Plots of dry weights of herring larvae against age, samples pooled by feed type

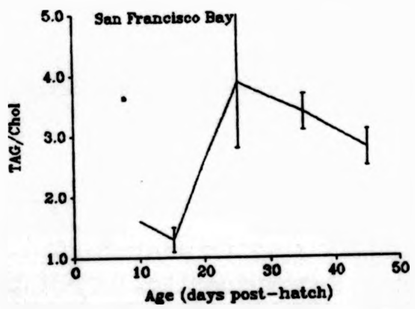
Medians joined by continuous plot
Vertical bars indicate upper and lower quartiles
The number of individuals in each sample was 90 unless indicated by numbers on the vertical bars





Plots of triacyglycerol/cholesterol ratios for herring larvae against age, samples pooled by feed type

Means joined by continuous plot
Vertical bars indicate standard deviation
The means and standard deviations were calculated from the results from three tanks



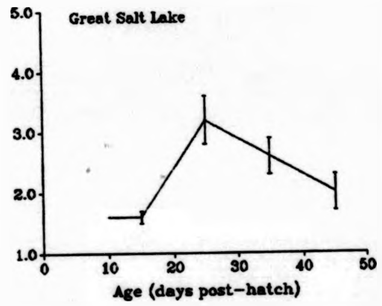
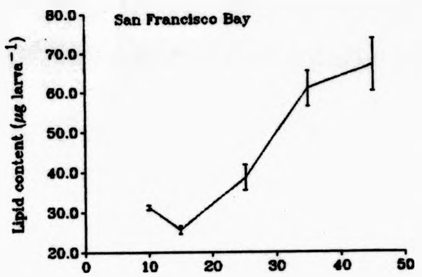
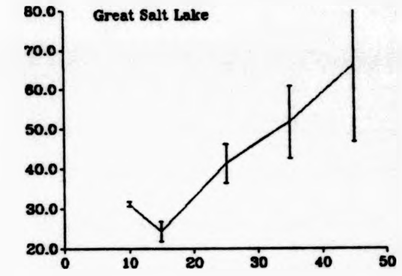


Fig 3-5 Plots of lipid content of herring larvae against age, samples pooled by feed type

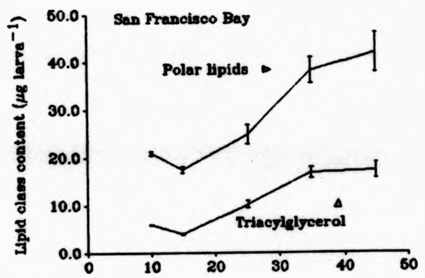
Means joined by continuous plot
Vertical bars indicate standard deviations
The means and standard deviations were calculated from analyses of duplicate samples from each of three tanks

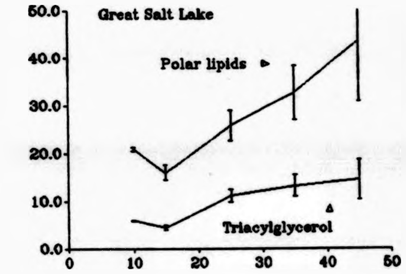




Plots of the amounts of polar lipids and triacylglycerol in herring larvae against age

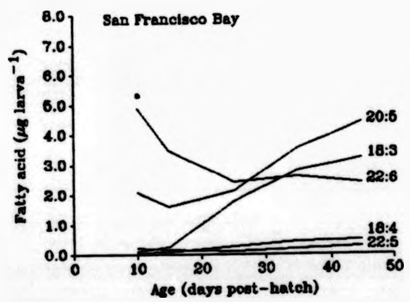
Means joined by continuous plot
Vertical bars indicate standard deviations
The means and standard deviations were calculated from analyses of duplicate samples from each of three tanks

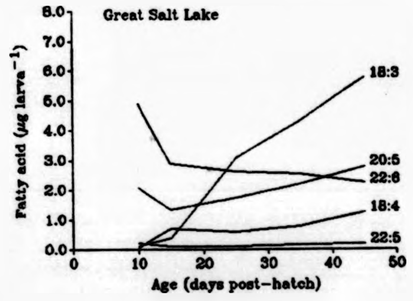




Plots of the amounts of various (n-3) fatty acids in herring larvae against age

Means joined by continuous plot, standard deviations are not shown for clarity
The means are calculated from the fatty acid analyses and lipid contents of samples from three tanks at each sample point





Chapter 4 The use of microencapsulated diets for nutritional studies on herring larvae

4.1 Introduction

At present the majority of marine fish larviculture utilises live feeds. Inert diets are readily accepted by salmonid young, but not by the larvae of marine species. Turbot have been weaned onto non-living feeds but had to be fed live prey until 22-25 days post-hatch (Bromley & Howell 1983). In a study in which cod larvae were reared in a saltwater pond, they did take a pelleted feed. However, it only formed a significant component of their diet after 90 days (i.e. post-metamorphosis) and after they had denuded the natural live prey (Øiestad *et al.* 1985).

Most marine fish are batch spawners with high annual fecundity and small eggs which release larvae of only a few millimetres in length (Russell 1976). The small size of marine larvae may be one reason for difficulties in designing suitable inert feeds for them. An exception are the larvae of the wolf fish which hatch at 20 mm and exist on yolk reserves until metamorphosis (Tilseth 1990). A survival rate of 53.7% over 108 days has been achieved with naturally spawned fry of this species fed on a mix of *Artemia* and dry diet at first feeding and on-grown on pelleted feeds (Moksness 1989).

4.1.1 The use of live diets for marine fish larviculture

The reliance on live feeds is a major constraint for the marine aquaculture industry, particularly in the Far East where mariculture is more advanced than in Western Europe. The most commonly used feed items are the euryhaline rotifer, *Brachionus plicatilis* and the brine shrimp, *Artemia*. Rearing the former animal is labour intensive, since algae

must be cultured to feed the rotifers (Teshima *et al.* 1981). To overcome this, the use of yeast as an algal replacement was pioneered by the Japanese. However, the rotifers produced were nutritionally deficient and when fed to larval fish, poor growth and survival resulted (Watanabe *et al.* 1983). Rotifer cultures are also prone to collapse and require careful husbandry.

Brine shrimp nauplii are easier to procure as they merely require hatching from cysts which are commercially available. However, as aquaculture has expanded, the price of cysts has increased. In recent years, the inoculation of lakes previously devoid of *Artemia* and the development of unexploited natural sources has improved supply (Sorgeloos 1980). Currently, the major problem is the high variability in nutritional quality of nauplii (see section 3.4.3). When the fish larvae become larger it maybe desirable to increase the size of *Artemia* being used (Léger *et al.* 1986). This can be achieved by harvesting natural populations, intensive culturing with algae or by rearing brine shrimps on cheaper substitutes such as rice bran (Johnson 1980). Whichever technique is adopted it is still likely to increase the cost of rearing the fish larvae.

4.1.2 The advantages of inert feeds for fish larviculture

Compared to live feeds, artificial diets have a number of advantages. These have been reviewed by Meyers (1979) but are worth repeating:-

i. The composition and size of the food may be altered to reflect the needs of the larvae.

ii. A continuity of food supply of recognized quality is assured.

iii. The introduction of infections and pollutants is eliminated.

iv. The reduction in labour cost and increased ease of handling result in greater efficiency.

v. The need to wean metamorphosed larvae onto an inert feed is removed.

For the purposes of nutritional research, an inert diet allows qualitative and quantitative dietary studies in a way which is not possible with live feeds. Diets enclosed by membranes may also reduce the levels of bacteria in the water which may interfere with nutritional studies (Jones *et al.* 1979) and radioactively labelled tracers may be easily incorporated into the diet (Jones *et al.* 1975).

4.1.3 The development of microencapsulated diets

In 1966, Chang *et al.* reported results from experiments on producing artificial cells containing aqueous solutions. Although a number of patents had described encapsulation systems prior to this paper, these involved the inclusion of an oily or solid phase, or were not bound by a true membrane. The procedures used by Chang *et al.* involved the emulsification of an aqueous solution of protein in an organic solvent and the subsequent formation of a membrane around the droplet by interfacial polymerization or coacervation. In the former process, the protein in the outer surface of the droplet was cross-linked by a polymerizing agent carried in the organic phase. A number of suitable cross-linking agents exist. Early work produced nylon encapsulated particles by the action of a dicarboxylic acid halide on an aliphatic diamine included in the aqueous compartment. Later procedures have eliminated the nylon component and rely on protein cross-linking alone. The cross-linking agent most commonly used is succinyl dichloride. Substances such as glutaraldehyde and dicarboxylic halides are extremely toxic and cannot be employed in aquaculture applications.

Providing the aqueous compartment contains a sufficiently high concentration of protein, a strong wall is formed. The strength of the boundary membrane also depends on the amino-acid spectrum of the protein used (Patent Spec. 1979).

Interfacial coacervation relies on the lower solubility of the polymer at the aqueous/organic interface to deposit a membrane on the droplet by a physical process. Cellulose nitrates are commonly used in this method.

By the addition of emulsifiers and the manipulation of mixing speed, temperature and component concentrations Chang *et al.* were able to produce capsules from 20–80 µm diameter. The capsules were mechanically strong and permeable to water so they could be dried and subsequently rehydrated.

In clinical research cross-linked albumin microspheres containing a radioactive tracer have been used as a means of studying circulatory systems in animals and subsequently numerous papers have described the use of this type of capsule for non-oral drug delivery e.g. Willmott *et al.* (1985). The advantages of using microcapsules include improved target specificity and controlled release of the entrapped medication (Davis *et al.* 1987). A review of some of the more mundane uses of microencapsulation such as in the production of carbon-less copy paper and 'scratch-and-sniff' patches is provided by Watanabe & Hayashi (1976).

The development of microencapsulated diets as algal supplements for the enrichment and on-growing of *Artemia* nauplii and for rearing penaeid prawns has been relatively successful and several commercial products are now on the market (Walford & Lam 1987, Jones *et al.* 1979). The range of preparation procedures and their relative

advantages and disadvantages have been reviewed by Langdon *et al.* (1985).

In contrast, the development of microencapsulated diets for marine fish larvae has been unsuccessful. Bivalves and penaeid prawns are able to mechanically rupture food particles to some extent by use of the crystalline style and gastric shield and by the gastric mill respectively (Reid 1981, Gibson 1981). This means that the microcapsule wall can be relatively strong and the use of nylon coated particles is not precluded (Jones *et al.* 1974).

Larval marine fish, however, rely largely on enzymic breakdown of their prey. *Artemia* nauplii, after capture by herring larvae are rapidly swallowed, moved to the hind-gut by peristaltic and ciliary action and there digested. This process can be followed under the microscope in anaesthetized larvae and it is clear that mechanical rupturing of the brine shrimp does not occur (personal observation). Microcapsules which are bounded by a strong, non-biodegradable membrane will probably not be digested by young fish larvae. Kanazawa *et al.* (1982b) demonstrated that red sea bream (1-10 days post-hatch) fed nylon encapsulated capsules showed poor growth and survival. Ayu larvae fed on rotifers until day 10 post-hatch and then transferred to nylon encapsulated capsules showed positive but suppressed growth compared to controls fed on live diet. The authors attributed these results to the poor digestibility of the capsules. Garatun-Tjeldstø *et al.* (1989) also reported negative growth and poor survival of cod larvae fed nylon coated microcapsules. Particles formed by cross-linking protein should be more digestible since the wall can be broken down by proteolytic enzymes.

4.1.4 Aims of this rearing trial

The experiments described in this section were designed to study the possibility of rearing herring larvae on cross-linked protein capsules with a view to carrying out long-term nutritional studies and/or using the capsules as a vector for radioactively labelled tracers.

In 1987 a large scale rearing experiment was carried out and in 1988 a small experiment was run to try and clarify the results from the previous year.

Encapsulated cod-roe (prepared by Frippak Feeds Ltd., Basingstoke, England.) was chosen as the inert feed. Cod-roe based feeds (encapsulated in hard fat rather than cross-linked) have been successful in salmon rearing and have also been partially successful for rearing cod and plaice larvae (Garatun-Tjeldstø *et al.* 1989). Cod-roe is rich in protein and long-chain PUFAs (Molvik *et al.* 1984) and should constitute a good basis for a formulated diet for marine fish larvae.

4.2 Materials and methods

The procedures used to culture *Nannochloropsis oculata*, rotifers and *Artemia* are described in sections 2.2 - 2.4.

4.2.1 Microencapsulated diet

Fresh cod-roe was microencapsulated by Frippak Feeds Ltd. and supplied in nitrogen filled canisters. Butylated hydroxy-anisole was included at 1% as an antioxidant. Forty grams of 50-90 µm and 120g of 90-500 µm capsules were supplied. Once opened the canisters of pellets were stored at -20°C.

4.2.2 Supply of herring larvae for spring 1987

Fertile male and female herring were caught on Ballantrae Bank (55°8'N 004°20'W) off the Firth of Clyde by the F.R.V. Clupea on March 13, 1987. Gonads were excised and eggs fertilized as detailed in section 2.1. Stacks of eggs were transferred in pails of clean seawater to the DAFS Marine Research Unit, Firemore Bay, Poolewe and incubated as detailed in section 3.2.1.

4.2.3 Tank layout

Ten, 235 l, black PVC, circular, flat-bottomed tanks were set up in a constant temperature room at the DAFS Marine Research Unit, Poolewe as shown in Fig 4-1. Each tank was fitted with a barrel outlet covered initially with 250 µm mesh. When the herring larvae were larger this was changed for 400 µm mesh in order to aid the clearance of feed from the tanks (Fig 4-2).

4.2.4 Water supply

The water supply to the rearing tanks was described in section 3.2.3.

4.2.5 Lighting

A double bank of 40 W fluorescent tubes (Philips daylight) were set 1 m above each tank (see section 3.2.4). Light measurements were recorded at 2 cm below the water surface and 6 cm above the base of

each tank using a LI-185B quantum/radiometer (Li-Cor Inc.). The lights were on a 14:10 h light:dark cycle.

The recorded meter readings were converted from mV to $\mu Em^{-2}s^{-1}$ using the formulae recommended by the manufacturer. For comparison with results quoted in the literature conversion to metre-candles (mc) was necessary. Exact conversion of photon to photometric units is complex but an approximate conversion was made by division by a factor of 0.012 as recommended for white fluorescent lighting by LiCor Inc.

4.2.6 Temperature

The temperature of incoming seawater was not under control but the trial was run in a constant temperature room set to 8°C. Water temperatures in each tank were recorded every morning, afternoon and evening.

4.2.7 Tank cleaning

The flow of seawater through the tanks helped to flush away unused food. However material did settle on the tank bases and had to be removed daily. Debris was collected into a pail by siphon and allowed to stand. Any live larvae were dipped out with a small beaker and returned to the rearing tank. The debris was then washed through a 250 µm mesh. Dead larvae were collected and stored in phosphate buffered saline containing 2% formalin. The numbers of dead larvae recovered were determined at the end of the experiment.

4.2.8 Experimental design

A few herring larvae hatched on April 5 with larger hatches daily from April 6 to April 9. Larvae were collected from the incubation tanks using a beaker and their numbers estimated by sub-sampling after gentle mixing. Groups of about 5000 larvae were placed into the rearing tanks as below:-

Tank no.	Larval hatch	Date larvae introduced
1-3	05/04 + 06/04	10/04
4-6	07/04	11/04
7-10	08/04	12/04

The tanks were designated the following diets:-

Tank no.	Larval hatch	Diet
1	06/04	Rotifers/*Artemia*
2	06/04	Wean
3	06/04	Rotifers/*Artemia*
4	07/04	Microdiet
5	07/04	Wean
6	07/04	Microdiet
7	08/04	Microdiet
8	08/04	Rotifers/*Artemia*
9	08/04	Wean
10	08/04	Starve

Feeding was begun immediately at a level of 500 particles l^{-1} $feed^{-1}$. This was steadily increased until by day 10 post-hatch 1000 particles l^{-1} were being given each morning and afternoon. There was insufficient time to assess the feed levels in the tanks and adjust the daily feeding rate accordingly.

Tanks assigned live feed were initially given rotifers. From day 28 post-hatch, *Artemia* were included in the feed. The proportion of rotifers in the diet was decreased and that of *Artemia* increased until by day 31 only brine shrimps were being given.

The average number of capsules gram^{-1} of microencapsulated feed was determined by manually counting subsamples from a distilled water suspension. Subsequently the amount of feed to use was determined by weight. Tanks assigned to microencapsulated feed were initially given particles in the size range 50–90 µm. From day 14 post-hatch the larger size range (90–500 µm) was used. Pellets were suspended in distilled water for 2 h prior to use. After draining off the distilled water the diet was resuspended in a little seawater and distributed on the surface of the rearing tanks.

Larvae assigned to the weaning program were reared on live feed, as detailed above until 19 days post-hatch. From day 20–26 the proportion of live feed was reduced and replaced with microencapsulated feed (in the size range 90–500 µm) until by day 27 post-hatch only pelleted feed was being used.

Larvae were sampled for length, weight and gut contents at 9, 14, 20, 26, 32 and 38 days post-hatch. Samples were collected for biochemical analyses at 9, 14, 20, 26 and 38 days post-hatch. Survival in tank 7 appeared to be poor and so samples for biochemical analyses were not taken from this tank at 14 or 20 days post-hatch.

The experiment had been planned to continue until 45 days post-hatch but had to be terminated after 38 days due to a shortage of microencapsulated feed which Frippak had not been able to manufacture in sufficient quantity for the original experimental plan.

4.2.9 Sampling of larvae and experimental diets

Larvae were attracted to a bench light mounted on the edge of the tank and collected in a small beaker. Towards the end of the study when the numbers of larvae remaining were small, this method became too time consuming. Water was thus removed from the tanks using a pail and the water level in the pail lowered by slow siphoning through a 250 µm mesh. This process was repeated until enough larvae were obtained for the analyses being undertaken.

The lengths and weights of larvae were determined as detailed in section 2.6. Whilst larval lengths were being recorded, a record of gut contents was made. The scores used were (+) food items in the gut, (-) empty gut or (?) possible food items (orange staining or distention of the gut wall).

Larvae for biochemical analysis were counted into a dish of seawater using a wide-bore pipette. They were then washed onto a 200 µm mesh and rinsed with distilled water. Larvae were transferred to labelled glass specimen bottles which were sealed with foil-lined lids and stored under liquid nitrogen. Sample size was initially 300 individuals but was reduced to 150 individuals as the larvae became larger. The samples were subsequently transferred to a -80°C freezer at the University of Stirling prior to analysis.

Rotifers were collected by passing the culture medium through a 200 µm mesh (to remove clumps of yeast and algal debris) and two 60 µm meshes to retain the rotifers. The trapped rotifers were then washed onto a 25 µm mesh, rinsed quickly with distilled water to remove salt and freeze dried. The samples were stored at -20°C prior to analysis.

Newly hatched brine shrimps were collected on a 250 µm mesh, washed with distilled water, freeze dried and stored at -20°C prior to analysis.

Samples of microencapsulated feed were collected and stored at -20°C.

4.2.10 Biochemical analyses

Analyses of lipid content, lipid classes and fatty acids were carried out. The lipid content and class analyses were performed on single samples from each tank at each sample point. The lipid remaining after these assays was pooled by diet and the triacylglycerol fraction isolated by TLC. Following direct transmethylation without internal standard, the fatty acid spectra were determined by gas-liquid chromatography. These methods are fully described in Chapter 2.

4.2.11 Statistical analysis of morphometric data

The standard length and dry weight data was initially analysed to yield the median and upper and lower quartiles for individual tanks and for tanks pooled by treatment.

The data sets for individual tanks were then analysed for goodness of fit to a normal distribution using the Kolmogorov-Smirnov test for small samples (Zar 1984). The data sets were acceptably normal ($p>0.05$). The data sets were then tested for homogeneity of variance using the F_{max} test (Sokal & Rohlf 1987). At each sample point there was marked heteroscedasticity. A variety of transformations were attempted but failed

to remove the differences. The experiment was designed to be analysed as a balanced two-factor design by ANOVA (data from tank 10 would not be included). However, this technique assumes normal distribution and homogeneity of variance in the data sets. Due to the heteroscedasticity of the data and the un-balanced design generated at day 38 by the total mortality in tank 9, the data was analysed by non-parametric means.

Length and dry weight data were considered separately. For each sample point i.e. 9, 14, 20, 26, 32 and 38 days post-hatch a Kruskall-Wallis test was carried out between data from tanks making up each treatment. This test was designed to show if intra-treatment differences existed.

Data sets were then pooled by treatment and analysed for inter-treatment differences using a Kruskall-Wallis test.

A non-parametric multiple comparison procedure was then carried out on pooled data for days 32 and 38. Only for these sample points were intra-treatment differences not highly significant. The procedure used was that of Dunn (1964) as described by Zar (1984).

4.3 Results

4.3.1 Light levels and tank design and layout

The light levels in each tank are given in Table IV,i. At no time did the water temperature in the tanks deviate from 8°C by more than 1°C.

4.3.2 Feeding incidence

Plate 4-1 shows the appearance of a sample of the microencapsulated diet after 2 h rehydration in distilled water.

At 9 days post-hatch, 100% of the larvae hatched on April 6 had visible vestiges of yolk-sacs whilst those larvae from subsequent hatches had completely depleted yolk-sacs (Table IV,ii).

There was a positive correlation between hatching date and the dates when larvae showed the earliest signs of feeding.

More than 95% of larvae on the live diet were feeding by 20 days post-hatch and the percentage of larvae with positively identified prey items in the gut never fell below 75% during the remainder of the trial.

Between 20 and 25% of the larvae fed microdiet showed possible signs of feeding at 14 days post-hatch. In the majority of cases this consisted of distended guts but in tank 7 at this age 10% showed positive signs of feeding. From day 20 onwards the proportion showing positive feeding varied from 36-75%. Although gut contents were not scored in terms of volume, it was noted that the amount of feed ingested was small when compared with larvae on the live diet. The digestibility of the microencapsulated diet was not assessed.

Larvae weaned from live to inert feed showed comparable levels of feeding to the live-fed larvae up to day 26. The weaning was almost complete at this stage but *Artemia* were still present in the rearing tanks since positive feeding records were entirely due to the presence of brine shrimps. The feeding incidence fell to 10-50% after the transition to a totally pelleted diet. By 38 days post-hatch the percentage showing positive feeding had risen to a comparable level to that in larvae in tanks 4, 6 and 7, fed only pellets since 5 days post-hatch.

4.3.3 Length and weight data

The morphometric data analysed to yield medians and quartiles are presented in Tables IV,iii to IV,vi. These results are represented graphically in Fig 4-3 and 4-4 for each tank. Fig 4-5 presents the data pooled by treatment. Also shown in Fig 4-5 are the modes and ranges at each sample point. The trends for changes in length and weight were consistent for tanks within the same treatment. The median standard length of live-fed larvae increased during the study from 10.9 to 14.8 mm (pooled data) whilst median dry weight initially fell from 0.275 to 0.201 mg (days 9 to 26) but then increased sharply reaching 0.435 mg by the end of the trial.

Larvae fed on the microdiet increased their median standard length from 11.1 to 12.6 mm between days 9 and 32, after this a decline occurred to 12.3 mm. The microdiet-fed larvae failed to put on weight but did not show the dramatic decrease in dry weight exhibited by the starving control fish.

Weaned larvae showed a greater increase in standard length compared to live-fed larvae over 9 to 26 days (11.1 to 13.1 mm). After weaning was complete the length continued to increase but at a slower rate than for live-fed larvae reaching 14.2 mm by day 38. The dry weights for weaned larvae remained relatively static up to day 26. The unpooled data however showed that the three tanks behaved differently over this period with fluctuating dry weights in two out of three tanks. After weaning was complete, median dry weight increased in tanks 2 and 5 but fell in tank 9. Unfortunately a mass mortality occurred in

this tank and the data set was not completed. The median dry weight from pooled samples thus increased from 0.245 to 0.347 mg after weaning.

The median standard length of starving larvae (Tank 10) fluctuated from day 9 to day 26 post-hatch whilst median dry weight fell dramatically reaching a minimum of 0.138 mg by day 20 post-hatch. All the starving control larvae had died by day 29.

The plots of modal data from samples pooled by treatment followed the median trends. The maximum and minimum values have been plotted to show the wide range which extreme individuals in the samples attained.

4.3.4 Statistical analysis of morphometric data

The results of Kruskall-Wallis tests for differences between the larval samples within treatment groups at each sampling point are given in Table IV,vii. There were highly significant differences ($p<0.01$) in standard length and dry weight for all treatments until day 26, these being especially noticeable in the early stages of the trial. At 32 and 38 days post-hatch, there were some significant ($p<0.05$) but not highly significant differences. The data from individual tanks were pooled by treatment for further analysis but the results for days 9-26 post-hatch must be treated cautiously and the intra-treatment differences found at days 32 and 38 also borne in mind.

The results for Kruskall-Wallis tests between data sets pooled by treatment are given in Table IV,viii. Differences in standard lengths became increasingly significant as the trial progressed whilst differences

in dry weight data were highly significant from 9 days post-hatch onwards.

The results of the multiple comparison procedure on data from days 32 and 38 post-hatch are given in Table IV,ix. The conclusion was that by the end of the trial the standard lengths and dry weights of herring larvae reared on live feed were not significantly different from those which had been weaned onto microcapsules. However, the standard lengths and dry weights of larvae which had been reared exclusively on microcapsules were lower than the other two treatment groups and the difference was highly significant.

4.3.5 Lipid content and lipid class composition

Table IV,x gives the results of lipid content and class analyses for the *Artemia* and microencapsulated feeds. The lipid class composition for rotifers was not determined due to the small samples available. *Artemia* possessed a high proportion of triacylglycerol (28.9%) but only 0.7% wax/sterol esters. Pigment comprised 6.6% of the total lipid. The content of phosphatidylethanolamine was greater than that of phosphatidylcholine. The pellets possessed a high proportion of free fatty acid. Phosphatidylcholine comprised 39.0% of total lipid but phosphatidylethanolamine was only present in trace amounts.

Results of lipid content and class analyses from the larval samples by tank are given in Tables IV,xii to IV,xxi. The larval samples collected in this study exhibited high levels of free fatty acids (12.0 to 36.4%). The levels of free fatty acids recorded in the literature from a variety of fish are given in Table IV,xi for comparison.

The triacylglycerol/cholesterol ratio was calculated from the class composition data. The relative lipid contents were calculated by combining the median dry weight of larvae and the absolute lipid content. The percentages of triacylglycerol and cholesterol relative to larval dry weight were also calculated. This data is summarised, pooled by treatment in Table IV,xxii.

Fig 4-6 illustrates the absolute and relative lipid contents. The absolute lipid content of live-fed larvae increased from 18.5 to 36.7 µg larva^{-1} by the end of the trial. The lipid content of microdiet fed larvae oscillated from day 9 to 27 in a similar manner to that of live-fed larvae but declined after day 27 to 16.4 µg larva^{-1} by day 38. The lipid content of weaned larvae initially followed a similar pattern to live-fed larvae. After the weaning was complete the lipid content of the larvae continued to increase but at a lower rate than for live-fed larvae.

The increases in absolute lipid content after day 26 shown by larvae fed live diet, or weaned onto microcapsules,. when expressed relative to larval dry weight became negative. This implies that other body components, most likely protein, were being laid down at a greater rate. The reverse trend was noted with starving larvae.

Fig 4-7 illustrates triacylglycerol and cholesterol levels relative to larval dry weight and the ratio of these two lipid classes. Expressing triacylglycerol and cholesterol contents relative to larval dry weight allows an assessment of the changes occurring in comparison with other body components. The triacylglycerol content of larvae from all the treatments decreased until day 20. Live-fed larvae then exhibited an increase whilst microcapsule-fed and starved larvae showed a decrease to trace levels. Weaned larvae demonstrated an increase but at a lower rate

than for larvae fed live diets. The minimum levels found in weaned larvae and in larvae fed exclusively on micropellets, were, at day 20 slightly lower than those found in larvae fed live diets.

Cholesterol levels tended to follow an inverse relationship to triacylglycerol levels. This was especially clear with starved larvae (tank 10) which showed a noticeable increase in cholesterol content relative to body weight.

The triacylglycerol/cholesterol ratios declined up to 20 days post-hatch to 0.3 or 0.4 for all treatments but then recovered for live-fed larvae reaching 1.2 by day 38. The ratio for weaned larvae also recovered but increased at a lower rate than for live-fed larvae. The ratio for microdiet fed larvae had reached zero by 38 days post-hatch, a level reached by the starving larvae at 26 days post-hatch.

4.3.6 Fatty acid analyses

The fatty acid spectra of the three diets employed are given in Table IV,xxiii. The majority of the fatty acids in rotifers were 16:0, 16:1, 18:1 and 18:2 with very low levels of 18:3(n-3), 20:5(n-3) and 22:6(n-3). The lipid content relative to dry weight was 8.7%.

Artemia had a higher lipid content at 13.8% and the levels of 18:3(n-3) and 20:5(n-3) were higher than in the rotifers at 15.9 and 8.7% respectively but 22:6(n-3) was not detected.

The fatty acid spectra and lipid content of microcapsules in the size range <90 µm was not significantly different to that of those in the size range 90-500 µm so the results were pooled. The lipid content was 6.4% and the levels of 20:5(n-3) and 22:6(n-3) were 8.3 and 10.9% respectively, 18:3(n-3) was present at 0.7%.

The results of fatty acid analyses of the triacylglycerol fractions of herring larvae fed on the three diets are given in Tables IV,xxiv to IV,xxvi. Amongst larvae fed on live diet, 16:0 decreased and 18:1(n-7) increased whilst there were substantial decreases in 22:6(n-3) and increases in 18:3(n-3). The major changes occurred between days 27 and 38. It is noteworthy that the diet had been changed from rotifers to *Artemia* between days 28 and 31.

The fatty acid spectra of larvae fed on microdiet from 5 days post-hatch was initially similar to those on live feed. The major changes from day 9-27 were an increase in 18:1(n-7) and 20:1 and an increase followed by a decrease in 20:5(n-3) and 22:6(n-3). By 38 days post-hatch, triacylglycerol was only present in trace amounts and so the fatty acids were not determined for this sample.

Larvae subject to weaning showed similar triacylglycerol fatty acid spectra to live-fed larvae until day 20. This pattern included an initial increase in 20:5(n-3) and 22:6(n-3). Weaning onto pellets was completed by day 27. There was an increase followed by a decrease in 20:5(n-3) and 22:6(n-3). By day 38 the level of 20:1 and 18:1(n-9) had increased significantly to 6.1 and 36.7% respectively.

4.3.7 Larval mortality and survival

The numbers of dead larvae recovered from the tanks during cleaning is shown in Fig 4-9. When corrected for larvae removed during sampling there was a massive discrepancy between the estimated numbers of larvae alive at the end of the study and those recovered (Fig 4-8). The values in Fig 4-9 may be cautiously interpreted to indicate the trends in mortality occurring during the trial. The greatest mortality

probably occurred between days 18-32. This was especially apparent in the starved control (tank 10) and also in tanks on live feed (1 and 3), weaning treatment (tank 2) and microcapsules (tank 4). Large mortalities were noted immediately after the transfer of the larvae into the tanks.

There was a wide variation between tanks in terms of survival. Larvae on live diets survived better than those weaned from live to pelleted feeds. Larvae fed pelleted feed throughout the experiment survived less well, but 2.7% did survive to the end of the experiment. In contrast, all the starved control larvae had died by day 32. Survival in tank 7 was poor, especially considering that larvae were not taken from this population for lipid analysis on two occasions. It was felt that disturbance might explain this. The mass mortality which occurred in tank 9 was probably caused by a failure of the water supply on that night. There appeared to be a greater amount of microcapsule debris on the base of this tank compared to the others and the decay of this material combined with a failure in the water supply may have caused a drop in dissolved oxygen. This tank was difficult to clean due to its location and this event highlights the need for good hygiene in this type of study.

4.4 Discussion

4.4.1 Lighting and temperature

These are two environmental factors which may affect fish larval growth and must thus be standardized in comparative growth experiments. When ranked in order of illumination intensity there was no apparent correlation between the tank position and growth performance of the larvae. Blaxter (1968b) quotes the illumination thresholds for

herring larvae feeding on *Artemia* as between 2.0×10^{-2} and 9.0×10^{-2} metre-candles. Thus the illumination in all the tanks used in this trial was substantially above this level.

The study was run at a constant temperature which was within the range normally found in Loch Ewe for this time of year (Gamble *et al.* 1974) and was thus comparable to the mesocosm study of Fraser *et al.* (1987) in this respect.

4.4.2 Sampling of larvae

The use of a light to attract larvae to the water surface for collection may bias the sample towards stronger larvae. The method used later on, when larval numbers were becoming low, of removing water from the tanks with a pail probably produced more representative samples but also caused much more disturbance.

4.4.3 Stocking density, food concentration and feeding incidence

The initial stocking density was twice that recommended as a maximum by Blaxter & Hunter (1982). However, large numbers of larvae were removed for morphometric and biochemical analyses during the course of the experiment so the larval density was rapidly reduced.

The concentration of feed given was greater than that used in many rearing studies although Houde (1975) employed up to 10,000 food organisms l^{-1} in rearing larval sea bream, *Archosargus rhomboidalis*. Herring larvae are able to initiate exogenous feeding at prey concentrations as low as 7.5 particles l^{-1} but the onset of feeding is delayed (Kiørboe *et al.* 1985). The actual feed concentration in the water was likely to have been higher in the live-fed tanks (since live animals

could stay up in the water column) than in the pellet-fed tanks (since the capsules tended to sediment out). It was noted however, that both rotifers and *Artemia* became moribund in the cold water and tended to collect at the bottom of the tanks within a few hours. The difficulty in comparing live and inert feeds for fish larvae is compounded by the fact that the experimental organisms are delicate and vigorous water currents cannot be used. Continuous feeding by mechanical means is recommended, at least for the inert feed but the mechanisms for this were not available in this experiment.

The low feeding incidence recorded at the first two sampling points may have been partly due to the fact that rotifers were broken down much more fully than *Artemia* during digestion. It is possible that feeding remains at this stage were overlooked.

There appeared to be a correlation between the onset of feeding and the hatching date. Since larvae from the hatch on April 6 had vestiges of yolk-sacs remaining at 9 days post-hatch, it seems likely that they had greater reserves of energy and so did not need to begin feeding as early as larvae from later hatches. However, the relative lipid and triacylglycerol contents of larvae at day 9 showed no clear trend with hatching date. Larvae hatched at the earlier date did not commence feeding even though live food was available. It is possible that the later hatch of larvae were at a more advanced stage physiologically and could begin feeding earlier because of this. It is interesting to note in relation to the wild situation that a difference of one or two days in hatching date may have a disproportionate effect on the time available to larvae before first feeding is essential and/or possible.

Larvae fed on microcapsules were undoubtedly taking the pelleted material but probably at a lower rate compared to live feed. An empirical relationship relating mouth width to the particle width for optimum feeding success in herring larvae is available (Beyer 1980) as given below :-

$$pw = 0.83\ mw - 0.057 \quad \text{(mm)}$$

where pw = optimum prey width (mm)
mw = larval mouth width (mm)

Beyers also quotes a relationship for jaw gape to fish length derived from Blaxter (1965) of :-

$$mj = 0.0382\ l - 0.0147 \quad \text{(mm)}$$

where mj = jaw gape (mm)
l = larval length (mm)

Assuming that jaw gape is synonymous with mouth width (if this is not true then optimal particle size becomes larger than jaw gape) and using values for the length increase of Clyde herring larvae growing under good nutritional conditions over 40 days of 8.3 to 15.9 mm (Fraser *et al.* 1987), then optimum prey width will increase from 194 µm to 435 µm. These values are larger than the prey widths recorded in selection experiments by Checkley (1982) which indicated a maximal prey width of 250 µm for a 16 mm larva.

The sizes of microcapsules used were chosen to be approximately similar to the widths of *Artemia* nauplii and to cover the optimal widths indicated by the theoretical calculations above.

The provision of the correct size of inert diets for fish larvae is further complicated by the development of size hierarchies within the larval population and the technical constraints of the diet manufacture.

The initial feeding incidence levels of weaned larvae were comparable to the live-fed larvae over this period. The decline in feeding incidence after the transfer to totally pelleted feed indicates that the larvae required some time before they learnt to take the inert diet.

4.4.4 Growth as measured by standard length and dry weight

The initial decrease in dry weight of larvae on the live diet was probably due to the nutritional inadequacy of the rotifers. It was notable that growth of larvae on the live diet increased dramatically once *Artemia* nauplii were included in the diet (after day 28). Subsequent experiments showed that spring-spawned herring larvae are capable of taking brine shrimp nauplii from first feeding (see Chapter 3).

The use of a monospecific diet of brine shrimps may not provide a good control. High mortalities in sea bass, *Dicentrarchus labrax*, have been linked with use of *Artemia* as the sole food source (Katavic 1986). This problem may be overcome by supplementing the feed with live-caught zooplankton. However the time and resources were not available for this in this trial.

Morphometric differences between laboratory reared and wild caught fish larvae are common (Blaxter 1975). However, we may cautiously compare the growth obtained in the live-fed controls with values quoted by Fraser *et al.* (1987) for larvae from the same stock reared in 300 m^3 mesocosms at Loch Ewe. Fraser *et al.* obtained daily increases in length and dry weight over the period 9-38 days post-hatch of 0.2 mm day^{-1} and 0.027 mg day^{-1} for well fed larvae. This compares with increases of 0.13 mm day^{-1} and 0.006 mg day^{-1} over a comparable period in this study. The relatively poor growth in this trial could be due to the nutritional factors mentioned above or to the effect of rearing the larvae in a

confined area. Container size has been found to affect the growth of larval jack mackerel, *Trachurus symmetricus* (Theilacker 1980).

The larvae reared on microencapsulated cod-roe grew better compared to the starving control but did not put on weight and the decline in median standard length after 28 days post-hatch indicated that mortality would have eventually eliminated the stock without positive growth.

The data from the weaning study was less clear. After weaning was completed, median dry weight remained stable (tank 9) or increased (tanks 2 and 5). This implies that weaned larvae were gaining much more benefit from the microencapsulated cod-roe diet than the larvae which had been reared exclusively on the micropellets. It is unclear from the data whether this trend would have continued or if growth of weaned larvae would have subsequently declined.

Statistical analysis indicated highly significant intra-treatment differences between 9 and 26 days post-hatch. These may have been generated by the use of stocks of larvae hatched on different days. Although this method allowed the exact age of the larvae to be calculated, the use of a mixed stock may have removed this variation.

Due to the levels of intra-treatment variation, the multiple comparison procedure was only carried out on pooled data for 32 and 38 days post-hatch. This confirmed that the live-fed larvae had achieved the best growth. By the end of the trial, larvae which had been weaned had slightly lower lengths and weights but these differences were not statistically significant. The microdiet-fed larvae had performed least well but had still survived and grown better than the starving control larvae.

The aquaculturist will be interested in measurement of the mean or median length or weight of the population and in maximizing this along with survival. However, the ecologist may be more interested in what happens to the extremes of the population. Enhanced recruitment of a year class to the mature stock can be the result of very small changes in the percentage survival of larvae (Jones 1989, Houde 1987) and from this point of view the use of average statistics in may be misleading. This is demonstrated here by the plots of modes and maxima and minima for the samples (Fig 4-5). Thus at day 38 for larvae reared on capsules, although the median and modal dry weight of the population had decreased since day 26, at least one larva had reached a weight comparable with the live-fed larvae. In ecological terms high performance of a few larvae may be more important than the growth of a complete population. This clearly complicates the interpretation of ecologically orientated experiments but is recommended as a point for consideration.

The inter-quartile ranges and the absolute ranges within treatments and tanks show that size hierarchies had developed amongst the larvae in this trial. This was most pronounced for dry weight measurements of live-fed larvae and is a common phenomenon in fish larval rearing studies (Blaxter 1975). The reasons why some larvae seem to grow so much better than others, even when the population trend is downwards, deserve investigation. Whether such hierarchical effects are important in natural populations is unclear.

4.4.5 Lipid content and lipid class analyses

Relatively little work has been carried out on the requirements of fish for different lipid classes. Kanazawa (1983a & 1983b) demonstrated

that the growth of red sea bream, knifejaw and Ayu larvae was markedly improved by the addition of 3% soybean or egg lecithin to microparticulate zein-coated diets. Since a diet containing pollack oil failed to have the same effect it was concluded that the lipid classes rather than the lack of (n-3)PUFAs were responsible.

The main commercial source of (n-3)PUFA is from fish oils which are predominantly composed of triacylglycerol (Sargent *et al.* 1989). It is possible that problems experienced in fish larval rearing on commercial feeds may be partly due to the low levels of phospholipids in them. Cod-roe is a rich natural source of phosphatidylcholine (Tocher & Sargent 1984) and this class is retained during microencapsulation as shown in this study (where it comprised 39% of the lipid from the microcapsules). Tocher & Sargent (1984) also found that phosphatidylethanolamine comprised 20.2% of cod-roe total lipid so its absence from the microdiet was surprising. It is possible that it was linked to protein in the encapsulation process since it has a free amine group. It would then not be extracted in the Folch-Lees procedure.

The high levels of free fatty acids in the larval samples were indicative of degradation. From the literature the maximum level of free fatty acid expected is about 10%. In this case the degradation could be attributed to a break-down of the -80°C freezer used to store the samples. Although the samples were rapidly transferred to another freezer they may have thawed out for several hours. In subsequent work samples were stored in chloroform:methanol (2:1 by vol) at -20°C. Free fatty acid levels in these samples were present only at trace levels (see Chapter 3).

Despite the degradation of the samples several parameters are still likely to be reliable. Total lipid content should only have been changed

by a small factor. Cholesterol is not subject to lipase attack and will not be affected. Triacylglycerols may be affected by lipase action but are much less susceptible than the phospholipids (Sargent pers. comm.).

Although lipid in live-fed larvae increased in absolute terms during the study, when expressed relative to body weight, the decrease after day 27 indicated that the accumulation of non-lipid material (probably protein) was more important at this stage. This trend was also noted in enclosure reared larvae (Fraser *et al.* 1987). The maximum relative lipid content (10.4% for live-fed larvae on day 26) was slightly less than that recorded by Fraser for well nourished larvae of a similar age reared in an enclosure. The maximum absolute lipid contents in this study were about one third of those recorded by Fraser. This again indicates that the growth exhibited by larvae on the control live diet was sub-optimal.

The decrease in absolute lipid content for larvae fed exclusively on the microdiet suggested that the feed was inadequate to support growth.

Since the absolute lipid content of weaned larvae continued to increase after their transfer onto the encapsulated feed, they were clearly able to gain more benefit from this feed than the larvae fed microcapsules from day 5 post-hatch.

The trend observed in starving larvae, of a conservation of lipid at the expense of other body components, was the opposite of that observed by Tocher *et al.* (1985).

4.4.6 The use of triacylglycerol/cholesterol ratios as a nutritional condition index

Triacylglycerol is the main energy storage lipid for fish. Thus the level of this lipid class is usually related to the nutritional condition of the larvae. It has been suggested that the ratio of triacylglycerol to

cholesterol would provide a useful condition index (Fraser 1989, Fraser *et al.* 1987).

In this study, changes in the two components of the proposed condition index were first assessed. The decreases in triacylglycerol content of live-fed and weaned larvae between days 9 and 20 may have been due to a low rate of capture success leading to a net loss of energy (Kiorbøe *et al.* 1985). A second reason might be the poor nutritional quality of the rotifers used as feed. However, the small increase in triacylglycerol reserves between day 20 and 26 (i.e. before *Artemia* were included in the diet) indicated that the larvae were obtaining sufficient nutrients to lay down some reserves.

The triacylglycerol levels in microdiet fed larvae supported the conclusion that the microdiet was unable to sustain growth beyond day 26.

The pattern for weaned larvae confirmed the conclusions drawn from the morphometric data. Weaned larvae were able to avoid a depletion in energy reserves as was suffered by those larvae fed exclusively on microcapsules.

The pattern for cholesterol content, relative to dry weight, in starving larvae implied that net catabolism of cholesterol was occurring at a lower rate than for other body components. Undernourished larvae from enclosure studies (Fraser *et al.* 1987) showed a similar increase in sterol levels which did not occur in well fed larvae. In this study live-fed larvae, judged to be in moderately good condition from morphometric and lipid content data, showed relatively low cholesterol levels at the termination of the trial.

Since the relative cholesterol levels for microcapsule reared larvae did not increase as much as in starved larvae, the conclusion that the microcapsules were providing some nourishment was supported.

Condition indices based on morphometric methods suffer from the constraints that wild-caught samples are often distorted during capture (McGurk 1985) or fixation (Hay 1981) whilst indices based on histological criteria tend to be time consuming (O'Connell 1980). For these reasons considerable effort has been directed towards biochemical condition indices. These should be less affected by capture damage and automation of the assays involved might be possible.

Fraser (1989) states that the advantage of using the triacylglycerol/cholesterol ratio as opposed to the triacylglycerol content alone, is that quantitative extraction of lipid from larvae would not be necessary. However, triacylglycerol determined relative to total lipid also does not require quantitative extraction. In the opinion of the author, the only apparent advantage of using the triacylglycerol/cholesterol ratio, is that the sensitivity of the index may be increased since the two components appear to have an inverse relationship. The Iatroscan system has been employed for the analysis of microgram quantities of lipid (Hakanson 1984). However, the difficulties experienced with the system (discussed in section 2.9) indicated that the use of HPTLC/densitometry might be more successful and that single fish larvae might be analysed by this method.

The trends shown by the ratio in this study confirmed the conclusions drawn earlier from morphometric and lipid content data. The fact that the ratio can fall as low as 0.3 and the larvae subsequently

Since the relative cholesterol levels for microcapsule reared larvae did not increase as much as in starved larvae, the conclusion that the microcapsules were providing some nourishment was supported.

Condition indices based on morphometric methods suffer from the constraints that wild-caught samples are often distorted during capture (McGurk 1985) or fixation (Hay 1981) whilst indices based on histological criteria tend to be time consuming (O'Connell 1980). For these reasons considerable effort has been directed towards biochemical condition indices. These should be less affected by capture damage and automation of the assays involved might be possible.

Fraser (1989) states that the advantage of using the triacylglycerol/cholesterol ratio as opposed to the triacylglycerol content alone, is that quantitative extraction of lipid from larvae would not be necessary. However, triacylglycerol determined relative to total lipid also does not require quantitative extraction. In the opinion of the author, the only apparent advantage of using the triacylglycerol/cholesterol ratio, is that the sensitivity of the index may be increased since the two components appear to have an inverse relationship. The Iatroscan system has been employed for the analysis of microgram quantities of lipid (Hakanson 1984). However, the difficulties experienced with the system (discussed in section 2.9) indicated that the use of HPTLC/densitometry might be more successful and that single fish larvae might be analysed by this method.

The trends shown by the ratio in this study confirmed the conclusions drawn earlier from morphometric and lipid content data. The fact that the ratio can fall as low as 0.3 and the larvae subsequently

recover (weaned larvae in this study) may limit its use in predicting the future survival of a population in the wild.

As with all condition measures, calibration poses considerable problems (Fraser 1989). It remains to be seen whether lipid based condition indices will prove any more useful than the established methods or other new techniques such as RNA/DNA ratios, which can also determine a biochemical condition factor for individual animals (Clemmesen 1988, Martin & Wright 1987).

4.4.7 Fatty acid analyses

The nutritional composition of the rotifer is markedly affected by the feed it is given in culture (Watanabe *et al.* 1983) and if fed on yeast alone it becomes deficient in (n-3)PUFA. The supplements of *Nannochloropsis oculata* given in this study were clearly inadequate to boost the levels of (n-3)PUFA in the rotifers. Large scale culturing of this algae was not possible in this trial. Since a supply of long-chain (n-3)PUFA is considered essential for rearing marine fish larvae (Kanazawa 1985), the rotifers probably provided an inadequate prey for the herring larvae. This may explain the relatively poor larval growth of the live-fed control larvae before day 27.

The levels of lipid and 20:5(n-3) present in the San Francisco Bay *Artemia* were higher but were still low in relation to the levels present in wild prey (see section 3.4.3). The problems of using brine shrimps as the only food source for rearing marine fish larvae have already been discussed.

The microcapsules contained considerably more long chain (n-3)PUFAs but the level of lipid was only 6.4% of dry weight. Cod-roe contains about 13.2% lipid (Tocher & Sargent 1984). It is likely that in manufacturing the microcapsules a considerable proportion of lipid is lost (since the capsules are prepared in cyclohexane, an organic solvent). This is a major problem with this type of encapsulated diet which has yet to be solved on a commercial scale.

The fatty acid spectra of the triacylglycerol fraction of the larvae tended to reflect the dietary fatty acids. The change of diet of live-fed larvae from rotifers to *Artemia* was not manifested until day 38 post-hatch. Thus a lag occurred before the dietary fatty acids were reflected in the triacylglycerol reserves. It is presumed that the relatively high levels of 20:5(n-3) and 22:6(n-3) in the triacylglycerol of live-fed larvae between 9 and 20 days post-hatch were due to efficient conservation of these fatty acids during a period in which dry weight did not increase significantly. After 28 days post-hatch, growth increased dramatically and it is likely that these fatty acids were diverted to the phospholipids for cell membrane formation. Thus the levels of 20:5(n-3) and 22:6(n-3) in the triacylglycerol reserves fell. The increases in 18:3(n-3) and 18:1(n-7) are explicable by their high levels in the *Artemia*.

The fatty acid spectra from the triacylglycerol fraction of herring larvae reared on microencapsulated cod-roe were initially similar to those of larvae fed live-diets. The overall fall in content of (n-3)PUFA from day 14-27 combined with the decrease in absolute triacylglycerol levels suggested that the microdiet was not contributing significantly to the nutrition of the larvae.

The increases in 20:5(n-3) and 22:6(n-3) in the triacylglycerol of weaned larvae between days 9 and 20 were presumably derived from the efficient retention of these fatty acids as in live-fed larvae. After weaning onto pellets (completed by day 27) the levels of 20:5(n-3) and 22:6(n-3) in triacylglycerol initially increased but then decreased. This probably indicated the preferential use of these fatty acids from the diet for growth. The increase in 20:1 reflected the presence of this fatty acid in the microdiet at a level of 5.2%.

In conclusion, the fatty acid data indicated that the dietary intake of microdiet was inadequate for larvae reared entirely on this feed. However, the presence of 20:1 in the triacylglycerol of larvae which had been weaned onto the microdiet indicated that the pellets were being ingested and were contributing significantly to their nutrition.

4.4.8 Mortality data and live recoveries

The low numbers of larvae noted in Tank 7 throughout the trial were thought to be caused by disturbance of the larvae due to the fact that this tank was adjacent to the door.

Mixing and sub-sampling of the larval populations was tried as a means of estimating daily survival but the amount of variation between replicate counts did not justify the disturbance caused. A suitable technique for measuring daily mortality of herring larvae in tanks of this size was not found. The collection of dead larvae underestimated mortality as the larvae are prone to rapid disintegration.

Mortality estimates (Fig 4-9) indicated that a high mortality occurred in most tanks, including the starved control larvae, between days 24 and 32. This may imply that a proportion of the larvae in all the tanks failed to initiate feeding successfully and suffered mortality once their energy reserves had been depleted.

4.4.9 Reasons for the poor performance of microcapsule reared larvae

There are a variety of reasons why diets may perform poorly for fish larval rearing. The problem may be regarded at two levels. The first is to get the fish to ingest sufficient of the feed and the second is to consider what happens once the feed is ingested.

The first stage may be analysed at the following levels:-

1. The fish must be interested enough in the feed to initiate an attack. Factors which may be important at this level include appearance and size of the feed, the movement of the feed and chemicals released by the feed.

2. Once attacked the feed must be retained and swallowed. Factors important at this level include the size and shape of the feed, the taste of the feed and its texture.

The second stage may be analysed at the following levels:-

3. Once ingested the particle must be digested. Factors important at this level include the coating of the particle and the susceptibility of the particle to digestion.

4. If all the above conditions can be met the particle must contain the correct nutrients to support growth.

In this study, records of gut contents indicated that the ingestion rate for pellets was considerably lower than for live feeds. The data collected in this study did not indicate whether this was due to a poor attack rate, or due to rejection of pellets after attack. The photomicrograph of the microdiet (Plate 4-1) shows that a high proportion

of the encapsulated material consisted of fragments and ruptured pellets. The microencapsulation process may further degrade the nutritional value of the smallest pellets by totally cross-linking the particle.

At present, there is considerable debate over the relative importance of digestive enzymes produced by fish larvae and those obtained from the diet. Low levels of endogenous proteolytic enzyme production by coregonid larvae have been correlated with difficulties experienced in rearing them on inert feeds (Lauf & Hofer 1984). A few attempts have been made to improve the performance of inert feeds by incorporating proteolytic enzymes into diets. However, the addition of bovine trypsin to diets for common carp fry only increased growth marginally (Dabrowski & Glogowski 1977). The problem of quantifying endogenous and exogenous proteolytic enzymes was tackled in a novel way by Hjelmeland *et al.* (1989) who measured the levels of trypsin and trypsinogen using a sensitive radio-immunoassay in herring larvae which had ingested inert polystyrene spheres. The levels found were compared to control larvae which had ingested live *Acartia tonsa* nauplii. Although ingestion of inert spheres did cause a slight increase in proteolytic enzyme activity, the ingestion of live prey caused a considerably greater increase. Either the live prey stimulate proteolytic enzyme secretion in a way that the inert spheres do not, or autolysis of the ingested prey accounts for the difference.

Very little work has been carried out on lipid digestion in fish larvae. Cousin *et al.* (1987) used histochemical methods to monitor the production of various digestive enzymes in turbot larvae fed on rotifers and *Artemia*. They found that lipase enzymes were only produced after day 15, which coincided with metamorphosis. This surprising result may

be due to the relative insensitivity of the assay used. The development of radioimmunoassays for the lipases of marine fish larvae would provide much useful information.

The ability of fish larvae to digest different lipid classes also requires investigation. Although phospholipid digestion has not been studied in fish (Henderson & Tocher 1987), phospholipids may be more easily digested than triacylglycerols due to their emulsification properties. This has serious implications for the formulation of commercial fish diets which currently incorporate large quantities of triacylglycerol as their lipid source (Sargent *et al.* 1989).

The results from larvae which were weaned onto the microencapsulated cod-roe diet were encouraging. It is possible that the ingestion of live prey at first feeding stimulates the development of the digestive system in a way in which inert feeds fail to do. A histochemical study of the ontogeny of digestive enzyme production in larvae reared solely on, and weaned onto the microdiet would be valuable.

4.5 Conclusions

It was considered that without development of weaning techniques, cross-linked protein diets would probably not support long-term rearing studies with herring larvae.

The growth of herring larvae reared on commercially microencapsulated cod-roe was compared with live-fed and starved controls and with that of larvae weaned from live to inert diet. Larvae reared on the inert diet survived better than the starving control. They

increased in length but failed to put on weight or lay down triacylglycerol reserves.

Larvae weaned onto the inert diet utilised microencapsulated cod-roe more successfully than those reared on microcapsules from first-feeding. At the end of the trial weaned larvae had slightly shorter median standard length and slightly reduced median dry weight compared to larvae reared on a live diet. The differences were not however statistically significant.

The levels of triacylglycerol and possibly the ratio of triacylglycerol to cholesterol appear to provide reasonable indices of nutritional condition. In future, it may be possible to quantify these lipid classes in individual larvae.

Evidence from the fatty acid analyses of the triacylglycerol fractions of larvae did not contradict the conclusions of Chapter 3, namely there was no evidence for efficient elongation and desaturation of 18:3(n-3) and 20:5(n-3) by herring larvae.

4.6 Feeding observation experiments

A short series of experiments were carried out during the spring of 1988 to investigate further the reasons for the failure of larvae to show positive weight gain on microencapsulated feed.

4.6.1 Materials and methods

Herring larvae were from the stock obtained as described in section 3.2.1. Larvae were reared on San Francisco Bay *Artemia*. Two vertical, transparent perspex cylinders each holding 29.6 l of seawater were set up. The cylinders were supplied with flowing seawater, filtered to 0.22 µm and were covered with a black polythene sleeve to reduce disturbance of the larvae. The polythene sleeve could be lowered to make observations. Tube 1 was fitted with an autofeeder capable of dispensing microencapsulated diet in suspension. The autofeeder was programmed to operate during the hours of light only. The light:dark cycle was as in the previous section.

As cod-roe was not available, the microcapsules were based on frozen mysids supplied by Xotic Pets Ltd. and encapsulated by Niel McDonald (Heriot-Watt University on contract to Frippak Ltd.). They were in the size range 90-250 µm and were washed in distilled water for 2 h prior to use.

Trial I - The effect of weaning larvae onto microcapsules.

One hundred larvae aged 24 days post-hatch were introduced to each cylinder and initially fed on *Artemia*. After 24 h, dead larvae were removed from the base of the tube and replaced with live animals. The

Artemia in tube 1 were gradually replaced over a period of days with microcapsules dispensed continually by the autofeeder as shown in the table below :-

	Concentration of prey items in water column		
	Tube 2	Tube 1	
Day	*Artemia* l^{-1}	*Artemia* l^{-1}	Capsules l^{-1}
1	2000	2000	-
2	2000	1600	400
3	2000	1200	800
4	2000	800	1200
5	2000	400	1600
6	2000	-	2000
7	2000	-	2000
8	2000	-	2000

The levels of *Artemia* in the tubes were assessed at 09:00 and 17:00 daily by taking cores of water and counting subsamples (four cores each counted four times per tube). Sufficient *Artemia* were added after this assessment to restore the concentration to the levels shown above. The amount of microcapsules dispensed by the autofeeder was assessed by combining data on the time taken for the capsules to sink to the base of the tube (3 h) with the number of pellets g^{-1} of dry feed.

At 12:00 and 20:00 daily, the gut contents of 20 larvae in each tube were scored visually. An empty gut scored 0, a full hind-gut 5 and a full gut 10. Behavioural observations were also made.

Trial II - The role of prey movement.

The observation tubes were set up as in trial I with larvae aged 35 days post-hatch which were acclimated for 24 h without food. A sample of newly hatched *Artemia* nauplii were killed by plunging them into water at 95°C and collecting the dead shrimps on a mesh. Dead *Artemia* were introduced continually to tube 1 by the autofeeder described above and

live *Artemia* added to tube 2 to give prey concentrations of 2000 l^{-1}. The stock of *Artemia* in the autofeeder was replenished every three hours. The experiment was run for 48 h with two sets of gut scores being made as above.

4.6.2 Results

Trial I - The average daily mortality was 2.3% and 1.8% for tubes 1 and 2 respectively. A few *Artemia* remained in the water column of tube 1 even after day 6 when only microcapsules were being dispensed.

Behavioural observations were made on several days and are summarized below.

The addition of pellets at the water surface caused herring larvae in this region to swim erratically although they settled down after a few moments. The pellets mixed into the top 0.3 m of water within 5 min and a few fish took up S-shaped attack postures but then lost interest. The remaining fish did not appear interested. After 10 min the water circulation had distributed the pellets throughout the upper 0.7 m of water. A few larvae were seen to attack pellets but they were rejected immediately after capture. After 1 h, the largest pellets were near the base of the tube. Larval activity appeared to have increased slightly and several more larvae went into S-shapes over the next hour. The level of activity of the larvae was considerably lower than in the control tube which contained live *Artemia*. The larvae preferentially selected the brine shrimps as opposed to the pellets, even when the density of shrimps was very low and that of the pellets high.

Numerical data are presented in Table IV,xxvii. The gut scores were analysed using the Mann-Whitney test. The z-statistic and the two-tailed probability of equalling or exceeding this value are given.

The mean gut scores of larvae in the control tube were somewhat variable during the trial but never fell below 1.7. As the change in diet in tube 1 from *Artemia* to microcapsules took place, the feeding levels fell. After the transfer to pelleted feed (day 6) the gut scores remained low (0 to 0.7) for 48 h but a slightly higher score was recorded at the final assessment. A few *Artemia* were still present in the tube at this time and were noted in the guts of some larvae. These animals were not included in the daily assessments of gut contents after day 6. Once weaning was complete the gut scores of microdiet-fed larvae were statistically, significantly lower than those of the control larvae.

Trial II - The dead *Artemia* formed dense clumps in the water column after being released from the autofeeder and did not disperse as well as the pellets. Initially, larvae showed no interest in the food cloud. After 30 min, the feed had become more dispersed and some larvae were seen to attack the dead shrimps. If the strike was successful, the particle was invariably swallowed and rejection behaviour was not observed. In many instances similar behaviour to that observed with the pellets was noted in that larvae took up S-shaped attack positions but then lost interest and drifted away.

The feeding levels in the control tube were comparable to those found in Trial I. At the first assessment the gut scores for tube 1 (being fed dead *Artemia*) were higher but this trend reversed for the next three assessments and the differences were statistically significant. The mean

gut scores of larvae fed dead *Artemia* were higher than for those fed micropellets in the previous trial.

4.6.3 Discussion

Herring larvae are primarily visual feeders and the manner of feeding involving the S-shaped attack position has been well documented (Rosenthal & Hempel 1970). It is also known that visual contrast of prey items is important for larval fish feeding. Dendrinos *et al.* (1984) were able to demonstrate that the capture success of Dover sole larvae and juveniles was improved by staining *Artemia* nauplii with black food dye.

The results from trial I suggested that even with gradual weaning, herring larvae aged 24 days post-hatch were reluctant to take the microencapsulated feed and selected out *Artemia*, even when they were present at low concentrations. From the behavioural observations, it is suggested that movement is an important cue in eliciting attack behaviour by the larvae. However, herring larvae will ingest non-motile particles, as demonstrated by the use of inert polystyrene spheres in a study of proteolytic enzyme secretion (Hjelmeland *et al.* 1989).

On the few occasions when micropellets elicited an attack, the particle was rejected after capture. Similar behaviour has been noted in larval guppies (*Poecilia reticulata*) fed nylon-encapsulated microcapsules (Jones *et al.* 1984). The pellets thus not only fail to stimulate a substantial number of attacks but appear to be unacceptable if they are captured. This may be due to their taste and/or texture. It was noted that capsules were at pH 4-5 (as measured by indicator solution) even after rehydration in distilled water for 2 h. This presumably derives from

the use of an acid cross-linking agent in the manufacture process and may make them unpalatable.

Trial II examined the role of prey movement further by comparing the feeding behaviour of larvae on dead and live *Artemia*. The use of freshly killed shrimps should have meant that the size, colour, texture and chemical exudates of the test and control diets were similar. Differences in the behaviour of the larvae in relation to the two diets were thus attributed to the lack of movement of the test diet. The gut scores for larvae fed dead *Artemia* were higher than those of larvae weaned onto pellets and this was explained by the fact that shrimps which were attacked were not rejected.

This area requires further investigation to determine if the response to movement is innate, or learned. The fact that willingness to ingest inert feeds is apparently correlated with larval size has already been mentioned but the reasons why older fish appear more willing to tackle non-motile food items are unclear.

4.6.4 Conclusions

Even with gradual weaning the feeding rate of herring larvae on microcapsules was low. This was attributed mainly to the static nature of the particles which appeared to elicit fewer attacks than motile prey. Particles which were attacked were usually rejected. A small percentage of the larvae did ingest a low number of capsules.

The feeding of larvae on freshly killed *Artemia* was at an intermediate level to that on live prey and microcapsules. Although the

number of attacks was less than on live prey, rejection behaviour of captured particles was not noted. It is hypothesised that prey movement is a major factor in eliciting attacks by herring larvae.

4.7 Overall conclusions to Chapter 4

The results of the trials in 1987 and 1988 suggest that herring larvae will take microencapsulated feeds to a limited extent. It was felt that the problems to be solved in rearing marine fish larvae on inert diets precluded their use, at the time, for long-term nutritional studies. The relatively good growth of herring larvae which had been weaned onto microencapsulated cod-roe suggested that this material may be useful as a feed for marine fish larvae.

The low capture rate of micropellets by herring larvae also precluded their use as an efficient vector for the delivery of radiolabelled lipids. A radically different approach to the design of a suitable vector was developed and is described in the following chapter.

Table IV,i Light levels in tanks

Tank	Surface ($\mu Em^{-2}s^{-1}$)	Basal	Surface (mc)	Basal
1	26.6	21.9	2217	1825
2	26.6	23.8	2217	1983
3	35.9	33.3	2992	2775
4	36.6	33.3	3050	2775
5	28.0	19.0	2333	1583
6	26.0	19.0	2167	1583
7	31.9	23.8	2658	1983
8	36.6	26.6	3050	2217
9	18.0	11.4	1500	950
10	23.3	16.2	1942	1350

Table IV,ii Feeding incidence in herring larvae fed on live or microencapsulated diet and in larvae being weaned from live to inert feed

The percentage of larvae with food in the gut (+) or with traces of possible feeding (?) from a sample of size (n) is given.
The larval ages are given in days post-hatch (dph).

Live fed larvae

Age (dph)	Tank 1			Tank 3			Tank 8		
	+	?	n	+	?	n	+	?	n
9			(21)			(21)			(21)
14		1	(21)			(21)	90	5	(21)
20	100		(21)	100		(14)	95		(21)
26	100		(19)	95		(20)	75	15	(20)
32	95		(20)	100		(20)	90		(20)
38	75	15	(20)	100		(20)	90		(19)

Microencapsulated diet fed larvae

Age (dph)	Tank 4			Tank 6			Tank 7		
	+	?	n	+	?	n	+	?	n
9			(21)			(21)			(14)
14		20	(21)		23	(13)	10	25	(21)
20	65		(17)	40	20	(20)	50	10	(21)
26	85	10	(20)	56	15	(18)	60	35	(20)
32	40	55	(20)	30	15	(20)	36	14	(14)
38	75	20	(20)	35	25	(20)	40	10	(20)

Weaned larvae

Age (dph)	Tank 2			Tank 5			Tank 9		
	+	?	n	+	?	n	+	?	n
9			(21)			(21)			(21)
14			(21)		40	(13)	85		(20)
20	83		(18)	100		(13)	85		(20)
26	80	10	(20)	75	15	(20)	95		(20)
32[a]	20	40	(20)	10	25	(20)	50	15	(20)
38	65	10	(20)	65	25	(20)	Mass mortality		

[a] Weaning completed by day 27 post-hatch
Tanks 1,2,3 hatched 06/04/87
Tanks 4,5,6 hatched 07/04/87
Tanks 7,8,9 hatched 08/04/87

Table IV,iii Standard lengths and dry weights for herring larvae reared on live feed

Data are expressed as medians (med) and lower (q1) and upper quartiles (q2). The number of larvae in the sample is also given (n).

Live fed larvae - data from individual tanks

Tank 1

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	21	10.6	10.3	11.2	21	0.285	0.239	0.348
14	21	11.4	11.1	12.0	21	0.206	0.189	0.229
20	21	12.1	11.3	12.5	21	0.202	0.154	0.232
26	19	12.8	11.9	13.1	19	0.210	0.143	0.252
32	20	13.8	13.2	14.3	19	0.318	0.263	0.360
38	20	14.8	13.7	15.3	20	0.409	0.284	0.448

Tank 3

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	21	10.8	10.4	11.0	20	0.226	0.212	0.243
14	21	11.9	11.6	12.2	21	0.227	0.201	0.248
20	14	12.7	12.2	12.8	14	0.254	0.224	0.296
26	20	12.1	11.4	12.8	20	0.196	0.160	0.268
32	20	13.9	13.4	14.4	20	0.339	0.311	0.415
38	20	14.8	14.1	15.7	20	0.436	0.327	0.575

Tank 8

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	21	11.3	10.9	11.6	21	0.291	0.268	0.326
14	21	12.0	11.7	12.8	21	0.231	0.206	0.262
20	21	12.1	11.8	13.2	14	0.216	0.186	0.307
26	20	12.7	12.0	13.6	20	0.192	0.167	0.297
32	20	14.2	13.3	15.3	20	0.320	0.281	0.478
38	20	15.0	13.7	15.9	20	0.477	0.279	0.602

Live fed larvae - pooled data

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	63	10.9	10.4	11.4	62	0.275	0.222	0.308
14	63	11.9	11.5	12.2	63	0.222	0.197	0.244
20	56	12.2	11.6	12.8	56	0.225	0.184	0.267
26	59	12.4	11.6	13.1	59	0.201	0.158	0.259
32	60	13.9	13.3	14.4	59	0.325	0.287	0.408
38	60	14.8	13.9	15.6	60	0.435	0.294	0.575

Table IV,iv Standard lengths and dry weights for herring larvae reared on micro-encapsulated cod-roe

Data are expressed as medians (med) and lower (q1) and upper quartiles (q2). The number of larvae in the sample is also given (n).

Microcapsule fed larvae - data from individual tanks

Tank 4

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	21	11.1	11.0	11.3	19	0.214	0.199	0.225
14	21	11.5	11.2	12.0	21	0.177	0.162	0.185
20	17	11.6	11.3	12.4	17	0.208	0.168	0.229
26	20	12.1	11.7	12.5	20	0.227	0.177	0.252
32	20	12.5	12.1	12.9	18	0.228	0.201	0.245
38	20	12.3	11.7	12.5	20	0.203	0.195	0.221

Tank 6

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	21	11.3	11.0	11.5	21	0.216	0.204	0.232
14	13	11.6	11.3	12.0	13	0.176	0.167	0.188
20	20	12.0	11.5	12.4	19	0.258	0.201	0.305
26	18	12.8	12.3	13.2	18	0.257	0.225	0.274
32	20	12.7	12.4	13.2	20	0.240	0.229	0.256
38	20	12.5	11.9	12.6	20	0.236	0.210	0.270

Tank 7

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	14	10.7	10.2	10.9	14	0.270	0.206	0.291
14	21	11.2	10.8	11.6	21	0.170	0.163	0.195
20	21	11.9	11.5	12.4	21	0.185	0.155	0.242
26	20	12.6	12.3	13.3	20	0.274	0.224	0.305
32	14	12.7	12.1	13.1	10	0.213	0.172	0.236
38	20	12.4	12.0	12.6	20	0.212	0.191	0.241

Microcapsule fed larvae - pooled data

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	56	11.1	10.9	11.4	54	0.217	0.202	0.245
14	55	11.5	11.1	11.7	55	0.176	0.165	0.190
20	58	11.9	11.5	12.4	57	0.208	0.178	0.266
26	58	12.5	12.0	12.9	58	0.250	0.220	0.276
32	54	12.6	12.1	13.1	48	0.232	0.203	0.251
38	60	12.3	12.0	12.6	60	0.211	0.195	0.236

Table IV,v Standard lengths and dry weights for herring larvae weaned from live feed onto microencapsulated cod-roe

Data are expressed as medians (med) and lower (q1) and upper quartiles (q2). The number of larvae in the sample is also given (n).

Weaned larvae - data from individual tanks

Tank 2

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	21	10.5	10.1	11.0	19	0.224	0.211	0.234
14	21	11.2	10.7	12.0	21	0.224	0.186	0.248
20	18	12.5	11.4	13.1	18	0.229	0.174	0.255
26	20	12.4	11.0	13.0	20	0.186	0.127	0.214
32	20	14.0	13.0	14.6	18	0.314	0.216	0.427
38	20	14.6	13.8	15.3	20	0.347	0.265	0.418

Tank 5

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	21	11.2	10.8	11.4	20	0.211	0.200	0.231
14	21	11.9	11.6	12.1	21	0.226	0.181	0.245
20	13	12.4	11.5	12.7	13	0.245	0.197	0.263
26	20	13.3	13.0	13.6	20	0.247	0.232	0.309
32	20	13.0	12.5	14.5	20	0.241	0.179	0.374
38	20	13.9	12.6	14.6	20	0.350	0.246	0.493

Tank 9

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	21	11.3	11.1	11.6	20	0.325	0.292	0.374
14	20	11.2	10.5	11.6	19	0.220	0.188	0.230
20	20	12.6	12.1	13.0	20	0.248	0.234	0.289
26	20	13.5	12.9	14.0	19	0.305	0.244	0.370
32	20	13.6	12.9	13.9	20	0.306	0.246	0.338
38	Mass mortality				Mass mortality			

Weaned larvae - pooled data

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	63	11.1	10.5	11.4	61	0.228	0.206	0.293
14	62	11.6	10.9	12.0	61	0.221	0.184	0.242
20	51	12.5	11.9	13.0	51	0.244	0.209	0.260
26	60	13.1	12.3	13.6	59	0.245	0.194	0.315
32	60	13.6	12.8	14.3	60	0.298	0.220	0.381
38	40	14.2	13.4	14.9	40	0.347	0.253	0.426

Table IV,vi Standard lengths and dry weights for starving herring larvae

Data are expressed as medians (med) and lower (q1) and upper quartiles (q2). The number of larvae in the sample is also given (n).

Starved larvae - Tank 10

Age (dph)	Standard length (mm)				Dry weight (mg)			
	n	med	q1	q2	n	med	q1	q2
9	21	11.3	11.0	11.6	19	0.322	0.231	0.358
14	20	11.6	11.3	11.8	20	0.175	0.160	0.197
20	13	11.1	10.7	11.6	13	0.138	0.120	0.150
26	12	11.8	11.0	11.9	12	0.141	0.136	0.148

Table IV,vii Intra-treatment differences tested by Kruskall-Wallis and Mann-Whitney tests

Data for standard lengths and dry weights of larvae sampled from replicate tanks within treatments were compared at each sample point using a Kruskall-Wallis test (Mann-Whitney test for weaned larvae at 38 days post-hatch).
The null hypothesis was that the differences between tanks within each treatment group were not significant.
NS indicates acceptance of the null hypothesis. The null hypothesis was rejected at a probability of 0.05, values below this become increasingly significant.
Age is given in days post-hatch.

Age	Treatment	Tanks	Probability	
			Length data	Weight data
9	Live fed	1,3,8	<0.01	<0.01
	Microdiet	4,6,7	<0.01	0.0342
	Wean	2,5,9	<0.01	<0.01
14	Live fed	1,3,8	<0.01	<0.01
	Microdiet	4,6,7	0.0344	NS
	Wean	2,5,9	<0.01	NS
20	Live fed	1,3,8	NS	0.0111
	Microdiet	4,6,7	NS	<0.01
	Wean	2,5,9	NS	NS
26	Live fed	1,3,8	NS	NS
	Microdiet	4,6,7	<0.01	0.0167
	Wean	2,5,9	<0.01	<0.01
32	Live fed	1,3,8	NS	NS
	Microdiet	4,6,7	NS	0.0421
	Wean	2,5,9	NS	NS
38	Live fed	1,3,8	NS	NS
	Microdiet	4,6,7	NS	0.0478
	Wean	2,5	0.0228	NS

Table IV,viii Inter-treatment differences tested by Kruskall-Wallis tests between larvae pooled by treatment

Measurements for standard lengths and dry weights of larvae pooled by treatment were compared at each sample point using a Kruskall-Wallis test.
The null hypothesis was that the differences between treatments were not significant.
NS indicates acceptance of the null hypothesis. The null hypothesis was rejected at a probability of 0.05, values below this become increasingly significant.
Age is given in days post-hatch.

Age	Treatments compared	Probability	
		Length data	Weight data
9	Live,Microdiet,Weaned,Starved	0.0442	<0.01
14	Live,Microdiet,Weaned,Starved	NS	<0.01
20	Live,Microdiet,Weaned,Starved	<0.01	<0.01
26	Live,Microdiet,Weaned,Starved	<0.01	<0.01
32	Live,Microdiet,Weaned	<0.01	<0.01
38	Live,Microdiet,Weaned	<0.01	<0.01

Table IV,ix Inter-treatment differences in standard lengths and dry weights as analysed by the procedure of Dunn (1964) described by Zar (1984)

The length and weight data pooled by treatment were examined independently where significant inter-treatment, but not highly significant intra-treatment differences had been indicated (Table IV,viii).
The null hypothesis was that there was no difference between two sets of data.
The null hypothesis was rejected at a probability of 0.05 ($Q_{0.05,3}$ = 2.394), values below this become increasingly significant.
Age is given in days post-hatch.

Age	Parameter	Comparison	Mean rank difference	SE	Q	Probability
32	Std. length	Live v Micro	70.145	9.457	7.417	<0.001
		Live v Wean	19.78	9.203	2.149	NS
		Wean v Micro	50.365	9.457	5.326	<0.001
32	Dry weight	Live v Micro	59.476	9.398	6.328	<0.001
		Live v Wean	21.799	8.865	2.459	0.0439
		Wean v Micro	37.677	9.363	4.024	<0.001
38	Std. length	Live v Micro	76.375	7.394	10.329	<0.001
		Live v Wean	17.438	8.267	2.109	NS
		Wean v Micro	58.938	8.267	7.129	<0.001
38	Dry weight	Live v Micro	72.114	8.442	8.542	<0.001
		Live v Wean	17.883	9.398	1.903	NS
		Wean v Micro	54.231	9.430	5.826	<0.001

Overall conclusion:- At 32 and 38 days post-hatch, the standard lengths of herring larvae reared on live feed and those which had been weaned onto microcapsules were not significantly different, but the differences between live-fed and microcapsule-fed and between weaned and microcapsule-fed larvae were highly significant.

At 32 days post-hatch, the dry weights of herring larvae reared on live feed and those which had been weaned onto microcapsules were just significantly different, but the differences between live-fed and microcapsule-fed and between weaned and microcapsule-fed larvae were highly significant.

At 38 days post-hatch, the dry weights of herring larvae reared on live feed and those which had been weaned onto microcapsules were not significantly different, but the differences between live-fed and microcapsule-fed and between weaned and microcapsule-fed larvae were highly significant.

Table IV,x Total lipid analyses from feeds used to rear herring larvae

Each set of results is based on three samples collected at varying times during the the course of the experiment.
The means and standard deviations were computed after arcsin transformation of percentage data.
T = Trace, lipid class was detected by HPTLC but below the resolution of Iatroscan analysis, class comprises <1.0% of total lipid.
- = Lipid class was not detected.

	Artemia	Microencapsulated cod-roe
Lipid content (% dry weight)	13.8 ± 1.0	6.4 ± 0.005
Lipid class composition (% total lipid)		
Pigment	6.6 ± 1.0	-
Sterol/Wax esters	0.7 ± 0.003	0.2 ± 0.003
Triacylglycerol	28.9 ± 0.4	13.2 ± 0.4
Free fatty acids	12.7 ± 0.05	27.2 ± 0.2
Diacylglycerol	T	T
Monoacylglycerol	T	T
Cholesterol	7.0 ± 0.001	13.4 ± 0.008
Phosphatidylethanolamine	24.3 ± 0.9	T
Phosphatidylserine/inositol	2.1 ± 0.002	T
Phosphatidylcholine	15.8 ± 0.6	39.0 ± 0.5
Sphingomyelin	1.9 ± 0.01	2.9 ± 0.004
Lysophosphatidylcholine	T	4.1 ± 0.02
Total neutral classes	55.9 ± 0.3	54.0 ± 0.5
Total polar classes	44.1 ± 0.3	36.0 ± 0.5

Table IV,xi Free fatty acid levels (% total lipid) in herring, whiting, capelin and halibut samples recorded in the literature

Species	Race	Age	Free fatty content (%)	Source
Herring	Atlantic	0 DPF[a]	6.2	Tocher *et al.* 1985
Herring	Atlantic	25 DPF	4.5	Tocher *et al.* 1985
Herring	Atlantic	1 DPH[b]	0.7	Fraser *et al.* 1987
Herring	Atlantic	26 DPH	2.6	Fraser *et al.* 1987
Herring	Baltic	juvenile	8.0	Shatunovsky 1970
Whiting		ripe roe	13.2	Tocher & Sargent 1984
Capelin		ripe roe	6.9	Tocher & Sargent 1984
Halibut	Atlantic	eggs	2.9	Falk-Petersen *et al.* 1989

a Days post-fertilisation
b Days post-hatch

Table IV,xii Lipid content and class analyses from herring larvae reared on live diets

Tank 1

Lipid class (% total lipid)	Age (days post-hatch) 9	14	20	26	38
Sterol/Wax esters	5.6	6.4	6.7	4.3	3.2
Triacylglycerol	15.2	12.8	6.6	7.9	12.4
Free fatty acids	26.2	22.2	20.7	24.7	28.0
Diacylglycerol	T	T	T	T	T
Monoacylglycerol	T	T	T	T	T
Cholesterol	14.4	12.0	16.7	15.8	12.3
Phosphatidylethanolamine	4.6	18.6	21.5	19.2	17.7
Phosphatidylserine/inositol	T	T	T	T	T
Phosphatidylcholine	29.2	23.2	22.5	22.1	26.0
Sphingomyelin	T	T	T	T	T
Lysophosphatidylcholine	T	T	T	T	T
Total neutral classes	61.5	52.9	50.4	52.6	56.0
Total polar classes	38.5	47.1	49.6	47.4	44.0
Triacylglycerol/Cholesterol ratio	1.1	1.1	0.4	0.5	1.0
Dry weight (mg larva^{-1})	0.285	0.206	0.202	0.210	0.409
Lipid content (µg larva^{-1})	18.9	21.4	17.5	26.3	38.4
Lipid content (% dry weight)	6.5	10.2	5.8	12.5	9.4
Triacylglycerol (% dry weight)	1.0	1.3	0.6	1.0	1.2
Cholesterol (% dry weight)	0.9	1.2	1.5	2.0	1.1

Table IV,xiii Lipid content and class analyses from herring larvae reared on live diets

Tank 3

Lipid class (% total lipid)	Age (days post-hatch) 9	14	20	26	38
Sterol/Wax esters	5.6	3.7	2.8	4.0	2.5
Triacylglycerol	14.7	12.0	5.5	5.2	13.8
Free fatty acids	30.7	26.7	27.7	18.7	23.4
Diacylglycerol	T	T	T	T	T
Monoacylglycerol	T	T	T	T	T
Cholesterol	15.0	13.1	13.0	16.3	13.5
Phosphatidylethanolamine	6.8	18.5	22.7	27.6	18.6
Phosphatidylserine/inositol	T	T	T	T	T
Phosphatidylcholine	27.2	20.6	21.7	23.1	26.0
Sphingomyelin	T	T	T	T	T
Lysophosphatidylcholine	T	T	T	T	T
Total neutral classes	66.0	55.6	49.0	44.2	53.2
Total polar classes	44.0	44.4	51.0	55.8	46.8
Triacylglycerol/Cholesterol ratio	1.0	0.9	0.4	0.3	1.0
Dry weight (mg larva^{-1})	0.226	0.227	0.254	0.196	0.436
Lipid content (pg larva^{-1})	15.5	17.0	18.0	20.7	28.9
Lipid content (% dry weight)	6.7	7.4	7.2	10.4	6.6
Triacylglycerol (% dry weight)	1.0	0.9	0.4	0.5	0.9
Cholesterol (% dry weight)	1.1	1.0	0.9	1.7	0.8

Table IV,xiv Lipid content and class analyses from herring larvae reared on live diets

Tank 8

Lipid class (% total lipid)	Age (days post-hatch) 9	14	20	26	38
Sterol/Wax esters	4.0	4.6	3.1	4.2	1.9
Triacylglycerol	15.1	11.1	5.8	5.8	18.0
Free fatty acids	34.0	24.3	28.6	19.7	20.4
Diacylglycerol	T	T	T	T	T
Monoacylglycerol	T	T	T	T	T
Cholesterol	14.3	12.9	15.3	16.0	11.5
Phosphatidylethanolamine	7.9	21.7	21.7	25.7	21.8
Phosphatidylserine/inositol	T	T	T	T	T
Pphosphatidylcholine	24.7	19.4	19.8	25.3	24.7
Sphingomyelin	T	2.6	3.4	] 3.2	] 1.8
Lysophosphatidylcholine	T	3.3	2.2		
Total neutral classes	67.4	52.9	52.8	45.7	51.8
Total polar classes	32.6	47.1	47.2	54.3	48.2
Triacylglycerol/Cholesterol ratio	1.1	0.9	0.4	0.4	1.6
Dry weight (mg larva^{-1})	0.291	0.231	0.216	0.192	0.477
Lipid content (µg larva^{-1})	21.1	25.2	15.3	16.3	42.7
Lipid content (% dry weight)	7.3	11.1	7.3	8.6	8.9
Triacylglycerol (% dry weight)	1.1	1.2	0.4	0.5	1.6
Cholesterol (% dry weight)	1.0	1.4	1.1	1.4	1.0

Table IV,xv Lipid content and class analyses from herring larvae reared on microencapsulated feed

Tank 4

Lipid class (% total lipid)	Age (days post-hatch) 9	14	20	26	38
Sterol/Wax esters	4.5	4.3	3.3	5.0	5.8
Triacylglycerol	14.6	8.9	4.0	8.2	T
Free fatty acids	30.9	23.2	21.6	23.5	23.0
Diacylglycerol	T	T	1.8	T	T
Monoacylglycerol	T	T	T	T	T
Cholesterol	15.8	15.8	15.7	15.7	20.3
Phosphatidyethanolamine	6.8	19.8	20.6	20.9	18.5
Phosphatidylserine/inositol	T	T	T	T	T
Phosphatidylcholine	26.3	22.1	26.6	20.2	26.9
Sphingomyelin	T	2.5	3.0	2.5	] 4.4
Lysophosphatidylcholine	T	3.3	2.2	4.0	
Total neutral classes	65.7	52.2	46.3	52.3	50.2
Total polar classes	34.3	47.8	53.7	47.7	49.8
Triacylglycerol/Cholesterol ratio	0.9	0.6	0.3	0.5	0
Dry weight (mg larva^{-1})	0.214	0.177	0.208	0.227	0.203
Lipid (µg larva^{-1})	17.3	19.2	11.6	16.8	14.9
Lipid (% dry weight)	8.2	10.7	5.5	7.3	7.5
Triacylglycerol (% dry weight)	1.2	1.0	0.2	0.6	T
Cholesterol (% dry weight)	1.3	1.7	0.9	1.2	1.5

Table IV,xvi Lipid content and class analyses from herring larvae reared on microencapsulated feed

Tank 6

Lipid class (% total lipid)	Age (days post-hatch) 9	14	20	26	38
Sterol/Wax esters	3.5	4.9	2.3	4.4	T
Triacylglycerol	16.6	8.7	5.3	6.4	T
Free fatty acids	27.1	26.8	23.2	22.8	36.4
Diacylglycerol	T	T	1.8	T	T
Monoacylglycerol	T	T	T	T	T
Cholesterol	15.7	16.7	15.4	14.8	14.8
Phosphatidylethanolamine	10.0	16.5	23.6	24.0	23.7
Phosphatidylserine/inositol	T	T	T	T	T
Phosphatidylcholine	26.8	20.0	24.0	22.8	25.2
Sphingomyelin	T	2.6	2.3	] 4.8	T
Lysophosphatidylcholine	T	3.3	2.2		T
Total neutral classes	63.2	57.0	46.2	48.4	51.1
Total polar classes	36.8	43.0	53.8	51.6	48.9
Triacylglycerol/Cholesterol ratio	1.1	0.5	0.3	0.4	0
Dry weight (mg larva^{-1})	0.216	0.176	0.258	0.257	0.236
Lipid (µg larva^{-1})	17.8	18.6	17.4	25.4	13.8
Lipid (% dry weight)	8.1	10.3	6.7	9.8	6.0
Triacylglycerol (% dry weight)	1.4	0.9	0.4	0.6	T
Cholesterol (% dry weight)	1.3	1.7	1.0	1.5	0.9

Table IV,xvii Lipid contnet and class analyses from herring larvae reared on microencapsulated feed

Tank 7

Lipid class (% total lipid)	Age (days post-hatch) 9	14	20	26	38
Sterol/Wax esters	2.8	No sample taken		4.7	10?0
Triacylglycerol	15.7			6.9	T
Free fatty acids	27.4			22.5	27.5
Diacylglycerol	T			4.7	T
Monoacylglycerol	T			T	T
Cholesterol	14.8	No sample taken		13.5	21.6
Phosphatidylethanolamine	10.1			13.7	13.7
Phosphatidylserine/inositol	T			T	T
Phosphatidylcholine	29.2			23.4	22.8
Sphingomyelin	T			] 6.8	] 4.4
Lysophosphatidylcholine	T				
Total neutral classes	60.7	No sample taken		56.1	59.1
Total polar classes	39.3			43.9	40.9
Triacylglycerol/Cholesterol ratio	1.1			0.5	0
Dry weight (mg larva^{-1})	0.270	0.170	0.185	0.274	0.212
Lipid (µg larva^{-1})	18.2	No sample taken		18.2	20.5
Lipid (% dry weight)	6.7			6.7	9.8
Triacylglycerol (% dry weight)	1.0			0.4	T
Cholesterol (% dry weight)	1.0	No sample taken		0.9	1.5

Table IV,xviii Lipid content and class analyses from herring larvae weaned from live to microencapsulated feed

Tank 2

Lipid class (% total lipid)	Age (days post-hatch) 9	14	20	26	38
Sterol/Wax esters	3.3	4.2	3.3	4.3	5.4
Triacylglycerol	16.3	11.8	4.7	6.2	6.7
Free fatty acids	25.7	26.8	17.8	18.8	26.9
Diacylglycerol	T	T	1.8	T	T
Monoacylglycerol	T	T	T	T	T
Cholesterol	13.0	13.0	19.2	17.2	20.8
Phosphatidylethanolamine	8.7	19.0	23.0	24.9	12.9
Phosphatidylserine/inositol	T	T	T	T	T
Phosphatidylcholine	33.0	23.0	22.9	23.2	27.4
Sphingomyelin	T	2.4	2.9	2.5	T
Lysophosphatidylcholine	T	3.8	3.4	3.0	T
Total neutral classes	58.2	51.8	45.8	46.5	59.6
Total polar classes	41.8	48.2	54.2	53.5	40.4
Triacylglycerol/Cholesterol ratio	1.3	0.9	0.2	0.4	0.3
Dry weight (mg larva^{-1})	0.224	0.224	0.229	0.186	0.347
Lipid (µg larva^{-1})	18.1	22.9	13.5	23.0	31.1
Lipid (% dry weight)	8.2	10.4	5.9	12.1	8.9
Triacylglycerol (% dry weight)	1.3	1.2	0.3	0.8	0.6
Cholesterol (% dry weight)	1.1	1.4	1.1	2.1	1.9

Table IV,xix Lipid content and class analyses from herring larvae weaned from live to microencapsulated feed

Tank 5

Lipid class (% total lipid)	Age (days post-hatch) 9	14	20	26	38
Sterol/Wax esters	4.2	5.8	2.2	3.2	6.0
Triacylglycerol	18.2	9.4	4.4	7.4	9.2
Free fatty acids	27.7	25.2	25.9	21.4	26.8
Diacylglycerol	T	T	1.8	T	T
Monoacylglycerol	T	T	T	T	T
Cholesterol	14.8	13.6	15.6	14.0	14.4
Phosphatidylethanolamine	6.3	19.6	24.8	26.1	17.3
Phosphatidylserine/inositol	T	T	T	T	
Phosphatidylcholine	28.8	19.6	20.8	23.5	26.4
Sphingomyelin	T	2.9	2.6	} 4.3	T
Lysophosphatidylcholine	T	3.9	3.8		T
Total neutral classes	65.0	54.0	48.0	46.0	56.4
Total polar classes	35.0	46.0	52.0	54.0	43.6
Triacylglycerol/Cholesterol ratio	1.2	0.7	0.3	0.5	0.6
Dry weight (mg larva^{-1})	0.211	0.226	0.245	0.247	0.350
Lipid (µg larva^{-1})	19.1	19.8	13.7	26.7	33.7
Lipid (% dry weight)	9.1	8.6	5.5	10.7	9.6
Triacylglycerol (% dry weight)	1.7	0.8	0.2	0.8	0.9
Cholesterol (% dry weight)	1.4	1.2	0.8	1.5	1.4

Table IV,xx Lipid content and class analyses from herring larvae weaned from live to microencapsulated feed

Tank 9

Lipid class (% total lipid)	Age (days post-hatch) 9	14	20	26	38
Sterol/Wax esters	3.9	3.2	2.0	2.1	100%
Triacylglycerol	13.8	9.4	6.5	5.4	mortality
Free fatty acids	29.7	24.1	30.0	20.7	
Diacylglycerol	T	T	T	2.1	
Monoacylglycerol	T	T	T	T	
Cholesterol	15.1	14.8	14.8	14.0	
Phosphatidylethanolamine	12.7	22.8	18.2	27.8	
Phosphatidylserine/inositol	T	T	T	T	
Phosphatidylcholine	24.7	20.1	26.4	23.4	
Sphingomyelin	T	2.5	2.7	] 4.9	
Lysophosphatidylcholine	T	3.1	4.0		
Total neutral classes	62.6	51.5	49.2	43.9	
Total polar classes	37.4	48.5	50.8	56.1	
Triacylglycerol/Cholesterol ratio	0.9	0.6	0.4	0.4	
Dry weight (mg larva^{-1})	0.325	0.220	0.248	0.306	
Lipid (µg larva^{-1})	18.3	21.7	17.0	28.1	
Lipid (% dry weight)	5.6	9.9	6.8	10.2	
Triacylglycerol (% dry weight)	0.8	0.9	0.4	0.6	
Cholesterol (% dry weight)	0.8	1.5	1.0	1.6	

Table IV,xxi Lipid content and class analyses from starving herring larvae

Tank 10

Lipid class (% total lipid)	Age (days post-hatch) 9	14	20	26	38
Sterol/Wax esters	4.3	6.4	4.6	7.1	100%
Triacylglycerol	16.9	5.6	5.7	T	mortality
Free fatty acids	25.2	21.0	16.0	12.0	
Diacylglycerol	T	3.0	1.2	T	
Monoacylglycerol	T	T	T	T	
Cholesterol	12.9	14.8	21.6	28.8	
Phosphatidylethanolamine	14.3	25.0	25.7	26.7	
Phosphatidylserine/inositol	T	T	T	T	
Phosphatidylcholine	26.4	19.3	19.2	21.8	
Sphingomyelin	T	2.5	3.0	] 3.4	
Lysophosphatidylcholine	T	2.5	3.0		
Total neutral classes	59.3	50.7	49.0	48.0	
Total polar classes	40.7	49.3	51.0	52.0	
Triacylglycerol/Cholesterol ratio	1.3	0.4	0.3	0	
Weight (mg larva^{-1})	0.322	0.175	0.138	0.141	
Lipid (µg larva^{-1})	18.6	13.7	10.6	13.1	
Lipid (% dry weight)	5.8	7.6	7.6	9.4	
Triacylglycerol (% dry weight)	1.0	0.4	0.4	T	
Cholesterol (% dry weight)	0.8	1.1	1.6	2.7	

Table IV,xxii Pooled data from lipid class analyses

Each set of results are derived from data for individual tanks (Tables IV,xii - IV,xxi) pooled by treatment.
Means and standard deviations were computed after arcsin transformation of percentage data.
Age is given in days post-hatch.

Lipid content (μg larva^{-1})

Age	9	14	20	26	38
Live-fed	18.5 ± 2.8	21.2 ± 4.1	16.9 ± 1.4	21.1 ± 5.0	36.7 ± 7.1
Microdiet	17.8 ± 0.5	18.9 ± 0.4	14.5 ± 4.1	20.1 ± 4.6	16.4 ± 3.6
Weaned	18.5 ± 0.5	21.5 ± 1.6	14.7 ± 2.0	25.9 ± 2.6	32.4 ± 1.8

Triacylglycerol (% total lipid)

Age	9	14	20	26	38
Live-fed	15.0 ± 0.002	12.0 ± 0.02	5.9 ± 0.02	6.3 ± 0.08	14.7 ± 0.2
Microdiet	15.6 ± 0.02	8.8 ± 0.0006	4.6 ± 0.05	7.1 ± 0.03	T
Weaned	16.1 ± 0.09	10.2 ± 0.05	5.2 ± 0.06	6.3 ± 0.04	7.9 ± 0.1

Cholesterol (% total lipid)

Age	9	14	20	26	38
Live-fed	14.6 ± 0.003	12.7 ± 0.008	15.0 ± 0.07	16.0 ± 0.001	12.4 ± 0.02
Microdiet	15.4 ± 0.006	16.2 ± 0.007	15.6 ± 0.0009	14.7 ± 0.02	18.8 ± 0.2
Weaned	14.3 ± 0.03	13.8 ± 0.02	16.5 ± 0.01	15.0 ± 0.06	17.5 ± 0.4

Lipid (% dry weight)

Age	9	14	20	26	38
Live-fed	6.8 ± 0.007	9.5 ± 0.1	7.8 ± 0.03	10.4 ± 0.1	8.3 ± 0.08
Microdiet	7.6 ± 0.03	10.5 ± 0.002	6.1 ± 0.03	7.9 ± 0.09	7.7 ± 0.1
Weaned	7.6 ± 0.1	9.6 ± 0.03	6.1 ± 0.02	11.0 ± 0.0002	9.2 ± 0.007

Triacylglycerol (% dry weight)

Age	9	14	20	26	38
Live-fed	1.0 ± 0.0008	1.1 ± 0.01	0.5 ± 0.007	0.6 ± 0.03	1.2 ± 0.03
Microdiet	1.2 ± 0.008	0.9 ± 0.001	0.3 ± 0.02	0.5 ± 0.007	T
Weaned	1.2 ± 0.04	1.0 ± 0.01	0.3 ± 0.009	0.7 ± 0.005	0.7 ± 0.02

Cholesterol (% dry weight)

Age	9	14	20	26	38
Live-fed	1.0 ± 0.003	1.2 ± 0.008	1.2 ± 0.02	1.7 ± 0.01	1.0 ± 0.003
Microdiet	1.2 ± 0.007	1.7 ± 0	0.9 ± 0.001	1.2 ± 0.002	1.3 ± 0.03
Weaned	1.1 ± 0.02	1.4 ± 0.004	1.0 ± 0.006	1.7 ± 0.01	1.6 ± 0.02

Triacylglycerol/cholesterol ratio

Age	9	14	20	26	38
Live-fed	1.1 ± 0.1	1.0 ± 0.1	0.4 ± 0	0.4 ± 0.1	1.2 ± 0.3
Microdiet	1.0 ± 0.1	0.6 ± 0.1	0.3 ± 0	0.5 ± 0.1	0 ± 0
Weaned	1.1 ± 0.2	0.7 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.2

Table IV,xxiii Fatty acid spectra of feeds used in herring larval rearing study

The results of analyses of three samples of each feed collected at regular intervals throughout the rearing trial are given.
The means and standard deviations were computed after arcsin transformation of percentage data.
T = Trace, fatty acid was detected but constitued <0.1% of total fatty acids.
- = Fatty acid was not detected.

	Rotifers mean ± sd	Artemia mean ± sd	Microcapsules mean ± sd
Lipid content (% dry weight)	8.7 ± 0.08	13.8 ± 0.03	6.4 ± 0.006

	Fatty acid composition (%)		
	Rotifers	Artemia	Microcapsules
Fatty acid (% total fatty acids)	mean ± sd	mean ± sd	mean ± sd
14:0	2.6 ± 0.03	1.2 ± 0.0007	3.2 ± 0.02
15:0	0.8 ± 0.04	0.3 ± 0.3	0.4 ± 0
16:0	8.6 ± 0.1	11.6 ± 0.08	26.0 ± 0.03
16:1	30.3 ± 0.05	10.6 ± 0.002	5.4 ± 0.03
16:2	T	0.7 ± 0.008	1.6 ± 0.04
16:3	0.8 ± 0.004	0.2 ± 0.6	T
16:4	T	-	-
18:0	5.5 ± 0.01	4.6 ± 0.1	4.4 ± 0.01
18:1(n-9)	22.3 ± 0.03	16.1 ± 0.1	15.2 ± 0.006
18:1(n-7)	9.0 ± 0.1	17.7 ± 0.2	7.5 ± 0.05
18:2(n-6)	5.5 ± 0.03	5.6 ± 0.02	4.7 ± 0.01
18:3(n-6)	0.9 ± 0.03	T	T
18:3(n-3)	0.5 ± 0.002	15.9 ± 0.05	0.7 ± 0.001
18:4(n-3)	T	1.6 ± 0.02	0.4 ± 0
20:0	0.1 ± 0.04	T	-
20:1	3.5 ± 0.003	T	5.2 ± 0.004
20:2(n-6)	-	-	-
20:3(n-6)	-	-	-
20:4(n-6)	0.6 ± 0.05	3.3 ± 0.003	1.0 ± 0.003
20:4(n-3)	0.3 ± 0.03	T	0.2 ± 0
20:5(n-3)	0.9 ± 0.0009	8.7 ± 0.002	8.2 ± 0.05
22:0	-	-	-
22:1	1.6 ± 0.002	-	1.1 ± 0.003
22:5(n-3)	-	-	0.6 ± 0.004
22:6(n-3)	T	-	10.8 ± 0.2
24:1	0.2 ± 0.2	-	0.8 ± 0
Total unknowns	6.0 ± 0.7	1.9 ± 0.2	2.6 ± 0.004
Total saturates	17.6 ± 0.1	17.7 ± 0.2	34.0 ± 0.06
Total monoenes	66.9 ± 0.08	44.4 ± 0.02	35.2 ± 0.06
Total (n-3)	1.7 ± 0.02	26.2 ± 0.06	20.9 ± 0.2
Total (n-6)	7.0 ± 0.07	8.9 ± 0.01	5.7 ± 0.02
(n-3)/(n-6)	0.2 ± 0	2.9 ± 0.006	3.7 ± 0.003

Table IV,xxiv Fatty acid spectra of triacylglycerol fractions from herring larvae reared on live diets

Fatty acids are given as percentages of total fatty acids in single samples.
Age is given in days post-hatch.
T = Trace, fatty acid was detected but constitued <0.1% of total fatty acids.
- = Fatty acid was not detected.

Age	9	14	20	27	38
Triacylglycerol (% dry weight)	1.0	1.1	0.5	0.7	1.2
	Fatty acid composition (% total fatty acids)				
Age	9	14	20	27	38
14:0	4.5	2.6	2.8	2.8	4.1
15:0	0.5	0.4	0.9	0.6	1.3
16:0	21.1	16.1	13.9	19.2	9.2
16:1	8.6	7.7	10.1	10.2	10.0
16:2	1.2	0.3	0.9	0.6	0.9
16:3	0.7	0.7	0.7	0.7	1.9
16:4	-	-	-	-	-
18:0	3.6	5.6	8.2	6.3	5.5
18:1(n-9)	19.4	19.3	20.7	21.8	25.6
18:1(n-7)	9.4	7.2	6.8	8.7	13.4
18:2(n-6)	2.1	0.9	1.4	1.0	4.0
18:3(n-6)	0.5	T	T	0.1	0.7
18:3(n-3)	1.4	1.0	0.7	0.7	10.3
18:4(n-3)	0.7	0.8	0.6	0.3	1.9
20:0	T	T	T	T	0.4
20:1	3.8	T	3.6	T	0.4
20:2(n-6)	T	T	T	T	T
20:3(n-6)	T	T	T	T	T
20:4(n-6)	0.4	0.6	0.6	0.5	1.6
20:4(n-3)	0.5	0.6	T	0.3	T
20:5(n-3)	4.1	6.5	5.4	3.5	3.4
22:0	-	-	-	T	-
22:1	1.3	1.2	1.2	1.2	1.1
22:5(n-3)	0.4	1.3	1.3	0.5	T
22:6(n-3)	12.0	20.1	14.1	9.4	0.9
24:1	0.2	1.0	1.2	1.2	1.1
Total unknowns	3.6	3.6	4.9	6.6	1.8
Total saturates	29.7	24.7	25.8	28.9	20.5
Total monoenes	42.7	36.4	43.6	43.1	51.6
Total (n-3)	19.1	30.3	22.1	14.7	16.5
Total (n-6)	3.0	1.5	2.0	1.6	6.3
(n-3)/(n-6)	6.4	20.5	11.1	9.2	2.6

Table IV,xxv Fatty acid spectra of triacylglycerol fractions from herring larvae reared on microencapsultated cod-roe diets

Fatty acids are given as percentages of total fatty acids in single samples.
Age is given in days post-hatch.
T = Trace, fatty acid was detected but constitued <0.1% of total fatty acids.
- = Fatty acid was not detected.

Age	9	14	20	27	38
Triacylglycerol (% dry weight)	1.2	1.0	0.3	0.5	Trace
	Fatty acid composition (% total fatty acids)				
Age	9	14	20	27	38
14:0	4.1	2.5	2.4	1.4	Not analysed
15:0	0.6	0.4	0.5	0.5	
16:0	23.5	16.1	21.1	22.0	
16:1	7.5	5.8	5.0	6.3	
16:2	0.9	0.8	0.7	0.6	
16:3	0.1	T	0.4	0.5	
16:4	-	-	-	-	
18:0	4.4	3.6	7.9	6.4	
18:1(n-9)	23.4	16.1	20.2	20.5	
18:1(n-7)	8.2	8.5	7.0	12.8	
18:2(n-6)	1.2	0.9	1.2	1.6	
18:3(n-6)	0.3	T	T	0.5	
18:3(n-3)	1.2	0.9	0.6	0.4	
18:4(n-3)	0.6	0.7	0.7	T	
20:0	0.1	T	T	T	
20:1	4.2	3.5	5.8	8.6	
20:2(n-6)	T	T	T	T	
20:3(n-6)	T	T	T	T	
20:4(n-6)	0.4	0.5	0.5	0.7	
20:4(n-3)	0.5	0.5	0.3	T	
20:5(n-3)	3.9	7.9	6.2	4.0	
22:0	0.1	-	T	-	
22:1	1.4	1.4	1.9	2.3	
22:5(n-3)	0.8	1.3	0.9	0.6	
22:6(n-3)	10.5	26.5	13.3	7.6	
24:1	0.8	0.6	1.1	1.1	
Total unknowns	1.3	1.5	2.3	1.6	
Total saturates	32.8	22.6	31.9	30.3	
Total monoenes	45.5	35.9	41.0	51.6	
Total (n-3)	17.5	37.8	22.0	12.6	
Total (n-6)	1.9	1.4	1.7	2.8	
(n-3)/(n-6)	9.2	27.0	12.9	4.5	

Table IV,xxvi Fatty acid spectra of triacylglycerol fractions from herring larvae reared weaned from live to microencapsulated diets

Fatty acids are given as percentages of total fatty acids in single samples.
Age is given in days post-hatch.
T = Trace, fatty acid was detected but constituted <0.1% of total fatty acids.
- = Fatty acid was not detected.

			Wean complete	v	
Age	9	14	20	27	38
Triacylglycerol (% dry weight)	1.3	1.0	0.3	0.7	0.8

	Fatty acid composition (% total fatty acids)				
Age	9	14	20	27	38
14:0	3.1	2.6	2.8	1.0	1.0
15:0	0.5	0.5	0.7	0.3	T
16:0	23.4	17.1	13.9	18.3	14.6
16:1	9.0	7.7	11.0	6.9	6.7
16:2	0.8	0.6	0.6	0.3	0.5
16:3	0.6	0.7	1.0	0.5	0.5
16:4	-	-	-	-	-
18:0	4.1	6.5	6.2	5.2	5.0
18:1(n-9)	20.0	19.2	21.2	12.5	36.7
18:1(n-7)	8.6	7.6	6.6	8.4	4.6
18:2(n-6)	1.3	1.0	0.3	0.2	2.3
18:3(n-6)	-	-	0.3	0.3	-
18:3(n-3)	1.1	0.9	0.9	T	0.6
18:4(n-3)	0.5	0.7	0.8	T	0.5
20:0	-	T	0.2	T	0.4
20:1	3.3	3.3	3.7	1.1	6.1
20:2(n-6)	T	T	T	T	T
20:3(n-6)	T	T	T	T	T
20:4(n-6)	0.4	C.5	0.9	1.4	0.6
20:4(n-3)	0.5	0.5	0.5	0.2	0.2
20:5(n-3)	3.5	5.8	6.5	8.4	4.5
22:0	-	-	0.2	T	0.2
22:1	1.6	1.1	1.1	T	2.1
22:5(n-3)	0.8	1.0	1.1	1.1	0.7
22:6(n-3)	9.8	17.8	15.4	25.4	9.2
24:1	0.5	0.9	1.1	0.5	1.2
Total unknowns	6.6	4.5	3.0	8.2	1.8
Total saturates	31.1	26.7	24.0	24.8	21.2
Total monoenes	43.0	39.8	44.7	29.4	57.4
Total (n-3)	16.2	26.7	25.2	35.1	15.7
Total (n-6)	1.7	1.5	1.5	1.9	2.9
(n-3)/(n-6)	9.5	17.8	16.8	18.5	5.4

Table IV,xxvii Results of gut scores from feeding observation experiments carried out during spring 1988

The mean gut scores for observation tube 2 (live-fed control) and observation tube 1 (test-diet) are given for morning and evening counts.
The data were tested using the Mann-Whitney test, the calculated statistic z is quoted.
The null hypothesis tested was that there was no difference in gut scores between tubes 1 and 2.
The null hypothesis was rejected at a probability (P) of 0.05, values below this become increasingly significant.
A positive z statistic implies that the gut scores of the control were higher, a negative z statistic that the gut scores of the test-diet larvae were higher.

Trial I

Day	Morning				Evening			
	Tube 2 Control	Tube 1 Test	z	P	Tube 2 Control	Tube 1 Test	z	P
1	1.9	1.4	0.65	NS	4.3	3.8	0.35	NS
2	3.2	3.5	-0.48	NS	3.8	4.2	-0.09	NS
3	3.3	2.4	1.86	NS	2.5	2.4	0.07	NS
4	3.6	3.5	-0.11	NS	3.5	2.9	1.41	NS
5	3.0	2.1	1.89	NS	3.8	3.0	1.78	NS
6	2.7	0.3	5.17	<0.01	3.3	0.7	4.99	<0.01
7	2.1	0.2	3.93	<0.01	2.3	0.1	3.81	<0.01
8	1.7	0	4.00	<0.01	2.2	1.0	2.39	0.0168

Trial II

Day	Morning				Evening			
	Tube 2 Control	Tube 1 Test	z	P	Tube 2 Control	Tube 1 Test	z	P
1	1.1	2.1	-1.67	NS	2.5	1.4	2.37	0.0179
2	3.3	1.2	3.21	<0.01	3.1	1.3	3.13	<0.01

Fig 4-1 Tank layout in the constant temperature room at the DAFS Marine Research Unit, Firemore Bay, Poolewe

Numbers shown indicate the tank numbers used throughout this study

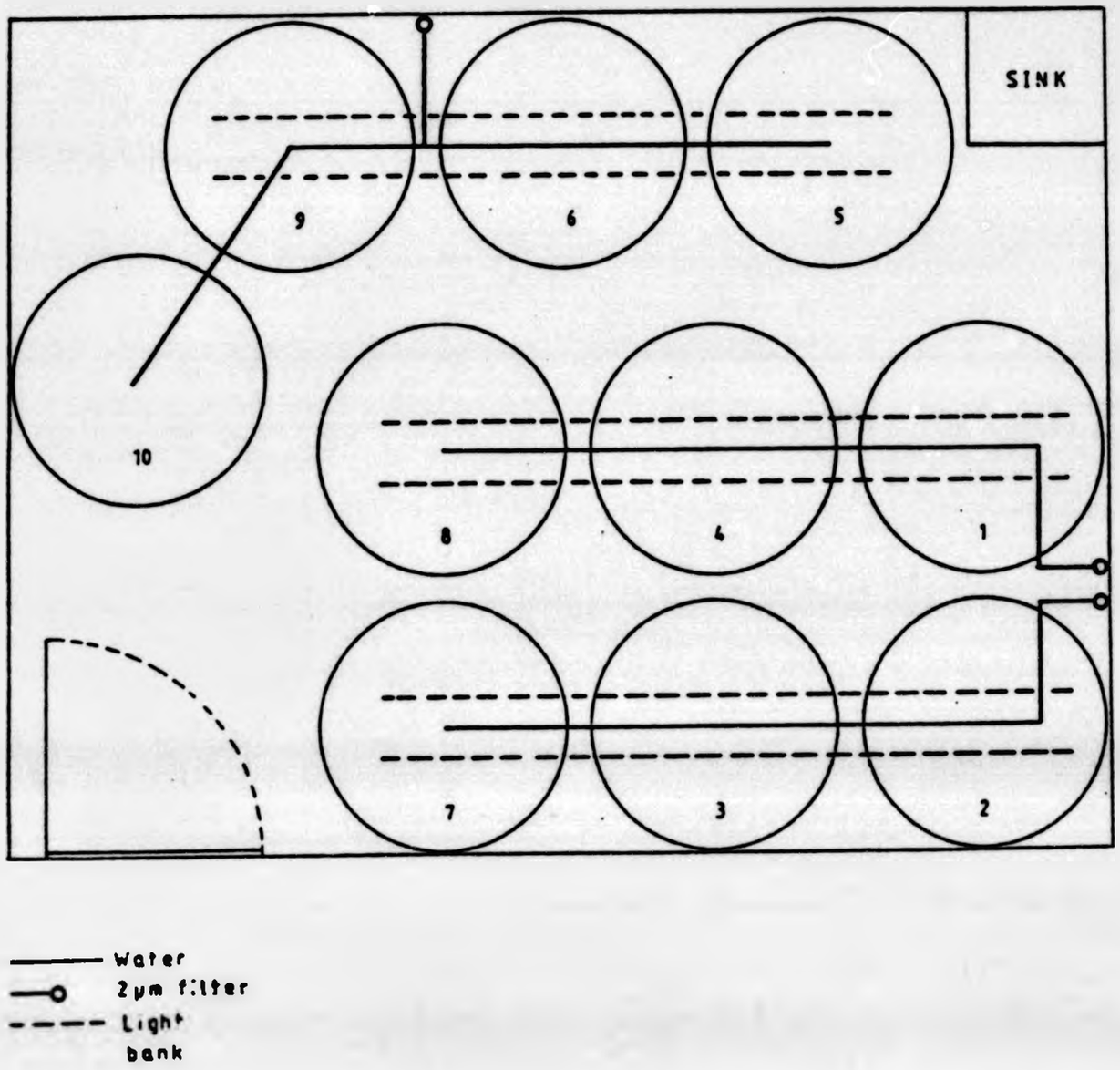


Fig 4-2 Design for barrel filter outlets fitted to each rearing tank

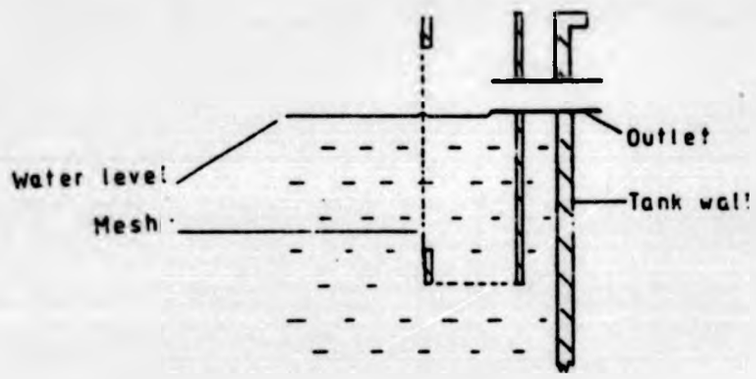


Fig 4-1 Tank layout in the constant temperature room at the DAFS Marine Research Unit, Firemore Bay, Poolewe

Numbers shown indicate the tank numbers used throughout this study

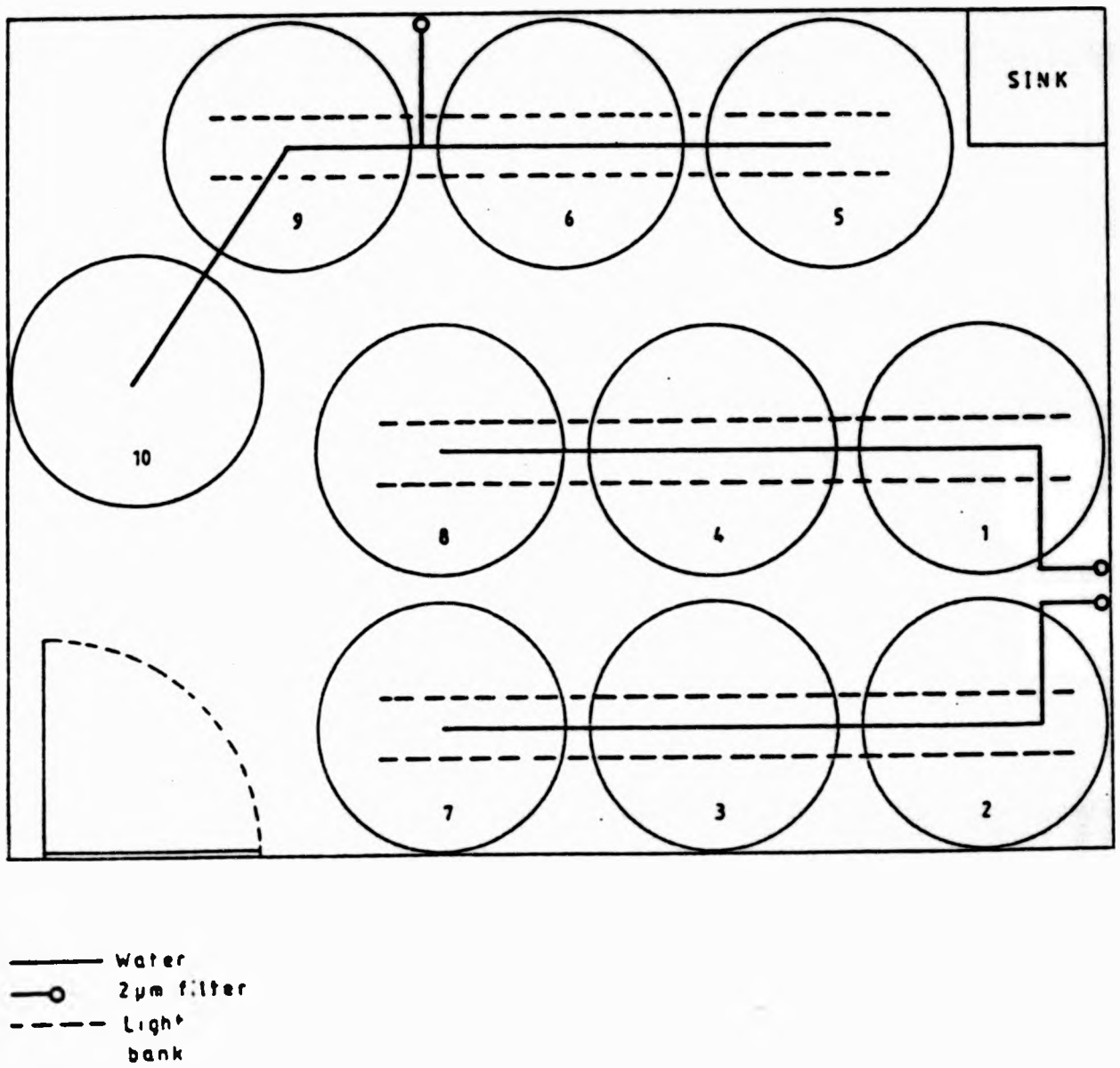
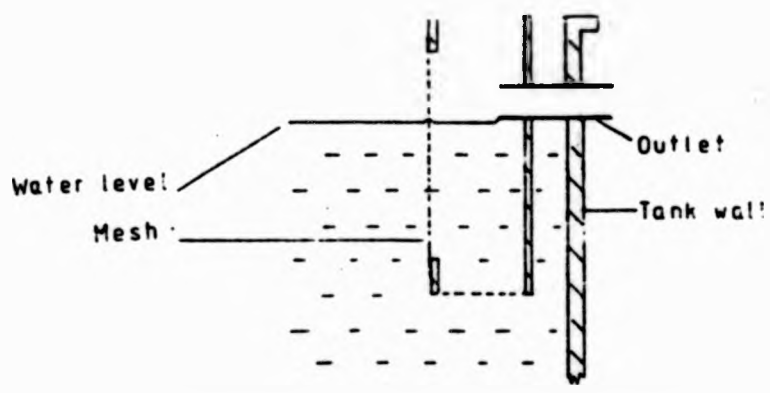


Fig 4-2 Design for barrel filter outlets fitted to each rearing tank



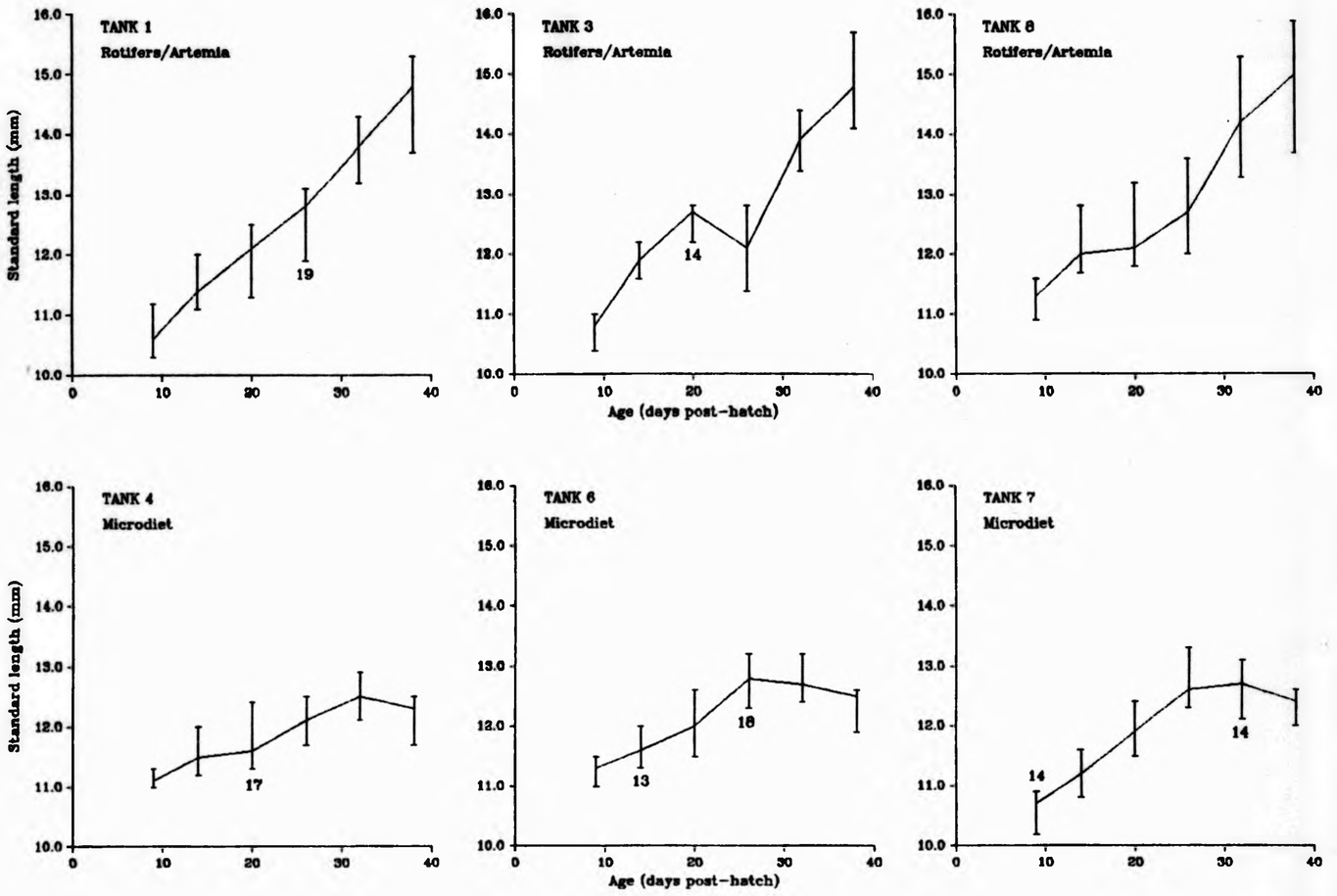
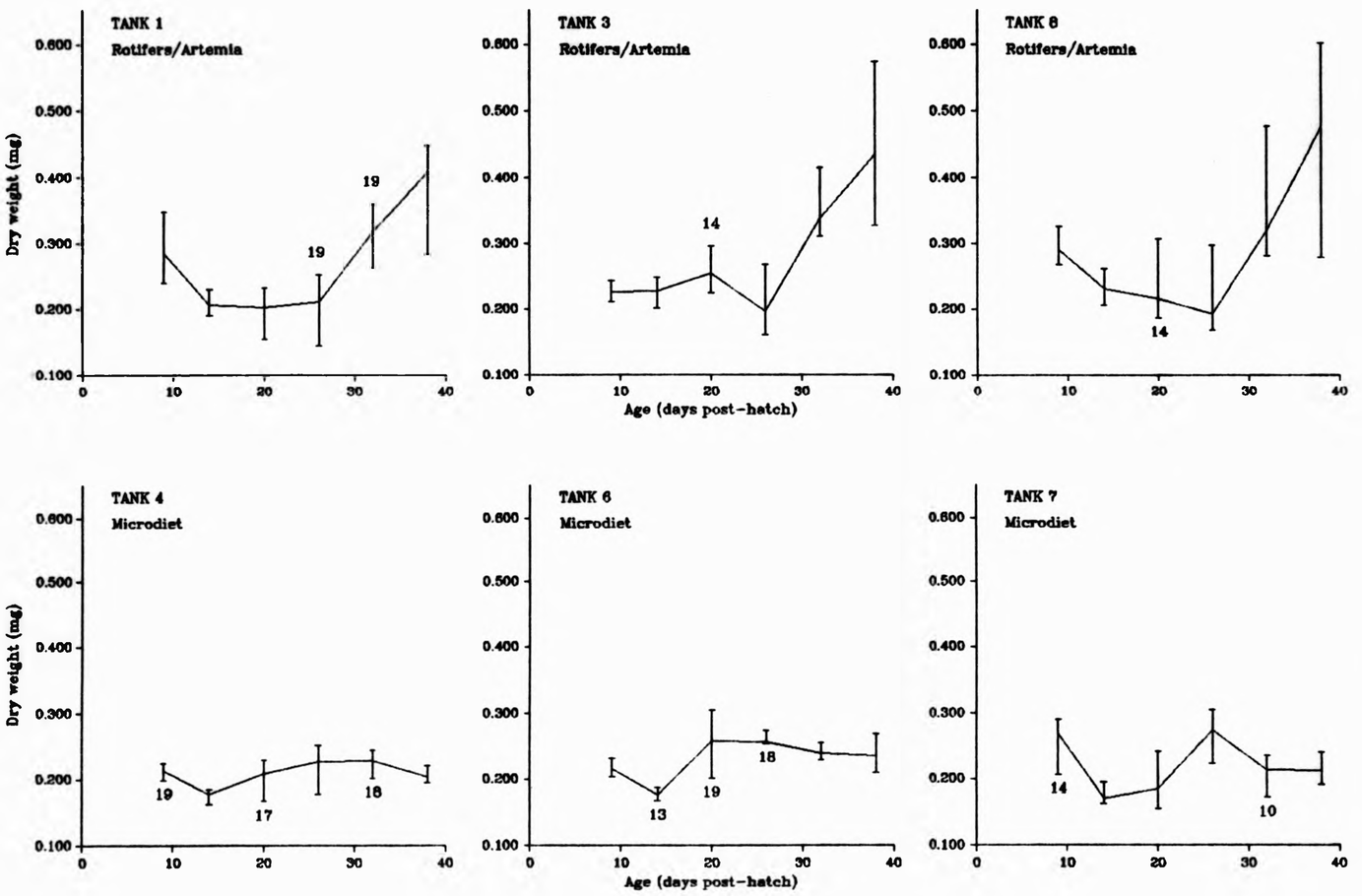


Fig 4-3 Plots of standard length against age for larvae from individual tanks

Medians joined by continuous plot
Vertical bars indicate the upper and lower quartiles
Sample size)20 unless indicated by numbers on vertical bars



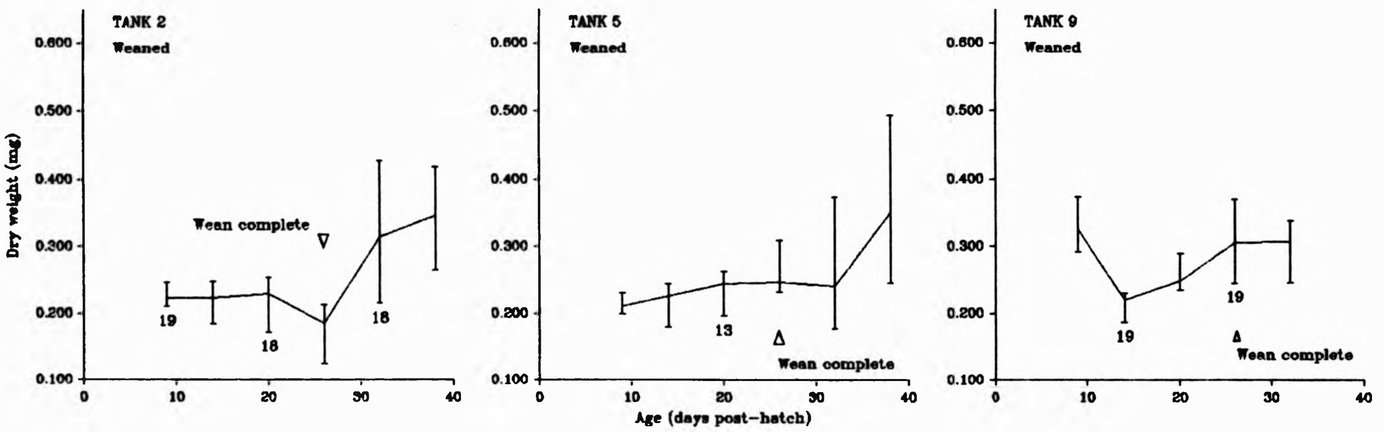


Fig 4-4 Plots of dry weight against age for larvae from individual tanks

Medians joined by continuous plot
Vertical bars indicate the upper and lower quartiles
Sample size)20 unless indicated by numbers on vertical bars

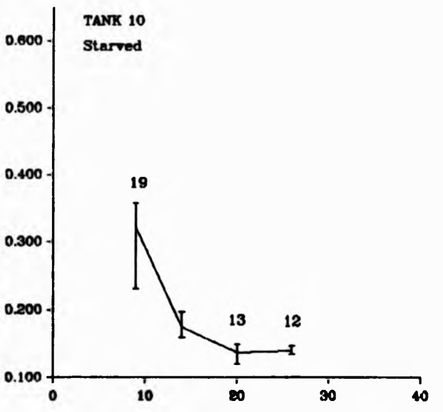
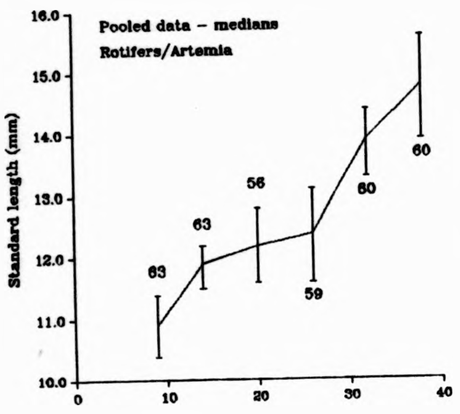
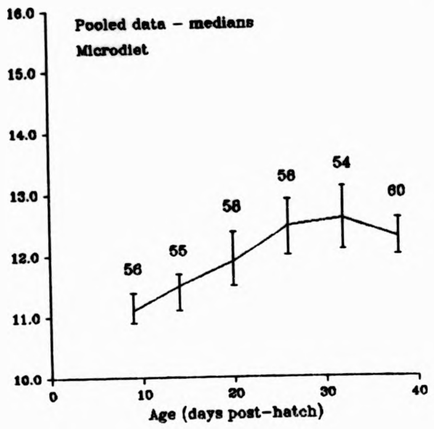


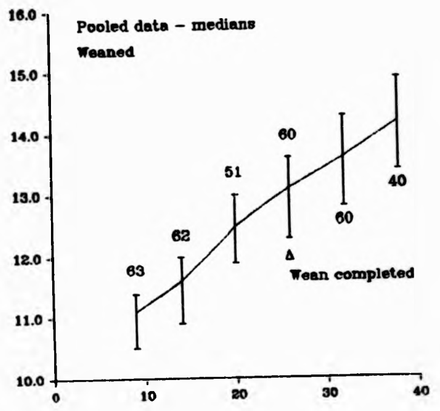
Fig 4-5 Plots of standard lengths and dry weights against age, samples pooled by treatment

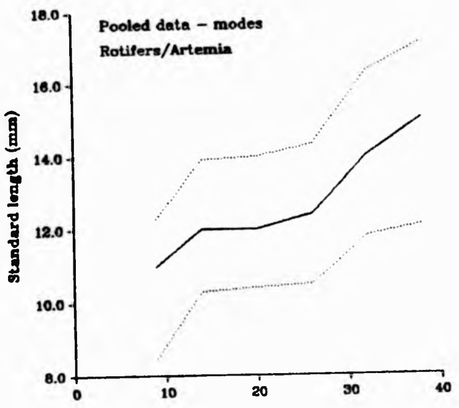
Upper graphs Medians joined by continuous plot
Vertical bars indicate the upper and lower quartiles
The number of measurements in the pooled samples is indicated by the numbers on the vertical bars

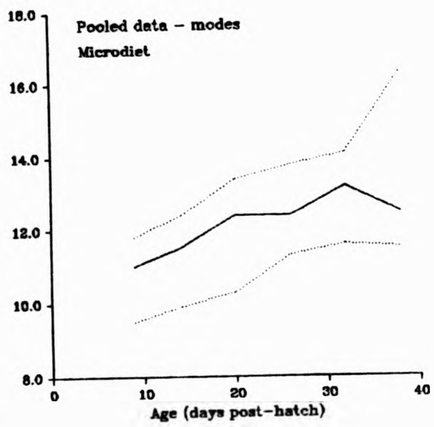
Lower graphs Modes joined by continuous plot, the maximum and minimum values in the datasets are shown by dashed lines
Sample numbers are as for the median plots

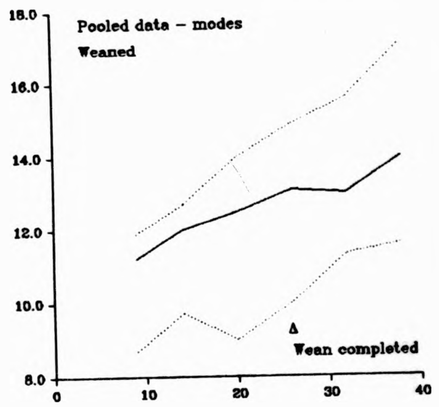

Pooled data – medians
Rotifers/Artemia
Standard length (mm)
16.0
15.0
14.0
13.0
12.0
11.0
10.0
0
10
20
30
40
63
63
56
59
60
60

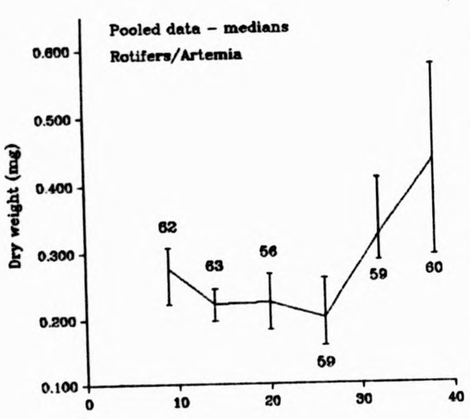

Pooled data – medians
Microdiet
16.0
15.0
14.0
13.0
12.0
11.0
10.0
0
10
20
30
40
56
55
58
58
54
60
Age (days post-hatch)

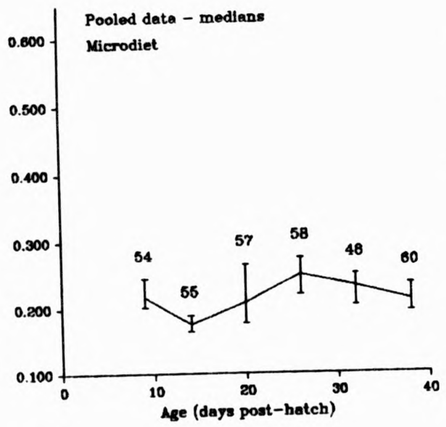

Pooled data – medians
Weaned
16.0
15.0
14.0
13.0
12.0
11.0
10.0
0
10
20
30
40
63
62
51
60
60
40
Δ
Wean completed

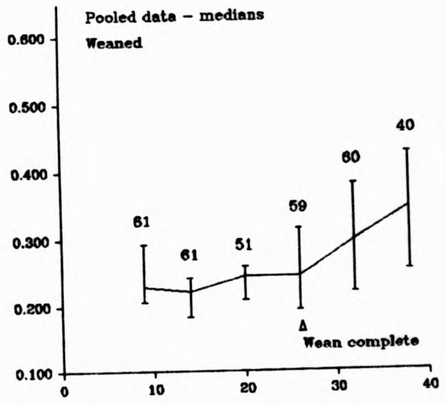

Pooled data – modes
Rotifers/Artemia
Standard length (mm)
18.0
16.0
14.0
12.0
10.0
8.0
0
10
20
30
40

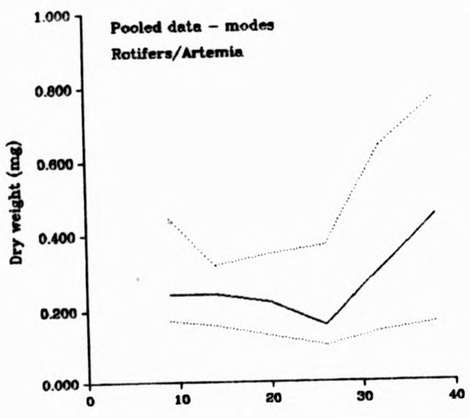

Pooled data – modes
Microdiet
18.0
16.0
14.0
12.0
10.0
8.0
0
10
20
30
40
Age (days post-hatch)

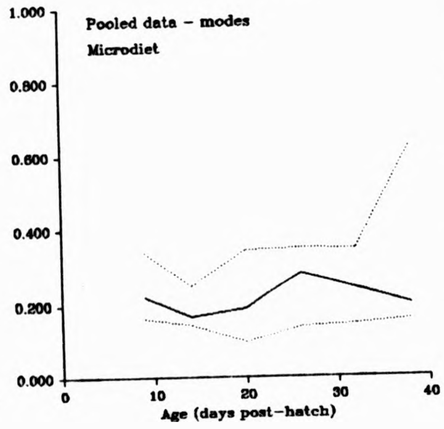

Pooled data – modes
Weaned
18.0
16.0
14.0
12.0
10.0
8.0
0
10
20
30
40
Δ
Wean completed

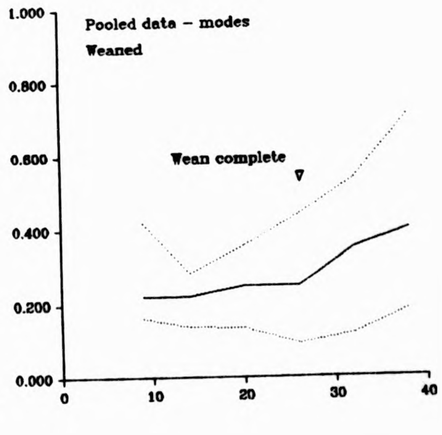

Pooled data – medians
Rotifers/Artemia
Dry weight (mg)
0.600
0.500
0.400
0.300
0.200
0.100
0
10
20
30
40
62
63
56
59
59
60


Pooled data – medians
Microdiet
0.600
0.500
0.400
0.300
0.200
0.100
0
10
20
30
40
54
55
57
58
48
60
Age (days post-hatch)


Pooled data – medians
Weaned
0.600
0.500
0.400
0.300
0.200
0.100
0
10
20
30
40
61
61
51
59
60
40
Δ
Wean complete


Pooled data – modes
Rotifers/Artemia
Dry weight (mg)
1.000
0.800
0.600
0.400
0.200
0.000
0
10
20
30
40


Pooled data – modes
Microdiet
1.000
0.800
0.600
0.400
0.200
0.000
0
10
20
30
40
Age (days post-hatch)


Pooled data – modes
Weaned
1.000
0.800
0.600
0.400
0.200
0.000
0
10
20
30
40
Wean complete
∇

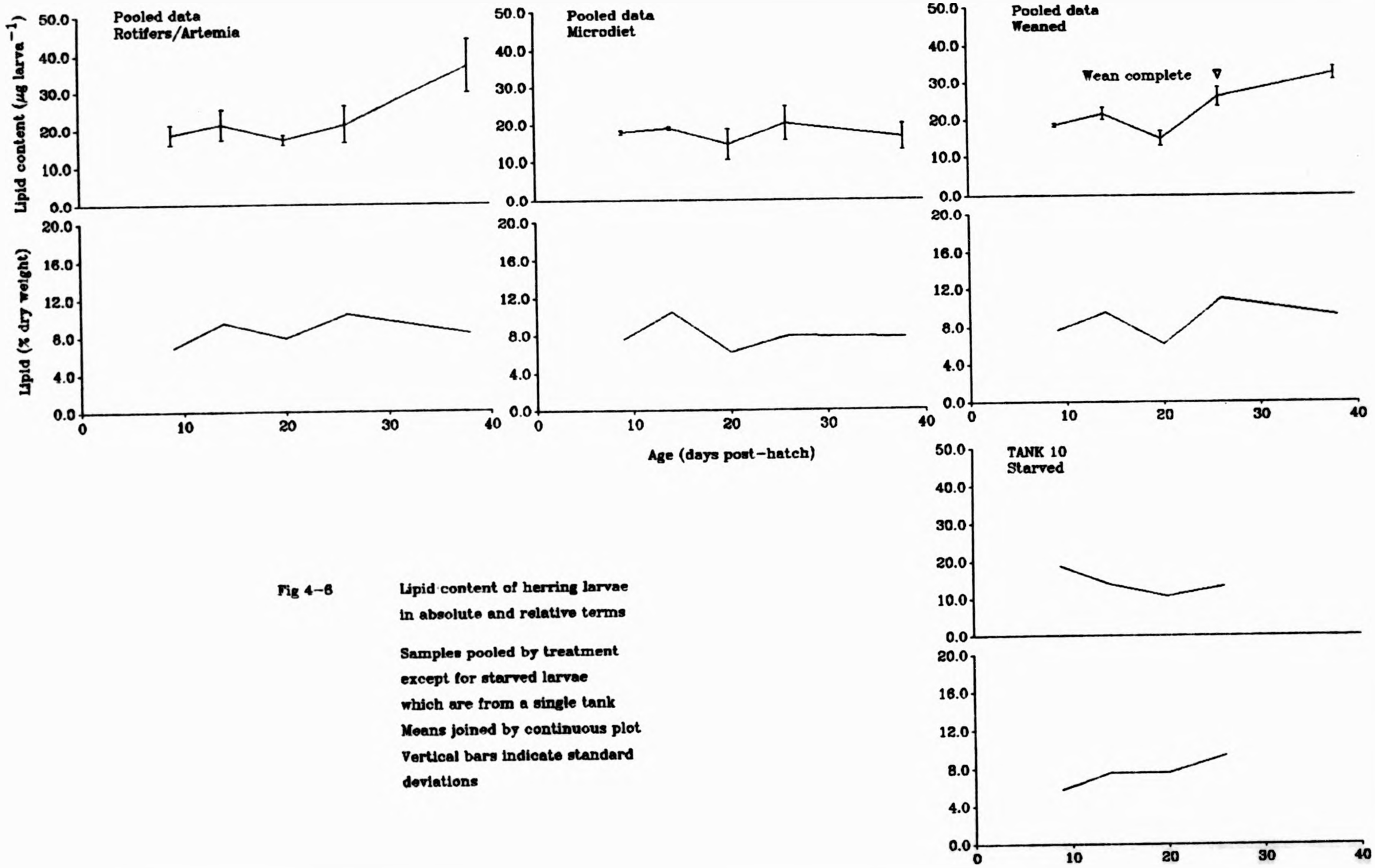


Fig 4–6 Lipid content of herring larvae in absolute and relative terms

Samples pooled by treatment except for starved larvae which are from a single tank
Means joined by continuous plot
Vertical bars indicate standard deviations

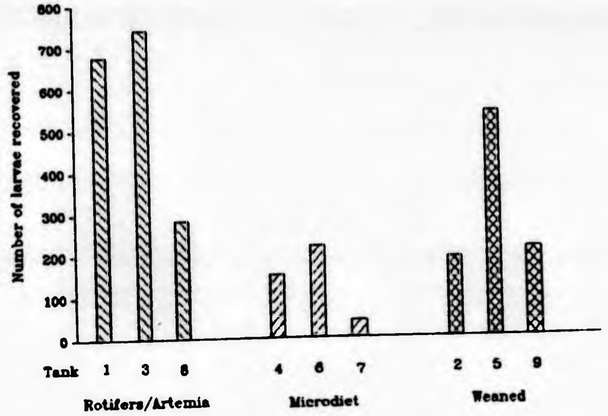
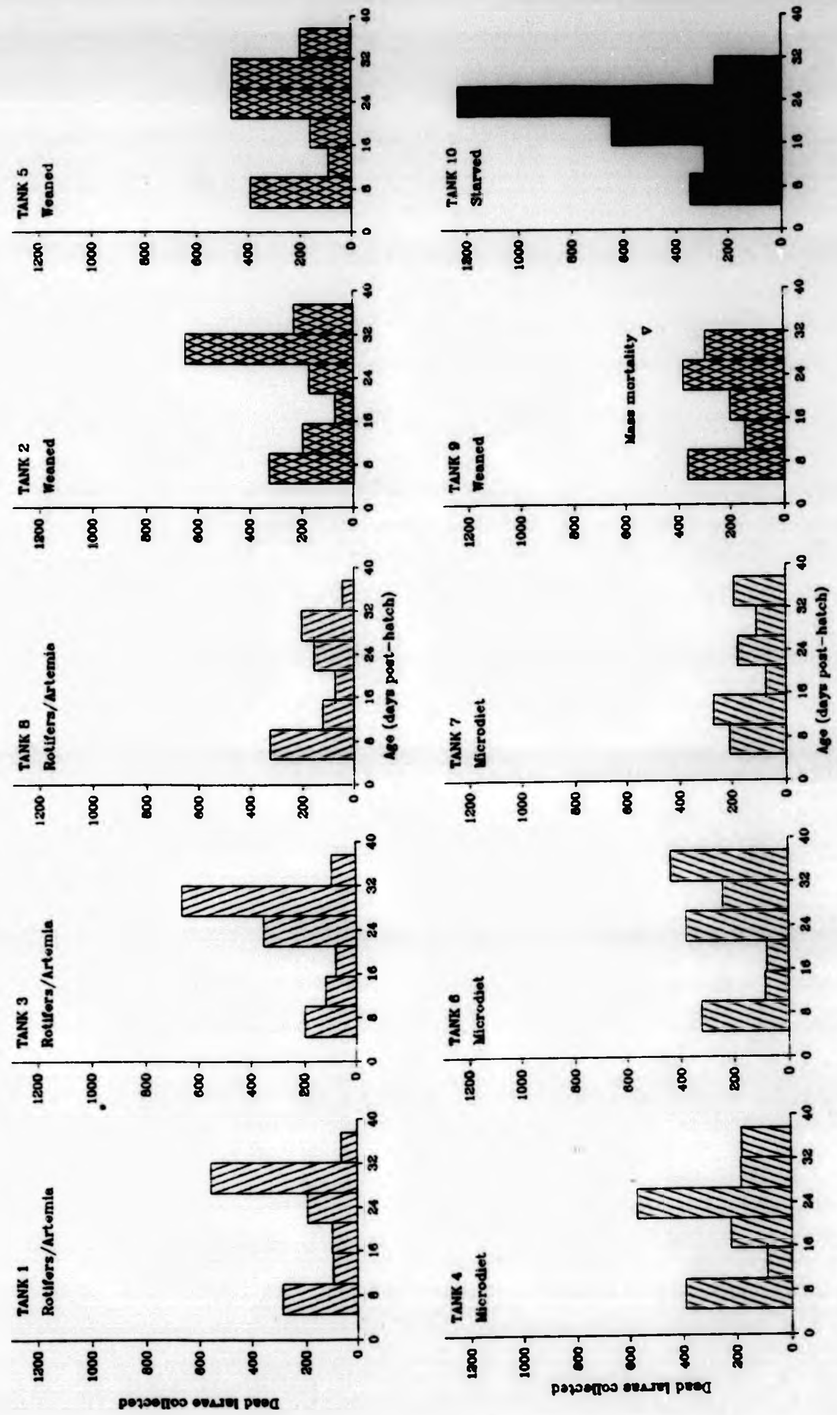


Fig 4-8 Numbers of live larvae recovered from tanks 1 - 8 on day 38
The number shown for tank 9 is an estimate based on the number of dead larvae collected after total mortality on day 32

Fig 4-9 Opposite

The numbers of dead larvae recovered from tanks 1 - 10 during the course of the rearing trial



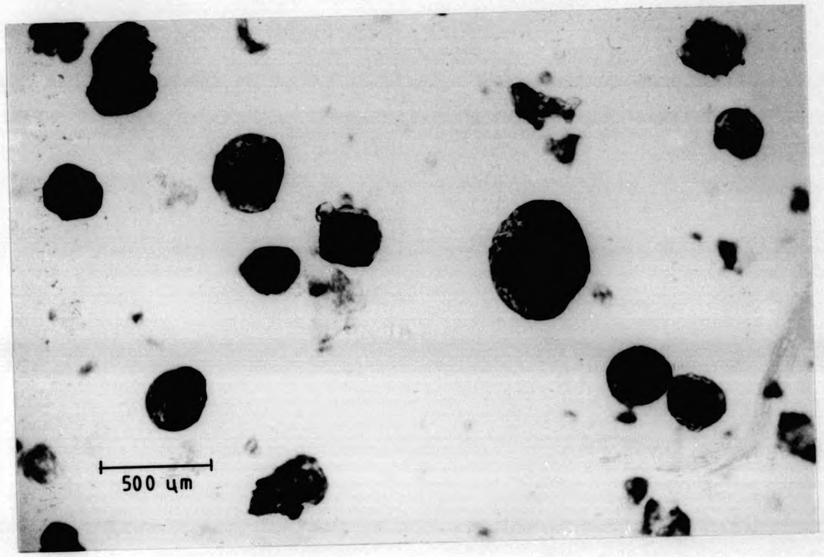


Plate 4-1 **Frippak microcapsules (90 - 500 µm) after rehydration in distilled water for 2 h**

Chapter 5 The use of liposomes for studying the polyunsaturated fatty acid nutrition of herring larvae

5.1 Introduction

It is known that marine fish larvae drink in order to replace water lost to their environment by osmosis. It was hypothesised that if larvae were placed in a suspension of fine particles, the particles might also be ingested. This could offer a route for the uptake of radioactively labelled metabolic tracers by marine fish larvae.

5.1.1 Osmoregulation in marine fish

Organisms living in an aqueous environment must deal with the problems caused by osmosis across semipermeable surfaces. For marine animals living in a hyperosmotic environment the problem is the loss of water from the body. Failure to compensate for this would result in the concentration of salts in the tissues eventually leading to death. The two modern classes of fish, the elasmobranchs and the teleosts overcome this in different ways. The elasmobranchs possess blood which is almost isotonic with seawater. This reduces water loss and may even cause a net water gain. The high osmolarity of the blood is due to its elevated urea and trimethylamine oxide content. However, a low concentration of monovalent ions are maintained in the blood by the action of salt secreting cells (known as chloride cells) in the rectal gland (Young 1981).

In adult marine teleosts, the blood has a lower osmolarity than seawater and there is thus net loss of water to the environment. The skin is almost entirely waterproof (excluding the gut and gill surfaces) due to its restricted vascularization and mucus coating. The areas across which water and salt movement most readily occur are the gills and gut which,

of necessity, must be permeable in order to carry out their primary functions of respiration and nutrient absorption respectively. Water lost from the body, mainly across the gills, is replaced by drinking and consequent water absorption from the gut. Water conservation is enhanced by reduced glomerular filtration, the kidney serving principally as an organ for excreting excess divalent ions (Foskett *et al.* 1983).

Divalent ions from ingested seawater are less readily absorbed and a large proportion are voided with the faeces. However, certain levels of these minerals are required for normal metabolism and their absorption from seawater is important in nutrition of marine fish (Steffens 1989). The monovalent salts pass more easily across the gut wall and thus the organism is now faced with an increased bodily salt content. In adult fish, the excess monovalent ions are excreted almost entirely by chloride cells located in the gills (Evans 1980). The discovery that salt influx as measured by radiotracers was five to ten times greater than could be accounted for by drinking alone complicated this model (Potts 1976). In fact the gills are the site of a major two-way ion flux, although the net effect is the loss of monovalent ions from the body. The exact nature and control of these fluxes remains to be elucidated but it is thought that chloride ions are actively excreted whilst net efflux of sodium ions is due to passive diffusion (Foskett *et al.* 1983).

The osmoregulation problems faced by marine teleost larvae are the same as those of adult fish. Shelbourne (1957) considered that the osmotic hazard facing larvae was considerably greater than that facing the adult. The original point was that larvae are unable to bear the metabolic cost of osmoregulation under starvation conditions and that they are more prone to starvation than adults. However, attempts to measure the cost of osmoregulation and to demonstrate that it does impose a

significant metabolic burden on adult fish have led to conflicting results. Potts *et al.* (1973) predicted that osmoregulation costs would be as low as 4% of total metabolism in flounder, *Platichthys flesus*, in seawater. The cost of osmoregulation was measured at 12% of total metabolic rate in 65 g red, hybrid tilapia (*Oreochromis mossambicus* x *O. hornorum*) (Ferry & Lutz 1987). For rainbow trout acclimated to 30 ppt, Rao (1968) claimed the cost of osmoregulation to be 27% of total metabolic rate whilst Furspan *et al.* (1984) quoted a value as high as 50% in resting catfish. It is unlikely that valid data relating to osmotic costs in larvae will become available until improvements can be made in the methodology for the measurement of this parameter.

Early work demonstrated that herring larvae are remarkably euryhaline (Holliday & Blaxter 1960) and this was later shown to be true of a range of species (Holliday 1969). A decrease in the degree of salinity tolerance occurs with age until, at metamorphosis the range is usually close to that of the adult. On abrupt transfer of larvae between salinities the osmotic concentration of the body tissues changes. Herring larvae (age un-specified) were able to function at internal salinities of 8.7 - 27.5 ppt (measured by freezing point determination) for up to 3 h. This ability allowed a respite for true osmoregulation to begin. The larvae were then able to return the body osmolarity to near normal levels (12.3 ppt) provided that the external salinity was in the range 20 - 40 ppt (Holliday & Blaxter 1960).

In early life stages of marine teleosts the adult structures concerned with water balance, i.e. the gills and kidney, have not yet developed and the mechanisms of osmoregulation in larvae are only now becoming clear (Alderdice 1988). It appears that larvae use similar methods to adults making use of drinking to replace water lost to the

environment and excreting excess salts via chloride cells located in the skin. There is evidence that drinking occurs as early as the embryo stage with the intake of perivitelline fluid facilitated by lateroventral pores (Guggino 1980). It has now been demonstrated that drinking occurs in the yolk-sac and first feeding stages of several marine fish (Mangor-Jensen & Adoff 1987, Tytler & Blaxter 1988b). As the rate of water loss from a larva due to osmosis is dependant on the osmotic concentration of the bathing medium, a direct relationship between drinking rate and external salinity should be found. This relationship has been demonstrated for herring, plaice and cod larvae. The drinking rates in larvae were higher compared to those for adults (on a mass-related basis), but less so than surface area comparisons would indicate. It thus seems likely that the larval skin is less permeable to water than adult gill epithelium (Tytler & Blaxter 1988a).

Although the changes in intestinal salt transport which occurred when adult flounder (a euryhaline teleost) were transferred into freshwater have been examined (Gibson *et al.* 1987), the above studies on larvae have all used external osmolarities higher than the internal osmolarity of the larvae. However, larvae are able to survive at salinities as low as 1-1.5 ppt for Clyde herring, 2-3 ppt for cod and 0-1 ppt for flounder (Yin & Blaxter 1987). At these salinities the larvae will be faced with the opposite set of problems i.e. net water influx and net ion loss. Since flounder may spawn near estuaries and herring spawn from oceanic to near freshwater conditions, the mechanisms for dealing with these environments merit further study.

The drinking process offers a route by which dissolved material can be accumulated in the gut. In experiments to measure the drinking rate in marine larvae (Tytler & Blaxter 1988a), the accumulation of a

fluorescent or radiolabelled, non-digestible marker dissolved in seawater was followed. It was reasoned that if digestible material were similarly accumulated, a proportion might be absorbed.

5.1.2 The development of liposomes for use in nutritional studies on bivalves

Artificial diets have the same advantages for nutritional studies on bivalves as they do for studies on fish and crustaceans (see section 4.1.2). Filter feeders are ideal organisms for feeding with inert diets and effort has been directed towards the use of cross-linked protein particles for this purpose (Langdon 1981). One of the problems with these diets is that up to 50% of the lipid in the feed mix is lost in the encapsulation process since the particles are prepared in cyclohexane, an organic solvent (Jones *et al.* 1979). In an attempt to overcome this problem lipid walled capsules, made by hardening Atlantic menhaden oil containing 10% ethyl cellulose were used to supplement alginate capsules used to feed *Crassostrea virginica* (Gmelin) spat. The lipid capsules also had improved retention characteristics for low molecular weight compounds such as vitamins. The prepared diets supported modest growth over 3 weeks (Langdon & Bolton 1984, Langdon & Siegfried 1984). However, this method of preparing capsules is relatively difficult and the digestibility of hardened menhaden oil, the majority of which is triglyceride, is likely to be low. Release of the entrapped components must thus be by mechanical rupturing of the capsule and the nutritional value of the wall material itself may be low.

Parker & Selivonchick (1986) used liposomes to feed juvenile Pacific oysters (for descriptions of liposomes see the following section). Both hydrophilic and hydrophobic compounds e.g. fatty acids, glucose and amino acids were incorporated into the liposomes. The bivalves took up

40% of the available dose in 24 h and labelled compounds were absorbed and further metabolized. The advantages of using liposomes for aquatic nutrition studies include their near neutral buoyancy and non-toxic composition.

The use of phospholipid vesicles for commercial scale delivery of lipids to bivalves, either as a complete diet or as a means of enriching microalgae deficient in essential fatty acids has yet to be realised although the technique is being developed for feeding penaeid shrimps.

5.1.3 Liposomes

Liposome technology provides a method for producing a fine suspension of lipid based particles. In addition, visual markers can be easily incorporated into the particles.

A liposome is defined as a vesicle comprised of phospholipid and aqueous compartments where the lipid exists primarily in bilayer form. Their formation is due to the amphipathic properties of polar lipids such as phosphatidylcholine, sphingomyelin, cardiolipin and phosphatidic acid. Stable structures composed of closed, bimolecular sheets intercalated with aqueous spaces spontaneously form when water is added to these compounds (Fig 5-1). The first liposome-like structures were observed by Bangham and co-workers in the early 1960s (Bangham *et al.* 1965). Similarities between electron micrographs of liposome preparations and natural biomembranes suggested that liposomes could model these structures. Since then liposomes have come to play an important role in research into cell membrane properties such as fluidity, permeability and lipid\protein interactions (Papahadjopoulos *et al.* 1988).

Liposomes are capable of encapsulating both hydrophilic and hydrophobic drugs (Mayer *et al.* 1986) and in certain cases will deliver

their load to target cells. The ability of liposomes to fuse with cells or to undergo phagocytosis is of prime importance in this process (Bangham 1981). Use has been made of liposomes in the development of orally administered drugs due to the protection from digestion to encapsulated material. The current status of liposome research has been reviewed by Papahadjopoulos *et al.* (1988).

Since the original work of Bangham *et al.* several different systems for the production of liposomes have been developed. These range from simple agitation or sonication of the components to detergent dialysis (Hope *et al.* 1986). The simpler methods tend to produce heterogenous collections of vesicles in terms of size and integrity. Closely defined size ranges may be produced by passing heterogenous collections of liposomes through polycarbonate membrane filters under pressure (Mayer *et al.* 1986b). Liposomes may be multiwalled (MLVs - multilamellar vesicles) or formed from a single bilayered wall. The latter category are further subdivided in terms of their size. Vesicles under 0.1 µm diameter are called small unilamellar vesicles (SUVs) whilst those over 0.1 µm are called large unilamellar vesicles (LUVs) (Szvoka & Papahadjopoulos 1981).

5.2 Materials and methods

The procedures used to culture *Nannochloropsis oculata*, rotifers, *Artemia* are described in sections 2.2 - 2.4.

5.2.1 Rearing herring larvae for autumn 1988

Fertile male and female herring were caught off Whiten Head (58°51'N 004°20'W) by the commercial trawler 'Sparkling Star' on August 31, 1988. Eggs were fertilized as described in section 2.1. Stacks

of fertilized eggs were stored at 10°C in clean, aerated seawater at the DAFS Marine Laboratory, Aberdeen. At 6 days post-fertilization, four stacks of 15 plates were placed in pails of clean seawater and transported to Stirling University. On arrival they were transferred into four, 100 l black PVC, circular, flat-bottomed tanks placed in a constant temperature room. Seawater (2 µm filtered) from the University aquarium recirculation system was run slowly through the tanks which were gently aerated. Lighting was on a 13:11 h light:dark time cycle and the experiments were run at 10°C.

On September 9 some larvae hatched prematurely. These larvae were not fully developed and died within two days. Aeration was removed at this stage to prevent further disturbance. The majority of the larvae hatched in two batches between September 10 and 14. All egg stacks were removed by the September 14 and larvae sorted into two stocks with mean hatching dates taken as September 11 and 13.

From day 5 post-hatch the herring larvae were fed a mix of rotifers and San Francisco Bay *Artemia* nauplii. The rotifer concentration was reduced steadily and finally omitted by day 15 post-hatch. Larvae were examined visually and noted to be feeding well by day 13 post-hatch. Food was added to the tanks at a rate of 2000 particles l^{-1} day^{-1}.

5.2.2 Rearing herring larvae for spring 1989

Mature herring were caught from Ballantrae Bank (55°8'N 004°20'W) by the F.R.V. Clupea. Eggs were removed and fertilized as described above on the afternoon of March 16. The stacks were transferred to SMBA Laboratory, Oban and incubated in running seawater. They were transferred to Stirling on March 30 and placed in the aquarium system

described above. Hatching occurred over the next three days thus giving a mean hatching date of April 1.

An explosion in the aquarium (see Plate 1) necessitated the simplification of the planned feeding schedule. From day 5 post-hatch, larvae were fed with rotifers at a rate of 2000 l^{-1} day^{-1} which had been reared on *Nannochloropsis oculata*. When the herring larvae were 9 days post-hatch the rotifer stocks crashed so the herring were fed Great Salt Lake *Artemia* at a rate of 2000 nauplii l^{-1} day^{-1} from then on.

5.2.3 Source of Baltic herring larvae used in spring 1989

A stock of Baltic herring larvae were obtained from The Danish Institute for Fisheries Research, Charlottenland. Eggs were fertilized on April 28 and flown directly to Scotland. After temperature acclimation, the stacks were placed in the aquarium system described above. The larvae hatched between May 11 and 12 and were kept as a single stock. The Baltic larvae were not fed as the rotifer population could not be restarted and the larvae were too small to take *Artemia* nauplii.

5.2.4 Production of liposomes

Liposomes were prepared by a modification of the method of Parker & Selivonchick (1986). Two ml of lipid solution [egg-yolk phosphatidyl-choline:cholesterol:stearylamine (7:1:2 by wt) in chloroform (10 mg ml^{-1})] were evaporated in a boiling tube under vacuum. Four ml of 0.2 µm filtered, autoclaved seawater containing 1% fluoroscein isothiocyanate (Sigma F7250) and 0.4 ml of sodium carbonate buffer pH 9.2 were added. The tube was flushed with nitrogen, sealed and sonicated in a Towson-Merser ultrasonic cleaning bath for 10 min at 35°C. The

suspension was dialysed against 2 l of distilled water containing 64 g sodium chloride overnight to remove unbound fluorescein. Larger volumes were prepared by scaling up the above procedure to a maximum of 50 mg total lipid. Liposomes containing radiolabelled lipid were prepared as above except that the radiotracer was added to the lipid mix prior to solvent removal (radiolabelled fatty acids were supplied dissolved in toluene by Amersham International, Bucks, England).

For experiments carried out after autumn 1988, antioxidant was also included. Compared to synthetic antioxidants such as butylated hydroxytoluene, vitamin E (α-tocopherol) was selected as least likely to harm the fish. The incorporation level is important as too high a concentration can itself act as an oxidant (Pokorny 1987). A level of 0.15% of total lipid weight was chosen as being suitable (Luotela *et al.* 1985). Vitamin E was supplied dissolved in soybean oil at 670 mg g^{-1} oil and the relevant quantity added to the liposome preparation prior to solvent removal. Thus for a 10 mg liposome preparation the amount of soybean oil added was 22.4 µg.

5.3 The production of liposomes and their stability

The experiments in this section were concerned with the production of liposomes, their characteristics and stability in seawater and the incorporation of radiolabelled fatty acids.

5.3.1 Materials and methods

Liposomes labelled with fluorescein were produced as detailed in section 5.2.4. Liposomes were suspended in 0.22 µm filtered, autoclaved seawater at 10°C and sampled at intervals up to 24 h. The liposomes were

examined using ultra-violet epifluorescence microscopy (450–490 nm) and their size determined using an eye-piece graticule. The settlement of the particles over 24 h was noted.

5.3.2 Results

The fatty acid spectrum of the phosphatidylcholine used is given in Table V,i. The major fatty acids present were 16:0, 18:0, 18:1(n-9) and 18:2(n-6) which together accounted for 93.2%. Polyunsaturated fatty acids of the (n-3) series were present at less than 1%.

Plate 5-1 shows liposomes labelled with fluorescein viewed under ultra-violet epifluorescence microscopy. The size of the particles (mean ± standard deviation) was 19.7 ± 27.9 µm (n=30) with a range of 250 µm downwards.

Over 24 h suspension in seawater, no obvious degradation of the liposomes was noted. A reduction in overall fluorescence of the liposomes was observed as fluorescein reacts chemically with water. Leaching of the marker from the particles to the seawater was not noted.

Liposome preparations were near neutrally buoyant. Static seawater suspensions of liposomes at 10°C took 12 h to clear but with gentle agitation the particles became resuspended.

5.3.3 Discussion

The egg-yolk phosphatidylcholine and cholesterol form the main components of the liposome wall. The presence of stearylamine is thought to encourage the separation of the bilayer membranes by imparting a positive charge. In addition stearylamine strongly binds the fluoroscein marker.

Compared to the size ranges quoted by Parker & Selivonchick (1986) of 8.0 - 0.4 µm, particles produced in this study were large. It seems likely that, rather than pure multi-lamellar vesicles, clumps were being produced by the fusion of smaller liposomes. A modification of the procedure to reduce the amount of stearylamine used might have resulted in smaller particles being produced (Howells pers. com.).

One problem in using liposomes for nutritional studies with labelled lipids is that the lipid from which the liposome is constructed may itself interfere with metabolic pathways. It has been shown, at least in rainbow trout, that long-chain PUFAs of the (n-3) series in the diet restrict the elongation and desaturation of both (n-3) and (n-6) fatty acids (Leger *et al.* 1981). Thus in a study designed to detect the synthesis of long-chain PUFAs of one series, it seems prudent to ensure that high levels of polyunsaturates of the other series are not included in the diet. This problem does not arise when defined inert diets are used where the dietary components can be refluxed with organic solvents prior to the addition of the fatty acid(s) being studied. One method of improving liposomes from this point of view would be to synthesise phosphatidylcholine as a di-molecular species containing only the radiolabelled fatty acid being studied. [1-C^{14}]di-16:0 phosphatidylcholine is produced commercially but the range of fatty acid molecules available is limited. Synthesis of [1-C^{14}]di-18:3(n-3) and [1-C^{14}]di-20:5(n-3) phosphatidylcholines was beyond the scope of this project. Although the majority of the fatty acids in the phospholipid used to generate liposomes were short chain, it must be borne in mind that the levels of 22:6(n-3) and 20:4(n-6) present at 0.5% and 3.0% respectively, might have had an effect on the elongation and desaturation ability of herring larvae, as measured in the experiments described later in this chapter.

Although many sophisticated systems for producing liposomes are now available, the equipment required tends to be expensive. Results from the other experiments in this section demonstrated that, for this study, the simple technique used was sufficient. Multi-lamellar vesicles are preferred for carrying lipid soluble substances as these liposomes have a high lipid/aqueous component ratio. If it is desired to carry hydrophilic substances, unilamellar vesicles are a better choice. In many cases the liposomes in this study are likely to have had fractures in the walls so producing a leaky aqueous compartment. If it had been wished to encapsulate water soluble material it would be necessary to improve the liposome production technique.

5.4 The incorporation and stability of radiolabelled fatty acids into liposomes

5.4.1 Materials and methods

The incorporation efficiency was determined, after overnight dialysis of the liposome preparations by taking 4 x 25 µl samples from the liposome suspension and 4 x 1 ml samples from the dialysate. Instagel scintillant (2.5 ml) was added to each sample and the radioactivity determined by liquid scintillation spectrophotometry. The incorporation efficiency was calculated by comparing the activity in the liposome suspension with that lost to the dialysate.

The stability of [1-C^{14}]18:3(n-3) in the liposome preparations used in the experiments described in sections 5.6 - 5.10 was assessed by recovering the particles from experimental solutions by repeated filtration through a 0.22 µm membrane. Lipid was extracted by the method of Folch *et al.* (1957) and the classes separated by TLC using hexane:ether:acetic acid (40:9:1 by vol). Autoradiography showed that all the radiolabel was

located in the free fatty acid band. The purity of the radiolabelled lipid was assessed by argentation TLC after conversion of the free fatty acids to methyl esters (see section 2.11.1)

The same procedure was followed for liposomes prepared with [1-C^{14}]20:5(n-3). However, argentation TLC was unable to resolve the pentaenoic and hexaenoic fatty acids so samples were analysed by packed column GLC (see section 2.11.2).

5.4.2 Results

The efficiency of incorporation of radiolabelled fatty acids into liposomes was high. Average incorporation of both [1-C^{14}]18:3(n-3) and [1-C^{14}]20:5(n-3) from six preparations after 24 h dialysis was 93.8 ± 6.2%.

The results of the stability assessment for [1-C^{14}]18:3(n-3) are given in Table V,ii and summarized in Table V,iii. The first table details the percentage of total radioactivity measured in each 0.5 cm band scraped from the chromatography plate. Tracks 'a','b' and 'c', 'd' represent replicate separations of the original labelled fatty acid and of that recovered after 24 h in seawater respectively. Tracks 'a' and 'b' indicate that the material supplied by Amersham was 91% pure. However, it must be borne in mind that argentation TLC does not identify individual fatty acids. Some radioactivity may reside in trienes other than 18:3(n-3). Around 1.5% of radioactivity occurred in a discrete band above the triaenoic fatty acids. The fatty acid(s) giving rise to this were not determined. In sample 'b', contamination (3.12%) was located in the bands containing fatty acids with four, five and six double bonds.

After 24 h in seawater, the fraction of radioactivity recovered from triene fatty acids had declined by between 2.90 and 6.56%. This was

compensated for by increased activity in monoenes, dienes and in the unidentified bands.

Table V,iv gives the results of the stability assessment for [1-C^{14}]20:5(n-3). The analyses were conducted using a packed column gas chromatograph allowing the radioactivity in individual fatty acids to be measured. Tracks 'e' and 'f' indicate that the original material supplied by Amersham was only 67.9% pure. Most of the remaining radioactivity was located in longer chain fatty acids. Track 'f' suggests that up to 6.15% of the total radioactivity in the starting material was present as 22:5(n-3) and 1.25% as 22:6(n-3). After 24 h in seawater the average counts present in 20:5(n-3) had not decreased. The percentage of C22PUFAs had increased whilst a significant increase in the radioactivity recovered from the saturated fatty acids 14:0 and 16:0 had occurred.

5.4.3 Discussion

The leaching rate in seawater of [1-C^{14}]18:3(n-3) and [1-C^{14}]20:5(n-3) incorporated into liposomes was not assessed due to the difficulty in complete recovery of the liposomes. Microscopic examination showed that, although repeated filtering removed the majority of liposome material, some particles still remained in suspension. However, the losses during dialysis over 24 h indicated that leaching would be low.

Neither of the two techniques available for the separation of radiolabelled fatty acids was ideal. Argentation chromatography is unable to separate individual fatty acids. If the precursor fatty acid is elongated but not desaturated, this will not be detected. Separation of individual fatty acids by packed-column gas chromatography is possible. However, a certain amount of carry-over between adjacent peaks was known to

occur. This may explain the spurious results in track 'e' (Table V,iv). A large amount of variation between replicate runs occurred and it was likely that small inter-sample differences would not be detected using this system.

The analyses of the original labelled fatty acids supplied by Amersham indicated that [1-C^{14}]20:5(n-3) contained large amounts of impurities. When carrying out metabolic studies even small amounts of contamination may cause problems. If the contaminant is a metabolic end-product, it may accumulate in the experimental organism and give spurious results. Further purification of 20:5(n-3) was considered impractical due to the difficulty in separating PUFAs by argentation TLC.

The double bonds in PUFAs are prone to oxidation (Pokorny 1987). Degradation of radiolabelled PUFAs will manifest itself in increased levels of radioactivity in less saturated fatty acids. After 24 h in seawater, [1-C^{14}]20:5(n-3) suffered more degradation than [1-C^{14}]18:3(n-3). It is likely that these changes would have been greater if antioxidant had not been included in the preparation, or the water temperature during the experiments had been higher. The use of ultrasound to generate the liposomes may have caused some damage. Lipid was sonicated in an atmosphere of nitrogen to prevent oxidation but the large amounts of energy imparted in this process may have caused isomerization in some of the molecules (Suslick 1989). Gentler liposome preparation techniques are available (Parker & Selivonchick 1986). These methods were tried but failed to generate small particles.

The stability of radiolabelled fatty acids in seawater without the presence of larvae was not examined. It is possible that some of the

degradation during the 24 h in seawater was due to digestion followed by egestion from the larvae. Since egested material became available to the larvae it was important to consider any degradation occurring at this stage.

5.4.4 Conclusion

[1-C^{14}]18:3(n-3) incorporated into liposomes was relatively stable over a period of 24 h suspension in seawater at 10°C. The amount of degradation experienced over this period was between 3 and 7%.

The purity of radiolabelled 20:5(n-3) supplied by Amersham was only 60 - 75%. When incorporated into liposomes, [1-C^{14}]20:5(n-3) itself was stable and degradation could not be detected over a period of 24 h. Some changes in the distribution of radioactivity within contaminating fatty acids did however occur.

5.5 The uptake of liposomes into the guts of herring larvae

The purpose of these experiments were to investigate whether herring larvae would ingest liposomes when placed in a suspension of these particles and to measure the rate of uptake.

5.5.1 Materials and methods

Larvae used were from the autumn 1988 stock (see section 5.2.1). Fluoroscein labelled liposomes were prepared as described in section 5.2.4. Seawater used throughout this experiment was at 969.3 ± 3.2 mosmol (34 ppt) and had been passed through a 0.22 µm filter. Twenty-five larvae aged 7-5 days post-hatch were collected from the stock tank and placed

in clean seawater overnight to allow the guts to clear of food. The following morning the larvae were transferred into 75 ml of clean seawater. Ten mg of liposomes suspended in 10 ml of seawater were added and the beaker placed in a chilled cool-box in the dark. Periodically single larvae were removed with a wide bore pipette, washed twice for 2 min in clean seawater and then anaesthetized in a bath containing benzocaine (500 mg l^{-1}). The larva was rapidly transferred to a cavity slide and observed by ultra-violet epifluorescence microscopy (450 - 490 nm) with and without additional transmitted light. Larvae which had been exposed to the liposome solution were compared with control larvae which had been kept in clean seawater. After 24 h in the liposomes some larvae were transferred to clean seawater and the guts allowed to clear for 7 h. These larvae were then observed as above.

This experiment was repeated with larvae aged 14 and 19 days post-hatch from the same stock and the following spring with Baltic larvae (see section 5.2.3) aged 4-2 days post-hatch.

5.5.2 Results

Control larvae showed a background level of fluorescence (which was too low to photograph) when viewed using epifluorescence microscopy. Examination of both rotifers and *Artemia* nauplii showed that this was due to the pigments in the brine shrimps. After 1 h in the liposome solution a significant increase in larval gut fluorescence was noted (Plate 5-2). Under high magnification the motion of individual particles could be followed with liposomes being swept by ciliary and peristaltic action and concentrated in the hindgut (Plate 5-3). After 4 h in the liposome suspension, fluorescent material was packed into the hind-gut and was extruded from the anus by peristaltic action within 5 min of

the larva being anaesthetized (Plate 5-4). The extruded liposomes appeared to be bound together and did not disperse into the water after egestion. After 10 h in the liposome suspension, fluorescence in the hindgut was significant even when viewed with added transmitted light under lower magnification.

The 7 h clearing period was sufficient to ensure egestion of all particulate material from the guts of larvae which had been in the liposome suspension for 24 h. On examination by ultra-violet epifluorescence microscopy fluorescent spots were observed in the hindgut near the anus.

Similar results were obtained in the replicate experiments with larvae at different ages including yolk-sac Baltic larvae.

5.5.3 Discussion

The observation that the pigments from *Artemia* nauplii could give rise to background fluorescence in the guts of larval herring would be important if direct measurements of ingestion rates were made from the fluorescence. It was found in initial work on this technique that calibration was difficult but the use of image analysis might make this a viable method of estimating the amounts of material ingested (Tytler P. pers. com.).

The bright spots observed in the anal area of the hind-guts are similar to those observed in the foregut of cod larvae and in the stomach of plaice larvae and may represent the sites of water absorption (Tytler & Blaxter 1988a). Little work has been carried out on the mechanisms or specialised structures involved in water absorption by marine fish larvae.

Fluorescent liposomes may prove a useful tool for observations on transport mechanisms in fish larval guts. Ciliated epithelial cells are uncommon in the digestive tract of teleost fish but have been recorded in first-feeding Baltic herring larvae (Iwai & Rosenthal 1981). In this study, liposomes appeared to be transported in the gut by a combination of ciliary and peristaltic action.

Herring larvae generally begin feeding on particulate prey towards the end of yolk-sac absorption (about 6-5 days post-hatch for spring spawned Clyde larvae and a day or so earlier for Baltic larvae). The successful transition from reliance on endogenous yolk supplies to external food is frequently regarded as one of the critical stages in fish larval life (Kiørboe *et al.* 1985). However, supporting field evidence has failed to demonstrate conclusively that particularly high mortalities of fish larvae occur at the time of yolk resorption (May 1974). In wild caught, first feeding cod larvae, amorphous green material is often noted in the gut (Kvenseth & Øiestad 1984). Blaxter & Hunter (1982) suggested that similar material in herring larvae is the remains of phytoplankton, accidentally taken by the larvae in a learning phase. However, it has been found in aquaculture that fish larvae reared in water containing cultures of microalgae survive better than those reared in 'clean' water (Dye pers. com.). It is unclear whether the green material found in larval guts indicates direct feeding on phytoplankton or is the pigmented remains of digested microzooplankton but fatty acid data does indicate that the material is of phytoplanktonic origin (Klungsøyr *et al.* 1989).

It has been shown that cod larvae will ingest bacteria and accumulate them in the midgut in yolk-sac larvae and in the hindgut in older larvae (Olafsen 1984). Providing the prey concentration is high, juvenile herring will filter *Artemia* nauplii from the water currents

passing across the gill rakers and this behaviour can occur in the dark (Batty *et al.* 1986). The demonstration in this experiment that herring larvae can also accumulate inert particles in the dark by drinking has implications for fish larval nutrition.

5.5.4 Conclusions

Herring larvae will ingest liposomes into their guts when placed in a seawater suspension of these particles. Reproducible, quantitative measurements of the rate of uptake by measuring epifluorescence were not possible using the equipment available at the time.

The role of microzooplankton and phytoplankton in the nutrition of marine fish larvae, especially at first feeding requires more study.

5.6 The rate of uptake of liposomes and water by herring larvae

If the rate of particle uptake can be measured it can be compared with the drinking rate necessary to replace water loss by osmosis. If the two measurements are closely related then this implies that particle ingestion is a direct result of drinking. Since herring larvae are regarded as being visual feeders (Blaxter & Hunter 1982) and the above experiments were conducted in the dark, this is what would be expected. Since direct measurement of liposome uptake was not possible, an attempt was made to use radiolabelled liposomes for this purpose.

Work in this section was carried out in conjunction with Dr P. Tytler (School of Natural Sciences, University of Stirling).

5.6.1 Materials and methods

Two experiments on the rate of liposome uptake were carried out using 17-15 days post-hatch Clyde larvae (see section 5.2.2) and 8-6 days post-hatch Baltic larvae (see section 5.2.3) from spring 1989 stocks.

Experiment I The rate of uptake of liposomes and water by Clyde-spawned herring larvae

Seawater was made up to nominal salinities of 34 and 17 ppt. The seawater at 34 ppt came from the aquarium re-circulation system and was reduced to 17 ppt by dilution with distilled water. The solutions were then filtered through 0.22 µm polycarbonate membranes [Millipore (U.K. Ltd.), London] to remove bacteria. Two batches of 70 larvae were removed from the stock tank and transferred into 2 l of each salinity overnight to allow gut clearance and acclimation to the new salinity. Thirty mg of liposomes were prepared incorporating 30 µCi of [1-C^{14}]18:3(n-3) as described in section 5.2.4.

The following day the larvae were transferred into 50 ml of seawater of the relevant salinity. Liposomes were removed from dialysis and sampled to determine their activity. Two ml of liposome solution were added to each batch of larvae and the beakers placed in the dark. The solutions were stirred gently every hour.

Periodically samples of ten larvae were removed from the beakers and placed in a tea-strainer. They were passed through three, 2 min washes in seawater at the relevant salinity. The larvae were then counted onto a disc of filter paper and placed in a scintillation vial. One ml of Soluene-350 (Packard) was added and the tissue left to digest for 24 h at room temperature. After this 2.5 ml of HionicFluor (Packard) were added

and the radioactivity present in each sample determined by liquid scintillation spectrophotometry.

Drinking rates were determined in a separate experiment using larvae from the same stock aged 16-14 days post-hatch. Seawater was made up to nominal salinities of 34 and 17 ppt and filtered as above. Two batches of 50 larvae were removed from the stock tank and transferred into 2 l of each salinity overnight to allow gut clearance and acclimation to the new salinity.

The following day the larvae were transferred into 50 ml of seawater of the relevant salinity and 125 µCi of [H^3]dextran (mw 70,000 Amersham) dissolved in 0.5 ml of distilled water added. The beakers containing larvae were placed in the dark. The solutions were stirred gently every hour.

Periodically, four replicate samples of two larvae were removed from the beakers and placed in a tea-strainer. They were washed and the radioactivity in them determined as above.

The osmolarity of the seawater from the three baths was determined using a Wescor 5100C vapour pressure osmometer.

Experiment II The rate of uptake of liposomes and water by 8-6 days post-hatch Baltic larvae

This was conducted following the same protocol as Experiment I except that:-

Liposome uptake was measured using 120 larvae at each salinity. Samples of 20 larvae were taken after 0.5, 1, 2, 3 and 5 h.

Drinking rates were determined simultaneously in separate beakers and 75 µCi of [H^3]dextran were added to each bath. The drinking rate determination used 120 larvae in each salinity and four replicate samples of five larvae were taken at each time point.

Uptake values were determined using the following equation:

$$\text{Uptake value} = \frac{(R_s - C)}{(R_r - C)N}$$

R_s is the total radioactivity of the sample in DPM
C is the background radioactivity determined from a sample of larvae not exposed to radiolabelled liposomes
R_r is the activity of the bathing solution in DPM μl^{-1}
N is the number of larvae in the sample

For measurements of the uptake of liposomes, the values are called water imbibed equivalents. These may then be directly compared with water uptake values as measured in the drinking rate experiments.

The higher levels of radioactivity used in drinking rate determinations allowed four replicates to be taken at each sample point. The rates of drinking and liposome uptake were calculated by linear regression of the uptake values. The median standard length (Experiments I and II) and dry weight (Experiment I only) were determined from samples of larvae at the same age and from the same stock. Absolute uptake rates were divided by larval median dry weight to yield a relative measure.

5.6.2 Results

There was no visual evidence that liposomes had settled out of suspension between sampling points. The results from Experiment I are given in Table V,v and displayed graphically in Fig 5-2. There was some variation in the osmolarities of the nominal salinities between measurement of drinking rate and liposome uptake as these two factors were measured on different days. The water imbibed equivalents exceeded the water uptake values by a factor of 2-300. The results of the regression analyses are given in Table V,vii. There was little relation between the water

imbibed equivalent rates (2.62 and 3.00 µl larva^{-1} h^{-1} at 551 and 967 mosmol respectively) and the external osmolarity. There was a direct relationship between drinking rates (0.00882 and 0.0143 µl larva^{-1} h^{-1} at 492 and 969 mosmol respectively) and the external osmolarity.

The results from Experiment II are given in Table V,vi and Fig 5-3 and rate measurements are detailed in Table V,viii. The 2 h drinking value at 493 mosmol was an outlier and was disregarded. Relative rates were not computed since the larvae still possessed yolk-sacs whose presence adds considerable weight to the larvae without contributing to gut volume. Once again the water imbibed equivalents exceeded the measured drinking rates but by a factor of 100. There was no direct relationship between water imbibed equivalent rate and external osmolarity but the drinking rate at 960 mosmol was greater than that at 493 mosmol (0.00785 and 0.00625 µl larva^{-1} h^{-1} respectively). The drinking rate at the higher osmolarity was not, however, twice that at the lower osmolarity.

5.6.3 Discussion

These experiments were based on the standard method for measuring drinking rates in aquatic organisms. The organism is immersed in a solution of an inert marker that is taken into the alimentary canal but not absorbed across the gut wall. After an interval, sufficient to allow the substance to enter the gut but not to be defecated, the amount of marker contained in the gut is measured and divided by its concentration in the external solution. Previous experiments had shown that the drinking rate tends to be linear up to 6 h for herring larvae and 1-2 h for cod larvae (Tytler & Blaxter 1988a). Thus the drinking rates should be measured within these initial periods. The method assumes that the only route of entry for the marker is via the mouth and that the marker is not

degraded. Dextran being a carbohydrate is biodegradable by micro-organisms in seawater, ultimately to carbon dioxide and water. The radiolabel will then be lost rapidly from the experimental system. All experiments were performed using seawater filtered to 0.22 µm to reduce the numbers of micro-organisms present. In addition larvae were washed overnight in the above seawater to try and reduce the numbers of bacteria associated with them. Dextran solutions were freshly made up.

The radiolabelled fatty acid used as a marker in the liposomes was not biologically inert and was assimilated by the larvae (see section 5.8). A certain percentage of the marker taken up would have been catabolized by ß-oxidation and thus, the water imbibed equivalent represents an underestimate of the total liposome material ingested and absorbed. It might be possible to gain a more accurate measure by encapsulating [^{3}H]dextran in liposomes but improvements in the method used for liposome production would be required to achieve this.

The rate of drinking has been shown to be related to the external salinity. This follows from the hypothesis that drinking is used in non-feeding larvae to replace water lost from the organism by osmosis. The losses due to osmosis will be greater the more hyperosmotic the bathing medium is. The drinking rates recorded in Experiment I showed this relationship. The results from Experiment II were not as clearly related to salinity but the drinking rate at the higher osmolarity was still greater. For 35 day old herring larvae Tytler & Blaxter (1988a) calculated drinking rates of 0.0192 and 0.0466 µl larva^{-1} h^{-1} (0.0647 and 0.155 µl mg^{-1} h^{-1} relative to dry weight) at 16 and 32 ppt respectively. The drinking rates from this study were slightly lower. Absolute drinking rates were related to size, the rate for the larger Clyde larvae being greater than that for the Baltic larvae. Changes in the permeability of the

larval skin with age may lead to changes in the rate of water loss. This aspect of osmoregulation in fish larvae has still to be investigated.

The original hypothesis was that liposome uptake was a passive result of the drinking behaviour described here. If this is correct then the water imbibed equivalent rates should be of the same order of magnitude as the drinking rates. The results clearly show that this was not the case as the uptake of liposomes exceeded that which could be explained by passive drinking by a factor of 100–300. Tytler & Blaxter (1988a) estimated the gut volume of 30 day old herring larvae to be 0.1 µl. Using this value, the maximum liposome ingestion rate found in this study would require 26 gut volumes h^{-1}. It is suggested that fish larvae ingest volumes of water considerably in excess of those required for osmoregulation in the process of feeding. Excess water is presumably egested via the anus. Attention should thus be focused on the mechanisms by which larvae limit the uptake of water from the gut since drinking rate *per se* is likely to play little part in controlling water absorption in feeding larvae.

There are several possible explanations for the high water imbibed equivalents:-

i. The liposomes settled out in the experimental beakers and the larvae were thus drinking a denser solution than was calculated.

ii. The presence of lipid vesicles in the water or in the guts of the larvae stimulated drinking.

iii. The liposomes were taken in by an additional route other than by the mouth alone.

The first possibility for explaining the discrepancy between water imbibed rates and drinking rates cannot be discounted. However, suspensions of liposomes in static seawater took 12 h to clear. In these

experiments not only was the water disturbed by the movements of the larvae but the solution was mixed every hour. In addition, if a gradual increase in the density of liposomes at the base of the experimental beaker were occurring over the experimental time, the rate of liposome uptake would be expected to be non-linear. This did not appear to be the case in any of the data sets.

Herring larvae are considered to be visual feeders (Blaxter 1967). Since the experiments in this section were conducted in the dark, active particulate feeding via visual cues was discounted. There is some evidence that fish larvae do take in particles from suspension at a greater rate than that explicable by drinking for osmotic reasons. This was found when the uptake of the bacterium *Aeromonas* by trout alevins was considered (Tytler pers. com.). Stimulation of drinking might come from olfactory cues or from the physical sensation of particles in the gut.

The final possibility was that uptake of liposomes occurred by a subsidiary route other than via the gut. Liposomes are capable of fusing with cells, a phenomenon which leads to their use in non-oral drug delivery (Papahadjopoulos *et al.* 1988). Liposomes could possibly be taken up from suspension by fusion across the larval skin. The experiment in the following section attempted to elucidate the route of uptake of radiolabelled fatty acid from liposomes in suspension.

5.7 Histological evidence for the site of uptake of radiolabelled fatty acid from liposomes in suspension

5.7.1 Materials and methods

This technique was attempted on a trial basis with larvae taken from the experiment described in section 5.8. Whilst being incubated with liposomes containing [1-C^{14}]18:3(n-3), a sample of larvae was removed hourly up to 6 h and then at 14, 21, 28 and 35 h. These larvae were placed in clean seawater for a further 7 h to ensure the guts were cleared of liposomes. After washing, they were then fixed and stored in phosphate buffered saline (pH 7.4) containing 3% (w/v) formaldehyde.

The larval samples were subsequently dehydrated by passing them through the following solutions:-

25%	ethanol	for 45 min
50%	ethanol	for 45 min
75%	ethanol	for 60 min
90%	ethanol	for 60 min
100%	ethanol	for 60 min
100%	ethanol	for 120 min
100%	ethanol	overnight

The larvae were then embedded in JB-4 resin (Polysciences Inc., Warrington) following the instructions of the manufacturer.

Microscope slides were thoroughly cleaned with acetone and dipped in a solution containing 0.5% (w/v) gelatin and 0.05% (w/v) chrome alum. The slides were dried at room temperature. Resin embedded larvae were serially sectioned to 3 µm and mounted on the slides which were then coated with photographic emulsion under safelight conditions (Ilford Nuclear Research, K5 emulsion warmed to 37°C). The specimens were then stored in a light-proof case with desiccant at -80°C for two months.

After this time the case was opened under safelight and the autoradiographs developed for 3 min in Kodak D-19. The slides were then fixed for 5 min in 30% sodium thiosulphate (w/v) followed by a 10 min

wash in distilled water. The sections were lightly stained with methylene blue to highlight the tissues and after drying cover-slips were sealed on. Areas of radioactivity are shown by the deposition of silver grains which appear black.

5.7.2 Results

Difficulties were experienced with the technique. In many cases the background on the autoradiographs was unacceptably high, in some cases the sections had torn and in many cases had failed to adhere to the gelatin-coated slides. In several instances the sections failed to stain with methylene blue and satisfactory results were only obtained from a few of the slides prepared.

Plate 5-5 shows a cross section of the foregut of a Clyde herring larva following a 1 h incubation in a seawater suspension of radiolabelled liposomes. Silver grains were deposited around the skin and especially on the fin. No activity was detected in the foregut. After a 5 h incubation, radioactivity could be detected in the liver as well as the skin. Examination under high power magnification indicated that most of the radioactivity at the skin was located sub-dermally (Plate 5-6). Radioactivity was detected in the hind-gut after 21 and 35 h incubations (Plates 5-7 and 5-8). Once again, activity on and just under the skin was high. In none of the sections was significant radioactivity noted in the notochord.

5.7.3 Discussion

The major problem with this technique is that the dehydration of tissues in ethanol is likely to have removed radiolabelled lipid. It is suggested that the solvent used, ethanol, will have removed neutral lipids, such as free fatty acids but did not remove phospholipid, presumably predominantly located in cell membranes. The results indicate that radiolabelled free fatty acid from the liposomes had become incorporated into phospholipids (as confirmed in section 5.8).

The results suggested that either absorption of radiolabelled fatty acid was occurring through the skin, or that the sub-dermal layer is particularly active with regard to lipid deposition. The activity noted in the liver (Plate 5-6) clearly demonstrates the importance of this organ in fatty acid metabolism whilst the activity found in the hind-gut cells (Plates 5-7 and 5-8) but not in the fore-gut, suggests that the hind-gut is the primary site for fatty acid absorption from the intestine.

Improved results might be obtained if the freeze-drying technique outlined by Becker & Bruce (1985) were adopted. However, this technique is designed for larger organisms and might be difficult to adapt to fish larvae.

5.7.4 Conclusions

The results of autoradiography on sections of herring larvae which had been incubated in suspensions of liposomes containing [1-C^{14}]18:3(n-3) suggested that absorption of the radiolabel across the skin might have occurred. Radioactivity was also found in the liver and hind-gut cells but not in the foregut.

5.8 The absorption and assimilation of radiolabelled fatty acid from liposomes by herring larvae

The autoradiographs from the previous section suggested that herring larvae can assimilate radiolabelled fatty acid from liposome suspensions. The experiments described in this section were designed to examine which lipid classes the radiolabelled fatty acids were assimilated into.

5.8.1 Materials and methods

Experiment III

Larvae used in this section were autumn spawned Clyde stock reared as detailed in section 5.2.1. Four hundred and fifty larvae aged 12-10 days post-hatch were removed from the stock tank and transferred into 2 l of 0.22 µm filtered seawater to which 0.1 g of penicillin G (Sigma) and 0.2 g of streptomycin sulphate (Sigma) had been added to reduce the levels of micro-organisms which might degrade the vesicles. Liposomes (40 mg) incorporating 25 µCi of [1-C^{14}]18:3(n-3) (Amersham CFA 146) were prepared and dialysed overnight as described in section 5.2.4. Filtered seawater (including antibiotics as before) was autoclaved * and subsequently bubbled with air passed through a sterile 0.22 µm filter.

The following day the larvae were removed from the clearing bath and transferred into 200 ml of the aerated, autoclaved seawater in a small beaker. The liposomes were removed from dialysis and four 25 µl samples taken to determine their activity. These samples were mixed with 2.5 ml of HionicFluor (Packard) and the radioactivity determined by liquid scintillation spectrophotometry. The liposomes were added to the beaker

containing larvae giving a total volume of 216 ml. The beaker was then placed in the dark and aerated gently with 0.22 µm filtered air to keep the liposomes in suspension and ensure that dissolved oxygen levels did not fall.

Every 7 h about 80 larvae were removed using a wide bore pipette. These were transferred via two washes into 200 ml of 0.22 µm filtered, autoclaved seawater and left for a further 7 h to ensure the guts had emptied. This was confirmed by examination of two larvae per sample under ultra-violet epifluorescence microscopy. Larvae were then counted onto a 68 µm mesh and washed with distilled water. The sample was transferred into 7.5 ml of chloroform:methanol (2:1) and stored at -20°C prior to analysis. In all five samples were collected.

Lipid was extracted from the samples using the method of Folch *et al.* (1957) and the lipid classes separated by TLC. Polar lipids were first separated half-way up the plate using chloroform:ethanol:water: triethylamine (30:35:6:35 by vol, Leray & Pelletier 1987). After drying the plate under vacuum, neutral lipids were separated using hexane:ether:acetic acid (40:9:1 by vol, Christie 1982). The bands containing radioactivity were located by autoradiography and identified by comparison with non-radiolabelled standards. Class bands were scraped into scintillation vials, 2.5 ml of Optifluor (Packard) added and the radioactivity present determined by liquid scintillation spectrophotometry.

Experiment IV

Larvae used were from the same stock as above but were aged 34-32 days post-hatch. The protocol followed was as in Experiment III except that the incubation beaker was replaced by six glass jars of 50 ml

capacity. The chambers were set up to see if carbon dioxide given off by the larvae during the experiment could be collected to determine the rates of β–oxidation. The apparatus was only partially successful. Sixty larvae were transferred into each chamber and 1.3 ml of liposome suspension added giving a total volume of 51.3 ml sample^{-1}. The chambers were sealed and slowly aerated. The liposome suspensions clumped in several of the chambers, settled out and smothered the larvae. This may have been due to traces of detergent on the glassware. Larvae were removed from chambers in which liposomes had not clumped after 7, 14 and 28 h incubations. The samples of larvae were washed, stored in solvent and subsequently analysed as in Experiment III.

5.8.2 Results

Larvae left for 7 h in clean water after being incubated in the liposome suspension had empty guts. Although no particles were seen, the hindguts did exhibit fluorescence and some discharge of fluorescein from the anus was noted.

Table V,ix gives the activity in each sample, the water imbibed equivalent and the class distribution of radioactivity in absolute and percentage terms for larvae from Experiment III. The uptake rates and absolute class distributions are shown graphically in Fig 5–4 whilst Fig 5–5 shows the percentage distribution relative to total radioactivity larva^{-1}. The total lipid class composition of larvae used in these experiments was not determined. For comparison data are given for well nourished, spring–spawned herring larvae (from Fraser *et al.* 1987).

The total radioactivity assimilated by each larva increased in a linear manner. Linear regression indicated that the equivalent of 0.57 µl of the liposome suspension was absorbed by each larva every hour.

The amount of radioactivity recovered from the free fatty acid fraction reached a maximum of 235 DPM larva^{-1} (5.1% of total radioactivity) after 32 h incubation. The majority of the radiolabelled fatty acid was recovered from lipid classes where one, two or three fatty acids are esterified to a backbone (a sterol in the case of sterol esters or glycerol for the other classes). The distribution of radioactivity expressed in relative terms indicated that there were no identifiable trends apart from a decline in the level of activity recovered from triacylglycerols with incubation time.

Radioactivity was detected in a band tentatively identified as phosphatidic acid (1,2-diacyl-*sn*-glycerol-3-phosphate) at levels of between 6.6 and 12.5%.

The results from Experiment IV are given in Table V,x but are not illustrated graphically. The assimilation rate was higher than in Experiment III (the water imbibed equivalent was 0.98 µl larva^{-1} h^{-1}). The relative distribution of radioactivity within lipid classes was similar to the previous experiment although the activity in cardiolipin, phosphatidic acid and phosphatidylserine was lower. The levels recovered from phosphatidylcholine were greater. Separation of sphingomyelin from phosphatidylcholine was achieved but sphingomyelin contained less than 1% of total radioactivity. A consistent decline in activity of triacylglycerols did not occur with incubation time.

5.8.3 Discussion

In Experiment III the water imbibed equivalents (Table V,ix) were considerably lower than those calculated in section 5.6.2. This was probably because these results were computed on the activity remaining

in the larvae after they had 7 h to clear the guts. The values thus account only for the radioactivity assimilated by the larvae and do not include gut contents. The values are still considerably greater than the drinking rates measured in section 5.6 and thus provide further evidence that the high water imbibed equivalents recorded were not due to settlement of the liposomes (since the suspension in this experiment was continually mixed).

The fact that the majority of the radioactivity recovered occurred in lipid classes in which fatty acids are esterified to other molecules demonstrated that [1-C^{14}]18:3(n-3) had been absorbed and metabolized by the larvae.

The detection of radioactivity in phosphatidic acid provided further confirmation that the radiolabelled fatty acid was being actively incorporated into body lipids. This class normally occurs only in trace amounts and is not detected as a significant mass in tissue lipid analyses. It is the precursor of all the other glycerophospholipids and of triacylglycerols and has a high turnover rate. Radioactivity was detected in this class in Experiment IV but at a much lower level. This difference is difficult to explain but the rates of metabolic turnover of lipids may vary with the age of the larvae. Further experiments would be needed to investigate this.

The lipid class composition of larvae from this experiment was not available. The comparison of the percentage incorporation data with a typical lipid class composition on a mass basis, from well nourished, spring-spawned herring larvae (derived from Fraser *et al.* 1987) is only approximate. The two larval stocks were raised in different rearing systems, fed on different diets and were spawned at different seasons.

Lipid class data for autumn spawned herring larvae is not available in the literature.

Fig 5-5 allows a comparison between the distribution of radioactivity within the lipid classes of incubated larvae and the un-labelled lipid class composition of 'typical' herring larvae. The majority of the counts were located in the band containing phosphatidylcholine and sphingomyelin. The latter class only occurs in minor amounts in herring larvae (1-4%) whilst phosphatidylcholine is the major lipid. It is likely that the majority of the counts were incorporated into phosphatidylcholine and this was confirmed by the results from Experiment IV. However, [1-C^{14}]18:3(n-3) was probably not preferentially incorporated into the polar lipids, phosphatidylcholine and phosphatidylethanolamine, since the high levels recovered from these classes reflect their abundance in the larval lipid.

The decline in the percentage of radioactivity recovered from triacylglycerol with time may reflect an overall decrease in the amounts of this class in the larvae. Triacylglycerol is the normal energy storage compound in herring larvae and declines with starvation (Fraser *et al.* 1987).

The results from Experiment IV confirmed the trends observed in Experiment III. The greater rate of radiolabel absorption compared to the previous experiment can be explained by the greater size of the larvae (34-32 compared to 12-10 days post-hatch).

5.8.4 Conclusions

When larvae were bathed in a suspension of liposomes containing [1-C^{14}]18:3(n-3) the labelled fatty acid was assimilated by the larvae and incorporated into the body lipids, predominantly into polar classes.

5.9 The elongation and desaturation of 18:3(n-3) by herring larvae

The ultimate aim of designing a vector to allow the assimilation of radiolabelled fatty acids by marine fish larvae is to carry out pulse-chase experiments. These are used to assess the ability of the larvae to modify the precursor substance. As explained previously, in relation to lipid nutrition, if a high rate of conversion of the precursor fatty acid to longer chain/more unsaturated fatty acids is demonstrated, it is generally accepted that the end-product is not an essential nutrient and need not necessarily be present in the diet, providing that enough of the precursor is available.

5.9.1 Materials and methods

Experiment V

Larvae from the spring 1989 Clyde spawned stock (reared as in section 5.2.2) were used in this experiment. Four hundred and fifty larvae aged 19-17 days post-hatch were removed from the stock tank and transferred into 2 l of 0.22 µm filtered seawater to which 0.1 g of penicillin G (Sigma) and 0.2 g of streptomycin sulphate (Sigma) had been added to reduce the levels of micro-organisms which might degrade the vesicles. Liposomes (20 mg) incorporating 40 µCi of [1-C^{14}]18:3(n-3)

(Amersham CFA 146) were prepared and dialysed overnight as described in section 5.2.4. Filtered seawater (including antibiotics as before) was autoclaved and subsequently bubbled with air passed through a sterile 0.22 µm filter.

The following day, 300 larvae were removed from the clearing bath and transferred into 75 ml of the aerated, autoclaved seawater in a small beaker. The liposomes were removed from dialysis and four 25 µl samples taken to determine their activity. The samples were mixed with 2.5 ml of HionicFluor (Packard) and the radioactivity determined by liquid scintillation counting. The liposomes were added to the beaker containing larvae giving a total volume of 82 ml. The beaker was then placed in the dark and aerated slowly with air filtered to 0.22 µm.

After 24 h, live larvae (274) were recovered from the beaker containing liposomes and transferred into 2 l of clean seawater filtered to 0.22 µm to allow liposomes in the guts to clear. After 7 h several larvae were checked microscopically to ensure that the guts had emptied. Fifty larvae were transferred into 7.5 ml of chloroform:methanol (2:1) and stored at -20°C for subsequent analysis. The remaining larvae (210) were transferred once more into 2 l of clean seawater and Great Salt Lake *Artemia* nauplii added at a concentration of 2000 l^{-1}. Lighting was continuous. After 24 h, most of the larvae in the beaker had *Artemia* in their guts. Further samples of larvae were collected from this beaker after 41 h and 89 h and stored as above.

Lipid was extracted from the larval samples by the method of Folch *et al.* (1957). Methyl esters were prepared by acid-catalysed transmethylation and separated by argentation TLC (see section 2.11.1).

Experiment VI

This experiment followed the same protocol as Experiment V except that:-

Larvae from the spring 1989 Baltic spawned stock (reared as in section 5.5.2) were used. Six hundred larvae aged 6-4 days post-hatch (still possessing yolk-sacs) were removed from the stock tank and transferred into the overnight clearing bath.

The following day, 500 larvae were removed from the clearing bath and transferred into 75 ml of seawater for incubation with the liposomes.

Three hundred and sixty live larvae were recovered after 24 h and transferred into the clearing bath.

One hundred larvae were transferred after 7 h into 7.5 ml of chloroform:methanol (2:1) and stored at -20°C for subsequent analysis. The remaining larvae (210) were transferred once more into 2 l of clean seawater, feed was not added. Further samples of 49 and 66 larvae were collected from this beaker after 17 h and 41 h and stored as above. The pulse chase was not run for as long as in Experiment V since rotifers were not available to feed the larvae and they were too small to take *Artemia*. Samples were analysed as in Experiment V.

5.9.2 Results

The radiolabelled 18:3(n-3) was 91% pure as described in section 5.4.2. As previously described, up to 3.5% radioactivity was recovered from the bands corresponding to :4, :5 and :6 unsaturated fatty acids. Bands 17 and 23 contained 1.62 and 1.16% of the total radioactivity in distinct spots, the identity of the fatty acids in these areas was not determined.

The results from Experiment V are detailed in Table V,xi and are summarised in Table V,xiii. The lipid content of the larvae did not fall appreciably over the 89 h pulse-chase but a decline in the amount of radioactivity present in the larvae did occur. The levels of radioactivity per larva indicated that 48.8% of the precursor fatty acid was oxidized over a period of 89 h. A comparison of the starting material with the argentation separation of methyl esters from 0, 41 and 89 h pulse-chase showed that saturates had increased from 0.03 to 3.32% but then declined to 2.62 and 1.2% respectively. The levels of labelled monoenes increased with time from 0.19% in the original 18:3(n-3) to 0.42, 0.61 and 1.57% after 0, 41 and 89 h pulse chase. The levels of labelled dienes increased slightly with time but this is probably not significant. At all times the majority of the radiolabel was recovered from the triene band but a decrease from 92% to 80% occurred over the 89 h pulse-chase. An increase of 3.27 to 8.27% occurred in :4, :5 and :6 fatty acids.

Results from Experiment VI are given in Table V,xii and are summarised in Table V,xiv. The lipid content of the larvae after 48 h pulse-chase was slightly lower than at the start of the experiment. The lipid content per larva was significantly less than from larvae in Experiment V due to their smaller size. As in Experiment V, the amount of radioactivity present in the larvae fell with time in the pulse-chase. The levels of radioactivity in the lipid indicated that 34.8% of the fatty acid was catabolized over a period of 41 h.

A comparison of the starting material with the argentation separation of lipid from 0, 17 and 41 h pulse-chase showed that labelled saturates had increased from 0.03 to 0.61, 2.5 and 3.51% respectively. The levels of labelled monoenes increased slightly with time from 0.19% in the original 18:3(n-3) to 0.34, 0.64 and 0.86% after 0, 17 and 41 h pulse chase.

The levels of dienes decreased slightly with time but this is probably not significant. At all times the majority of the radiolabel was recovered from the triene band but a small decrease from 92.07% to 87.26% occurred over the 47 h pulse-chase. A slight increase of 3.27 to 3.73% occurred in :4, :5 and :6 fatty acids.

5.9.3 Discussion

In Experiment V the relatively constant lipid content of the larvae over the 89 h pulse-chase indicated that the larvae had recommenced feeding.

The number of larvae in each sample was selected to ensure sufficient radioactivity was present to enable the analyses to be carried out. In the event, the levels of radioactivity were high enough that replicates of smaller samples could have been taken. However, the greater levels of radioactivity present ensured a higher probability of detecting molecules which had been elongated and desaturated (assuming they are in the minority).

The increase in labelled mono- and dienes was probably due recycling of [C^{14}]acetate derived from the labelled fatty-acid by β-oxidation.

It is hypothesised that elongation and desaturation of 18:3(n-3) would result in production of the PUFAs, 20:5(n-3) and 22:6(n-3). This is because the intermediate molecules in the conversion only occur in small amounts in herring larval lipid (for a discussion of the metabolic pathways involved, see Appendix I). Argentation TLC is not capable of detecting molecules which have only been elongated as it separates the fatty acids in terms of the number of double bonds contained in them. The increase in radiolabelled :4, :5 and :6 fatty acids with pulse-chase cannot be taken

as proof that elongation and desaturation of 18:3(n-3) had occurred due to the presence of radioactivity in these bands in the original 18:3(n-3). However, since the total reduction in activity in groups which did decrease was 11.05% over the 89 h pulse-chase, a percentage increase in all the other groups of fatty acids (:4, :5 and :6 fatty acids, monoenes, dienes and unidentified material) of 2.21% would occur assuming the levels of the other components remained static. The increase in labelled :4, :5 and :6 fatty acids was actually 4.25% so there is evidence that a low rate of elongation/desaturation had occurred. The end product may have been 18:4(n-3), as indicated by the results in Chapter 3.

In Experiment VI the Baltic larvae were not being fed and consequently a decrease in the lipid content of the larvae occurred over the pulse-chase. A consistent increase in labelled :4, :5 and :6 fatty acids did not occur during the pulse-chase. Thus the tentative conclusion from Experiment V could not be supported but it is probable that the negative results were due to the fact that the Baltic larvae were not actively growing.

Pulse-chase experiments using radiolabelled metabolic precursors are a powerful method of assessing the ability of an organism to modify the precursor. There is however, considerable difficulty in interpreting the results in terms of what constitutes an 'essential' nutrient. The capacity of the organism to modify a precursor can be affected by its previous dietary regime. Olsen *et al.* (1990) found that the degree of elongation and desaturation of radiolabelled 18:3(n-3) and 18:2(n-6) injected into adult *Tilapia* was related to the levels of long-chain PUFA in their diet. For a fish larva reared on a luxus of long-chain (n-3)PUFA, there is little reason to believe that it must, of necessity, be actively

synthesising these fatty acids by elongation and desaturation of dietary precursors. The enzymes involved may always be present but be inhibited by long-chain PUFA (Leger *et al.* 1981) or enzyme production may be induced by declining long-chain PUFA levels in the fish.

In order to test whether a nutrient is essential, the organism must be reared on a diet deficient in the nutrient. Thus, for studies on marine fish larvae, the use of pulse-chase experiments does not eliminate the need for a means of feeding the larvae defined diets over longer periods of time. In the strictest sense, the pulse-chase experiments can only confirm pathways indicated by longer term defined diet studies. The problem of designing inert diets with defined composition for marine fish larvae still remains (see Chapters 2 and 4).

It had been hoped to use three stocks of herring larvae reared on rotifers which had been fed yeast [low 20:5(n-3) and 22:6(n-3)], *Nannochloropsis oculata* [high 20:5(n-3) and low 22:6(n-3)] and *Pavlova lutheri* [high 20:5(n-3) and 22:6(n-3)](Scott & Middleton 1979). Cultures of these algae and rotifers were set up during the early spring of 1989. However, an accident in the aquarium (Plate 1) destroyed the algal stocks and closed a large section of the facility thus necessitating the scaling down of the experiment. A single stock of larvae were used in Experiment V and were reared on Great Salt Lake *Artemia*, whilst the Baltic larvae in Experiment VI were unfed (see 5.5.3). The fatty acid spectra for the *Artemia* used are given in section 5.2.2. This strain is relatively low in 20:5(n-3) and lacks 22:6(n-3). The results from Chapter 3 indicated that the level of 20:5(n-3) in this *Artemia* strain was insufficinet to maintain the levels of long-chain n-3PUFAs in the herring larvae. Thus if herring larvae are capable of elongating and desaturating 18:3(n-3) to

20:5(n-3) and 22:6(n-3) these activities should have been at a detectable level, at least in Experiment V.

5.9.4 Conclusions

The results demonstrated that the experimental method was successful for carrying out pulse chase experiments with marine fish larvae. In order to obtain unambiguous results, the purity of the radiolabelled 18:3(n-3) must be increased and the pulse-chase study should be run in conjunction with longer term rearing experiments using live diets with semi-defined fatty acid spectra. More rigorous analytical methods are required which are capable of measuring the radioactivity in identified fatty acids.

The results indicated that a high rate of conversion of 18:3(n-3) to longer chain, more unsaturated fatty acids does not occur in herring larvae, but that limited conversion may occur, probably to 18:4(n-3).

5.10 The elongation and desaturation of 20:5(n-3) by herring larvae

Whilst some work on the essentiality of 18:3(n-3) to marine fish is recorded in the literature, few studies have considered the role of 20:5(n-3) and whether it can be elongated and desaturated to 22:6(n-3). The results of rearing studies (Chapter 3) suggested that efficient elongation and desaturation of 20:5(n-3) does not occur. To investigate this further, two experiments were run using [1-C^{14}]20:5(n-3) as the labelled tracer.

5.10.1 Materials and methods

Experiment VII

Larvae from the spring 1989 Clyde spawned stock (reared as in section 5.2.2) were used in this experiment. Four hundred and fifty larvae aged 20-18 days post-hatch were removed from the stock tank and transferred into 2 l of 0.22 µm filtered seawater to which 0.1 g of penicillin G (Sigma) and 0.2 g of streptomycin sulphate (Sigma) had been added to reduce the levels of micro-organisms which might degrade the vesicles. Liposomes (20 mg) incorporating 22 µCi of [1-C^{14}]20:5(n-3) (Amersham CFA 654) were prepared and dialysed overnight as described in section 5.2.4. Filtered seawater (including antibiotics as before) was autoclaved and subsequently bubbled with air passed through a sterile 0.22 µm filter.

The following day 300 larvae were removed from the clearing bath and transferred into 75 ml of the aerated, autoclaved seawater in a small beaker. The liposomes were removed from dialysis and four 25 µl samples taken to determine their activity. The samples were mixed with 2.5 ml of HionicFluor (Packard) and the radioactivity determined by liquid scintillation counting. The liposomes were added to the beaker containing larvae giving a total volume of 90 ml. The beaker was then placed in the dark and aerated slowly with 0.22 µm filtered air.

After 24 h, live larvae (170) were recovered from the beaker containing liposomes and transferred into 2 l of clean 0.22 µm filtered seawater to allow the guts to clear. After 7 h, several larvae were checked microscopically to ensure that the guts were empty. Thirty larvae were transferred into 7.5 ml of chloroform:methanol (2:1) and stored at -20°C for subsequent analysis. The remaining larvae (130) were transferred

once more into 2 l of clean seawater and fed Great Salt Lake *Artemia* nauplii at a concentration of 2000 l^{-1}. Lighting was continuous. Further samples of larvae were collected from this beaker after 41 h and 89 h and stored as above.

Lipid was extracted from the larval samples by the method of Folch *et al.* (1957). Methyl esters were prepared from total lipid by acid-catalysed transmethylation and separated by packed column chromatography (see section 2.11.2).

Experiment VIII

This followed the same protocol as Experiment VII except the larvae were aged 26-24 days post-hatch. Out of the 300 larvae placed into the beaker containing liposomes only 83 survived. Thirty-eight of these were sampled after 7 h in clean seawater and the remaining 33 live larvae were sampled after 41 h in clean water with *Artemia*. The experiment was then terminated.

Lipids were extracted and analysed as described for Experiment VII.

5.10.2 Results

The results from Experiment VII are presented in Table V,xv. The number of larvae sampled, their lipid and [C^{14}] activities and the distribution of radiolabel amongst the fatty acids is given.

The lipid content $larva^{-1}$ was initially higher than in the Experiment V but the value after 89 h pulse-chase was similar. The levels of radioactivity $larva^{-1}$ in this experiment were 2184, 2999 and 1660 DPM after 0, 41 and 89 h pulse-chase respectively. The percentage of radiolabel recovered in 20:5(n-3) fell from 75.34 (original material) to

between 31.28 and 34.10% in incubated larvae. This decline was partly compensated for by an increase in the radioactivity recovered from shorter chain fatty-acids, particularly 14:0, 16:0 and 18:0 + 18:1 which accounted for between 15 and 23% of the activity recovered. The levels of radioactivity recovered from the C22PUFAs were similar to those in the original 20:5(n-3) except for 41 h pulse chase when they were higher. A noticeable increase in activity in 22:1 appeared to have occurred. After 41 and 89 h pulse chase the level of radioactivity in 22:5(n-3) had increased from 6.15% to 10.25%. Only a small increase in labelled 22:6(n-3) occurred [1.25% in the original 20:5(n-3) to 1.47, 3.34 and 1.97% after 0, 41 and 89 h pulse-chase respectively].

The results from Experiment VIII are presented in Table V,xvi. They were similar to those from Experiment VII except that the percentage activity recovered from 22:5(n-3) after 41 h pulse-chase was less than in the original radiotracer. The increase in activity in C22PUFAs was less marked than in the previous experiment but that from 22:1 was greater.

The lipid content of the larvae increased slightly during the pulse-chase. The radioactivity larva^{-1} was less after 41 h pulse-chase than at 0 h pulse-chase. The activity recovered from 20:5(n-3) fell from 75.34 (original material) to 33.11 and 46.75% in incubated larvae. A consistent decrease with time did not occur as in the experiments using [1-C^{14}]18:3(n-3). The decline in activity recovered from 20:5(n-3) was again partly accounted for by increases in the levels of labelled shorter chain fatty-acids, in particular 14:0, 16:0 and 18:0 + 18:1 which accounted for between 13 and 16% of the activity recovered. The levels of radioactivity in the saturates 14:0 and 16:0 was very similar to that in lipid recovered from liposome suspensions after 24 h immersion in seawater (see section 5.4). However, liposomes recovered from seawater

did not contain appreciable amounts of radiolabelled 18:0 and 18:1. The levels of radioactivity recovered from 22:6(n-3) after 41 h pulse-chase (2.66%) were slightly higher than in the starting material (1.25%)

5.10.3 Discussion

The decrease in total radioactivity larva^{-1} in Experiment VII was not consistent with time as in the pulse-chase experiments using [1-C^{14}]18:3(n-3). This implies that the assimilation of the labelled fatty acid from the liposomes was not homogenous i.e. some larvae took up more label than others. The small numbers of larvae in the samples may have exacerbated the heterogenous distribution. The catabolism of 20:5(n-3) was considerably greater than for 18:3(n-3) over a comparable time period. This result was unexpected since it was assumed that the longer chain PUFA would be retained more tenaciously.

The small increase in activity in 22:6(n-3) cannot be taken as definite proof of its synthesis. Since activity was present in 22:6(n-3) as a contaminant in the starting material, the percentage changes could have been generated by the large decreases which occurred in 20:5(n-3).

The levels of radioactivity in the saturates 14:0 and 16:0 were very similar to that in lipid recovered from liposome suspensions after 24 h immersion in seawater (see section 5.4) and could have derived from this source. However, liposomes recovered from seawater did not contain appreciable amounts of radiolabelled 18:0 and 18:1. The activity in these fatty acids recovered from larvae is presumably due to recycling of [C^{14}]acetate derived from the labelled 20:5(n-3) by ß-oxidation. It must also be remembered that chain shortening and saturation of fatty acids, although not recorded in the literature, might occur.

The counts recorded in 22:1 must be regarded cautiously since the gas chromatograph peak for this fatty acid was close to that of 20:5(n-3) and carry-over of radioactivity may have occurred. High levels of 22:1 were presumably derived from the elongation of radiolabelled 18:1 which itself would have derived from recycling of [C^{14}]acetate. The appearance of large amounts of 22:1 is unusual since this fatty acid normally occurs in small amounts in fish tissue. It is present in large amounts in copepods and is normally catabolized via mitochondrial ß-oxidation by herring (Henderson & Sargent 1985).

The trends observed in Experiment VIII tended to confirm those from Experiment VII except that an increase in activity in 22:5(n-3) was not observed.

Larval survival in these two experiments, and especially in Experiment VIII was poor. It does appear that older, larger larvae are more delicate and must be handled with greater care. However, those in Experiment VII were the same age as those in the initial [1-C^{14}]18:3(n-3) pulse-chase, which survived well.

5.10.4 Conclusions

In order to obtain unambiguous results the purity of the radiolabelled 20:5(n-3) must be increased and the pulse-chase study should be run in conjunction with longer term rearing experiments using defined live diets.

The results indicated that conversion of 20:5(n-3) to longer chain, more unsaturated fatty acids does occur in herring larvae but that the rate is low. There was evidence that elongation to 22:5(n-3) occurs and it is possible that it is low activity of delta-4 desaturase which is limits

conversion of this fatty acid to 22:6(n-3). These results agree with those obtained from the rearing study using live diets described in Chapter 3.

5.11 Overall conclusions to Chapter 5

Measurements of the uptake of liposomes by herring larvae from seawater suspensions indicated that active mechanisms were involved as the rate of uptake was far in excess of that explicable by passive accumulation associated with drinking to replace water loss by osmosis.

There was a rapid assimilation of radiolabelled fatty acid from the liposomes into the body tissues and thence into the body lipids. Low levels of activity were recovered from the free fatty fraction of larval lipids whilst high levels were recovered from other classes. Fatty acids were probably esterified into lipid classes in a non-preferential manner but the abundance of phospholipids in the lipid pool led to high levels of activity in these lipids.

The use of liposomes as a vector for the incorporation of radiolabelled fatty acids into herring larvae in pulse-chase experiments was demonstrated.

Pulse-chase experiments with [1-C^{14}]18:3(n-3) indicated that a high rate of elongation and desaturation of this fatty acid does not occur in herring larvae but limited conversion, probably to 18:4(n-3) can occur.

Pulse-chase experiments with [1-C^{14}]20:5(n-3) indicated that elongation of this fatty acid can occur in herring larvae but that delta-4 desaturase activity is limited. The rate of conversion is probably too low to be of nutritional importance.

Table V,i Fatty acid profile of egg-yolk phosphatidylcholine

Analysis of Sigma P6013.

Fatty acid	%
14:0	0.1
16:0	32.1
16:1	2.4
16:3	0.1
18:0	1.9
18:1 n-9	31.9
18:2 n-6	17.3
20:2 n-6	0.3
20:3 n-6	0.1
20:4 n-6	3.0
22:6 n-3	0.5
Unidentified	0.3

Table V,ii Stability of [1-C^{14}]18:3(n-3) incorporated into liposomes

Radioactivity located in 0.5 cm bands of methyl-esters separated by degree of unsaturation using argentation thin-layer chromatography. Counts are expressed in terms of percentage of total DPM after correction for quench and background.
The separation of polyunsaturated methyl-esters with more than 3 double bonds was poor. Thus :4, :5 and :6 fractions are treated as a single band.

Band	No. of double bonds	Initial 18:3(n-3) a	Initial 18:3(n-3) b	Lipid recovered after 24 h in seawater c	Lipid recovered after 24 h in seawater d
1	:0	0.07	0.01	0.04	0.03
2		0.05	0.02	0.04	0.04
3		0.05	0.02	0.06	0.05
4		0.06	0.03	0.09	0.05
5	:1	0.15	0.05	0.11	0.17
6	:1	0.07	0.02	0.17	0.16
7	:1	0.08	0.06	0.07	0.08
8	:1	0.09	0.11	0.10	0.08
9		0.09	0.04	0.11	0.17
10	:2	0.14	0.05	0.31	0.19
11	:2	0.11	0.07	0.21	0.02
12	:2	0.15	0.04	0.39	0.34
13	:2	0.12	0.04	0.35	0.27
14	:2	0.22	0.17	1.08	0.78
15		0.24	0.77	0.55	0.44
16		0.10	0.15	0.30	0.29
17		0.19	0.32	0.40	0.32
18		0.20	0.01	0.41	0.30
19		0.19	0.07	0.37	0.27
20		0.16	0.16	0.83	0.47
21		1.47	1.61	2.01	2.26
22	:3	2.96	1.16	3.52	3.28
23	:3	90.95	90.83	83.83	85.81
24		1.35	0.92	1.78	1.53
25	:4	0.48	0.64	1.00	0.94
26	:5	0.09	0.41	0.34	0.27
27	:6	0.07	0.90	0.28	0.22
28	:4	0.02	0.65	0.41	0.38
29	:5	0.02	0.16	0.48	0.33
30	:6	0.01	0.23	0.18	0.15
31	:4	0.02	0.09	0.07	0.06
32	:5	0.01	0.04	0.03	0.03
33	Origin	0.02	0.15	0.08	0.22

Table V,iii Summary of results of the determination of the stability of [1-C^{14}]18:3(n-3) incorporated into liposomes

The values given in this table were derived by summation of the percentage radioactivity recovered from bands as given in Table V,ii. The separation of polyunsaturated methly-esters with more than 3 double bonds was poor so :4, :5 and :6 fractions are treated as a single group.

No. of double bonds	Initial 18:3(n-3)		Lipid recovered after 24 h in seawater	
	a	b	c	d
:0	0.07	0.01	0.04	0.03
:1	0.39	0.24	0.45	0.49
:2	0.74	0.37	2.34	1.60
:3	93.91	91.99	87.35	89.09
:4 :5 :6	0.72	3.12	2.79	2.38
Origin	0.02	0.15	0.08	0.22
Unidentified	4.15	4.12	6.95	6.19

Table V,iv Stability of [1-C^{14}]20:5(n-3) incorporated into liposomes

The distribution of radioactivity in the fatty acids was determined by a Pye-104 packed column gas chromatograph.
Counts are expressed in terms of percentage of total DPM after correction for quench and background.
] = Failure to resolve the fatty acids indicated

Fatty acid	Initial 20:5(n-3)		Lipid recovered after 24 h in seawater	
	e	f	g	h
14:0	-	-	3.81	4.19
16:0	-	-	2.45	2.54
16:1	-	-	] 0.16	-
16:2	] 0.23	-	-	0.52
16:3	-	-	-	0.32
16:4	-	-	] 0.56	] 1.02
18:0	-	-	-	0.12
18:1	-	-	-	1.32
18:2	-	-	2.37	0.12
18:3(n-3)	-	-	0.72	1.17
18:4(n-3)	-	-	0.55	1.24
20:1	-	1.20	74.94	63.05
20:4(n-6)	60.48	75.34	0.49	8.07
20:5(n-3)	25.57	9.05	7.70	8.71
22:1	7.02	7.01	5.19	6.17
22 PUFAs	] 6.70	6.15	1.06	1.44
22:5(n-3)		1.25		
22:6(n-3)				

Table V,v Experiment I - Uptake of liposomes containing [1-C^{14}]18:3(n-3) compared with drinking rate in 17-15 days post-hatch Clyde herring larvae

Liposome uptake				Water uptake			
Nominal salinity (ppt)	Osmolarity (mosmol)	Time (h)	Water imbibed equivalent (µl $larva^{-1}$)	Osmolarity (mosmol)	Time (h)	Water uptake (µl $larva^{-1}$) mean ± sd	
17	551	1	2.7	492	1	0.009	0.003
		2	5.3		2	0.012	0.003
		3	7.4		3	0.022	0.014
		4	11.5		4	0.045	0.014
		5	12.7		5	0.038	0.009
34	967	1	4.5	969	1	0.016	0.003
		2	7.6		2	0.041	0.020
		3	10.9		3	0.057	0.010
		4	13.2		4	0.074	0.019
		5	16.7		5	0.073	0.016

Table V,vi Experiment II - Uptake of liposomes containing [1-C^{14}]18:3(n-3) compared with drinking rate of 8-6 days post-hatch Baltic herring larvae

Liposome uptake				Water uptake			
Nominal salinity (ppt)	Osmolarity (mosmol)	Time (h)	Water imbibed equivalent (µl $larva^{-1}$)	Osmolarity (mosmol)	Time (h)	Water uptake (µl $larva^{-1}$) mean ± sd	
17	493	0.5	0.51	493	0.5	0.002	0.001
		1	1.03		1	0.009	0.005
		2	1.78		2	0.025	0.007
		3	2.98		3	0.017	0.003
		5	4.58		5	0.032	0.006
34	960	0.5	0.62	960	0.5	0.004	0.001
		1	1.16		1	0.008	0.002
		2	2.20		2	0.020	0.004
		3	3.09		3	0.027	0.004
		5	4.37		5	0.039	0.003

Table V,vii Experiment I - Morphometric data for larval stock, water imbibed equivalents, water uptake rates and relative rates

Standard lengths and dry weights of larvae

	Std. length (mm)	Dry wt (mg)	Number in sample
Median	13.0	0.305	30
Upper quartile	13.6	0.418	
Lower quartile	12.1	0.225	

Linear regression equations for liposome uptake (water imbibed equivalent) and drinking in various salinities

Nominal salinity (ppt)	Water imbibed equivalent	r^2	Drinking rate	r^2
17	$y = 0.06 + 2.62x$	0.980	$y = -0.000674 + 0.00882x$	0.576
34	$y = 1.58 + 3.00x$	0.997	$y = 0.00929 + 0.0143x$	0.655

True and relative liposome uptake rates (water imbibed equivalents) and drinking rates at various salinities

Nominal salinity (ppt)	Water imbibed equivalent rate ($\mu l\ larva^{-1}\ hr^{-1}$) ± std. error	Relative water imbibed equivalent ($\mu l\ mg^{-1}\ hr^{-1}$)
17	2.62 ± 0.22	8.59
34	3.00 ± 0.09	9.84

Nominal salinity (ppt)	Drinking rate ($\mu l\ larva^{-1}\ hr^{-1}$) ± std. error	Relative drinking rate ($\mu l\ mg^{-1}\ hr^{-1}$)
17	0.00882 ± 0.00184	0.0289
34	0.0143 ± 0.00260	0.0469

Table V,viii Experiment II - Morphometric data for larval stock, water imbibed equivalents, water uptake rates and relative rates

Standard lengths and dry weights of larvae

	Std. length (mm)	Dry wt (mg)	Number in sample
Median	8.2	a	20
Upper quartile	8.4	a	
Lower quartile	8.0	a	

Linear regression equations for liposome uptake (water imbibed equivalent) and drinking in various salinities

Nominal salinity (ppt)	Water imbibed equivalent	r^2	Drinking rate	r^2
17	y = 0.08 + 0.91x	0.995	y = 0.000142 + 0.00625x	0.884
34	y = 0.36 + 0.83x	0.985	y = 0.00153 + 0.00785x	0.949

True and relative liposome uptake rates (water imbibed equivalents) and drinking rates at various salinities

Nominal salinity (ppt)	Water imbibed equivalent rate ($\mu l\ larva^{-1}\ hr^{-1}$) ± std. error	Relative water imbibed equivalent ($\mu l\ mg^{-1}\ hr^{-1}$)
17	0.91 ± 0.03	a
34	0.83 ± 0.06	a

Nominal salinity (ppt)	Drinking rate ($\mu l\ larva^{-1}\ hr^{-1}$) ± std. error	Relative drinking rate ($\mu l\ mg^{-1}\ hr^{-1}$)
17	0.00625 ± 0.000605	a
34	0.00785 ± 0.000429	a

a Dry weights were not determined for these larvae as they possessed yolk-sacs which would distort the relative results.

Table V,ix Experiment III - Incorporation of radioactivity by 12-10 days post-hatch autumn spawned herring larvae incubated in a suspension of liposomes containing [1-C^{14}]18:3(n-3)

Specific activity of bathing solution = 228,148 DPM ml^{-1}

Sample	Time in[a] liposomes (h)	Larvae in sample	Lipid content (µg $larva^{-1}$)	Counts (DPM $larva^{-1}$)	Water imbibed equivalent (µl $larva^{-1}$)
1	7	75	18.1	770	3.4
2	14	75	18.5	1613	7.1
3	21	77	17.7	2573	11.3
4	28	85	14.7	3153	13.8
5	35	75	15.2	4579	20.1

Linear regression for water imbibed equivalents

$y = -0.87 + 0.57x$ $r^2 = 0.98$

Incorporation of radioactivity into lipid classes DPM $larva^{-1}$

Time in liposomes (h)	7	14	21	28	32
Class					
Sterol/Wax esters	5	10	14	16	23
Triacylglycerol	56	97	124	135	165
Free fatty acids[b]	18	83	113	158	235
Cholesterol	55	85	132	158	190
Phosphatidic acid	69	171	226	394	302
Phosphatidylethanolamine	103	256	292	195	563
Phosphatidylinositol	42	145	252	259	307
Phosphatidylserine	59	84	144	139	298
Phosphatidylcholine[c]	363	682	1276	1699	2496
Total neutrals	79	190	251	309	423
Total polars	691	1423	2322	2844	4156

Incorporation as percentage of total counts per sample

Time in liposomes (h)	7	14	21	28	32	Std[d]
Class						
Sterol/Wax esters	0.6	0.6	0.5	0.5	0.5	2.0
Triacylglycerol	7.3	6.0	4.8	4.3	3.6	14.3
Free fatty acidsa[b]	2.3	5.1	4.4	5.0	5.1	2.0
Cholesterol	7.1	5.3	5.1	5.0	4.2	-
Phosphatidic acid	9.0	10.6	8.8	12.5	6.6	-
Phosphatidylethanolamine	13.4	15.9	11.3	6.2	12.3	19.5
Phosphatidic acid	5.5	9.0	9.8	8.2	6.7	] 4.9[e]
Phosphatidylserine	7.7	5.2	5.6	4.4	6.5	
Phosphatidylcholine[c]	47.0	42.2	49.6	53.9	54.5	56.3
Total neutrals	10.2	11.7	9.7	9.8	9.2	18.3
Total polars	89.7	88.2	90.2	90.2	90.8	80.7

a 'Time in liposomes' does not include 7 h in clean water between removal of larvae from liposomes and transfer into chloroform:methanol (2:1)

b This class resolved as several bands on autoradiograph.

c Sphingomyelin failed to resolve from phosphatidylcholine.

d Typical lipid class composition (as % total lipid) for well nourished, 9-12 days post-hatch, spring spawned herring larvae, derived from Fraser *et al.* (1987) and corrected to exclude sterols.

e Phosphatidylinositol and phosphatidylserine were not separated in the analysis of Fraser *et al.* (1987)

Table V,x Experiment IV - Incorporation of radioactivity by 34-32 days post-hatch autumn spawned herring larvae incubated in a suspension of liposomes containing [1-C^{14}]18:3(n-3)

Specific activity of bathing solution = 168,838 DPM ml^{-1}

Sample	Time in[a] liposomes (h)	Larvae in sample	Lipid content (μg $larva^{-1}$)	Counts (DPM $larva^{-1}$)	Water imbibed equivalent (μl $larva^{-1}$)
1	7	48	50.8	2322	13.8
2	14	43	44.2	3210	19.0
3	28	45	48.9	5124	30.4

Linear regression for water imbibed equivalents

$y = 5.56 + 0.98x$ $\quad r^2 = 0.81$

Incorporation of radioactivity into lipid classes DPM $larva^{-1}$

Time in liposomes (hrs)	7	14	28
Class			
Sterol/Wax esters	12	25	52
Triacylglycerol	185	197	366
Free fatty acids[b]	150	174	410
Cholesterol	13	10	21
Phosphatidic acid	8	22	23
Phosphatidylethanolamine	237	368	627
Phosphatidylinositol	68	127	158
Phosphatidylserine	39	64	72
Phosphatidylcholine	1593	2203	3375
Sphingomyelin	17	20	20
Total neutrals	347	396	828
Total polars	1975	2814	4296

Incorporation as percentage of total counts per sample

Time in liposomes (h)	7	14	28	Std[c]
Class				
Sterol/Wax esters	0.5	0.8	1.0	3.4
Triacylglycerol	8.0	6.1	7.1	17.0
Free fatty acids[b]	6.5	5.4	8.0	2.6
Cholesterol	0.6	0.3	0.4	-
Phosphatidic acid	0.3	0.7	0.4	-
Phosphatidylethanolamine	10.2	11.5	12.2	22.8
Phosphatidylinositol	2.9	4.0	3.1	] 4.9
Phosphatidylserine	1.7	2.0	1.4	
Phosphatidylcholine	68.6	68.6	65.9	46.3
Sphingomyelin	0.7	0.6	0.4	3.0
Total neutrals	15.0	12.3	16.1	23.0
Total polars	85.0	87.7	83.9	75.2

a 'Time in liposomes' does not include 7 h in clean seawater between removal of larvae from liposomes and transfer into chloroform:methanol (2:1).

b This class resolved as several bands on autoradiograph.

c Typical lipid class composition (as % total lipid) for well nourished, 36-41 days post-hatch, spring spawned herring larvae, derived from Fraser *et al.* (1987) and corrected to exclude sterols.

Table V,xi Experiment V - Elongation/desaturation of [1-C^{14}]18:3(n-3) by 18-20 days post-hatch spring spawned Clyde herring larvae in a pulse-chase experiment

Pulse time in liposomes (h)	Time in clean water (h)	Chase time (h)	Number of larvae	Lipid content (µg $larva^{-1}$)	Radioactivity (DPM $larva^{-1}$)
24	7	0	50	31.2	8438
24	7	41	50	28.0	6541
24	7	89	96	30.9	4319

Counts located in 0.5 cm bands scrapped from a plate carrying radiolabelled methyl-esters separated by degree of unsaturation using argentation TLC.
Counts are expressed in terms of percentage of total DPM after correction for quench and background.
The bands containing fatty acids were identified by comparison with non-radioactive standards run alonside.

The separation of polyunsaturated methyl-esters with more than 3 double bonds was poor. Thus :4, :5, and :6 fractions are treated as a single band.

Band	No. of double bonds	Original 18:3(n-3)	Chase time (h) 0	41	89
1		0.01	0.02	0.05	0.02
2	:0	0.01	0.35	1.01	0.25
3	:0	0.02	1.97	1.61	0.95
4		0.03	0.29	0.32	1.21
5		0.04	0.07	0.10	1.24
6	:1	0.03	0.12	0.06	0.53
7	:1	0.11	0.21	0.18	0.41
8	:1	0.05	0.09	0.37	0.63
9		0.03	0.04	0.31	0.25
10		0.04	0.04	0.07	0.15
11	:2	0.06	0.13	0.05	0.20
12	:2	0.05	0.06	0.11	0.05
13	:2	0.07	0.03	0.11	0.06
14		0.06	0.04	0.04	0.10
15		0.12	0.05	0.06	0.10
16		0.23	0.20	0.08	0.06
17		1.62	1.32	0.99	1.30
18		0.76	0.68	0.96	1.06
19	:3	1.01	9.35	2.97	20.93
20	:3	0.35	50.00	41.70	32.35
21	:3	41.82	26.25	33.39	19.35
22	:3	48.89	3.49	8.20	7.47
23		1.16	0.92	0.94	2.78
24	:4	0.62	1.06	0.80	0.89
25	:5	0.39	0.40	1.99	1.40
26	:6	0.23	0.52	0.04	2.71
27	:4	0.41	0.94	0.72	1.32
28	:5	0.93	0.58	0.54	0.87
29	:6	0.35	0.20	0.81	0.68
30	:4	0.13	0.18	0.43	0.20
31	:5	0.14	0.09	0.20	0.18
32	:6	0.07	0.05	0.16	0.02
33	Origin	0.16	0.25	0.26	0.01

Table V,xii Experiment VI - Elongation/desaturation of [1-C^{14}]18:3(n-3) by 5-7 days post-hatch Baltic herring larvae

Pulse time in liposomes (h)	Time in clean water (h)	Pulse chase time (h)	Number of larvae	Lipid content (µg $larva^{-1}$)	Radioactivity (DPM $larva^{-1}$)
24	7	0	100	15.3	4893
24	7	17	49	16.5	3640
24	7	41	56	14.6	3188

Counts located in 0.5 cm bands scrapped from a plate carrying radiolabelled methyl-esters separated by degree of unsaturation using argentation TLC.
Counts are expressed in terms of percentage of total DPM after correction for quench and background.
The bands containing fatty acids were identified by comparison with non-radioactive standards run alongside.

The separation of polyunsaturated methyl-esters with more than 3 double bonds was poor. Thus :4, :5, and :6 fractions are treated as a single band.

Band	No. of double bonds	Original 18:3(n-3)	Chase time (h) 0	17	47
1		0.01	0.02	0.04	0.04
2	:0	0.01	0.03	1.46	2.14
3	:0	0.02	0.58	1.04	1.37
4		0.03	1.07	0.08	0.12
5		0.04	0.33	0.11	0.13
6	:1	0.03	0.05	0.17	0.19
7	:1	0.11	0.08	0.29	0.36
8	:1	0.05	0.21	0.18	0.31
9		0.03	0.26	0.05	0.06
10		0.04	0.09	0.04	0.05
11	:2	0.06	0.07	0.12	0.15
12	:2	0.05	0.17	0.09	0.08
13	:2	0.07	0.07	0.04	0.03
14		0.06	0.06	0.04	0.04
15		0.12	0.09	0.08	0.07
16		0.23	0.46	0.16	0.13
17		1.62	1.38	1.32	1.25
18		0.76	0.62	0.63	0.72
19	:3	1.01	0.67	0.53	0.56
20	:3	0.35	19.52	1.29	1.59
21	:3	41.82	60.26	76.97	72.50
22	:3	48.89	8.99	10.31	12.61
23		1.16	0.95	1.02	1.41
24	:4	0.62	0.28	0.79	1.06
25	:5	0.39	0.52	0.26	0.30
26	:6	0.23	0.41	0.43	0.50
27	:4	0.41	0.44	0.33	0.50
28	:5	0.93	1.24	0.71	0.60
29	:6	0.35	0.30	0.30	0.23
30	:4	0.13	0.19	0.25	0.31
31	:5	0.14	0.09	0.31	0.12
32	:6	0.07	0.09	0.12	0.11
33	Origin	0.16	0.43	0.45	0.35

Table V,xiii Experiment V - Summary of the results of elongation/desaturation of [1-C^{14}]18:3(n-3) by 20-18 days post-hatch spring spawned Clyde herring larvae in a pulse-chase experiment

The values given in this table were derived by summation of the percentage of radioactivity recovered from bands as given in Table V,x.

No. of double bonds	Original 18:3(n-3)	Chase time (h) 0	41	89
:0	0.03	3.32	2.62	1.20
:1	0.19	0.42	0.61	1.57
:2	0.18	0.22	0.27	0.31
:3	92.07	89.03	86.26	80.10
:4 :5 :6	3.27	4.02	5.69	8.27
Unidentified	4.26	2.99	4.55	8.55

Table V,xiv Experiment VI - Summary of the results of elongation/desaturation of [1-C^{14}]18:3(n-3) by 7-5 days post-hatch spring spawned Baltic herring larvae in a pulse-chase experiment

The values given in this table were derived by summation of the percentage of radioactivity recovered from bands as given in Table V,xi.

No. of double bonds	Original 18:3(n-3)	Chase time (h) 0	17	47
:0	0.03	0.61	2.50	3.51
:1	0.19	0.34	0.64	0.86
:2	0.18	0.31	0.25	0.26
:3	92.07	89.44	89.10	87.26
:4 :5 :6	3.27	4.23	3.50	3.73
Unidentified	4.26	5.07	4.01	4.38

Table V,xv Experiment VII - Elongation/desaturation of [1-C^{14}]20:5(n-3) by 20-18 days post-hatch spring spawned Clyde herring larvae in a pulse-chase experiment

Time in liposomes (h)	Time in clean water (h)	Pulse chase time (h)	Number of larvae	Lipid content (µg $larva^{-1}$)	Radioactivity (DPM $larva^{-1}$)
24	7	0	30	41.3	2184
24	7	41	30	44.7	2999
24	7	89	29	31.4	1660

The percentage distribution of radioactivity in methyl-esters separated on a Pye-104 packed column gas chromatograph.

Fatty acid	Original 20:5(n-3)	Pulse chase time (h) 0	41	89
14:0	-	4.15	2.99	1.91
16:0	-	5.60	4.58	] 5.44
16:1	-	1.39	1.18	
16:2	-	0.29	0.49	0.61
16:3	-	0.50	] 0.67	0.13
16:4	-	0.52		0.45
18:0	-	] 7.18	] 9.38	] 16.03
18:1	-	0.52	1.36	0.41
18:2	-	2.48	2.15	3.34
18:3(n-3)	-	1.62	1.73	1.21
18:4(n-3)	-	2.80	] 3.50	2.41
20:1	-	1.14		1.45
20:4(n-6)	1.20	43.07	31.28	34.10
20:5(n-3)	75.34	14.55	14.48	10.20
22:1	6.87	7.58	12.62	6.39
22 PUFAs	7.01	5.14	10.25	10.25
22:5(n-3)	6.15	1.47	3.34	1.97
22:6(n-3)	1.25			
Total C22PUFAs	14.41	15.69	26.21	18.67

Table V,xvi Experiment VIII - Elongation/desaturation of [1-C^{14}]20:5(n-3) by 27-25 days post-hatch spring spawned Clyde herring larvae in a pulse-chase experiment

Time in liposomes (h)	Time in clean water (h)	Pulse chase time (h)	Number of larvae	Lipid content (µg $larva^{-1}$)	Radioactivity (DPM $larva^{-1}$)
24	7	0	38	36.8	1911
24	7	41	38	38.5	1886

The percentage distribution of radioactivity in methyl-esters separated on a Pye-104 packed column gas chromatograph.

Fatty acid	Original 20:5(n-3)	Pulse chase time (h) 0	41
14:0	-	1.20	2.20
16:0	-	5.32	3.31
16:1	-	0.60	0.28
16:2	-	0.84	-
16:3	-	0.27	-
16:4	-	0.52	0.57
18:0	-	] 9.36	] 7.66
18:1	-		
18:2	-	1.74	1.01
18:3(n-3)	-	2.83	2.75
18:4(n-3)	-	2.22	1.74
20:1	-	3.32	1.17
20:4(n-6)	1.20	1.88	1.26
20:5(n-3)	75.34	33.11	46.75
22:1	6.87	16.93	16.04
22 PUFAs	7.01	8.77	9.36
22:5(n-3)	6.15	7.62	4.18
22:6(n-3)	1.25	2.54	2.66
Total C22PUFAs	14.41	18.93	16.20

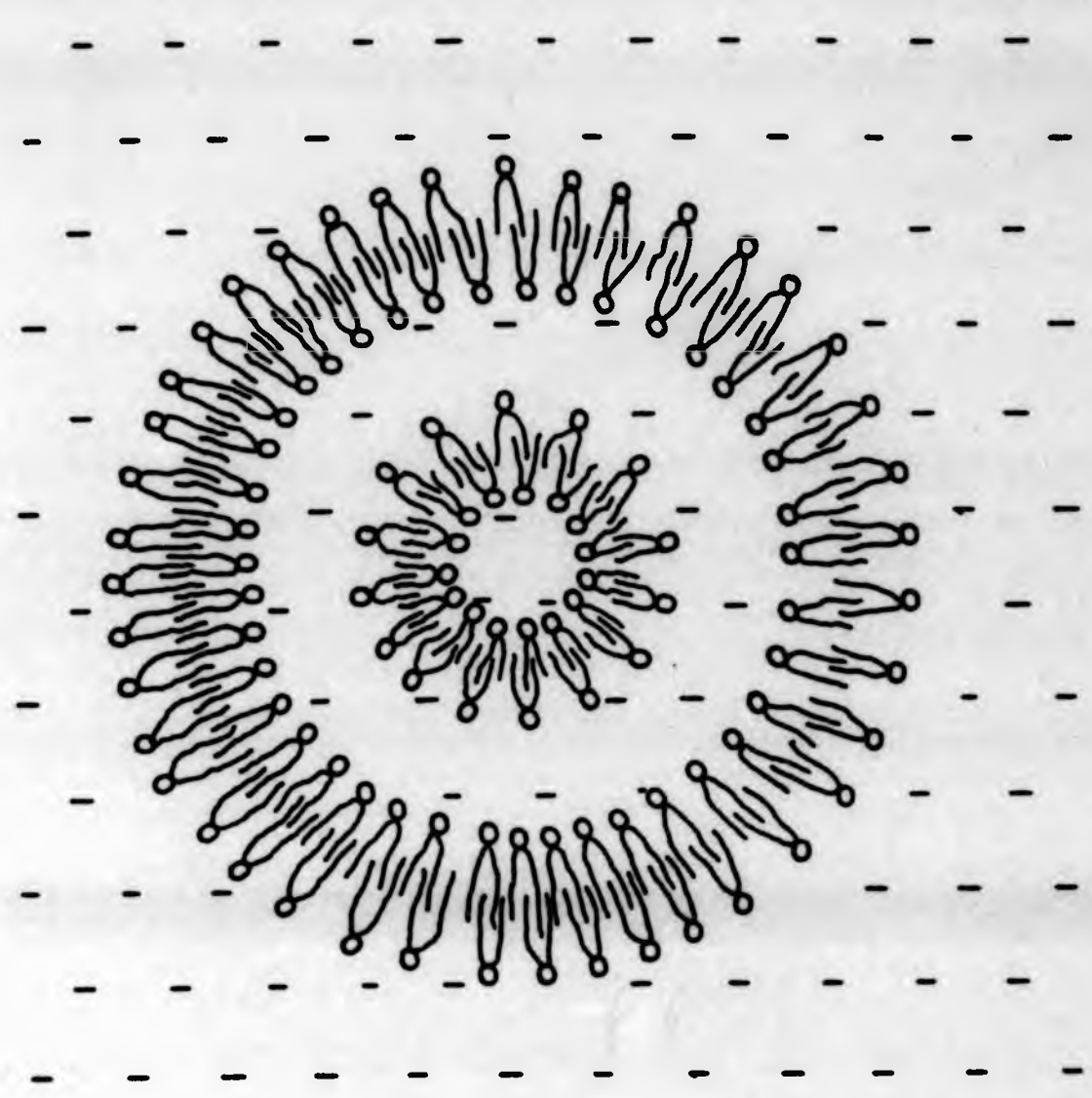


Fig 5-1 Structure of a multi-lamellar vesicle (liposome)

Phospholipid forms the main component of the liposome wall. Cholesterol and other charged lipids are included to aid separation of the walls, these molecules are not shown in the diagram

Phospholipid molecule

Water and aqueous soluble material

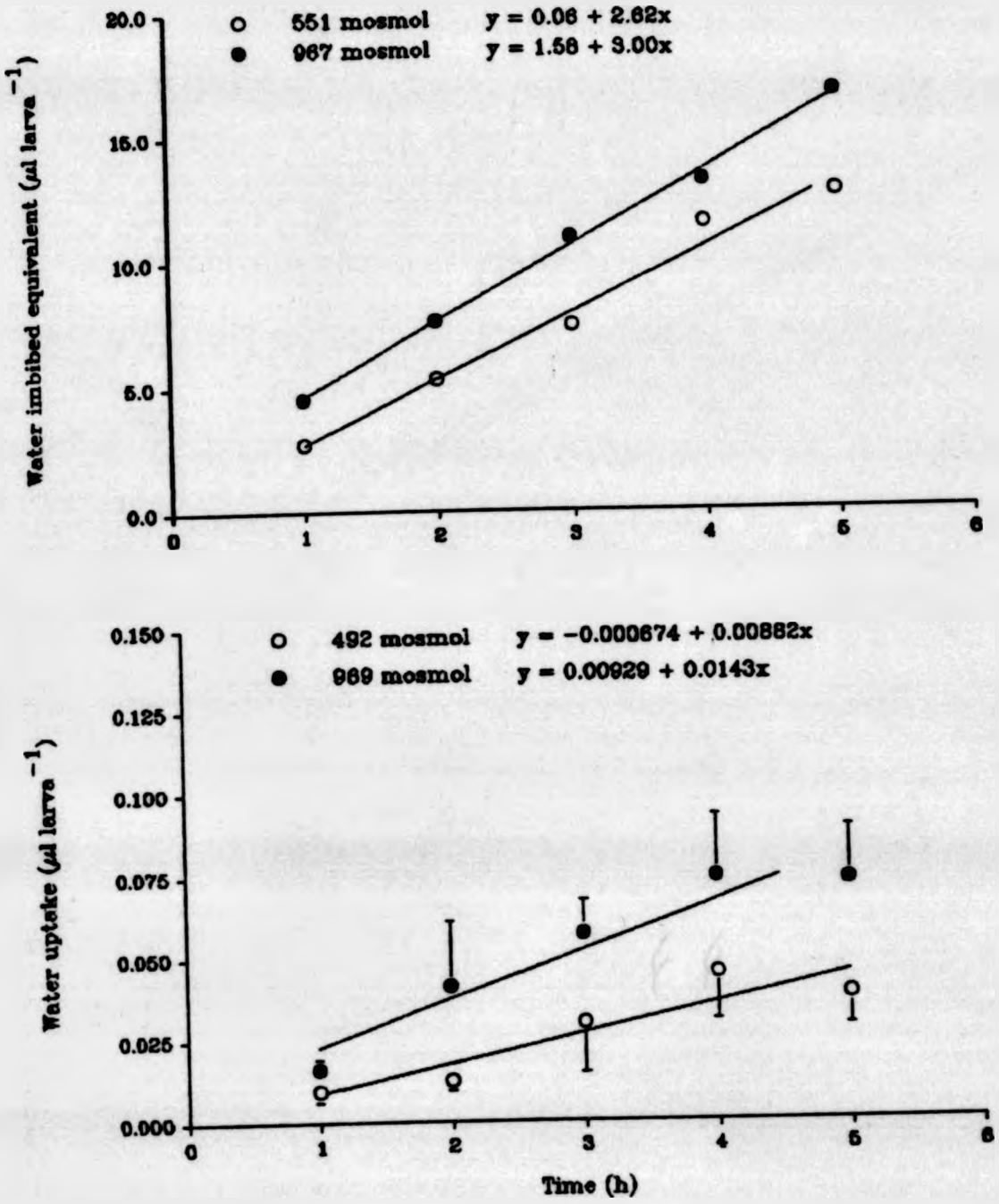


Fig 6–2 **Experiment I**
Plots of water imbibed equivalents and water uptake values with time for 16–17 days post–hatch Clyde herring larvae

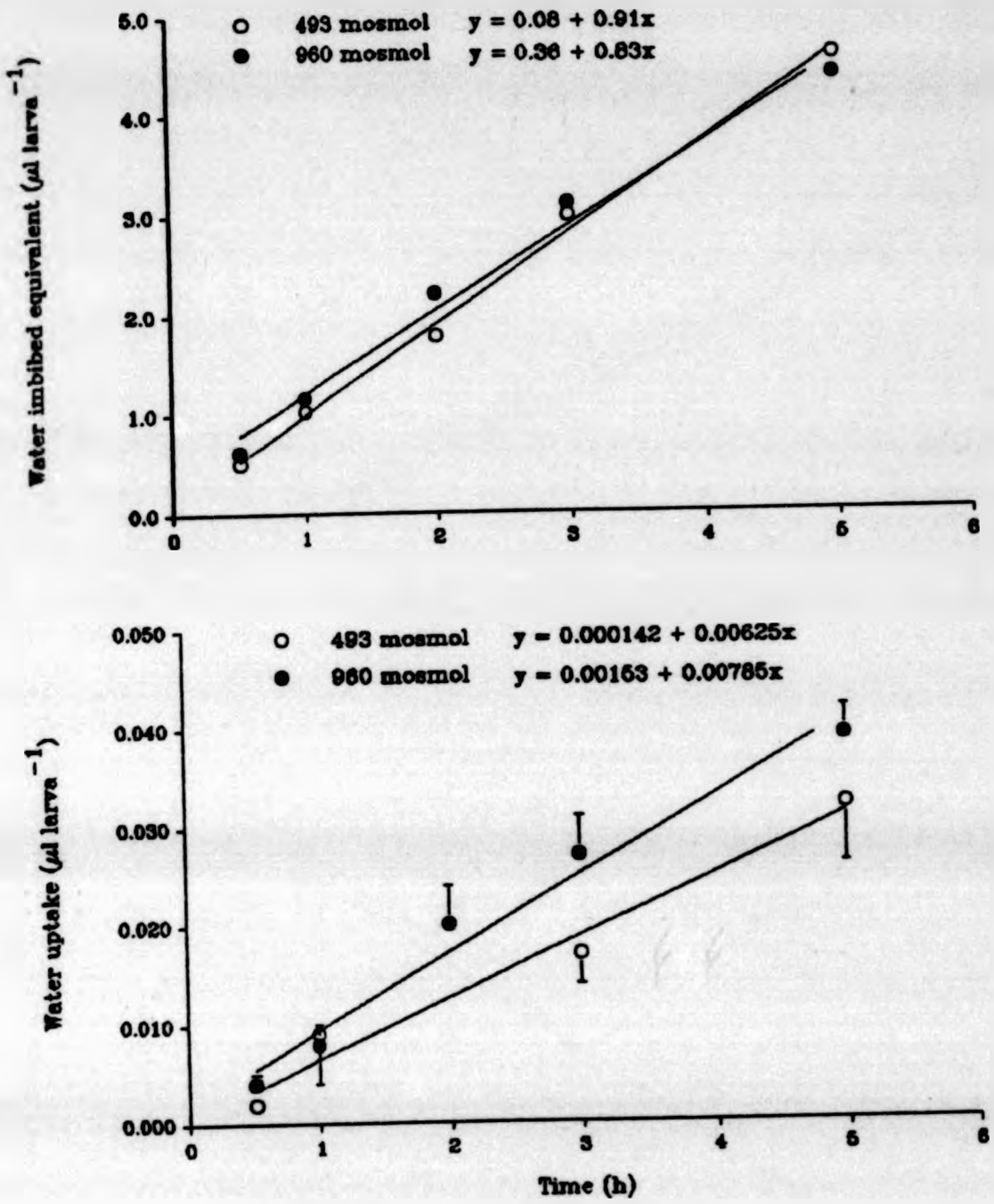


Fig 5-3 Experiment II
Plots of water imbibed equivalents and water uptake values with time for 5-8 days post-hatch Baltic herring larvae

Fig 5-4 Experiment III - Plots of the incorporation of radioactivity from [1-C^{14}]18:3(n-3) incorporated into liposomes into the lipid classes of 17-15 days post-hatch herring larvae

The radioactivity incorporated from the liposomes into the lipid classes is shown in absolute terms as disintegrations per min (DPM)

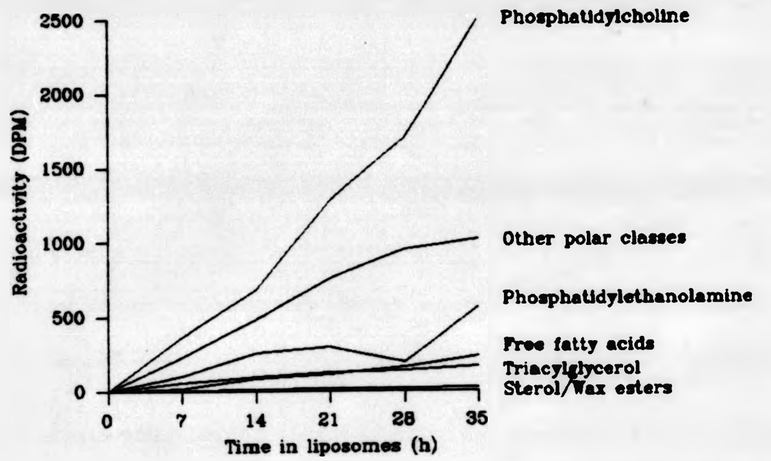


Fig 5-5 Plots of the incorporation of radioactivity form [1-C^{14}]18:3(n-3) incorporated into liposomes into the lipid classes of 17-15 days post-hatch herring larvae

The radioactivity incorporated into various lipid classes is shown in relative terms as the percentage of total activity at each sample point

Std Typical lipid class composition (as % total lipid) for well nourished, 36-41 days post-hatch, spring spawned herring larvae, derived from Fraser *et al.* (1987) and corrected to exclude sterols

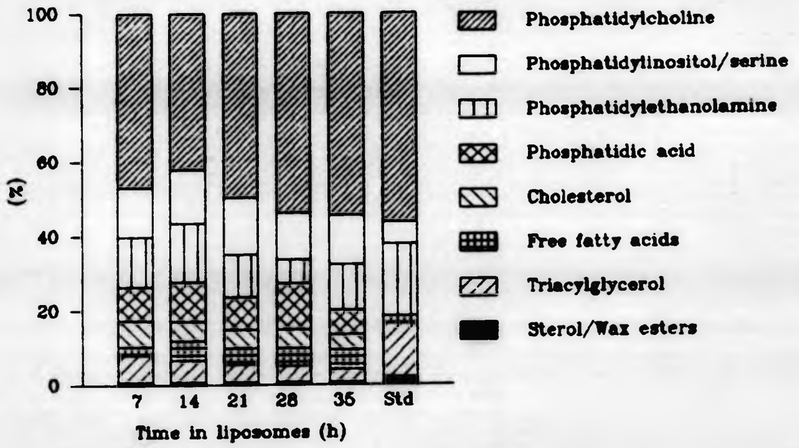


Plate 5-1 Photomicrograph of fluoroscein labelled liposomes suspended in seawater viewed using ultra-violet, epifluorescence microscopy (450-490 nm)

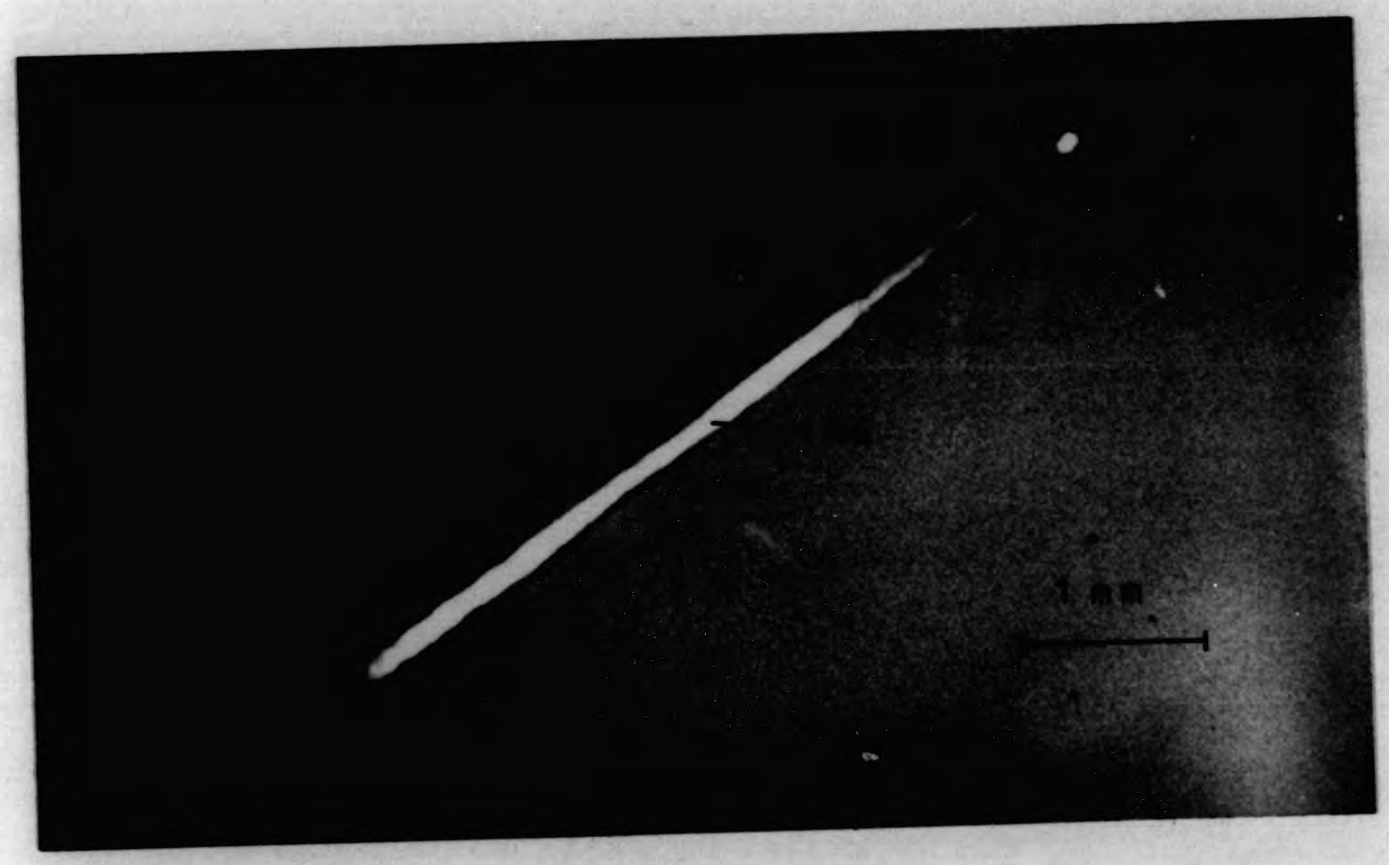


Plate 5-2 Clyde herring larva (aged 7-5 days post-hatch) viewed under epifluorescence microscopy after being in a seawater suspension of fluorescein labelled liposomes for 1 h
IL = ileocaecal valve; MG = midgut; HG = hindgut

Plate 5-3 Clyde herring larva (aged 19 days post-hatch) viewed under epifluorescence microscopy after being in a seawater suspension of fluorescein labelled liposomes for 1 h; fluorescent material is being concentrated in the hindgut
HG = hindgut; C = chromatophore; A = anus; FL = fluorescent liposome material

Plate 5-4 Clyde herring larva (aged 7-5 days post-hatch) viewed under epifluorescence microscopy after being in a seawater suspension of fluorescein labelled liposomes for 4 h; fluorescent material is being exuded from the anus by peristaltic action
HG = hindgut; C = chromatophore; A = anus; FL = fluorescent liposome material

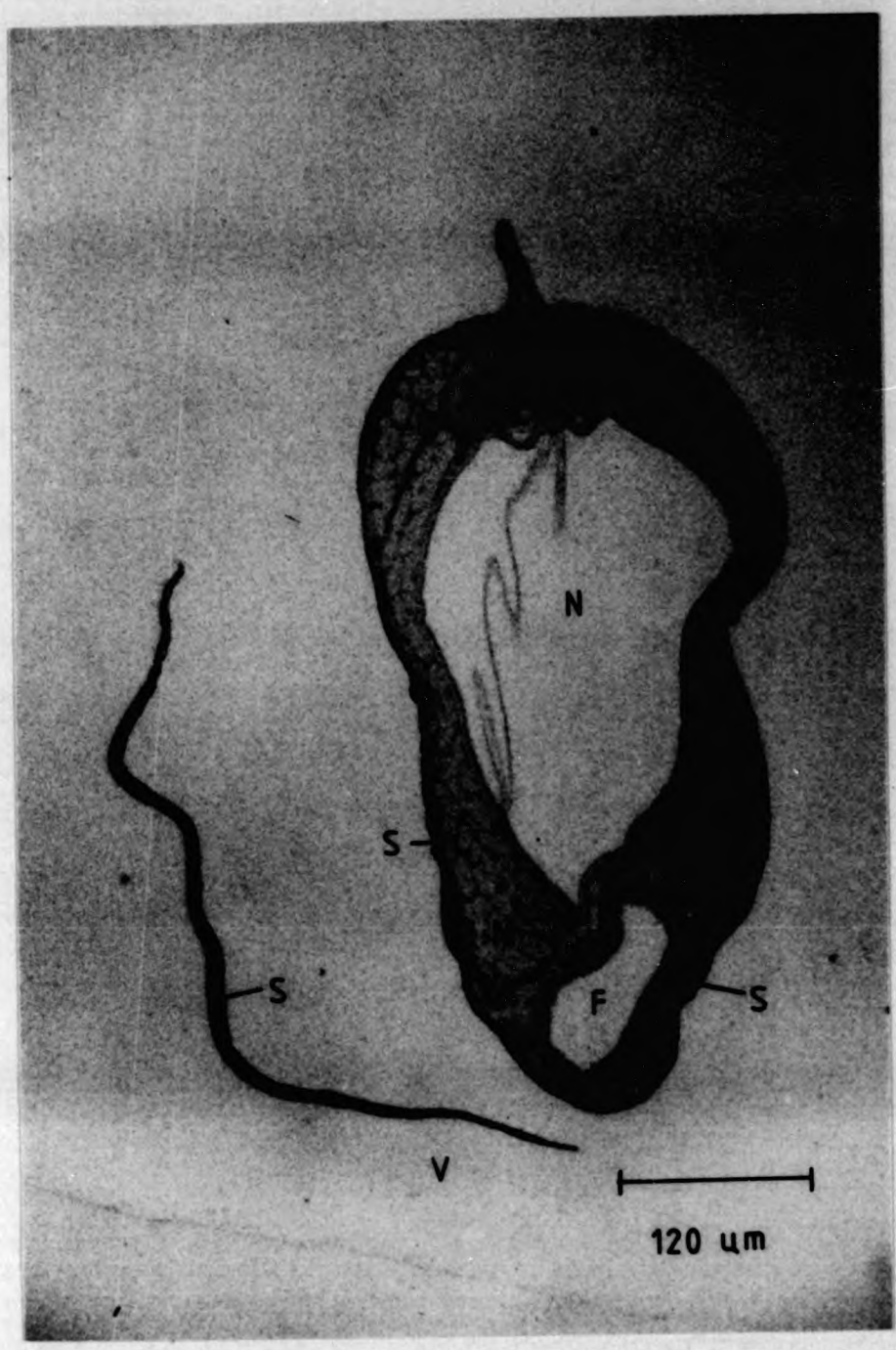


Plate 5-5 Autoradiograph of cross section through fore-gut of a Clyde herring larva after 1 h incubation in a seawater suspension of [1-C^{14}]18:3(n-3) containing liposomes
N = notochord; M = muscle blocks; F = foregut; V = ventral fin; S = deposited silver grains

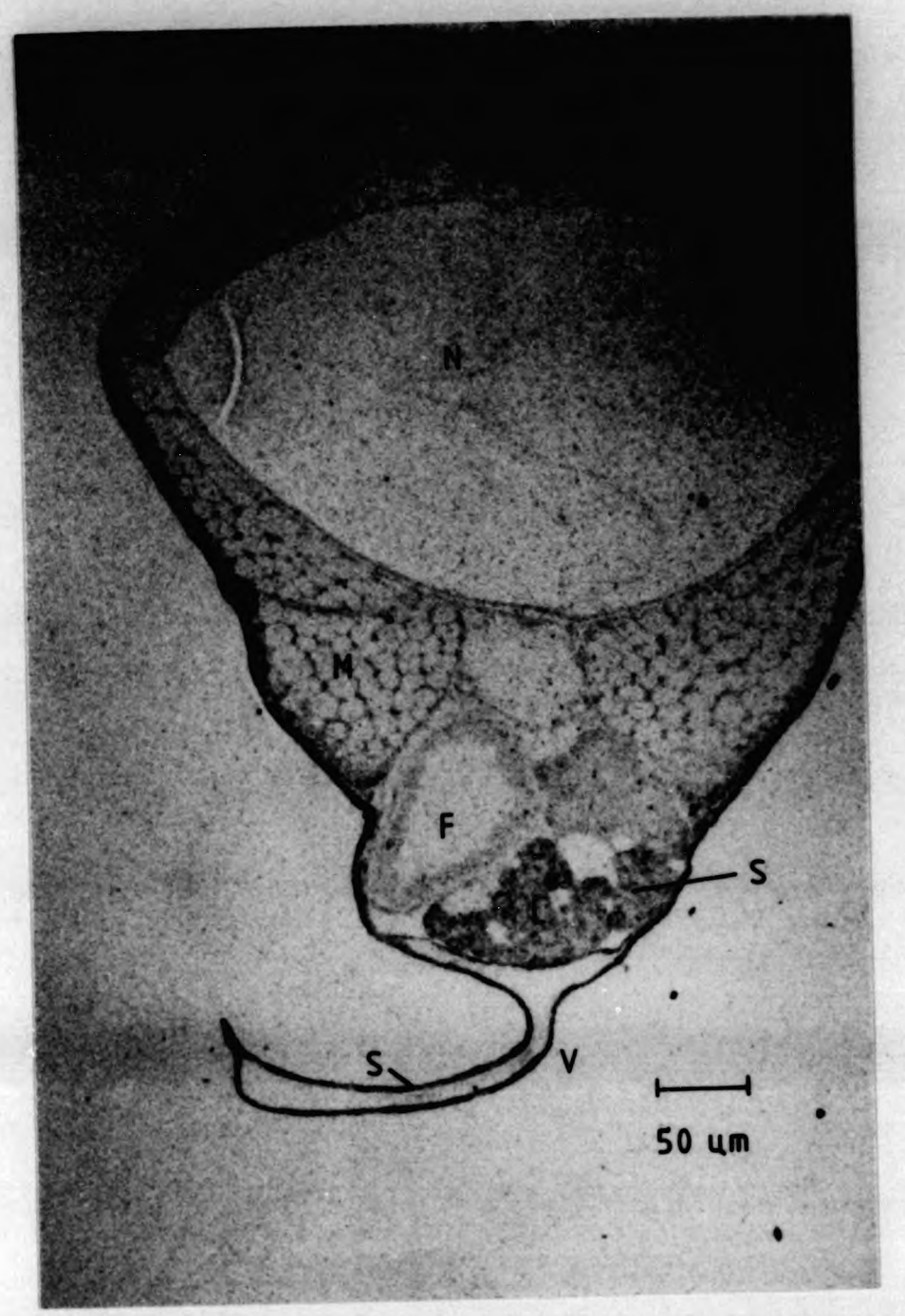


Plate 5-6 Autoradiograph of cross section through fore-gut and liver of a Clyde herring larva after 5 h incubation in a seawater suspension of [1-C^{14}]18:3(n-3) containing liposomes
N = notochord; M = muscle blocks; F = foregut; V = ventral fin; L = liver; S = deposited silver grains

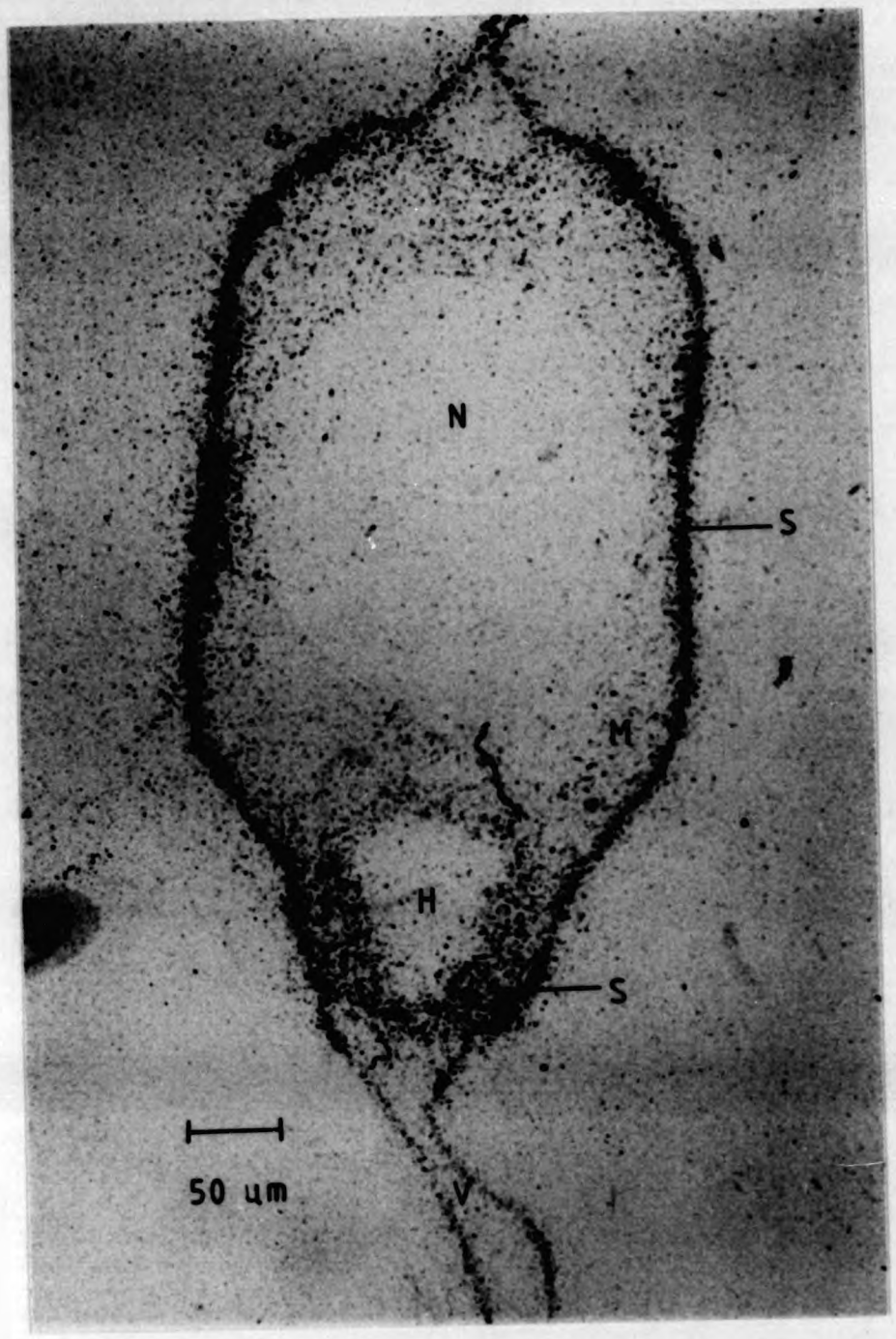


Plate 5-7 Autoradiograph of cross section through hind-gut of a Clyde herring larva after 21 h incubation in a seawater suspension of [1-C^{14}]18:3(n-3) containing liposomes
N = notochord; M = muscle blocks; H = hindgut; V = ventral fin;
S = deposited silver grains

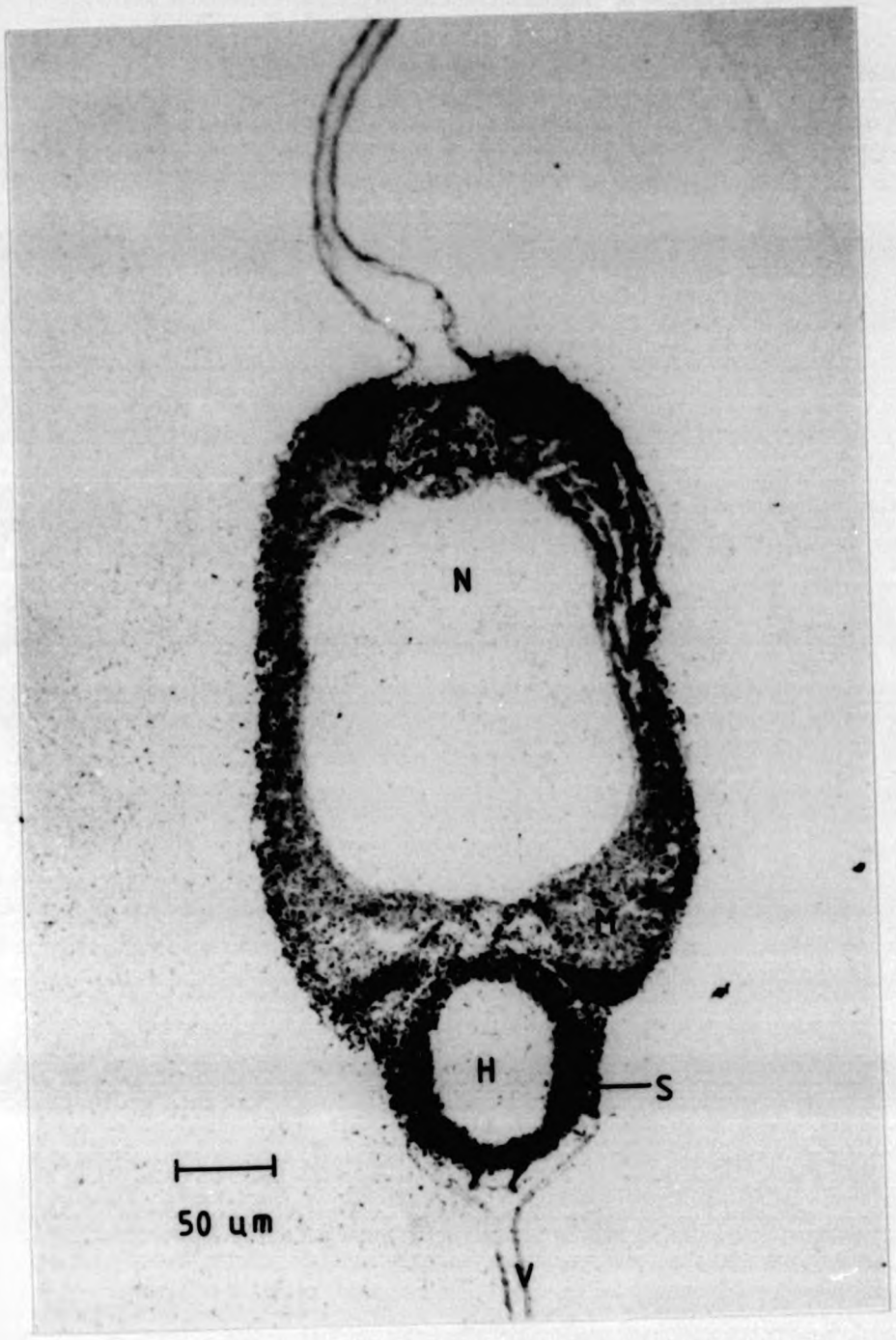


Plate 5-8 Autoradiograph of cross section the hind-gut of a Clyde herring larva after 35 h incubation in a seawater suspension of [1-C^{14}]18:3(n-3) containing liposomes
N = notochord; M = muscle blocks; H = hindgut; V = ventral fin;
S = deposited silver grains

Chapter 6 Overall conclusions and the future of lipid nutritional studies on marine fish larvae

Using radiolabelled fatty acids, it was demonstrated that herring larvae do not appear to be capable of elongating and desaturating 18:3(n-3) to longer-chain PUFAs but are capable of limited conversion of 20:5(n-3) to 22:6(n-3). However, the results from rearing experiments using live diets demonstrated that the rate of conversion was insufficient to maintain 22:6(n-3) at the levels found in first-feeding larvae. It has been shown in wild-caught cod and herring larvae, that a significant decline in the levels of long-chain PUFAs with age does not occur (Klungsøyr *et al.* 1989, Fraser *et al.* 1987).

It is thus concluded, that for artificial rearing of herring larvae, adequate levels of both 20:5(n-3) and 22:6(n-3) must be supplied in the diet. It is highly probable that a similar situation appertains to the larvae of other cold-water marine fish, some of which may, in the future become important aquaculture species.

6.1 Dietary levels of long-chain (n-3)PUFAs required for growth of marine fish larvae

The present study failed to define the dietary levels of 20:5(n-3) and 22:6(n-3) required for optimal growth of herring larvae. Using enriched rotifers, Izquierdo *et al.* (1989) found that the optimal level of dietary long-chain (n-3)PUFAs for red seabream larvae was 0.4% (wet weight) or 3.46% on a dry weight basis. It is noteworthy that this is 20% higher than the calculated requirement of juvenile red seabream.

It is possible that the requirement by cold-water marine fish larvae is even higher. The natural zooplankton diet of these larvae contains up to 20% lipid on dry weight basis, most of which is phospholipid. Fatty

acids comprise around 60% of the phospholipid mass of which about half are (n-3)PUFAs. Thus it may be estimated that (n-3)PUFAs comprise up to 6% of the natural diet. It has already been outlined that the use of high levels of marine fish triacylglycerols in artificial diets might lead to problems of digestibility and it has also been noted that phospholipid may be an essential dietary component for fish larvae. The development of inert starter feeds for marine fish larvae should probably concentrate on the use of marine fish roe as a basic ingredient. Roe is the only natural source of material rich in long-chain (n-3)PUFAs and phospholipids and is, *a priori*, an ideal mixture of the nutrients required by fish larvae (Sargent *et al.* 1989).

6.2 The potential for using liposomes in nutritional studies on aquatic organisms

There appears to be considerable potential for the use of liposomes in nutritional studies on aquatic organisms which are too small or delicate for injection techniques.

It may be possible to use liposomes to budget the use of various fatty acids by marine fish larvae. Fatty acids in the diet can follow a number of routes after absorption. The fatty acid may be esterified and used as a structural component, or stored for later use. Alternatively the fatty acid may be modified by elongation and/or desaturation and then passed to storage or structural compounds. The final possible fate is mitochondrial or peroxisomal ß-oxidation. AcetylCoA liberated may then be passed to the tricarboxcylic acid cycle or used for the anabolism of saturated fatty acids which then enter the pathways mentioned above. AcetylCoA is also the basic unit for the synthesis of cholesterol and the ketone bodies (acetoacetate, ß-hydroxybutyrate and acetone). In addition

the tricarboxylic acid cycle intermediates, α-ketoglutarate and oxaloacetate may be used for the synthesis of amino acids.

In this study, the radioactive carbon was carried as the carboxyl terminus of the labelled fatty acids. Since β-oxidation proceeds from the carboxyl end of the fatty acid, the radiotracer is passed into the above pathways. If the [C^{14}]acetate is transferred to the tricarboxcylic acid cycle, it will ultimately be excreted as [C^{14}]carbon dioxide. These pathways are illustrated in Fig 6-1.

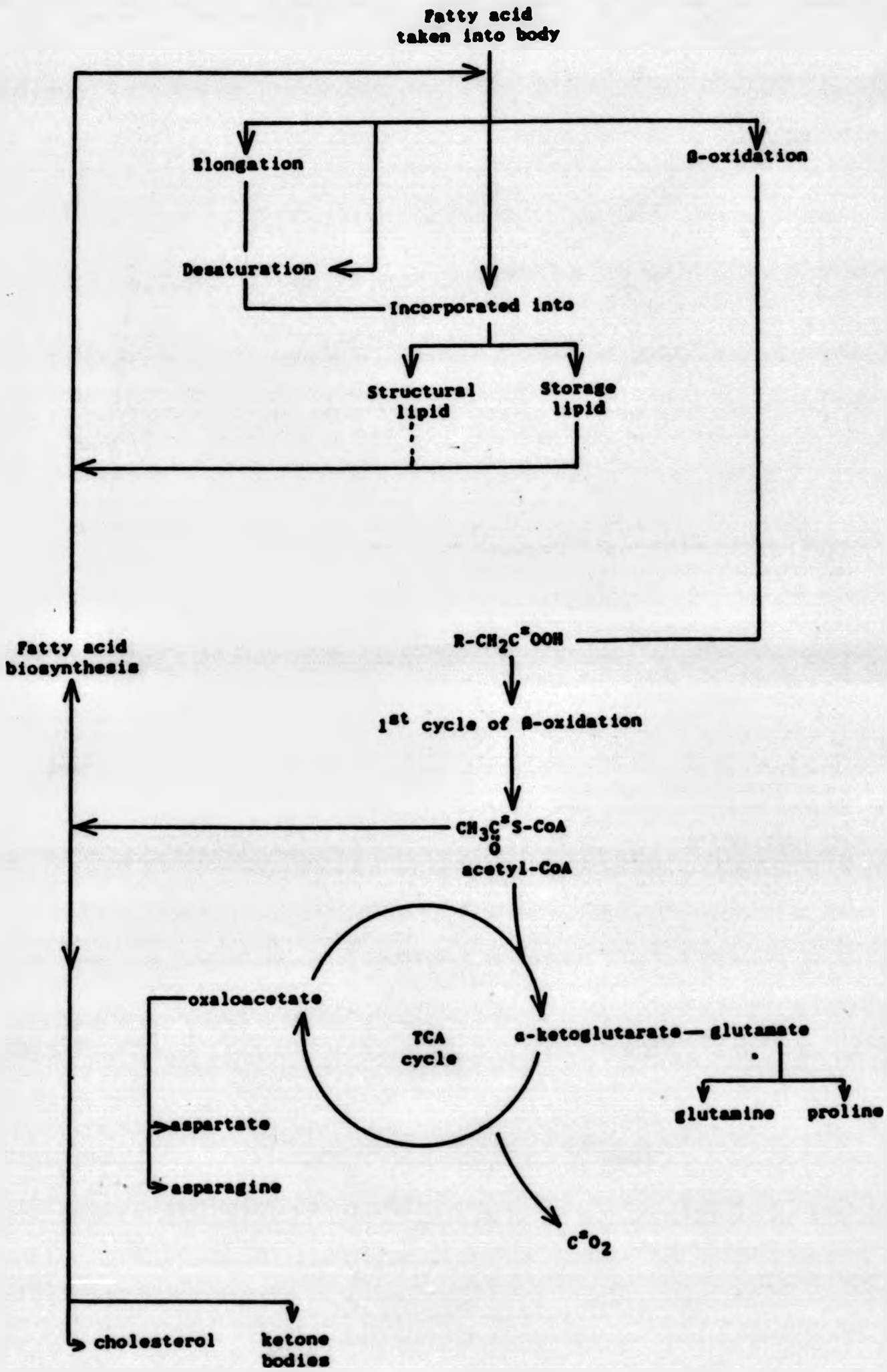
Several systems have been used to collect excreted carbon dioxide from aquatic organisms (Harrison 1984, Kanazawa *et al.* 1978). A prototype collection system for use with fish larvae was tried in this study (section 5.8) but was only partially successful. However, [C^{14}]carbon dioxide was detected indicating that a proportion of the radiolabelled fatty acids assimilated by the herring larvae from the liposomes was being respired. It should be possible to measure the extent to which different fatty acids are used for energy or are retained by the larvae, given the design of a suitable metabolism chamber. It is hypothesised that (n-6)fatty acids and possibly unsaturated fatty acids will be preferentially respired and (n-3)PUFAs retained. The patterns of use may however depend on the nutritional status of the larva and its activity pattern.

The unique properties of liposomes mean that they may prove to be powerful tools in nutritional research on aquatic organisms. They are easy to prepare and the use of modern techniques means that specific size ranges can be generated. The particles are near neutrally buoyant and only require gentle mixing to keep them in suspension. The technology for encapsulating non-lipid compounds is now quite advanced. It should be possible to use liposomes as a vector for investigating the metabolic fate of a wide range of nutrients in marine fish larvae.

The higher than expected uptake efficiencies noted in this study require further investigation but mean that smaller samples of larvae or reduced amounts of economically expensive radiotracers can be employed in metabolic studies.

Finally, liposomes might provide a means of directly supplementing the diet of marine fish larvae in culture. This may be particularly relevant at first feeding when the larvae require an exogenous source of energy but are insufficiently experienced to capture live prey items efficiently.

Fig 6-1 The metabolic fate of fatty acids



The above pathways are derived from mammalian biochemistry.
C* Radioactively labelled carbon

Appendix I The structure of lipids

This section is intended to provide an outline of compounds mentioned in the text and an introduction to fatty acid metabolism. For more detailed discussions see Gurr & James (1975) and Zubay (1988). For information on nomenclature refer to IUPAC-IUB (1977).

Lipids

Definition - A heterogenous group of substances being insoluble in water but soluble in non-polar solvents. This definition includes various pigments, fatty acids and their esters, sterols, hydrocarbons (aliphatic and polycyclic) and the fat soluble vitamins. In common usage, the term usually excludes pigments and vitamins.

The major lipid classes found in herring larvae are sterol and wax esters, triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids, cholesterol, phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin, cardiolipin and lyso-phosphatidylcholine. The structures of these compounds will be discussed whilst those of classes found in other organisms will be mentioned.

It must be borne in mind that the structures discussed are those of the molecules after extraction from tissue. *In vivo*, lipids are rarely present as 'free' molecules, they are usually associated with proteins or carbohydrates. For example, inter-cellular transport of esterified lipids takes place as lipoprotein and chylomicron complexes whilst unesterified fatty acids are transported bound to serum albumin. An understanding of the architecture of cell membranes cannot be attained without understanding the inter-actions of the lipids and proteins found in them.

Fatty acids

Definition - Aliphatic monocarboxylic acids liberated by hydrolysis from naturally occurring fats and oils.

Saturated fatty acids are formed from a carbon backbone without double bonds whilst unsaturated fatty acids carry fewer hydrogen atoms and consequently possess double bonds. The nomenclature of fatty acids has been confused with a plethora of alternative trivial and systematic names. Recently some consensus has emerged and the following system is recommended.

The fatty acid is referred to by a short-hand notation [e.g. 18:2(n-6)] in which the first number describes the total number of carbon atoms present in the backbone. Due to their method of synthesis, most natural fatty acids are even-numbered but odd-numbered fatty acids do occur.

Saturated fatty acids may be straight chain, branched or contain a cyclic group. The majority of branched chain fatty acids are mono-methyl substituted acids. If the branch exists as the terminus, the fatty acid is termed *iso*-, if the branch occurs on the third carbon from the methyl terminus, the fatty acid is termed *anteiso*-. Branched and cyclic fatty acids are commonly found in bacteria as are a wide range of keto, hydroxy and epoxy acids.

The second number defines the total number of double bonds. For most naturally derived fatty acids, the double bonds are methylene

interrupted. It is thus necessary only to specify the location of the first double bond to describe the molecule. The letter 'n' (formerly 'omega') is used to specify the number of carbon atoms counting in from the methyl terminus. In some instances, e.g. when discussing the action of animal desaturase enzymes, it is more informative to specify the location from the carboxyl terminus (since animals, unlike plants, are only able to desaturate carbon atoms between the existing double bond and the carboxyl terminus). The number of carbon atoms is then defined as 'delta' (Fig Appendix I-1). In general, the double bonds are of the *cis* form but *trans*-isomers do occur (e.g. in some seed oils and milk products).

As mentioned before, fatty acids are normally found as esterified compounds in nature. High levels of unesterified fatty acids in lipids extracted from tissues are frequently indicative of sample degradation.

The neutral lipid classes (Fig Appendix I-2)

Definition - A group of compounds with widely differing chemical, but similar physical properties. They lack ionized or polar groups and are thus readily extracted into apolar solvents.

The major sterol found in herring larvae is cholesterol, a tetracyclic ring compound. Various other sterols occur in plants e.g. ergosterol and ß-sitosterol.

Sterol esters consist of a fatty acid esterified to a sterol in position 3 of the primary ring.

Small amounts of wax esters are found in herring larvae. These consist of a fatty acid esterified to a fatty alcohol. Wax esters are frequently major components of copepods particularly in bathypelagic and polar environments but are not accumulated to any great extent by predators higher in the food web (Sargent *et al.* 1981).

The major neutral lipid in herring larvae is triacylglycerol. Three fatty acids are esterified to the trihydric alcohol, glycerol. Partial glycerides (mono- and diacylglycerols) occur in small amounts.

Carbon atoms of glycerol are numbered stereospecifically according to the standard Fischer projection and prefixed -*sn*- to distinguish the name from non-stereospecific nomenclature.

Complex glycerides (estolides) may be encountered in some fungi and seeds in which further fatty acids are linked to hydroxy fatty acids, themselves esterified to glycerol.

Alkyl-diacylglycerides (1-alkyl-2,3-diacyl-*sn*-glycerols) are found in large amounts in the lipids of some marine organisms and consist of a long-chain alkyl group linked via an ether linkage to position 1 of glycerol. The neutral plasmalogens are related compounds detected in small amounts only in some animal tissues. In these, the alkyl group is joined to position 1 of glycerol, via a vinyl ether bond.

The polar lipid classes (Fig Appendix I-3)

Definition - A group of compounds with similar chemical and physical properties. They possess ionized or polar groups and are thus extracted into more polar solvents than the neutral lipids.

Polar lipids can be further divided into the glycerophospholipids, glycoglycerolipids and the sphingolipids.

The glycerophospholipids possess glycerol as their alcohol. There is an enormous range of glycerophospholipids in nature but those containing choline, ethanolamine, serine and inositol esterified to phosphoric acid at the *sn*-3 position of the glyceride are the main ones found in herring larvae. Small amounts of cardiolipin also occur. Phosphatidic acid is a biosynthetic intermediate and normally occurs only in trace amounts.

The glycerophospholipids of micro-organisms contain a wide range of short chain branched and cyclic fatty acids.

Glycoglycerolipids are common in plant tissues. They consist of 1,2-diacyl-*sn*-glycerols joined via a glycosidic link at position 3 to carbohydrate moieties. The carbohydrate may be mono, di, tri or tetra galactose or glucose. These molecules are thus termed monogalactosyl-diacylglycerol, digalactosyldiacylglycerol etc. In addition a unique sulpholipid is found in the chloroplasts of plants in which sulphonic acid is linked to position 6 of the monosaccharide moiety.

A large variety of glycoglycerolipids are found in bacteria containing glucose, galactose, mannose, rhamnose or glucuronic acid as the carbohydrate moiety.

Glycoglycerolipids have been isolated in small quantities from mammals but were not detected in larval herring lipids in this study.

The sphingolipids contain ceramides consisting of a fatty acid linked to a long-chain di- or trihydroxy base via the amine group. Sphingosine is normally the most important base in animals whereas phytosphingosine is found in plants. The free hydroxy bases are toxic and only occur in trace amounts. Sphingomyelin is formed from the esterification of a ceramide to phosphocholine and is found in herring larvae. Sphingolipids are important components of the myelin sheath in mammals and also occur in plasma as components of low-density lipoprotein.

Cerebrosides (glycosphingolipids) consist of a ceramide linked to glucose, galactose or polysaccharide. They are found in neural tissue but were not detected in herring larval samples.

A variety of other complex lipids can be found in various organisms but are outside the scope of this review.

Anabolism of fatty acids (see Fig Appendix I-4)

The pathways involved have been elucidated using microbiological and mammalian systems but there is no evidence that the *de novo* synthesis of fatty acids in fish occurs by a different mechanism (Henderson & Sargent 1985) though rates and substrate specificities may vary. In eukaryotes, *de novo* synthesis of fatty acids occurs in the cytosol by the repeated addition of two carbon containing acetate groups. The mechanisms involved have been reviewed by Wakil *et al.* (1983). The major products of these reactions are the saturated fatty acids 16:0 and 18:0.

Animals do not possess desaturase enzymes which are able to produce double bonds at a greater distance than nine carbon atoms from the carboxyl terminus. Thus, 18:0 may be converted by delta-9 desaturase into 18:1(n-9) and further chain elongated and desaturated within the

(n-9) series of fatty acids. Normally, the longer chain members of this series are present in low concentrations. However, when (n-3) and (n-6) fatty acids are not supplied in sufficient quantities in the diet, PUFAs of the (n-9) series, especially 20:3(n-9), are accumulated and their presence may be used to indicate essential fatty acid deficiency.

Since animals cannot desaturate beyond the delta-9 carbon, fatty acids of the (n-3) and (n-6) series must be supplied in the diet. In addition, this means that inter-conversion of fatty acids of the (n-9), (n-6) and (n-3) series is not possible. Pathways exist for the elongation and desaturation of dietary 18:2(n-6) and 18:3(n-3) to the longer chain members of each family. For animals, long-chain PUFAs appear to be necessary for normal metabolism (they are not found to a large extent in bacteria). However, the effectiveness of the enzymes required for each elongation or desaturation step varies with species and this determines which of the polyunsaturated fatty acids are essential dietary components. Further, it must be pointed out that fatty acids of the different series may act as competitive analogues for the elongation/desaturation enzymes. It is thus the overall balance of fatty acids in the diet, combined with the current nutritional and physiological status of the organism which will determine the relative rates of bioconversion of the fatty acids and whether essential fatty acid deficiency pathologies will develop.

Catabolism of fatty acids

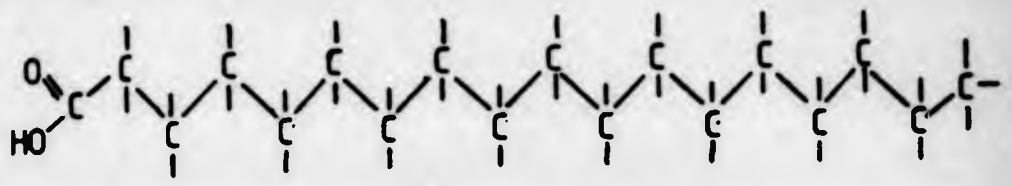
Fatty acids are catabolized in the mitochondria by ß-oxidation, essentially the reverse of the anabolic process. Partial ß-oxidation may also occur in the peroxisomes but the complete catabolism of fatty acids is not possible in these organelles due to the inability of acyl-CoA oxidase to react with hexanoyl-CoA or butryl-CoA. ß-oxidation of saturated fatty acids involves the sequential removal of two carbon units from the carboxyl terminus. Oxidation of unsaturated fatty acids requires the additional enzymes enoyl-CoA isomerase, 2,4-dienoyl-CoA isomerase and 2,4-dienoyl-CoA reductase. Thus retro-conversion of PUFAs to shorter chain molecules is theoretically possible and probably occurs in peroxisomes. Retro-conversion of PUFAs has not been studied to any great extent in fish.

The two carbon acetate units liberated by ß-oxidation may be fed into the tricarboxylic acid cycle, yielding energy, carbon dioxide and water or used for anabolism of a variety of compounds.

Oxidation of fatty acids from the methyl terminus has been detected in rat liver microsomes. The quantitative importance of this pathway is unclear.

Fig Appendix I-1

18:0
A saturated fatty acid

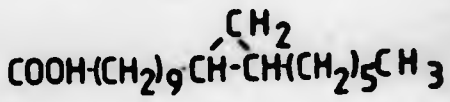


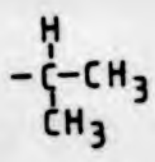
carboxyl terminus

methyl terminus

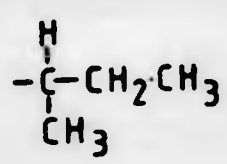
Some of the configurations found in saturated fatty acids

11,12 methyleneoctadecanoic acid
A cycloprane fatty acid from bacterial lipid

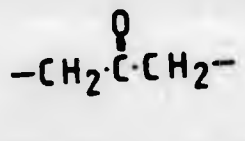




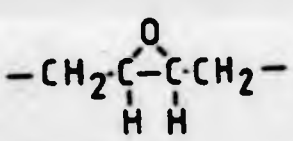
iso-acids



anteiso-acids

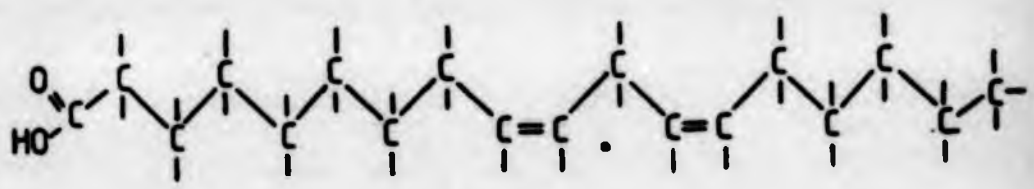


ketone



epoxy

18:2(n-6)
An unsaturated fatty acid



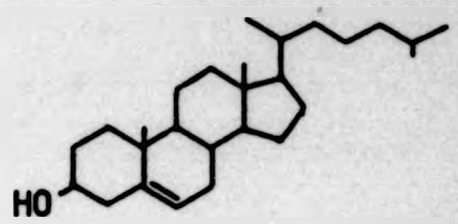
Δ — — n

Alternative names

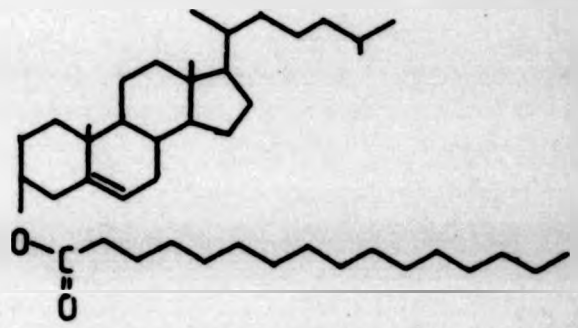
Linoleic acid
cis, cis-9,12 octadecadienoic acid
Δ-9,12 octadecadienoic acid
18:2(ω 6)

Fig Appendix I-2

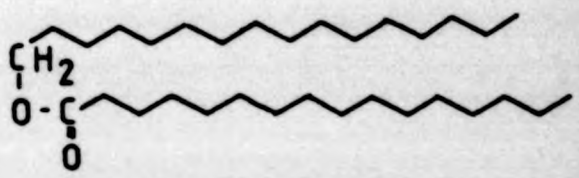
Cholesterol



Cholesterol ester



Wax ester



Triacylglycerol
1,2,3-triacyl-sn-glycerol

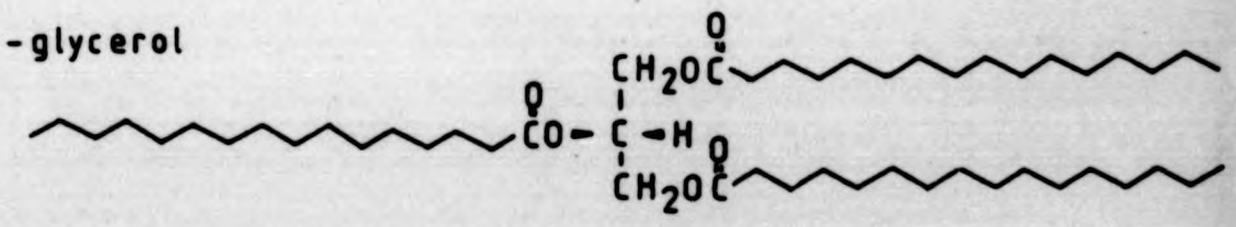
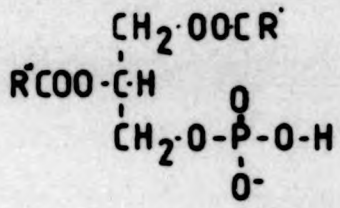


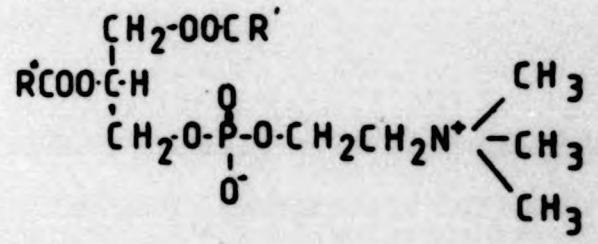
Fig Appendix I-3

R = fatty acid

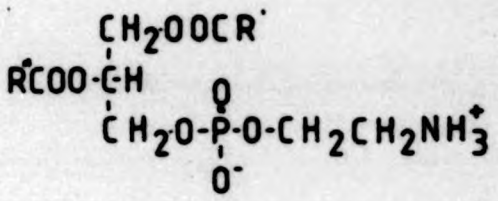
Phosphatidic acid



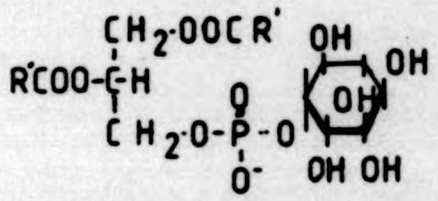
Phosphatidylcholine



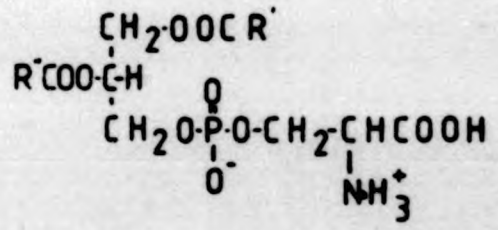
Phosphatidylethanolamine



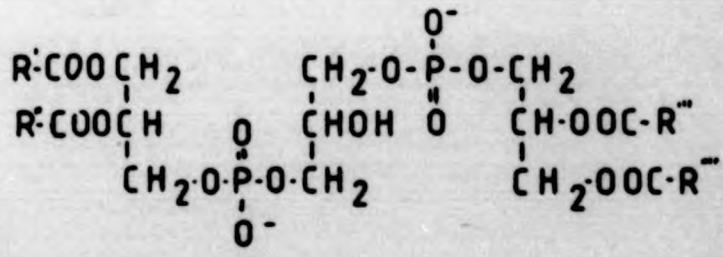
Phosphatidylinositol



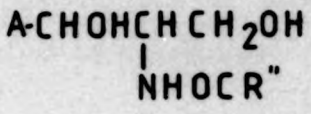
Phosphatidylserine



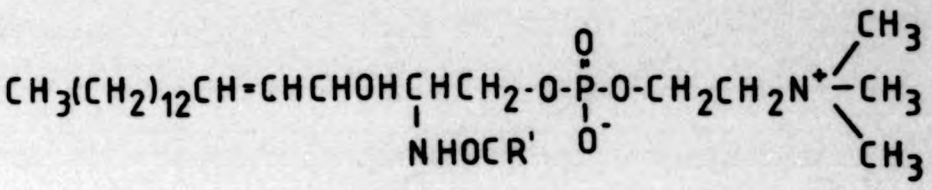
Cardiolipin



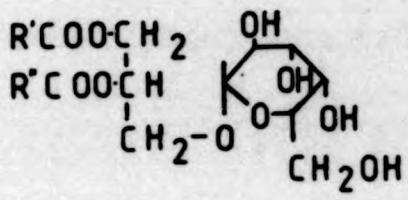
Ceramide



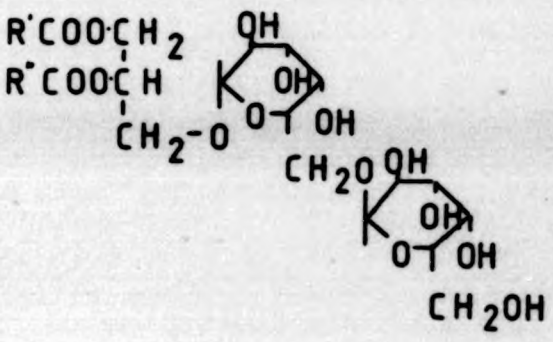
Sphingomyelin



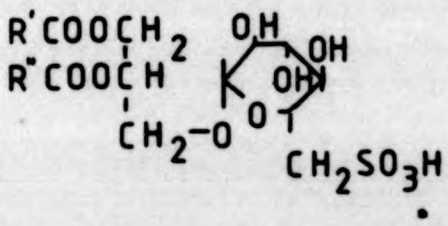
Monogalactosyldiacylglycerol



Digalactosyldiacylglycerol



Sulphoquinovosyldiacylglycerol



A = long chain di- or trihydroxy alkyl base

R = fatty acid

Fig Appendix I-4

Desaturation and elongation of (n-9),(n-6) and (n-3) fatty acids in animals

16:0 → 18:0 → 18:1 → 18:2 → 20:2 → 20:3 → 22:3 → 22:4 n-9 series

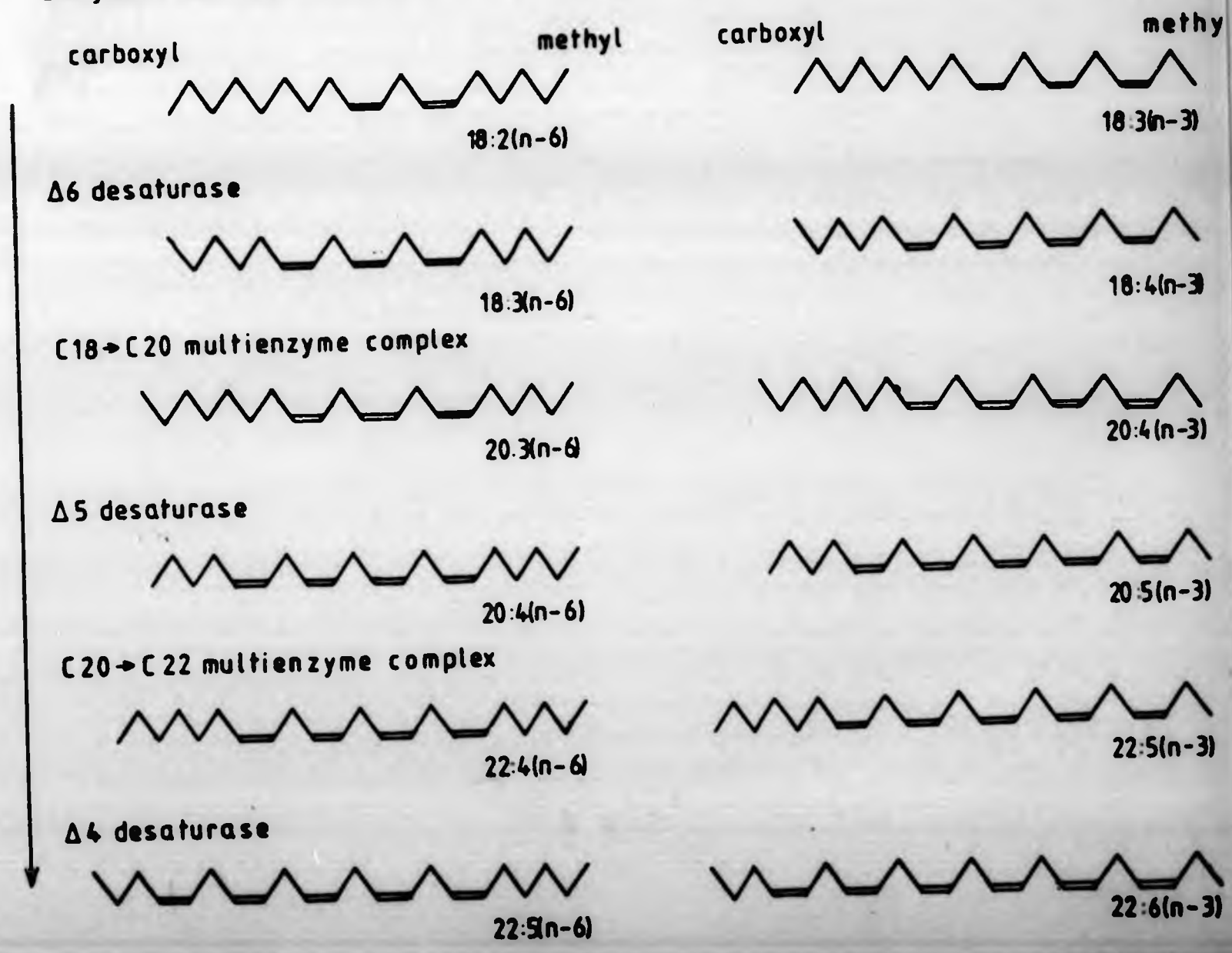
de novo

diet → 18:2 → 18:3 → 20:3 → 20:4 → 22:4 → 22:5 n-6 series

18:3 → 18:4 → 20:4 → 20:5 → 22:5 → 22:6 n-3 series

(adapted from Henderson & Sargent 1985)

Enzymes involved in desaturation and elongation of (n-6) and (n-3) fatty acids



Appendix II Program for the computation of lipid class spectra for the Iatroscan system

This program computes the lipid class distribution from the data derived from the Iatroscan TLC-FID system when run in accordance with the separate polar and neutral separation method of Fraser, Tocher & Sargent (1985) for the analysis of lipid samples containing wax esters/sterol esters, cholesterol, triglyceride, free fatty acid, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, phosphatidylcholine and lyso-phosphatidylcholine.

Installation

The program can be obtained on disc from the author. It was written in BBC basic on model B system fitted with a single, 40 track disc drive and connected to an Epson compatible printer. The program is menu driven.

Before analysing data, calibration curves must be entered.

1. After loading the program remove the master disc from the disc drive and insert a new, formatted disc.
2. Select ITEM 1 from the MAIN MENU to access the CALIBRATION MENU.
3. Each lipid class is assigned a code, the list of available codes is displayed with ITEM 1 from the CALIBRATION MENU. These codes are used throughout the program and should be noted down.
4. To enter new calibration curves select ITEM 2 from the CALIBRATION MENU.
5. Select the relevant lipid class code and enter the curve as related peak areas (detector response) and amount of lipid, type 999 as the peak area after the last item. The first pair of numbers entered should be the minimum values. The program reads the calibration by linearizing the curve between the points entered. Thus the recommended technique is to plot out the calibration data on graph paper initially. Fit a curve to the data, divide the abscissa into equal sections and use the corresponding lipid weights to enter the calibration curve into the program.
6. When all the curves have been entered (for cholesteryl oleate, triglyceride, cholesterol, oleic acid, phosphatidylethanolamine and phosphatidylcholine) it is necessary to enter the minimum and maximum peak areas for each curve. The program will then reject data entered which lies outside the calibrations.
7. Any calibration curve may be checked by selecting ITEM 3 from the CALIBRATION MENU and entering the relevant lipid class code.
8. Select ITEM 4 from the CALIBRATION MENU to return to the MAIN MENU.

The program is now set up for data analysis. The disc used in the above steps contains the calibration curve information and is also used to store data, DO NOT REMOVE the disc whilst the program is running.

Entering the data to be analysed

9. Select ITEM 2 from the MAIN MENU.
10. Data can be analysed for up to ten replicate separations per sample (i.e. 20 rods). The integrated trace from each rod should be assigned a consecutive number e.g. a lipid sample was analysed

three times, number the neutral separations 1, 2 and 3; number the polar separations 4, 5 and 6.

11. Input the rod (trace) number, the class codes and the relevant peak area (detector response). When all the peaks in the trace have been entered type END as the class code.
12. Select whether you wish to enter data for another separation, to check the data entered or to return to the MAIN MENU.
13. Data entered may also be checked by selecting ITEM 4 from the MAIN MENU.

Analysing the data

14. Select ITEM 3 from the MAIN MENU.
15. You will be asked for the numbers of the rods (traces) you wish to analyse. The total number of rods should be 20 or less, the series should contain equal numbers of polar and neutral separations. From the example in stage 10, you would enter 1, 2, 3, 4, 5, and 6. If you were unhappy with the separation of the peaks on trace 5, you could delete this, but you must also delete a neutral separation. You might analyse 1, 2, 4 and 6 or 1, 3, 4 and 6.
16. The program now compares the peak areas you have entered with the calibration curves stored earlier. The areas are converted into amounts of lipid. For pairs of rods, the proportions of polar lipid classes and total neutral lipids are calculated from the polar separation. The proportions of neutral lipid classes are then calculated from the neutral separation and reduced in accordance with the total neutral lipid present. Finally the mean and standard deviation of the results from the replicate analyses are computed.
11. Select whether you wish to have a printed copy of the results or not. If you do, ensure that your printer is turned ON and is ON LINE.
12. Return to the MAIN MENU.

Known bugs

With some disc drive systems the disc library will become filled after the data for 20 traces has been entered. To overcome this assign consequent traces the same numbers (see stage 10) as traces which have already been analysed. The program will overwrite the old data. Note that the old data will be lost so a printed copy of the results should have been obtained.

The means and standard deviations in the current version of the program are computed on non arcsin transformed data. This will lead to slight statistical errors in the final results.

```
10REM.LIST OR RODS
20 DIM analist$(10)
30REM.MAXMINS FOR DATA ENTRY
40 DIMmaxmaester(2):DIM maxmintag(2):DIM maxminfree(2):DIM maxminchol(2)
50 DIM maxmainps(2):DIM maxmainpc(2): DIM maxmainneut(2): DIM maxmainpol(2)
60REM.AMOUNTS OF LIPID FROM CALBS.
70 DIM maxester(10):DIM sterols(10):DIM tag(10)
80 DIM freefatty(10):DIM cholest(10):DIM pc(10)
90 DIM ps(10):DIM pi(10):DIM lpc(10):DIM sm(10)
100 DIM pe(10):DIM neut(10):DIM pol(10)
110REM.TOTAL LIPIDS FROM TRACES
120DIM tpol(10):DIM tlip(10):DIM tneut(10)
130REM.PERCENT LIPID CLASSES
140DIM pneut(10):DIM ppol(10):DIM pmax(10): DIM pster(10)
150DIM ptag(10):DIM pfree(10):DIM pchol(10)
160DIM ppc(10):DIM pps(10):DIM ppi(10):DIM plpc(10)
170DIM psm(10):DIM ppe(10)
180PROCINTRO
190REM.MAIN MENU SETUP
200DEF PROCINTRO
210CLS:@%=10
220PRINTTAB(7,4)"REMOVE PROGRAM DISC"
230PRINTTAB(7,5)"INSERT CALIBRATION DISC"
240PRINTTAB(13,8)"MAIN MENU"
250PRINTTAB(13,9)"---------"
260PRINTTAB(2,11)"1.) CALIBRATION CURVE ALTERATIONS."
270PRINTTAB(2,12)"2.) DATA INPUT."
280PRINTTAB(2,13)"3.) DATA PROCESSING."
290PRINTTAB(2,14)"4.) CHECK DATA INPUT."
300PRINTTAB(2,15)"5.) END."
310PRINTTAB(2,17)"TYPE REQUIRED OPTION No. ";
320menopt=GET
330IF menopt=49 THEN PROCCUR
340IF menopt=50 THEN PROCDAT
350IF menopt=51 THEN PROCCAL
360IF menopt=52 THEN PROCCHECK
370IF menopt=53 THEN CLS:*BASIC:NEW
380IF menopt<49 OR menopt>53 THEN 320
390ENDPROC
400REM.CALIBRATION CURVES
410DEF PROCCUR
420CLS:PRINTTAB(8,8)"CALIBRATION MENU"
430PRINTTAB(8,9)"---------------"
440PRINTTAB(2,11)"1.) SUMMARY OF AVAILABLE CURVES"
450PRINTTAB(7,12)"AND FILE CODES."
460PRINTTAB(2,13)"2.) INPUT NEW CALIBRATION CURVE."
470PRINTTAB(2,14)"3.) CHECK CALIBRATION CURVE."
480PRINTTAB(2,15)"4.) MAIN MENU."
490PRINTTAB(2,18)"TYPE REQUIRED OPTION No. ";
500menopt=GET
510IF menopt=49 THEN 560
520IF menopt=50 THEN 760
530IF menopt=51 THEN 1320
540IF menopt=52 THEN PROCINTRO
550IF menopt<49 OR menopt>52 THEN 500
560REM.AVAILABLE CURVE LIST
570CLS:PRINTTAB(2,3)"AVAILABLE CALIBRATION CURVE LIST"
580PRINTTAB(2,4)"-----------------------------"
590PRINTTAB(2,8)"TOTAL POLAR (P)."
600 PRINTTAB(2,9)"TOTAL NEUTRAL (N)."
610PRINTTAB(2,10)"PHOSPATIDYLCHOLINE (PC)."
620PRINTTAB(2,11)"PHOSPATIDYLETHANOLAMINE (PE)."
630PRINTTAB(2,12)"PHOSPATIDYLINOSITOL/SERINE (as PE)."
640PRINTTAB(2,13)"LYSO-PHOSPHATIDYLCHOLINE (as PE)."
650PRINTTAB(2,14)"SPHINGOMYELIN (as PE)."
660PRINTTAB(2,14)"SPHINGOMYELIN (as PE)."
670PRINTTAB(2,15)"STEROL ESTERS (NS)."
680PRINTTAB(2,16)"WAX ESTERS (as NS)."
690PRINTTAB(2,17)"CHOLESTEROL (NC)."
700PRINTTAB(2,18)"TRIACYLGLYCEROLS (NT)."
710PRINTTAB(2,19)"FREE FATTY ACIDS (NF)."
720PRINTTAB(2,21)"HIT SPACE-BAR TO CONTINUE ";
730menopt=GET
740 IF menopt=32 THEN PROCCUR
750IF menopt<>32 THEN 730
760REM. CALIBRATION CURVE FILE INPUT
770 CLS:PRINTTAB(2,11)"STEROL ESTERS (NS)."
780PRINTTAB(2,12)"TRIACYLGLYCEROLS (NT)."
790PRINTTAB(2,13)"CHOLESTEROL (NC)."
800PRINTTAB(2,15)"PHOSPHATIDYLCHOLINE (PC)."
810PRINTTAB(2,16)"PHOSPHATIDYLETHANOLAMINE (PE)."
820PRINTTAB(2,18)"TOTAL NEUTRALS (N)."
830PRINTTAB(2,19)"TOTAL POLARS (P)."
840INPUTTAB(2,8)"CALIBRATION CURVE FILE CODE  "AS
850*ACCESS NS
860*ACCESS NT
870*ACCESS NF
880*ACCESS NC
890*ACCESS PC
900*ACCESS PE
910*ACCESS N
920*ACCESS P
930Z=OPENOUT AS
940REPEAT
950CLS:PRINTTAB(7,10)"CALIBRATION INPUT OPTION"
960PRINTTAB(7,11)"----------------------"
970PRINTTAB(2,20)"TYPE '999' AS AREA CO-ORD TO STOP"
980INPUTTAB(6,15)"AREA OF PEAK",X
990INPUTTAB(4,16)"AMOUNT OF LIPID",Y
1000PRINT#Z,X,Y
1010UNTIL X=999
1020CLOSE#Z
```

```
1030*ACCESS NS L
1040*ACCESS NT L
1050*ACCESS NF L
1060*ACCESS NC L
1070*ACCESS PC L
1080*ACCESS PE L
1090*ACCESS P L
1100*ACCESS N L
1110PRINT TAB(2,22)"HIT SPACE-BAR TO ENTER ANOTHER CURVE."
1120PRINT TAB(2,23)"OR ANOTHER KEY TO CONTINUE."
1130menopt=GET
1140IF menopt=32 THEN 770
1150IF menopt<>32 THEN 1160
1160CLS:PRINTTAB(1,5)"Please give curve codes and max."
1170PRINTTAB(4,6)"and min. areas for each."
1180PRINTTAB(3,7)"Type 'END' as code to stop."
1190PRINT TAB(1,10)"Code":PRINT TAB(13,10)"Max. area":PRINT TAB(26,10)"Min. are
a"
1200*ACCESS MAXMINS
1210X=OPENOUT "MAXMINS"
1220cnt=14:REPEAT
1230INPUT TAB(2,C)Es
1240INPUTTAB(14,C)Y
1250INPUTTAB(28,C)W
1260PRINT£X,Es,Y,W
1270C=C+1
1280UNTIL Es="END"
1290CLOSE£X:*ACCESS MAXMINS L
1300PROCCUR
1310REM.TO CHECK CURVE
1320CLS:INPUTTAB(2,11)"REQUIRED CALIBRATION CURVE CODE  "Ys
1330Q=OPENIN Ys
1340REPEAT
1350INPUT£Q,K
1360PRINT K
1370UNTIL K=999
1380CLOSE£Q
1390PRINTTAB(2,28)"PRESS SPACE-BAR TO RETURN TO MENU."
1400menopt=GET
1410 IF menopt=32 THEN PROCCUR
1420 IF menopt<>32 THEN 1400
1430ENDPROC
1440REM.DATA INPUT
1450DEF PROCDAT
1460REM. CHECK IF AREA IN CURVE
1470CLS
1480Z=OPENIN "MAXMINS"
1490REPEAT
1500INPUT£Z,Ts,K,I
1510IF Ts="NS" THEN maxminster(1)=K
1520IF Ts="NS" THEN maxminster(2)=I
1530IF Ts="NT" THEN maxmintag(1)=K
1540IF Ts="NT" THEN maxmintag(2)=I
1550IF Ts="NF" THEN maxminfree(1)=K
1560IF Ts="NF" THEN maxminfree(2)=I
1570IF Ts="NC" THEN maxminchol(1)=K
1580IF Ts="NC" THEN maxminchol(2)=I
1590IF Ts="PE" THEN maxminpe(1)=K
1600IF Ts="PE" THEN maxminpe(2)=I
1610IF Ts="PC" THEN maxminpc(1)=K
1620IF Ts="PC" THEN maxminpc(2)=I
1630IF Ts="N" THEN maxminneut(1)=K
1640IF Ts="N" THEN maxminneut(2)=I
1650IF Ts="P" THEN maxminpol(1)=K
1660IF Ts="p" THEN maxminpol(2)=I
1670UNTIL Ts="END"
1680CLOSE£Z
1690cnt=0
1700CLS:PRINTTAB(10,1)"DATA INPUT OPTION"
1710PRINTTAB(10,2)"================"
1720PRINTTAB(2,4)"TYPE IN ROD No."
1730INPUTTAB(5,5)"(ie. RUN £) "Os
1740CLOSE£Q
1750L=OPENOUT Os
1760REPEAT
1770cnt=cnt+1
1780IF cnt<10 THEN 1790 ELSE 1810
1790PRINTTAB(0,cnt+6)"PEAK "¦cnt"  I.D. CODE       AREA "
1800GOTO1820
1810PRINTTAB(0,cnt+6)"PEAK "¦cnt" I.D. CODE       AREA "
1820INPUTTAB(19,cnt+6) Is
1830INPUTTAB(31,cnt+6) S
1840IF Is="NS" THEN 1860 ELSE 1850
1850IF Is="NS" THEN 1860 ELSE 1870
1860IF S>maxminster(1) OR S<maxminster(2) THEN 2030 ELSE 2070
1870IF Is="NT" THEN 1880 ELSE 1890
1880IF S>maxmintag(1) OR S<maxmintag(2) THEN 2030 ELSE 2070
1890IF Is="NF" THEN 1900 ELSE 1910
1900IF S>maxminfree(1) OR S<maxminfree(2) THEN 2030 ELSE 2070
1910IF Is="NC" THEN 1920 ELSE 1930
1920IF S>maxminchol(1) OR S<maxminchol(2) THEN 2030 ELSE 2070
1930IF Is="PS" THEN 1980 ELSE 1940
1940IF Is="PI" THEN 1980 ELSE 1950
1950IF Is="LPC" THEN 1980 ELSE 1960
1960IF Is="SM" THEN 1980 ELSE 1970
1970IF Is="PE" THEN 1980 ELSE 1990
1980IF S>maxminpe(1) OR S<maxminpe(2) THEN 2030 ELSE 2070
1990IF Is="PC" THEN 2000 ELSE 2010
2000IF S>maxminpc(1) OR S<maxminpc(2) THEN 2030 ELSE 2070
2010IF Is="N" THEN 2020 ELSE 2070
2020IF S>maxminneut(1) OR S<maxminneut(2) THEN 2030 ELSE 2070
```

```
2030CLS:PRINT TAB(7,7)"Last area entered was of#"
2040PRINT TAB(7,8)"of the calibration curves."
2050PRINT TAB(6,12)"Hit any key to reenter data.";
2060IF GET THEN PROCINTRO
2070PRINT #L,I$,S
2080 UNTIL I$="END"
2090CLOSE#L
2100PRINTTAB(5,22)"TYPE 'Y' FOR ANOTHER ROD No. "
2110PRINTTAB(5,23)"OR 'C' TO CHECK DATA ENTERED."
2120PRINTTAB(5,24)"(or SPACE-BAR for main menu.).";
2130menopt=GET
2140IF menopt=89 THEN PROCDAT
2150IF menopt=32 THEN PROCINTRO
2160IF menopt=67 THEN PROCCHECK
2170IF menopt<>89 OR menopt<>32 OR menopt<>67 THEN 2130
2180ENDPROC
2190REM. TO CHECK DATA
2200DEF PROCCHECK
2210CLS:CLOSE#0
2220PRINTTAB(2,1)"Type run number you wish to check"
2230PRINTTAB(2,2)"in double-quotes"
2240INPUTTAB(24,2)A$
2250Y=OPENIN A$
2260REPEAT
2270INPUT#Y,I$,S
2280PRINTTAB(11);I$,S
2290UNTIL I$="END"
2300CLOSE#Y
2310PRINTTAB(6,23)"Hit any key to continue"
2320IF GET THEN PROCCUR
2330ENDPROC
2340REM. DATA PROCESSING OPTION
2350REM. ROD NUMBERS FOR ANALYSIS
2360DEF PROCCAL
2370CLS:CLOSE#0
2380PRINTTAB(7,1)"DATA PROCESSING OPTION"
2390cnt=1
2400IF cnt=1 THEN PRINTTAB(5,7)"TYPE IN RUN Nos.FOR ANALYSIS"
2410PRINTTAB(6,8)"Type END after last entry"
2420REPEAT PRINTTAB(5,cnt+9)cnt"."
2430INPUTTAB(20,cnt+9)analist$(cnt)
2440cnt=cnt+1
2450UNTIL cnt=11 OR analist$(cnt-1)="END"
2460PRINTTAB(5,22)"Press SPACE bar to continue"
2470PRINTTAB(7,23)"or 'C' to correct list."
2480menopt=GET
2490IF menopt=32 THEN 2520
2500IF menopt=67 THEN PROCCAL
2510IF menopt<>67 OR menopt<>32 THEN 2480
2520cnt=0
2530REPEAT
2540cnt=cnt+1
2550Q=OPENIN analist$(cnt)
2560REPEAT
2570IF analist$(cnt)="END" THEN 2920
2580INPUT#Q,R$,W
2590REM. TO CONVERT AREA TO AMOUNTS
2600IF R$="END"THEN 2900
2610IF R$="PS" OR R$="PI" OR R$="LPC" OR R$="SM" THEN 2670
2620IF R$="NW"THEN 2650
2630G=OPENIN R$
2640GOTO 2680
2650G=OPENIN "NS"
2660GOTO2680
2670G=OPENIN "PE"
2680A=0:B=0:E=0:F=0:H=0
2690REPEAT
2700INPUT#G,A,B
2710IF A=999 THEN 2740
2720IF A>=W AND E<=W THEN H=F+(((B-F)/(A-E))*(W-E))
2730E=A:F=B
2740UNTIL A=999
2750CLOSE#G
2760REM. TO STORE AMOUNTS OF LIPID
2770IF R$="NW" THEN waxester(cnt)=H
2780IF R$="NS" THEN sterols(cnt)=H
2790IF R$="NT" THEN tag(cnt)=H
2800IF R$="NF" THEN freefatty(cnt)=H
2810IF R$="NF" THEN cholest(cnt)=H
2820IF R$="PC" THEN pc(cnt)=H
2830IF R$="PS" THEN ps(cnt)=H
2840IF R$="PI" THEN pi(cnt)=H
2850IF R$="LPC" THEN lpc(cnt)=H
2860IF R$="SM" THEN sm(cnt)=H
2870IF R$="PE" THEN pe(cnt)=H
2880 IF R$="N" THEN neut(cnt)=H
2890IF R$="P" THEN pol(cnt)=H
2900UNTIL R$="END"
2910CLOSE#0
2920UNTIL analist$(cnt)="END"
2930REM.PROPORTIONS OF NEUTRAL CLASSES IN NEUTRAL FRACTION (NEUTRAL TRACE)
2940cnt=0
2950REPEAT
2960cnt=cnt+1
2970IF cnt=11 OR analist$(cnt)="END" THEN 3180
2980REM.PROPORTIONS OF NEUTRAL/POLAR CLASSES IN TOTAL LIPID (POLAR TRACE)
2990tpol(cnt)=pc(cnt)+ps(cnt)+pi(cnt)+lpc(cnt)+sm(cnt)+pe(cnt)
3000tlip(cnt)=tpol(cnt)+neut(cnt)
3010ppol(cnt)=tpol(cnt)/tlip(cnt)*100
3020pneut(cnt)=neut(cnt)/tlip(cnt)*100
3030REM.PROPORTIONS OF POLAR CLASSES
```

```
3040ppc(cnt)=pc(cnt)/tpol(cnt)*ppol(cnt)
3050pps(cnt)=ps(cnt)/tpol(cnt)*ppol(cnt)
3060ppi(cnt)=pi(cnt)/tpol(cnt)*ppol(cnt)
3070ppe(cnt)=pe(cnt)/tpol(cnt)*ppol(cnt)
3080psm(cnt)=sm(cnt)/tpol(cnt)*ppol(cnt)
3090plpc(cnt)=lpc(cnt)/tpol(cnt)*ppol(cnt)
3100REM.PROPORTIONS OF NEUTRAL CLASSES
3110tneut(cnt)=waxester(cnt)+sterols(cnt)+tag(cnt)+freefatty(cnt)+cholest(cnt)
3120pwax(cnt)=waxester(cnt)/tneut(cnt)*pneut(cnt)
3130pster(cnt)=sterols(cnt)/tneut(cnt)*pneut(cnt)
3140ptag(cnt)=tag(cnt)/tneut(cnt)*pneut(cnt)
3150pfree(cnt)=freefatty(cnt)/tneut(cnt)*pneut(cnt)
3160pchol(cnt)=cholest(cnt)/tneut(cnt)*pneut(cnt)
3170ptag(cnt)=tag(cnt)/tneut(cnt)*pneut(cnt)
3180UNTIL cnt=11 OR analist$(cnt)="END"
3190REM.TO SUMMATE CLASSES
3200meancnt=cnt-1
3210FOR M=1 TO meancnt
3220sumlip=sumlip+tlip(M)
3230sumpol=sumpol+ppol(M)
3240sumneut=sumneut+pneut(M)
3250sumpc=sumpc+ppc(M)
3260sumps=sumps+pps(M)
3270sumpi=sumpi+ppi(M)
3280sumpe=sumpe+ppe(M)
3290sumsm=sumsm+psm(M)
3300sumlpc=sumlpc+plpc(M)
3310sumwax=sumwax+pwax(M)
3320sumsterol=sumsterol+pster(M)
3330sumtag=sumtag+ptag(M)
3340sumffa=sumffa+pfree(M)
3350sumchol=sumchol+pchol(M)
3360NEXT M
3370REM.TOCALCULATE MEANS
3380M=M-1
3390meanlip=sumlip/M
3400meanpol=sumpol/M
3410meanneut=sumneut/M
3420meanpc=sumpc/M
3430meanps=sumps/M
3440meanpi=sumpi/M
3450meanlpc=sumlpc/M
3460meansm=sumsm/M
3470meanwax=sumwax/M
3480meansterol=sumsterol/M
3490meantag=sumtag/M
3500meanffa=sumffa/M
3510meanchol=sumchol/M
3520REM. TO CALCULATE S.D. FROM MEANS
3530FOR M=1 TO meancnt
3540devlip=devlip+(tlip(M)-meanlip)^2
3550devneut=devneut+(pneut(M)-meanneut)^2
3560devpol=devpol+(ppol(M)-meanpol)^2
3570devpc=devpc+(ppc(M)-meanpc)^2
3580devps=devps+(pps(M)-meanps)^2
3590devpi=devpi+(ppi(M)-meanpi)^2
3600devpe=devpe+(ppe(M)-meanpe)^2
3610devlpc=devlpc+(plpc(M)-meanlpc)^2
3620devsm=devsm+(psm(M)-meansm)^2
3630devwax=devwax+(pwax(M)-meanwax)^2
3640devsterol=devsterol+(pster(M)-meansterol)^2
3650devffa=devffa+(pfree(M)-meanffa)^2
3660devtag=devtag+(ptag(M)-meantag)^2
3670devchol=devchol+(pchol(M)-meanchol)^2
3680NEXT M
3690M=M-1
3700sdlip=SQR(devlip/M)
3710sdpol=SQR(devpol/M)
3720sdneut=SQR(devneut/M)
3730sdpc=SQR(devpc/M)
3740sdps=SQR(devps/M)
3750sdpi=SQR(devpi/M)
3760sdpe=SQR(devpe/M)
3770sdsm=SQR(devsm/M)
3780sdlpc=SQR(devlpc/M)
3790sdwax=SQR(devwax/M)
3800sdsterol=SQR(devsterol/M)
3810sdtag=SQR(devtag/M)
3820sdffa=SQR(devffa/M)
3830sdchol=SQR(devchol/M)
3840CLS:PRINTTAB(0,12)"DO YOU WANT A PRINTED COPY ?"
3850PRINTTAB(0,13)"Y/N."
3860menopt=GET
3870 IF menopt=78 THEN CLS:PROCPRINT
3880 IF menopt=89 THEN 3900
3890IF menopt<>78 OR menopt<>89 THEN 3860
3900REM. TO ACTIVATE PRINTER
3910CLS
3920*FX5,1
3930*FX6
3940PRINTTAB(0,2)"TYPE IN TITLE"
3950INPUTTAB(0,3)A$
3960VDU2
3970PRINT A$
3980PROCPRINT
3990ENDPROC
4000REM. TO PRINT OUT
4010DEF PROCPRINT
4020@%=&20106
4030PRINT"NO. OF RODS ANALYSED ";meancnt
```

```
4050PRINT
4060PRINT"NEUTRAL LIPID (%)     "¦meanneut¦¦"  +/-  "¦sdneut
4070PRINT"POLAR LIPID (%)       "¦meanpol¦¦"  +/-  "¦sdpol
4080PRINT
4090PRINT"PC (%)                "¦meanpc¦¦"  +/-  "¦sdpc
4100PRINT"PE (%)                "¦meanpe¦¦"  +/-  "¦sdpe
4110PRINT"PI (%)                "¦meanpi¦¦"  +/-  "¦sdpi
4120PRINT"PS (%)                "¦meanps¦¦"  +/-  "¦sdps
4130PRINT"LYSO-PC (%)           "¦meanlpc¦¦"  +/-  "¦sdlpc
4140PRINT"SPHINGOMYELIN (%)     "¦meansm¦¦"  +/-  "¦sdsm
4150PRINT
4160PRINT"WAX ESTERS (%)        "¦meanwax¦¦"  +/-  "¦sdwax
4170PRINT"STEROL ESTERS (%)     "¦meansterol¦¦"  +/-  "¦sdsterol
4180PRINT"TRIACYLGLYCEROL (%)   "¦meantag¦¦"  +/-  "¦sdtag
4190PRINT"FREE FATTY ACIDS (%)  "¦meanffa¦¦"  +/-  "¦sdffa
4200PRINT"CHOLESTEROL (%)       "¦meanchol¦¦" +/-  "¦sdchol
4210PRINT
4220VDU3
4230PRINT"HIT 'SPACE-BAR' FOR MAIN MENU."
4240menopt=GET
4250IF menopt=32 THEN RUN
4260IF menopt<>32 THEN 4240
4270ENDPROC
```

Appendix III Program for the computation of fatty acid spectra from GLC traces

This program calculates the fatty acid distribution from gas-chromatograph data for samples run with, or without internal standard.

Installation

The program can be obtained on disc from the author. It was written in BBC basic on a model B system fitted with a single, 40 track disc drive and connected to an Epson compatible printer. The program is menu driven.

Section 1 (lines 10-180) should be stored on disc in file "GLC". Section 2 (lines 10-1980) should be stored in file "GLC1" and section 3 (lines 10-2140) in file "GLC3".

Type CHAIN"GLC" [RETURN] to begin the program.

Running the program.

1. On loading a menu giving a choice between analysis of traces with internal standard and without internal standard is presented.
2. After selecting which program to load, a spreadsheet is presented consisting of the fatty acids found in herring lipid samples, spaces for the amount of starting lipid, artefacts, total counts (and for internal standard analyses, the amount of internal standard used and the internal standard peak area).
3. Enter the integrated area for each fatty acid present in the gas-chromatograph trace followed by [RETURN]. If a fatty acid is absent press [RETURN] only.
4. Enter the data for the amount of lipid used and the total counts of the trace.
5. Enter the integrated areas of peaks to be ignored (artefacts). These peaks will be totalled and subtracted from the total counts before analysis. Press [RETURN] after entering the final artefact.
6. A choice of correcting data or continuing with the analysis will be presented.
7. Data correction is achieved by moving the cursor around the spreadsheet using the UP and DOWN ARROW keys ONLY. Press [RETURN] to correct an item, enter the correct data and press [RETURN] once more.
8. After the cursor is moved below the final item of the spreadsheet, the choice of correcting data or proceeding with the analysis is presented.
9. After selecting to continue with analysis, a choice of obtaining a hard copy or screen copy only is presented. Since the current version of the program does not allow data to be stored on disc, it is recommended that a hardcopy is always obtained.
10. After selecting hardcopy, enter a title for the results.
11. Ensure the printer is turned ON and is ON LINE.
12. The program first subtracts the total artefacts from the total counts entered. The proportion of each fatty acid in percentage terms is then computed. For samples containing internal standard, the ratio of total fatty acid peak areas/internal standard peak area is related directly to the amounts of internal standard and total lipid used. The program computes the yield of fatty acids in absolute terms. The results will include a summary of the amount of non-fatty acid material (called non-sapon) and fatty acids (sapon) in the sample.

The amounts of fatty acids in absolute and relative terms are presented along with a calculation of the percentage distribution had no internal standard been used.
The total saturates, monoenes, (n-3), (n-6) and the (n-3)/(n-6) ratio is also given.
After printing the results, the choice of entering another trace or of ending the session is presented.

Known bugs

Use of the LEFT and RIGHT arrow keys when within the DATA CORRECTION mode (see stage 7) will cause the program to freeze. It will be necessary to turn off the computer and reboot the program.

Developments

Complete redrafting of this program is currently being undertaken. The new version will speed up data input by changing the method of artefact handling, allow user defined fatty acids and unknowns to be included, allow the data to be stored to disc for reanalysis and combine the data entry and correction menus into a single spreadsheet.

SECTION 1

```
10REMMenu setup
20CLS:VDU 23,1,0;0;0;0;
30TIME=0
40PRINTTAB(9,2)"GLC trace analysis"
50PRINTTAB(17,3)"by"
60PRINTTAB(14,4)"Clive Fox"
70REPEAT UNTIL TIME>150
80CLS
90PRINTTAB(14,3)"MAIN MENU"
100PRINTTAB(14,4)"_________"
110PRINTTAB(12,6)"Select option"
120PRINTTAB(0,10)"1. Trace contains no internal standard."
130PRINTTAB(0,12)"2. Trace contains internal standard,"
140PRINTTAB(3,13)"amount of starting lipid known."
150menu=GET
160IF menu<49 OR menu>50 THEN 150
170IF menu=49 CHAIN"GLC1"
180IF menu=50 CHAIN"GLC2"
```

SECTION 2

```
10DIM AX(31)
20DIM PERA(26)
30MODE3
40PROCINTRO
70DEF PROCINTRO
80VDU3:CLS
90VDU 23,1,1;0;0;0;
100PRINTTAB(20,0)"GLC TRACE ANALYSIS - no internal standard"
110PRINTTAB(2,3)"14:0"
120PRINTTAB(2,4)"15:0"
130PRINTTAB(2,5)"16:0"
140PRINTTAB(2,6)"16:1"
150PRINTTAB(2,7)"16:2"
160PRINTTAB(2,8)"16:3"
170PRINTTAB(2,9)"16:4"
180PRINTTAB(2,10)"18:0"
190PRINTTAB(2,11)"18:1 n-9"
200PRINTTAB(2,12)"18:1 n-7"
210PRINTTAB(2,13)"18:2 n-6"
220PRINTTAB(2,14)"18:3 n-6"
230PRINTTAB(2,15)"18:3 n-3"
240PRINTTAB(40,3)"18:4 n-3"
250PRINTTAB(40,4)"20:0"
260PRINTTAB(40,5)"20:1"
270PRINTTAB(40,6)"20:2 n-6"
280PRINTTAB(40,7)"20:3 n-6"
290PRINTTAB(40,8)"20:4 n-6"
300PRINTTAB(40,9)"20:4 n-3"
310PRINTTAB(40,10)"20:5 n-3"
320PRINTTAB(40,11)"22:0"
330PRINTTAB(40,12)"22:1"
340PRINTTAB(40,13)"22:5 n-3"
350PRINTTAB(40,14)"22:6 n-3"
360PRINTTAB(40,15)"24:1"
370PRINTTAB(2,18)"Amount of lipid used (ug.)"
380PRINTTAB(2,19)"Total counts"
390PRINTTAB(60,19)"Tot. artefacts"
400PRINTTAB(2,20)"Artefacts"
410H=1:G=7
420INPUTTAB(12,G)AX(H)
430H=H+1:G=G+1
440IF H<14 GOTO 420 ELSE 450
450H=14:G=7
460INPUTTAB(50,3)AX(H)
470H=H+1:G=G+1
480 IF H<27 GOTO 460 ELSE 490
490H=27:G=18
500INPUTTAB(40,G)AX(H)
510H=H+1:G=G+1
520IF H<29 GOTO 500 ELSE 530
530 REPEAT
540PRINTTAB(40,3)SPC(20):INPUTTAB(40,G)B
550 IF B=0 THEN 590
560 AX(29)=AX(29)+B
570 PRINTTAB(65,3);AX(29)
580UNTIL B=0
590VDU 23,1,0;0;0;0;
600PRINTTAB(0,23)SPC(19)"SELECT '1' TO CONTINUE, '2' TO CORRECT ENTRY"
610select=GET
620IF select<49 OR select>50 THEN 610
630IF select=49 THEN PROCCAL
640IF select=50 THEN PROCCOR
650ENDPROC
```

```
660DEF PROCCAL
670REMCalculates peak areas
680H=1
690A%(28)=A%(28)-A%(29)
700ident=ident+A%(H)
710H=H+1
720IF H<27 THEN 700 ELSE 730
730 H=1
740PERA(H)=A%(H)/A%(28)*100
750H=H+1
760IF H<27 THEN 740 ELSE 770
770REMCalculate ident percent etc.
780total=ident
790unident=A%(28)-ident
800punident=unident/A%(28)*100
810pident=ident/A%(28)*100
820CLS:PRINT"Do you want a printed copy (Y/N)?"
830copy=GET
840IF copy=78 THEN 930
850IF copy=89 THEN 870
860IF copy <>89 OR copy <>78 THEN 830
870*FX3,1
880*FX6
890PRINTTAB(0,2)"Type in title"
900INPUTTAB(0,3)A$
910 VDU2
920PRINT A$
930@%=&2010F
940PRINTTAB(0,5)SPC(20);"  Percent"
960PRINT
970PRINT"Ident. peaks",pident
980PRINT"Unident. peaks",punident
990PRINT
1000PRINT"14:0",PERA(1)
1010PRINT"15:0",PERA(2)
1020PRINT"16:0",PERA(3)
1030PRINT"16:1",PERA(4)
1040PRINT"16:2",PERA(5)
1050PRINT"16:3",PERA(6)
1060PRINT"16:4",PERA(7)
1070PRINT"18:0",PERA(8)
1080PRINT"18:1 n-9",PERA(9)
1090PRINT"18:1 n-7",PERA(10)
1100PRINT"18:2 n-6",PERA(11)
1110PRINT"18:3 n-6",PERA(12)
1120PRINT"18:3 n-3",PERA(13)
1130PRINT"18:4 n-3",PERA(14)
1140PRINT"20:0",PERA(15)
1150PRINT"20:1",PERA(16)
1160PRINT"20:2 n-6",PERA(17)
1170PRINT"20:3 n-6",PERA(18)
1180PRINT"20:4 n-6",PERA(19)
1190PRINT"20:4 n-3",PERA(20)
1200 PRINT"20:5 n-3",PERA(21)
1210 PRINT"22:0",PERA(22)
1220 PRINT"22:1",PERA(23)
1230 PRINT"22:5 n-3",PERA(24)
1240 PRINT"22:6 n-3+24:0",PERA(25)
1250 PRINT"24:1",PERA(26)
1260PRINT
1270PRINT"Total sats.",PERA(1)+PERA(2)+PERA(3)+PERA(8)+PERA(15)+PERA(22)
1280PRINT"Total monoenes",PERA(4)+PERA(9)+PERA(10)+PERA(16)+PERA(23)+PERA(26)
1290PRINT"Total n-3",PERA(13)+PERA(14)+PERA(20)+PERA(21)+PERA(24)+PERA(25)
1300PRINT"Total n-6",PERA(11)+PERA(12)+PERA(17)+PERA(18)+PERA(19)
1310ratio=(PERA(13)+PERA(14)+PERA(20)+PERA(21)+PERA(24)+PERA(25))/(PERA(11)+PER
A(12)+PERA(17)+PERA(18)+PERA(19))
1320PRINT"n-3/n-6",ratio
1330VDU3
1340PRINT
1350PRINT"                    SELECT '1' TO ENTER ANOTHER TRACE, '2' TO END"
1360menu=GET
1370IF menu=49 THEN RUN
1380IF menu=50 THEN CLS:*BASIC
1390IF menu<49 OR menu>50 THEN 1360
1400ENDPROC
1410DEF PROCCOR
1420PRINTTAB(15,23)SPC(50)
1430PRINTTAB(10,0)"Use edit keys to move cursor, press RETURN to correct an ent
ry"
1440VDU 23,1,1;0;0;0;
1450G=3
1460*FX 4,1
1470VDU 31,12,8
1480move=GET
1490IF move=138 THEN 1520
1500IF move=139 THEN 1550
1510IF move<138 OR move>139 THEN 1930
1520G=G+1
1530IF G<16 GOTO 1470 ELSE 1570
1540GOTO 1470
1550G=G-1
1560IF G<3 THEN 1520 ELSE 1470
1570VDU 31,50,(G-13)
1580move=GET
1590 IF move=138 THEN 1620
1600 IF move=139 THEN 1650
1610IF move<138 OR move>139 THEN 1930
1620G=G+1
1630IF G<29 GOTO 1570 ELSE 1670
1640GOTO 1570
1650G=G-1
```

```
1640IF 8<16 THEN 1470 ELSE 1570
1670VDU 31,40,(8-11)
1680move=GET
1690 IF move=138 THEN 1720
1700 IF move=139 THEN 1750
1710IF move<138 OR move>139 THEN 1930
1720G=G+1
1730IF G<31 GOTO 1670 ELSE 1770
1740GOTO 1670
1750G=G-1
1760IF G<29 THEN 1570 ELSE 1670
1770VDU 31,40,(8-11)
1780move=GET
1790IF move=138 THEN 590
1800IF move=139 THEN 1910
1810IF move<138 OR move>139 THEN 1820
1820PRINTTAB(2,23)"Input correct data and press RETURN"
1830A%(29)=0
1840REPEAT
1850PRINTTAB(60,23)SPC(10):INPUTTAB(60,23)B
1860IF B=0 THEN PRINTTAB(60,23)SPC(10):PRINTTAB(65,20)SPC(20):GOTO 590
1870A%(29)=A%(29)+B
1880PRINTTAB(40,20)SPC(20):PRINTTAB(40,20);B
1890PRINTTAB(65,20)SPC(20):PRINTTAB(65,20);A%(29)
1900UNTILB=0
1910G=G-1
1920GOTO 1670
1930PRINTTAB(2,23)"Input correct data and press RETURN"
1940INPUTTAB(60,23)A%(G-2)
1950IF G<16 THEN PRINTTAB(12,G)SPC(10):PRINTTAB(12,G);A%(G-2):PRINTTAB(60,23): P
C(10):GOTO 1520
1960IF G>=16 AND G<29 THEN PRINTTAB(50,G-13)SPC(10):PRINTTAB(50,G-13);A%(G-2):P
RINTTAB(60,23)SPC(10):GOTO1620
1970IF G>=29 AND G<33 THEN PRINTTAB(40,G-11)SPC(10):PRINTTAB(40,G-11);A%(G-2):P
RINTTAB(60,23)SPC(10):GOTO 1720
1980ENDPROC
```

SECTION 3

```
10DIM A%(31)
20DIM PERA(26)
30DIM A(26)
40DIM PA(26)
50MODE3
60PROCINTRO
70DEF PROCINTRO
80VDU5:CLS
90VDU 23,1,1;0;0;0;
100PRINTTAB(20,0)"GLC TRACE ANALYSIS - with internal standard"
110PRINTTAB(2,3)"14:0"
120PRINTTAB(2,4)"15:0"
130PRINTTAB(2,5)"16:0"
140PRINTTAB(2,6)"16:1"
150PRINTTAB(2,7)"16:2"
160PRINTTAB(2,8)"16:3"
170PRINTTAB(2,9)"16:4"
180PRINTTAB(2,10)"18:0"
190PRINTTAB(2,11)"18:1 n-9"
200PRINTTAB(2,12)"18:1 n-7"
210PRINTTAB(2,13)"18:2 n-6"
220PRINTTAB(2,14)"18:3 n-6"
230PRINTTAB(2,15)"18:3 n-3"
240PRINTTAB(40,3)"18:4 n-3"
250PRINTTAB(40,4)"20:0"
260PRINTTAB(40,5)"20:1"
270PRINTTAB(40,6)"20:2 n-6"
280PRINTTAB(40,7)"20:3 n-6"
290PRINTTAB(40,8)"20:4 n-6"
300PRINTTAB(40,9)"20:4 n-3"
310PRINTTAB(40,10)"20:5 n-3"
320PRINTTAB(40,11)"22:0"
330PRINTTAB(40,12)"22:1"
340PRINTTAB(40,13)"22:5 n-3"
350PRINTTAB(40,14)"22:6 n-3"
360PRINTTAB(40,15)"24:1"
370PRINTTAB(2,17)"Total counts"
380PRINTTAB(2,18)"Internal std."
390PRINTTAB(2,19)"Amount of lipid used (ug.)"
400PRINTTAB(2,20)"Amount of internal std. used (ug.)"
410PRINTTAB(60,20)"Tot. artefacts"
420PRINTTAB(2,21)"Artefacts"
```

```
430H=1:G=3
440INPUTTAB(12,G)AX(H)
450H=H+1:G=G+1
460IF H<14 GOTO 440 ELSE 470
470H=14:G=3
480INPUTTAB(50,G)AX(H)
490H=H+1:G=G+1
500 IF H<27 GOTO 480 ELSE 510
510H=27:G=17
520INPUTTAB(40,G)AX(H)
530H=H+1:G=G+1
540IF H<31 GOTO 520 ELSE 550
550 REPEAT
560PRINTTAB(40,G)SPC(20):INPUTTAB(40,G)B
570 IF B=0 THEN 610
580 AX(31)=AX(31)+B
590 PRINTTAB(65,G);AX(31)
600UNTIL B=0
610VDU 23,1,0;0;0;0;
620PRINTTAB(0,23)SPC(15)"SELECT '1' TO CONTINUE, '2' TO CORRECT ENTRY"
630select=GET
640IF select <49 OR select >50 THEN 630
650IF select=49 THEN PROCCAL
660IF select=50 THEN PROCCOR
670ENDPROC
680DEF PROCCAL
690REMCalculates peak areas
700H=1
710AX(27)=AX(27)-(AX(28)+AX(31))
720ident=ident+AX(H)
730H=H+1
740IF H<27 THEN 720 ELSE 750
750fact=AX(30)/AX(28)
760 H=1
770PERA(H)=AX(H)/AX(27)*100
780A(H)=AX(H)*fact:PA(H)=A(H)/AX(29)*100
790H=H+1
800IF H<27 THEN 770 ELSE 810
810REMCalculate total lipid etc.
820total=ident
830unident=AX(27)-ident
840unident=unident*fact:punident=unident/AX(29)*100
850ident=ident*fact:pident=ident/AX(29)*100
860tot=AX(27)*fact:ptot=tot/AX(29)*100
870nonsap=AX(29)-tot:pnonsap=nonsap/AX(29)*100
880IFtot>AX(29) THEN 890 ELSE 960
890CLS:PRINT"                                        ERROR"
900PRINT
910PRINT"        Total amount of lipid calculated is greater than that used."
920PRINT"        Implies that less than correct volume of standard was added."

930PRINT
940PRINT
950GOTO1510
960CLS:PRINT"Do you want a printed copy (Y/N)?"
970copy=GET
980IF copy=78 THEN 1070
990IF copy=89 THEN 1010
1000IF copy <>89 OR copy <>78 THEN 970
1010*FX5,1
1020*FX6
1030PRINTTAB(0,2)"Type in title"
1040INPUTTAB(0,3)A$
1050 VDU2
1060PRINT A$
1070@%=&2010F
1080PRINTTAB(0,5)SPC(20);"  Percent          Amount          Percent"
1090PRINT,SPC(20)" no int. std.       (ug.)          (by wt.)"
1100PRINT
1110PRINT"Sapon."SPC(15),tot,ptot
1120PRINT"Non-sapon."SPC(15),nonsap,pnonsap
1130PRINT"Ident. peaks",total/AX(27)*100,ident,pident
1140PRINT"Unident. peaks",100-(total/AX(27)*100),unident,punident
1150PRINT
1160PRINT"14:0",PERA(1),A(1),PA(1)
1170PRINT"15:0",PERA(2),A(2),PA(2)
1180PRINT"16:0",PERA(3),A(3),PA(3)
1190PRINT"16:1",PERA(4),A(4),PA(4)
1200PRINT"16:2",PERA(5),A(5),PA(5)
1210PRINT"16:3",PERA(6),A(6),PA(6)
1220PRINT"16:4",PERA(7),A(7),PA(7)
1230PRINT"18:0",PERA(8),A(8),PA(8)
1240PRINT"18:1 n-9",PERA(9),A(9),PA(9)
1250PRINT"18:1 n-7",PERA(10),A(10),PA(10) - -
1260PRINT"18:2 n-6",PERA(11),A(11),PA(11)
1270PRINT"18:3 n-6",PERA(12),A(12),PA(12)
1280PRINT"18:3 n-3",PERA(13),A(13),PA(13)
1290PRINT"18:4 n-3",PERA(14),A(14),PA(14)
1300PRINT"20:0",PERA(15),A(15),PA(15)
1310PRINT"20:1",PERA(16),A(16),PA(16)
1320PRINT"20:2 n-6",PERA(17),A(17),PA(17)
1330PRINT"20:3 n-6",PERA(18),A(18),PA(18)
1340PRINT"20:4 n-6",PERA(19),A(19),PA(19)
1350PRINT"20:4 n-3",PERA(20),A(20),PA(20)
1360 PRINT"20:5 n-3",PERA(21),A(21),PA(21)
1370 PRINT"22:0",PERA(22),A(22),PA(22)
1380 PRINT"22:1",PERA(23),A(23),PA(23)
1390 PRINT"22:5 n-3",PERA(24),A(24),PA(24)
1400 PRINT"22:6 n-3",PERA(25),A(25),PA(25)
1410 PRINT"24:1",PERA(26),A(26),PA(26)
```

```
1420PRINT
1430PRINT"Total sats.",PERA(1)+PERA(2)+PERA(3)+PERA(8)+PERA(15)+PERA(22),A(1)+A
(2)+A(3)+A(8)+A(15)+A(22),PA(1)+PA(2)+PA(3)+PA(8)+PA(15)+PA(22)
1440PRINT"Total monoenes",PERA(4)+PERA(9)+PERA(10)+PERA(16)+PERA(23)+PERA(26),A
(4)+A(9)+A(10)+A(16)+A(23)+A(26),PA(4)+PA(9)+PA(10)+PA(16)+PA(23)+PA(26)
1450PRINT"Total n-3",PERA(13)+PERA(14)+PERA(20)+PERA(21)+PERA(24)+PERA(25),A(13
)+A(14)+A(20)+A(21)+A(24)+A(25),PA(13)+PA(14)+PA(20)+PA(21)+PA(24)+PA(25)
1460PRINT"Total n-6",PERA(11)+PERA(12)+PERA(17)+PERA(18)+PERA(19),A(11)+A(12)+A
(17)+A(18)+A(19),PA(11)+PA(12)+PA(17)+PA(18)+PA(19)
1470ratio=(A(13)+A(14)+A(20)+A(21)+A(24)+A(25))/(A(11)+A(12)+A(17)+A(18)+A(19))
1480PRINT"n-3/n-6",ratio,ratio,ratio
1490VDU5
1500PRINT
1510PRINT"                    SELECT '1' TO ENTER ANOTHER TRACE, '2' TO END"
1520menu=GET
1530IF menu=49 THEN RUN
1540IF menu=50 THEN CLS:*BASIC
1550IF menu<49 OR menu>50 THEN 1520
1560ENDPROC
1570DEF PROCCOR
1580PRINTTAB(15,23)SPC(50)
1590PRINTTAB(10,0)"Use edit keys to move cursor, press RETURN to correct an ent
ry"
1600VDU 23,1,1;0;0;0;
1610G=3
1620*FX 4,1
1630VDU 31,12,8
1640move=GET
1650IF move=138 THEN 1680
1660IF move=139 THEN 1710
1670IF move<138 OR move>139 THEN 2090
1680G=G+1
1690IF G<16 GOTO 1630 ELSE 1730
1700GOTO 1630
1710G=G-1
1720IF G<3 THEN 1680 ELSE 1630
1730VDU 31,30,(G-13)
1740move=GET
1750 IF move=138 THEN 1780
1760 IF move=139 THEN 1810
1770IF move<138 OR move>139 THEN 2090
1780G=G+1
1790IF G<29 GOTO 1730 ELSE 1830
1800GOTO 1730
1810G=G-1
1820IF G<16 THEN 1630 ELSE 1730
1830VDU 31,40,(G-12)
1840move=GET
1850 IF move=138 THEN 1880
1860 IF move=139 THEN 1910
1870IF move<138 OR move>139 THEN 2090
1880G=G+1
1890IF G<33 GOTO 1830 ELSE 1930
1900GOTO 1830
1910G=G-1
1920IF G<29 THEN 1730 ELSE 1830
1930VDU 31,40,(G-12)
1940move=GET
1950IF move=138 THEN 610
1960IF move=139 THEN 2070
1970IF move<138 OR move>139 THEN 1980
1980PRINTTAB(2,23)"Input correct data and press RETURN"
1990A%(31)=0
2000REPEAT
2010PRINTTAB(60,23)SPC(10):INPUTTAB(60,23)B
2020IF B=0 THEN PRINTTAB(60,23)SPC(10):PRINTTAB(40,21)SPC(20):PRINTTAB(60,21)SP
C(20):GOTO 610
2030A%(31)=A%(31)+B
2040PRINTTAB(40,21)SPC(20):PRINTTAB(40,21);B
2050PRINTTAB(65,21)SPC(20):PRINTTAB(65,21);A%(31)
2060UNTILB=0
2070G=G-1
2080GOTO 1830
2090PRINTTAB(2,23)"Input correct data and press RETURN"
2100INPUTTAB(60,23)A%(G-2)
2110IF G<16 THEN PRINTTAB(12,8)SPC(10):PRINTTAB(12,8);A%(G-2):PRINTTAB(60,23)SP
C(10):GOTO 1680
2120IF G>=16 AND G<29 THEN PRINTTAB(50,G-13)SPC(10):PRINTTAB(50,G-13);A%(G-2):P
RINTTAB(60,23)SPC(10):GOTO1780
2130IF G>=29 AND G<34 THEN PRINTTAB(40,G-12)SPC(10):PRINTTAB(40,G-12);A%(G-2):P
RINTTAB(60,23)SPC(10):GOTO 1880
2140ENDPROC
```

References

Ackman R.G. (1981) Problems in introducing new chromatographic techniques for lipid analyses. *Chem. Ind.* 17 Oct. 39-46

Ackman R.G. (1982) Fatty acid composition of fish oil. In: NUTRITIONAL EVALUATION OF LONG CHAIN FATTY ACIDS IN FISH OIL (eds. Barlow S.M. & Stanley M.E.) Academic Press, London. 25-88

Ackman R.G. & Tocher C.S. (1968) Marine phytoplankter fatty acids. *J. Fish. Res. Board Can.* 25 1603-1620

Alderdice D.F. (1988) Osmotic and ionic regulation in teleost eggs and larvae. In: FISH PHYSIOLOGY. (eds. Hoar W.S., Randall D.J.) Academic Press Inc. Vol. XI. Part A. 163-251

Applegate K.R. & Glomset J.A. (1986) Computer-based modeling of the conformation and packing properties of docosahexaenoic acid. *J. Lipid Res.* 27 658-680

Ash R. (1985) Protein digestion and absorption. In NUTRITION AND FEEDING IN FISH. (eds. Cowey C.B., Mackie A.M. Bell J.G.) Academic Press Inc., London. 69-93

Baker D,H. (1986) Problems and pitfalls in animal experiments designed to establish dietary nutrient requiremnets for essential nutrients. *J. Nutr.* 116 2339-2349

Bainbridge V. & Forsyth D.C.T. (1971) The feeding of herring larvae in the Clyde. *Rapp. P.-V. Réun. Cons. int. Explor. Mer.* 160 104-113

Ballaer E.V., Amat F., Hontoria F. & Léger P. & Sorgeloos P. (1985) Preliminary results on the nutritional evaluation of omega3-HUFA enriched *Artemia* nauplii for larvae of the sea bass, *Dicentrarchus labrax*. *Aquaculture* 49 223-229

Bangham A.D. (1981) Introduction. In: LIPOSOMES: FROM PHYSICAL STRUCTURE TO THERAPEUTIC APPLICATIONS. (ed. Knight) Elsevier/North-Holland, Biomedical Press. 1-17

Bangham A.D., Standish M.M. & Watkins J.C. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* 13 238-252

Batty R.S., Blaxter J.H.S. & Libby D.A. (1986) Herring (*Clupea harengus*) filter-feeding in the dark. *Mar. Biol.* 91 371-375

Bautista M.N. & De La Cruz M.C. (1988) Linoleic (omega6) and linolenic (omega3) acids in the diet of fingerling milkfish (*Chanos chanos* Forsskal). *Aquaculture* 71 347-358

Becker W. & Bruce A (1985) Autoradiographic studies with fatty acids and some other lipids: A review. *Prog. Lipid Res.* 24 325-346

Bell M.V., Simpson C.M.F. & 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

Bell M.V., Henderson R.J., Pirie B.J.S. & Sargent J.R. (1985a) Effects of dietary polyunsaturated fatty acid deficiencies on mortality, growth and gill structure in the turbot, *Scophthalmus maximus*. *J. Fish Biol.* 26 181-191

Bell M.V., Henderson R.J. & Sargent J.R. (1985b) Changes in the fatty acid composition of phospholipids from turbot (*Scophthalmus maximus*) in relation to dietary polyunsaturated fatty acid deficiencies. *Comp. Biochem. Physiol.* 81B 193-198

Bell M.V., Henderson R.J. & Sargent J.R. (1986) Minireview - The role of polyunsaturated fatty acids in fish. *Comp. Biochem. Physiol.* 83B 711-719

Bell M.V. & Tocher D.R. (1989) Molecular species composition of the major phospholipids in brain and retina from rainbow trout (*Salmo gairdneri*). *Biochem. J.* 264 909-915

Benjits F., Van Voorden E. & Sorgeloos P. (1975) Changes in the biochemical composition of the early larval stages of the brine shrimp, *Artemia salina* L. *Proc. Eur. Symp. Mar. Biol. 10th*, Ostend, Belgium, Sept. 17-23, 1975, Universa Press, Wetteren, Belgium. 1 1-9

Ben-Amotz A., Fishler R. & Schneller A. (1987) Chemical composition of dietary species of marine unicellular algae and rotifers with emphasis on fatty acids. *Mar. Biol.* 95 31-36

Beyer J.E. (1980) Feeding success of clupeoid fish larvae and stochastic thinking. *Dana* 1 65-91

Blaxter J.H.S. (1965) The feeding of herring larvae and their ecology in relation to feeding. *Calif. Coop Oceanic Fish. Invest. Rep. 10 79-88*

Blaxter J.H.S. (1968a) Rearing herring larvae to metamorphosis and beyond. *J. Mar. Biol. Assoc. U.K.* 48 17-28

Blaxter J.H.S. (1968b) Visual thresholds and spectral sensitivity of herring larvae. *J Exp. Biol.* 48 39-53

Blaxter J.H.S. (1975) Reared and wild fish - how do they compare? *Proc. Eur. Symp. Mar. Biol. 10th*, Ostend, Belgium, Sept. 17-23, 1975, Universa Press, Wetteren, Belgium. 1 11-26

Blaxter J.H.S. & Hunter J.R. (1982) The biology of Clupeoid fishes. *Adv. Mar. Biol.* 20 1-223

Bloch K. & Vance D. (1977) Control mechanisms in the synthesis of saturated fatty acids. *Annu. Rev. Biochem.* 46 263-298

Briggs M.R.P., Jauncey K. & Brown J.H. (1988) The cholesterol and lecithin requirements of juvenile prawn (*Macrobrachium rosenbergii*) fed semi-purified diets. *Aquaculture* 70 121-129

Bromley P.J. & Howell B.R. (1983) Factors influencing the survival and growth of turbot larvae, *Scophthalmus maximus* L., during the change from live to compound feeds. *Aquaculture* 31 31-40

Bruggeman E., Baeza-Mesa M., Bossuyt E. & Sorgeloos P. (1979) Improvements in the decapsulation of *Artemia* cysts. *European Maricult. Soc. Spec. Publ.* 4 309-315

Castell J.D. (1979) Review of lipid requirements of finfish. *Proc. World Symp. on Finfish Nutrition and Fishfeed Technology*, Hamburg 20-23 June 1978. 1 59-84

Castell J.D., Sinnhuber R.O., Wales J.H. & Lee D.J. (1972a) Essential fatty acids in the diet of rainbow trout (*Salmo gairdneri*): Growth, feed conversion and some gross deficiency symptoms. *J. Nutr.* 102 77-86

Castell J.D., Lee D.J. & Sinnhuber R.O. (1972b) Essential fatty acids in the diet of rainbow trout (*Salmo gairdneri*): Lipid metabolism and Fatty acid composition. *J. Nutr.* 102 93-100

Castell J.D., Sinnhuber R.O., Lee D.J. & Wales J.H. (1972c) Essential fatty acids in the diet of rainbow trout (*Salmo gairdneri*): Physiological symptoms of EFA deficiency. *J. Nutr.* 102 87-92

CCAP CATALOQUE 1988 Freshwater Biological Association, Ambleside, Cumbria (eds. Thompson A.S., Rhodee J.C., Rettman I.)

Chang T.M.S., MacIntosh F.C. & Mason S.G. (1966) Semipermeable aqueous microcapsules I. Preparation and Properties. *Can. J. Physiol. Pharmacol.* 44 115-128

Chapman D. (1982) BIOLOGICAL MEMBRANES. Academic Press, London. 526 pp.

Checkley .M.Jr. (1982) Selective feeding by Atlantic herring (*Clupea harengus*) larvae on zooplankton assemblages. *Mar. Ecol. Prog. Ser.* 9 245-253

Christie W.W. (1972) Quantitative esterification of lipids on thin-layer adsorbents. *Analyst* 97 221-223

Christie W.W. (1982) LIPID ANALYSIS (2nd ed) Pergamon Press, Oxford, England. 207 pp

Clark J., Quayle K.A., MacDonald N.L. and Stark J.R. (1988) Metabolism in marine flatfish - V. Chitinolytic activities in Dover sole, *Solea solea* (L.) *Comp. Biochem. Physiol.* 90B 379-384

Claus C., Benjits F., Vandeputte G. & Gardner W. (1979) The biochemical composition of the larvae of two strains of *Artemia salina* (L.) reared on two different algal foods. *J. Exp. Mar. Biol. Ecol.* 36 171-183

Clemmesen C.M. (1988) A RNA and DNA fluorescence technique to evaluate the nutritional condition of individual marine fish larvae. *Meeresforsch* 32 143-143

Conklin D.E., D'Abramo L.R., Bordner C.E. & Baum N.A. (1980) A successful purified diet for the culture of juvenile lobsters: the effect of lecithin. *Aquaculture* 21 243-249

Conover W.J. (1980) PRACTICAL NONPARAMETRIC STATISTICS. (2nd ed), John Wiley & Sons Inc., New York. 494 pp

Cousin J.C.B., Baudin-Laurencin F. & Gabaudan J. (1987) Ontogeny of enzymatic activities in fed and fasting turbot, *Scophthalmus maximus* L. *J. Fish Biol.* 30 15-33

Cowey C.B. (1976) Use of synthetic diets and biochemical criteria in the assessment of nutrient requirements of fish. *J. Fish. Res. Board Can.* 33 1040-1045

Cowey C.B., Adron J.W., Owen J.M. & Roberts R.J. (1976b) The effect of different dietary oils on tissue fatty acids and tissue pathology in turbot *Scophthalmus maximus*. *Comp. Biochem. Physiol.* 53B 399-403

Cowey C.B., Mackie A.M. & Bell J.G. (1985) NUTRITION AND FEEDING IN FISH. Academic Press, London. 489 pp.

Cowey C.B., Owen J.M., Adron J.W. & Middleton C. (1976a) Studies on the nutrition of marine flatfish. The effect of different dietary fatty acids on the growth and fatty acid composition of turbot (*Scophthalmus maximus*). *Br. J. Nutr.* 36 479-486

Cowey C.B. & J.R. Sargent (1977) Lipid nutrition in fish. *Comp. Biochem. Physiol.* 57B 269-273

Csengeri I., Majoros F., Olah J. & Farkas T. (1977) Influence of essential fatty acid-containing oils on the fatty acid composition of liver lipids in the common carp. *European Maricult. Soc. Spec. Publ.* 4 87-106

Dabrowski K.R. (1986) Mini-review: Ontogentical aspects of nutritional requirements in fish. *Comp. Biochem. Physiol.* 85A 639-655

Dabrowski K. & Glogowski J. (1977) A study of the application of proteolytic enzymes to fish food. *Aquaculture* 12 349-360

Davies J. (1985) The role of dietary fibre in fish nutrition. In: RECENT ADVANCES IN AQUACULTURE Vol II (eds. Muir J.F. & R.J. Roberts). Croom-Helm, London. 219-249

Davis S.S., Mills S.N. & Tomlinson E. (1987) Chemically cross-linked albumin microspheres for the controlled release of incorporated rose bengal after intramuscular injection into rabbits. *J. Controlled Release* 4 293-302

De Los Santos Jr.C., Sorgeloos P., Lavina E. & Bernardino A. (1980) In: THE BRINE SHRIMP ARTEMIA, Vol 3 (ed. Personne G., Sorgeloos P., Roels O. & Jaspers E.), Universa Press, Wetteren, Belgium. 159-163

Dendrinos P. & Thorpe J.P. (1987) Experiments on the artificial regulation of the amino acid and fatty acid contents of food organisms to meet the assessed nutritional requirements of larval, post-larval and juvenile Dover sole [*Solea solea*(L.)] *Aquaculture* 61 121-154

Dendrinos P., Dewan S. & Thorpe J.P. (1984) Improvement in the feeding efficiency of larval, postlarval and juvenile Dover sole (*Solea solea* L.) by the use of staining to improve the visibility of *Artemia* used as food. *Aquaculture* 38 137-144

Doyle M.J. (1977) A morphological staging system for the larval development of the herring, *Clupea harengus* L. *J. Mar. Biol. Assoc. U.K.* 57 859-867

Dratz E.A. & Deese A.J. (1986) The role of docosahexaenoic acid (22:6omega3) in biological membranes: Examples from photoreceptors and model membrane bilayers. In: HEALTH EFFECTS OF POLYUNSATURATED FATTY ACIDS IN SEAFOODS. (eds. Simopoulos A.P., Kifer R.R. & Martin R.E.), Academic Press Inc., New York. 319-351

Eggers D.M. (1977) The nature of prey selection by planktivorous fish. *Ecology* 58 46-59

Evans D.H. (1980) Osmotic and ionic regulation by freshwater and marine fishes. In: ENVIRONMENTAL PHYSIOLOGY OF FISHES. (ed. Ali M.A.), Plenum Press, N.Y. 93-122

Falk-Petresen S., Falk-Petersen I.-B., Sargent J.R. & Haug T. (1986) Lipid class and fatty acid composition of eggs from the Atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture* 52 207-211

Falk-Petersen S., Sargent J.R., Fox C., Falk-Petersen I-B., Haug T. & Kjørsvik E. (1989) Lipids in Atlantic halibut (*Hippoglossus hippoglossus*) eggs from planktonic samples in Northern Norway. *Mar. Biol.* 101 553-556

Farkas T., Csengeri I., Majoros & Olah J. (1977) Metabolism of fatty acids in fish. 1. Development of essential fatty acid deficiency in the carp, *Cyprinus carpio* Linnaeus 1758. *Aquaculture* 11 147-157

Ferry R. & Lutz P. (1987) Energy partitioning in fish: The activity-related cost of osmoregulation in a euryhaline cichlid. *J. Exp. Biol.* 128 63-85

Folch J., Lees M. & Sloane Stanley G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226 497-509

Forster J.R.M. & Wickins J.F. (1967) I.C.E.S. Maricult. Committee E:13, 9 pp

Foskett J.K., Bern H.A., Machen T.E. & Conner M. (1983) Chloride cells and the hormonal control of teleost fish osmoregulation. *J. Exp. Biol.* 106 255-281

Fraser A.J. (1986) Synthesis, transfer and utilisation of lipids and fatty acids in experimental marine food chains. PhD. thesis. University of Aberdeen

Fraser A.J. (1989) Triacylglycerol content as a condition index for fish, bivalve and crustacean larvae. *Can. J. Fish. Aquatic Sci.* 46 1868-1873

Fraser A.J., Tocher D.R. & Sargent J.R. (1985) Thin-layer chromatography - flame ionization detection and the quantitation of marine neutral lipids and phospholipids. *J. Exp. Mar. Biol. Ecol.* 88 91-99

Fraser A.J., Sargent J.R., Gamble J.C. & MacLachlan P.M. (1987) Lipid class and fatty acid composition as indicators of the nutritional condition of larval Atlantic herring. *Am. Fish. Soc. Symp.* 2 129-143

Fraser A.J., Gamble J.C. & Sargent J.R. (1988) Changes in lipid content, lipid class composition and fatty acid composition of developing eggs and unfed larvae of cod (*Gadus morhua*) *Mar. Biol.* 99 307-313

Furspan P., Prange H.D. & Greenwald I. (1984) Energetics and osmoregulation in the catfish, *Ictalurus punctatus*. *Comp. Biochem. Physiol.* 77A 773-778

Gamble J.C. & Davies J.M. (1982) Application of enclosures to the study of marine pelagic systems. In: MARINE MESOCOSMS, BIOLOGICAL AND CHEMICAL RESEARCH IN EXPERIMENTAL ECOSYSTEMS (eds. George G.D. & Reeve M.R.), Springer-Verlag, New York Inc. 25-48

Gamble J.C., Davies J.M. & Steele J.H. (1977) Loch Ewe bag experiment, 1974. *Bull. Mar. Sci.* 27 146-175

Gamble J.C., MacLachlan P., Nicoll N.T. & Baxter I.G. (1981) Growth and feeding of Atlantic herring larvae reared in large plastic enclosures. *Rapp. P-v. Réun. Cons. int. Explor. Mer.* 178 121-134

Gamble J.C. & Houde E.D (1984) Growth, mortality and feeding of cod (*Gadus morhua* L.) larvae in enclosed water columns and in laboratory tanks. In: THE PROPAGATION OF COD *Gadus morhua* L.: AN INTERNATIONAL SYMPOSIUM, (eds. Dahl E., Danielssen D.S., Moksness E. & Solemdal P.), Arendal, Norway, 14-17 June 1983, *Flødevigen rapporter* 1 123-143

Garatun-Tjeldstø O., Opstad I., Hansen T. & Huse I. (1989) Fish roe as a major component in start-feed for marine fish larvae. *Aquaculture* 79 353-362

Gatten R.R., Sargent J.R. & Gamble J.C. (1983) Diet induced changes in fatty acid composition of herring larvae reared in enclosed ecosystems. *J. Mar. Biol. Assoc. U.K.* 63 575-584

German B., Bruckner G. & Kinsella J. (1983) Evidence against a PGF_{4a} prostaglandin structure in trout tissue - a correction. *Prostaglandins* 26 207-210

Gibson R. (1981) Feeding and digestion in decapod crustaceans. *Proc. Second Int. Conf. on Aquacult. Nutr.: Biochem. Physiol. Approaches to Shellfish Nutr.*, Reheboth Beach, Delaware, 27-29 Oct. 1981, (eds. Pruder G.D., Langdon C.J. & Conklin D.E.) Louisiana State University Press, Baton Rouge. *World Maricult. Soc. Spec. Publ.* 2 59-70

Gibson J.S., Ellory J.C. & Lahlou B. (1987) Salinity acclimation and intestinal salt transport in the flounder: The role of the basolateral cell membrane. *J. Exp. Biol.* 128 371-382

Gier J.De. (1988) The use of liposomes in the search for an understanding of the significance of membrane lipid diversity. *Biochem. Soc. Trans.* 16 912-914

Girrard J.P., Thomson A.J. & Sargent J.R. (1977) Adrenalin induced turnover of phosphatidic acid and phosphatidylinositol in chloride cells from the gills of *Anguilla anguilla*. *FEBS Lett.* 73 267-270

Goodrich T.D. & Morita R.Y. (1977) Bacterial chitinase in the stomachs of marine fishes from Yaquina Bay, Oregon, U.S.A. *Mar. Biol.* 41 355-360

Govoni J.J., Boehlert G.W. & Watanabe Y. (1986) The physiology of digestion in fish larvae. *Env. Biol. Fishes* 16 59-77

Greene D.H.S. & Selivonchick D.P. (1987) Lipid metabolism in fish. *Prog. Lipid Res.* 26 53-85

Guggino W.B. (1980) Water balance in embryos of *Fundulus heteroclitus* and *F. bermudae* adapted in seawater. *Am. J. Physiol.* 238 R42-R49

Gurr M.I. & James A.T. (1975) LIPID BIOCHEMISTRY. (2nd ed), Chapman and Hall, London. 244 pp

Hakanson J.L. (1984) The long and short term feeding condition in field-caught *Calanus pacificus*, as determined from the lipid content. *Limnol. Oceanogr.* 29 794-804

Hanson B.J., Cummins K.W., Cargill A.S. & Lowry R.R. (1985) Lipid content, fatty acid composition and the effect of diet on fats of aquatic insects. *Comp. Biochem. Physiol.* 80B 257-276

Harrison P.T.C. (1984) A technique for investigating food utilization in individual small aquatic invertebrates. *Comp. Biochem. Physiol.* 77A 769-771

Hay D.E. (1981) Effects of capture and fixation on gut contents and body size of Pacific herring larvae. *Rapp. P.-v. Réun. Cons. int. Explor. Mer.* 178 395-400

Hazel J.R. (1984) Effects of temperature on the structure and metabolism of cell membranes in fish. *Am J. Physiol.* 246 R460-R470

Henderson R.J. & Sargent J.R. (1985) Fatty acid metabolism in fish. In NUTRITION AND FEEDING IN FISH. (eds. Cowey C.B., Mackie A.M. & Bell J.C.), Academic Press Inc. (London) Ltd. 349-364

Henderson R.J. & Tocher D.R. (1987) The lipid composition and biochemistry of freshwater fish. *Prog. Lipid Res.* 26 281-347

Henderson R.J., Bell M.V. & Sargent J.R. (1985) The conversion of polyunsaturated fatty acids to prostaglandins by tissue homogenates of the turbot, *Scophthalmus maximus* (L.) *J. Exp. Mar. Biol. Ecol.* 85 93-99

Hettler W. (1981) Spawning and rearing Atlantic menhaden. *Prog. Fish. Cult.* 43 80-84

Hinchcliffe P.R. & Riley J.P. (1972) The effect of diet on the component fatty-acid composition of *Artemia salina*. *J. Mar. Biol. Assoc. U.K.* 52 203-211

Hjelmeland K., Pedersen B.H. & Milssen E.M. (1989) Trypsin content in intestines of herring larvae (*Clupea harengus*) ingesting inert polystyrene spheres or live crustacea prey. *Mar. Biol.* 98 331-335

Holliday F.G.T. (1969) The effects of salinity on the eggs and larvae of teleosts. In: FISH PHSIOLOGY. (eds. Hoar W.S. & Randall D.J.) Academic Press Inc. (London) Ltd. I 293-311

Holliday F.G.T. & Blaxter J.H.S. (1960) The effects of salinity on the developing eggs and larvae of the herring. *J. Mar. Biol. Assoc. U.K.* 39 591-603

Hope M.J., Bally M.B., Mayer L.D., Janoff A.S. & Cullis P.R. (1986) Generation of multilamellar and unilamellar phospholipid vesicles. *Chem. Phys. Lipids* 40 89-107

Houde E.D. (1975) Effects of stocking density and food density on survival, growth and yield of laboratory-reared larvae of sea bream *Archosargus rhomboidalis* (L.)(Sparidae) *J. Fish Biol.* 7 115-127

Houde E.D. (1987) Fish early life dynamics and recruitment variability. *Am. Fish. Soc. Symp.* 2 17-29

Houde E.D. & Schekter R.C. (1980) Feeding by marine fish larvae: developmental and functional responses. *Env. Fish. Biol.* 5 315-334

Howell B.R. (1979) Experiments on the rearing of larval turbot, *Scophthalmus maximus* L. *Aquaculture* 18 215-225

IUPAC-IUB Commission on Biochemical Nomenclature (1977) The nomenclature of lipids. *Eur. J. Biochem.* 79 11-21

Iwai T. & Rosenthal H. (1981) Ciliary movement in guts of early clupeoid and salangid larvae. *Mar. Ecol. Prog. Ser.* 4 365-367

Izquierdo M.S., Watanabe T., Takeuchi T., Arakawa T. & Kitajima C. (1989) Requirement of larval red seabream *Pagrus major* for essential fatty acids. *Nippon Suisan Gakkaishi* 55 859-867

Jobling M. (1983) Ashort review and critique of methodology used in fish growth and nutrition studies. *J. Fish. Biol.* 23 685-703

Johnson D.A. (1980) Evaluation of various diets for optimal growth and survival of selected life stages of *Artemia* In: THE BRINE SHRIMP *Artemia* (eds. Persoone G., Sorgeloos P., Roels O. & Jaspers E.), Wetteren Press, Belgium. 3 185-192

Jones R. (1989) Towards a general theory of population regulation in marine teleosts. *J. Cons. Int. Explor. Mer.* 45 176-189

Jones D.A., Munford J.G. & Gabbott P.A. (1974) Microcapsules as artificial food particles for aquatic feeders. *Nature* 247 233-235

Jones D.A., Möller T.H., Campbell R.J., Munford J.G. & Gabbott P.A. (1975) Studies on the design and acceptibility of micro-encapsulated diets for marine particle feeders. *Proc. Eur. Symp. Mar. Biol. 10th*, Ostend, Belgium, Sept. 17-23, 1975, Universa Press, Wetteren, Belgium. 1 229-239

Jones D.A., Kanazawa A. & Rahman S.A. (1979) Studies on the presentation of artificial diets for rearing the larvae of *Penaeus japonicus* Bate. *Aquaculture* 17 33-43

Jones D.A., Holland D.L. & Jabborie S. (1984) Current status of microencapsulated diets for aquaculture. *Appl. Biochem. and Biotechnology.* 10 275-288

June F.C. & Carlson F.T. (1971) Food of the young Atlantic menhaden *Brevcoortia tyrannus* in relation to metamorphosis. *Fish. Bull. U.S.* 68 493-512

Kanazawa A. (1985) Essential fatty acid and lipid requirement of fish. In: NUTRITION AND FEEDING IN FISH. (eds. Mackie A.M., Bell J.C.), Academic Press, London. 281-298

Kanazawa A., Teshima S. & Ono K. (1978) Relationship between essential fatty acid requirements of aquatic animals and the capacity for bioconversion of linolenic acid to highly unsaturated fatty acids. *Comp. Biochem. Physiol.* 63B 295-298

Kanazawa A., Teshima S. & Ono K. (1979) Relationship between essential fatty acid requirements of aquatic animals and the capacity for bioconversion of linolenic acid to highly unsaturated fatty acids. *Comp. Biochem. Physiol.* 63B 295-298

Kanazawa A., Teshima S., Sakamoto M. & Awal Md.A. (1980) Requirements of *Tilapia zillii* for essential fatty acids. *Bull. Jap. Soc. Sci. Fish.* 46 1353-1356

Kanazawa A., Teshima S., Imatanaka N., Imada & Inoue A. (1982a) Tissue uptake of radioactive eicosapentaenoic acid in the red sea bream. *Bull. Jap. Soc. Sci. Fish.* 48 1441-1444

Kanazawa A., Teshima S., Inamori S., Sumida S. & Iwashita T. (1982b) Rearing of larval red sea bream and Ayu with artificial diets. *Mem. Fac. Fish., Kagoshima Univ.* 31 185-192

Kanazawa A., Teshima S., Inamori S. & Matsubara H. (1983a) Effects of dietary phospholipids on growth of the larval red sea bream and knife jaw. *Mem. Fac. Fish., Kagoshima Univ.* 32 109-114

Kanazawa A., Teshima S., Kobayashi T., Takae M., Iwashati T & Uehara R. (1983b) Necessity of dietary phospholipids for growth of the larval ayu. *Mem. Fac. Fish., Kagoshima Univ.* 32 115-120

Kapoor B.G., Smit H. & Verighina I.A. (1975) The alimentary canal and digestion in teleosts. *Adv. Mar. Biol.* 13 109-239

Katavic I. (1986) Diet involvement in mass mortality of sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture* 58 46-54

Kattner G. & Krause M. (1987) Changes in lipids during the development of *Calanus finmarchicus* s.l. from Copepodid I to adult. *Mar. Biol.* 96 511-518

Kayama M., Tsuchiya Y. & Mead J.F. (1963) A model experiment of aquatic food chain with special significance in fatty acid conversion. *Bull. Jap. Soc. Sci. Fish.* 29 452-458

Kean J.C., Castell J.D., Boghen A.G., D'Abramo L.R. & Conklin D.E. (1985) A re-evaluation of the lecithin and cholesterol requirements of juvenile lobster (*Homarus americanus*) using crab protein-based diets. *Aquaculture* 47 143-149

Kiørboe T., Munk P. & Støttrup J.G. (1985) First feeding by larval herring *Clupea harengus* L. *Dana* 5 95-107

Kirk R. (1987) A HISTORY OF MARINE FISH CULTURE IN EUROPE AND NORTH AMERICA. Fishing News Books Ltd., Surrey, England. 192 pp

Kissil G.W., Youngson A. & Cowey C.B. (1987) Capacity of the European eel (*Anguilla anguilla*) to elongate and desaturate dietary linoleic acid. *J. Nutr.* 117 1379-1384

Klungsøyr J., Tilseth S., Wilhelmsen S., Falk-Petersen S. & Sargent J.R. (1989) Fatty acid composition as an indicator of food intake in cod larvae *Gadus morhua* from Lofoten, Northern Norway. *Mar. Biol.* 102 183-188

Koven W.M., Kissil G.Wm. & Tandler A. (1989) Lipid and n-3 requirement of *Sparus aurata* larvae during starvation and feeding. *Aquaculture* 79 185-191

Kramer J.K.G., Thompson B.K. & Farnworth E.R. (1986) Variation in the relative response factor for triglycerides on Iatroscan chromarods with fatty acid composition and sequence of analyses. *J.Chromatogr.* 355 221-228

Kvenseth P.G. & Øiestad V. (1984) Large-scale rearing of cod fry on the natural food production in an enclosed pond. In: THE PROPAGATION OF COD *Gadus morhua* L.: AN INTERNATIONAL SYMPOSIUM, (eds. Dahl E., Danielssen D.S., Moksness E. & Solemdal P.), Arendal, Norway, 14-17 June 1983, *Flødevigen rapporter* 1 645-655

Lamptey M.S. & Walker B.L. (1976) A possible essential role for dietary linolenic acid in the development of the young rat. *J. Nutr.* 106 86-93

Landau M. & Riehm J.P. (1985) The nutritional chemistry of the decapsulated cysts of four geographical strains of the brine shrimp *Artemia salina* (L.) *Comp. Biochem. Physiol.* 81A 551-554

Langdon C.J. (1981) New techniques and their application to studies on bivalve nutrition. In: *Proc. Second Int. Conf. Aquacult. Nutr., Biochem. Physiol. Approaches to Shellfish Nutr.* (eds. Pruder G.D., Langdon C.J. & Conklin D.E.) Reheboth Beach, Delaware, 27-29 Oct. 1981, Louisiana State University Press, Baton Rouge. *World Mariculture Society. Special Publication* 2 305-320

Langdon C.J. & Bolton E.T. (1984) A microparticulate diet for a suspension-feeding bivalve mollusc, *Crassostrea virginica* (Gmelin). *Exp. Mar. Biol. Ecol.* 82 239-258

Langdon C.J. & Siegfreid C.A. (1984) Progress in the development of artificial diets for bivalve filter feeders. *Aquaculture* 39 135-153

Langdon C.J., Levine D.M. & Jones D.A. (1985) Review: Microparticulate feeds for marine suspension-feeders. *J. Microencapsulation* 2 1-11

Lauff M. & Hofer R. (1984) Proteolytic enzymes in fish development and the importance of dietary enzymes. *Aquaculture* 37 335-346

Leger C. (1985) Digestion, absorption and transport of lipids. In NUTRITION AND FEEDING IN FISH. (eds. Cowey C.B., Mackie A.M. & Bell J.G.), Academic Press, London. 299-332

Leger C., Fremont L. & Boudon M. (1981) Fatty acid composition of lipids in the trout - 1. Influence of dietary fatty acids on the triglyceride fatty acid desaturation in serum, adipose tissue, liver, white and red muscle. *Comp. Biochem. Physiol.* 69B 99-105

Léger P., Bengston D.A., Simpson K.L. & Sorgeloos P. (1986) The use and nutritional value of *Artemia* as a food source. *Oceanogr. Mar. Biol. Ann. Rev.* 24 521-623

Leong R. (1971) Induced spawning of northern anchovy *Engraulis mordax* Girard. *Fish. Bull.* 69 357-360

Leray C. & Pelletier X. (1987) Thin-layer chromatography of human platelet phospholipids with fatty acid analysis. *J. Chromatogr.* 420 411-416

Luotela M.T., Westermarck T.W. & Luotela J.E.I. (1985) Effect of α-tocopherol on the peroxidation of cod-liver oil. *Life Chem. Rep.* 3 159-163

MacDonald N.L., Stark J.R. and Austin B. (1986) Bacterial microflora in the gastro-intestinal tract of Dover sole (*Solea solea* L.) with emphasis on the possible role of bacteria in the nutrition of the host. *FEMS Microbiol. Letters* 35 107-111

Mangor-Jensen A. & Adoff G.R. (1987) Drinking activity of the newly hatched larvae of cod *Gadus morhua* L. *Fish Physiol. Biochem.* 3 99-103

Martin F.D. & Wright D.A. (1987) Nutritional state analysis and its use in predicting striped bass recruitment: Laboratory calibration. *Am. Fish. Soc. Symp.* 2 109-114

May R.C. (1974) Larval mortality in marine fishes and the critical period concept. In THE EARLY LIFE HISTORY OF FISH (ed. Blaxter J.H.S.), Springer Verlag, Berlin. 3-19

Mayer L.D., Bally M.B., Hope M.J. & Cullis P.R. (1986a) Techniques for encapsulating bioactive agents into liposomes. *Chem. Phys. Lipids* 40 333-345

Mayer L.D., Hope M.J. & Cullis P.R. (1986b) Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta* 858 161-168

McGurk M.D. (1985) Multivariate analysis of morphometry and dry weight of Pacific herring larvae. *Mar. Biol.* 86 1-11

McVey J.P. (1983) HANDBOOK OF MARICULTURE. CRC Press Inc., Boca Raton, Florida. 441 pp

Mearns K.J. (1986) Sensitivity of brown trout (*Salmo trutta* L.) and Atlantic salmon (*Salmo salar* L.) fry to amino acids at the start of exogenous feeding. *Aquaculture* 55 191-200

Meyers S.P. (1979) Formulation of water-stable diets for larval fishes. *Proc. World Symp. on Finfish Nutrition and Fishfeed Technology*, Hamburg 20-23 June, 1978 Vol. II pp 13-20

Miller J.T., Crowder L.B., Rice J.A. & Marschall E.A. (1988) Larval size and recruitment mechanisms in fishes: Toward a conceptual framework. *Can. J. Fish. Aquat. Sci.* 45 1657-1670

Minkoff G. (1987) The effect of secondarily enriched rotifers on the growth and survival of marine fish larvae. PhD thesis, No. 956, Inst. of Aquaculture, University of Stirling. 168 pp

Moksness E., Gjøsaeter J., Reinert A., Fjalstein I.S. (1989) Startfeeding and ongrowing of wolf fish (*Anarhicas lupus*). *Aquaculture* 77 221-228

Molvik G., Hjelmeland K., Ringø E. & Raa J. (1984) Properties of a new artificial diet for fish larvae, including cod *Gadus morhua* L. In: THE PROPAGATION OF COD *Gadus morhua* L.: AN INTERNATIONAL SYMPOSIUM, (eds. Dahl E., Danielssen D.S., Moksness E. & Solemdal P.), Arendal, Norway, 14-17 June 1983, *Flødevigen rapporter* 1 203-211

Morris R.J., Ferguson C.F., Raymont J.E.G. 1973 Preliminary studies on the lipid metabolism of *Neomysis integer*, involving labelled feeding experiments. *J. Mar. Biol. Assoc. U.K.* 53 657-664

Needleman P., Turk J., Jakschik B.A., Morrison A.R. & Lefkowith J.B. (1988) Arachidonic acid metabolism. *Ann. Rev. Biochem.* 35 69-102

Neuringer M., Connor W.E., Van Petten C. & Barstad L. (1984) Dietary omega-3 fatty acid deficiency and visual loss in infant rhesus monkeys. *J. Clin. Invest.* 73 272-276

Neuringer M., Anderson G.J. & Connor W.E. (1988) The essentiality of n-3 fatty acids for the development and function of the retina and brain. *Ann. Rev. Nutr.* 8 517-541

O'Connell C.P. (1980) Percentage of starving northern anchovy, *Engraulis mordax*, larvae in the sea as estimated by histological methods. *Fish. Bull.* 78 475-489

Øiestad V. & Mokness E. (1981) Study of growth and survival of herring larvae (*Clupea harengus* L.) using plastic bag and concrete basin enclosures. *Rapp. P.-v. Reun. Cons. int. Explor. Mer.* 178 144-149

Øiestad V., Kvenseth P.G. & Folkvord A. (1985) Mass production of Atlantic cod juveniles *Gadus morhua* in a Norwegian saltwater pond. *Trans. Am. Fish. Soc.* 114 590-595

Olafsen J.A. (1984) Ingestion of bacteria by cod (*Gadus morhua* L.) larvae. In: THE PROPAGATION OF COD *Gadus morhua* L.: AN INTERNATIONAL SYMPOSIUM, (eds. Dahl E., Danielssen D.S., Moksness E. & Solemdal P.), Arendal, Norway, 14-17 June 1983, *Flødevigen rapporter* 1 627-643

Olsen R.E. & Henderson R.J. (1989) The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. *J. Exp. Mar. Biol. Ecol.* 129 189-197

Olsen R.E., Henderson R.J. & McAndrew B.J. (1990) The conversion of linoleic acid and linolenic acid to longer chain polyunsaturated fatty acids by *Tilapia (Oreochromis) nilotica in vivo*. *Fish Physiol. Biochem.* 8 261-270

Oshima T., Ratnayake W.M.N & Ackman R.G. (1987) Cod lipids, solvent systems and the effect of fatty acid chain length and unsaturation on lipid class analysis by Iatroscan TLC-FID. *J. Am. Oil Chem. Soc.* 64 219-223

Owen J.M., Adron J.W. Sargent J.R. & Cowey C.B. (1972) Studies on the nutrition of marine flatfish. The effect of dietary fatty acids on the tissue fatty acids of the plaice *Pleuronectes platessa*. *Mar. Biol.* 13 160-166

Owen J.M., Adron J.W., Middleton C. & Cowey C.B. (1975) Elongation and desaturation of dietary fatty acids in turbot, *Scophthalmus maximus* L., and rainbow trout, *Salmo gairdnerii* Rich. *Lipids* 10 528-531

Papahadjopoulos D., De Gier J., Anner B.M., Rey H.G., Meda P., Nicolau C., Richards R.L., Hayre M.D., Hockmeyer W.T. & Alving C.R. (1988) Liposomes: From membrane model to therapeutic applications. *Biochem. Soc. Trans.* 16 909-922

Parker R.S. & Selivonchick D.P. (1986) Uptake and metabolism of lipid vesicles from seawater by juvenile Pacific oysters (*Crassostrea gigas*). *Aquaculture* 53 215-228

Patent Specification (1979) Application No. 7943754

Peters K. & Richards F.M. (1977) Chemical cross-linking: Reagents and problems in studies of membrane structure. *Ann. Rev. Biochem.* 46 523-551

Pokorny J. (1987) Major factors affecting the autoxidation of lipids. In: AUTOXIDATION OF UNSATURATED LIPIDS. (ed. Chan H.W.-S.), Academic Press Inc. (London) Ltd. 141-206

Potts W.T.W. (1976) Ion transport and osmoregulation in marine fish. In: PERSPECTIVES IN EXPERIMENTAL BIOLOGY. (ed. Spencer-Davies P.), Pergamon, Oxford. I 65-75

Potts W.T.W., Fletcher C.R. & Eddy F.B. (1973) An analysis of sodium and chloride fluxes in the flounder *Platichthys flesus*. *J. Comp. Physiol.* 87 21-28

Purcell J.E. & Grover J.J. (1990) Predation and food limitation as causes of mortality in larval herring at a spawning ground in British Columbia. *Mar. Ecol. Prog. Ser.* 59 55-61

Rao G.M.M. (1968) Oxygen consumption of rainbow trout (*Salmo gairdneri*) in relation to activity and salinity. *Can. J. Zool.* 46 781-787

Read G.H.L. (1981) The response of *Penaeus indicus* (crustacea: Penaeidea) to purified and compounded diets of varying fatty acid composition. *Aquaculture* 24 245-256

Reid R.G.B. (1981) Aspects of bivalve feeding and digestion relevent to aquaculture nutrition. *Proc. Second Int. Conf. on Aquacult. Nutr.: Biochem. Physiol. Approaches to Shellfish Nutr.*, (eds. Pruder G.D., Langdon C.J. & Conklin D.E.), Reheboth Beach, Delaware, 27-29 Oct. 1981, Louisiana State University Press, Baton Rouge. *World Mariculture Soc. Spec. Publ.* 2 59-70

Reinitz G.L., Orme L.E., Lemm C.A. & Hitzel F.N. (1978) Influence of varying lipid concentration with two protein concentrations in diets for rainbow trout (*Salmo gairdneri*). *Trans. Am. Fish. Soc.* 107 751-754

Rezanka T. (1989) Very long-chain fatty acids from the animal and plant kingdoms. *Prog. Lip. Res.* 28(3) 147-188

Robbins K.R., Horace W.N. & Baker D.H. (1979) Estimation of nutrient requirements from growth data. *J. Nutr.* 109 17110-1714

Rosenthal H. & Hempel G. (1970) Experimental studies in feeding and food requirements of herring larvae (*Clupea harengus* L.) In: MARINE FOOD CHAINS (ed. Steele J.H.), Oliver Boyd, Edinburgh. 334-364

Russell F.S. (1976) THE EGGS AND PLANKTONIC STAGES OF BRITISH MARINE FISH. Academic Press, London. 524 pp

Sakamoto M., Holland D.L. & Jones D.A. (1982) Modification of the nutritional composition of *Artemia* by incorporation of polyunsaturated fatty acids using micro-encapsulated diets. *Aquaculture* 28 311-320

Salem N.Jr., Kim H. & Yergey J.A. (1986) Docosahexaenoic acid: membrane function and metabolism. In: HEALTH EFFECTS OF POLYUNSATURATED FATTY ACIDS IN SEAFOODS. (eds. Simopoulos A.P., Kifer R.R. & Martin R.E.), Academic Press Inc., New York. 263-317

Samulski M.A. & Walker B.L. (1982) Maternal dietary fat and polyunsaturated fatty acids in the developing foetal rat brain. *J. Neurochem.* 39 1163-1168

Sandermann H. (1978) Regulation of membrane enzymes by lipids. *Biochem. Biophys. Acta* 515 209-237

Sargent J.R. & Whittle K.J. (1981) Lipids and hydrocarbons in the marine food chain. In: ANALYSIS OF MARINE ECOSYSTEMS (ed. Longhurst A.R.), Academic Press, London. 491-534

Sargent J.R., Gatten R.R. & Henderson R.J. (1981) Marine wax esters. *Pure Appl. Chem.* 53 867-871

Sargent J., Henderson R.J. & Tocher D.R. (1989) The lipids In: FISH NUTRITION (ed. Halver J.E.) Academic Press Inc (London) Ltd. 153-218

Satoh S., Poe W.E. & Wilson R.P. (1989) Studies on the essential fatty acid requirement of channel catfish, *Ictalurus puntatus*. *Aquaculture* 79 121-128

Schnack D. (1981) Studies on the mortality of Pacific herring larvae during their early development using artificial *in situ* containments. *Rapp. P.-v. Réun. Cons. Int. Explor. Mer.* 178 135-142

Scott A.P. & Middleton C. (1979) Unicellular algae as a food for turbot (*Scophthalmus maximus* L.) larvae - the importance of dietary long-chain polyunsaturated fatty acids. *Aquaculture* 18 227-240

Shatunovskiy M.I. (1970) Features of the qualitative composition of lipids in the eggs, young and spawning females of the spring and autumn Baltic herring in the Gulf of Riga. *J. Ichthyol.* 10 772-779

Shelbourne J.E. (1957) Site of chloride regulation in marine fish larvae. *Nature* 180 920-922

Sherr E., Sherr B. (1988) Role of microbes in pelagic food webs: A revised concept. *Limnol. Oceanogr.* 33 1225-1227

Simopoulos A.P., Kifer R.R. & Martin R.E. (eds.) (1986) HEALTH EFFECTS OF POLYUNSATURATED FATTY ACIDS IN SEAFOODS. Academic Press Inc., New York.

Sokal R.R. & Rohlf F.J. (1987) INTRODUCTION TO BIOSTATISTICS (2nd ed), W.H. Freeman & Co., New York. 363 pp

Sorgeloos P. (1980) The use of the brine shrimp *Artemia* in aquaculture. In: THE BRINE SHRIMP *Artemia* Vol. 3 (eds. Persoone G., Sorgeloos P., Roels O. & Jaspers E.), Wetteren Press, Belgium. 25-46

Sorgeloos P., Bossuyt E., Lavens P., Léger P., Vanhaecke P. & Versichele D. (1983) In HANDBOOK OF MARICULTURE, (ed. McVEY J.P.) CRC Press, Boca Raton, Florida. I 71-96

Steele J.H., Farmer D.M. & Henderson E.W. (1977) Circulation and temperature structure in large marine enclosures. *J. Fish. Res. Board Can.* 34 1095-1104

Steffens W. (1989) PRINCIPLES OF FISH NUTRITION. Ellis Horwood Ltd., Chichester, England. 384 pp.

Stephens G.C. & Manahan D.T. (1984) Technical advances in the study of nutrition of marine molluscs. *Aquaculture* 39 155-164

Stickney R.R. & Hardy R.W. (1989) Lipid requirements of warmwater species. *Aquaculture* 79 145-156

Suslik K.S. (1989) The chemical effects of ultrasound. *Sci. Am.* 260 (2) 62-68

Szoka F. & Papahadjopoulos D. (1981) Liposomes: Preparation and characterization. In: LIPOSOMES: FROM PHYSICAL STRUCTURE TO THERAPEUTIC APPLICATIONS. (ed. Knight), Elsevier/North-Holland Biomedical Press. 1-17

Tacon A.C.J. & 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. & Bell J.G.), Academic Press, London. 489 pp.

Takeuchi T. & Watanabe T. (1976) Nutritive value of omega-3 highly unsaturated fatty acids in pollock liver oil for rainbow trout. *Bull. Jap. Soc. Sci. Fish.* 42 907-919

Takeuchi T. & Watanabe T. (1977) Effect of eicosapentaenoic acid and docosahexaenoic acid in pollock liver oil on growth and fatty acid composition of rainbow trout. *Bull. Jap. Soc. Sci. Fish.* 43 947-953

Takeuchi A & Watanabe T. (1978) Growth-enhancing effect of cuttlefish liver oil and short-necked clam oil on rainbow trout and their effective components. *Bull. Jap. Soc. Sci. Fish.* 44 733-738

Takeuchi T., Arai S., Watanabe T. & Shimma Y. (1980) Requirement of eel *Anguilla japonica* for essential fatty acids. *Bull. Jap. Sci. Fish.* 46 345-353

Takeuchi T., Satoh S. & Watanabe T. (1983) Requirement of *Tilapia nilotica* for essential fatty acids. *Bull. Jap. Soc. Sci. Fish.* 49 1127-1134

Tandler A., Watanabe T., Satoh S. & Fukusho K. (1989) The effect of food deprivation on the fatty acid and lipid profile of red seabream (*Pagrus major*) larvae. *Br. J. Nutr.* 62 349-362

Teshima S., Kanazawa A. & Sakamoto M. (1981) Attempt to culture the rotifers with microencapsulated diets. *Bull. Jap. Soc. Sci. Fish.* 47 1575-1578

Theilacker G.H. (1980) Rearing container size affects morphology and nutritional condition of larval jack mackeral (*Trachurus symmetricus*) *Fish. Bull.* 78 789-791

Thongrod S., Takeuchi T., Satoh S. & Watanabe T. (1989) Requirement of fingerling white fish *Coregonus lavaretus maraena* for dietary n-3 fatty acids. *Nippon Suisan Gakkaishi* 55 1983-1987

Tilseth S. (1990) New marine fish species for cold-water farming. *Aquaculture* 85 235-245

Tinoco J., Williams M.A., Hincenbergs I. & Lyman R.L. (1971) Evidence for non-essentiality of linolenic acid in the diet of the rat. *J. Nutr.* 101 937-946

Tocher D.R. & Sargent J.R. (1984) Analyses of lipids and fatty acids in ripe roes of some Northwest European marine fish. *Lipids* 19 492-499

Tocher D.R. & Harvie D.G. (1988) Fatty acid compositions 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

Tocher D.R., Fraser A.J., Sargent J.R. & Gamble J.C. (1985a) Fatty acid composition of phospholipids and neutral lipids during embryonic and early larval development in Atlantic herring (*Clupea harengus* L) *Lipids* 20 69-74

Tocher D.R., Fraser A.J., Sargent J.R. & Gamble J.C. (1985b) Lipid class composition during embryonic and early larval development in Atlantic herring (*Clupea harengus*, L.) *Lipids* 20 84-89

Tocher D.R., Sargent J.R. & Frerichs G.N. (1988) The fatty acid compositions of established fish cell lines after long-term culture in mammalian sera. *Fish Physiol. Biochem.* 5 219-277

Tocher D.R., Carr J. & Sargent J.R. (1989) Polyunsaturated fatty acid metabolism in fish cells: Differential metabolism of (n-3) and (n-6) series acids by cultured cells originating from a freshwater teleost fish and from a marine teleost fish. *Comp. Biochem. Physiol.* 94B 367-374

Tytler P. & Blaxter J.H.S. (1988a) The effects of external salinity on the drinking rates of the larvae of herring, plaice and cod. *J. Exp. Biol.* 138 1-15

Tytler P. & Blaxter J.H.S. (1988b) Drinking in yolk-sac stage larvae of the halibut, *Hippoglossus hippoglossus* (L). *J. Fish Biol.* 32 439-494

Tzoumas A. (1988) The nutritional value of various *Artemia* strains for rearing sole larvae. MSc. thesis. Institute of Aquaculture. University of Stirling.

Vanhaecke P. & Sorgeloos P. (1980) International study on *Artemia* IV. The biometrics of *Artemia* strains from different geographical origins. In: THE BRINE SHRIMP *Artemia*. (eds. Persoone G., Sorgeloos P., Roels O. & Jaspers E.), Universa Press, Wetteren, Belgium. 3 394-405

Verreth J., Storch V. & Segner H. (1987) A comparitive study on the nutritional quality of decapsulated *Artemia* cysts, micro-encapsulated egg diets and enriched dry feeds for *Clarias gariepinus* (Burchell) larvae. *Aquaculture* 63 269-282

Vitiello F. & Zanetta J.-P. (1978) Thin-layer chromatography of phospholipids. *J. Chromatogr.* 166 637-640

Wakil S.J., Stoops J.K. & Joshi V.C. (1983) Fatty acid synthesis and its regulation. *Ann. Rev. Biochem.* 52 537-579

Walford J. & Lam T.J. (1987) Effect of feeding with microcapsules on the content of essential fatty acids in live foods for the larvae of marine fishes. *Aquaculture* 61 219-229

Watanabe T. (1982a) Lipid nutrition in fish. *Comp. Biochem. Physiol* 73B 3-15

Watanabe Y. (1982b) Intracellular digestion of horseradish peroxidase by the intestinal cells of teleost larvae and juveniles. *Bull. Jap. Soc. Sci. Fish.* 48 37-42

Watanabe T., Koobayashi I., Utsue O. & Ogino C. (1974) Effect of dietary methyl linolenate on fatty acid composition of lipids in rainbow trout. *Bull. Jap. Soc. Sci. Fish.* 40 387-392

Watanabe A. & Hayashi T. (1976) Microencapsulation techniques of Fuji Photo Film Co. Ltd., and their applications. In: MICROENCAPSULATION (ed. Nixon J.R.), Marcel Dekker N.Y. pp 13-38

Watanabe T., Kitajima C., Arakawa T., Fukusho K. & Fujita S. (1978) Nutritional quality of rotifer *Brachionus plicatilis* as a living feed from the viewpoint of essential fatty acids for fish. *Bull. Jap. Soc. Sci. Fish.* 44 1109-1114

Watanabe T., Oowa F., Kitajima C. & Fujita S. (1980) Relationship between dietary value of brine shrimp *Artemia salina* and their content of w3 highly unsaturated fatty acids. *Bull. Jap. Soc. Sci. Fish.* 46 35-41

Watanabe T., Kitajima C. & Fujita S. (1983) Nutritional values of live organism used in Japan for mass propagation of fish: A review. *Aquaculture* 34 115-143

Watanabe T., Ohta M., Kitajima C. & Fujita S. (1982) Improvement of dietary value of brine shrimp *Artemia salina* for fish larvae by feeding them on omega3 highly unsaturated fatty acids. *Bull. Jap. Soc. Sci. Fish.* 48 1775-1782

Watanabe T., Throngrod S., Takeuchi T., Satoh S., Kubota S.S., Fujimaki Y. & Cho C.Y. (1989a) Effect of dietary n-6 and n-3 fatty acids on growth, fatty acid composition and histological changes of white fish *Coregonus lavaretus maraena*. *Nippon Suisan Gakkaishi* 55 1977-1982

Watanabe T., Arakawa T., Takeuchi T & Satoh S. (1989b) Comparison between eicosapentaenoic and docosahexaenoic fatty acid efficiency in juvenile striped jack *Pseudocaranx dentex Nippon Suisan Gakkaishi* 55 1989-1995

Ways P. & Hanahan D.J. (1964) Characterization and quantification of red cell lipids in normal man. *J. Lipid Res.* 5 318-328

Wickins J.F. (1972) The food value of brine shrimp, *Artemia salina* L., to larvae of the prawn, *Palaemon serratus* Pennant. *J. Exp. Mar. Biol. Ecol.* 10 151-170

Williams S., Lovell R.T. & Hawke J.P. (1985) Value of menhaden oil in diets of Florida pompano. *Prog. Fish. Cult.* 47 159-165

Willmott N., Cummings J. & Florence A.T. (1985) *In vitro* release of adriamycin from drug-loaded albumin and haemoglobin microspheres. *J. Microencapsulation* 2 293-304

Witt U., Quantz G., Kuhlmann D. & Kattner G. (1984) Survival and growth of turbot larvae *Scophthalmus maximus* L. reared on different food organisms with special regard to long-chain polyunsaturated fatty acids. *Aquacult. Engin.* 3 177-190

Yamada K., Kobayashi K. & Yone Y. (1980) Conversion of linolenic acid to omega-3-highly unsaturated fatty acids in marine fishes and rainbow trout. *Bull. Jap. Soc. Sci. Fish.* 46 1231-1233

Yin M.C. & Blaxter J.H.S. (1987) Temperature, salinity tolerance, and buoyancy during early development and starvation of Clyde and North Sea herring, cod, and flounder larvae. *J. Exp. Mar. Biol. Ecol.* 107 279-290

Yone Y. & Fujii M. (1975) Studies on nutrition of red sea bream - XI Effect of omega3 fatty acid supplement in a corn oil diet on growth rate and feed efficiency. *Bull. Jap. Soc. Sci. Fish.* 41 73-77

Young J.Z. (1981) THE LIFE OF VERTEBRATES. Clarendon Press, Oxford. 136-137

Yu T.C. & Sinnhuber R.O. (1972) Effect of linolenic acid and docosahexaenoic acid on growth and fatty acid composition of rainbow trout (*Salmo gairdneri*). *Lipids* 7 450-454

Yu T.C. & Sinnhuber R.O. (1975) Effect of dietary linolenic and linoleic acids upon growth and lipid metabolism of rainbow trout (*Salmo gairdneri*) *Lipids* 10 63-81

Yu T.C. & Sinnhuber R.O. (1976) Growth response of rainbow trout (*Salmo gairdneri*) to dietary n-3 and n-6 fatty acids. *Aquaculture* 8 309-317

Zar J.H. (1984) BIOSTATISTICAL ANALYSIS (2nd ed), Prentice-Hall International Inc., New Jersey. 718 pp

Zeitoun I.H., Halver J.E., Ullrey D.E. & Tack P.I. (1973) Influence of salinity on protein requirements of rainbow trout (*Salmo gairdneri*) fingerlings. *J. Fish. Res. Board Can.* 30 1867-1873

Zeitoun I.H., Ullrey D.E., Halver J.E., Tack P.I. & Magee W.T. (1974) Influence of salinity on protein requirements of coho salmon (*Onchorhynchus kisutch*) smolts. *J. Fish. Res. Board Can.* 31 1145-1148

Zubay G. (1988) BIOCHEMISTRY. (2nd ed), Macmillan Publishing Co., New York. 1266 pp