EFFECT OF SUPPLEMENTARY ENZYMES ON THE GROWTH AND FEED UTILISATION OF GILTHEAD SEA BREAM, *SPARUS AURATA* L.

Thesis submitted for the degree of Doctor of Philosophy

by

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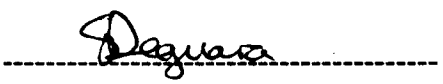
Declaration

I hereby declare that this thesis has been compiled by myself and is the result of my own investigations. It has neither been accepted nor submitted for any other degree. All the sources of information have been duly acknowledged.



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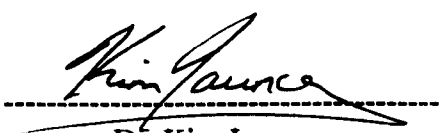

Dr. Kim Jauncey
Principal Supervisor

To my wife Lucienne and our Families

ABSTRACT

A series of five experiments were carried out to determine the effect of supplementary enzymes on growth performance and feed utilisation of juvenile gilthead sea bream, *Sparus aurata*, fed diets in which soybean meal (SBM) partially replaced fish meal (FM).

In the first of these experiments the addition of cocktails containing 1 g/kg low pH active protease and 1 g/kg α-galactosidase or 1 g/kg high pH active protease and 1 g/kg α-galactosidase to a 320 g/kg SBM, 260 g/kg FM pressed diet were both found to significantly ($P<0.05$) improve performance of fish fed these diets compared to fish fed the unsupplemented diet and a 320 g/kg FM, 220 g/kg SBM diet. This improvement in performance was not obtained when fish were fed 440 g/kg SBM, 230 g/kg FM diets with the same enzyme combinations. In some parameters performance of fish decreased as the SBM level in the diets was increased.

The significant improvements observed in Experiment 1, with addition of enzyme cocktails to the 320 g/kg SBM diet, were not repeated in any of the subsequent experiments. The second experiment was aborted due to abnormal feeding behaviour of the fish. In the third experiment, in which the enzymes employed in Experiment 1 were used individually at 1 g/kg in 320 g/kg SBM diets, no significant differences in specific growth rate (SGR), food conversion ratio (FCR) or protein efficiency ratio (PER) were noted in comparison to fish fed the unsupplemented diet. This was also the case with fish fed diets to which the two enzyme cocktails had been added at enzyme inclusion levels of 0.5 g/kg each. Although no significant differences were found, feeding the

diet with low pH protease alone appeared to increase performance compared to fish fed the unsupplemented diet, and the results of fish fed diets with high pH protease alone or with α-galactosidase alone indicated that there was a decrease in performance compared to fish fed the unsupplemented diet.

In Experiment 4 fish fed 320 g/kg SBM diets with 0.5, 1.0 and 1.5 g/kg low pH protease showed similar SGRs, FCRs and PERs which appeared to show an improved performance (although not significantly so) compared to fish fed diets with 1.0 g/kg α-galactosidase used together with either 0.5 or 1.0 g/kg low pH protease.

In the final experiment fish were fed 320 g/kg SBM extruded diets to which 0, 0.33, 0.66, 1.00 and 1.33 g/kg of low pH protease had been added. Although no significant differences in SGR, FCR or PER were obtained, fish fed the diets containing 0.66 and 1.33 g/kg protease appeared to improve performance compared to fish fed any of the other diets or a diet containing 320 g/kg FM and 220 g/kg SBM. Fish fed the other 320 g/kg SBM supplemented diets gave similar results.

A histological study of the position of nuclei in hepatocytes and the presence of fat globules around hepatopancreatic tissue in liver samples taken from fish fed the various experimental diets failed to show any relationships with either SBM level or enzyme inclusion in the diet.

A series of analyses on the distribution of activities of six enzymes in the digestive tract of sea bream indicated that relative activities differed from one enzyme to another and from one region to another. In an investigation into the variation of pH in various parts of the digestive tract after one or two feeds, it was observed that within the first 24 hours after feeding the pH in the stomach decreased to a minimum value of 2.5 and the pH in the rest of the intestine varied between 6.5 and 7.7.

From a series of gastric evacuation trials which were performed, it was found that the time of day sea bream were fed a meal influenced the gastric evacuation rate, with fish fed in the afternoon taking longer to evacuate the meal than fish fed a similar meal in the morning. Doubling the size of a meal did not double the gastric evacuation time. Instead, the time to evacuate a given percentage of the larger meal only increased by 1.4 and 1.6 times in fish fed the pressed and extruded feeds respectively compared to fish fed the smaller meal. When the sea bream were fed multiple meals it was found that the evacuation rate of an earlier meal was increased by a subsequent meal.

A series of trials investigating the distribution in consumption of a population of sea bream fed a single meal indicated that there was a wide variation in the amount of food consumed by each fish in the population and it was observed that even fish of the same size consumed very different quantities of food.

Before any definite conclusions can be drawn regarding the use of the three enzymes tested in these experiments to improve growth and feed utilisation in FM-substituted diets, further investigations need to be carried out in an attempt to obtain more significant results. This thesis has shown that additional research into the mode of action of these enzymes is required as well as studies into how the digestive physiology of the sea bream may affect the use of these (and other) supplementary enzymes.

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CHAPTER 1

INTRODUCTION

1.1 WORLD AQUACULTURE

The demand for aquatic products as a source of food for human consumption and for other uses, such as reduction to fish meal, has grown steadily since the end of World War 2 (Beveridge, 1987). This demand has led to an expansion in the exploitation of these resources. The increase in wild catches was greatest during the two decades after 1950 until the early 1970s, after which lower and more erratic increases were recorded (Barlow, 1989). It is generally agreed that many of the major world fisheries are already exploited at or beyond their maximum sustainable yields (Rhodes, 1993; Aiken and Sinclair, 1995), although other authors believe that an increase is possible (Grainger and Garcia, 1996).

The output of aquaculture has been increasing steadily since the middle of the 1960s when there was a trend towards the production on an industrial scale of aquatic species in ponds, tanks and cages (Steffens, 1989).

The aquaculture industry is currently the fastest growing meat producing industry in the world. In 1993, 188 million metric tons (mmt) of farmed meat were produced in the world, compared to 147 mmt in 1984. The highest production was in fact pig meat, followed by beef/veal with a 1993 production of 75.2 and 50.2 mmt respectively, these values representing a 31 and 6.8% growth over the 1984 productions respectively. The production of fish and crustaceans increased from 4.9 to 12.1 million metric tons in the same period, an increase of 146% (Tacon, 1996).

The total aquaculture production, including algae and other species was 27.8 mmt in 1995. Of this total volume 6.9 mmt was accounted for by seaweed and 20.9 mmt by fish and shellfish. This latter value accounted for 18.6% of the total global harvest of fish and shellfish which was 113 mmt in 1995. In this year Asian countries alone

contributed 90% of the world aquaculture production of fish and shellfish, followed by Europe with 6.7% (FAO, 1997). Asia not only produces the bulk of aquaculture production but is also the main site of growth of the industry, with a 184% increase in production over the period 1984 to 1994 compared to only 27% within developed countries (Tacon, 1996).

As the world population grows, there is agreement that aquaculture will have to supply the shortfall of finfish and shellfish required to at least maintain the 1989 global per capita consumption with a total estimated consumption to reach 162 mmt in the year 2025 (Ruckes, 1994; Aiken and Sinclair, 1995; New, 1995; Avault, 1996; Pillay, 1996; Anon., 1997a, b).

1.2 THE FEED INDUSTRY

Seventy to 80% of the world finfish and crustacean aquaculture production is carried out within extensive or semi-intensive systems where food is provided for by using fertilisation and/or supplementary feeding or by just using the natural productivity of the rearing enclosure (Tacon, 1996).

As the level of intensification increases, and as the industry gets bigger and more developed, there is an increasing dependency on the use of artificial formulated feeds, especially for carnivorous species. Along with the current growth of the industry as a whole, it is not surprising therefore, that the aquafeed industry is one of the fastest expanding agribusinesses in the world (Tacon, 1996).

Smith and Guerin (1995) put the total aquafeed production in 1994 at 4.25 mmt of which 60% was produced in Asia and 21% in Europe. 40% of this production was for carnivorous finfish, 35% for non-carnivorous finfish and 25% for shrimp. It is estimated that the total production by the year 2000 will be 7.5 mmt. Aquafeed

production represented about 1% of the total animal feed production in the world in 1995 which reached 560 mmt. The top feed users were the poultry and pigs industries with 32% and 31% of the total production respectively.

1.2.1 FEED MANUFACTURE

The technology used in the manufacture of dry pelleted feeds is part of a whole process involving numerous steps and procedures. The pellet produced by this process can be one of two general types. Pressed pellets are produced in a pellet mill by a process called steam pelleting, and extruded pellets (also called expanded pellets) are produced in an extruder (single-screw or twin-screw).

1.2.1.1 Steam Pelleting

In the process of steam pelleting soft feed mix is forced through holes in a metal die to form compacted pellets which are then cut as they emerge from the die. Steam may be added to the feed mix either in a conditioning chamber or in the pelleting chamber itself. The moisture content of the feed can be increased up to 16% (Maier and Gardecki, 1993). The temperature of the feed mixture is increased up to 60 or 70°C (Springate, 1991), although temperatures of up to 90°C may also be reached (Hastings and Higgs, 1980), but the pressure developed in a pellet mill is much less than that developed in an extruder. After cooling and drying, the resulting pellet density is such that it sinks rapidly in water.

1.2.1.2 Extrusion

In the production of extruded pellets, feed is driven through the decreasing diameter of a tough barrel by one or two augers (single and twin-screw extruders respectively). Water and/or steam can be applied in a conditioning chamber or at various stages in the

extrusion barrel which can be surrounded by a jacket carrying steam or cold water to heat and cool the feed. The moisture content of the feed mix may range from 10 to 40% (Gehrig, 1989). Temperature ranges in the extruder may vary from 80 to 250°C (Melcion, 1987), although in the production of fish feeds the usual temperatures are in the range 80 to 90°C in single screw extruders and up to 120°C in twin screw extruders (Evers, Pers. Comm., 1994). Pressure at the end of the barrel can vary from 30 to 120 atmospheres (Melcion, 1987; Autin, 1997), such that as the pellet leaves the die, the reduction in pressure results in a sudden expansion of water vapour in the pellet, causing air pockets to form and a 'puffing' or expansion of the product. The pellet obtained by extrusion has a density lower than that obtained by steam pelleting, and is such that it floats or slowly sinks in water (Hardy, 1989).

1.2.1.3 Extrusion *vs.* Steam Pelleting

Extrusion and the final product has several advantages over steam pressing and the compressed pellets it forms.

- The extrusion process allows a wider range of ingredients to be used in the formulations (Botting, 1991; Kearns, 1993).
- Extruded pellets can be formulated with a higher energy content as a result of the much higher lipid which they can take up (Anon., 1987; Springate, 1991; Autin, 1997), and by also increasing the digestibility of carbohydrates, they increase the utilisation of protein (Hilton and Slinger, 1983; Anon., 1985; Tacon and Jackson, 1985; Roberts, 1989; Clarke, 1990; Autin, 1997).
- Extruded feeds have improved physical attributes: can be made with variable densities, have better water stability, have lower dust levels, are softer, have improved mechanical durability and have improved storage life (Hilton *et al.*, 1981; Clarke, 1990; Botting, 1991; Kearns, 1993).

- Extrusion brings about sterilisation of the ingredients (Kearns, 1993; Anon., 1989a), and destroys numerous antinutritional factors (Anon., 1985.; Clarke, 1990).
- The above allow a better food utilisation and reduction in pollution (Hilton *et al.*, 1981; Anon., 1989a; Roberts, 1989; Botting, 1991; Johnson *et al.*, 1993; Robert *et al.*, 1993).

The extrusion process also has a number of drawbacks compared to steam pressing.

- The higher temperatures and pressures used in the manufacture of extruded feed destroy a number of heat-labile nutrients (Hardy, 1989; Putnam, 1990).
- The purchase of equipment and the manufacture of pellets by extrusion is much more expensive (Botting, 1991; Autin, 1997; Kearns, 1993).

1.3 FISH MEAL

On a global scale increasing attention is being paid to the availability of two very important ingredients used in fish feed manufacture, fish meal and fish oil, on the market.

The aquaculture industry is currently heavily dependent on these two ingredients. In fact, in 1994 aquaculture used 1.15 mmt of fish meal, equivalent to 15.5% of the total world production of 7.44 mmt, and 346,000 metric tonnes of fish oil, equivalent to 23.7% of total world production of 1.46 mmt (FAO, 1996). With the further expansion of the aquaculture industry, usage of these commodities is expected to be equivalent to the use of 25 to 30% and 30 to 50% of the total world fish meal and fish oil supplies respectively by the next decade, assuming supplies remain constant (Rumsey, 1994; Smith and Guerin, 1995; IFOMA, 1996).

Good quality fish meal is an excellent source of highly digestible protein containing a well balanced profile of essential amino acids which come close to satisfying the

requirements of many species of fish. It is also a good source of energy, essential fatty acids (especially highly unsaturated fatty acids), certain minerals and vitamins and has been attributed with unidentified growth factors. Fish meal also has the additional quality of high palatability (FAO, 1983).

Numerous reports have shown that the quality of the fish meal produced can be improved and that there can be important variations in fish meals from different sources (Danielssen *et al.*, 1989; Marki, 1990; Dallimore, 1993; Ostrowski *et al.*, 1993; Pike, 1993; Deguara, 1997; Moksness *et al.*, 1995; Saldivar, 1996; Aksnes and Mundheim, 1997). Several fish meal manufacturers have undertaken and advertised measures to improve their fish meal production quality (Anon., 1992c; Anon., 1993; Anon., 1994a; Anon., 1994b; Pastor, 1995; Anon., 1995; Anon., 1996a, b).

Notwithstanding the improvements in quality, increasing prices and restricted availability have brought about an emphasis on reducing the dependence on fish meal by using alternative protein sources.

The greatest promise has been shown by plant legumes and oilseeds, such as soybean, sunflower and rapeseed. Numerous other ingredients have been used and tested, and a few examples of such investigations in fish are shown in Table 1.1. A more detailed analysis of experiments where soybean meal alone has been used to replace fish meal is given below in Section 1.4.3.

1.4 SOYBEAN MEAL

From the point of view of economics, market availability and nutritional value, the prime candidate for replacing fish meal protein in aquaculture diets is soybean (*Glycine max* L.). Soybean meal is already a very important ingredient in all formulated feeds, and is becoming increasingly so. Soybean made up to 48.4% (111 mmt) of all oil seed

Table 1.1 Selected experiments carried out on alternative protein sources in the fish farming industry.

Ingredient/s tested	Species	Reference
Fish silage	Rainbow trout, *Oncorhynchus mykiss*	Stone *et al.*, 1989
Krill, *Euphausia superba*	Red tilapia	Lou and Chen, 1980
One of or combinations of: animal by-products, blood, bone, feather, meat, poultry meals	European sea bass, *Dicentrarchus labrax.* Palmetto bass, *Morone saxatilis x M. chrysops* Red drum, *Sciaenops ocellatus* Nile tilapia, *Oreochromis niloticus*	Langer and Metailler, 1989; Alexis, 1997 Gallagher and LaDouceur, 1995 Meilahn *et al.*, 1996 Rodriguez-Serna *et al.*, 1996
Terrestrial worms	Rainbow trout, *O. mykiss*	Tacon *et al.*, 1983c; Stafford and Tacon, 1984
Single Cell Protein	*O. mossambicus*	Davies and Wareham, 1988
Pito brewery waste	*Tilapia busumana*	Oduro-Boateng and Bart-Plange, 1988
Green alga, *Cladophora glomerata*	*Sarotherodon niloticus*	Appler and Jauncey, 1983
Green gram, *Phaseolus aureus*	Nile tilapia, *O. niloticus*	De Silva and Gunasekera, 1989
One of or combinations of: Copra, corn gluten, cotton, groundnut, leucaena, linseed, lupin, mustard, rapeseed, sesame, soybean, sunflower meals, malt protein flour	*Sarotherodon mossambicus* Chinook salmon, *O. tshawytscha* Rainbow trout, *O. mykiss* Channel catfish, *Ictalurus punctatus* Common carp, *Cyprinus carpio*	Jackson *et al.*, 1982 Higgs *et al.*, 1982, 1983 Smith *et al.*, 1988; Gomes and Kaushik, 1989; Moyano *et al.*, 1992; Morales *et al.*, 1994, Sanz *et al.*, 1994; Akiyama *et al.*, 1995; Stickney *et al.*, 1996 Webster *et al.*, 1992 Hasan *et al.*, 1997
Combinations of: Bone and meat, blood, carob seed germ, chicken offal, corn gluten, feather, poultry, soybean. *Spirulina*, squilla meals, dried brewer's yeast, malt protein flour	Coho salmon, *O. kisutch* Nile tilapia, *O. niloticus* Rainbow trout, *O. mykiss* Common carp, *C. carpio* Australian snapper, *Pagrus auratus* Silver sea bream, *Rhabdosargus sarba* *O. niloticus*, *Clarias gariepinus* Red sea bream, *Pagrus major* Palmetto bass, *M. saxatilis x M. chrysops*	Higgs *et al.*, 1979 Tacon *et al.*, 1983b Alexis *et al.*, 1985; Yamamoto *et al.*, 1995 Nandeesha *et al.*, 1989 Quartararo *et al.*, 1992 El-Sayed, 1994 Sadiku, 1995 Yamamoto *et al.*, 1996 Webster *et al.*, 1997

production in 1993, followed by cottonseed (13.4%), rapeseed (11.4%), groundnuts (10.9%) and sunflower (8.9%). In this year 79.7 mmt of soybean meal/cake were produced from all the soybean available (FAO, 1994).

1.4.1 SOYBEAN MEAL AS A PROTEIN SOURCE

Although the protein content of soybean meal (SBM) is less than that of fish meal (FM)(Table 1.2), the essential amino acid profiles of processed SBM products compare well with that of FM when considered on a percentage of protein basis, and, with the exception of methionine, would seem to meet the requirements of channel catfish (*I. punctatus*), chinook salmon (*O. tshawytscha*) and gilthead sea bream (*S. aurata*)(Table 1.3).

The dry matter, crude protein, crude lipid and gross energy digestibilities of various soybean products have been found to vary quite a bit, but are generally lower than those of fish meals (Table 1.4). As seen in Table 1.4, a high variation in digestibilities have been measured even in the same species of fish and with the same type of soybean product.

1.4.2 THE ANTI-NUTRITIONAL FACTORS IN SOYABEAN MEAL

Notwithstanding the favourable nutritional composition of SBM and of other plant materials, their use is often restricted by the presence of the numerous antinutritional factors (ANFs) they contain. The ANFs present in SBM can be divided into those which are destroyed by heat and those which are not.

Table 1.2 Proximate composition of herring fish meal and some soybean products.

	Herring fish meal (mechanically extracted)	Soybean seed (steam cooked, full fat)	Soybean meal (solvent extracted)	Soybean meal (solvent extracted without hulls)
International feed number	5-02-000	5-04-597	5-04-604	5-04-612
Dry matter content (%)	92	90	90	90
Crude protein content (%)	72.0	38.0	44.0	48.5
Crude lipid content %)	8.4	18.0	1.1	0.9
Crude fibre content (%)	0.6	5.0	7.3	3.4
Ash content (%)	10.4	4.5	6.3	5.8
Calculated crude carbohydrate content (%)	0.6	24.5	31.3	31.4

Table 1.3 Essential amino acid content of herring fish meal and some soybean meal products and amino acid requirements of channel catfish, *Ictalurus punctatus*, chinook salmon, *O. tshawytscha* and the gilthead sea bream, *Sparus aurata*. The essential amino acid contents and requirements are expressed as a percentage of protein[1].

	Amino acid composition as a percentage of protein (%)				Amino acid requirements as a percentage of dietary protein[1]		
Amino acid	Herring fish meal (mechanically extracted)	Soybean seed (steam cooked, full fat)	Soybean meal (solvent extracted)	Soybean meal (solvent extracted without hulls)	Channel catfish, *I. punctatus*.	Chinook salmon., *O. tshawytscha*.	Gilthead sea bream, *Sparus aurata*.
International feed number	5-02-000	5-04-597	5-04-604	5-04-612			
Arginine	6.31	6.66	7.70	7.57	4.3	6.0	5.1
Histidine	2.29	2.26	2.70	2.52	1.5	1.8	1.8
Isoleucine	4.35	4.21	4.61	4.41	2.6	2.2	3.6
Leucine	7.21	6.92	7.93	7.48	3.5	3.9	5.8
Lysine	7.74	5.89	6.48	6.35	5.1	5.0	6.0
Methionine	2.89	1.21	1.30	1.40	2.3	1.6	2.6
Cystine[2]	1.03	0.89	1.59	1.55			1.1
Phenylalanine	3.76	4.53	5.05	5.03	5.0	5.1	3.3
Tyrosine[2]	3.06	3.29	3.57	3.63			3.2
Threonine	4.03	3.71	4.05	3.90	2.0	2.2	3.9
Tryptophan	1.07	1.37	1.45	1.42	0.5	0.5	0.6[3]
Valine	5.97	5.32	4.59	5.26	3.0	3.2	4.4

1. Data obtained from NRC (1993). The amino acid requirements for channel catfish and chinook salmon were obtained by using chemically defined diets. The requirements for the gilthead sea bream are values for carcass composition of 63 g fish as obtained in this work.
2. Not essential amino acid.
3. Obtained from Luquet and Sabaut (1974).

Table 1.4 Selected recent data showing the variation in apparent digestibility coefficients of various fish meal and soybean meal products for a number of fish species.

Ingredient	International number	Species	Apparent digestibility coefficient (%)				Reference
			Dry matter	Protein	Lipid	Gross energy	
Menhaden FM	5-02-009	Catfish, *Mystus nemurus*	83.74	88.23		95.56	Khan, 1994
		Hybrid striped bass, *M. saxatilis* x *M. chrysops*		76.9	67.6	92.1	Sullivan and Reigh, 1995
		Red drum, *Sciaenops ocellatus*	76.79	95.87		60.14	Gaylord and Gatlin, 1996
		Red drum, *S. ocellatus*	97.00	97.80		77.88	McGoogan and Reigh, 1996
Portuguese brown FM (sardine)		Rainbow trout, *O. mykiss*	89.4 78.0	92.3 86.6		93.8 69.7	Gomes *et al.*, 1995
Danish herring FM		Gilthead sea bream, *S. aurata*		95.8		94.1	Nengas *et al.*, 1995
Fish meal		Gilthead sea bream, *S. aurata.*		83	95	80	Lupatsch *et al.*, 1997
Oil extracted SBM		Catfish, *M. nemurus*	91.55	86.00		67.89	Khan, 1994
Full fat toasted SBM		Rainbow trout, *O. mykiss*	75.4	86.4		80.2	Gomes *et al.*, 1995
Full fat toasted and micronised SBM		Rainbow trout, *O. mykiss*	86.4	96.3		90.7	Gomes *et al.*, 1995
Full fat SBM		Gilthead sea bream, *S. aurata*		75.7	84.6	61.9	Nengas *et al.*, 1995
Hexane extracted SBM		Gilthead sea bream, *S. aurata*		90.9	62.9	44.7	Nengas *et al.*, 1995
Extracted SBM	5-04-604	Hybrid striped bass, *M. saxatilis* x *M. chrysops*	44.49	79.95		55.24	Sullivan and Reigh, 1995
		Red drum, *S. ocellatus*	41.41	80.24		37.77	McGoogan and Reigh, 1996
Dehulled SBM	5-04-612	Red drum, *S. ocellatus*		86.1	62.7	63.3	Gaylord and Gatlin, 1996
SBM		Gilthead sea bream, *S. aurata*		86-88		72	Lupatsch *et al.*, 1997

1.4.2.1 The Heat-Labile ANFs

1.4.2.1.1 Proteolytic Enzyme Inhibitors

SBM contains two classes of protease inhibitors. Soybean trypsin inhibitors (STI) are polypeptides, each of which inhibits one trypsin molecule. Soybean proteinase inhibitors (SPI), which are smaller than STIs, contain independent reaction sites for one enzyme of trypsin and for one enzyme of chymotrypsin. These protein inhibitors also inhibit other proteases such as elastase and a number of serine proteases.

Protease inhibitors can constitute up to 8.3% of the total protein of soybeans (Nitsan, 1991) and have been attributed to be responsible for 30 to 50% of the growth inhibitor effect of raw soybeans in rats (Rackis, 1965; Kakade *et al.*, 1973).

The overall effect of these inhibitors is an inhibition of growth and a depression in protein digestibility. They have been observed to cause an enlargement of the pancreas, involving an increase in size and number of acinar cells, and an increase in the synthesis of pancreatic enzymes in a number of animals (Gertler and Nitsan, 1970; Yanatori and Fujita, 1976; Roy and Schneeman, 1981; Grant *et al.*, 1989).

STIs are more sensitive to heat treatment (Birk, 1989) and the low pH and pepsin activity in the gastric juice than are the SPIs (Krogdahl and Holm, 1981).

1.4.2.1.2 Lectins (or Haemagglutinins)

Lectins are chemicals that are able to bind to carbohydrate-containing molecules with a high degree of specificity towards the sugar component. They have the ability to agglutinate red blood cells and other cells from various species of animals. Defatted soybean flour contains several haemagglutinins comprising from 10 to 30 g/kg of the total protein (Liener, 1979).

About 60% of the lectins survive the intestinal transit in rats and becomes bound to the intestinal epithelium where they cause disruption of the brush border (Pusztai *et al.*, 1990), atrophy of the microvilli (Jindal *et al.*, 1984), and reduce the viability of epithelial cells (Ishiguro, 1992). Other effects linked to lectins are inhibition of the dissaccharidases and proteases in the intestine (Jindal, 1982; Rouanet, 1983), degenerative changes in the liver and kidneys and possibly pancreas (de Aizpurue and Russell-Jones, 1988), and an interference with absorption of non-heme iron (Hisayasu *et al.*, 1992) and lipid (Khalifa *et al.*, 1992) from the diets.

Lectins are more labile to heat and processing than are trypsin inhibitors, and a properly processed soybean meal product should have less than 0.05 micrograms of lectin per gram (Visser, 1992).

1.4.2.1.3 Goitrogens

Some reports have identified the goitrogenic agent to be a low-weight oligopeptide (Konijn *et al*, 1972), while another report has attributed at least part of the effect to soyasapogenol (Suwa and Kimura, 1981).

These molecules cause enlargement of the thyroid gland, and the bulk of the evidence suggests that these agents act by inhibiting the uptake of iodine by the thyroid gland. (Sharpless et a., 1939, Wilgus *et al.*, 1941). In fact, Suwa *et al.* (1979) found that these effects were counteracted by the administration of iodine.

1.4.2.1.4 Urease

Urease present in soybeans has not been found to cause any growth depression in chickens (Vohra and Kratzer, 1991).

1.4.2.1.5 Anti-Vitamins

Raw soybean contains the enzyme lipoxygenase which oxidises and destroys carotene (Sumner and Dounce, 1939). Dudly (1971) also reported finding a factor in soybean protein isolate that decreased carotene absorption in chicks by increasing the amount of residue-bound carotene passing through the intestine.

Apart from being inherently deficient in vitamin B12, soybean has also been reported to contain a substance that increases the requirement for vitamin B12 (Edelstein and Guggenheim, 1970; Williams and Spray, 1973).

Unheated soybean meal has been reported to cause rickets (Carlson *et al.*, 1964; Miller *et al.*, 1965; Jensen and Mraz, 1966), although the effect was reversed by adding more calcium and phosphorus to the diet.

Isolated soybean protein has been demonstrated to increase chick requirement for α-tocopherol (Fisher *et al.*, 1969). Murillo and Gaunt (1975) suggested that the α-tocopherol oxidase present in soybean may be responsible for the destruction of this vitamin.

1.4.2.2 Heat-Stable ANFs

1.4.2.2.1 Oligosaccharides

Oligosaccharides are low molecular weight carbohydrates containing α-galactosidic and β-fructosidic linkages. The oligosaccharides raffinose (a trisaccharide), stachyose (a tetrasaccharide) and verbascose (a pentasaccharide) have been attributed as the causative factors for flatulence, the first two being the important causative agents of flatulence in soybean (Murphy, 1963; Steggerda *et al.*, 1966; Rackis *et al.*, 1970; Cristofaro *et al.*, 1974; Rackis, 1975). However, a number of studies have indicated

that oligosaccharides are not the only factors which cause flatulence (Wagner *et al.*, 1976; Hellendorn, 1979; Fleming, 1982; Savitri and Desikachar, 1985).

The range of oligosaccharides found in soybeans varies with the cultivar. Their reported ranges (on a dry weight basis) for raffinose has been found to be between 4 and 20 g/kg, and for stachyose, 14 to 80 g/kg (Smith and Circle, 1972; Hymowitz and Collins, 1974; Cegla and Bell, 1977; Savitri and Desikachar, 1985; Knudsen and Li, 1991).

Monogastrics lack the necessary α-galactosidase activity required to break down oligosaccharides (Gitzelmann and Auricchio, 1965; Cummings *et al.*, 1986). Intact oligosaccharides are metabolised by the microflora in the lower intestine producing gases such as carbon dioxide, hydrogen and methane which may bring about nausea, diarrhoea, abdominal rumbling and the ejection of rectal gases (Calloway *et al.*, 1966; Champ *et al.*, 1990), and have also been attributed to increase the microbial population in the gut (Champ *et al.*, 1990; Veldman *et al.*, 1993). The presence of oligosaccharides has been suspected of causing an osmotic effect leading to fluid retention and an increased rate of passage of digesta that affects absorption of nutrients (Reddy, 1984; Wiggins, 1984; Low, 1985; Huisman and Le Guen, 1991; Veldman *et al.*, 1993).

Numerous processing techniques have been attempted to eliminate oligosaccharides. Omosaiye *et al.* (1978) successfully used ultrafiltration of aqueous extracts of soybeans to remove up to 96% of the oligosaccharides. Germination removed both raffinose, stachyose and verbascose by 144 hours (East *et al.*, 1972; Rao and Belavady, 1978; Jood *et al.*, 1985, Savitri and Desikachar, 1985). Cooking of soybean has been found by a number of authors to bring about an increase in the oligosaccharide level compared to the raw beans (Venkataraman and Jaya, 1975; Rao and Belavady, 1978; Savitri and Desikachar, 1985). On the other hand Venkataraman and Jaya (1975) found that

cooking lowered flatus, and germination either kept the same amount of flatus or increased it. A number of investigations have been carried out using enzymes to destroy oligosaccharides (see Section 1.5.3).

According to Visser (1992), properly processed concentrates should contain only 0.5 g/kg raffinose and 7 g/kg stachyose.

1.4.2.2.2 Phytate

Phytate is a cyclic compound containing six phosphate groups and occurs in soybean to the extent of 10 to 15 g/kg (Liener, 1994).

While binding 40 to 60% of phosphorus in soybean, phytate also chelates with di- and trivalent metals, such as calcium, magnesium, zinc and iron, forming unavailable poorly soluble compounds (Davies and Reid, 1979; Reddy *et al.*, 1982; Heaney *et al.*, 1991; Hurrell *et al.*, 1992). Phytate also interacts strongly with the basic residues of proteins, and has been shown in vitro to inhibit the action of a number of digestive enzymes, such as pepsin (Vaintraub and Bulmuga, 1991), trypsin (Caldwell, 1992) and α-amylase (Thompson and Yoon, 1984).

A number of enzyme preparations have been used to eliminate the antinutritional effect of phytate. These experiments are outlined in Section 1.5.3.

1.4.2.2.3 Saponins

Saponins constitute a family of structurally related compounds containing a steroid or triterpenoid aglycone linked to one or more oligosaccharide moieties. Whole soybeans contain approximately 56 g/kg of saponins (Fenwick and Oakenfull, 1981).

Saponins are responsible for imparting a bitter taste to plant materials containing high levels. They can cause intestinal membrane destabilisation and cell lysis (Johnson *et al.*, 1986). Unlike some other plant saponins, soybean saponins have been found to be

relatively innocuous to chicks, rats and mice, even when fed at three times the level normally found in soybean (Ishaaya, 1969), and are hydrolysed by bacterial enzymes in the lower intestine in these animals (Gestetner *et al.*, 1968). Soya bean saponins appear to have only a weak effect on active nutrient transport (Johnson *et al.*, 1986), and although in vitro studies have indicated an ability to inhibit various enzymes, including trypsin and chymotrypsin, this activity is eliminated by preincubation with other proteins (Sautier *et al.*, 1979).

1.4.2.2.4 Antigenic Factors

It has been suggested that the components most likely to be responsible for allergenicity are glycinin and β-conglycinin (Killshaw and Sissons, 1979; Liener, 1994).

Antigenic factors cause an immune response in the host animal if they are absorbed into the bloodstream. They can cause damage to the intestinal mucosa, resulting in negative effects on digestion and absorption processes, and have been known to cause diarrhoea, weight losses and occasional death (Kilshaw and Sissons, 1979; Miller *et al.*, 1984; Seegraber and Morril, 1986; Toullec and Guilloteau, 1989).

Contradictory reports have been presented as to the stability of these antigens to different processing methods (Kilshaw and Sissons, 1979; Visser, 1992).

1.4.2.2.5 Tannins and Other Phenolic Compounds

Tannins are water soluble phenolic compounds having a molecular weight between 500 and 3,000. The tannin content of soybeans is about 450 mg/kg (Rao and Prabhavati, 1982).

Tannins and other phenolic compounds are associated with the adverse flavours, odours and colours occurring in oilseed protein sources (Sossulski, 1979). Tannins form complexes with proteins, carbohydrates and other polymers, such as digestive enzymes,

mucosal proteins and glycoproteins (Rao and Prabhavathi, 1982). These affinities lead to a reduction in nutrient digestibility (Griffiths and Mosely, 1980; Jansman *et al.*, 1989; Marquardt, 1989). Tannins have also been attributed to cause damage in the gut wall and interfere with the absorption of some minerals (Mitjavila *et al.*, 1977).

1.4.2.2.6 Oestrogens

Natural oestrogens found in soybeans have been reported to bring about an oestrogenic response in experimental mammals (Magee, 1963; Farmakalidis and Murphy, 1984). These oestrogens can be removed by hexane and ethanol-water extraction and properly processed soybean products should be inactive (Visser, 1992).

1.4.2.2.7 Lysinoalanine

Lysine is converted to lysinoalanine under severe alkaline conditions (Van Beek *et al.*, 1974; Karayiannis *et al.*, 1979). The conversion of lysine to lysinoalanine leads to a decrease in the digestibility of proteins and a decrease in biologically available lysine (Hayashi and Kameda, 1980; Robbins *et al.*, 1980; Abe *et al.*, 1984). Lysinoalanine has been reported to bring about lesions in the kidney of rats (Robbins *et al.*, 1980). Other effects have been reported which tend to indicate that different species of animals have different reactions to this toxin (Woodward and Short, 1963; De Groot *et al.*, 1976). According to Visser (1992) properly processed soybean products should contain no free lysinoalanine and about 40 mg/kg bound lysinoalanine.

1.4.2.3 Experiments Involving Purified ANFs in Fish

As can be seen in Table 1.5, the amount of work carried out on soybean ANFs using purified products is very limited, concentrating on proteolytic enzyme inhibitors and phytic acid. In these experiments there is a clear effect of these ANFs on fish

Table 1.5 Selected experiments carried out on soybean ANFs in fish species.

ANF and inclusion details	Species/size	Results	Reference
3.7, 7.3, 11.0, 14.7 g/kg SPI	300 g rainbow trout, *O. mykiss*	As SPI level increased the fish intestinal trypsin activity was markedly reduced, and there was an increase in protein and lipid excretion. The authors suggest that a limited compensation to SPI activity may be possible.	Berg Lea *et al.*, 1989
3.7, 7.3, 11.0, 14.7 g/kg SPI	265 g rainbow trout, *O. mykiss*	As SPI level increased the fish intestinal trypsin activity was markedly reduced. Protein digestibility was markedly affected by SPI level, but lipid digestibility was not. The authors suggest that a limited compensation to SPI activity may be possible.	Krogdahl *et al.*, 1994
1.05, 3.15, 4.2 g/kg trypsin inhibitor	180 g Atlantic salmon, *S. sailor*	As fish were fed diets with increased trypsin inhibitor the growth, digestibility and intestinal trypsin activity were significantly reduced, although a limited compensation in trypsin activity was observed	Olli *et al.*, 1994
5 g/kg phytic acid	26 g rainbow trout, *O. mykiss*	Addition of phytic acid reduced fish growth and feed conversion by 10% compared to fish fed unsupplemented diet. Authors concluded that reduced growth was related to a reduction in protein availability rather than to a reduction in zinc or copper availability.	Spinelli *et al.*, 1983
1.62, 6.46, 25.8 g/kg phytic acid	0.85 g chinook salmon, *O. tshawytscha*	Fish fed 25.8 g/kg phytic acid diets suffered depressed performance and health compared to fish fed the other diets.	Richardson *et al.*, 1985
15 g/kg phytic acid	3 g *O. aureus*	Fish fed the 15 g/kg phytic acid diets showed reduced performance and zinc bioavailability compared to fish fed the unsupplemented diet.	McClain and Gatlin, 1988
5, 10 g/kg phytic acid	6.5 g channel catfish, *I. Punctatus*	Fish fed phytic acid did not show less growth than fish fed unsupplemented diets	Gatlin and Phillips, 1989

Table 1.5 (continued) Selected experiments carried out on soybean ANFs in fish species.

ANF and inclusion details	Species/size	Results	Reference
11g, 22 g/kg phytic acid	5 g channel catfish, *I. punctatus*	Fish fed the diet with supplemental 22 g/kg phytate performed significantly less than fish fed diets without phytic acid and 11 g/kg supplemental phytic acid. Fish vertebral zinc content was significantly reduced as the level of phytate in the diets increased in the diet containing 50 mg/kg zinc but not the diet containing 150 mg/kg zinc.	Satoh *et al.*, 1989
5, 10 g/kg phytic acid	4 g common carp, *C. carpio*	As phytic acid was increased fish performance, protein utilisation and digestibility and mineral bioavailability decreased compared to fish fed the phytate-free diet.	Hossain and Jauncey, 1993

performance and nutrient utilisation. Phytate was generally found to reduce performance but not only by affecting mineral availability but also protein digestibility and utilisation. In the case of protein inhibitors the authors noticed that while high levels of inhibitor significantly reduced intestinal trypsin activity, an increased trypsin production may be able to compensate for the effect of inhibitors but only to a limited extent.

1.4.3 EXPERIMENTS WITH SOYBEAN MEAL REPLACING FISH MEAL IN FISH DIETS

The literature describing experiments in which SBM has been substituted for fish meal is often inconsistent and contradictory. Different experiments invariably involved the use of various types and qualities of SBM and FM and experimental protocols, making it difficult to draw definite clear-cut conclusions.

Tables 1.6 and 1.7 present a summary of many of the experiments which have been carried out in which SBM alone has been used to replace FM in fish diets. Table 1.6 presents experiments investigating the effect of replacing FM with different types and levels of SBM on fish performance. Table 1.7 presents experiments investigating the effect on fish performance of further supplementation with amino acids diets in which SBM has replaced FM.

The following general trends can be made out in the experiments carried out on the use of this alternative protein source.

1) SBM cannot completely replace fish meal in the diets of most fish, but can sometimes be included at quite a high level.
2) Larger fish accept higher replacements of FM than do smaller fish.

Table 1.6 Selected experiments in which various types and quantities of SBM were used to replace FM in fish diets.

Soybean meal type and Inclusion details	**Species, size**	**Results**	**Reference**
210 g/kg SBM	2.1 g milkfish, *Chanos chanos*	Fish fed the SBM diet gave better performance than fish fed a 640 g/kg Peruvian FM diet.	Pena de la *et al.*, 1987
500 g/kg full fat SBM treated at two temperatures and moisture conditions for different times	23 g carp, *C. carpio*	Fish fed all the experimental diets gave lower growth than the control 620 g/kg FM diet, but better growth than fish fed the unheated SBM diet. The best growth was obtained with a temperature of 90 to 95°C with saturated steam for 30 minutes.	Abel *et al.*, 1984
300 g/kg cyclohexane extracted SBM heated at 100°C for different times	36 g sea bass, *Dicentrarchus labrax*	Heating the SBM for 12 mins followed by heating for 8 mins gave better fish growth than the control diet.	Amerio *et al.*, 1989
Trials 1,2: 160, 340, 440 g/kg SBM	Trial 1: 100 to 150 g hybrid striped bass , *M. saxatilis x M. chrysops* Trial 2: 5 g, *M. saxatilis x M. chrysops*	Trial 1: Growth of fish fed experimental diets was only slightly less than that of fish fed the control 470 g/kg menhaden FM die Trial 2: All treatments gave inferior fish performance than fish fed the control diet.	Gallagher, 1994
730g/kg full fat SBM	26 g rainbow trout, *O. mykiss*	Fish fed the test diet performed better those fed the control 250 g/kg herring FM diet.	Reinitz *et al.*, 1978
310, 400, 480, 560, 650 g/kg dehulled solvent extracted SBM	15.2 g rainbow trout, *O. mykiss*	Fish fed all experimental diets gave inferior performance to fish fed the control 250 g/kg herring FM diet.	Reinitz, 1980

Table 1.6 (continued) Selected experiments in which various types and quantities of SBM were used to replace FM in fish diets.

Soybean meal type and Inclusion details	Species, size	Results	Reference
Trial 1: 500 g/kg puffed full fat SBM, 320 g/kg full fat SBM, 360 g/kg extruded hexane extracted SBM, 270 g/kg alcohol extracted SBM, 260 g/kg extracted SBM Trial 2: 250 g/kg hexane extracted SBM, 320 g/kg puffed full fat SBM	Trial 1: 33 g rainbow trout, *O. mykiss* Trial 2: 70 g rainbow trout	Trial 1: All treatments gave improved fish performance over control 350 g/kg FM diet. Best growth was obtained with fish fed the 320 g/kg full fat SBM diet. Trial 2: SBM diets gave better results than the control diet, with fish fed the full fat diet giving the best performance.	Tacon *et al.*, 1983a
120 and 250 g/kg SBM	20 g rainbow trout, *O. mykiss*	Fish fed these diets showed better growth than control 570 g/kg herring meal diet.	Alexis *et al.*, 1985
200, 400, 600 g/kg hydrothermically treated SBM	27 g rainbow trout, *O. mykiss*	The 200 g/kg SBM diet gave a significantly higher fish growth than fish fed the other diets and the control 300 g/kg.	Beckmann and Pfeffer, 1989
130, 250, 500 g/kg SBM	1 g rainbow trout, *O. mykiss*	50% mortalities were recorded with fish fed the 500 g/kg SBM (0 FM) diet. The 130 g/kg SBM diet gave equal fish growth to control 350 g/kg FM diet, but fish fed the 250 g/kg SBM inclusion showed reduced growth.	Dabrowski *et al.*, 1989
300, 400, 500 g/kg defatted and extruded defatted SBM	12 g rainbow trout, *O. mykiss*	Fish performances decreased as the SBM level was increased, and all gave lower performances than the fish fed the control 640 g/kg white FM diet.	Pongmaneerat and Watanabe, 1992
Trial 1: 300 g/kg solvent extracted SBM Trial 2: 300 g/kg non-extruded, extruded SBM	5 g rainbow trout, *O. mykiss*	Trial 1: Fish fed this diet gave slightly lower performance than control 550 g/kg FM diet. Trial 2: These diets gave lower fish performances than the control diet.	Pongmaneerat and Watanabe, 1993
400 g/kg specially processed solvent extracted SBM, solvent extracted SBM, ethanol extracted SBM, alkali treated SBM	5 g rainbow trout, *O. mykiss*	The specially processed solvent extracted SBM diet gave better fish growth than the control 280 g/kg LT FM diet. All other diets gave lower fish growth than the control diet.	Rumsey *et al.*, 1993

Table 1.6 (continued) Selected experiments in which various types and quantities of SBM were used to replace FM in fish diets.

Soybean meal type and Inclusion details	Species, size	Results	Reference
200, 300, 400, 500 g/kg defatted SBM	5 g rainbow trout, *O. mykiss*	With all except the 300 g/kg SBM diet fish growth was superior but feed efficiency less than fish fed the control 550 g/kg sardine FM diet.	Watanabe and Pongmaneerat, 1993
300g/kg full fat SBM, 320 g/kg full fat extruded SBM, 240 g/kg solvent extracted SBM, 210 g/kg solvent extracted SBM treated with infra red	38 g rainbow trout, *O. mykiss*	Fish fed all diets except the full fat extruded SBM diet performed better than fish fed the control 570 g/kg brown FM diet.	Oliva-Teles *et al.*, 1994
220, 420, 620 g/kg soy protein concentrate, 240, 420 g/kg soy flour	83 g rainbow trout, *O. mykiss*	Diets with 420 and 620 g/kg soy protein concentrate gave equal fish growth and better PER than the 650 g/kg Norwegian herring FM diet, but lower FE. Use of soy flour reduced fish performance compared to the control diet.	Kaushik *et al.*, 1995
25, 50, 75% replacement of FM protein with a commercial soybean protein concentrate and hexane extracted SBM	0.5 g *O. mossambicus*	All hexane extracted SBM diets gave inferior fish performance than the control diet. Fish fed the 50, 75% protein concentrate diets gave superior performance to fish fed the control diet.	Davies *et al.*, 1989
Combinations of boiled and unboiled SBM, defatted SBM	10 g *O. niloticus*	The best growth was given by fish fed the diet containing boiled SBM only, followed by fish fed the defatted SBM diet. Both these diets gave better fish growth than the control 600 g/kg tilapia FM diet.	Wee and Shu, 1989
170 g/kg defatted SBM, 180 g/kg full fat SBM	5.1 g tilapia, *O. niloticus* x *O. aureus*	The diet with defatted SBM diet gave better fish performance than fish fed the control 360 g/kg FM diet.	Shiau *et al.*, 1990
420, 600 g/kg SBM	77 g *O. niloticus* x *O. aureus*	Fish fed the experimental diets gave equal performances to the control 200 g/kg FM diet	Viola *et al.*, 1988

Table 1.6 (continued) Selected experiments in which various types and quantities of SBM were used to replace FM in fish diets.

Soybean meal type and Inclusion details	**Species, size**	**Results**	**Reference**
170, 340 g/kg dehulled, hexane extracted SBM	925 g Atlantic salmon, *Salmo salar*	Growth of fish fed the 170 g/kg SBM diet was slightly higher than that of fish given the control 560 g/kg Norse-LT94 FM diet..	Olli *et al.*, 1995
25, 50, 75, 100% replacement of FM with solvent extracted SBM	7.4 g red drum, *S.s ocellatus*	25% replacement improved fish growth over control 520 g/kg menhaden FM diet. The other diets reduced fish growth, and the fish receiving the diet with 100% SBM replacement showing reduced consumption and 50% mortalities in 4 weeks.	Reigh and Ellis, 1992
200 g/kg dehulled solvent extracted SBM heated at 108°C for various times	45 g yellowtail, *Seriola quinqueradiata.*	The best fish performance was obtained with feeding fish a diet containing SBM which had been treated with 30 minutes heating. All experimental diets gave lower fish performance than fish fed the control 750 g/kg brown FM diet.	Shimeno *et al.*, 1992
200, 300, 400, 500 g/kg defatted SBM	Trial 1: 350 g yellowtail, *S. quinqueradiata* Trial 2: 39 g yellowtail	Trial 1: growth of the fish fed the 400 g/kg SBM diet equalled that of the control 550 g/kg sardine FM control. The other inclusions gave lower growth and all diets gave lower feed utilisation. Trial 2: The performance of fish fed the 200 g/kg SBM diet was only slightly lower than that of fish fed the control diet.	Viyakarn *et al.*, 1992
Trial 1:100, 200, 300 g/kg defatted SBM Trial 2: 300 g/kg defatted SBM	Trial 1: 160 g yellowtail., *S. quinqueradiata* Trial 2: 1200 g yellowtail	Trial 1: Performances of fish fed all treatments were lower than that of fish fed the control 560 g/kg FM diet. Trial 2: Fish performance was reduced slightly compared to fish fed the control diet.	Watanabe *et al.*, 1992.

Table 1.6 (continued) Selected experiments in which various types and quantities of SBM were used to replace FM in fish diets.

Soybean meal type and Inclusion details	Species, size	Results	Reference
200, 300 g/kg defatted SBM	230 g yellowtail, *S. quinqueradiata.*	Fish fed the experimental diets performed slightly better than fish fed the control 720 g/kg brown FM diet.	Shimeno *et al.*, 1993a
300 g/kg SBM, fermented defatted SBM, 230 g/kg soy protein concentrate	42 g yellowtail, *S. quinqueradiata*	Fish fed the fermented diets performed better those fed the unfermented SBM, but less than fish fed the control 750 g/kg brown FM diet. Fish fed the soy concentrate diet gave slightly better performance than fish fed the control diet.	Shimeno *et al.*, 1993b
300 g/kg heated defatted, raw defatted, heated full fat, extruded SBM, 200 g/kg soy protein concentrate, 160g/kg soy protein separate, soy protein peptide	18 g yellowtail, *S. quinqueradiata.*	All diets gave lower fish performances than fish fed the 700 g/kg brown FM control. The soy protein diets gave the best fish performance of all the experimental diets.	Shimeno *et al.*, 1995

Table 1.7 Selected experiments in which amino acids were supplemented to diets in which SBM was used to replace FM in fish diets.

Soybean meal type and Inclusion details	Species, size	Results	Reference
360 g/kg hexane extracted SBM, 410 g/kg methanol treated hexane extracted SBM, essential amino acid mixture, nonessential amino acid mixture	3.5 g carp, *C. carpio*	Supplementation of amino acid mixture brought fish performance up to 90% of that of fish fed the 450 g/kg white FM control. Methanol increased fish performance over fish fed the untreated SBM diets.	Murai *et al.*, 1986
410 g/kg SBM, methionine, essential amino acid mixture	3.4 g carp, *C. carpio.*	Addition of methionine to the SBM diet increased fish growth over the unsupplemented diet, but the essential amino acid mixture was not sufficient to improve growth without the addition of methionine, and the non essential amino acid mix lowered fish performance compared to the unsupplemented diet.	Murai *et al.*, 1989a
560 g/kg dehulled, hexane solvent extracted SBM, 3 g/kg L-methionine, DL-methionine, acetylmethionine and 4 g/kg Dl-methionine hydroxy analogue	8 g sunshine bass, *M. chrysops* x *M. saxatilis*	Performances of fish fed all supplemented diets except for the analogue were significantly higher than fish fed the basal diet, but lower than that of fish fed the 570 g/kg menhaden FM control.	Keembiyehetty and Gatlin, 1997
800 g/kg dehulled SBM, various individual or mixtures of amino acids	9 g rainbow trout, *O. mykiss*	Addition of lysine, histidine and various other mixtures to the basal diet improved fish growth relative to the unsupplemented diet.	Rumsey and Ketola, 1975
260 g/kg SBM, cystine and tryptophan, lysine, cystine, lysine and arginine	33 g rainbow trout, *O. mykiss*	Fish fed the diet supplemented with 10 g/kg cystine and 5 g/kg tryptophan gave equal performance to fish fed the 280 g/kg FM control. Fish fed diets with other combinations of amino acids gave inferior performance to fish fed the control diet.	Dabrowska and Wojno, 1977
480 g/kg soya flour, 480 g/kg methanol treated soya flour, essential and nonessential amino acid mixtures	0.7, 5.1, 8.9 g rainbow trout, *O. mykiss*	In all cases fish performance were inferior to the control 560 g/kg FM diet. However, ethanol treatment and addition of amino acids did improve fish performances.	Murai *et al.*, 1989b

Table 1.7 (continued) Selected experiments in which amino acids were supplemented to diets in which SBM was used to replace FM in fish diets.

Soybean meal type and Inclusion details	**Species, size**	**Results**	**Reference**
600 g/kg dehulled hexane extracted SBM, methionine, lysine, essential amino acid mixture	50 g rainbow trout, *O. mykiss*	All diets gave inferior fish performance to control 580 g/kg Chilean brown herring FM diet. Using the amino acid mixture improved fish performance compared to using methionine or methionine and lysine supplemented diets.	Davies & Morris, 1997
420 g/kg hexane extracted SBM, 500 g/kg full fat SBM, methionine	0.8 g *O. niloticus*	Addition of methionine of up to 7 g/kg to the 420 g/kg SBM diet still gave inferior performances to the control 450 g/kg brown or white FM diets. Addition of 5 g/kg methionine to the 500 g/kg SBM diet gave as good a growth as the brown FM diet, but lower than the white FM diet.	Tacon *et al.*, 1983b
240 and 320 g/kg protein diets in which 30% of the FM was replaced by hexane extracted SBM, methionine	1.2 g *O. niloticus* x *O. aureus*	In fish fed the 240 g/kg protein diet, addition of 2 g/kg methionine gave a performance slightly better than fish fed the control 350 g/kg blue whiting FM control, but addition of 2.6 g/kg methionine to the 320 g/kg protein diet gave a lower performance than the control 470g/kg blue whiting FM control. Additions of methionine improved fish performance over the performances of fish fed unsupplemented SBM diets.	Shiau *et al.*, 1987
100, 200, 300 g/kg soy protein concentrate, essential amino acid mix	Trial 1: 35 g yellowtail, *S. quinqueradiata* Trial 2: 129 g yellowtail. Trial 3: 189 g yellowtail	Trial 1: Fish performance was comparable but lower than that of fish fed the 650 g/kg brown FM control; fish fed the diet with 200 g/kg soy protein gave the best performance. Trial 2: In this trial fish fed the supplemented 200 g/kg soy protein diet had better growth than fish fed the control diet. Trial 3: The addition of amino acids gave as good a performance as fish fed the control diet.	Takii *et al.*, 1989

3) Essential amino acid supplementation generally does improve performance of fish compared to unsupplemented diets.
4) The methods and conditions used in SBM processing are important parameters in determining performance.
5) Palatability is generally not affected by the introduction of SBM, except in the cases where a very high percentage of the FM is replaced, in which cases significant mortalities are often recorded.

It must be kept in mind that even in those cases where a superior performance to a control diet is not obtained, the cheaper price of the SBM diets used might still give a better economic return.

1.5 SUPPLEMENTARY ENZYMES IN FEEDS

Industrially produced enzymes are currently being used in numerous ways such as in detergents, brewing, cheese making and in feed production for farmed animals. The majority of industrial enzymes are hydrolytic in nature (Collier and Hardy, 1986a; Daniels, 1990; Walsh and Headon, 1994). According to Walsh and Headon (1994), the estimated annual world-wide sales of bulk enzymes is around US$600 million. The largest enzyme type sold are the proteases, at roughly half the total volume of enzymes produced, followed by the carbohydrases (Daniels, 1990; Walsh and Headon, 1994).

Bacteria, yeasts and moulds are fertile sources of a wide range of enzymes. Plant enzymes are limited to cereal amylases, although some proteases such as papain and bromelain are also extracted from plants. Similarly, enzymes of animal origin are still limited although rennet, lipases and trypsins have been produced (Daniels, 1990).

1.5.1 ENZYME COCKTAILS

Currently, most enzymes used in feeds are targeted against the cereals wheat, barley, oats, rye and triticale (Bedford, 1993a). It has been found that using purified enzymes does not bring about as good an improvement in performance as using a number of different enzymes together ('cocktails') although one particular enzyme may be the main component of the preparation (Clifford, 1989; GrootWassink *et al.*, 1989; Inborr, 1989, 1990; Graham and Inborr, 1993a).

The number of enzymes and enzyme cocktails available for use in the feed industry is always increasing, as new enzymes are produced and species and diet-specific concoctions are developed. In the experiments referred to below a number of purified enzymes as well as numerous commercially produced enzyme cocktails were used. Amongst these commercial preparations used in these experiments were Avizyme SX, Avizyme TX, Porzyme SP, Porzyme TP, Porzyme SF, Zymo-Best and Kemzyme.

1.5.2 ENZYMES AND FEED INGREDIENTS

1.5.2.1 Intestinal Viscosity

An important component of oats, barely, rye and to some extent also wheat, which has limited their use beyond a certain extent in monogastric feeds are the non-starch polysaccharides, consisting of components such as cellulose, arabinoxylans, β-glucans and pectins, which monogastrics are virtually unable to digest (Graham and Bedford, 1992; Bedford, 1993b). The bulk of the non-starch polysaccharides in oats and barley are β-glucans and, in wheat and rye, arabinoxylans (Henry, 1985; Annison, 1990).

Apart from these components being themselves indigestible and interfering with the accessibility of other digestible materials, there are other deleterious effects related to

the property of intestinal viscosity (Antoniou and Marquardt, 1982; White *et al.*, 1983; Graham and Bedford, 1992; Wang *et al.*, 1992). Unimpeded movement of enzymes, substrates and products by diffusion is essential for efficient digestion and absorption. However, if the viscosity of the intestinal contents increases, the rate of diffusion decreases (Fengler and Marquardt, 1988). The larger the size of the solute, the greater the extent to which its rate of diffusion decreases (Campbell *et al.*, 1983). The animal has some capacity to compensate for such a reduction in digestive capacity through increased production of digestive enzymes, although this capacity may be limited (Ikegami *et al.*, 1990).

An increased viscosity resulted in a decreased throughput of material in the intestine of birds fed rye and barley-based diets and consequently the feed intake was also reduced (Salih *et al.*, 1991). A reduced feed transit rate leads to a reduced flushing rate which allows intestinal bacteria to multiply and migrate to the upper parts of the intestine (Salih *et al.*, 1991). These bacteria will consume some of the products of digestion. Some intestinal bacteria also produce bile degrading enzymes, further reducing lipid digestion and since some bile acids are also thought to stabilise pancreatic proteases, protein digestion may also be affected (Feighner and Dashkevicz, 1988). In fact, addition of either bile acids or antibiotics has been found to alleviate many of the above problems (Campbell *et al.*, 1983).

The aspect of intestinal viscosity has now become the accepted mechanism by which such cereals influence digestion and performance (Graham and Bedford, 1992).

1.5.2.2 Using Supplemental Enzymes

The use of supplemental enzymes in feeds has shown that lower cost raw ingredients or cheaper less-processed materials, such as those mentioned above, can be used with equal and even better performance than more expensive materials. The use of enzymes

with feeds containing these more expensive feeds has also been found to bring about an improvement in food utilisation. Enzymes thus enable new ingredients to be introduced into feed formulations, and thereby increase the choice and flexibility of the feed manufacture.

The beneficial effect of supplemental enzymes in diets containing various ingredients has been reported in broiler chickens by Jensen *et al* (1957), Burnett (1966), Herstad and McNab (1974), Hesselman *et al.* (1982), Classen *et al.* (1985), Collier and Hardy (1986b), Hesselman and Aman (1986), Pettersson *et al.* (1987), Pettersson and Aman (1988, 1989), Edney *et al.* (1989), GrootWassink *et al.* (1989), Cantor (1990), Cave *et al.* (1990), Classen *et al.* (1991), Graham and Inborr (1993a), Potter (1991), Bedford and Classen (1992a), Graham and Bedford (1992), Walsh and Headon (1994) and Finnfeeds (1995a, b, c), and in starter pigs by Thomke *et al.* (1980), Grammer *et al.* (1982), Newman *et al.* (1983), Graham *et al.* (1988), Nasi (1988a), Adams (1989), Inborr (1990), Graham and Inborr, (1993a) and Finnfeeds (1996a, b).

The improvement in performance and productivity was also demonstrated for layers (Nasi, 1988b; Adams, 1989; Graham and Bedford, 1992), larger swine (Classen *et al.*, 1991; Graham and Inborr, 1993a) and in growing turkeys (Salmon *et al.*, 1986).

The addition of supplemental enzymes has also been found to reduce the amount of digestive disorders and litter problems in these animals (Nahm and Carlson, 1988a; Cantor, 1990; Inborr, 1990; Pettersson *et al.*, 1990; Classen *et al.*, 1991; Graham and Inborr, 1993a; Graham and Bedford, 1992; Herstad and McNab, 1974; Finnfeeds, 1996b).

A number of these studies have indicated that the response to enzyme supplementation decreases with animal age, possibly a result of increasing digestive capacity with a larger enzyme production and a more developed microbial population (Clifford, 1989;

Inborr, 1989; Classen *et al.*, 1991; Harker, 1991; Graham and Bedford, 1992; Bedford, 1993b).

Improved results have also been observed following pre-treatment of feed materials in order to give the enzymes more time to act on their substrates (Nasi, 1988a). According to Inborr (1990) enzyme pre-treatment of feeds and feed raw materials is possibly the best way to increase efficiency and performance of the feeds.

The beneficial use of supplemental enzymes has been strongly connected to their effect on viscosity. Reduced digesta viscosity has been shown to explain 50 to 80% of the improvements in broiler chick performances fed enzyme supplemented wheat-rye and barley based diets (Graham and Inborr, 1993b). The relationship between intestinal viscosity, performance and the effect of enzyme addition was demonstrated by Bedford and Classen (1992a, b), Hesselman and Aman (1986), Classen *et al.* (1991), Wang *et al.* (1992) and Finnfeeds (1995a, b, c). Improvement in digestibility of the nutritional components in feeds has been found to be not only limited to that of the non-starch polysaccharides but also the other organic components such as starch and protein, a feature that has been attributed to the effect of reduced intestinal viscosity and release of nutrients from the plant cells.

While barley, rye, triticale and oat-based diets can cause a high intestinal viscosity in poultry and pigs, this is not so in the case of maize, sorghum and tapioca-based diets, and the benefit of enzymes in the case of diets containing these ingredients probably lies in the breakdown of the cell walls which releases the contents to the host's enzymes (Bedford, 1993a).

No evidence has been found that indicates that the host enzymes are inhibited by the addition of exogenous enzymes (Graham and Inborr, 1993a). Instead, Shields *et al.*

(1980) and Owsley *et al.* (1986) found evidence suggesting that addition of exogenous enzymes actually stimulated host enzyme production.

1.5.3 ENZYMES AND ANTI-NUTRITIONAL FACTORS

Enzymes are also being used to try and reduce the effect of antinutritional factors in the ingredients being used in feed formulation. The work carried out so far has been limited to phytic acid and oligosaccharides.

Phytase enzymes have been considered as possible feed additives to improve phosphorus availability of feeds (Zyta, 1992). Walsh and Headon (1994) reported an increase in absorption and retention of phosphorus in trials in which pigs were fed phytase-supplemented feeds, as did Beers and Jongbloed (1992). Knorr *et al.* (1981) observed a reduction in phytic acid when phytase and phosphatase were added to whole-wheat flour dough.

Although oligosaccharide digestibility was increased, Veldman *et al.* (1993) concluded that there was no beneficial effect to be derived from adding an α-galactosidase of fungal origin (*A. niger*, var. Teighem) to a piglet feed containing velasse.

α-galactosidase from germinating guar seeds (*Cyamopsis tetragonolobus*) hydrolysed the raffinose and stachyose in soybean milk (Shivanna *et al.*, 1989), as did the α-galactosidase from the mould *Mortierella vinacea* (Thananunkul *et al.*, 1976) and that obtained from *Aspergillus saitoi* (Sugimoto and Buren, 1970). Somiari and Balogh (1992) used α-galactosidase from *A. niger* to reduce the raffinose and stachyose content in cowpea flour by 95 and 82% respectively.

1.5.4 ENZYME STABILITY

Enzymes added to feeds have to withstand manufacturing processes, storage before and after addition to a feed, and intestinal conditions such as proteolytic and acid attack. The enzymes used should have temperature and pH optima at or at least close to the physiological conditions in which they are to function. The search for stability starts even before the enzymes themselves are produced. Stability is altered and influenced by careful selection of the source and production technique.

Low-temperature drying is the most effective method of improving storage. Many enzymes can be stabilised by simply adding common salt and various sugars. Stability can also be conferred by chemical cross-binding, although the same result can be obtained by allowing the enzymes to bind to a substrate and then remove water (Graham and Inborr, 1993b).

Liquid-based enzymes are less stable than dry enzymes (Graham and Inborr, 1993b; Nissinen, 1994). Storing at a suitable pH and under refrigeration greatly improves liquid enzyme stability. Further stability can be obtained by pre-treatments such as absorbing liquid enzymes onto carriers providing substrate to which the enzymes can bind (Inborr, 1990).

Results from shelf life stability trials suggest that suitably stabilised enzymes can be stored at room temperature for up to six months without any significant loss in activity (Classen *et al.*, 1991). Only a small reduction in the main enzyme activities was recorded by Graham and Inborr (1993b) after 50 weeks storage at room temperature. Vitamins, minerals, trace elements and oxidising agents usually found in concentrated premixes have been shown to cause no significant loss in enzyme activity during the first two months of mixing together (Inborr, 1990). A high recovery was also obtained by Graham and Inborr (1993b) even after 31 weeks.

Stabilised enzymes can withstand pelleting processes at high temperatures (up to 95°C) without loss of effectiveness (Inborr, 1990; Bedford, 1993c; Graham and Inborr, 1993b). Current enzymes will probably not be able to survive the conditions experienced in the expansion of feed (Bedford, 1993c; Graham and Inborr, 1993b; Nissinen, 1994) and are added after the pellets leave the pellet mill.

When properly stabilised and mixed into feed, enzyme activity remains high for several hours in acidic (HCl) conditions and have been found to be able to withstand pepsin and trypsin activity (Graham and Inborr, 1993b). Again, liquid enzymes have been found to be more susceptible to breakdown in the intestine than dry enzymes (Graham and Inborr, 1993b).

1.5.5 SUPPLEMENTARY ENZYMES IN AQUACULTURE TRIALS

Few trials have so far been performed, or at least published, on the effect on fish performance of supplemental enzymes in feeds. Table 1.8 summarises the experiments carried out on supplementary enzymes in aquaculture studies.

From Table 1.8 it can be seen that the diets with supplementation of enzymes generally improved the performance of the fish and increased digestibility over those shown by fish fed unsupplemented diets.

1.6 ENZYMES IN THE DIGESTIVE TRACT OF FISH

During the early stages of their development fish have an incompletely developed digestive tract. Various studies have shown that the organs of the intestine complete development as the fish grows older, and the enzymatic capacity of the digestive tract increases along with this development (Kawai and Ikeda, 1973a, b; Lauff and Hofer, 1984; Buddington, 1985; Clark *et al.*, 1985; Baragi and Lovell, 1986; Cousin *et al.*,

Table 1.8 Experiments involving supplemented enzymes in aquaculture trials.

Supplemented Enzyme	Species, size	Results	Reference
Various volumes of carp intestine and hepatopancrease enzyme extracts	1.5 mg carp, *C. carpio*	Performance of fish fed all experimental diets was well below that obtained by fish fed zooplankton.	Dabrowska *et al.*, 1979
Commercial amylase preparation	13 g Atlantic salmon, *S. salar*	There were no significant differences between the performance of fish the fed supplemented and unsupplemented diets.	Carter *et al.*, 1992a
2.5 g/kg proteases (Coralases) and 2.5 g/kg amylases added to diet	3.2 mg *Menidia beryllina*	Growth of fish fed the enzyme supplemented diet was slightly lower than unsupplemented diet, but survival was higher. Both gave equal growth to fish fed live food but had lower survival.	Ashraf *et al.*, 1993
0.4, 1.2, 3.6 g/kg enzyme mix (Kemzyme dry) to diet with 40% or protein supplied by cotton seed meal	38 g rainbow trout, *O. mykiss*	No differences between fish fed any of the diets were found.	Cardenete *et al.*, 1993
2 Multienzyme premixes	Carp, *C. carpio*	Protease and diastase activity in intestine were found to have increased, and while diastase activity in the hepatopancreas increased protease activity decreased.	Ye *et al.*, 1993
1 mg/kg enzyme mix (proteases, carbohydrases) to 340 g/kg soybean meal diet	95 g Atlantic salmon, *S. salar*	Fish fed supplemented diet gave better performance than fish fed unsupplemented diet and control 660 g/kg LT fish meal diet.	Carter *et al.*, 1994
0.5, 1.0, 1.5 g/kg cocktail containing amylase, protease, β-glucanase, β-glucosidase, cellulase (Polizyme)	46 g carp, *C. carpio*	The fish fed the 1.5 g/kg supplemented diet gave a significantly better performance than the fish fed the unsupplemented diet.	Bogut *et al.*, 1995
Phytase treated soybean meal	1.9 g rainbow trout, *O. mykiss*	Use of phytase treated soybean diets improved performance of fish over fish fed the untreated soybean meal diets, increased phosphorus availability and reduced phosphorus in effluent.	Cain and Garling, 1995

Table 1.8 (continued) Experiments involving supplemented enzymes in aquaculture trials.

Supplemented Enzyme	Species, size	Results	Reference
1000 U/kg phytase (*Aspergillus niger*)	Rainbow trout, *O. mykiss*	Digestibility and utilisation of phosphorus were improved with addition of phytase to the diets.	Rodehutscord and Pfeffer, 1995
500 and 1000 U/kg phytase (*A. niger*)	40 g Common carp, *C. carpio*	Supplementation with phytase improved utilisation of phosphorus and reduced phosphorus excretion.	Schaefer *et al.*, 1995
Multienzyme premix	Carp, *C. carpio*	Supplementation improved performance and digestibility in fish fed these diets. Protease and diastase activity in the intestine were found to have increased with supplementation, and while diastase activity in the hepatopancreas increased protease activity decreased.	Ye *et al.*, 1995
1 g/kg Enzyme mix containing a protease and a carbohydrase to 494 g/kg SBM diet	4 g mirror carp, *C. carpio*	Fish fed the supplemented diet performed better than fish fed the unsupplemented diet and control 250 g/kg FM diet.	Feord, Pers. Comm., 1996
1 g/kg Enzyme mix (Pescazyme 5602) to 630 g/kg SBM diet	4 g *O niloticus*	Fish fed the supplemented diet performed better than fish fed the unsupplemented diet and 120 g/kg FM control.	Finnfeeds 1996c
1 g/kg Enzyme mix (Pescazyme 5602) to 505 g/kg SBM diet	5 g common carp, *C. carpio*	Fish fed the supplemented diet performed better than fish fed the unsupplemented diet but less than fish fed the control 100 g/kg FM diet.	Finnfeeds 1996d
500, 1000, 2000 and 4000 Units/kg microbial phytase	6.5 g channel catfish, *I. punctatus*	All fish fed supplemented diets showed improved performance compared to fish fed the unsupplemented diet and reduced amount of faecal phosphorus. The best result was obtained with the fish fed the 1000 Units/kg supplemented diet.	Robinson *et al.*, 1996

Table 1.8 (continued) Experiments involving supplemented enzymes in aquaculture trials.

Supplemented Enzyme	Species, size	Results	Reference
0.5 g porcine pancreatic enzyme (pancreatin) to microdiet fed with and without live food	20 day old sea bass, *D. labrax*	Addition of enzymes to microdiet fed alone did not improve growth of fish, but addition to microdiet combined with live food feeding increased growth of fish over fish fed the unsupplemented microdiet and unsupplemented diet with live food feeding.	Kolkovski *et al.*, 1997
Various combinations of α-amylase (250, 500, 750 IU/g) and trypsin (70 μM/g)	0.60 g *P. japonicus*	All diets gave better performance in shrimp compared to shrimp fed the unsupplemented diet. Amylase activities in the hepatopancreas increased as the level of supplemented amylase increased as did the protease activity in the tyrpsin supplemented diets.	Maugle *et al.*, 1983a
30 and 60 IU α-amylase	0.64 g *P. japonicus*	Fish fed the supplemented diets showed improved performance over shrimp fed the unsupplemented diets, starch digestibility was increased as enzyme inclusion increased. Amylase activity of the hepatopancreas increased but protease activity generally decreased.	Maugle *et al.*, 1983b
1 g/kg Microbial enzyme	Prawn	Prawn fed the supplementated diet had a better performance over prawns fed the unsupplemented diet.	Zhong *et al.*, 1994
2.5 g/kg enzyme mixture (Porozyme) added to low and high canola meal diet	0.96 g prawn, *P. monodon*	Supplemented diets improved performance of prawns over those fed the unsupplemented diets, in the case of prawns fed the low canola meal diet performance was better than prawns fed the control 590 g/kg squid meal diet.	Buchanan *et al.*, 1997

1987; Umeda *et al.*, 1987; Segner *et al.*, 1989; Dabrowski and Culver, 1991; Kolkovski *et al.*, 1993; Cahu and Infante, 1995; Gawlicka *et al.*, 1995; Kuz'mina, 1996; Peres *et al.*, 1996).

A number of authors have found good correlations between the activities of proteases, carbohydrases and lipases and the natural feeding habits of fish (Fish, 1960; Olatunde and Ogunbiyi, 1977; Hsu and Wu, 1979; Hofer and Schiemer, 1981; Ray, 1988; Olatunde *et al.*, 1991; Sabapathy and Teo, 1993; Ugolev and Kuz'mina, 1994) although this was not the case in the investigations carried out by Stickney and Shumway (1974), Ni *et al.* (1990) and Chakrabarti *et al.* (1995).

Research by various authors has also shown that the digestive enzyme complement of a fish is influenced by the nutritional composition of the diet and by changes in the nutritional composition of the diet (Kawai and Ikeda, 1972; 1973a; Olatunde and Ogubiyi, 1977; Hofer, 1979a, b, 1982; Reimer, 1982; Hofer and Sturmbauer, 1985; Buddington and Hilton, 1987; Phadate and Srikar, 1988; Achene *et al.*, 1989; Dabrowski *et al.*, 1989, 1992; Filioglou and Alexis, 1989; Dabrowski and Culver, 1991; Caruso *et al.*, 1993; Cahu and Infante, 1994, 1995a, b, 1997; Yamamoto and Akiyama, 1995; Peres *et al.*, 1996).

A large number of analyses have been carried out to determine the enzymes present in a wide range of fish species, and few differences exist as to the types of enzymes present, although the activities of the enzymes present in the different parts of the intestine may vary. Comparing activities of enzymes is not easy because of the many different assay methods, such as reagents and temperatures, which have been used in the various determinations. Table 1.9 and 1.10 give a limited list of enzymes found in some of these investigations in the stomach and intestine respectively. Most work has been carried out on pepsin, trypsin and chymotrypsin (generally determined by most authors)

Table 1.9 Digestive enzymes present in the stomach of fish. A figure in brackets denotes the determined pH optimum.

Enzyme	Species	Reference
Pepsin	Cod, *Gadus callarias*	Labarre *et al.*, 1951
	Tilapia mossambica, European perch, *Perca fluviatilis*	Fish, 1960
	Eel, *Anguilla japonica* (2.5-3.3)	Moroshita *et al.*, 1964
	Nile tilapia, *O. nilotica* (2.1)	Moriarty, 1973
	Bonefish, *Carassius auratus gibelio* (4.5)	Jany, 1976
	Catfish, *C. gariepinus* (3.0)	Uys and Hecht, 1987
	Dolphin fish, *Coryphaena hippurus*	Divakaran and Ostrowski, 1990
	Sea bass, *Lates calcarifer*, rabbitfish, *Siganus canaliculatus*	Sabapathy and Teo, 1993
	Atlantic salmon, *S. salar*	Einarsson *et al.*, 1996
	Turbot, *Scophthalmus maximus* (2.0), redfish, *Sebastes mentella* (2.0)	Munilla-Moran and Saborido-Rey, 1996a
Trypsin and Chymotrypsin	Rabbitfish, *S. canaliculatus*	Sabapathy and Teo, 1993
Elastase	Sea bass, *L. calcarifer*	Sabapathy and Teo, 1993
Leucine aminopeptidase	Dover sole, *Solea solea*	Clark *et al.*, 1987
	Rabbitfish, sea bass, *L. calcarifer*	Sabapathy and Teo, 1993
Amylase	Herring, *Clupea harengus*	Battle, 1935
	Gizzard shad, *Dorosoma cepedianum*	Bodola, 1966
	Mullet, *Liza parsia*	Das *et al.*, 1987
	Ambassis nama , Ambassis. ranga, Colisa fasciata	Ray, 1988
	Clarias isheriensis	Fagbenro, 1990
	Rabbitfish, *S. canaliculatus*	Sabapathy and Teo, 1993
	African bony-tongue, *Heterotis niloticus*	Ugwumba, 1993
Maltase	Red sea bream, *P. major*	Kawai and Ikeda, 1971
	African bony-tongue, *H. niloticus*	Ugwumba, 1993
Laminarinase	Rabbitfish, *S. canaliculatus*	Sabapathy and Teo, 1993
Hyaluronidase	Japanese mackerel, *Scomber japonicus*	Yamamato and Kitamikado, 1971

Table 1.9 (continued) Digestive enzymes present in the stomach of fish.

Enzyme	Species	Reference
Chitinase	Some elasmobranchs, insect-feeding teleosts, the dipnoan *Polypterus*	Okutani, 1966; Micha *et al.* 1973
	Red sea bream, *P. major*	Kono *et al.*, 1987
	Coryphaenoides rupestris, Etmopterus spinax, Raja radiata	Fange *et al.*, 1979
	Rainbow trout, *O. mykiss*	Lindsay *et al.*, 1984
	Cod, *G. morhua*	Lindsay and Gooday, 1985
	Sea bass, *L. calcarifer*, rabbitfish, *S. canaliculatus*	Sabapathy and Teo, 1993
Chitobiase	Rainbow trout, *O. mykiss*	Lindsay *et al.*, 1984
	Cod, *G. morhua*	Lindsay and Gooday, 1985
Cellulase	Numerous fish species	Stickney and Shumway, 1974
	Clarias isheriensis	Fagbenro, 1990
Lipase	A number of tilapia species	Al-Hussaini and Kholy, 1953
	Gizzard shad, *D. cepedianum*	Bodola, 1966
	Mullet, *L. parsia*	Das *et al.*, 1987
Esterases	Rainbow trout, *O. mykiss*	Kitamikado and Tachino, 1960

Table 1.10 Digestive enzymes present in the intestine of fish. A figure in brackets denotes the determined pH optimum.

Enzyme	Species	Reference
Trypsin and Chymotrypsin[1]	Rainbow trout, *O. mykiss*	Croston, 1965
	Nile tilapia, *T. nilotica*	Moriarity, 1973
	Sea bass, *D. labrax* (8.2, 8.2)	Alliot *et al.*, 1974
	Bonefish, *C. auratus gibelio* (9.0, 9.0)	Jany, 1976
	Dover sole, *S. solea* (8.0, 7.5)	Clark *et al.*, 1985
	Catfish, *C. gariepinus* (8.2, 7.8)	Uys and Hecht, 1987
	Atlantic cod, *G. morhua* (8.0, 8.0)	Simpson *et al.*, 1989
	Skipjack (7.9, 7.8)	Joakimsson and Nagayama, 1990
	Rabbitfish, *S. canaliculatus* (8.0, 8.0)	Sabapathy and Teo, 1995
	Atlantic salmon, *S. salar*	Einarsson *et al.*, 1996
Elastase	Angler, *Loophius piscatorius*	Lansing *et al.*, 1953
	Thinness secundodorsalis	Zendzian and Barnard, 1967
	Chimaera monstrosa	Nilsson and Fange, 1969
	African lungfish, *Protopterus aethiopicus*	Walsh, 1970a, b
	Dover sole, *S. solea*	Clark *et al.*, 1985
	Sea bass, *D. labrax*, hybrid striped bass, *M. chrysops x M. saxatilis*	Eshel *et al.*, 1993
	Rabbitfish, *S. canaliculatus*, sea bass, *L. calcarifer*	Sabapathy and Teo, 1993
Leucine aminopeptidase	Catfish, *Ameiurus bebulosus*	Fraisse *et al.*, 1981
	Carp, *C. carpio* (7.4)	Khablyuk and Proskuryakov, 1983
	White sturgeon, *Acipenser transmontanus*	Buddington and Doroshov, 1986
	Dover sole, *S. solea* (8.3)	Clark *et al.*, 1987
	Turbot, *S. maximus*	Cousin *et al.*, 1987
	Skipjack (8.5)	Joakimsson and Nagayama, 1990
	Sea bass, *D. labrax*, hybrid striped bass, *M. chrysops x M. saxatilis*	Eshel *et al.*, 1993
	Rabbitfish, *S. canaliculatus* (7.0-9.0)	Sabapathy and Teo, 1995

1. Values in brackets are for tryspin and chymotrypsin respectively.

Table 1.10 (continued) Digestive enzymes present in the intestine of fish.

Enzyme	Species	Reference
Carboxypeptidase A	Spiny Pacific dogfish, *Squalus acanthias*	Prahl and Neurath, 1966
	Japanese mackarel, *S.r japonicus*	Ooshiro, 1968
	Sea bass, *D. labrax*	Alliot *et al.*, 1974
	Myxine glutinosa	Nilsson and Fange, 1970
	Dover sole, *S. solea*	Clark *et al.*, 1985
	White sturgeon, *A. transmontanus*	Buddington and Doroshov, 1986
	Turbot, *S. maximus*	Munilla-Moran *et al.*, 1990
	Sea bass, *D. labrax*, hybrid striped bass, *M. chrysops x M. saxatilis*	Eshel *et al.*, 1993
Carboxypeptidase B	Spiny Pacific dogfish, *S. acanthias*	Prahl and Neurath, 1966
	Tuna	Zendzian and Barnard, 1967
	Sea bass, *D. labrax*	Alliot *et al.*, 1974
	Dover sole, *S. solea*	Clark *et al.*, 1985
	White sturgeon, *A. transmontanus*	Buddington and Doroshov, 1986
	Turbot, *S. maximus*	Munilla-Moran *et al.*, 1990
	Sea bass, *D. labrax*, hybrid striped bass, *M. chrysops x M. saxatilis*	Eshel *et al.*, 1993
Collagenase	19 species	Yoshinaka *et al.*, 1978
	Dover sole, *S. solea*	Clark *et al.*, 1985
	Dolphin fish, *C. hippurus*	Divakaran and Ostrowski, 1990
	Sea bass, *D. labrax*, hybrid striped bass, *M. chrysops x M. saxatilis*	Eshel *et al.*, 1993
Pepsin	*Clarias isheriensis*	Fagbenro, 1990
	Rabbitfish, *S. canaliculatus*, sea bass, *L. calcarifer*	Sabapathy and Teo, 1993

Table 1.10 (continued) Digestive enzymes present in the intestine of fish. A figure in brackets denotes the determined pH optimum.

Enzyme	Species	Reference
Amylase	European perch, *P. fluviatilis*	Fish, 1960
	Nile tilapia, *T. nilotica* (7-8)	Moriarty, 1973
	Rainbow trout, *O. mykiss* (6.9)	Fal'ge and Shpannkhof, 1976
	Mullet, *L. parsia*	Das *et al.*, 1987
	Catfish, *C. gariepinus* (7.8)	Uys and Hecht, 1987
	Dolphin fish, *C. hippurus*	Divakaran and Ostrowski, 1990
	Grass carp, *Ctenopharyngodon idella* (6.4); common carp, *C. carpio* (6.4); silver carp, *Hypophthalmichthys molitrix* (6.8-7.2); bighead carp, *Aristichthys nobilis* (6.8-7.2)	Ni *et al.*, 1992
	Rabbitfish, *S. canaliculatus,* sea bass, *L. calcarifer*	Sabapathy and Teo, 1993
	Common carp, *C. carpio* (7.6)	Tang *et al.*, 1994
	Redfish, *S. mentella* (4.5-5.5), Turbot, *S. maximus* (7.0)	Munilla-Moran and Saborido-Rey, 1996b
Other carbohydrases (maltase, trehalase, sucrase, glucosidases, lactase, collobiase, salicinase)	Red sea bream., *P. major*	Kawai and Ikeda, 1971
	Carp, *C. carpio*	Kawai and Ikeda, 1972
	Rainbow trout, *O. mykiss*	Buddington and Diamond, 1987
	Coregonus lavaretus	Segner *et al.*, 1989
	Clarias isheriensis	Fagbenro, 1990
	Rabbitfish, S. canaliculatus, sea bass, *L. calcarifer*	Sabapathy and Teo, 1993
	African bony-tongue, *H. niloticus*	Ugwumba, 1993
	Pike, *Esox lucius*; perch, *P. fluviatilis*; bream, *Abramis brama*; roach, *Rutilus rutilus*	Kuz'mina, 1996
Laminarinase	*Tilapia macrochira*	Piavaux, 1973
	Chondrostoma nasus; Oreochromis sp	Sturmbauer, 1991
	Tropheus moorii; Simochromis diagramma; Petrochromis orthognathus; Eretmodus cyanosticus	Sturmbauer *et al.*, 1992
	Rabbitfish, *S. canaliculatus*	Sabapathy and Teo, 1993

Table 1.10 (continued) Digestive enzymes present in the intestine of fish. A figure in brackets denotes the determined pH optimum.

Enzyme	Species	Reference
Amyloglucosidase	Carp, *C. carpio*, Catfish, *A. bebulosus*	Fraisse *et al.*, 1981
Chitinase	*Chimaera monstrosa*	Fange *et al.*, 1976
	Rainbow trout, *O. mykiss*	Lindsay *et. al.*, 1984
	Dover sole, *S. solea* (9.5)	Clark *et al.*, 1984
	Cod, *G. morhua*	Lindsay and Gooday, 1985
	Rabbitfish, *S. canaliculatus*	Sabapathy and Teo, 1993
Chitobiase	Dover sole, *S. solea* (5)	Clark *et al.*, 1984
	Rainbow trout, *O. mykiss*	Lindsay *et. al.*, 1984
	Cod, *G. morhua*	Lindsay and Gooday, 1985
Cellulase	Grass carp, *C. idella*	Lindsay and Harris, 1980
	Clarias isheriensis	Fagbenro, 1990
	Aplodactylus punctatus	Ojeda and Caceres, 1995
Lipase	Carp, *C. carpio* (7-7.5)	Schlottke, 1938
	Tilapia mossambica	Nagase, 1964
	Anchovy, *Engraulis mordax*, Jack mackerel, *Trachurus symmetricus*	Patton *et al.*, 1975
	Turbot, *S. maximus*	Cousin *et al.*, 1987
	Mullet. *L. parsia*	Das *et al.*, 1987
	Dolphin fish, *C. hippurus*	Divakaran and Ostrowski, 1990
	Clarias isheriensis	Fagbenro, 1990
	Grass carp, *C. idella*	Das and Tripathi, 1991
Esterase and Phosphodiesterase	Rainbow trout, *O. mykiss* (9.0)	Imura, 1974a, b
	Rainbow trout, *O. mykiss* (7.6)	Fal'ge and Shpannkhof, 1976
	Turbot, *S. maximus*	Cousin *et al.*, 1987

Table 1.10 (continued) Digestive enzymes present in the intestine of fish. A figure in brackets denotes the determined pH optimum.

Enzyme	Species	Reference
Alkaline phosphatase	Carp, *C. carpio*, Catfish, *A. bebulosus* White sturgeon, *A. transmontanus* *Coregonus lavaretus* Pike, *Esox lucius*; perch, *P. fluviatilis*; bream, . *brama*; roach, *R. rutilus*	Fraisse *et al.*, 1981 Buddington and Doroshov, 1986 Segner *et al.*, 1989 Kuz'mina, 1996
Alkaline RNase	3 teleosts and 3 elasmobranchs Cod, *G. morhua* Rainbow trout, *O. mykiss* (8.5)	Zendzian and Barnard, 1967 Overnell, 1973 Imura, 1974a, b
DNase	Rainbow trout, *O. mykiss* White sturgeon, *A. transmontanus*	Imura, 1974a Buddington and Doroshov, 1986

and amylase, and the examples given in the Tables for these enzymes are limited to 10. It is therefore clear to see that for most other enzymes the amount of work is still small. Less work has been carried out on the pH optima of these enzymes, and the results of research in this area are also shown in Table 1.9 and 1.10 when available. Table 1.11 presents the measured pH profile of different parts of the intestines in some fish and in some cases the variation of pH in the different parts of the gut with time after feeding. Again difficulties in carrying out comparisons arise due to the different food being eaten and the timing of the determination, with some authors capturing fish from the wild thereby giving no indication of how long the food has been in the intestine.

Only a limited number of investigations have been carried out to study the variation in enzyme activity as food is being processed in the intestine of fish (Moriarty, 1973; Norris *et al.*, 1973; Fal'ge and Shpannkhof, 1976; Onishi *et al.*, 1976; Takii *et al.*, 1985; Danulat, 1987; Uys *et al.*, 1987; Getachew, 1989; Tian and Lin, 1993; Einarsson *et al.*, 1996).

1.7 GASTRIC EVACUATION

Investigations on gastric, or stomach, evacuation have been carried out to be able to estimate the food consumption by natural fish populations. This data is then used to quantify predation and to determine the extent of any feeding interactions between species so as to construct food chains with the final aim of aiding fish stock management (Bromley, 1994). Gastric evacuation studies have been used as a practical means of determining the feeding rates of fish.

Table 1.11 Variation of the pH in the various parts of fish intestines. Where no time is given in the 'Time after feeding' column, values given are those recorded immediately after capture.

Species	pH							Reference
	Time after feeding	Stomach	Pyloric caeca	Upper Intestine	Middle intestine	Lower intestine.	Rectum	
Bluegill, *Lepomis macrochirus*	0 1	5 1-2						Norris *et al.*, 1973
Nile tilapia, *T. nilotica*	0 6 feeding	7 1.4-2.0		5.5-6.0	7.5	8.2	8	Moriarty, 1973
Scarus jonesi	Feeding nonfeeding		6.8 7.2	6.9 7.5		7.5 7.6	8.2	Smith and Paulson, 1974
Scarus gibbus	Feeding		6.4	6.5		6.4	7.5	Smith and Paulson, 1974
Tilapia guineensis		1-4		6.4-7.4	8-8.5			Payne, 1978
Sarotherodon melanotheron		2-4		6.4-7.4	8-8.5			Payne, 1978
Mullet, *L. falcipinnis*		2-5						Payne, 1978
Mullet, *L. dumerili*		7-8.5						Payne, 1978
Mullet, *M. cephalus, M. curema*		8.5						Payne, 1978
Goldfish, *Carassius auratus*	0 2 4 7 24			6.9 7.4 7.7 7.3 7.1	7.1 7.5 7.9 7.5 7.6	7.1 7.5 7.9 7.4 7.2	7.3 7.4 7.8 7.2 7.1	Maier and Tullis, 1984
Oreochromis mossambicus	0 2 4 6 8	1.1 4.7 3.9 3.4 1.2		7.5 7.7 7.4 7.5 7.8	8 8.5 8.2 8 8.2	7.1 7.8 8 8.1 7.9	7.2 7.6 7.6 7.6 7.5	Maier and Tullis, 1984

Table 1.11 (continued) Variation of the pH in the various parts of fish intestines. Where no time is given in the 'Time after feeding' column, values given are those recorded immediately after capture.

Species	pH							Reference
	Time after feeding	Stomach	Pyloric caeca	Upper Intestine	Middle intestine	Lower intestine.	Rectum	
Atlantic salmon, *S. salar*	Feeding nonfeeding			4.6 6.5	7.8	7.7		Usher *et al.*, 1990
Labeo rohita	0 0.5 2.5	7.4 7.5-7.7 7.5-7.6		7.5 7.8 7.7	7.2 7.8 7.7	7.4 7.8 7.7	7.4 7.7 7.7	Pandey *et al.*, 1992
Heteropneustes fossilis	0 0.5 2.5	7.4-7.7 7.7 7.4		7.3 7.3 7.6	7.4 7.4 7.3	7.3 7.8 7.3	7.1 7.8 7.3	Pandey *et al.*, 1992
Anabus testudineus	0 0.5 2.5	5.9 6.6-6.9 6.4-7	5.9 6.5 6.4	6.2 7.4 7.1	6.3 7.8 7.4	6.4 7.5 7.6	6.2 7.2 7.6	Pandey *et al.*, 1992
Aplodactylus punctatus	Day Night	2.74 2.06		7.68-7.83 6.63-6.83				Ojeda and Caceres, 1995
Channa striatus		5.3		8	7.1	6.9		Chakrabarti *et al.*, 1995
Aristichthys nobilis		5.5		6.6	6.9	7.2		Chakrabarti *et al.*, 1995
Catla catla		7		5.9	8.1	7.9		Chakrabarti *et al.*, 1995
Labeo calbasu		5.6		6.5	6.4	6		Chakrabarti *et al.*, 1995
Labeo rohita		6.5		5.7	5.6	5.9		Chakrabarti *et al.*, 1995
Cirrhinus mrigala		5.9		7	7.3	7.8		Chakrabarti *et al.*, 1995
Silver carp, *H. molitrix*		6.5		7.3	7.9	5.8		Chakrabarti *et al.*, 1995
Carp, *C. carpio*		6.2		6.1	6	5.6		Chakrabarti *et al.*, 1995
Puntius javanicus		6.9		7.6	7.33	5.4		Chakrabarti *et al.*, 1995

The amount of food eaten in a meal, the satiation time and the subsequent return of appetite have been studied in a number of fish (Brett and Higgs, 1970; Brett, 1971; Windell *et al.*, 1972; Elliott, 1975a, b; Jobling *et al.*, 1977; Grove *et al.*, 1978; Flowerdew and Grove, 1979; Gwyther and Grove, 1981; Grove *et al.*, 1985; Bromley, 1987; Paul *et al.*, 1990; Sims *et al.*, 1996. These investigations have all indicated that the appetite of the fish is closely related to the stomach fullness, although other research has pointed out that environmental factors and systemic factors such as temperature, salinity, circulating nutrients or the respiratory rate may also be involved in the control of appetite (Stewart *et al.*, 1967; Muir and Niimi, 1972; Colgan, 1973; Niimi and Beamish, 1974; De Silva and Perera, 1976; Vahl, 1979; Fletcher, 1984; Booth, 1993).

Gastric evacuation studies are relevant to an aquaculture industry in which the farmer aims to optimise the use of the food being given to the animal being cultured. Apart from the importance of the formulation and manufacturing processes involved, the feeding strategy employed must take into consideration the natural processes taking place in the digestive tract. The mechanisms and factors affecting the amount of food taken up and its movement through the gut must be understood so as to maximise feed utilisation by making maximum use of the digestive capacity of the intestine.

1.7.1 FACTORS AFFECTING GASTRIC EVACUATION

The following is a summary of the main factors affecting the gastric evacuation rate in fish. Unlike the case with temperature and the energy content of the food items, there are conflicting claims as to the relationships between fish size, meal size, feeding history, multiple meals and gastric evacuation. Undoubtedly, much of these conflicting reports have arisen due to the highly variable experimental set-up used in the trials and investigations.

1.7.1.1 Temperature

There is a general agreement that as temperature increases so does the gastric evacuation rate (Hofer *et al.*, 1982; Ross and Jauncey, 1981; Ryer and Boehlert, 1983; Nagata, 1989; Rogers and Burley, 1991; Santulli *et al.*, 1993) and a number of authors have found that the relationship between temperature and the rate of gastric evacuation was empirically described by an exponential function (Elliott, 1972, 1991; Persson, 1979, 1981, 1982; Smith *et al.*, 1989; Parrish and Margraf, 1990; Dos Santos and Jobling, 1991a; He and Wurstbaugh, 1993; Jensen and Berg, 1993; Buckel and Canover, 1996; Paakkonen and Marjomaki, 1997). Vondracek (1987) described the relationship between temperature and gastric evacuation with a curvilinear function.

1.7.1.2 Fish Weight

Several researchers have reported that gastric evacuation varied with fish weight, with larger fish taking more time to evacuate a given meal, expressed as a percentage of the body weight, than smaller fish (Pandian, 1967; Gerald, 1973; Swenson and Smith, 1973; Jobling *et al.*, 1977; Grove *et al.*, 1978; Flowerdew and Grove, 1979; Ross and Jauncey, 1981; De Silva and Owoyemi, 1983). On the other hand, other researchers have reported that fish size in fact had no significant effect on evacuation rate (McKone, 1971; Jobling, 1980b, 1987; Persson, 1981; Garber, 1983; Rosch, 1987; Dos Santos and Jobling, 1991b; Buckel and Conover 1996).

1.7.1.3 Meal Size

An increase in meal size (as a percentage body weight) increases both the residence time of food in the stomach and the amount of food evacuated per unit time according to

Beamish (1972), Swenson and Smith, (1973), Jobling *et al.* (1977), Flowerdew and Grove (1979), Grove *et al.* (1985), Bromley (1987), Beyer *et al.* (1988), Macpherson *et al.* (1989), Ruggerone (1989a), Smith *et al.* (1989), Paul *et al.* (1990), Dos Santos and Jobling (1991b, 1992), Rogers and Burley (1991), Sims *et al.* (1996), Paakkonen and Marjomaki (1997). However, this relationship was not found to be the case in investigations carried out by Brett and Higgs (1970), Elliott (1972), Persson (1979, 1981), Talbot *et al.* (1984) and Bromley (1988).

1.7.1.4 Feeding History

Pre-prandial starvation of fish resulted in a larger consumption when compared to pre-prandially fed fish in trials carried out by Brett (1971) and Talbot *et al.* (1984). Windell (1966), Western (1971), Elliott (1972) and Talbot *et al.* (1984) reported that gut evacuation rate decreased as the length of time fish were starved increased, contrary to what was observed by Goddard (1974). Windell (1966), Western (1971) and Swenson and Smith (1973) found that force feeding fish resulted in a decrease in the evacuation rate. Meanwhile, Noble (1973), Corrazza and Nickam (1983) and Talbot *et al.* (1984) found that post-prandial starvation lead to a reduction in the evacuation rate.

1.7.1.5 Interaction Between Meals

According to Johnston *et al.* (1994), sequential meals do not mix to any extent in the stomach, an observation also reported by Fletcher *et al.* (1984). Elliott (1991) found that interactions between meals led to an increase in gastric evacuation rates for meals consumed earlier, and a decrease in evacuation rates for later meals. Similar interactions between evacuation rates of consecutive meals had also been observed previously by Noble (1973), Persson (1984), Fletcher *et al.* (1984), Talbot *et al.* (1984),

Rosch (1987) and Ruggerone (1989b). However, Elliott (1972) and Sarokon (1975) found no difference in gastric evacuation rates when feeding multiple meals in trials they carried out.

1.7.1.6 Energy Content of Food

Lee and Putnam (1973) showed that the daily ration of rainbow trout increases if the energy content of the available food is decreased, a result confirmed by Grove *et al.* (1978, 1985), and numerous experiments have shown that high-energy diets are emptied from the stomach more slowly than diets of low energy content (Grove *et al.*, 1978; Flowerdew and Grove, 1979; Jobling, 1980, 1981; Dos Santos and Jobling, 1988), similar to what has been found to occur in mammals (Hunt and Stubbs, 1975; McHugh and Moran, 1979; Hunt, 1980).

1.7.2 GASTRIC EVACUATION MODELS

Quite a number of mathematical models have been developed in order to explain the physiological basis of gastric evacuation in fish. Most of the models so far developed have assumed that evacuation is a smooth continuous process, but different models are based on different assumptions.

The main models are:

- The exponential model implying that the stomach contents are depleted at a constant rate (Brett and Higgs, 1970; Elliott, 1972; Persson, 1979, 1981, 1982; Brodeur, 1984; Jobling, 1986; Persson, 1986; Brodeur and Pearcy, 1987; Ruggerone, 1989a;

Smith *et al.*, 1989; Elliott, 1991; Jensen and Berg, 1993; Andrade *et al.*, 1996; Sims *et al.*, 1996; Paakkonen and Marjomaki, 1997).

- The linear model indicating that the quantity of food leaving the stomach is constant with time (Swenson and Smith, 1973; Brodeur, 1984; Vandracek, 1987; Cortes and Gruber, 1992; He and Wurtsbaugh, 1993; Nelson and Ross, 1995).

- The square root model implying that the evacuation rate is dependent on the amount of food present in the stomach (Jobling, 1982; Nagata, 1989; Nelson and Ross, 1995).

Within the limits of the assumptions of the models it has been generally observed that particular models do fit a certain set of conditions, but more than one model is needed to accurately estimate evacuation rates at different combinations of temperature, fish size, meal size, etc.

The evacuation of food from the stomach of fish may actually involve phases and not occur in a continuous manner (Rosch, 1987; Grove *et al.*, 1985; Jobling, 1987; Vondracek, 1987; Hopkins and Larson, 1990). There is also increasing evidence that the pattern of emptying of the different components of the diet is actually different in fish (Kionka and Windell, 1972; Dos Santos and Jobling, 1991a) as in other monogastrics (Heading *et al.*, 1976; Hinder and Kelly, 1977).

1.7.3 MEASURING GASTRIC EVACUATION RATES

A number of methods of measuring gastric evacuation rates and residual stomach content have been developed. The main methods used are the following:

- Serial slaughter of fish after which the stomach contents are removed for analysis (Noble, 1973; Brett and Higgs, 1970; Persson, 1979, 1982; De Silva and Owoyemi, 1983; Smith *et al.*, 1989; Elliott, 1991; Jensen and Berg, 1993; Santulli *et al.*, 1993; Buckel and Conover, 1996; Paakkonen and Marjomaki, 1997).

- Following the passage of various radioactive isotopes (Kevern, 1966; Storebakken *et al.*, 1981; Jorgensen and Jobling, 1988; Storebakken and Austreng, 1988a, b; Forseth *et al.*, 1992).

- Following the passage of X-ray dense materials (Sims *et al.*, 1996; Edwards, 1971, Jobling *et al.*, 1977; Flowerdew and Grove, 1979; Ross and Jauncey, 1981; Talbot and Higgins, 1983; Fletcher *et al.*, 1984; Talbot *et al.*, 1984; Wetherbee *et al.*, 1987; Jorgensen and Jobling, 1988).

- Mechanically evacuating gastric contents (Foster, 1977; Hayward and Bushmann, 1993; Rogers and Burley, 1991; Dos Santos and Jobling, 1991b; He and Wintsbaugh, 1993; Bromley 1988; Hopkins and Larson, 1990; Brodeur, 1984; Parrish and Margraf, 1990).

1.8 THE GILTHEAD SEA BREAM, *SPARUS AURATA*, LINNEAUS

The gilthead sea bream, *Sparus aurata* L. (Plate 1.1), belongs to the Family Sparidae (Superorder Teleostea, Order Perciforme), which are known generically as sea breams. The *S. aurata* is a temperate, euryhaline fish (Chervinski, 1984, Castello-Orvay, 1993) which is found along the coasts of the Mediterranean Sea, the Black Sea and the East Atlantic from England to Senegal (Saroglia, 1983). It inhabits rocky and sandy bottoms of coastal water and river deltas (FAO, 1976).

The gilthead sea bream is bilaterally compressed. It can reach a size of 70cm with a corresponding body weight of 4 to 5 kilograms (Saroglia, 1983). It is a protrandrous hermaphrodite and spawning takes place between October and December, usually in deep waters (Barnabe, 1990; Suau and Lopez, 1976; Eisawy and Wassef, 1984).

1.8.1 PRODUCTION OF THE GILTHEAD SEA BREAM IN THE MEDITERRANEAN

Two species of fish, the gilthead sea bream, *Sparus aurata*, and the sea bass, *Dicentrarchus labrax*, currently equally dominate the aquaculture finfish production in the Mediterranean, with only about 2% of production being from another 10 species of fish (Stephanis, 1996).

Sea bream has long been extensively bred in lagoons in Egypt, France, Greece, Tunisia, Italy, Turkey and Spain and in 'valli' in Italy (FAO 1979; Saroglia, 1983; MEDRAP, 1985; Boatto *et al.*, 1989; Barnabe, 1990; Carrieri *et al.*, 1990).

Since the 1970s when artificial reproduction techniques were set up for the gilthead sea bream, numerous reports have been published on attempts to grow gilthead sea bream in

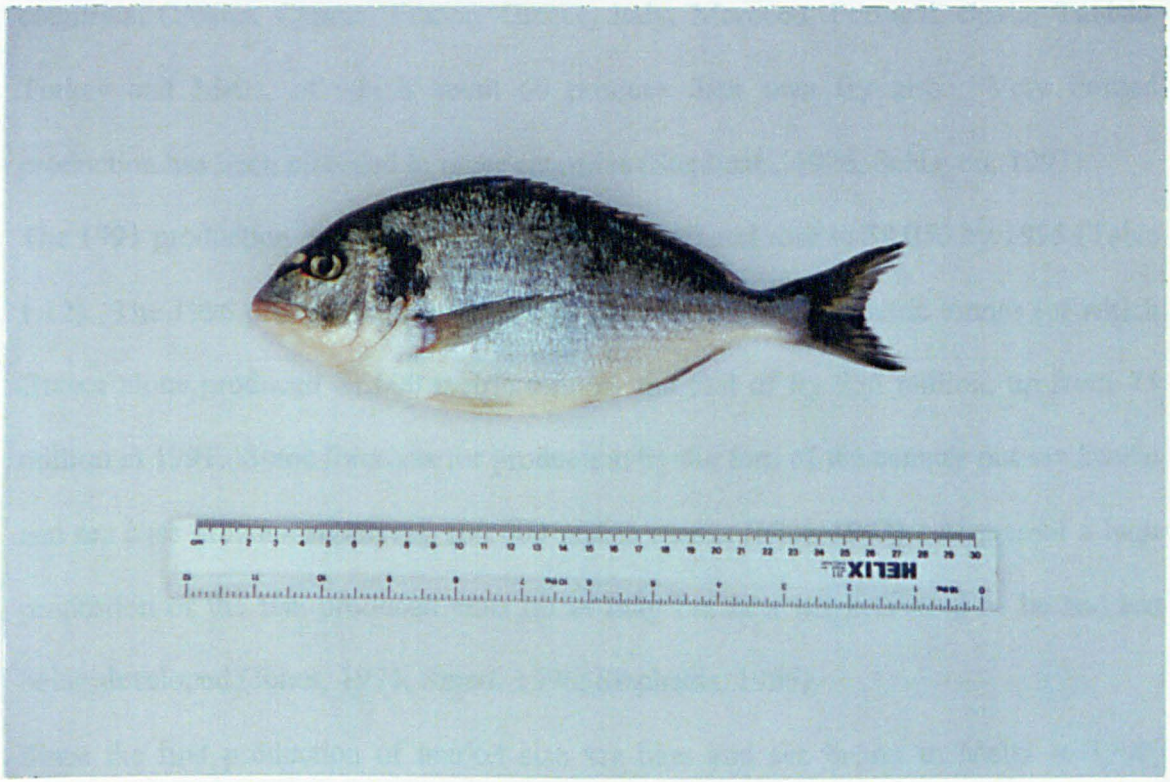


Plate 1.1 A harvest size gilthead sea bream, *Sparus aurata*, of 330 g.

a more intensive way in ponds, tanks, raceways and cages. Nowadays there are only a few land-based farms while the major part of the ongrowing takes place in sea cages. There is also an increasing tendency for systems to be established offshore.

More than 500 sea bass and sea bream production units exist in the Mediterranean in 11 countries: Croatia, Cyprus, France, Greece, Italy, Morocco, Portugal, Spain, Tunisia, Turkey and Malta, of which about 60 produce their own fry also. Very limited production has been recorded in other countries (Stephanis, 1996, Schiavon, 1997).

The 1991 production of these two species was 8,460 and rose to 38,050 by 1995 (Table 1.12). The 1996 production of market size fish reached 56,000 metric tonnes (of which Greece alone produced 21,000 metric tonnes) and that of fry 236 million, up from 75 million in 1991. Some forecasts for production by the turn of the century put sea bream and sea bass growers supplying 100,000 metric tonnes (Hjul, 1997). At present a high proportion of the fish produced ends up in Italy but new markets need to be and are being developed (Jones, 1994; Smart, 1996; Stephanis, 1996).

Since the first production of market size sea bass and sea bream in Malta in 1990, production has increased to near the 2000 metric tonnes mark and is expected to increase. Due to the exposed sites around Malta, the ongrowing systems used are heavy duty systems consisting of large net cages of typical volumes from 2,000 to 4,500m^3, such as Dunlop Tempest rubber cages and Farmocean cages. A number of smaller units are used for prefattening in less exposed sites inshore.

1.8.2 NATURAL DIET OF THE GILTHEAD SEA BREAM

In the wild the gilthead sea bream has a diverse diet, consisting of numerous classes of organisms (Po river delta, Ferrari and Chieragato, 1981). Fry smaller then 30mm have a prevalently planktophagous diet with cirripeda nauplii, copepod and polychaete larvae

Table 1.12 Sea bass and sea bream production in the Mediterranean from 1991 to 1995 (from Stephanis, 1996).

Country	Production (metric tonnes)				
Year	**1991**	**1992**	**1993**	**1994**	**1995**
France	600	1,200	2,400	3,200	3,200
Greece	2,500	6,000	8,500	13,000	17,000
Italy	2,500	2,900	3,400	4,000	6,000
Portugal	300	380	500	700	1,050
Spain	1,200	2,000	2,600	3,200	3,950
Total E.U.	7,100	12,480	17,400	24,100	31,200
Croatia	400		300	1,200	1,700
Cyprus	60	70	170	210	350
Malta			300	1,100	1,300
Morocco	200	300	470	600	1,000
Tunisia	450	500	650	700	300
Turkey	250	1,200	1,500	2,000	2,000
Other		100	100	100	200
Total Mediterranean	8,460	14,650	20,890	30,010	38,050

being the most important items. Over 30mm (to 85mm) in size the sea bream diet changes and they feed preferentially on polychaetes and amphipods and macrophagous detritus.

Wassef and Eisawy (coast of Alexandria, 1985) found that the diet of 10 to 15cm fish consisted mainly of polychaete worms, small bivalves and isopods. Above this size there was a decrease in the consumption of polychaetes, gastropods and isopods, and an increase in the occurrence of bivalves, barnacles, crabs and prawns. As fish size increased there was a decrease in consumption of small, soft-bodied animals, and an increase in barnacles, crabs, echinoderms and ascidians. Other molluscs and some teleosts were also found to have been consumed by the fish, while ascidians, algae and bryozoa were occasionally found in the guts also. These authors noted that the fish seemed to consume the greatest proportion of food between 8 a.m. and 12 p.m.

Andrade *et al.* (1996) caught sea bream from commercially used ponds and a lagoon. These fish were 19.9 and 19.5cm long (138 and 110g) respectively. Eighteen different prey types were identified in fish caught from the ponds and 28 in fish caught from the lagoon. Polychaetes, mysids and pelleted food comprised the main dietary components of the pond fish while molluscs, crustaceans and polychaetes formed the main dietary components of the wild sea bream. The stomach of both sets of fish were found to contain plant/algae fragments.

1.8.3 THE DIGESTIVE TRACT OF THE GILTHEAD SEA BREAM

The gut of the gilthead sea bream has been studied by Elbal and Agulleiro (1986) and Cataldi *et al.* (1987). The intestine consists of a short and wide oesophagus which continues into a Y-shaped stomach. The intestine consists of an anterior intestine which carries four pyloric caeca (to the base of which the ductus pancreaticus and the ductus

hepaticus discharge) and a posterior intestine. A funnel-like valve marks the passage to the intestine's terminal region (Figure 1.1). The pancreas is composed of small masses spread along the upper intestine; in the adult, pancreatic infiltrations can be seen in the liver. The liver is composed of two lobes. The gall bladder is located dorsally and appears as a bag as long as the entire abdominal cavity. The relative length of the intestine of the gilthead sea bream was found to be 0.5 to 0.6 times the length of the body.

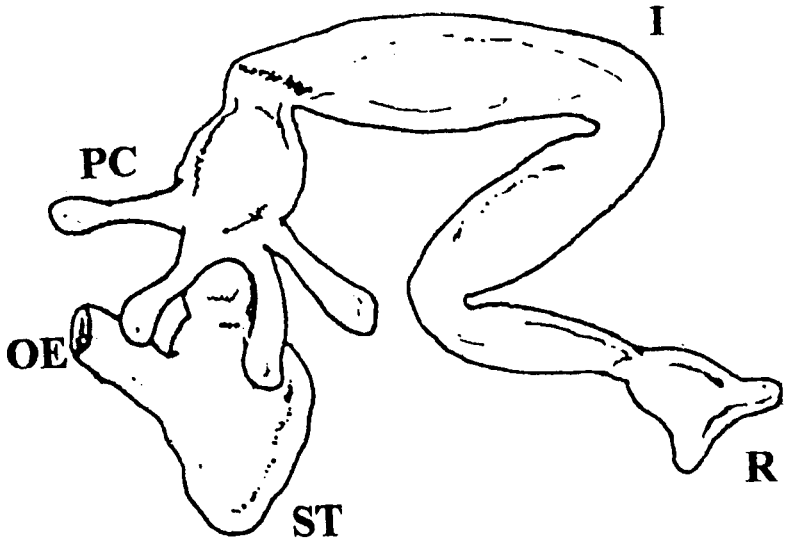
1.8.4 ENZYME ACTIVITY IN THE DIGESTIVE TRACT OF THE SEA BREAM

Gutierrez *et al.* (1985) determined the pH and temperature optima of the acid and alkaline phosphatases present in the intestine and pyloric caeca of *S. aurata.* The optimal pHs for these two enzymes were found to be 5.5 and 10.5 to 10.7 respectively, while the optimal temperatures were 40 to 45°C and 34 to 35°C respectively.

Alkaline protease activity, with a pH optimum in the range 8 to 10, was detected in the intestine of larval *S. aurata* by Moyano and Sarasquete (1993). Amylase activity with a similar pH optimum range as that for the proteases was also determined. Esterase and aminopeptidase activities were also detected in this study. Moyano *et al.* (1996) also detected protease, amylase and acid and alkaline phosphatase activity in sea bream larvae, while Sarasquete *et al.* (1993) detected trypsin, phosphatase and ATPase activities.

Munilla-Moran and Saborido-Rey (1996a) determined a pH optimum of 2.0 for the protease activity of the stomach of the sea bream and a pH optimum of 9.5 to 10.0 for the protease activity obtained from intestinal extracts. Optimal temperatures for

Figure 1.1 A schematic drawing of the digestive tract of *Sparus aurata*: oesophagus (OE), stomach (ST), pyloric caeca (PC), intestine (I) and rectum (R)(from Elbal and Agulleiro, 1986).



stomach protease activity was between 35 and 40°C and between 45 and 50°C for intestinal protease activity.

These authors also investigated amylase activity in the sea bream (Munilla-Moran and Saborido-Rey, 1996b). They detected amylase activity both in stomach and in intestinal extracts, but possessing only one pH optimum range between 7.0 and 7.5. The optimum temperatures of the starch hydrolysis activity of the extracts from these two parts of the intestine were 40 and 40 to 45°C respectively.

1.8.5 SATIATION AND GASTRIC EVACUATION IN THE SEA BREAM

Apostolopoulos and Klaoudatos (1986) found that 60g wild sea bream required about 10 days to acclimate to new surroundings and reach a constant satiation amount of food. Acclimated 65g fish required more food to become satiated the longer they were left hungry, up to 24 hours, after which no further increase in satiation quantity was observed.

The gastric evacuation rates of fresh polychaetes and pelleted food in sea bream were found to vary by Andrade *et al.* (1996). The estimated gastric evacuation rates were 6.24%/h and 7.97% for polychaetes and pellets respectively, with 2.6% remaining in the fish stomachs of the former after 12 hours and 1.1% in fish stomachs after 9 hours in the latter.

1.8.6 THE USE OF SUPPLEMENTARY ENZYMES IN SEA BREAM DIETS

Some work has been carried out on the use of supplementing enzymes in the diets of sea bream larvae but not larger fish.

Tandler and Kolkovski (1991) added 0.5 g/kg pancreatin to the diets of 8 to 22 day old larvae and found that addition of pancreatin increased digestibility by 30%, and had a positive effect on protein digestibility. Kolkovski *et al.* (1993a) also found a similar improvement in digestibility when they added 0.5 g/kg pancreatin to diets of 20 to 45 day old sea bream larvae as well as an increase in neutral lipid and protein assimilation. Koven *et al.* (1993) observed a marked effect on fatty acid incorporation in 45 day larvae when they were fed a diet containing 5 g/kg porcine lipase.

Kolkovski *et al.* (1993b) added 0.5 and 1.0 g/kg pancreatin to the microdiets of 20 to 32 day old larvae. Both supplementary enzymes equally improved the fish performance over those of fish fed the unsupplemented diet, but lower than the performance of fish fed live food.

The addition of 0.5 g/kg of an enzyme mixture consisting of papain and lipase to microcapsules fed to 6 and 12 day old sea bream larvae did not result in any significant changes in the degree of capsule breakdown (Fernandez-Diaz and Yufera, 1995).

1.8.7 REPLACING FISH MEAL IN SEA BREAM DIETS

Table 1.13 summarises the work that has been published with various ingredients being used to replace fish meal in the diets of sea bream.

These trials, although small in number, have shown that FM can successfully be replaced, to a certain extent, by numerous ingredients, including SBM. In the one case when all the fish meal was taken out of the diet, high mortalities were recorded (Amaral, 1994).

Table 1.13 Summary of experiments in which FM has been replaced by alternative protein sources in gilthead sea bream diets.

Fish meal replacer details	Fish weight	Results	Reference
290, 460 and 640 g/kg SBM	Trial 1: 2 g Trial 2: 75 g	Trial 1: Good results were obtained when fish were fed the diets in containing 290 and 460 g/kg SBM. Fish fed the diet with 640 g/kg SBM gave lower growth than fish fed the 480 g/kg FM control. Trial 2: Satisfactory results were obtained with all diets, with the fish fed the diet with 460 g/kg SBM actually giving a better performance than fish fed the control diet.	Millan *et al.*, 1989
Three different meat and bone meals as 40% of the protein, commercial protein product as 15% of protein	5 g	There were no differences in the performance of fish fed the meat and bone meal diets when compared to the fish fed the 740 g/kg white FM control.	Davies *et al.*, 1993
70, 85 and 100% of FM was replaced with various combinations of wheat, solvent extracted SBM and hydrolysed feather meal	2.3 g	Performance of the fish fed the experimental diets were all lower than that of the control 670 g/kg FM diet, with a decrease in performance as the level of FM decreased. A high mortality was observed in the fish fed the diet in which there was 0 FM.	Amaral, 1994
40, 55 and 70% of FM was replaced by various combinations of wheat, solvent extracted SBM and hydrolysed feathermeal	11 g	There were no significant differences in fish performance between the three experimental diets and the control 670 g/kg FM diet.	Bekkevold, 1994
100, 200 and 300 g/kg SBM	40 g	Using 100 and 200 g/kg of SBM in the diets slightly improved performance of the fish compared to fish fed the control 770 g/kg FM diet. As dietary SBM levels increased there was an increase in lipid deposition and a decreased glycogen deposition in the liver.	Robaina *et al.*, 1995
Trial 1: Solvent extracted SBM 120, 240, 360 and 480 g/kg inclusion levels. Trial 2: 35% of fish meal replaced by full fat SBM cooked for different times, solvent extracted SBM, commercial soy protein concentrate	Trial 1: 6.2 g Trial 2: 1.6 g	Trial 1: 120, 240 g/kg inclusion levels did not significantly affect growth of fish, but 360 and 480 g/kg inclusion levels significantly reduced growth compared to fish fed the 740 g/kg white FM control. Trial 2: Only the fish fed the full fat soybean meal cooked for 20 mins at 110°C gave a performance equal to the fish fed the 720 g/kg white FM control.	Nengas *et al.* 1996

1.9 AIMS OF THIS WORK

For the aquaculture industry to maintain its present rate of growth, towards a higher level of intensification, the field of nutrition has an important part to play. Nutrition involves all aspects involved in supplying the required nutrients to an organism, from ingredient quality to manufacturing technology to feeding management to cellular activity, with the final aim of maximising the use of food.

The use of fish meal as one of the ingredients still plays an important part in helping farmers make good utilisation of food. However, due to pressure from limited availability and high relative prices, the search for alternative protein sources is well under way.

Soybean meal has shown itself a promising and likely candidate for this role of fish meal replacer. Research has shown that other alternatives also exist, and combining ingredients is probably the realistic approach. Economics has the biggest say in any industrial venture, and in time the farmer may well have to accept a lower yield per unit time, with possibly a poorer conversion, to remain profitable.

Among the major problems with the plant ingredients considered are antinutritional factors and non-starch polysaccharides. The market offers numerous products which have undergone special production conditions and processes - at a cost – in which these factors are eliminated or reduced.

A solution to these problems lies in the use of supplemental enzymes. The large number of specialised enzyme cocktails on the market testify to their successful use in the poultry and pig industry. The small amount of work published so far on the use of enzymes in aquaculture feeds does suggest their potential and it is well worth the time

and money for the aquaculture industry to consider this technology in combination with development of alternative protein sources.

Knowledge of the processes and changes taking place in the digestive tract of fish can help to assess the potential and limitations of the use of ingredients and additives, such as enzyme supplements, and understand what factors affect digestion and the movement of food in the intestine. Such information is very limited in the case of the gilthead sea bream. This information may then be used to understand better how to determine feed formulations and feeding strategies to be used in aquaculture ventures.

The experiments and investigations carried out in this work had the following aims:

1. To determine to what extent solvent extracted soybean meal can replace fish meal in gilthead sea bream, *Sparus aurata*, diets.
2. To determine whether supplemental enzymes have an impact on the utilisation of diets in which soybean meal has replaced fish meal.
3. To determine which of the combinations of enzymes used gives the best results in terms of growth and feed utilisation.
4. To determine the individual contribution of each of the enzymes used in the combinations and the effect of using different dietary inclusion levels of these enzymes.
5. To study the use of supplemental enzymes in both pressed and extruded diets.
6. To determine the relative activities of a number of enzymes in different parts of the digestive tract of the sea bream.
7. To investigate the variation in pH along the length of the digestive tract after one and two feeds.

8. To study the effect of the time of feeding, feeding rates and feeding frequencies on the gastric evacuation rates in the gilthead sea bream.
9. To investigate the effect of feeding at different feeding rates on the individual consumption in a population of gilthead sea bream.

CHAPTER 2

GENERAL MATERIALS AND METHODS

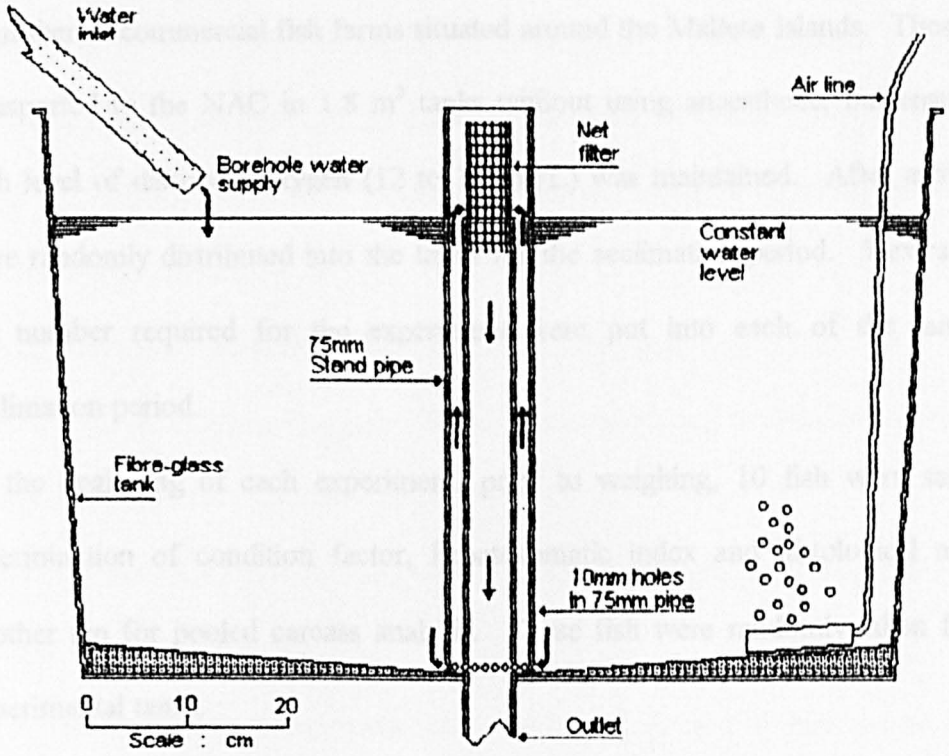
This chapter presents the materials and methods used that are common to all experiments. Where relevant, details pertaining to a particular experiment are presented in the appropriate section in the Chapter dealing with that experiment.

2.1 EXPERIMENTAL TANKS

All experiments were carried out in the aquarium facilities of the National Aquaculture Centre (NAC), Malta. 21 Fibreglass tanks (Figure 2.1) were utilised in the experiments, except in Experiment 5, in which 24 tanks were used. The volume of water in these tanks was 0.27 m^3 (length, 0.9 m, width, 0.6 m, depth, 0.5 m). Borehole water was supplied by an inlet pipe, connected to a ring circuit to ensure equal pressure throughout the system, at a rate of 6 L/min, such that complete water exchange was achieved in about 45 minutes. Outflow was through a central pipe of 50 mm diameter. A self-cleaning system was created by surrounding the central pipe with an outer 75 mm diameter standpipe which projected above the level of the water, but had 10 mm slits at its bottom, such that a water current was created along the sloping bottom of the tank. Aeration was provided by an air stone in each tank. The tanks were cleaned once a week during which all pipes and surfaces were scrubbed. Cleaning was carried out three hours after the morning feed.

Before the start of each experiment the tanks were randomly allocated to the different treatments such that each treatment had three replicates or, in the case of Experiment 5, four replicates.

Figure 2.1 Cross-section of experimental tank showing main features and water-flow pattern.



2.2 EXPERIMENTAL FISH AND HANDLING

Juvenile gilthead sea bream, *Sparus aurata,* for the five experiments were obtained from a number of commercial fish farms situated around the Maltese Islands. These fish were transported to the NAC in 1.8 m^3 tanks without using anaesthetic, but ensuring that a high level of dissolved oxygen (12 to 16 mg/L) was maintained. After arrival the fish were randomly distributed into the tanks for the acclimation period. 5 extra fish above the number required for the experiment were put into each of the tanks for the acclimation period.

At the beginning of each experiment, prior to weighing, 10 fish were sacrificed for determination of condition factor, hepatosomatic index and histological analysis and another ten for pooled carcass analysis. These fish were randomly taken from all the experimental tanks.

At the start of each experiment fish in each tank were counted and bulk-weighed and fish surplus to the required number removed. After the initial weighing all fish in each experimental tank were bulk-weighed every two weeks. Any mortalities which occurred during the experiment were recorded.

After the final weighing, 5 fish from tanks fed the same diet were randomly selected for determination of condition factor, hepatosomatic index and histological analysis and another five for pooled carcass analysis.

2.3 THE DIETS AND FEEDING REGIME

The diets used in the experiments were formulated, using a RAPP least cost feed formulation program (ATH Matematik-Konsult, Korsfararvagen 140, S-181 40 Lidingo,

Sweden), and manufactured by the commercial feed producing company Ewos. The first and second set of diets were manufactured by Ewos SA (N-620, Km 99, Duenas, Palencia 34210) in Spain with a Norvidan double-pelletiser type pellet mill, and all the other diets at the Ewos Technology Centre (Unit 1, Kingsthorne Park, Houstoun Industrial Estate, Livingston EH54 5DB) in Scotland, where the pressed diets were made using a California Pellet Mill (model CL) and the extruded diets with a Wenger twin screw extruder (model TX-57).

The enzymes used were supplied by Finnfeeds International (PO Box 777, Marlborough, Wiltshire, SN8 1XN) in England. Three enzymes (having temperature optima between 50 and 60°C) were used in the experiments:

- A protease with a pH optimum at 3.0. This is designated as 'low pH protease' in the text.
- A protease with a pH optimum at 8.5. This is designated as 'high pH protease' in the text.
- An α-galactosidase, with a pH optimum at 5.0.

Dry enzymes (with wheat as the carrier) were added to the feed mix prior to pelleting, and liquid enzymes (in aqueous solution) were mixed with oil and sprayed onto the pellets post-extrusion.

The sacks of food were kept in cold storage (4°C) until used.

The acclimation period was never less than two weeks, its termination being assessed by the feeding activity of the fish. The fish were fed twice daily at 0830 and 1600. In the first two experiments, the fish were fed 6 days of the week, and in experiments 3 to 5 the fish were not fed on the afternoon of the seventh day only.

2.4 WATER QUALITY AND ENVIRONMENTAL PARAMETERS

Dissolved oxygen was determined with an Oxyguard Handy Mk I twice a week, temperature with a digital thermometer weekly, and ammonia, nitrite and nitrate once every two weeks with a Hach Salt Water Master kit.

For each parameter except dissolved oxygen, samples were taken three hours after the morning feed from the water body of three randomly selected tanks. Dissolved oxygen readings were taken one hour after the termination of the afternoon feed and aeration adjusted such that the dissolved oxygen levels in all tanks were similar at any time. The dissolved oxygen level was maintained above 5.0 mg/L throughout the experiments.

Temperatures varied from 20.5 to 22.4°C throughout the five experiments, with a maximum variation of $\pm$ 0.5°C within a particular experiment. Levels of ammonia, nitrite and nitrate did not go above 0.6, 0.15 and 10 mg/L respectively at any point during the experiments. The photoperiod maintained throughout the experiments was L:D 12:12, operated by a fully automated switching system. Lights came on at 0630 and switched off at 1830.

2.5 FAECAL COLLECTION

Chromic oxide was used in the experimental diets to act as the indicator for analysis of digestibility coefficients. Faeces for digestibility studies were collected during a two to four week period in each experiment. The faeces collected during Experiments 1 to 4 were pooled.

With the restrictions on the facilities available, the following method of faecal collection was used: faeces were collected using 1.5 mm plastic mesh inserted into the central pipe (see Figure 1). Although this is not the best method of faecal collection, due to the possibility of leaching, this was kept to a minimum by collection of faecal material after 1 hour of insertion of the mesh. Faeces collected in this manner were dried at 105°C and kept in a desiccator until analysed.

Apparent Digestibility Coefficients (ADC)(Maynard and Loosli, 1969) were calculated using the following formula:

ADC (%) = 100 - {100 * (%Cr_2O_3 in food/%Cr_2O_3 in faeces) * (%nutrient in faeces/%nutrient in food)}.

All calculations were based on dry matter values.

2.6 INTESTINAL DRY MATTER CONTENTS

The intestinal dry matter contents of 6 randomly sampled fish from each diet were determined in Experiments 1 and 3. The fish sampled for this purpose were killed with lethal dose of anaesthetic (0.6 mL/L 2-phenoxyethanol) dissected while still fresh and the intestinal contents were collected from the point immediately behind the pyloric caeca to the anus by forcing out the contents with a pair of tweezers. The collected samples were then dried at 105°C.

2.7 LABORATORY ANALYSIS

2.7.1 CHEMICAL ANALYSIS

Fish sampled for chemical analysis were frozen, then thawed before blending (whole). Representative samples of each feed were randomly taken from the feed sacks and then ground in an IKA Analytical Mill (A10). Material which passed through a 1 mm sieve was used for the analyses.

Crude protein (Kjeldahl method), crude lipid (Soxhlet method), moisture (oven drying), ash (incineration), crude fibre (acid and alkali digestion) and phosphorus (molybdovanadate spectrophotometry) analyses were performed according to standard methods of the AOAC (1990) and ISO (1978, 1981, 1983). 3,5-Dinitrosalicylic acid was used to determine crude carbohydrate content (James, 1995). The chromic oxide content of feeds and faeces were analysed by Atomic Absorption Spectroscopy in Experiment 1 (on a Varian AA-1275 Series), and by the wet acid digestion method (Furukawa and Tsukahara, 1966) in the other experiments. Trypsin inhibitor activity was determined by the method of Smith *et al.* (1980) and protein solubility of the soybean meal by the method of Araba and Dale (1990). All the above analyses were carried out at the NAC in Malta.

Amino acid analysis was carried out following acid hydrolysis on an LKB Biochrom 4151 Alpha plus amino acid analyser (column used was an Ultiopac8 cation-exchange resin, 202 x 4.6 mm internal diameter) following manufacturers specifications, and energy content on a Gallenkamp Autobomb (CBA-350-K). Both of these analyses were carried out at Stirling University.

All analyses were carried out in duplicate or triplicate.

2.7.2 FISH CONDITION FACTOR AND HEPATOSOMATIC INDEX

The fish sampled for these parameters were analysed while still fresh.

The Fulton's Condition Factor (Steffens, 1989) of the fish was determined using the following formula:

Condition Factor = 100 * fish weight (g)/(total length in cm)^3.

The Hepatosomatic Index (HI)(Pfeffer *et al*, 1991) was calculated using the following formula:

Hepatosomatic index = 100 * liver weight (g)/body weight (g).

2.8 ASSESSMENT OF GROWTH AND FEED UTILIZATION

Growth and feed performance were described using the parameters below (Steffens, 1989; De Silva and Anderson, 1995), all calculations based on an as fed, wet basis.

The specific growth rate denotes the average daily growth as a percentage of the initial weight, and is calculated as follows:

Specific Growth Rate (SGR)(%/day) = 100 * (Log_e average final weight (g) - Log_e average initial weight (g))/number of days.

The daily food intake was calculated using the following formula:

Food intake (g/100g fish/day) = 100 x (daily feed intake per fish (g))/((final average weight (g) + initial average weight (g))/2).

A number of parameters are utilised to determine the value of feeds for providing the necessary requirements for growth. The food conversion ratio which is recognised and derived by almost all fish farmers is calculated as follows:

Food Conversion Ratio (FCR) = food given (g)/increase in biomass of fish (g).

Protein efficiency ratio is utilised to give a measure of how well the protein source in the diet provides for the essential amino acid requirements of the fish and how well the diet is balanced for energy and protein. This is calculated using the following formula:

Protein Efficiency Ratio (PER) = increase in biomass of fish (g)/protein given (g).

Since PER measures the deposition of fat as well as protein, it has been recognised that the apparent net protein utilisation gives a better measure of the actual use of the protein in the feed and is calculated as follows:

Apparent Net Protein Utilisation (ANPU)(%) = 100 * protein deposition (g)/protein given (g).

The efficiency of dietary lipid and energy utilisation by the fish are calculated in a similar way as for protein using the following equations:

Apparent Net Lipid Utilisation (ANLU)(%) = 100 * lipid deposition (g)/lipid given (g).

Energy Efficiency (EE)(%) = 100 * energy deposition (MJ)/energy given (MJ).

2.9 STATISTICAL ANALYSIS

Statistical analysis was performed using the BMDP statistical software package (Version PC90).

The results from the replicates for each treatment were used to provide the data for the statistical analysis including the standard deviations. Homogeneity of variances between samples was tested using the Levene's test (Dixon *et al.*, 1988). Multiple comparisons

between means were made using the Student-Newman-Keuls test. All percentage and ratio data were transformed to arcsine values prior to analysis (Zar, 1984). In the case where a homogeneity of variances was not found, the non-parametric Kruskal-Wallis test was performed. The significance levels of the tests was taken as 0.05.

CHAPTER 3

EXPERIMENT 1

Growth and feed utilisation of gilthead sea bream, *Sparus aurata* L., fed different levels of soybean meal with and without supplementary enzymes

3.1 INTRODUCTION AND AIMS OF THIS EXPERIMENT

SBM is a prospective substitute for FM in aquaculture diets. However, as seen in Section 1.4.3, the literature has clearly shown that using SBM to replace FM is not always effective, at least beyond a certain level of inclusion. Only two trials have been published on investigations into the use of SBM to replace FM in juvenile gilthead sea bream diets (Millan *et al.*, 1989; Robaina *et al.*, 1995), and more research is required into the levels accepted by this fish.

In the present experiment the effect of replacing FM with SBM in pressed gilthead sea bream diets was investigated. Three SBM levels were used, 220, 320 and 440 g/kg with corresponding FM contents of 320, 260 and 230 g/kg respectively.

Supplementary enzymes have been used in the poultry and pig industry for quite some time to reduce the antinutritional properties of particular components in ingredients used to substitute traditionally higher quality materials and to improve the general digestion of the feed (see Sections 1.5.2 and 1.5.3). The use of supplemental enzymes in the feeds of fish has given encouraging results in trials carried out so far (see Sections 1.5.5 and 1.8.6), and could well provide a means of improving fish performance.

Two enzyme cocktails were supplemented to the 320 and 440 g/kg SBM diets used in this experiment to investigate, first of all whether the enzymes had any effect on the performance of gilthead sea bream, and secondly to see which of these two cocktails gave the best results. The cocktails used were either 1 g/kg of a low pH protease and 1 g/kg of α-galactosidase or 1 g/kg of a high pH protease and 1 g/kg α-galactosidase.

The activities of the proteases used in these experiments were not disclosed, but proteases break down peptide links of proteins. Proteases are generally very specific in

their action and often exert their activity only at a particular point in the protein molecule depending on the nature of the chemical bonds on either side of the bond concerned. Proteases are exopeptidases if they are capable of acting on peptide bonds at the end of the protein and endopeptidases if they can act on peptide bonds within the protein molecule (Figure 3.1(a)). α-Galactosidase catalyses the hydrolysis of α-(1,6)-linked galactose moieties from raffinose and stachyose to produce galactose and sucrose (Figure 3.1(b)).

3.2 MATERIALS AND METHODS

Other details pertaining to experimental tanks, experimental fish and handling, diet production, water quality, faecal collection, laboratory analysis, calculations and statistical analysis are as described in Chapter 2.

3.2.1 EXPERIMENTAL FISH

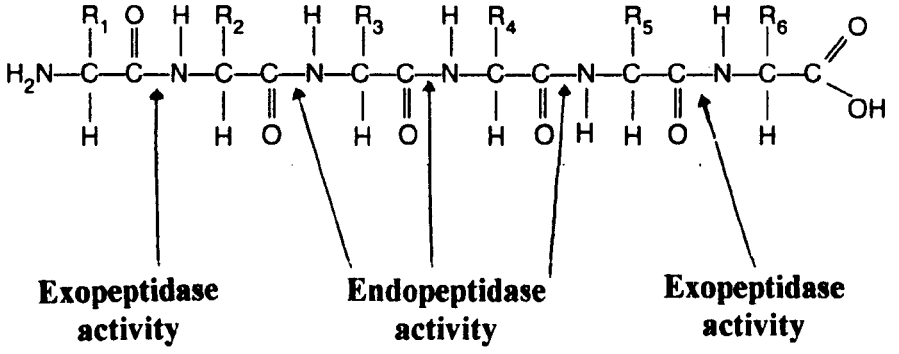
40 fish of 50 g average weight were used in each of the tanks in this experiment and each treatment had three replicates. The duration of the experiment, apart from the acclimation period, was 12 weeks.

3.2.2 THE DIETS AND FEEDING REGIME

The formulations of the diets used in this experiment are given in Table 3.1. The nutritional composition and amino acid contents of the soybean meal and fish meal used are given in Table 3.2. The percentage inclusion levels of the supplementary enzymes added to the diets and the nutritional composition of the diets themselves (3 mm pellets) are given in Table 3.3. Table 3.4 gives the amino acid composition of the diets.

Figure 3.1 (a)Diagram showing different points at which endopeptidases and exopeptidases hydrolyse protein chains. (b)Structures of raffinose and stachyose showing points where α-galactosidase hydrolyses linkages.

(a)



(b)

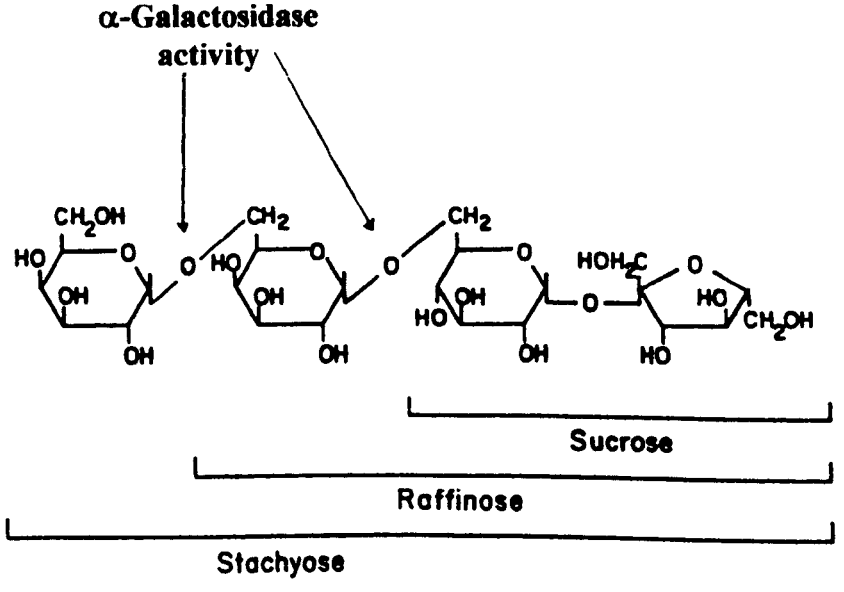


Table 3.1 Formulations of diets used in Experiment 1.

Diets	1	2 to 4	5 to 7
Ingredient	Inclusion (g/kg)		
Fish meal[1]	317	260	229
Dehulled hexane extracted soybean meal[2]	220	320	440
Blood meal[3]	50	33	0
Corn[4]	88	58	0
Feather meal[5]	100	100	76
Fish oil[6]	64	79	100
Limestone	30	30	30
Molasses[7]	40	60	60
Vitamins and minerals[8]	11	11	11
Whole wheat[9]	75	45	45
Chromic oxide	5	5	5

1. Source: Spain.
2. Source: Spain.
3. Source: Daka Ltd., Canada.
4. Source: Suprex Ltd., Scotland.
5. Source: Canada.
6. Source: UFP Ltd., Scotland.
7. Source: Spain.
8. Ewos Premix prepared by Roche Products Ltd., England.
9. Source: Scotland.

Table 3.2 Nutritional compositions of the soybean meal and fish meal used in the formulation of the diets in Experiment 1.

	Soybean meal	Fish meal
Moisture (g/kg)	126	75
Crude protein (g/kg)	486	654
Crude lipid (g/kg)	7	73
Ash (g/kg)	58	168
Crude fibre (g/kg)	34	5
Crude carbohydrate (g/kg)	300	16
Phosphorus (g/kg)	7	23
Protein solubility (%)	79.37	
Trypsin inhibitor activity (mg/g)	1.11	
Amino acid[1]	(g/100 g protein)	
Alanine	5.09	6.71
Arginine[2]	4.83	6.23
Aspartic acid	7.94	9.64
Cystine	1.06	1.54
Glutamic acid	11.83	14.29
Glycine	2.56	6.82
Histidine[2]	1.85	3.50
Isoleucine[2]	3.17	4.98
Leucine[2]	4.98	7.73
Lysine[2]	4.22	8.73
Methionine[2]	0.70	2.31
Phenylalanine[2]	3.30	4.26
Proline	3.11	4.54
Serine	3.40	4.62
Tyrosine	1.67	3.40
Threonine[2]	2.91	4.52
Valine[2]	3.15	5.58

1. No data is available for the essential amino acid tryptophan because it is destroyed during acid hydrolysis.
2. Essential amino acid

Table 3.3 Inclusion levels of enzymes and nutritional compositions of the diets used during Experiment 1.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed
Fish meal inclusion (g/kg)	320	260	260	260	230	230	230
Soybean meal inclusion (g/kg)	220	320	320	320	440	440	440
Low pH protease (g/kg)	0	0	1	0	0	1	0
High pH protease (g/kg)	0	0	0	1	0	0	1
α-galactosidase (g/kg)	0	0	1	1	0	1	1
Enzyme form	Dry	Dry	Dry	Dry	Dry	Dry	Liquid
Moisture (g/kg)	81	84	84	84	87	81	86
Crude protein (g/kg)	485	476	470	464	459	456	459
Crude lipid (g/kg)	129	128	124	124	128	142	110
Ash (g/kg)	114	116	100	113	112	103	109
Crude fibre (g/kg)	19	15	17	20	24	19	21
Crude carbohydrate (g/kg)	178	183	197	193	182	200	214
Phosphorus (g/kg)	12	11	10	10	11	10	10
Trypsin inhibitor activity (mg/g)	0.28	0.24	0.34	0.14	0.29	0.17	0.54
Chromic oxide (g/kg)	5	5	5	5	5	5	5
Energy content (kJ/g)	22.03	21.70	21.77	21.39	21.37	21.43	20.98
Protein/gross energy ratio (g/MJ)	22.00	21.93	21.60	21.67	21.49	21.27	21.89

Table 3.4 Amino acid contents of gilthead sea bream[1] carcass and the diets used in Experiment 1[2].

	Carcass	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Fish meal inclusion (g/kg)		320	260	260	260	230	230	230
Soybean meal inclusion (g/kg)		220	320	320	320	440	440	440
Low pH protease (g/kg)		0	0	1	0	0	1	0
High pH protease (g/kg)		0	0	0	1	0	0	1
α-galactosidase (g/kg)		0	0	1	1	0	1	1
Enzyme form		Dry	Dry	Dry	Dry	Dry	Dry	Liquid
	(g/100g protein)							
Alanine	5.35	5.10	4.24	4.61	4.44	3.92	3.86	3.94
Arginine[3]	5.10	4.28	4.70	5.23	6.57	4.89	4.05	4.52
Aspartic acid	8.01	6.92	6.93	8.31	8.64	8.40	7.38	7.17
Cystine	1.08	0.95	1.11	1.19	1.20	1.09	1.10	1.06
Glutamic acid	10.38	10.20	11.45	11.99	11.73	11.36	11.77	10.35
Glycine	6.12	4.36	4.61	4.54	4.83	4.64	4.03	4.27
Histidine[3]	1.83	2.03	2.31	2.26	2.25	1.97	1.94	2.17
Isoleucine[3]	3.62	2.78	2.79	3.10	2.90	2.86	2.27	3.08
Leucine[3]	5.83	6.65	6.57	6.88	6.91	6.42	5.60	5.84
Lysine[3]	6.01	5.04	4.84	4.86	5.14	5.18	4.98	4.61
Methionine[3]	2.56	0.56	0.49	0.60	0.64	0.71	0.72	0.53
Phenylalanine[3]	3.26	4.13	4.19	4.25	4.18	3.99	3.95	4.05
Proline	4.13	4.47	4.17	4.67	4.57	4.18	4.61	3.95
Serine	3.53	4.67	4.63	5.01	5.15	4.42	4.14	4.20
Tyrosine	3.17	1.99	2.08	2.50	2.41	2.24	2.17	2.22
Threonine[3]	3.91	3.35	3.60	3.78	3.83	4.69	3.57	3.70
Valine[3]	4.39	3.23	3.51	3.61	3.57	3.11	3.15	3.46

1. Average weight 63.28 g.
2. No data is available for the essential amino acid tryptophan because it is destroyed during acid hydrolysis.
3. Essential amino acid.

During the acclimation period the fish were fed a mixture of all the feeds. Throughout the acclimation period and the experiment the fish were fed to satiation. Food was offered to the fish in portions spread over the area of the tank with satiation at each feed being determined as the point at which the fish did not consume offered pellets after 5 minutes.

3.3 RESULTS

3.3.1 ASSESSMENT OF GROWTH AND FEED PERFORMANCE

(Table 3.5, Figures 3.2 to 3.7)

Going from a low content of soybean meal (SBM) to a high level of SBM (diet 1 to 2 to 5) a clear decrease in performance was seen in this experiment in terms of fish growth and feed utilisation, although this was not always significant. The specific growth rates (SGR), apparent net lipid utilisation (ANLU) and energy efficiencies (EE) of fish fed diets 1 and 2 were both significantly higher than that of fish fed diet 5. Fish fed diet 1 also showed significantly higher ANPU and EE than fish fed diet 2. .

The inclusion of low pH protease and α-galactosidase at the 320 g/kg SBM level (diet 3) significantly improved fish performance over the fish fed diet 2, which contained the same amount of SBM but no enzymes, and over fish fed diet 1 in SGR, protein efficiency ratio (PER), apparent net protein utilisation (ANPU), ANLU and EE. Fish fed diet 4, containing high pH protease and α-galactosidase, also showed significantly better results for SGR, ANPU, ANLU and EE than fish fed diets 1 and 2. Feeding of both diet 3 and 4 gave fish performances which were significantly better than when the

Table 3.5 Assessment of growth and feed performance in Experiment 1. Data are presented as means with the standard deviation in brackets. Means in a row followed by the same superscript are not significantly different ($P < 0.05$).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed
Fish meal inclusion (g/kg)	320	260	260	260	230	230	230
Soybean meal inclusion (g/kg)	220	320	320	320	444	444	444
Low pH protease (g/kg)	0	0	1	0	0	1	0
High pH protease (g/kg)	0	0	0	1	0	0	1
α-galactosidase (g/kg)	0	0	1	1	0	1	1
Initial weight (g)	52.42^{a} (1.84)	51.75^{a} (1.52)	51.79^{a} (1.98)	49.63^{a} (3.20)	51.67^{a} (2.03)	50.80^{a} (0.14)	50.38^{a} (2.06)
Final weight (g)	86.60^{cd} (1.17)	80.53^{bc} (2.12)	98.80^{e} (7.02)	89.61^{d} (5.20)	72.05^{b} (4.44)	73.39^{b} (3.62)	57.68^{a} (3.31)
Specific growth rate (SGR) (%/day)	0.60^{c} (0.06)	0.53^{c} (0.04)	0.77^{d} (0.04)	0.71^{d} (0.03)	0.39^{b} (0.03)	0.44^{b} (0.06)	0.16^{a} (0.05)
% Mortalities	1.67^{a} (2.89)	0.00^{a} (0.00)	0.00^{a} (0.00)	0.83^{a} (1.44)	4.17^{a} (1.44)	0.83^{a} (1.44)	0.83^{a} (1.44)
Food intake (g/100g fish/day)	1.78^{b} (0.14)	1.82^{b} (0.05)	1.98^{c} (0.05)	2.07^{c} (0.07)	1.56^{a} (0.05)	1.79^{b} (0.09)	1.52^{a} (0.10)
Food conversion ratio (FCR)	2.50^{a} (0.22)	2.82^{a} (0.11)	2.18^{a} (0.07)	2.46^{a} (0.06)	3.30^{a} (0.23)	3.50^{a} (0.59)	8.09^{b} (2.59)
Protein efficiency ratio (PER)	0.83^{c} (0.08)	0.75^{bc} (0.03)	0.98^{d} (0.03)	0.88^{cd} (0.02)	0.66^{b} (0.05)	0.64^{b} (0.10)	0.29^{a} (0.08)
Apparent net protein utilisation (ANPU)(%)	15.64^{cd} (1.37)	14.23^{bc} (0.46)	18.28^{e} (0.48)	16.68^{de} (1.12)	12.56^{b} (0.74)	13.81^{bc} (1.78)	6.38^{a} (1.29)
Apparent net lipid utilisation (ANLU)(%)	24.10^{d} (3.52)	16.60^{c} (1.73)	46.29^{f} (1.73)	34.30^{e} (5.81)	0.67^{b} (3.10)	3.01^{b} (3.67)	-23.96^{a} (5.44)
Energy efficiency (EE)(%)	15.47^{d} (1.69)	12.57^{c} (0.71)	22.08^{f} (0.66)	17.85^{e} (0.46)	7.27^{b} (1.21)	8.36^{b} (2.02)	-1.91^{a} (1.80)

Figure 3.2 Assessment of growth and feed performance in Experiment 1: specific growth rate. Bars indicate one standard deviation.

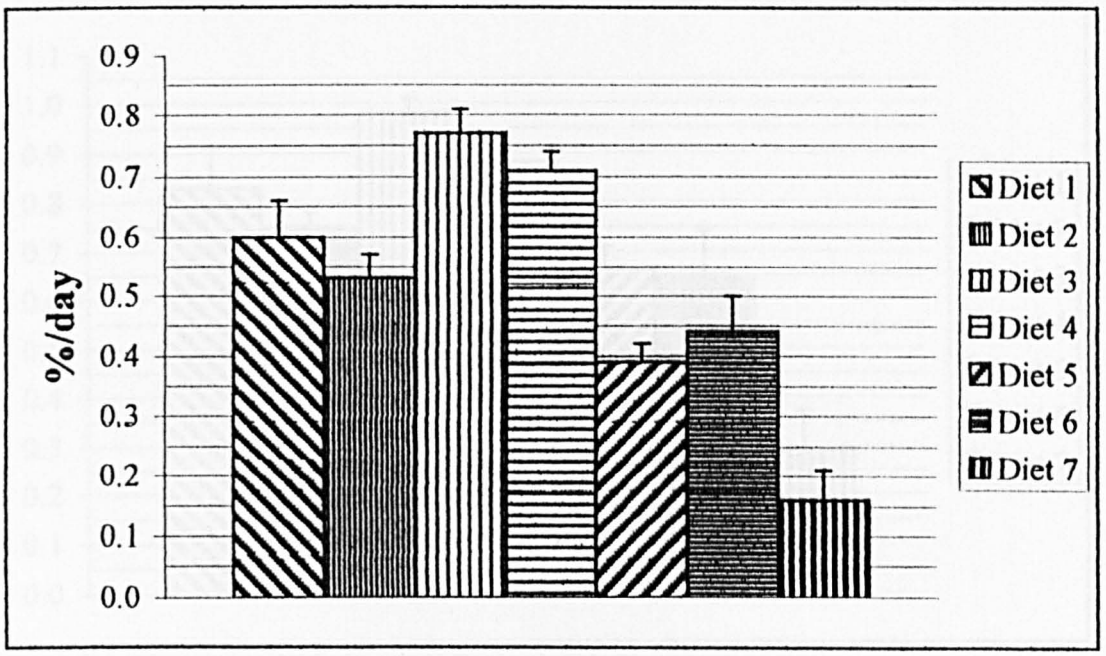


Figure 3.3 Assessment of growth and feed performance in Experiment 1: food conversion ratio. Bars indicate one standard deviation.

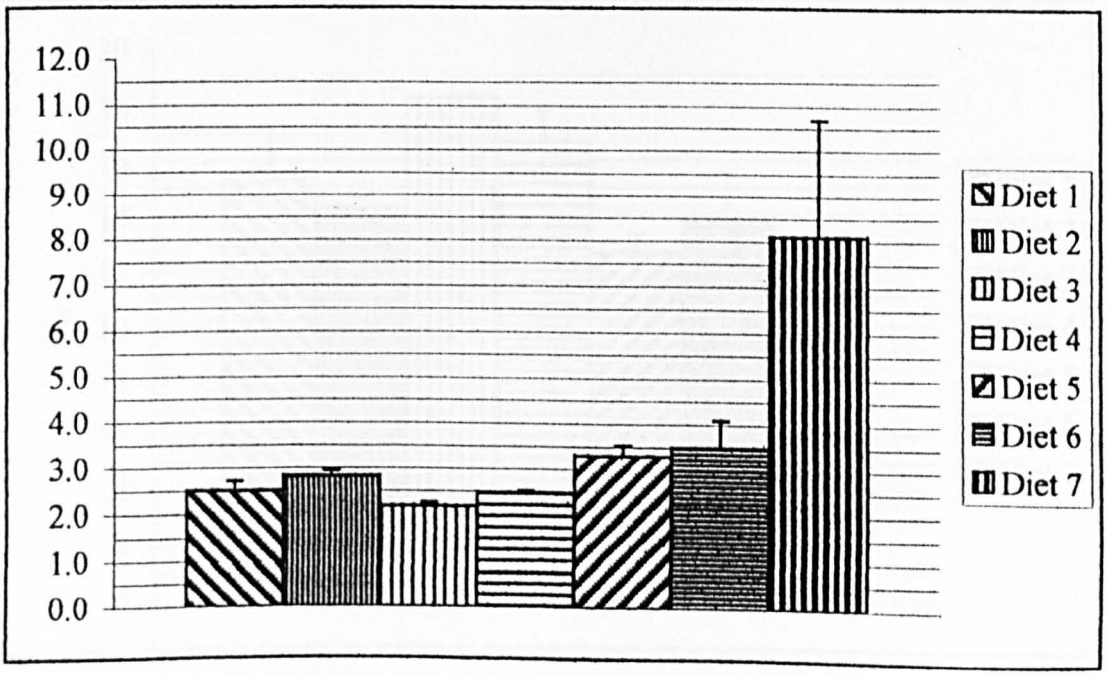


Figure 3.4 Assessment of growth and feed performance in Experiment 1: protein efficiency ratio. Bars indicate one standard deviation.

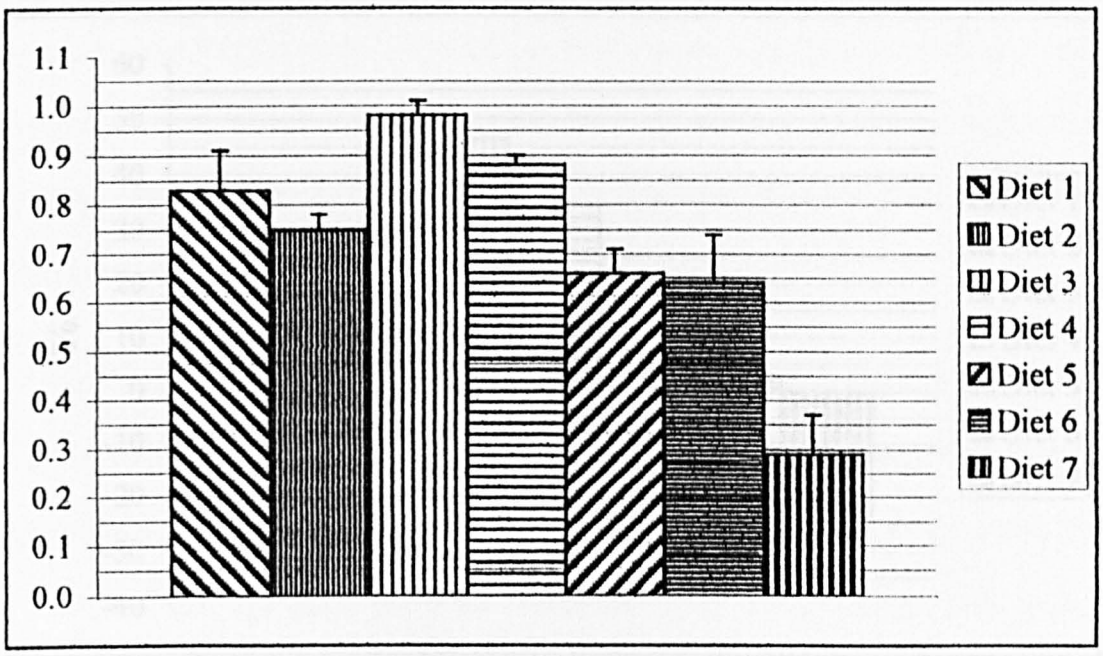


Figure 3.5 Assessment of growth and feed performance in Experiment 1: apparent net protein utilisation. Bars indicate one standard deviation.

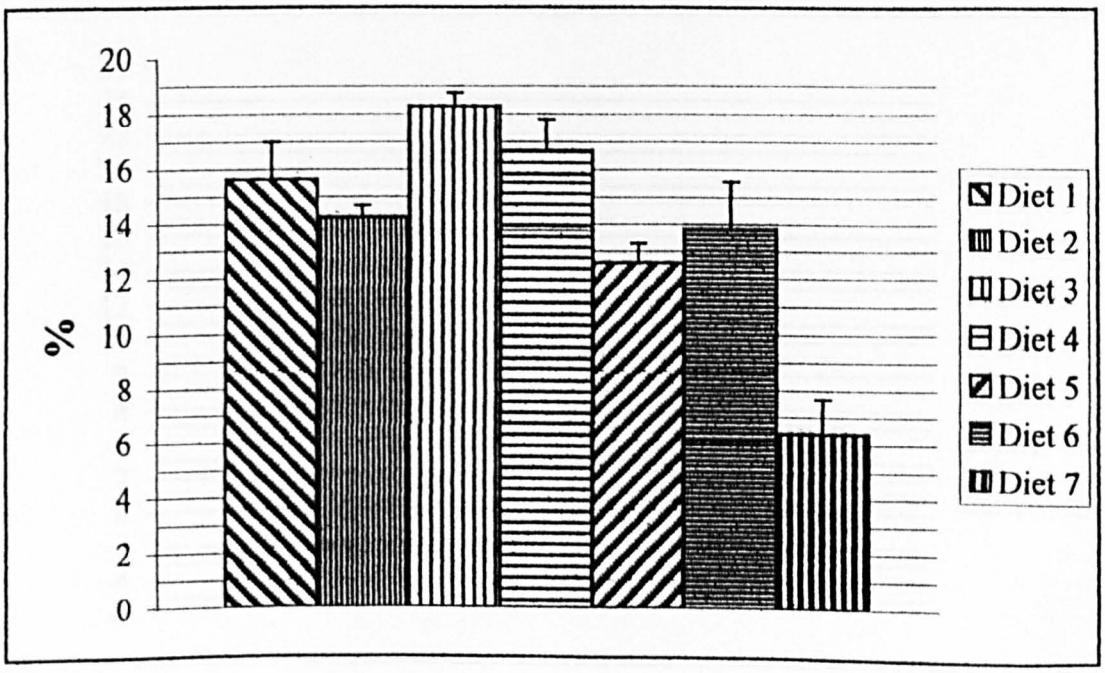


Figure 3.6 Assessment of growth and feed performance in Experiment 1: apparent net lipid utilisation. Bars indicate one standard deviation.

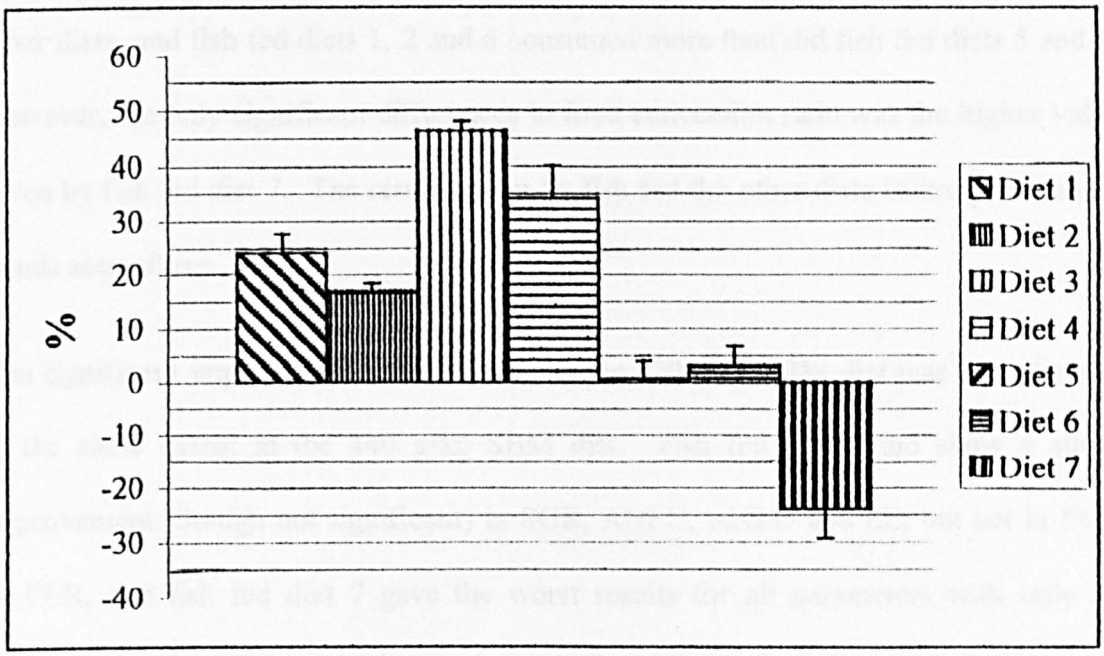
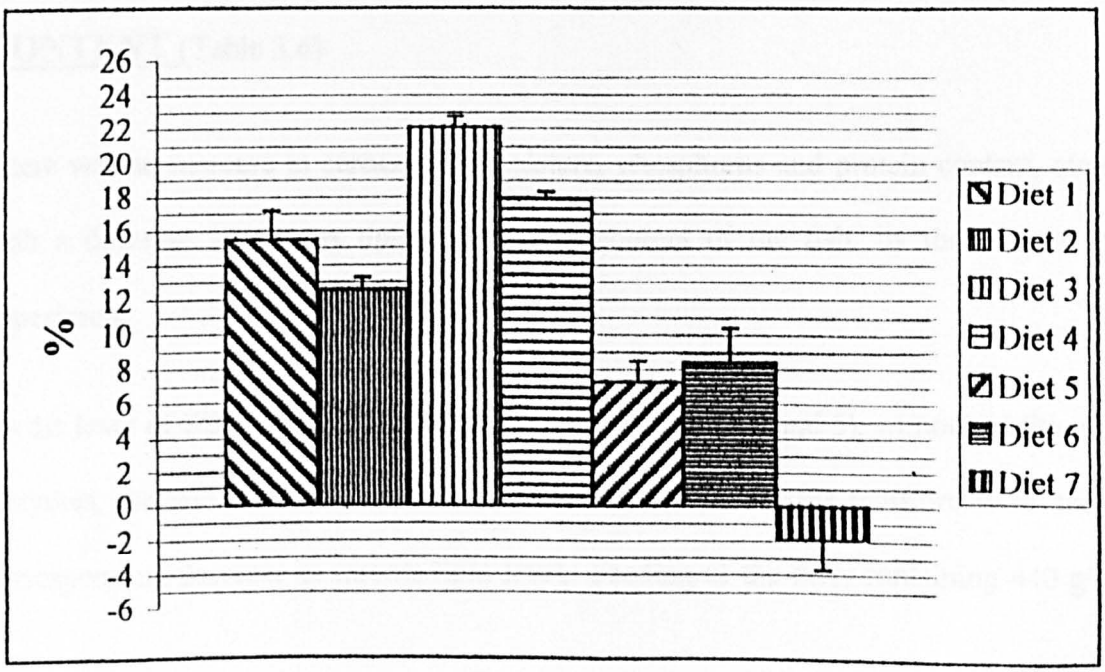


Figure 3.7 Assessment of growth and feed performance in Experiment 1: energy efficiency. Bars indicate one standard deviation.



fish were fed diets 5 to 7 in all the above parameters. Feeding of diet 3 also resulted in significantly better ANLU and EE than feeding fish diet 4.

Fish fed diets 3 and 4 consumed significantly more food than did fish fed any of the other diets, and fish fed diets 1, 2 and 6 consumed more than did fish fed diets 5 and 7. However, the only significant differences in food conversion ratio was the higher value given by fish fed diet 7. The results given by fish fed the other diets followed the same trends seen above.

The significant impact of enzyme addition to the 320 g/kg SBM diet was not mirrored to the same extent in the 440 g/kg SBM diet. Fish fed diet 6 did show a slight improvement (though not significant) in SGR, ANPU, ANLU and EE, but not in FCR or PER, and fish fed diet 7 gave the worst results for all parameters with only an average growth of 7.3 g over the whole experimental period and a negative ANLU and EE.

3.3.2 CARCASS COMPOSITION, CONDITION FACTOR, HEPATOSOMATIC INDEX AND INTESTINAL DRY MATTER CONTENT (Table 3.6)

There was an increase in carcass ash, moisture, phosphorus and protein content, along with a decrease in carcass lipid and energy content of the fish, by the end of the experiment.

As the level of SBM in the formulation increased (diets 1, 2 and 5), without addition of enzymes, the fish fed these diets showed an increase in carcass moisture level and a corresponding decrease in carcass lipid level. Feeding of the diets containing 440 g/kg

Table 3.6 Effect of dietary treatments on the body (whole) composition of fish in Experiment 1. Condition factor, hepatosomatic index and final intestinal percentage dry matter content are presented as means with the standard deviation in brackets. Means in a row followed by the same superscript are not significantly different ($P < 0.05$).

	Initial	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type		Pressed	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed
Fish meal inclusion (g/kg)		320	260	260	260	260	260	260
Soybean meal inclusion (g/kg)		220	320	320	320	320	320	320
Low pH protease (g/kg)		0	0	1	0	0	1	0
High pH protease (g/kg)		0	0	0	1	0	0	1
α-galactosidase (g/kg)		0	0	1	1	0	1	1
Moisture (g/100g)[1]	64.60	65.74	66.75	64.79	66.55	67.35	67.24	67.40
Protein (g/100g)[1]	16.28	17.27	17.29	17.41	17.73	17.02	17.89	17.06
Lipid (g/100g)[1]	14.55	11.89	11.49	13.58	12.36	10.67	10.63	10.19
Ash (g/100g)[1]	3.71	4.22	4.25	3.90	4.07	4.35	4.17	4.07
Phosphorus (g/100g)[1]	0.66	0.76	0.74	0.68	0.71	0.73	0.72	0.69
Energy content (kJ/g)[1]	9.97	9.32	9.04	9.65	9.22	8.88	9.05	8.92
Condition factor	1.48^{a} (0.16)	1.46^{a} (0.09)	1.42^{a} (0.06)	1.49^{a} (0.03)	1.44^{a} (0.13)	1.38^{a} (0.04)	1.29^{a} (0.13)	1.32^{a} (0.15)
Hepatosomatic index	1.73^{b} (0.41)	1.32^{ab} (0.19)	1.41^{ab} (0.24)	1.37^{ab} (0.14)	1.40^{ab} (0.33)	1.34^{ab} (0.32)	1.20^{a} (0.21)	1.09^{a} (0.30)
Intestinal dry matter content (g/100g)		18.12^{bc} (3.14)	19.69^{c} (3.19)	15.60^{ab} (3.28)	14.04^{ab} (2.83)	15.03^{ab} (1.49)	15.03^{ab} (1.45)	12.51^{a} (3.57)

1. Values are averages of pooled carcass samples.

SBM, with and without enzymes (diets 5, 6 and 7), resulted in fish having the highest carcass moisture contents and the lowest lipid contents. Fish fed diet 3 containing 320 g/kg SBM and low pH protease and α-galactosidase showed the lowest carcass moisture content of all the experimental fish, but the highest lipid content.

There were also decreases in fish condition factor (K) and hepatosomatic indexes (HSI) by the end of the experiment. A decrease in the condition factor of fish is seen as the SBM level of the diets fed goes up from 220 g/kg to 440 g/kg although this is not significant, and there was also a decrease in HSI of the fish fed diets containing 440 g/kg SBM (diets 5 to 7) compared to fish fed diets containing 320 g/kg SBM (diets 2 to 4), again not being significantly so.

As regards the intestinal dry matter contents of the guts, fish fed diet 2 gave the highest value, being significantly different from fish fed all the other diets except diet 1, which in turn was significantly higher than that found in fish fed diet 7. Feeding 320 g/kg SBM diets with enzymes to the fish (diets 3 and 4) reduced the intestinal dry matter content compared to fish fed the unsupplemented diet 2. A similar reduction in intestinal dry matter content was also observed in fish fed the high pH and α-galactosidase supplemented diet 7 (440 g/kg SBM) compared to fish fed the unsupplemented diet 5.

3.3.3 APPARENT DIGESTIBILITY COEFFICIENTS (Table 3.7)

Fish fed diet 3, containing 320 g/kg SBM with supplemental low pH protease and α-galactosidase, gave the best apparent digestibility coefficients (ADC) for protein, lipid, organic matter, energy and phosphorus of all the diets, followed by fish fed diet 4 in lipid, organic matter and phosphorus ADCs. The carbohydrate ADC was the highest in

Table 3.7 Effect of dietary treatments on apparent digestibility coefficients (ADC) in Experiment 1 calculated using faeces collected during weeks 7 and 8[1].

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed
Fish meal inclusion (g/kg)	320	260	260	260	260	260	260
Soybean meal inclusion (g/kg)	220	320	320	320	320	320	320
Low pH protease (g/kg)	0	0	1	0	0	1	0
High pH protease (g/kg)	0	0	0	1	0	0	1
α-galactosidase (g/kg)	0	0	1	1	0	1	1
Protein ADC (%)	84.54	86.97	87.73	86.52	85.58	81.24	85.34
Lipid ADC (%)	94.22	94.59	97.14	96.20	93.69	91.90	95.16
Carbohydrate ADC (%)	86.68	77.06	84.66	82.25	71.12	72.16	92.05
Energy ADC (%)[2]	88.83	88.67	90.78	89.62	87.13	84.08	90.19
Organic matter ADC (%)	67.34	73.27	78.76	77.96	67.16	62.59	74.78
Phosphorus ADC (%)	43.86	45.66	59.52	53.68	23.80	5.67	28.24

1. Values are averages of pooled faecal samples.
2 Energy calculated using the following values: protein, 23.4 kJ/g; lipid, 39.8 kJ/g; carbohydrate, 17.2 kJ/g.

fish fed diet 7 and the lowest ADCs for all nutritional components was given by fish fed diet 6. All diets fed except diet 6 gave higher protein ADCs than the fish fed the 220 g/kg SBM diet 1.

The phosphorus ADCs shown by the fish fed diets 5 to 7 containing 440 g/kg SBM were all lower than that shown by fish fed the 220 g/kg SBM diet 1, while fish fed diets containing 320 g/kg showed the highest values of all the diets used.

The carbohydrate ADC of the fish decreased as the level of dietary SBM increased (diets 1, 2 and 5). Feeding fish the 320 g/kg SBM enzyme supplemented diets 3 and 4 improved the ADC of lipid, carbohydrate, organic matter, energy and phosphorus compared to fish fed the unsupplemented diet 2.

On the other hand, feeding fish enzyme supplemented 440 g/kg SBM diets (diets 6 and 7) gave mixed results when compared to the fish fed the unsupplemented diet 5. From these three diets, fish fed diet 7 gave higher ADC values for all components except protein, while fish fed diet 6 gave lower ADCs in all parameters except carbohydrate.

3.4 DISCUSSION

The results of this experiment show some very interesting results from the point of view of using supplemental enzymes, with significant improvements in the performance of fish fed the enzyme supplemented diets, to an extent, as shall be seen in the following discussion.

For the purpose of simplification, the discussion of this experiment has been divided into two parts, the first discussing the impact of increasing the soybean meal (SBM)

level in the diet at the expense of fish meal (FM), and the second analysing the effect of adding the enzyme mixes on the performance of the fish.

3.4.1 REPLACING FISH MEAL IN GILTHEAD SEA BREAM DIETS

The following discussion will consider only the results of fish fed diets 1, 2 and 5.

As the level of SBM was increased from 220 g/kg (diet 1) to 320 g/kg (diet 2) to 440 g/kg (diet 5) a progressive reduction in fish performance was observed in all the nutritional parameters studied. In terms of specific growth rate (SGR), fish fed the first two of these diets grew significantly faster than the fish fed diet 5. Fish fed these two diets both gave significantly higher apparent net lipid utilisations (ANLU) and energy efficiencies (EE) than did fish fed diet 5, but at the same time fish fed diet 1 gave significantly higher values than fish fed diet 2 as well for these parameters. In the case of protein efficiency ratio (PER) and apparent net protein utilisation (ANPU) only fish fed diet 1 gave significantly higher values than fish fed diet 5.

While no significant differences were recorded in food conversion ratio (FCR) between fish fed these diets, the values themselves vary a lot, with fish fed diet 2 giving an FCR 113% and fish fed diet 5 132% of the FCR given by fish fed diet 1.

As seen below, the work of other authors in which SBM has been used to replace FM in gilthead sea bream diets did not show the same decreasing trend in fish performance as seen in this experiment. Instead, a number of these trials have shown a positive effect of replacing FM with SBM.

Millan *et al.* (1989), working with 75 g *Sparus aurata* obtained better growth and feed utilisation with a diet containing 450 g/kg SBM (200 g/kg FM) than with the control diet containing 0 g/kg SBM and 475 g/kg FM. This diet gave a better fish performance

than did the other experimental diets containing 290 and 640 g/kg SBM (300 and 100 g/kg FM respectively). In another trial with 1 g gilthead sea bream fed the same diets, the same authors obtained inferior performance to the fish fed the control diet, but the fish fed the 450 g/kg diet still gave the best results.

Robaina *et al.* (1995) obtained slightly better results with 40 g gilthead sea bream fed diets containing 100 and 200 g/kg SBM (690 and 610 g/kg FM respectively) than fish fed a control diet containing no SBM and 770 g/kg FM, which in turn gave better performance than fish fed a 300g SBM, 540 g/kg FM diet. The best performance was obtained with fish fed the 100 g/kg SBM diet.

With smaller gilthead sea bream of initial weight 6.2 g, Nengas *et al.* (1996) obtained equal results when they fed the fish a diet containing 740 g/kg white FM, 0 g/kg SBM and a diet containing 240 g/kg SBM and 560 g/kg FM. A 120 g/kg SBM diet used in this trial gave a lower fish performance than both of these diets as did fish fed a diet containing 480 g/kg SBM (440 g/kg FM).

A number of other experiments with the gilthead sea bream have been carried out in which the amount of FM in the diet was reduced and substituted with other ingredients. These experiments show that the FM content in the gilthead sea bream diet can be reduced to quite an extent without any loss in performance.

Experiments were carried out by Amaral (1994) and Bekkevold (1994) in which 2.3 and 11 g gilthead sea bream respectively were fed diets in which FM was gradually reduced from 670 to 0 g/kg using combinations of wheat, SBM, hydrolysed feather meal and blood meal.. The latter author did not find any significant differences in the performance of fish fed diets containing decreasing inclusions of FM (from 400 to 200 g/kg) compared to the control 670 g/kg FM diet, but Amaral obtained a decrease in performance as FM content was reduced from 200 to 0 g/kg with high mortality

occurring with the fish fed the FM-free diet. In this latter work, the fish fed the 200 g/kg SBM diet gave an inferior performance to fish fed the control 670 g/kg FM diet. Davies *et al.* (1993) fed 5 g gilthead sea bream a number of diets in which various meat and bone meals made up to 400 g/kg of the diet (470 g/kg white FM) but did not obtain any differences in performances when compared to fish fed the control 740 g/kg FM diet.

It could be argued that in the above experiments the FM content of the successful diets used in the above experiments was still high; in the work of Robaina *et al.* (1995) and Nengas *et al.* (1996), the lowest FM content of the diets used were in fact 540 and 440 g/kg respectively, higher than the 320 g/kg used in diet 1 of Experiment 1. On the other hand, the diet giving the best results in the work of Millan *et al.* (1989) contained 450 g/kg SBM and only 200 g/kg FM. The work of Bekkevold indicated that FM replacement to a high degree is possible, to a lower level of inclusion than actually used in Experiment 1, although more than one ingredient was used to effect this substitution and the control diet contained 670 g/kg FM.

The results obtained in Experiment 1 are not the first to have shown this negative relationship between SBM level in the diet and performance, and has been observed by numerous other authors in various species of fish. These include the work of Reinitz (1980), Alexis (1990) and Pongmaneerat and Watanabe (1992) on rainbow trout (*O. mykiss*), Watanabe *et al.* (1992) on yellowtail (*S. quinqueradiata*), Webster *et al.* (1992b) on blue catfish, (*I. furcatus*), El-Sayed (1994) on silver sea bream (*R. sarba*) and Davis *et al.* (1995) on red drum (*S. ocellatus*).

On the other hand, numerous authors have recorded improved or at least equal performance in one parameter or more in fish fed diets in which SBM replaced FM, such as in the work of Davies *et al.* (1989)(*O. mossambicus*),Webster *et al.*

(1992a)(blue catfish, *I. furcatus*), Reigh and Ellis (1992)(red drum, *S. ocellatus*), Viyakarn *et al.* (1992)(yellowtail, *S. quinqueradiata*), Shimeno *et al.* (1993a)(yellowtail, *S. quinqueradiata,*), Watanabe and Pongmaneerat (1993)(rainbow trout, *O. mykiss*), Gallagher (1994)(hybrid striped bass, *M. saxatilis* x *M. chrysops,*)Kaushik *et al.* (1995)(rainbow trout, *O. mykiss*), Olli *et al.* (1995)(Atlantic salmon, *S. salar*) and Stickney *et al.* (1996)(rainbow trout, *O. mykiss*),.

The three diets being considered here can be divided into two, diets 1 and 2 which only differed significantly in ANLU and EE , and fish fed diet 5. Fish fed diet 1 and 2 ate significantly more than fish fed diet 5 on a daily intake basis. Although consumption of diet 2 was slightly more than the consumption of fish fed diet 1, fish fed diet 2 gave an inferior performance to fish fed diet 1. Other authors have also noticed a reduction in food consumption when they fed fish diets containing increasing levels of SBM in at least one of their experimental diets (Balogun and Ologhobo, 1989; Reigh and Ellis, 1992; Davis *et al.*, 1995; Kubitza *et al.*, 1997) but others have even reported increases in consumption when SBM substituted FM in fish diets (Viyakarn *et al.*, 1992; Watanabe *et al.*, 1992; Watanabe and Pongmaneerat, 1993).

The main preoccupations with using SBM to replace FM in fish diets is the essential amino acid balance of this ingredient and the presence of antinutritional factors.

There were no major differences between the three diets 1, 2 and 5 in this experiment in terms of nutritional composition, although crude protein level and gross energy content decreased slightly as the level of SBM in the diet increased. The lipid and carbohydrate levels were very similar in these three diets. The amino acid contents show up some small differences between these three diets. However, these amino acid contents do not follow any regular trend going from the low to the high SBM containing diets.

In the literature there has been variable successes with adding supplemental amino acids to diets in which SBM has been used to replace FM (in particular methionine) to compensate for the amino acid differences between the two ingredients. While some authors successfully improved the performance of diets in which SBM has replaced FM (Dabrowska and Wojno, 1977; Shiau *et al.*, 1987; Webster *et al.*, 1995), quite a number of others have not (Pantha, 1982; Murai *et al.*, 1986; Shiau *et al.*, 1988; Viola *et al.*, 1988; Davies and Morris, 1997; Keembiyehetty and Gatlin, 1997).

The trypsin inhibitor activities (TIA) of the three diets being considered were all below the 0.3 mg/g diet level found by a number of authors to have an effect on growth and feed performance. Rumsey *et al.* (1993) suggested that TIA levels below 5 mg/g had little effect on rainbow trout, *O. mykiss*, Wilson and Poe (1985) suggested a maximum level of 3.2 mg/g for channel catfish, *I. punctatus*, Webster *et al.* (1992b) recommended a value of below 3.2 mg/g for blue catfish, *I. furcatus* and Wee and Shu (1989) recommended a maximum of 1.6 mg/g for Nile tilapia, *O. niloticus*.

The protein solubility of the SBM used in this experiment was 79.4% a value which lies between the 73 and 86% range in which Araba and Dale (1990) obtained the best growth in their trials with chicks. Protein solubility was used by these authors to assess the degree of heating, with values below this range indicating a possible overprocessing and reduction of amino acid (such as lysine and arginine) digestibility and availability.

The phosphorus contents of the three diets 1, 2 and 5 were similar (although it decreased slightly going from diet 1 to 5), but the decreasing phosphorus apparent digestibility coefficients (ADCs) of the fish fed these three diets clearly indicates that something was drastically affecting the digestibility of phosphorus in fish fed diet 5 compared to fish fed the other two diets. It would have been expected that a sequential

decrease be obtained for phosphorus ADCs considering the increasing proportion of phytate in the diets as the SBM level in the diet increased.

With increasing SBM level in the diet the oligosaccharide level is also expected to increase, and therefore so too the antinutritional effects associated with it. Oligosaccharides are strongly suspected to cause an osmotic effect leading to fluid retention and a reduced passage time and thereby reducing digestibility. Additionally, the oligosaccharides can be metabolised by the microflora in the intestine allowing increased microbial growth which also utilise nutrients to the detriment of the host. The former of these effects is generally assessed by measuring the intestinal dry matter content. An increased oligosaccharide presence would result in an increased moisture content of the intestinal contents, i.e. less dry matter. The measurements taken for dry matter content in Experiment 1 are not clear. While there was a lower dry matter content in the intestines of fish fed diet 5 than fish fed either diet 1 or 2, it would have been expected that fish fed diet 2 had intestinal dry matter contents lower than did fish fed diet 1.

The effect on digestibility is even more difficult to assess. It is impossible to distinguish between fish digestion and microbial digestion which may be taking place in the digestive tract of the fish. The digestibilities of the different components of the diets indicates that there were little differences in protein, lipid or energy digestibilities. This is somewhat surprising considering the differences in performance obtained by the fish. Two factors could have explain this result. First of all, the digestive physiology could have become adapted to the feed intake so as to get as much out of the ingested food as possible. The second reason could lie in the activity of the bacteria in the gut, increased by the higher oligosaccharide content of the diet, consuming more of the nutrients in the gut and therefore contributing to the observed ADC values. Evidence of

bacterial fermentation (by the production of volatile fatty acids) has been obtained by a number of authors (Rimmer and Wiebe, 1987; Lesel, 1993; Clements *et al.*, 1994; Kandel *et al.*, 1994; Kihara *et al.*, 1995; Smith *et al.*, 1996). Genovese *et al.* (1992) did not find any changes in intestinal microflora when fish were fed various diets containing SBM as compared to fish fed a SBM-free diet. On the other hand, Shivokene *et al.* (1985), Lesel (1988) and Lesel *et al.* (1988) found that the bacterial content of the intestine did vary with variation in diet composition. Wada *et al.* (1991) found that when humans were given soybean oligosaccharide extract (containing stachyose and raffinose) there was an increase in the intestinal microflora. However, to make matters even more complicated, Kitamikado *et al.* (1993) found that a number of oligosaccharide preparations actually had antibacterial activities.

The lower digestibility of the carbohydrates of the fish fed diets with increasing SBM level could also be related to the increasing proportion of carbohydrates being oligosaccharides, which would make less of the carbohydrate actually digestible in the first place.

Olli and Krogdahl (1995) fed Atlantic salmon, *S. salar*, diets in which 0 to 200 g/kg of commercially available soybean molasses (containing 17.2 and 4.1 g/100 g of stachyose and raffinose respectively) were added. Fat digestibility was significantly reduced as the level of molasses in the diet increased, but protein digestibility was not affected. Faecal dry matter contents found by these authors varied from 13.4 to 15.1 g/100 g, with the fish fed the control diet having an intestinal dry matter content of 14.2 g/100 g, but with no clear trend with increase in molasses inclusion level. The decrease in fat digestibility observed by these authors could be at least partially be explained by the fact that some bacteria are able to deconjugate bile salts (Feord, Pers. Comm., 1996)

and thus, if an increase in the intestinal microflora takes place, this effect would be enhanced.

Arnesen *et al.* (1989) fed rainbow trout, *O. mykiss* and Atlantic salmon, *S. salar*, various diets containing from 0 to 80 g/kg alcohol soluble carbohydrates. These carbohydrates were found to have a negative influence on the utilisation of nutrients in the Atlantic salmon, and to bring about a lower dry matter percentage in the faeces of both species. However, values were not given by these authors.

An increase in True Metabolisable Energy was observed by Coon *et al.* (1990) when they fed roosters diets containing 80% ethanol-extracted SBM compared to a SBM diet. These authors also observed an increased transit time as well as a higher apparent digestibility of the major carbohydrates of SBM in roosters fed the extracted SBM diet.

From trials carried out by Leske *et al.* (1991) with broilers and roosters using 80% ethanol-extracted SBM and the extract obtained, these authors also found that using extracted SBM increased the True Metabolisable Energy compared to feeding diets containing the unextracted SBM or to feeding the extracted SBM diet to which raffinose or the extract itself had been re-added. Additionally, the roosters fed the SBM diet and the diet to which the extract had been added again had excreta containing more water than those fed the extracted SBM diet.

The presence of antinutritional factors may account for part of the inferior performance of fish fed diets 2 and 5 compared to fish fed diet 1, but another factor probably played a part as well. The lower consumption of food by fish fed diet 5 meant that the daily energy intake was lower than that of fish fed either diet 1 or 2. A lower intake of energy and nutrients leaves less available for growth after the metabolic requirements are satisfied. This means there is less utilisation of nutrients for growth which is thereby translated into a poorer utilisation of the food in a lower PER, etc. This lower

energy availability was also represented in a lower condition factor (though not found to be significant) and lower carcass lipid (and inversely higher carcass moisture contents) and energy contents of fish fed diet 5 compared to fish fed either diets 1 and 2. The carcass protein contents of the fish fed these three diets did not differ greatly between them.

Numerous authors have also found that carcass composition varies when they change the level of SBM in the diet. In the work of Pongmaneerat and Watanabe (1992) with rainbow trout, *O. mykiss*, carcass protein and moisture levels of fish fed the higher SBM containing diets (300g to 500 g/kg) were all lower, while carcass lipid levels were all higher than fish fed the control diet containing 640 g/kg FM.

However, in the work of Watanabe and Pongmaneerat (1993), the carcass protein and moisture levels in rainbow trout, *O. mykiss*, decreased slightly as the SBM level in the diets fed increased from 0 to 500 g/kg, while carcass lipid content decreased.

In the experiment carried out be Kaushik *et al.* (1995) with rainbow trout, *O. mykiss*, carcass lipid content increased and moisture content decreased, while protein content did not differ between fish as the SBM levels in the diets increased from 0 to 620 g/kg.

El-Sayed found that when he fed silver sea bream, *R. sarba*, diets containing 0 to 840 g/kg SBM, the carcass protein level decreased as the SBM level increased while the carcass moisture and lipid levels were not significantly different between fish fed the various diets.

Feeding two sets of SBM products to *O. mossambicus* Davies *et al.* (1989) found two different trends in carcass lipid contents with increased SBM level, in one case decreasing and in the other increasing as the SBM level increased (from 110 to 360 g/kg SBM and from 80 to 240 g/kg SBM respectively). Carcass protein and moisture contents were not different whatever the SBM level used in the diets fed.

Shimeno *et al.* (1993a) did not find differences in any of the carcass components when they fed yellowtail, *S. quinqueradiata*, diets containing 0 to 300 g/kg SBM, while Shimeno *et al.* (1992)(yellowtail, *S. quinqueradiata*), Webster *et al.* (1992)(channel catfish, *I. punctatus*) and Olli *et al.* (1995)(Atlantic salmon, *S. salar*), found differences between the carcass compositions of these fish fed the various diets, but no clear pattern or trend with increasing SBM level.

In the experiment carried out by Olli *et al.* (1995) Atlantic salmon, *S. salar*, fed a diet containing 170 g/kg SBM had a condition factor of 1.4 but a condition factor of 1.1 when fish were fed diets containing 0 and 340 g/kg SBM.

The hepatosomatic index (HSI) of the fish fed diets 1, 2 and 5 in Experiment 1 did not show trend related to the SBM level in the diet, although that of fish fed diet 2 was the highest obtained in fish fed any of the diets used in this experiment.

Alexis (1990) with rainbow trout, *O. mykiss*, fed diets containing 190 to 450 g/kg SBM did not find differences in HSI nor did Kaushik *et al.* (1995) with increasing levels of SBM from 220 to 620 g/kg. Similarly, Olli *et al.* (1995) with Atlantic salmon, *S. salar* fed diets containing 0 to 340 g/kg SBM did not find any differences in HSI.

Pongmaneerat and Watanabe (1992) found that the HSI of rainbow trout, *O. mykiss*, fed diets containing 300 to 500 g/kg SBM decreased as the level of SBM increased, as did yellowtail, *S. quinqueradiata*, fed diets containing from 200 to 500 g/kg SBM.

Amaral (1994) found that the condition factor of 2.4 g gilthead sea bream decreased (from 2.6 to 2.4), as did the HSI (from 2.9 to 2.1) as the level of FM in the diet was reduced from 200 to 100 to 0 g/kg. Bekkevold (1994) however, found that there were no differences in the condition factor of the 11 g gilthead sea bream he used in his experiment (with an average value of 1.7) when he fed diets with decreasing levels of FM from 670 to 400 to 300 to 200 g/kg. The HSI dropped from 2.5 with the high FM

diet to 2.1 with the 400 g/kg FM diet to increase again to 2.3 as the FM was further replaced.

Robaina *et al.* (1995) obtained similar HSIs when they fed 40 g gilthead sea bream diets with 0, 100 and 200 g/kg SBM (with an average value of 1.3), but the HSI increased to 1.4 when the fish were fed a diet containing 300 g/kg SBM.

The results of the digestibility study carried out in this experiment were similar to that obtained by a number of other researchers, but not others, who had carried out similar experiments.

Watanabe *et al.* (1992) obtained similar values for protein ADC with yellowtail, *S. quinqueradiata*, when fed diets containing from 100 to 300 g/kg SBM (460 to 260 g/kg FM) and the control 560 g/kg FM, 0 g/kg SBM diet, the average value being 86%. Lipid ADC values obtained were only different in fish fed the 300 g/kg SBM, 260 g/kg FM from an average of 87% down to 74%. Carbohydrate digestibility decreased as the SBM level in the diet fed was increased from 0 g/kg which was 65% down to 55% with the fish fed the 300 g/kg SBM diet, as did energy ADC from 82% to 75%.

Watanabe and Pongmaneerat (1993), working with rainbow trout, *O. mykiss*, observed no differences in protein ADCs in fish fed diets containing 300 to 500 g/kg SBM (250 to 50 g/kg FM) when compared to the fish fed the control 550 g/kg FM, 0 g/kg SBM diet which gave an ADC of 92%. Crude starch digestibility was found to decrease from 90% to 73% as the SBM level of these diets increased, and energy ADCs also decreased from 91% of the fish fed the control diet down to a value of 86% given by the fish fed the highest SBM containing diet.

Similar results were obtained by Pongmaneerat and Watanabe (1992) again working with rainbow trout, *O. mykiss*. Protein ADC averaged around 93% but did not differ from that of the fish fed the control diet of 640 g/kg FM (0 g/kg SBM) as the SBM

levels of the experimental diets increased from 300 to 500 g/kg (340 to 140 g/kg FM). Both energy and starch digestibilities decreased as the level of the SBM in the diets fed increased. Fish fed the control diet gave an energy and starch ADC of 93 and 92% respectively, which decreased down to 83 and 71% respectively when fish were fed the diets containing 500 g/kg SBM.

When Shimeno *et al.* (1995) replaced FM in yellowtail, *S. quinqueradiata*, diets with a number of different types of SBM to give diets containing up to 300 g/kg SBM (480 g/kg FM) they obtained protein ADCs in the range of 55% up to a value of 74%, which was slightly better than the value of 72% obtained by fish fed the 700 g/kg FM control. However all fish fed the experimental diets gave lower sugar digestibilities (between 40 and 55%) than that given by fish fed the control diet (which was 56%).

In a similar trial with yellowtail, *S. quinqueradiata*, using various types of SBM at an inclusion level of up to 300 g/kg SBM (530 g/kg FM), protein ADCs of the fish fed these diets varied from 76 to 83% and carbohydrate ADCs from 36 to 57% (Shimeno *et al.*, 1993). Fish fed the control 750 g/kg FM diet gave protein and carbohydrate ADCs of 79 and 47% respectively.

Rainbow trout, *O. mykiss*, fed diets containing up to 300 g/kg SBM of different sources (400 g/kg FM) all gave protein digestibilities which were higher than the value of 77% given by the fish fed the control 570 g/kg FM diet, with the maximum value being 88% (Oliva-Teles *et al.*, 1994). On the other hand, energy digestibilities for all fish fed these diets was similar, averaging around 88%.

In the work of Robaina *et al.* (1995) where gilthead sea bream were fed diets containing 100 to 300 g/kg SBM (690 to 540 g/kg FM) only the fish fed the 100 g/kg SBM diet gave a slightly higher protein ADC than fish fed the control 770 g/kg FM diet, but all fish given the experimental diets gave higher lipid ADCs than the fish fed the control

diet. The highest values obtained were 94 and 98% for protein and lipid digestibilities respectively.

Bekkevold (1994), also working with gilthead sea bream, fed diets in which FM was replaced by a number of other ingredients from 400 g/kg down to 200 g/kg. This author found that the protein ADC shown by the fish decreased as the FM level in the diet decreased, from a value of 86% down to 78%.

As was seen in the case of carcass composition, condition factor and hepatosomatic index, and as can be seen from the results obtained by other authors for digestibilities, there is a large amount of variability in the effect of SBM on these parameters. Such a variability was also seen in growth and feed utilisation of fish, and the results of this trial with the gilthead sea bream further show that more work is required in order to understand better what factors are actually determining the observed results.

3.4.2 THE EFFECT OF SUPPLEMENTARY ENZYMES

The addition of the two enzyme mixes to the 320 g/kg SBM 2, diets 3 and 4, both gave a very good improvement in performance of the fish fed these two diets. In the case of fish fed diet 3, containing supplemental low pH protease and α-galactosidase, this improvement was significant in all parameters studied except FCR. Fish fed diet 4, containing high pH protease and α-galactosidase, also gave a significantly higher SGR, ANPU, ANLU and EE than did fish fed diet 2. Although the FCRs of diets 3 and 4 were not found to be significantly different from that of fish fed diet 2, their FCRs were only 77 and 87% respectively that of fish fed diet 2.

Feeding these two diets not only gave a better performance than fish fed the unsupplemented diet 2, but also a better performance than fish fed the higher FM, lower SBM containing diet 1. All parameters studied were better than those of fish fed diet 1,

diet 3 giving significantly better SGR, PER, ANPU, ANLU and EE than fish fed diet 1, and diet 4 significantly higher SGR, ANLU, EE. Fish fed these two diets had improved FCR, although not shown to be significant, with fish fed diet 3 showing an FCR 87% that of fish fed diet 1, but fish fed diet 4 being only slightly better.

The same improvement in performance was not seen to such an extent in fish fed diets 6 and 7, having supplemental low and high pH protease enzymes respectively together with α–galactosidase added to the 440 g/kg SBM diet 5. In fact, the results of fish fed diet 7 gave the lowest performance in all parameters, and shall be discussed further below.

Fish fed diet 6 did show an improvement in SGR, ANPU, ANLU and EE over fish fed the unsupplemented diet 5, but an inferior FCR (none being significant). Both of these diets gave lower performances than fish fed the unsupplemented 320 g/kg SBM diet 3 and fish fed any of the other diets except diet 7.

The effect of increasing SBM level has already been seen above. For the moment, the performance of diets 3 and 4 shall be considered with respect to fish fed diets 1 and 2.

Fish fed diets 3 and 4 consumed almost the same quantity of food but significantly more than fish fed the unsupplemented diets 1 and 2 (an effect of supplementary enzymes also seen by Hesselman *et al.* (1982), Maugle *et al.* (1983b), Hesselman and Aman (1986), Cave *et al.* (1990) and Carter *et al.* (1994)). The difference in performance can not only be attributed to the higher food consumption *per se*, because there was also a better utilisation of the food as expressed in the nutritional parameters mentioned above.

The reason why the fish fed diets 3 and 4 ate more food than fish fed either diets one or two is unclear when considered in terms of SBM content relative to FM content alone.

This means that additional to the nutritional improvement brought about by the enzymes, there is also apparently an increase in palatability. While small differences in the formulations used could somehow explain part of the differences in consumption between fish fed diets 1 and fish fed diets 2, 3 and 4, they obviously do not explain any differences between the three diets 2, 3 and 4. This means that either the carrier containing the enzyme had an effect or else there was some sort of enzyme activity even before the food was given to the fish, or just on impact with the water, possibly increasing the amount of attractants which might influence palatability and subsequent consumption. However, it is not possible to determine this with the available data but would be interesting to investigate further.

An increased level of consumption presents the fish with a higher amount of energy over the metabolic requirement available for growth. If more energy is available in the diet, protein sparing might occur. That this energy was made available is seen in the higher carcass lipid content of diets 3 and 4 over fish fed diets 1 and 2, the former having the highest carcass lipid content and energy content of all fish used in this experiment. However, fish fed diet 4 did not have as high a carcass energy content as did fish fed diet 1. The better energy utilisation of fish fed diets 3 and 4 compared to fish fed diets 1 and 2, is shown in the better ANLU and EE, and that protein sparing occurred compared to fish fed diets 1 and 2 is evident in the higher PERs and ANPUs.

The fish fed diets 3 and 4 showed higher protein, lipid, organic matter and energy ADCs than fish fed diets 1 and 2. That there was still a lower carbohydrate digestibility in fish fed diets 3 and 4, but improved over that of fish fed diet 2, indicates that the enzymes were not completely successful in eliminating the effects of additional SBM on carbohydrate digestibility. The addition of these enzymes also improved the

phosphorus utilisation of the fish fed diets 3 and 4 over those fed diets 1 and 2, a result which would have important environmental implications.

The enzymes used in this experiment had different pH optima (Feord, Pers. Comm., 1996). The low pH protease had an optimum activity around pH 3.0 and maintained up to 50% of this activity in the range 1.6 to 4.0. The high pH protease had its optimum pH around 8.5, with up to 50% of this activity maintained between pHs 7.2 and 10.2. The α–galactosidase had its optimum at 5.0 and maintained up to 50% of this activity between 3.4 and 6.8.

This means that both the low pH protease and the α–galactosidase were active in the stomach of the gilthead sea bream where the pH ranged from 2.5 to 5.5 (as reported in Table 10.1 (Chapter 10). The α–galactosidase would also have been active in the rest of the intestine, albeit with reduced activity. The high pH protease was not active in the stomach but was active in the rest of the intestine, although probably with a reduced activity throughout since the pH in the intestine ranged from 6.5 to 7.9 within the first 24 hours of feeding.

These pH optima and activity distribution could partly explain the better performance for all parameters of the low pH protease mixture compared to the high pH protease mixture. Since the pH of the stomach was closer to the optimum of the low pH protease than that of the intestine for the high pH protease, this enzyme would have been able to catalyse protein digestion to a higher degree than the high pH protease would. The α–galactosidase would have been more active in the stomach. In addition to the actual breakdown of proteins by the proteases, these enzymes would also indirectly assist in the breakdown of other components of the food, including other proteins, by making the food more accessible to the action of other enzymes, including the α–galactosidase itself. But, while the α–galactosidase could benefit by the action of the low pH

protease in the stomach, it could not benefit so effectively from the activity of the high pH protease in the rest of the intestine.

The specific mode of activity of the proteases in feed is unknown (Feord, Pers. Comm., 1997) and therefore the actual activity of the two proteases is not available. Apart from pH effects, differences in activities between these two enzymes could also help explain the differences obtained.

This discussion indicates that even when working at suboptimal pH conditions (to say nothing about suboptimal temperatures) these enzymes still gave a very good improvement in performance. Hence, it follows that if the conditions in the digestive tract could be made to approach the optimum pH for the enzymes an even greater effect could be obtained, with the subsequent implications for ingredients inclusion levels, etc. The other way around this is to use a different enzyme which could work more effectively in the known conditions in the intestine, although more work would be required to obtain the relevant information.

The intestinal dry matter contents of fish fed diets 3 and 4 were significantly lower than that found in fish fed diet 2, and both lower than that found in fish fed diet 1. If it is accepted that oligosaccharides cause a higher osmotic pressure and hence cause the intestinal contents to carry more water, it would be expected that while diet 2 should have a lower intestinal dry matter content relative to diet 1 as a result of a higher oligosaccharide content, in view of the expected activities of the α–galactosidase, fish fed diets 3 and 4 should have a higher dry matter content relative to fish fed diet 2 if not diet 1 also. Fish fed diet 4 had a lower intestinal dry matter content then fish fed diet 3, and this would agree with the above discussion in relation to fish fed diet 3 since there is possibly less activity of the α–galactosidase.

The results shown by fish fed the 440 g/kg SBM diets 5, 6 and 7 do not follow the same trends seen in fish fed diets 3 and 4 relative to fish fed diet 2. Fish fed diet 6 consumed significantly more than fish fed diet 5, an effect also seen for fish fed diets 3 and 4. Although growth was higher than fish fed diet 5, the food was not utilised so well, or at least not very more efficiently than fish fed diet 5. In fact the FCR was higher, and the PER slightly lower. However, these fish managed to put down more protein than fish fed diet 5 and although the PER was slightly lower than that of fish fed diet 5, the ANPU was slightly higher. These fish fed diet 6 also showed a slightly higher ANLU and EE than fish fed diet 5 although the carcass lipid contents were very similar.

Fish fed diet 6 showed lower ADC values for all the nutritional components analysed, except for carbohydrate, than fish fed diet 5 (and indeed of all diets). The small rise in carbohydrate ADC could have been due to the activity of the α–galactosidase, but why there was a drop in protein, lipid, organic matter, energy and phosphorus ADCs is unclear, although these would help to explain how the fish fed diet 6 had a higher FCR and lower PER than fish fed diet 5. These fish also showed a lower condition factor and HSI than fish fed diet 5.

It is difficult to understand the effect of the additional enzymes on the performance obtained, small as it is. Theoretically one of two effects would have been expected. First, the higher level of SBM in the diets (440 g/kg) could have given more scope, so to speak, for the enzymes to bring about a positive effect compared to the unsupplemented diet. The second possibility may have been the reason behind the results obtained, whereby the higher quantity of SBM actually restricted the activity of the enzymes, such that the amount of enzyme present was not sufficient to bring about an effect with 440 g/kg SBM as it had been with the 320 g/kg.

As for fish fed diet 7, although the daily consumption was only slightly less than fish fed diet 5, the performance is greatly below what would be expected. Assuming there were no effect of the enzymes, it would be expected that a performance similar to that of fish fed diet 5 would be obtained. Diet 7 was the only diet to which liquid enzymes had been added instead of dry enzymes. Why this should have had such a negative effect is also not known. The increased water as a result of addition of the liquid would not have had an effect and the moisture content of the diet was similar to that of diet 5. What remains is the reduced food intake due to reduced palatability, again for an unknown reason, since liquid enzymes have been used frequently in experiments without such an adverse effect being noted.

Fish fed diet 7 showed signs of the lower energy intake as a result of the reduced consumption. Although carcass lipid and energy contents were similar to that of fish fed diets 5 and 6, they showed one of the lowest condition factors and the lowest hepatosomatic index. The negative ANLU and EE indicate that over the experimental period there was little conversion of the dietary energy into body lipid. Protein utilisation was also very low, with a very low PER and ANPU.

The digestibilities of fish fed diet 7 were high, contrary to what might be expected. However, a low intake of food could possibly have been compensated for by an increased digestibility of the small amount of food consumed. If this was the case, it is clear that the amount of food ingested was still too little to allow for much growth once the metabolic requirements were met.

The few experiments that have been carried out on the use of supplementary enzymes in fish diets have generally shown a positive result as compared to fish fed unsupplemented diets. The number of references describing 'unsuccessful' experiments are few.

Although Carter *et al.* (1992a) did not observe a significant effect on either growth or feed utilisation when they fed 13g Atlantic salmon (*S. salar*) a diet containing a commercial amylase preparation, Carter *et al.* (1994) did with 95g fish. These authors fed the fish a diet containing 340 g/kg SBM, 440 g/kg FM to which a cocktail of proteases (trypsin (porcine); alkaline protease (*Bacillus* sp.); acid protease (*Rhizopterus* sp.)) and carbohydrases (amylase (malt), amylase (bacterial) and cellulase) had been added at 1 g/kg. The SGR and FCR of fish fed the enzyme supplemented diet were superior (1.08%/day and 0.76 respectively) to fish fed the unsupplemented SBM diet (0.66%/day and 1.13 respectively) and even better than that of fish fed the control 660 g/kg FM diet (0.89%/day and 0.81 respectively).

No significant effect of addition of 0.5 g/kg pancreatin to a microdiet was observed on the growth of 20 day old sea bass, *D. labrax*, larvae (Kolkovski *et al.* 1997) when compared to fish fed an unsupplemented diet. However the addition of pancreatin increased lipid and protein deposition in the carcass especially in the presence of *Artemia.*

11 day old silverside, *M. beryllina* larvae were fed a basal microbound diet to which a cocktail of corolases and amylases were added at 5 g/kg (Ashraf *et al.* 1993). Growth of fish was similar to the fish fed the unsupplemented diet but survival was improved from 60 to 73%.

Bogut *et al.* (1995) fed 46 g carp, *C. carpio*, a 360 g/kg protein diet to which 0.5, 1.0 and 1.5 g/kg of an enzyme cocktail (Polyzyme) containing amylase, protease, β-glucanase, β-glucosidase and cellulase. The fish fed the 1.5 g/kg Polyzyme diet had significantly better growth and feed utilisation (values not available).

With 38g rainbow trout, *O. mykiss*, Cardenete *et al.* (1993) found no differences in performance between fish fed a basal diet of which 40% of the protein was provided by

cotton seed meal and to which a commercial enzyme cocktail (Kemzyme Dry) was added at 0.4, 1.2 and 3.6 g/kg.

Kolkovski *et al.* (1993) fed 20 to 32 day old gilthead sea bream, *S. aurata*, a microdiet to which 0.5 and 1.0 g/kg of pancreatin were added. Fish fed the two supplemented diets both gave equal growth which was significantly higher than that of fish fed the unsupplemented diet. Survival of the fish fed the enzyme supplemented diet was increased (from 14 to 18%), as was the assimilation rate (by 30%), compared to fish fed the unsupplemented microdiet.

Fernandez-Diaz and Yufera (1995) added an enzyme mixture consisting of papain and lipase (*Candida cilindracea*) at 0.5 g/kg to microcapsules fed to 6 and 12 day old gilthead sea bream, *S. aurata*, larvae. The enzymes were not found to have a significant effect in helping the larvae to break down the microcapsules.

Common carp, *C. carpio*, fry of 0.5 g fed a basal diet to which 1.9 and 5.7 g/kg bovine trypsin had been added showed only slight improvements in food utilisation over fish fed the basal diet, with the fish fed the higher trypsin containing diet giving the best results (Dabrowski and Glogowski (1977). FCR, PER and ANPU improved from 1.74, 1.34 and 19.6% to 1.71, 1.40 and 20.4% respectively.

Finnfeeds International have carried out a number of trials using commercial enzyme cocktails. In one trial carried out by Finnfeeds (1996c) on 4 g Nile tilapia, *O. niloticus*, the addition of 1 g/kg Pescazyme 5602 (which also contained a protease) to a diet containing 630 g/kg SBM, 0 g/kg FM, obtained values of 3.16%/day for SGR and 1.47 for FCR in fish fed this diet which was not only superior to the performance of fish fed the unsupplemented diet (with values of 2.78%/day and 1.84 respectively) but also of the fish fed a 470 g/kg SBM, 120 g/kg FM diet (with values of 2.94%day and 1.70 respectively).

With 5 g common carp, *C. carpio*, fed a basal diet containing 505 g/kg SBM, 0 g/kg FM to which the same enzyme (at 1 g/kg) had been added, the improvement over the unsupplemented diet was also obtained with an improvement in SGR from 2.08 to 2.38%/day and FCR from 1.66 to 1.40, but the performance of these fish was still below that of fish fed a 410g g/kg SBM, 100 g/kg FM control (with an SGR of 2.52%/day and FCR of 1.32)(Finnfeeds, 1996d).

In yet another trial, this time with 4 g mirror carp, *C. carpio*, an enzyme mix of protease and carbohydrase was included at 1 g/kg to the diet (Feord, Pers. Comm., 1996). In this experiment, both the fish fed the 490 g/kg SBM, 50 g/kg FM diet and the supplemented diet performed better than fish fed the 250 g/kg FM, 200 g/kg SBM control diet. However, addition of enzymes still slightly improved the SGR of the fish fed the supplemented diet from 1.58 to 1.61%/day and the FCR from 1.97 to 1.94.

Cain and Garling (1995) fed 2 and 15 g rainbow trout, *O. mykiss*, diets containing 310 g/kg SBM which had or had not been pretreated with phytase. In both sizes of fish used, the weight gain of the fish increased, from 646 to 757% and from 273 to 375% respectively, FCR decreased from 0.93 to 0.86 and from 1.15 to 0.85 respectively. Protein utilisation of the larger fish increased from 27 to 39% when fish were fed the phytase treated SBM diet. Phosphorus discharged in the faeces was decreased in both sizes of fish fed the pretreated SBM diet compared to the fish fed the untreated SBM diet from 2.55 to 1.88 g/kg diet fed and from 1.73 to 0.34 g/kg diet fed respectively.

Feeding phytase supplemented diets (500 to 2000 units/kg) to 6.5 g catfish, *I. punctatus*, increased growth from 51 g/fish for the fish fed the unsupplemented diet to up to 63 g/fish and reduced FCR from 1.99 down to a minimum value of 1.70 (Robinson *et al.*, 1996).

Rodehutscord and Pfeffer (1995) fed 8.7 and 29 g rainbow trout, *O. mykiss*, a diet containing 630 g/kg of soybean products and to which phytase (*Aspergillus niger*, 1000 Units/kg) had been added. Addition of phytase increased the growth of fish compared to the fish fed the unsupplemented diets, of the smaller fish from 12.1 to 18.1 g/fish and the larger fish from 5 to 5.5 g/fish. FCRs were also improved when fish were fed the supplemented diet from 1.41 to 1.25 and from 3.02 to 2.67 in the small and larger fish respectively. Phosphorus utilisation of the fish fed the phytase supplemented diet was increased, from 17 to 49% and from 6 to 25% for the two sizes of fish respectively.

Schaefer *et al.* (1995) fed 40 g common carp, *C. carpio* a 530 g/kg SBM, 150 g/kg FM diet to which two levels of *A. niger* phytase had been added (500 and 1000 units/kg). The average weight gain of the fish fed the unsupplemented diet was 108 g/fish, which increased to 136 and 147 g/fish as the level of phytase was increased. FCRs of the fish decreased from 1.4 to 1.2 to 1.1 and ANPU increased from 18.2 to 23.2 to 24.8% as phytase level in the diet increased. The phosphorus utilisation increased from 30% in the fish fed the unsupplemented diet to 42 and 47% when fish were fed the two supplemented diets respectively.

The work done on crustaceans involving supplementary enzymes has been even less than that carried out on fish. Buchanan *et al.* (1997) working with 1 g *P. monodon* improved growth, FCR and PER by adding 2.5 g/kg Porzyme (Finnfeeds) to 200 and 640 g/kg canola meal (460, 180 g/kg squid meal respectively) containing diets. These increases in growth were from 2.29 to 2.40 g per prawn and from 1.79 to 2.29 g for each of the canola levels respectively. FCRs decreased from 2.49 to 1.98 and 3.06 to 2.31 respectively, and the PERs increased from 0.84 to 1.09 and from 0.80 to 1.02 respectively. Some of these results were even better than for prawn fed a diet

containing 590 g/kg squid meal (0 g/kg canola meal) which gave a growth of 2.33 g per prawn, an FCR of 2.28 and a PER of 0.87.

Maugle *et al.* (1983) fed 0.6 g *P. japonicus* a basal diet to which various combinations of bovine α–amylase and trypsin were added. Growth was increased in all shrimps fed supplemented diets and so was FCR except in one case, from values of 4.7%/day and 4.9 respectively for the shrimp fed the unsupplemented diets, to up to 7.7%/day and 3.7 for the shrimp fed the supplemented diets. In another trial with 0.6 g *P. japonicus* Maugle *et al.* (1983b) fed diets containing three combinations of casein and starch to which either 30 IU of 60 IU of α–amylase had been added. Enzyme addition at both levels increased growth performance and feed efficiency of those shrimp fed these diets. These authors also found that amylase supplementation increased the digestibility of carbohydrates by the shrimp fed the supplemented diet from 90 to 96%.

Most of these trials reported above have been carried out on fry or on fingerlings of even smaller size than that used in this experiment. Nonetheless, the evidence in this experiment, has shown that larger fish can also show a positive response to the use of supplemental enzymes.

This positive effect not only improved performance of fish over that of fish fed unsupplemented diets but can also improve performance over fish fed diets containing higher FM inclusion levels. However, the results obtained with fish fed 440 g/kg SBM diets indicate that more work is necessary to find the enzyme levels required to bring about the same level of performance seen with fish fed enzyme-supplemented 320 g/kg SBM diets.

CHAPTER 4

EXPERIMENT 2

Determination of the effect of using low pH protease, high pH protease and α-galactosidase alone on the growth and feed utilisation of gilthead sea bream, *Sparus aurata* L

4.1 INTRODUCTION AND AIM OF THIS EXPERIMENT

Numerous authors have found that using enzyme cocktails, rather than individual purified enzymes, resulted in higher improvements in performance than when individual enzymes were used, even if one particular enzyme formed the major acting component (Clifford, 1989; GrootWassink *et al.*, 1989; Inborr, 1989, 1990; Graham and Inborr, 1993a). Improvements in performance in the investigations carried out with aquaculture species have been obtained both with the use of single enzymes and with mixes of two or more enzymes (see Section 1.5.5).

However, more often than not the actual contributions of the individual enzymes in the cocktail remain unknown, and further research is required before the actual activities and mechanisms whereby each enzyme acts are elucidated. This information could lead to a better understanding of the factors affecting the digestion of certain ingredients and processes taking place in the digestive tract. Thus the determination of the effect of xylanases and β-glucanases on non-starch polysaccharides led to the understanding of how viscosity affects digestion in poultry and pigs (see Section 1.5.2). This information could then be used to develop new products or modes of application.

Following the successful use of the two enzyme cocktails in improving performance of gilthead sea bream fed the 320 g/kg SBM diets in Experiment 1, the aim of this experiment was to determine the individual contributions of the low pH protease, the high pH protease and the α-galactosidase to these results.

4.2 MATERIALS AND METHODS

Other details pertaining to experimental tanks, experimental fish and handling, diet production, water quality, laboratory analysis, calculations and statistical analysis are as described in Chapter 2.

4.2.1 EXPERIMENTAL FISH

40 fish of 70 g average weight were used in each of the tanks in this experiment. Each treatment had three replicates. The experiment was abandoned after 10 weeks as a result of the poor feeding behaviour and the inconsistent performance of the fish.

4.2.2 THE DIETS AND FEEDING REGIME

The formulations of the diets used in this experiment are given in Table 4.1. The nutritional compositions of the soybean meal and fish meal used are given in Table 4.2. The percentage inclusion levels of the supplementary enzymes added to the diets and the nutritional compositions of the diets themselves (2 mm pellets) are given in Table 4.3.

After the abortion of the experiment, the Ewos Technology Centre in Scotland carried out analysis on the diets for peroxides and a number of biogenic amines.

For the first week of acclimation the fish were fed the food they had been receiving on the farm of origin. The fish were then fed their particular diet according to the tank. The acclimation period had to be extended a further two weeks above the normal period of acclimation due to poor feeding behaviour. During the acclimation period and the experiment, the following regime was used: 61 to 80 g fish fed at 1.6 % body weight/day; 81 to 100 g fish fed at 1.5 % body weight/day, 101 to 200 g fish fed at 1.4% body weight/day.

Table 4.1 Formulations of diets used in Experiment 2.

Diets	1	2 to 7
Ingredient	Inclusion (g/kg)	
Fish meal[1]	317	260
Dehulled hexane extracted soybean meal[2]	220	320
Blood meal[3]	50	80
Corn[4]	88	50
Feather meal[5]	100	60
Fish oil[6]	64	88
Limestone	30	30
Molasses[7]	40	40
Vitamins and minerals[8]	11	17
Whole wheat[9]	75	50
Chromic oxide	5	5

1. Source: Spain.
2. Source: Spain.
3. Source: Daka Ltd., Canada.
4. Source: Suprex Ltd., Scotland.
5. Source: Canada.
6. Source: UFP Ltd., Scotland.
7. Source: Spain.
8. Ewos Premix prepared by Roche Products Ltd., England.
9. Source: Scotland.

Table 4.2 Nutritional compositions of the soybean meal and fish meal used in the formulation of the diets in Experiment 2.

	Soybean meal	Fish meal
Moisture (g/kg)	116	88
Crude protein (g/kg)	470	645
Crude lipid (g/kg)	13	88
Ash (g/kg)	56	159
Crude fibre (g/kg)	24	2
Crude carbohydrate (g/kg)	322	18
Phosphorus (g/kg)	6	19
Protein solubility (%)	78.14	

Table 4.3 Inclusion levels of enzymes and nutritional compositions of the diets used during Experiment 2.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed
Fish meal inclusion (g/kg)	320	260	260	260	260	260	260
Soybean meal inclusion (g/kg)	220	320	320	320	320	320	320
Low pH protease (g/kg)	0	0	0	1	0	0	1
High pH protease (g/kg)	0	0	1	0	0	1	0
α-galactosidase (g/kg)	0	0	0	0	1	1	1
Enzyme form	Dry	Dry	Dry	Dry	Dry	Dry	Dry
Moisture (g/kg)	72	76	78	81	83	84	80
Crude protein (g/kg)	487	469	455	455	468	463	467
Crude lipid (g/kg)	123	130	142	127	129	127	120
Ash (g/kg)	111	116	111	120	100	106	105
Crude fibre (g/kg)	15	18	18	18	19	23	19
Crude carbohydrate (g/kg)[1]	193	192	196	199	201	197	209
Phosphorus (g/kg)	10	11	10	11	11	11	10
Gross energy (kJ/g)[2]	19.61	19.43	19.67	19.13	19.54	19.27	19.32
Protein/gross energy ratio (g/MJ)	24.83	24.15	23.12	23.77	23.95	24.02	24.21

1 Calculated as 100% - (%moisture + %cr. protein + %cr. Lipid + %ash + %cr. fibre).
2 Energy calculated using the following values: protein, 23.4 kJ/g; lipid, 39.8 kJ/g; carbohydrate, 17.2 kJ/g.

After the ten weeks of the experiment, the two tanks which had given the worst results until that time in the experiment were given an extruded commercial diet (BOCM Pauls Keystart Fingerling 2.5 mm (16 g/100 g lipid, 50 g/100 g protein) for 1 week, after which the fish in these two tanks were weighed.

4.3 RESULTS

4.3.1 ASSESSMENT OF GROWTH AND FEED PERFORMANCE

(Table 4.4, Figures 4.1 to 4.3)

Feeding fish the 320 g/kg SBM diet with supplemental low pH protease (diet 4) gave a significant improvement in SGR and PER over feeding fish the control 220 g/kg SBM diet 1, the unsupplemented 320 g/kg soybean meal diet 2 and all the other diets. Daily food intake was not significantly different between treatments. Fish fed diet 4 gave the lowest FCR, which was, together with diets 1,2, 3, 5 and 6 significantly better than that of fish fed diet 7.

In this experiment feeding the fish a 320 g/kg SBM diet (diet 2) without enzyme supplementation improved the performance in terms of SGR, FCR and PER compared to fish fed the 220 g/kg SBM diet 1. However, apart from the fish fed diet 4 which had low pH protease only, feeding diets with any supplementation with enzymes reduced fish performance, and in some cases significantly so (SGR, diets 5, 6 and 7; FCR, diet 7; FE, diets 5 to 7; PER, diets 5 to 7) compared to fish fed diet 2.

Feeding fish diet 5, with a supplementation of α-galactosidase only, reduced performance compared to the addition of the proteases (diets 3 and 4), and its

Table 4.4 Assessment of growth and feed performance in Experiment 2. Data are presented as means with the standard deviation in brackets. Means in a row followed by the same superscript are not significantly different ($P < 0.05$).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed
Fish meal inclusion (g/kg)	320	260	260	260	260	260	260
Soybean meal inclusion (g/kg)	220	320	320	320	320	320	320
Low pH protease (g/kg)	0	0	0	1	0	0	1
High pH protease (g/kg)	0	0	1	0	0	1	0
α-galactosidase (g/kg)	0	0	0	0	1	1	1
Initial weight (g)	71.52^{a} (1.26)	71.99^{a} 0.92)	71.42^{a} (1.31)	71.69^{a} (1.42)	71.75^{a} (0.67)	71.93^{a} (1.50)	71.13^{a} (0.98)
Final weight (g)	89.75^{cd} (3.64)	93.89^{d} (2.25)	89.89^{cd} (0.61)	101.89^{e} (3.37)	85.67^{bc} (2.15)	84.25^{b} (4.43)	76.60^{a} (1.78)
Specific growth rate (SGR)(%/day)	0.32^{cd} (0.07)	0.38^{d} (0.05)	0.33^{cd} (0.04)	0.50^{e} (0.03)	0.25^{bc} (0.05)	0.21^{b} (0.05)	0.11^{a} (0.05)
% Mortalities	3.33^{a} (5.77)	1.68^{a} (1.44)	0.83^{a} (1.44)	1.67^{a} (1.44)	2.50^{a} (2.50)	3.33^{a} (2.89)	2.50^{a} (4.33)
Food intake (g/100g fish/day)	1.51^{a} (0.05)	1.47^{a} (0.01)	1.51^{a} (0.02)	1.48^{a} (0.02)	1.52^{a} (0.03)	1.50^{a} (0.10)	1.53^{a} (0.12)
Food conversion ratio (FCR)	4.21^{a} (1.06)	3.50^{a} (0.48)	4.00^{a} (0.46)	2.59^{a} (0.20)	5.34^{a} (1.00)	6.89^{a} (1.61)	13.65^{b} (5.39)
Protein efficiency ratio (PER)	0.51^{cd} (0.13)	0.61^{d} (0.09)	0.55^{cd} (0.06)	0.85^{e} (0.08)	0.41^{bc} (0.06)	0.33^{b} (0.07)	0.18^{a} (0.07)

Figure 4.1 Assessment of growth and feed performance in Experiment 2: specific growth rate. Bars indicate one standard deviation.

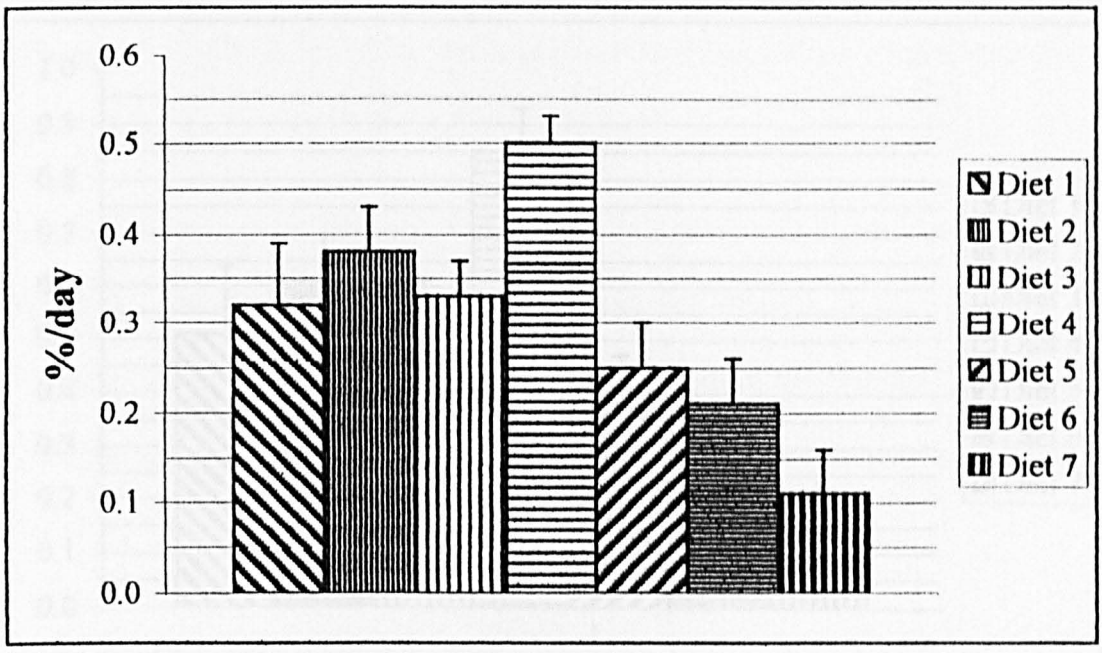


Figure 4.2 Assessment of growth and feed performance in Experiment 2: food conversion ratio. Bars indicate one standard deviation.

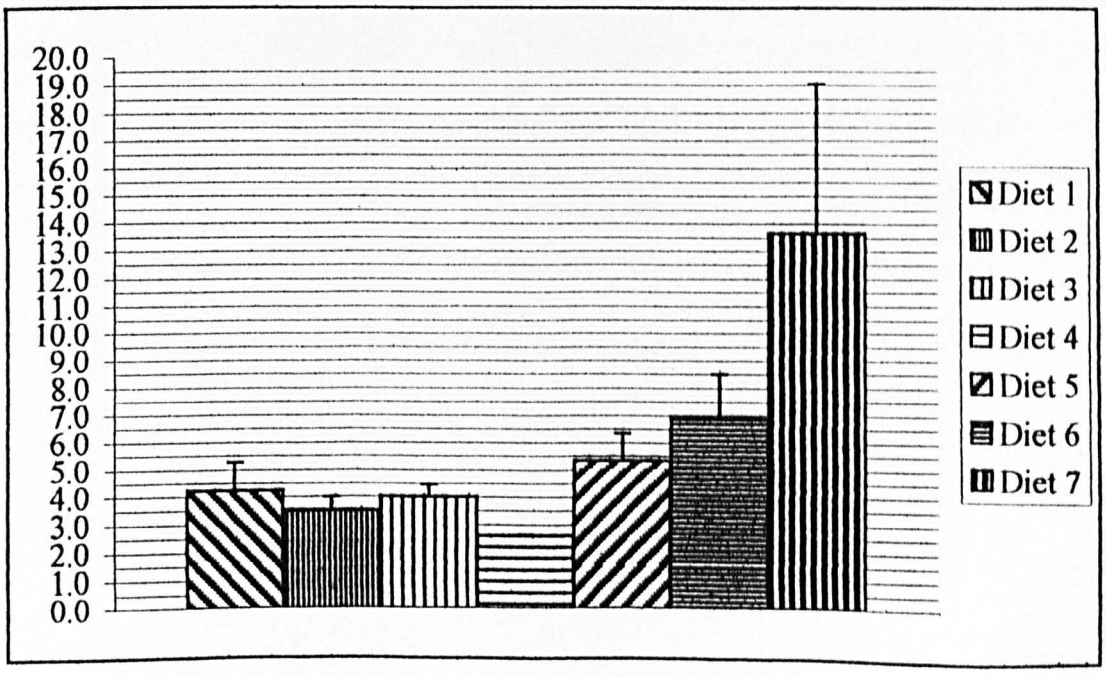
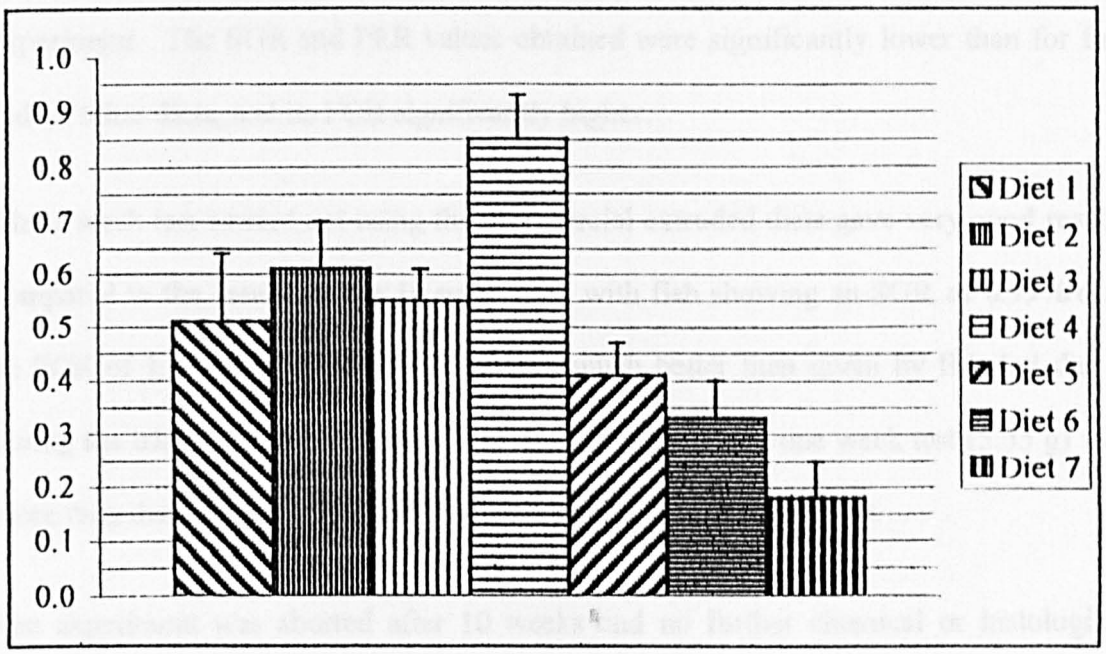


Figure 4.3 Assessment of growth and feed performance in Experiment 2: protein efficiency ratio. Bars indicate one standard deviation.



combination with the proteases (diets 6 and 7) further reduced growth and feed utilisation, significantly so in SGR and PER.

Feeding diet 7 resulted in the worst fish performance of all the diets tested in this experiment. The SGR and PER values obtained were significantly lower than for fish fed all other diets, and its FCR significantly higher.

The 1 week test carried out using the commercial extruded diets gave very good results compared to the results of the 10 week trial, with fish showing an SGR of 0.99%/day, an FCR of 1.33 and a PER of 1.47, values much better than given by fish fed diet 4 during the trial. The average growth of the fish used in this one week test (5.55 g) was more than that shown by fish fed diet 7 during the whole experiment.

The experiment was aborted after 10 weeks and no further chemical or histological analysis were conducted with the fish.

The Ewos Technology Centre analysed the experimental feeds and the commercial extruded feed used in this experiment for peroxides and the biogenic amines cadaverine, histamine, putrescine and tyramine, the results of which are shown in Table 4.5. Of the diets analysed, the commercial extruded diet contained the greatest amount of biogenic amines. This diet had the greatest amount of cadaverine and tyramine and one of the highest putrescine contents but the lowest histamine content. On the other hand, diets 2 to 6 had similar but lower amine contents which were comparatively lower than those in either diet 1 or 7 which had the highest contents of putrescine and histamine respectively.

Table 4.5 Peroxide and biogenic amine contents of experimental diets and the commercial extruded diet used during Experiment 2.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Commercial diet
Pellet type	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed	Pressed	Extruded
Fish meal inclusion (g/kg)	320	260	260	260	260	260	260	
Soybean meal inclusion (g/kg)	220	320	320	320	320	320	320	
Low pH protease (g/kg)	0	0	0	1	0	0	1	
High pH protease (g/kg)	0	0	1	0	0	1	0	
α-galactosidase (g/kg)	0	0	0	0	1	1	1	
Peroxide (g/kg)	nf[1]	nf	nf	nf	nf	nf	nf	nf
Cadaverine (g/kg)	0.36	0.26	0.23	0.23	0.24	0.24	0.31	0.59
Histamine (g/kg)	0.35	0.32	0.32	0.35	0.33	0.32	0.37	0.13
Putrescine (g/kg)	0.23	0.13	0.12	0.12	0.12	0.12	0.23	0.20
Tyramine (g/kg)	0.20	0.10	0.10	0.10	0.11	0.11	0.21	0.30
Total amines (g/kg)	1.14	0.81	0.77	0.80	0.80	0.79	1.12	1.22

1 not found.

4.4 DISCUSSION

The aim of this experiment was to determine the effect of each of the individual enzymes and the extent to which each of the three enzymes used in Experiment 1 was contributing to the observed performance seen in fish fed diets 3 and 4 compared to fish which had been fed diets 1 and 2. This experiment included four diets used in Experiment 1, namely diets 1, 2, 6 and 7, and the singly supplemented diets 3, 4 and 5 with high pH protease, low pH protease and a-galactosidase respectively. The experiment was aborted after 10 weeks and no further analysis carried out.

The fish were brought from one of the local farms and given the same food which they had been consuming on the farm, with no problems in feeding, while the fish acclimated to the new environment. When this phase of the acclimation was stopped, the experimental diets were used. It was at this point that the problems started with fish not taking up the food as vigorously as they did the original food. While a period of acclimation to the new food is expected, previous experience had shown that food uptake should resume the normal behaviour in a relatively short period. This was not the case here, and the acclimation period had to be extended for a further two weeks. The experiment was then commenced with feeding being carried out over a long period of time so that the fish could consume the offered pellets before some more pellets were given. The fish allowed the pellets to fall to the bottom of the tank before they attempted to consume them. Fish fed diet 4 consumed the food at the fastest rate of all the fish fed the various diets, although still slowly, with diets 6 and 7 being consumed at the slowest rate of all.

The feed had arrived in Malta in two batches, one by plane, and the other batch by ship. The former batch was used first, with the second batch being kept in cold store in the meantime. When the second batch was used (after week 8), there was still no overall

effect on feeding behaviour. Hence, the two batches of food elicited the same response (or rather, lack of it) from the normally vigorous sea bream.

The fish taken from the farm were all from the same batch of fish. On the farm from which the fish had been obtained the same batch of fish as the fish used in this experiment had reached a size of 135g a couple of weeks before the experiment was aborted. During week 9 of the experiment a number of fish from the slowest growing two tanks of diet 7 were checked for pathological problems. No such problems were detected, and the pathologist at the NAC could not offer any explanation after examining the healthy fish. Fish mortalities were low throughout the whole experiment, with no apparent pattern in the mortalities that were recorded.

The water quality of the tanks was the same as it had been in Experiment 1. The possibility that the tanks themselves had an effect on performance was eliminated since these same tanks had been used in Experiment 1 and were used in subsequent trials and additionally, or rather ironically, the tanks occupied by fish fed diet 7 were actually occupied by fish fed the best performing diets 3 and 4 in Experiment 1.

Therefore, during week 10 two of the worst performing tanks, one from diet 7 and one from diet 6 were fed a commercial extruded diet. These fish were not given an acclimation period, nor apparently was one required, since the fish immediately attacked the food in a normal feeding behaviour for the sea bream, and the results of this test speaks for itself.

Since the fish were healthy and the water quality was within the normal parameters of previous and subsequent experiments, the one week test carried out and the complete lack of enthusiasm in feeding of the experimental diets, it was concluded that the problem lay with the diets themselves. What and where things did not go right is not clear or obvious. Was something 'wrong' with the ingredients, the manufacture itself

or the transport? The same ingredients used in the first experiment were used in Experiment 2 and had been used in other experiments (Lopez, Pers. Comm., 1997) and the same manufacturing process and machine were used. Storage in the NAC was as it had been in Experiment 1 and was for subsequent trials. Analysis of the nutritional compositions of the diets does not give any indication of differences in the diets, nor would any be expected. The only other difference in the diet preparations themselves was the use or not of enzymes. Had the enzyme preparations used had any effect, the diets 2 to 7 would have been affected the same, and at least fish fed these diets should have given the same results. No such effect was seen in the same diets used in Experiment 1.

According to the results obtained before the experiment was aborted, fish fed diet 4, containing only low pH protease, gave a significantly higher SGR and PER, and a lower FCR to fish fed any of the other diets. This was followed in performance by fish fed the unsupplemented 320 g/kg SBM diet 2. The most inferior performance was given by fish fed diet 7 and then fish fed diet 6, both these diets containing the successful enzyme mixes used in Experiment 1.

According to the results of the first experiment fish fed diet 7 would have been expected to give better results than fish fed diet 6, which in turn should have given better results that fish fed diet 1, the 220 g/kg SBM diet. In actual fact, fish fed diets 6 and 7 in this experiment gave the lowest performance.

From the analysis carried out by the Ewos Technology Centre no peroxides were present in any of the feeds and no rancid off-smells had been detected in the food sacks. The commercial extruded diet contained the highest amount of tyramine, and cadaverine of all the diets, one of the highest putrescine contents but the lowest

histamine level. Diets 2 to 6 had similar biogenic amine contents which were comparatively lower than the amine contents of diets 1 and 7.

The levels of biogenic amines found in the diets used are below the levels which have been found by a number of authors to cause a loss in performance. Pike (1993) obtained lower performance and feed utilisation when he fed Atlantic salmon, *S. salar*, diets containing 2.1 and 3.9 g/kg total amines compared to fish fed a diet containing only 0.4 g/kg amines (of which 0.3 g/kg were cadaverine). This author observed a 10% reduction in feed consumption when the fish were fed the diet containing the highest amine content compared to the fish fed the diet containing only 0.4 g/kg.

Aksnes and Mundheim (1997) obtained reduced growth and feed utilisation in Atlantic halibut, *Hippoglossus hippoglossus*, fed two diets containing 0.67 and 0.97 g/kg dry matter of cadaverine, 0.49 and 0.69 g/kg dry matter histamine and 0.30 and 0.43 g/kg dry matter respectively, compared to fish fed a diet containing 0.34, 0.25 and 0.15 g/kg dry matter of these three amines respectively. Feed consumption was not affected by the diet used.

Dietary levels of putrescine up to 4 g/kg have not been found to influence the growth of rainbow trout by Cowey and Cho (1992), while Fairgrieve *et al.* (1994) found that diets supplemented with 2.0, 0.5 and 0.5 g/kg dry matter of histamine, putrescine and cadaverine respectively had no effect on rainbow trout, *O. mykiss*, performance and feed consumption compared to fish fed the unsupplemented diet. In a trial carried out on Atlantic salmon, *S. salar*, fed diets containing 0 to 1.5g/kg histamine performance was found to be similar (Lopez, Pers. Comm., 1997).

If time had permitted, it would have been fruitful to attempt to determine to a better extent the cause of the reduced palatability of the food to the fish. Among the analysis that could have been carried out could have been to determine if the SBM used was not

overprocessed using the Cresol Red Dye-binding method (Olomucki and Bornstein, 1960) and the Coomassie Blue method (Bradford, 1976) and to determine the contents in the diets of phenols and tannins. The protein solubility of the SBM used in this trial was well within the recommended range given by Araba and Dale (1990). Fish meal quality could be further analysed be determining lysine availability (Booth, 1971), the quantity of S-H and S-S bonds (Opstvedt *et al.*, 1984), total volatile basic nitrogen (Woyewoda *et al.*, 1986) and pepsin digestible protein (Olley and Pirie, 1966). Histological analysis of the stomach and intestine could also have been carried out to see if there were any abnormal changes in structure.

CHAPTER 5

EXPERIMENT 3

Determination of the effect of using low pH protease, high pH protease and α-galactosidase alone and lower cocktail enzyme inclusion levels on the growth and feed utilisation of gilthead sea bream, *Sparus aurata* L.

5.1 INTRODUCTION AND AIMS OF THIS EXPERIMENT

Since Experiment 2 had to be aborted, Experiment 3 maintained the aim of determining the individual contributions of the low pH protease, high pH protease and α-galactosidase to the improved performance of gilthead sea bream, *Sparus aurata*, fed the 320 g/kg soybean meal diets in Experiment 1.

Apart from the observation that enzyme cocktails in animals feeds have been found to give a higher improvement than individual enzymes, it has also been noted that varying the enzyme inclusion levels can have an important influence on the performance of the animals being fed. The results obtained from such investigations have been quite varied. Some authors have found that as the inclusion level was increased so to was the improvement in performance obtained (Hesseman *et al.*, 1982; Maugle *et al.*, 1983a, b; Adams, 1989; Schaefer *et al.*, 1995). Other authors found that beyond a certain inclusion level no further improvement in performance was seen (Collier and Hardy, 1986b; Bedford and Classen, 1992a; Kolkovski *et al.*, 1993). A number of authors have observed that as the inclusion level was increased the improvement in performance actually decreased (Collier and Hardy, 1986b; Bedford and Classen, 1992a) and sometimes even increased again (Bedford and Classen, 1992a; Robinson *et al.*, 1996). The reasons behind some of the observed results in dose-response trials are still unknown. A better knowledge of the activities of the enzymes would contribute to understanding of these results.

To investigate the effect of using a lower dose of each of the enzymes on the growth and feed utilisation of the sea bream, 0.5 g/kg instead of 1 g/kg (used in Experiment 1) of each enzyme in the cocktails were used.

5.2 MATERIALS AND METHODS

Other details pertaining to experimental tanks, experimental fish and handling, diet production, water quality, faecal collection, laboratory analysis, calculations and statistical analysis are as described in Chapter 2.

5.2.1 EXPERIMENTAL FISH

35 fish of 90 g average weight were used in each of the tanks in this experiment. Each treatment had three replicates. The duration of the experiment, apart from the acclimation period, was 14 weeks.

5.2.2 THE DIETS AND FEEDING REGIME

The formulation of the diets used in this experiment is given in Table 5.1. The nutritional compositions and amino acid contents of the soybean meal and fish meal used are given in Table 5.2. The percentage inclusion levels of the supplementary enzymes added to the diets and the nutritional compositions of the diets themselves (4.2 mm pellets) are given in Table 5.3. Table 5.4 gives the amino acid composition of the diets.

During the acclimation period the fish were fed diet 1. During the acclimation period and the experiment, the following regime was used: 81 to 120 g fish fed at 1.6 % body weight/day; 121 to 140 g fish fed at 1.5 % body weight/day; 141 to 160 g fish fed at 1.4% body weight/day, 161 to 180 g fish fed at 1.3% body weight/day.

Table 5.1 Formulation of diets used in Experiment 3.

Diets	All
Ingredient	Inclusion (g/kg)
Fish meal[1]	260
Dehulled hexane extracted soybean meal[2]	320
Blood meal[3]	80
Corn[4]	50
Feather meal[5]	60
Fish oil[6]	88
Limestone	30
Molasses[7]	40
Vitamins and minerals[8]	17
Whole wheat[9]	50
Chromic oxide	5

1. Austral SuperPrime (<500 mg/kg histamine), Source: South America.
2. HiPro soya, Source: Grosvenor Ltd., Scotland.
3. Source: Daka Ltd., Canada.
4. Source: Suprex Ltd., Scotland.
5. Source: Canada.
6. Source: UFP Ltd., Scotland.
7. Source: Spain.
8. Ewos Premix prepared by Roche Products Ltd., England.
9. Source: Scotland.

Table 5.2 Nutritional compositions of the soybean meal and fish meal used in the formulation of the diets in Experiment 3.

	Soybean meal	Fish meal
Moisture (g/kg)	122	83
Crude protein (g/kg)	440	670
Crude lipid (g/kg)	18	75
Ash (g/kg)	69	147
Crude fibre (g/kg)	34	7
Crude carbohydrate (g/kg)	310	17
Phosphorus (g/kg)	7	24
Protein solubility (%)	76.20	
Trypsin inhibitor activity (mg/g)	2.38	
Amino acid[1]	(g/100 g protein)	
Alanine	3.78	5.44
Arginine[2]	6.02	4.86
Aspartic acid	9.64	7.94
Cystine	0.69	0.86
Glutamic acid	15.62	10.69
Glycine	3.88	5.35
Histidine[2]	2.34	2.78
Isoleucine[2]	4.16	4.03
Leucine[2]	6.36	6.42
Lysine[2]	5.35	6.83
Methionine[2]	0.67	1.54
Phenylalanine[2]	4.37	3.50
Proline	4.66	4.69
Serine	3.33	2.78
Tyrosine	2.49	2.61
Threonine[2]	3.10	3.27
Valine[2]	4.80	3.49

1. No data is available for the essential amino acid tryptophan because it is destroyed during acid hydrolysis.
2. Essential amino acid

Table 5.3 Inclusion levels of enzymes and nutritional compositions of the diets used during Experiment 3.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type	Pressed	Extruded	Pressed	Pressed	Pressed	Pressed	Pressed
Fish meal inclusion (g/kg)	260	260	260	260	260	260	260
Soybean meal inclusion (g/kg)	320	320	320	320	320	320	320
Low pH protease (g/kg)	0	0.5	0	1.0	0	0	0.5
High pH protease (g/kg)	0	0	1.0	0	0	0.5	0
α-galactosidase (g/kg)	0	0.5	0	0	1.0	0.5	0.5
Enzyme form	Dry	Liquid	Dry	Dry	Dry	Dry	Dry
Moisture (g/kg)	87	77	82	86	80	84	86
Crude protein (g/kg)	465	465	470	462	464	464	460
Crude lipid (g/kg)	119	113	117	118	116	117	117
Ash (g/kg)	112	110	111	112	113	111	111
Crude fibre (g/kg)	21	19	23	18	21	17	18
Crude carbohydrate (g/kg)	193	211	200	201	206	205	205
Phosphorus (g/kg)	10	10	10	10	10	10	10
Trypsin inhibitor activity (mg/g)	0.57	0.55	0.76	0.49	0.66	0.62	0.63
Chromic oxide (g/kg)	4	4	5	5	5	5	5
Energy content (kJ/g)	21.29	21.14	21.19	21.08	21.02	21.10	20.85
Protein/gross energy ratio (g/MJ)	21.86	22.01	22.18	21.90	22.07	21.99	22.04

Table 5.4 Amino acid contents of gilthead sea bream[1] carcass and the diets used in Experiment 3[2].

	Carcass	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Fish meal inclusion (g/kg)		260	260	260	260	260	260	260
Soybean meal inclusion (g/kg)		320	320	320	320	320	320	320
Low pH protease (g/kg)		0	0.5	0	1.0	0	0	0.5
High pH protease (g/kg)		0	0	1.0	0	0	0.5	0
α-galactosidase (g/kg)		0	0.5	0	0	1.0	0.5	0.5
Enzyme form		Dry	Liquid	Dry	Dry	Dry	Dry	Dry
	(g/100 g protein)							
Alanine	5.35	5.22	5.54	5.34	4.95	5.13	4.91	5.35
Arginine[3]	5.10	5.11	5.81	5.31	4.78	5.32	4.94	5.33
Aspartic acid	8.01	8.80	9.82	9.32	9.12	9.21	8.60	9.40
Cystine	1.08	1.14	1.12	1.01	0.84	0.90	0.94	0.88
Glutamic acid	10.38	12.03	12.95	12.01	11.81	12.16	12.00	12.54
Glycine	6.12	5.03	5.26	5.07	4.49	4.88	4.64	5.05
Histidine[3]	1.83	3.04	2.99	3.03	3.12	3.13	2.79	3.04
Isoleucine[3]	3.62	3.80	4.23	3.85	3.42	3.67	3.74	4.06
Leucine[3]	5.83	7.77	7.80	7.71	6.87	7.45	7.00	7.47
Lysine[3]	6.01	6.01	6.27	6.29	5.49	6.05	5.86	6.39
Methionine[3]	2.56	1.28	1.33	1.12	1.15	1.31	1.20	1.46
Phenylalanine[3]	3.26	4.64	4.57	4.79	4.34	4.37	4.13	4.62
Proline	4.13	4.61	4.27	4.75	3.92	5.27	4.44	4.92
Serine	3.53	3.69	3.77	3.83	3.38	3.63	3.47	3.75
Tyrosine	3.17	2.52	2.63	2.32	2.20	2.26	2.35	2.56
Threonine[3]	3.91	3.49	3.47	3.37	3.07	3.33	3.09	3.41
Valine[3]	4.39	5.79	6.05	5.71	5.61	5.69	5.60	5.80

1. Average weight 63.28 g.
2. No data is available for the essential amino acid tryptophan because it is destroyed during acid hydrolysis.
3. Essential amino acid.

5.3 RESULTS

5.3.1 ASSESSMENT OF GROWTH AND FEED PERFORMANCE

(Table 5.5, Figures 5.1 to 5.6)

The addition of individual enzymes or combinations of enzymes to diets did not bring about any significant changes in fish SGR, FCR and PER compared to fish fed the unsupplemented diet 1. Fish fed the diet with low pH protease (diet 4) gave superior results in all the parameters compared to fish fed the unsupplemented diet 1, although these improvements were not significant. Feeding the diet with α-galactosidase alone (diet 5) gave the worst fish performance for these parameters, although adding it to high pH protease (in diet 6) had a positive effect on fish performance over feeding the high pH protease diet alone (diet 3). Of all the diets tested, the superior performance for the above parameters was given by feeding fish the extruded diet 2 containing both low pH protease and α-galactosidase at 0.5 g/kg each although this was not significant in the case of SGR, FCR or PER. Feed consumption per day of fish fed the various diets was not different.

The ANPU of fish fed diet 5, containing α-galactosidase alone, was the lowest and was significantly lower than that of fish fed diets 2, 4 and 7. The highest ANPU was obtained by feeding the extruded diet, and feeding the low pH protease diet 4 and diet 7 containing both low pH protease and α-galactosidase improved results over fish fed the unsupplemented diet 1 and over the ANPU of fish fed the high pH protease containing diets 3 and 6.

Table 5.5 Assessment of growth and feed performance in Experiment 3. Data are presented as means with the standard deviation in brackets. Means in a row followed by the same superscript are not significantly different ($P < 0.05$).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type	Pressed	Extruded	Pressed	Pressed	Pressed	Pressed	Pressed
Fish meal inclusion (g/kg)	260	260	260	260	260	260	260
Soybean meal inclusion (g/kg)	320	320	320	320	320	320	320
Low pH protease (g/kg)	0	0.5	0	1.0	0	0	0.5
High pH protease (g/kg)	0	0	1.0	0	0	0.5	0
α-galactosidase (g/kg)	0	0.5	0	0	1.0	0.5	0.5
Initial weight (g)	89.20^{a} (1.90)	92.39^{a} (0.97)	90.26^{a} (1.82)	89.72^{a} (1.04)	91.21^{a} (0.96)	91.24^{a} (2.03)	89.32^{a} (2.38)
Final weight (g)	170.90^{a} (4.69)	183.67^{a} (7.48)	170.28^{a} (8.68)	175.61^{a} (2.71)	168.23^{a} (8.14)	174.69^{a} (5.52)	171.91^{a} (0.38)
Specific growth rate (SGR)(%/day)	0.66^{a} (0.02)	0.70^{a} (0.04)	0.65^{a} (0.04)	0.68^{a} (0.01)	0.60^{a} (0.09)	0.66^{a} (0.04)	0.67^{a} (0.03)
% Mortalities	0.95^{a} (1.65)	0.00^{a} (0)	0.95^{a} (1.65)	0.95^{a} (1.65)	2.33^{a} (4.04)	0.95^{a} (1.65)	0.95^{a} (1.65)
Food intake (g/100g fish/day)	1.39^{a} (0.05)	1.33^{a} (0.07)	1.39^{a} (0.05)	1.36^{a} (0.03)	1.38^{a} (0.04)	1.37^{a} (0.04)	1.39^{a} (0.02)
Food conversion ratio (FCR)	1.83^{a} (0.09)	1.72^{a} (0.09)	1.90^{a} (0.11)	1.78^{a} (0.04)	1.97^{a} (0.20)	1.86^{a} (0.15)	1.84^{a} (0.07)
Protein efficiency ratio (PER)	1.18^{a} (0.06)	1.25^{a} (0.07)	1.12^{a} (0.07)	1.19^{a} (0.06)	1.10^{a} (0.11)	1.16^{a} (0.09)	1.19^{a} (0.04)
Apparent net protein utilisation (ANPU)(%)	22.91^{ab} (1.16)	25.63^{b} (1.12)	22.90^{ab} (1.05)	25.42^{b} (1.28)	21.99^{a} (1.82)	22.38^{ab} (1.39)	25.22^{b} (0.72)
Apparent net lipid utilisation (ANLU)(%)	64.58^{c} (3.51)	56.26^{b} (3.73)	44.43^{a} (3.74)	68.24^{c} (1.32)	40.43^{a} (5.61)	54.82^{b} (4.86)	57.99^{b} (2.24)
Energy efficiency (EE)(%)	29.26^{bc} (1.57)	27.82^{bc} (1.50)	24.10^{a} (1.52)	31.42^{c} (0.54)	22.34^{a} (2.38)	26.09^{b} (2.00)	28.54^{bc} (0.96)

Figure 5.1 Assessment of growth and feed performance in Experiment 3: specific growth rate. Bars indicate one standard deviation.

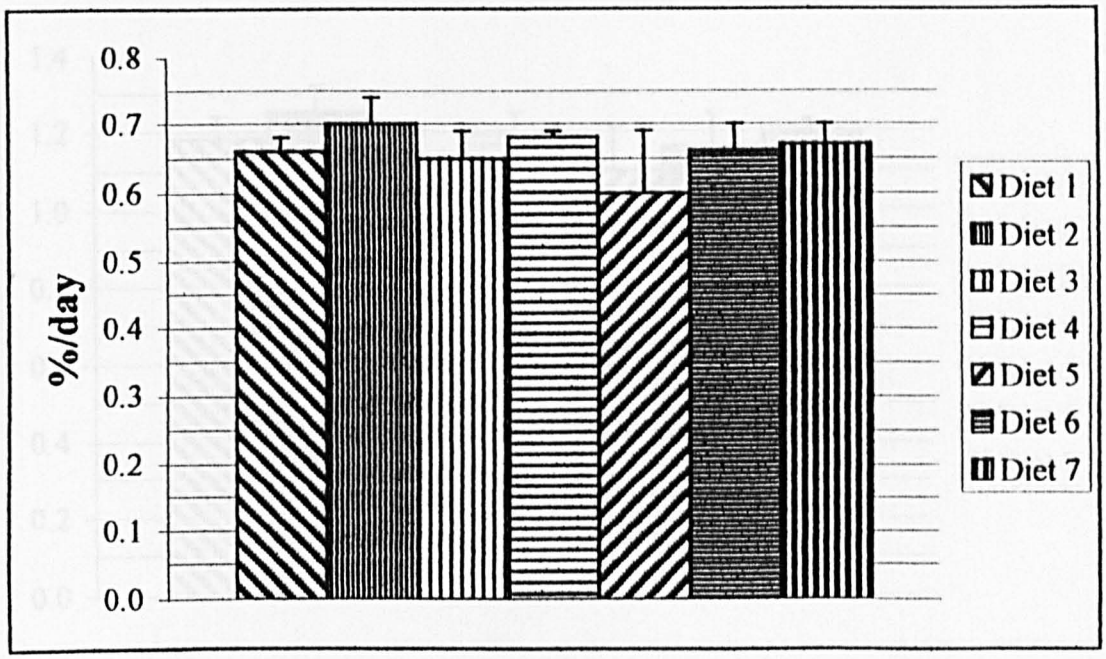


Figure 5.2 Assessment of growth and feed performance in Experiment 3: food conversion ratio. Bars indicate one standard deviation.

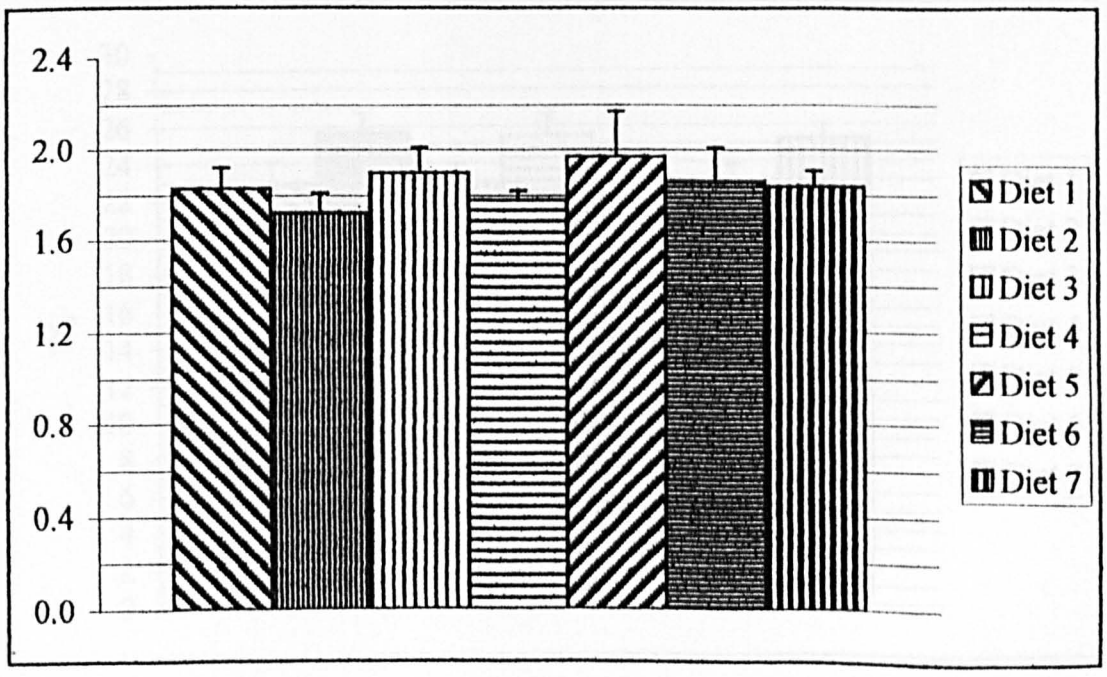


Figure 5.3 Assessment of growth and feed performance in Experiment 3: protein efficiency ratio. Bars indicate one standard deviation.

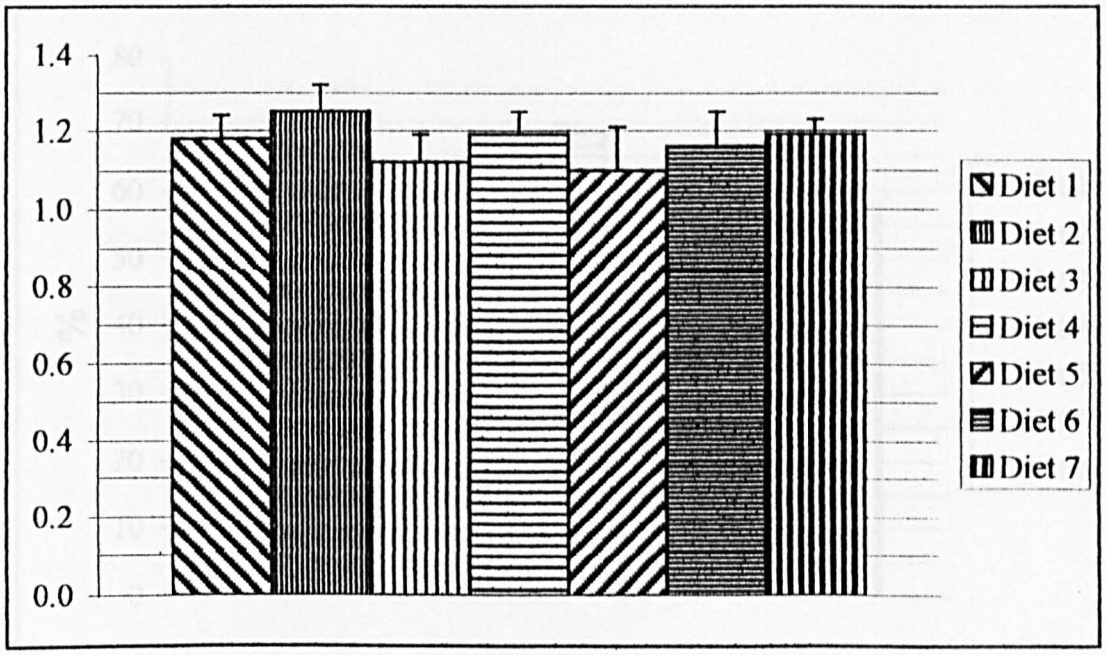


Figure 5.4 Assessment of growth and feed performance in Experiment 3: apparent net protein utilisation. Bars indicate one standard deviation.

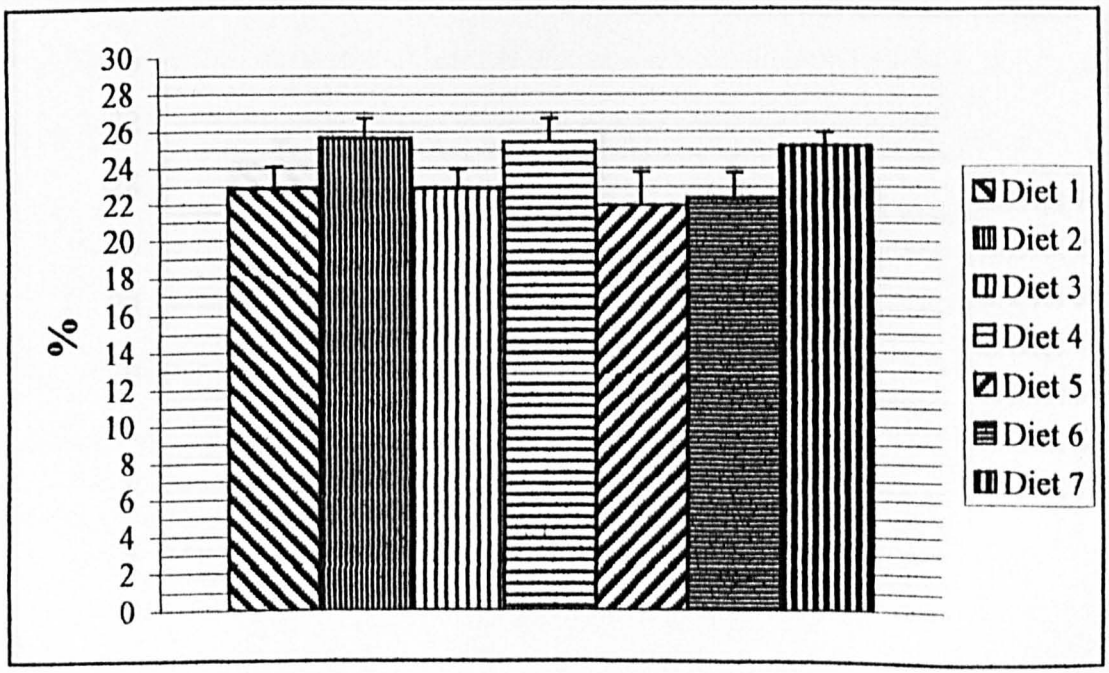


Figure 5.5 Assessment of growth and feed performance in Experiment 3: apparent net lipid utilisation. Bars indicate one standard deviation.

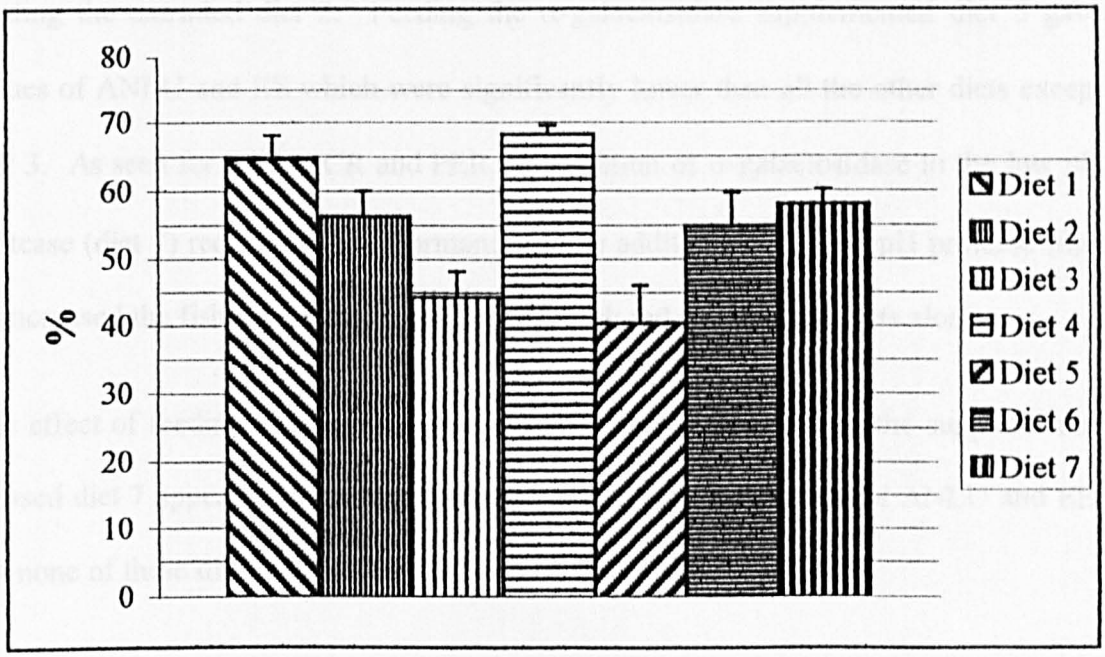
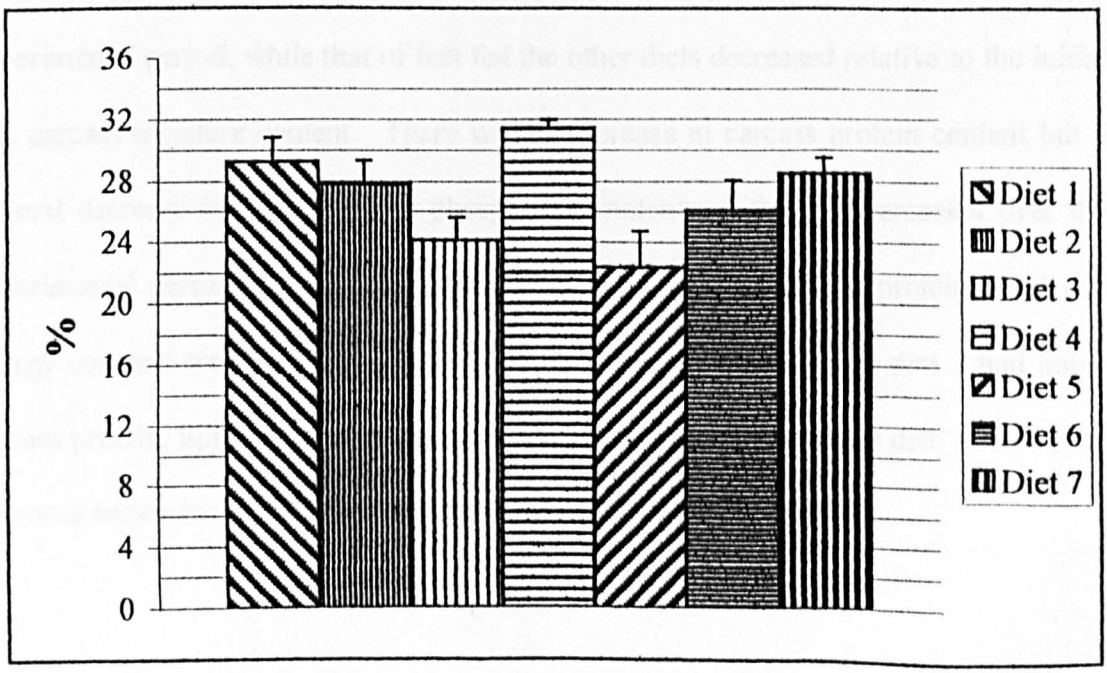


Figure 5.6 Assessment of growth and feed performance in Experiment 3: energy efficiency. Bars indicate one standard deviation.



Feeding diet 4 gave an ANLU value significantly higher than feeding all the other diets except diet 1, and EE significantly higher than feeding diets 3, 5 and 6. In both these parameters, feeding the low pH protease cocktail (diet 7) also gave a similar result to feeding the extruded diet 2. Feeding the α-galactosidase supplemented diet 5 gave values of ANLU and EE which were significantly lower than all the other diets except diet 3. As seen for SGR, FCR and PER, the addition of α-galactosidase to the low pH protease (diet 7) reduced fish performance, while addition to the high pH protease (diet 6) increased the fish performance compared to fish fed the protease diets alone.

The effect of feeding the supplemented extruded diet 2 compared to the supplemented pressed diet 7 appeared to be an improvement in all parameters except ANLU and EE, but none of these differences were found to be significant.

5.3.2 CARCASS COMPOSITION, CONDITION FACTOR, HEPATOSOMATIC INDEX AND INTESTINAL DRY MATTER CONTENT (Table 5.6)

The carcass moisture content of fish fed diets 5 and 6 increased by the end of the experimental period, while that of fish fed the other diets decreased relative to the initial fish carcass moisture content. There was an increase in carcass protein content but a general decrease in lipid, ash and phosphorus contents of the fish carcasses over the experimental period for fish fed all the diets. The highest carcass protein, lipid and energy contents were found in fish fed diet 4. Fish fed the extruded diet 2 had lower carcass protein, lipid and energy contents than the fish fed the pressed diet 7 containing the same enzymes, but had a higher carcass moisture content.

Table 5.6 Effect of dietary treatments on the body (whole) composition of fish in Experiment 3. Condition factor, hepatosomatic index and final intestinal moisture content are presented as means with the standard deviation in brackets. Means in a row followed by the same superscript are not significantly different ($P < 0.05$).

	Initial	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type		Pressed	Extruded	Pressed	Pressed	Pressed	Pressed	Pressed
Fish meal inclusion (g/kg)		260	260	260	260	260	260	260
Soybean meal inclusion (g/kg)		320	320	320	320	320	320	320
Low pH protease (g/kg)		0	0.5	0	1.0	0	0	0.5
High pH protease (g/kg)		0	0	1.0	0	0	0.5	0
α-galactosidase (g/kg)		0	0. 5	0	0	1.0	0.5	0.5
Moisture (g/100g)[1]	65.84	64.01	65.40	65.52	63.27	66.33	65.90	64.76
Protein (g/100g)[1]	15.95	17.61	18.15	18.01	18.50	17.75	17.48	18.47
Lipid (g/100g)[1]	14.30	14.17	12.65	12.26	14.29	11.98	13.15	13.44
Ash (g/100g)[1]	3.39	3.39	3.07	3.40	3.22	3.31	2.74	2.78
Phosphorus (g/100g)[1]	0.53	0.57	0.49	0.53	0.51	0.51	0.42	0.44
Energy content (kJ/g)[1]	9.70	10.34	9.70	9.60	10.53	9.42	9.63	9.88
Condition factor	1.54^{a} (0.09)	1.63^{ab} (0.10)	1.60^{ab} (0.11)	1.56^{a} (0.12)	1.59^{ab} (0.11)	1.59^{ab} (0.03)	1.72^{b} (0.13)	1.59^{ab} (0.07)
Hepatosomatic index	1.96^{b} (0.43)	1.48^{a} (0.19)	1.29^{a} (0.19)	1.33^{a} (0.32)	1.58^{ab} (0.26)	1.60^{ab} (0.36)	1.64^{ab} (0.37)	1.44^{a} (0.24)
Intestinal dry matter content (g/100g)		14.16^{a} (1.79)	12.22^{a} (1.08)	13.85^{a} (1.13)	14.47^{a} (1.76)	14.97^{a} (2.86)	14.00^{a} (2.42)	13.95^{a} (2.42)

1. Values are averages of pooled carcass samples.

Feeding diet 5, containing α-galactosidase only, resulted in the highest carcass moisture content of all the fish, and a corresponding lower lipid content. Feeding diet 7, containing both low pH protease and α-galactosidase, resulted in an increase in carcass moisture but lower lipid and energy contents compared to the carcass compositions of fish fed the low pH protease diet 4. On the other hand, feeding of the high pH protease and α-galactosidase diet 6 increased carcass moisture, lipid and energy composition compared to the high pH protease diet 3.

The condition factors of all fish increased over the experimental period. The condition factor of fish fed diet 6 was significantly higher than the condition factor of fish fed diet 3. The HSI of the fish, whatever the diet fed, decreased over the experimental period, the decreases obtained by feeding diets 1, 2, 3 and 7 being significant.

The HSI of fish fed the high pH protease supplemented diet 3 was lower than that of fish fed the low pH protease supplemented diet 4. Fish fed the high pH protease mixture diet 6 had a higher HSI than fish fed the low pH protease mixture diet 7. Fish fed the extruded diet 2 were found to have the lowest HSI of all the fish.

There were no significant differences in intestinal dry matter content in fish fed the different feeds, with the highest value being found in fish fed diet 5, and the lowest in fish fed diet the extruded diet 2.

5.3.3 APPARENT DIGESTIBILITY COEFFICIENTS (Table 5.7)

Fish fed the diets in which enzymes were supplemented all showed an increase in ADC of protein, carbohydrate and organic matter over the unsupplemented diet 1. Feeding the extruded diet 2 gave the highest ADC values for organic matter, carbohydrate and phosphorus, fish fed diet 4 gave the highest protein and energy ADC and fish fed diet 7

Table 5.7 Effect of dietary treatments on apparent digestibility coefficients (ADC) in Experiment 3 calculated using faeces collected during weeks 11 and 12[1].

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type	Pressed	Extruded	Pressed	Pressed	Pressed	Pressed	Pressed
Fish meal inclusion (g/kg)	260	260	260	260	260	260	260
Soybean meal inclusion (g/kg)	320	320	320	320	320	320	320
Low pH protease (g/kg)	0	0.5	0	1.0	0	0	0.5
High pH protease (g/kg)	0	0	1.0	0	0	0.5	0
α-galactosidase (g/kg)	0	0.5	0	0	1.0	0.5	0.5
Protein ADC (%)	89.68	90.81	91.18	92.13	90.41	90.05	90.17
Lipid ADC (%)	96.64	95.58	95.73	96.05	95.55	95.63	96.81
Carbohydrate ADC (%)	78.30	86.46	83.90	85.26	84.40	83.64	82.96
Energy ADC (%)[2]	90.62	91.26	91.87	92.79	91.43	91.17	91.30
Organic matter ADC (%)	79.83	83.16	81.41	83.01	81.67	80.67	80.55
Phosphorus ADC (%)	50.44	63.93	53.36	54.91	51.99	49.02	55.25

1. Values are averages of pooled faecal samples.
2. Energy calculated using the following values: protein, 23.4 kJ/g; lipid, 39.8 kJ/g; carbohydrate, 17.2 kJ/g.

the highest lipid ADC. The addition of α-galactosidase to the proteases (diets 6 and 7) reduced the protein, carbohydrate and organic matter ADC of fish fed these diets, but slightly improved lipid and phosphorus ADC in the case of the fish fed the low pH protease diet (diet 7) compared to the fish fed the protease only diets. Feeding the fish the extruded diet 2 improved all ADCs compared to the fish fed the corresponding pressed diet 7, except for the ADC of lipid.

5.4 DISCUSSION

The number of significant differences obtained between the results of fish fed the various enzyme supplemented diets in this experiment were few and mostly indicated an inferior performance when compared to fish fed the unsupplemented diet 1. In fact, of all the supplemented pressed diets tested, only fish fed diet 4 with 1.0 g/kg low pH protease appeared to give improvement in performance (not significant) compared to fish fed the unsupplemented diet 1.

After the successful use of the three enzymes in Experiment 1 the negative impact of using the 1.0 g/kg of the high pH protease and 1.0 g/kg of α-galactosidase was not expected. However, it must be kept in mind that these enzymes were used individually in this experiment, rather than in combination as had been done in Experiment 1. It is generally accepted that using enzyme cocktails is better than using enzymes alone (Clifford, 1989; GrootWassink *et al.*, 1989; Inborr, 1989; Graham and Inborr, 1993a), but the reason for a decrease in performance when fish were fed diets with only high pH protease or α-galactosidase individually is unclear,

Most of the literature available concerning studies involving enzymes comes from the poultry and pig industries. Positive effects have been achieved both with individual

enzymes (Herstad and McNab, 1975; Hesselman *et al.*, 1982; Hesselman and Aman, 1986; Pettersson *et al.*, 1987; Classen *et al.*, 1988; Edney *et al.*, 1989; GrootWassink *et al.*, 1989; Cantor, 1990; Cave *et al.*, 1990; Classen *et al.*, 1991; Bedford and Classen, 1992b; Veldman *et al.*, 1993; Wang *et al.*, 1992), as well as using more than one enzyme in combination (Burnett, 1966; Collier and Hardy, 1986b; Graham *et al.*, 1988; Adams, 1989; Clifford, 1989; Inborr, 1989; Pettersson *et al.*, 1990; Annison, 1990; Bedford and Classen, 1992a; Graham and Bedford, 1992; Irish and Balnave, 1993; Finnfeeds 1995a, b, c, 1996a, b). Unlike the present experiment, the majority of these experiments have reported significant positive effects.

Similarly, an improvement in performance in the investigations carried out involving fish and crustaceans have been obtained with the use of single enzymes (Dabrowski and Glogowski, 1977; Maugle *et al.*, 1983a, b; Rodehutscord and Pfeffer, 1995; Schaefer *et al.*, 1995; Robinson *et al.*, 1996) and with mixes of two or more enzymes (Maugle, 1983a; Ashraf *et al.*, 1993; Kolkovski *et al.*, 1993; Bogut *et al.*, 1994; Carter *et al.*, 1994; Feord, 1996, Pers. Comm.; Finnfeeds, 1996c, d; Buchanan *et al.*, 1997).

The negative effects of the high pH protease and the α-galactosidase on fish performance is difficult to explain and the reason is actually unknown. It can only be hypothesised that the activity of these two enzymes is somehow releasing a product or products, such as other antinutritional factors, which lead to an eventual decrease in nutrient utilisation. Another hypothesis is that bacteria are making use of the new products formed by these two enzymes to increase their own biomass at the detriment of the fish itself, which would explain why there were no important differences in digestibilities of fish fed these two diets compared to fish fed other diets. This hypothesis could be tested by using antibiotics in combination with the enzymes.

Unlike in Experiment 1, the addition of the two sets of enzymes together (diets 6 and 7) to the 320 g/kg SBM diet did not bring about significant improvements in fish performance compared to fish fed the unsupplemented diet 1. The lower inclusion levels of each of these enzymes possibly explains the lack of effect with 0.5 g/kg inclusion levels of each enzyme being insufficient to bring about a measurable effect.

The effect of dose on the performance of the animal being used has been found to be quite variable. The literature describing experiments in which different doses of enzymes were added to feeds shows quite varied results, from negative to no change at all in the performance of animals to continuous increases in performance with increasing inclusion level to increases followed by drops with a continued increase in inclusion level.

However, the difficulty in assessing dose effects in the published trials is that more often than not, apart from the actual enzymes often not being stated, the activities of the enzymes are not given. To make matters even more complicated, enzymes are obtained from different sources and, even when enzyme activities are given, since different assay methods are used by different investigators to determine these activities, comparisons are still difficult to make.

As usual, reports about experiments in which increasing levels of supplemental enzymes were used indicate an improvement in performance of the animal over the experimental period. Thus, Hesselman *et al.*(1982), Maugle *et al.* (1983a, b), Collier and Hardy (1986b), Adams (1989), Bedford and Classen (1992), Kolkovski *et al.* (1993), Bogut *et al.* (1994) and Robinson *et al.* (1996) found that performance increased as the level of the supplemental enzymes these authors used in the diets increased.

However, Cardenete *et al.* (1993) did report that when they fed 38 g rainbow trout, *O. mykiss*, diets containing 0.4, 1.2 and 3.6 g/kg of a commercial enzyme cocktail (Kemzyme Dry), there were no differences in performance between fish fed the experimental diets and fed the control diet.

Pettersson *et al.* (1987) fed broiler chickens diets containing 0.08, 0.24 and 0.72 g/kg of a commercial β-glucanase. After 22 days the FCRs of the chickens fed the supplemented diets did not differ from that of chickens fed the control diet, but the SGRs of the chickens fed the experimental diets were actually lower than that of the birds fed the control diet, as seen in this experiment. However, the differences in performances seen by Pettersson *et al.* (1987) were smaller than that seen in this experiment.

As mentioned in the Introduction, the use of the extruded diet 2 would have been expected to increase fish growth and feed utilisation compared to fish fed the pressed diet 7 containing the same enzymes. Although the differences were not always significant, the fish fed this diet appeared to bring about increases in SGR, FCR, PER and ANPU of all the diets, and showed higher protein, carbohydrate, organic matter and phosphorus ADCs.

As seen in Experiment 1, intestinal dry matter content could be used as an indication of oligosaccharide contents and α-galactosidase activity. The lower intestinal dry matter content of fish fed the extruded diet 2 compared to that of fish fed diet 1 could have resulted from the liberation of bound oligosaccharides as a result of the higher cooking temperatures occurring during extrusion (Venkataraman and Jaya, 1975; Rao and Belavady, 1978; Savitri and Desikachar, 1985) and insufficient α-galactosidase to digest them. The intestinal dry matter content of fish fed diet 5 showed an increase compared to fish fed diet 1 (but not much higher than that found in fish fed diet 4), as

expected due to the α-galactosidase activity on the oligosaccharides, but this was not found in fish fed diets 6 and 7, possibly a result of insufficient α-galactosidase. The intestinal dry matter contents of fish fed diets 3 and 4 were similar to that of fish fed the unsupplemented diet 1, and in fact, it would have been expected that the intestinal dry matter contents of fish fed these three diets be similar since no α-galactosidase was present.

The results of this trial have shown the lack of information available with respect to what the supplemental enzymes are really acting on, what the end products of these reactions are and what their effect on the organism might be. Although the reasons are not known, this experiment has clearly demonstrated that, at least in the gilthead sea bream, the three enzymes used give different effects when used in combination or individually and that more work should be carried out on this aspect of enzyme utilisation.

CHAPTER 6

EXPERIMENT 4

Investigation into the use of increasing levels of the low pH protease and the effect of additional α-galactosidase on the growth and feed utilisation of gilthead sea bream, *Sparus aurata* L.

6.1 INTRODUCTION AND AIMS OF THIS EXPERIMENT

Following the results of Experiment 1 and 3 it was decided to continue investigating the use of the low pH protease in gilthead sea bream diets. As already mentioned previously, enzyme inclusion levels can have an important effect on the resulting performance of the animal being fed. One of the aims of this experiment was to determine the effect of feeding pressed 320 g/kg SBM diets containing increasing levels, 0.5, 1.0 and 1.5 g/kg, of low pH protease on the performance of sea bream.

In addition, in order to further investigate the negative impact of the α-galactosidase seen in Experiment 3, the effect of adding 1.0 g/kg α-galactosidase to the diets containing 0.5 and 1.0 g/kg low pH protease on fish performance was also investigated.

Finally, two extruded diets, one of which contained 1.0 g/kg low pH protease, were used to determine whether the use of supplemental enzymes also brought about performance improvements in fish fed extruded diets.

6.2 MATERIALS AND METHODS

Other details pertaining to experimental tanks, experimental fish and handling, diet production, water quality, faecal collection, laboratory analysis, calculations and statistical analysis are as described in Chapter 2.

6.2.1 EXPERIMENTAL FISH

30 fish of 85 g average weight were used in each of the tanks in this experiment. Each treatment had three replicates. The duration of the experiment, apart from the acclimation period, was 12 weeks.

6.2.2 THE DIETS AND FEEDING REGIME

The formulation of the diets used in this experiment is given in Table 6.1. The nutritional compositions and amino acid contents of the soybean meal and fish meal used are given in Table 6.2. The percentage inclusion levels of the supplementary enzymes added to the diets and the nutritional compositions of the diets themselves (4.2 mm pellets) are given in Table 6.3. Table 6.4 gives the amino acid compositions of the diets.

During the acclimation period the fish were fed diet 2. During the acclimation period and the experiment, the following regime was used: 81 to 120 g fish fed at 1.6 % body weight/day; 121 to 140 g fish fed at 1.5 % body weight/day; 141 to 160 g fish fed at 1.4% body weight/day.

6.3 RESULTS

6.3.1 ASSESSMENT OF GROWTH AND FEED PERFORMANCE

(Table 6.5, Figures 6.1 to 6.6)

No significant differences were obtained between the fish fed the seven experimental diets for SGR, FCR and PER. There were no differences in daily food consumption of fish fed the various diets. When fish were fed diets 4 and 5, in which α-galactosidase had been added to the proteases, there was a drop in performance of the fish compared to the α-galactosidase-free diets 1 and 2 in the above parameters.

The ANPUs of fish fed diets 1 and 7 were significantly higher than those for the other diets and also significantly different between themselves, with fish fed diet 7 giving the highest value. Addition of α-galactosidase to the diets containing low pH proteases

Table 6.1 Formulation of diets used in Experiment 4.

Diets	All
Ingredient	Inclusion (g/kg)
Fish meal[1]	260
Dehulled hexane extracted soybean meal[2]	320
Blood meal[3]	80
Corn[4]	40
Feather meal[5]	60
Fish oil[6]	110
Limestone	30
Molasses[7]	40
Vitamins and minerals[8]	15
Whole wheat[9]	40
Chromic oxide	5

1. Norse LT94.
2. HiPro soya, Source: Grosvenor Ltd., Scotland.
3. Source: Daka Ltd., Canada.
4. Source: Suprex Ltd., Scotland.
5. Source: Canada.
6. Source: UFP Ltd., Scotland.
7. Source: Spain.
8. Ewos Premix prepared by Roche Products Ltd., England.
9. Source: Scotland.

Table 6.2 Nutritional compositions of the soybean meal and fish meal used in the formulation of the diets in Experiment 4.

	Soybean meal	Fish meal
Moisture (g/kg)	105	77
Crude protein (g/kg)	428	660
Crude lipid (g/kg)	11	100
Ash (g/kg)	61	132
Crude fibre (g/kg)	66	2
Crude carbohydrate (g/kg)	321	17
Phosphorus (g/kg)	5	19
Protein solubility (%)	67.02	
Trypsin inhibitor activity (mg/g)	1.30	
Amino acid[1]	(g/100 g protein)	
Alanine	4.69	6.78
Arginine[2]	7.26	7.19
Aspartic acid	11.63	9.05
Cystine	1.24	0.82
Glutamic acid	19.66	13.25
Glycine	4.75	7.37
Histidine[2]	2.69	2.59
Isoleucine[2]	5.04	4.78
Leucine[2]	7.85	7.61
Lysine[2]	6.04	7.84
Methionine[2]	0.61	2.57
Phenylalanine[2]	5.70	4.79
Proline	5.41	5.39
Serine	5.23	4.42
Tyrosine	3.13	3.35
Threonine[2]	4.12	4.69
Valine[2]	5.27	5.40

1. No data is available for the essential amino acid tryptophan because it is destroyed during acid hydrolysis.
2. Essential amino acid

Table 6.3 Inclusion levels of enzymes and nutritional compositions of the diets used during Experiment 4.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type	Pressed	Pressed	Pressed	Pressed	Pressed	Extruded	Extruded
Fish meal inclusion (g/kg)	260	260	260	260	260	260	260
Soybean meal inclusion (g/kg)	320	320	320	320	320	320	320
Low pH protease (g/kg)	0.5	1.0	1.5	0.5	1.0	0	1.0
α-galactosidase (g/kg)	0	0	0	1.0	1.0	0	0
Enzyme form	Dry	Dry	Dry	Dry	Dry	Liquid	Liquid
Moisture (g/kg)	89	67	63	74	47	93	95
Crude protein (g/kg)	444	467	467	467	478	454	453
Crude lipid (g/kg)	142	139	142	135	139	136	143
Ash (g/kg)	110	110	112	111	113	106	107
Crude fibre (g/kg)	19	25	24	27	25	22	22
Crude carbohydrate (g/kg)	195	206	189	189	204	193	184
Phosphorus (g/kg)	7	8	8	8	8	7	8
Trypsin inhibitor activity (mg/g)	0.53	0.53	0.58	0.72	0.63	0.57	0.55
Chromic oxide (g/kg)	4	4	5	4	5	4	5
Energy content (kJ/g)	21.15	21.80	21.71	21.55	22.17	20.93	21.46
Protein/gross energy ratio (g/MJ)	20.99	21.42	21.53	21.68	21.58	21.68	21.09

Table 6.4 Amino acid contents of gilthead sea bream[1] carcass and the diets used in Experiment 4[2].

	Carcass	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Fish meal inclusion (g/kg)		260	260	260	260	260	260	260
Soybean meal inclusion (g/kg)		320	320	320	320	320	320	320
Low pH protease (g/kg)		0.5	1.0	1.5	0.5	1.0	0	1.0
α-galactosidase (g/kg)		0	0	0	1.0	1.0	0	0
Enzyme form		Dry	Dry	Dry	Dry	Dry	Liquid	Liquid
	(g/100 g protein)							
Alanine	5.35	5.71	5.18	5.22	5.45	5.17	5.92	6.34
Arginine[3]	5.10	5.91	5.63	5.56	5.66	5.28	6.48	6.36
Aspartic acid	8.01	9.88	9.65	8.61	9.48	8.94	10.34	10.77
Cystine	1.08	1.43	1.25	1.22	1.15	1.02	1.20	1.69
Glutamic acid	10.38	14.32	13.13	13.07	13.82	12.67	15.51	15.95
Glycine	6.12	5.77	5.41	5.33	5.52	5.16	6.02	6.29
Histidine[3]	1.83	2.65	2.58	2.52	2.59	2.46	2.88	2.96
Isoleucine[3]	3.62	4.29	3.94	3.86	4.01	4.29	4.78	4.85
Leucine[3]	5.83	8.37	8.29	7.37	7.69	7.00	8.23	8.30
Lysine[3]	6.01	5.63	5.49	5.44	5.33	5.14	6.32	5.33
Methionine[3]	2.56	1.40	1.31	1.30	1.40	1.19	1.46	1.48
Phenylalanine[3]	3.26	5.04	4.49	4.64	4.70	4.28	5.42	5.57
Proline	4.13	5.34	5.26	4.69	4.90	4.43	4.94	5.40
Serine	3.53	5.18	4.74	4.82	4.87	4.68	5.67	5.48
Tyrosine	3.17	2.68	2.73	2.87	2.74	3.13	3.26	3.38
Threonine[3]	3.91	4.19	4.17	3.80	4.03	3.80	4.54	4.70
Valine[3]	4.39	5.64	5.19	5.05	5.53	5.02	6.20	6.54

1. Average weight 63.28 g.
2. No data is available for the essential amino acid tryptophan because it is destroyed during acid hydrolysis.
3. Essential amino acid.

Table 6.5 Assessment of growth and feed performance in Experiment 4. Data are presented as means with the standard deviation in brackets. Means in a row followed by the same superscript are not significantly different ($P < 0.05$).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type	Pressed	Pressed	Pressed	Pressed	Pressed	Extruded	Extruded
Fish meal inclusion (g/kg)	260	260	260	260	260	260	260
Soybean meal inclusion (g/kg)	320	320	320	320	320	320	320
Low pH protease (g/kg)	0.5	1.0	1.5	0.5	1.0	0	1.0
α-galactosidase (g/kg)	0	0	0	1.0	1.0	0	0
Initial weight (g)	83.17[a] (0.50)	84.83[a] (0.87)	84.74[a] (0.85)	84.94[a] (1.54)	85.39[a] (1.66)	84.86[a] (1.02)	84.57[a] (1.26)
Final weight (g)	156.55[a] (4.57)	161.53[a] (4.94)	160.85[a] (5.46)	156.75[a] (3.46)	158.60[a] (5.36)	157.35[a] (4.35)	159.91[a] (3.02)
Specific growth rate (SGR)(%/day)	0.75[a] (0.02)	0.76[a] (0.06)	0.76[a] (0.03)	0.73[a] (0.02)	0.74[a] (0.03)	0.74[a] (0.02)	0.76[a] (0.03)
% Mortalities	3.33[a] (3.34)	1.11[a] (1.92)	0.00[a] (0.00)	1.11[a] (1.92)	0.00[a] (0.00)	0.00[a] (0.00)	4.44[a] (1.93)
Food intake (g/100g fish/day)	1.38[a] (0.01)	1.41[a] (0.03)	1.39[a] (0.03)	1.41[a] (0.03)	1.41[a] (0.04)	1.41[a] (0.04)	1.40[a] (0.01)
Food conversion ratio (FCR)	1.66[a] (0.05)	1.61[a] (0.05)	1.63[a] (0.08)	1.73[a] (0.09)	1.71[a] (0.07)	1.73[a] (0.06)	1.67[a] (0.10)
Protein efficiency ratio (PER)	1.36[a] (0.04)	1.34[a] (0.05)	1.32[a] (0.06)	1.24[a] (0.08)	1.23[a] (0.06)	1.27[a] (0.04)	1.33[a] (0.08)
Apparent net protein utilisation (ANPU)(%)	26.14[b] (0.61)	24.13[a] (0.22)	22.87[a] (0.99)	21.90[a] (1.35)	22.80[a] (0.84)	23.94[a] (0.72)	27.88[c] (1.25)
Apparent net lipid utilisation (ANLU)(%)	62.49[c] (1.36)	64.45[c] (0.59)	64.10[c] (2.47)	65.07[c] (2.90)	63.97[c] (2.15)	44.00[a] (1.60)	57.14[b] (2.93)
Energy efficiency (EE)(%)	32.64[b] (0.75)	31.85[b] (0.29)	31.13[b] (1.26)	29.74[b] (1.40)	30.27[b] (0.99)	25.77[a] (0.86)	32.17[b] (1.61)

Figure 6.1 Assessment of growth and feed performance in Experiment 4: specific growth rate. Bars indicate one standard deviation.

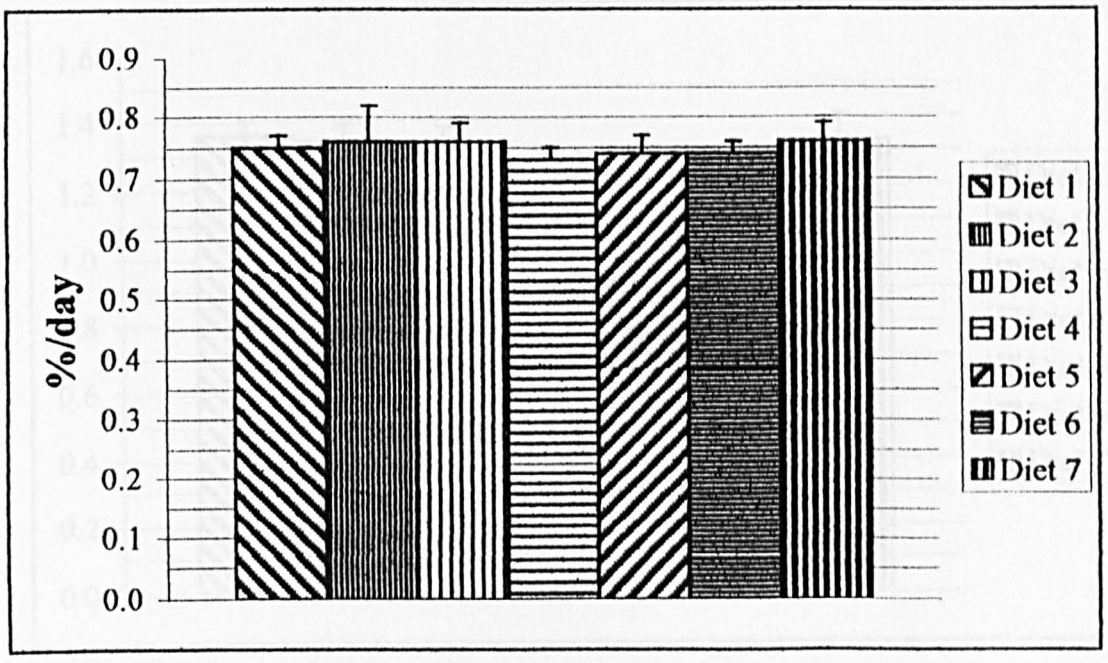


Figure 6.2 Assessment of growth and feed performance in Experiment 4: food conversion ratio. Bars indicate one standard deviation.

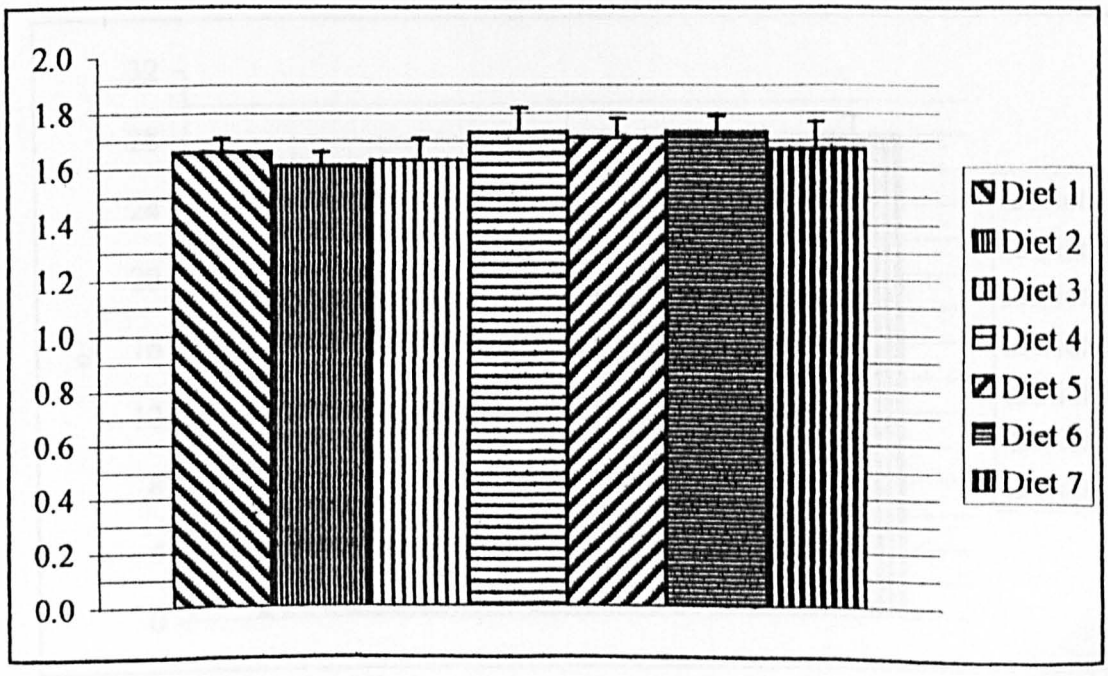


Figure 6.3 Assessment of growth and feed performance in Experiment 4: protein efficiency ratio. Bars indicate one standard deviation.

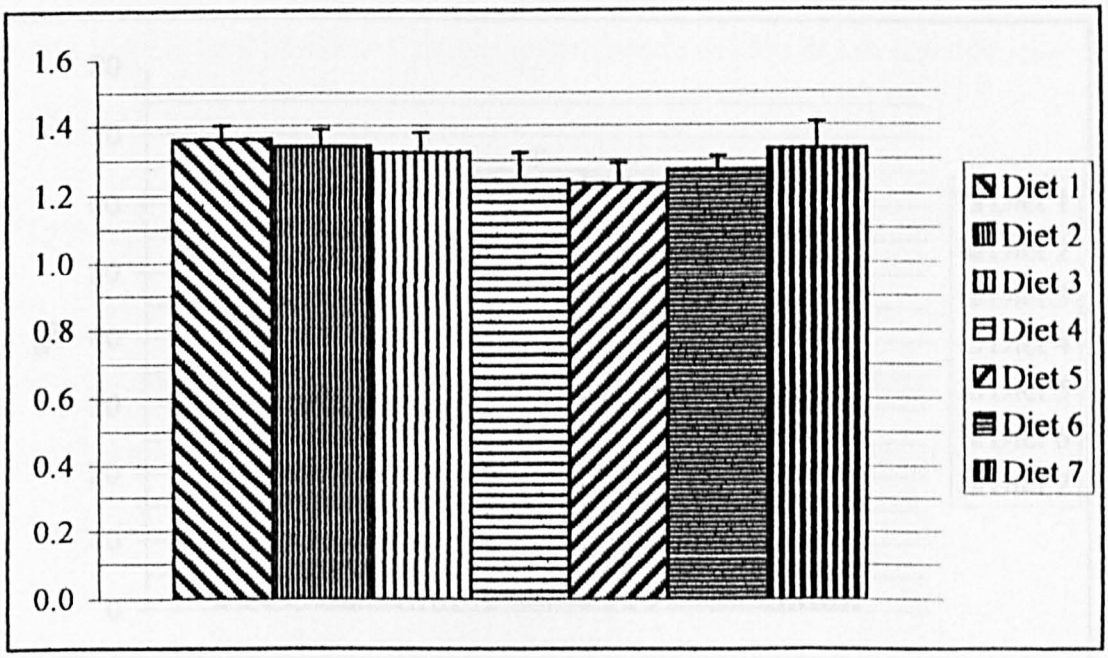


Figure 6.4 Assessment of growth and feed performance in Experiment 4: apparent net protein utilisation. Bars indicate one standard deviation.

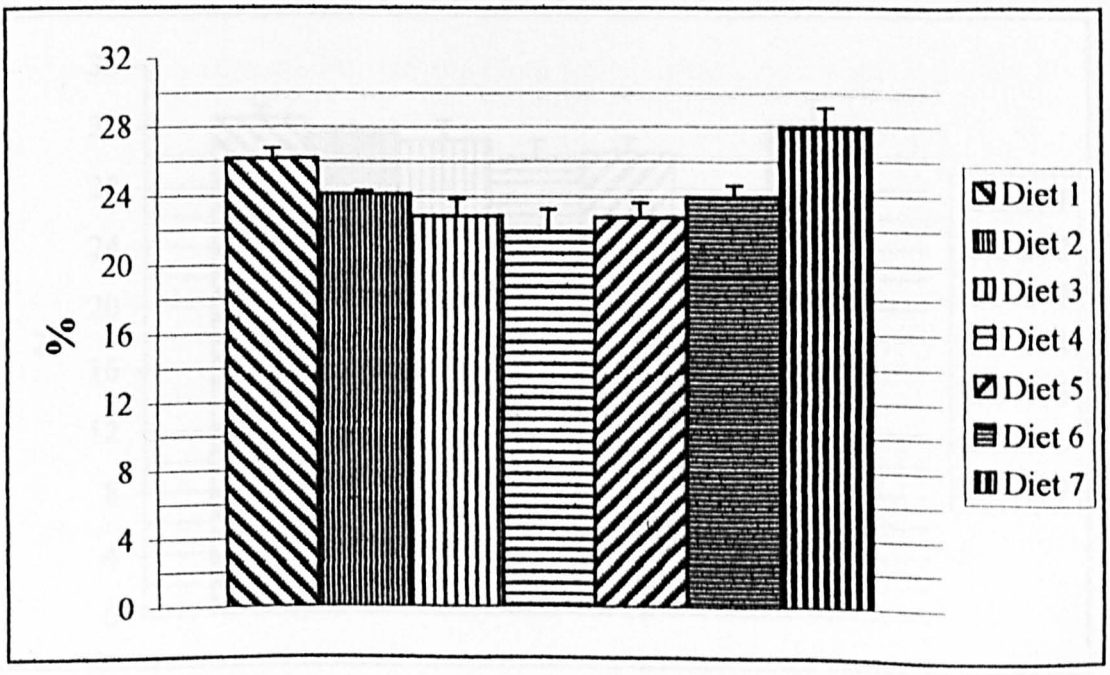


Figure 6.5 Assessment of growth and feed performance in Experiment 4: apparent net lipid utilisation. Bars indicate one standard deviation.

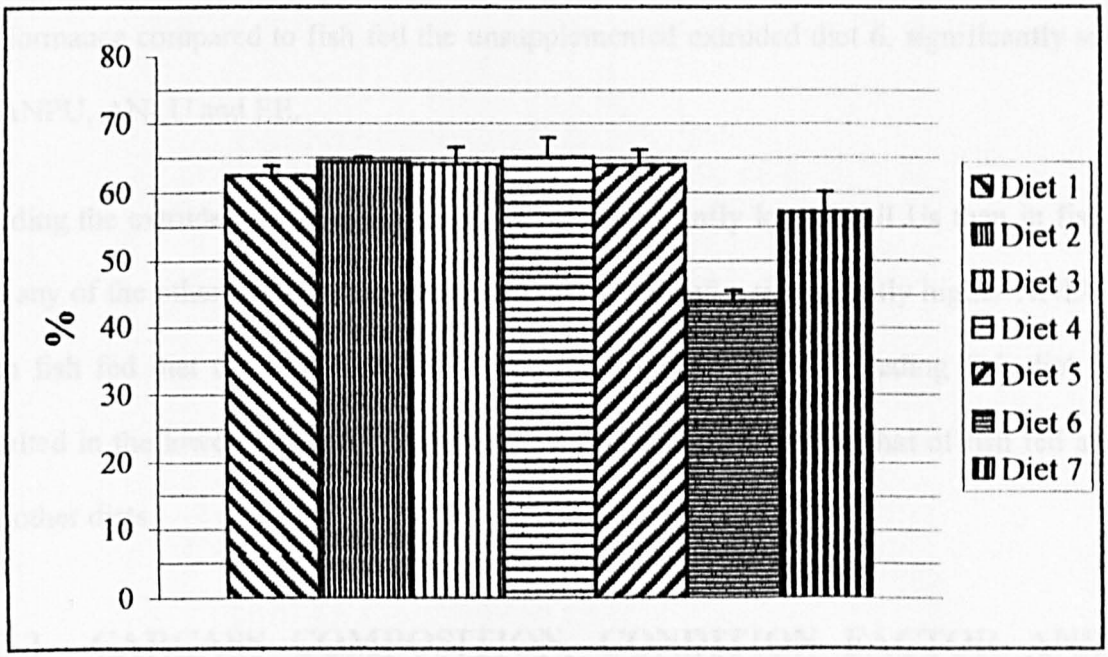
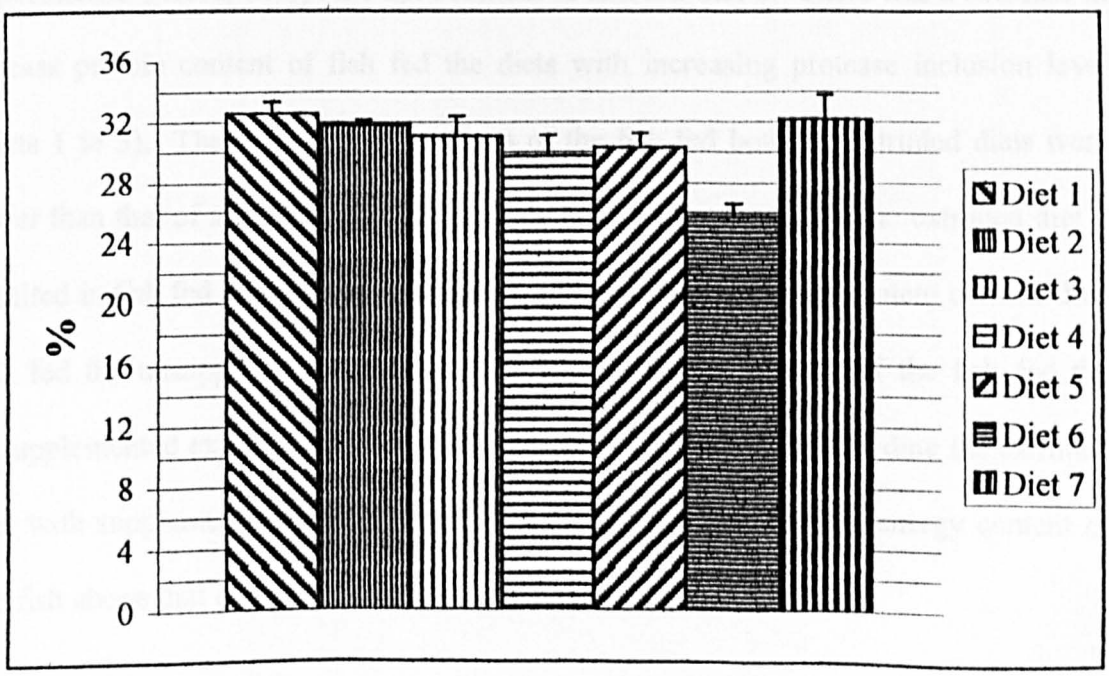


Figure 6.6 Assessment of growth and feed performance in Experiment 4: energy efficiency. Bars indicate one standard deviation.



(diets 4 and 5) further reduced the ANPUs of the fish fed these diets, significantly in the case of the 0.5 g/kg protease inclusion level.

Addition of enzyme to the extruded feed (diet 7) produced an increase in fish performance compared to fish fed the unsupplemented extruded diet 6, significantly so in ANPU, ANLU and EE.

Feeding the extruded diets 6 and 7 resulted in significantly lower ANLUs than in fish fed any of the other diets, although fish fed diet 7 showed a significantly higher ANLU than fish fed diet 6. Fish fed diet 4 gave the highest ANLU. Feeding fish diet 6 resulted in the lowest value for EE, a value significantly lower than that of fish fed all the other diets.

6.3.2 CARCASS COMPOSITION, CONDITION FACTOR AND HEPATOSOMATIC INDEX (Table 6.6)

Fish fed all the diets showed a decrease in carcass moisture content over the experimental period. The carcass protein, lipid and energy contents increased over the experimental period, except the lipid content of fish fed diet 6. There was a decrease in carcass protein content of fish fed the diets with increasing protease inclusion level (diets 1 to 3). The carcass lipid contents of the fish fed both the extruded diets were lower than that of all the pressed diets. Addition of the protease to the extruded diet 7 resulted in fish fed with this diet having a higher carcass lipid and protein content than fish fed the unsupplemented diet 6. The carcass energy content of the fish fed the unsupplemented extruded diet 6 was the lowest of all the fish, and feeding the extruded diet with supplementation of protease (diet 7) increased the carcass energy content of the fish above that of fish fed the corresponding pressed diet 2.

Table 6.6 Effect of dietary treatments on the body (whole) composition of fish in Experiment 4. Condition factor and hepatosomatic index are presented as means with the standard deviation in brackets. Means in a row followed by the same superscript are not significantly different ($P < 0.05$).

	Initial	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type		Pressed	Pressed	Pressed	Pressed	Pressed	Extruded	Extruded
Fish meal inclusion (g/kg)		260	260	260	260	260	260	260
Soybean meal inclusion (g/kg)		320	320	320	320	320	320	320
Low pH protease (g/kg)		0.5	1.0	1.5	0.5	1.0	0	1.0
α-galactosidase (g/kg)		0	0	0	1.0	1.0	0	0
Moisture (g/100g)[1]	68.84	65.94	66.18	65.74	66.97	65.43	67.06	65.16
Protein (g/100g)[1]	15.80	17.37	16.99	16.53	16.69	17.07	17.15	18.13
Lipid (g/100g)[1]	11.97	13.24	13.20	13.30	13.39	13.42	11.23	12.72
Ash (g/100g)[1]	3.52	3.70	3.54	3.50	3.11	3.30	3.82	3.81
Phosphorus (g/100g)[1]	0.48	0.59	0.56	0.50	0.45	0.49	0.59	0.60
Energy content (kJ/g)[1]	8.89	9.73	9.45	9.65	9.41	10.01	8.69	9.53
Condition factor	1.48^{a} (0.09)	1.72^{b} (0.08)	1.69^{b} (0.11)	1.68^{b} (0.16)	1.61^{b} (0.13)	1.67^{b} (0.14)	1.63^{b} (0.06)	1.62^{b} (0.13)
Hepatosomatic index	1.12^{a} (0.21)	1.57^{ab} (0.34)	1.66^{b} (0.50)	1.55^{ab} (0.33)	1.26^{ab} (0.40)	1.48^{ab} (0.25)	1.42^{ab} (0.25)	1.52^{ab} (0.28)

1. Values are averages of pooled carcass samples.

Both condition factor and hepatosomatic index of the fish increased over the experimental period, with the former significantly so for all the fish, but only in the case of fish fed diet 2 in the case of the HSI. Fish fed diets with 0.5 and 1 g/kg protease (diets 1 and 2 respectively) had higher conditions factors and HSI than the fish fed the diets with both protease and α-galactosidase (diets 4 and 5). Fish fed the extruded diet 7 with protease showed higher HSI than fish fed the unsupplemented extruded diet 6 but lower condition factor and HSI than fish fed the diet with the same inclusion level of protease (diet 2).

6.3.3 APPARENT DIGESTIBILITY COEFFICIENTS (Table 6.7)

The highest protein and organic matter ADCs were found in fish fed diet 1, followed by fish fed diet 2. The protein, organic matter and energy ADC given by fish fed diet 7 were the lowest for all the diets, with the unsupplemented extruded diet 6 giving the lowest values for the lipid and phosphorus ADCs. The lowest carbohydrate ADC was given by fish fed diet 3.

Fish fed diets 4 and 5 with both protease and α-galactosidase showed decreased protein, organic matter and phosphorus ADCs compared to fish fed diets 1 and 2 with protease only. Fish fed diet 4 gave the highest lipid, carbohydrate and energy ADCs of all fish. The fish fed the extruded diet 7 with protease showed lower ADCs than the fish fed the corresponding pressed diet 2 for all components.

Table 6.7 Effect of dietary treatments on apparent digestibility coefficients (ADC) in Experiment 4 calculated using faeces collected during weeks 9 and 10[1].

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Pellet type	Pressed	Pressed	Pressed	Pressed	Pressed	Extruded	Extruded
Fish meal inclusion (g/kg)	260	260	260	260	260	260	260
Soybean meal inclusion (g/kg)	320	320	320	320	320	320	320
Low pH protease (g/kg)	0.5	1.0	1.5	0.5	1.0	0	1.0
α-galactosidase (g/kg)	0	0	0	1.0	1.0	0	0
Protein ADC (%)	91.68	91.67	91.07	90.41	89.73	88.39	87.70
Lipid ADC (%)	89.42	90.57	90.05	91.28	90.35	88.86	90.22
Carbohydrate ADC (%)	87.79	87.89	82.69	93.65	87.77	89.41	85.00
Energy ADC (%)[2]	91.18	91.49	90.41	92.01	90.48	89.63	89.04
Organic matter ADC (%)	78.72	77.87	77.81	76.86	75.37	74.19	73.51
Phosphorus ADC (%)	58.07	60.95	56.95	54.77	49.01	42.51	48.87

1. Values are averages of pooled faecal samples.
1. Energy calculated using the following values: protein, 23.4 kJ/g; lipid, 39.8 kJ/g; carbohydrate, 17.2 kJ/g.

6.4 DISCUSSION

As in Experiment 3, the number of significant differences obtained in this experiment were few and none were found between SGRs, FCRs or PERs of the fish fed the various diets.

There were few differences between fish fed diets with the three levels of low pH protease with fish fed diet 1 containing 0.5 g/kg protease having the highest PER and fish fed diet 2 the lowest FCR. The addition of α-galactosidase to 0.5 and 1.0 g/kg protease (diets 4 and 5 respectively) clearly did not improve the performance of fish compared to fish fed the protease-only diets 1 and 2 respectively.

As seen in the discussion of Experiment 3, inclusion levels can have an important effect on the resulting performance of the fish. In this experiment the three doses of protease used did not result in large differences in performance of fish fed the three diets (1, 2 and 3). The results of this experiment indicate that similar improvements would be obtained whether fish were fed diets containing 0.5, 1.0 or 1.5 g/kg low pH protease.

A similar result has also been obtained by a number of other authors whereby a similar improvement in performance is obtained whatever the dose used in their trials. Thus, in a trial by Collier and Hardy (1986b) with early weaned pigs, addition of 0.5 and 1.0 g/kg of a commercial enzyme (FC2 containing a neutral proteinase, α amylase and β-glucanase) gave similar improvements in SGR and FCR over pigs fed the unsupplemented diet.

Kolkovski *et al.* (1993) also found no differences in the improvement in performance when they fed gilthead sea bream, *S. aurata*, larvae diets with 0.5 and 1.0 g/kg pancreatin compared to fish fed the control diet, although survival of fish fed the 1.0 g/kg pancreatin diet was better.

This levelling off of performance of fish fed the increased levels of protease could indicate that the substrate on which the enzyme was acting became limited. This limitation could either be because of a small quantity of the enzyme substrate or substrates actually being present or a physical limitation due to components of the ingredients in the feed itself.

On the other hand, these results do not agree with those of other authors working with different inclusion levels of enzymes who found that as the level of the enzyme in the diets increased, there was also an increase in performance of the animal being fed these diets.

Hesselman *et al.* (1982) found that as the level of commercial β-glucanase increased from 0.05 to 0.1 to 0.5 g/kg in the diet of broiler chickens both SGR and FCR improved also.

Adams (1989) reported an improved SGR and FCR in weaned pigs to which 0.5 and 0.75 g/kg of a commercial Kemzyme had been added to a basal diet. However, while the increase in SGR brought about by the addition of the larger dose of enzyme was higher than that resulting from feeding the pigs the 0.5g/kg supplemented diet, the FCRs of both feed doses were the same.

This trend was also seen in fish by Schaefer *et al.* (1995) who fed 40g carp, *C. carpio*, diets to which 500 and 1000 Units of phytase had been added, and also in shrimp. Feeding 0.64g *P. japonicus* two diets containing 30 and 60 IU amylase/g increased performance compared to shrimp fed the control diets, with a larger increase being obtained with shrimp fed the higher enzyme containing diet (Maugle *et al.*, 1983b). And a similar result was also obtained when 0.60g *P. japonicus* were fed diets containing 250 and 500 U amylase/g (Maugle *et al.*, 1983a).

The results for fish fed diets 6 and 7 show that enzymes can also bring about a positive effect in gilthead sea bream fed extruded feeds as well as pressed feeds. This improvement was evident in all the nutritional parameters studied, though not in the digestibilities. When performance is compared to fish fed the unsupplemented diets the improvement seen in fish fed the 1.0 g/kg protease extruded diet 7 was of the same scale as seen in Experiment 3 with fish fed the same quantity of enzyme added to a pressed diet.

The negative impact of addition of α-galactosidase to diets with 0.5 and 1.0 g/kg low pH protease on fish performance was very evident. The negative effect of the α-galactosidase used alone on fish performance was seen in Experiment 3. These results indicate, quite clearly, that rather than the α-galactosidase bringing about a positive effect on the food in the intestine by reducing stachyose and raffinose, it is causing something or producing something which eventually leads to inferior growth and feed utilisation (although not evident in the digestibility values). The reasons behind this are unclear and the same hypotheses expressed in the discussion of Experiment 3 apply here.

The results obtained in this experiment with the use of the diets with low pH protease and the α-galactosidase were similar, to an extent, what was found by Maugle *et al.* (1983b). These authors used α-amylase and trypsin separately and in combination in 0.6 g *P. japonicus* feeds. All shrimp fed the supplemented diets gave better performances than shrimp fed the unsupplemented diet (unlike what had been seen with the α-galactosidase in Experiment 3), which grew 4.7%/day and had an FCR of 4.9. Shrimp fed the diet containing amylase alone and trypsin alone gained 5.9 and 6.5%/day respectively, and obtained FCRs of 4.8 and 4.2 respectively. Shrimp fed the diet containing both enzymes (at the same inclusion levels as used when applied

individually) grew 6.1%/day and gave an FCR of 5.1 which indicated a reduction in performance compared to shrimp fed the diet with supplemental trypsin only.

Since equal levels of α-galactosidase were used in both diets 4 and 5, the similar results of fish fed these diets confirm the similar effects on performance of fish fed diets containing 0.5 and 1.0 g/kg protease.

These results reaffirm the need to investigate further the activities of these enzymes. They also show that not all supplemental enzymes are beneficial whatever their theoretical uses, although experiments obviously have to be carried out before this conclusion can be reached.

CHAPTER 7

EXPERIMENT 5

Influence of increasing levels of supplemental low pH protease on the growth and feed utilisation of gilthead sea bream, *Sparus aurata* L.

7.1 INTRODUCTION AND AIMS OF THIS EXPERIMENT

The salmonid feed industry is based on the use of extruded feeds containing high lipid levels. Until recently, the gilthead sea bream industry was based on the use of pressed feeds. The development of extruded feeds for the sea bream industry has followed the success achieved in the salmon and trout industries which utilise the beneficial effects of extruded feeds over pressed feeds (see Section 1.2.1.3) and, with time, a greater volume of extruded feeds is appearing on the market and it is predicted that the proportion of pressed feeds will continue to decrease. This trend has increased the emphasis on research into the use of extruded feeds in gilthead sea bream, including ways and means of improving growth and feed utilisation.

The results of the two extruded diets used in Experiment 4 had indicated that the low pH protease had an effect on fish growth and feed utilisation and the main aim of this experiment was to further study the effect of feeding extruded diets containing enzymes on the performance of gilthead sea bream. This was carried out by using increasing levels of low pH protease in the diets. Five enzyme levels were used, 0, 0.33, 0.66, 1.00 and 1.33 g/kg, which were added to a 320 g/kg SBM, 260 g/kg FM diet. In addition, a 220 g/kg SBM, 320 g/kg FM extruded diet was used to investigate the impact of a higher level of SBM in extruded sea bream feeds (which has not yet been studied) and compare the performance of fish fed this diet with the fish fed diets containing supplementary enzymes.

7.2 MATERIALS AND METHODS

Other details pertaining to experimental tanks, experimental fish and handling, diet production, water quality, faecal collection, laboratory analysis, calculations and statistical analysis are as described in Chapter 2.

7.2.1 EXPERIMENTAL FISH

30 fish of 90 g average weight were used in each of the tanks in this experiment. Each treatment had four replicates, although one tank each of diets 1 and 4 was lost due to a parasitic infection. The duration of the experiment, apart from the acclimation period, was 12 weeks.

7.2.2 THE DIETS AND FEEDING REGIME

The formulations of the diets used in this experiment are given in Table 7.1. The nutritional compositions and amino acid contents of the soybean meal and fish meal used are given in Table 7.2. The percentage inclusion levels of the supplementary enzyme added to the diets and the nutritional compositions of the diets themselves (3 mm pellets) are given in Table 7.3. Table 7.4 gives the amino acid compositions of the diets.

During the acclimation period the fish were fed diet 2. During the acclimation period and the experiment, the following regime was used: 81 to 100 g fish fed at 1.5 % body weight/day; 101 to 120 g fish fed at 1.4 % body weight/day; 121 to 140 g fish fed at 1.3% body weight/day.

Table 7.1 Formulations of diets used in Experiment 5.

Diets	1	2 to 6
Ingredient	Inclusion (g/kg)	
Fish meal[1]	320	260
Dehulled hexane extracted soybean meal[2]	220	320
Blood meal[3]	40	40
Dicalcium phosphate	3	5
Feather meal[5]	80	80
Fish oil[6]	170	175
Vitamins and minerals[7]	13	13
Whole wheat[8]	150	102
Chromic oxide	5	5

1. Norse LT94.
2. HiPro soya, Source: Grosvenor Ltd., Scotland.
3. Source: Daka Ltd., Canada.
4. Source: Suprex Ltd., Scotland.
5. Source: Canada.
6. Source: UFP Ltd., Scotland.
7. Ewos Premix prepared by Roche Products Ltd., England.
8. Source: Scotland.

Table 7.2 Nutritional compositions of the soybean meal and fish meal used in the formulation of the diets in Experiment 5.

	Soybean meal	Fish meal
Moisture (g/kg)	113	77
Crude protein (g/kg)	455	673
Crude lipid (g/kg)	18	95
Ash (g/kg)	68	137
Crude fibre (g/kg)	32	6
Crude carbohydrate (g/kg)	299	10
Phosphorus (g/kg)	7	18
Protein solubility (%)	81.43	
Trypsin inhibitor activity (mg/g)	1.24	
Amino acid[1]	(g/100 g protein)	
Alanine	6.13	4.87
Arginine[2]	6.18	4.41
Aspartic acid	10.75	6.12
Cystine	2.22	1.42
Glutamic acid	16.17	10.89
Glycine	4.08	4.69
Histidine[2]	2.37	1.58
Isoleucine[2]	4.45	3.54
Leucine[2]	6.87	5.70
Lysine[2]	6.07	6.07
Methionine[2]	1.67	2.22
Phenylalanine[2]	4.95	3.30
Proline	5.00	3.98
Serine	4.41	3.16
Tyrosine	2.94	2.56
Threonine[2]	3.98	3.35
Valine[2]	4.41	3.82

1. No data is available for the essential amino acid tryptophan because it is destroyed during acid hydrolysis.
2. Essential amino acid

Table 7.3 Inclusion levels of low pH protease and nutritional compositions of the diets used during Experiment 5.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Pellet type	Extruded	Extruded	Extruded	Extruded	Extruded	Extruded
Fish meal inclusion (g/kg)	320	260	260	260	260	260
Soybean meal inclusion (g/kg)	220	320	320	320	320	320
Low pH protease (g/kg)	0	0	0.33	0.66	1.00	1.33
Enzyme form	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
Moisture (g/kg)	55	80	77	79	78	75
Crude protein (g/kg)	455	437	446	437	438	439
Crude lipid (g/kg)	198	206	202	203	203	207
Ash (g/kg)	79	73	73	72	73	72
Crude fibre (g/kg)	12	16	16	17	15	18
Crude carbohydrate (g/kg)	205	190	191	187	203	184
Phosphorus (g/kg)	10	9	9	9	9	9
Trypsin inhibitor activity (mg/g)	0.40	0.59	0.40	0.52	0.51	0.50
Chromic oxide (g/kg)	5	4	4	4	5	4
Energy content (kJ/g)	23.76	23.40	23.31	23.20	23.18	23.29
Protein/gross energy ratio (g/MJ)	19.16	18.69	19.15	18.84	18.91	18.83

Table 7.4 Amino acid contents of gilthead sea bream[1] carcass and the diets used in Experiment 5[2].

	Carcass	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Fish meal inclusion (g/kg)		320	260	260	260	260	260
Soybean meal inclusion (g/kg)		220	320	320	320	320	320
Low pH protease (g/kg)		0	0	0.33	0.66	1.00	1.33
Enzyme form		Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
	(g/100 g protein)						
Alanine	5.35	4.11	4.54	4.75	4.86	4.90	4.57
Arginine[3]	5.10	4.26	4.46	5.31	5.59	5.23	5.45
Aspartic acid	8.01	6.43	7.17	8.24	8.17	8.49	8.43
Cystine	1.08	1.35	1.69	1.85	1.85	1.86	1.34
Glutamic acid	10.38	10.54	10.98	12.41	12.71	12.51	13.08
Glycine	6.12	4.34	4.23	4.61	4.88	5.01	4.89
Histidine[3]	1.83	1.79	1.85	2.15	2.14	2.03	2.26
Isoleucine[3]	3.62	3.32	3.38	3.98	4.22	4.07	4.05
Leucine[3]	5.83	5.86	6.02	6.77	6.87	6.89	7.02
Lysine[3]	6.01	4.86	4.77	5.82	5.97	5.93	5.78
Methionine[3]	2.56	0.91	0.92	1.07	1.27	1.28	1.08
Phenylalanine[3]	3.26	3.48	3.73	4.12	4.31	4.35	4.23
Proline	4.13	4.55	4.68	5.08	5.59	5.51	5.11
Serine	3.53	3.81	4.14	4.30	4.88	4.65	4.55
Tyrosine	3.17	2.24	2.29	2.62	2.46	2.60	2.69
Threonine[3]	3.91	3.29	3.52	3.82	3.67	3.93	3.57
Valine[3]	4.39	4.24	4.26	4.76	5.27	5.03	5.10

1. Average weight 63.28 g.
2. No data is available for the essential amino acid tryptophan because it is destroyed during acid hydrolysis.
3. Essential amino acid.

7.3 RESULTS

7.3.1 ASSESSMENT OF GROWTH AND FEED PERFORMANCE

(Table 7.5, Figures 7.1 to 7.6)

No significant differences were found in SGR, FCR and PER of fish fed any of the diets in this experiment. As the level of SBM in the diet was increased from 220 g/kg (diet 1) to 320 g/kg (diet 2) there was a reduction in performance in the SGR, FCR and PER shown by the fish fed these two diets, although this was not found to be significant. No differences in daily feed consumption on a body weight per day basis were found. The addition of low pH proteases at 0.33 g/kg did not improve the performance of fish given this diet, but feeding diets with 0.66 g/kg and 1.33 g/kg did, both appeared to give higher results for these parameters than did the fish fed diet 1, though not significantly so. Enzyme inclusion at 0.10 g/kg gave a similar fish performance to fish fed diets 2 and 3, and these fish did not perform better in these parameters than fish fed diet 1.

Fish fed diet 4 gave a significantly higher ANPU than fish fed any of the other diets, followed by the ANPU of fish fed diet 6 which in turn was significantly higher than that of fish fed diets 2 and 3.

Feeding fish diets 2 and 6 resulted in significantly higher ANLUs than fish fed the other diets, followed by fish fed diet 4 which in turn gave a significantly higher ANLU than fish fed diets 1 and 3. The EE given by fish fed diet 6 was significantly higher than the EEs given by fish fed any of any other diets, with fish fed diets 2 and 4 in turn giving significantly higher values than fish fed either diet 1, 3 or 5. The lowest value of EE was given by fish fed diet 1.

Table 7.5 Assessment of growth and feed performance in Experiment 5. Data are presented as means with the standard deviation in brackets. Means in a row followed by the same superscript are not significantly different ($P < 0.05$).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Pellet type	Extruded	Extruded	Extruded	Extruded	Extruded	Extruded
Fish meal inclusion (g/kg)	320	260	260	260	260	260
Soybean meal inclusion (g/kg)	220	320	320	320	320	320
Low pH protease (g/kg)	0	0	0.33	0.66	1.00	1.33
Initial weight (g)	90.76^{a} (0.96)	89.74^{a} (0.96)	90.25^{a} (1.46)	90.27^{a} (0.83)	90.00^{a} (0.79)	90.10^{a} (0.58)
Final weight (g)	139.65^{a} (3.11)	133.77^{a} (3.73)	134.87^{a} (5.23)	142.69^{a} (2.51)	136.15^{a} (3.12)	139.93^{a} (3.97)
Specific growth rate (SGR)(%/day)	0.51^{a} (0.03)	0.48^{a} (0.02)	0.48^{a} (0.04)	0.55^{a} (0.03)	0.49^{a} (0.02)	0.53^{a} (0.04)
% Mortalities	0.00^{a} (0.00)	0.00^{a} (0.00)	0.83^{a} (1.67)	0.00^{a} (0.00)	0.00^{a} (0.00)	1.67^{a} (1.92
Food intake (g/100g fish/day)	1.32^{a} (0.02)	1.29^{a} (0.04)	1.30^{a} (0.03)	1.32^{a} (0.04)	1.30^{a} (0.04)	1.30^{a} (0.04)
Food conversion ratio (FCR)	2.16^{a} (0.18)	2.38^{a} (0.17)	2.38^{a} (0.27)	2.06^{a} (0.14)	2.33^{a} (0.10)	2.10^{a} (0.20)
Protein efficiency ratio (PER)	1.02^{a} (0.08)	0.98^{a} (0.06)	0.95^{a} (0.10)	1.11^{a} (0.08)	0.98^{a} (0.04)	1.10^{a} (0.10)
Apparent net protein utilisation (ANPU)(%)	17.47^{abc} (1.31)	16.13^{a} (1.03)	16.94^{ab} (1.64)	22.37^{d} (1.53)	19.22^{bc} (0.79)	20.02^{c} (1.80)
Apparent net lipid utilisation (ANLU)(%)	28.37^{a} (2.45)	42.54^{c} (2.69)	28.68^{a} (3.45)	35.77^{b} (2.64)	31.73^{ab} (1.57)	46.26^{c} (3.04)
Energy efficiency (EE)(%)	18.35^{a} (1.57)	23.46^{b} (1.36)	19.45^{a} (1.92)	24.04^{b} (1.54)	20.71^{a} (0.90)	26.91^{c} (2.11)

Figure 7.1 Assessment of growth and feed performance in Experiment 5: specific growth rate. Bars indicate one standard deviation.

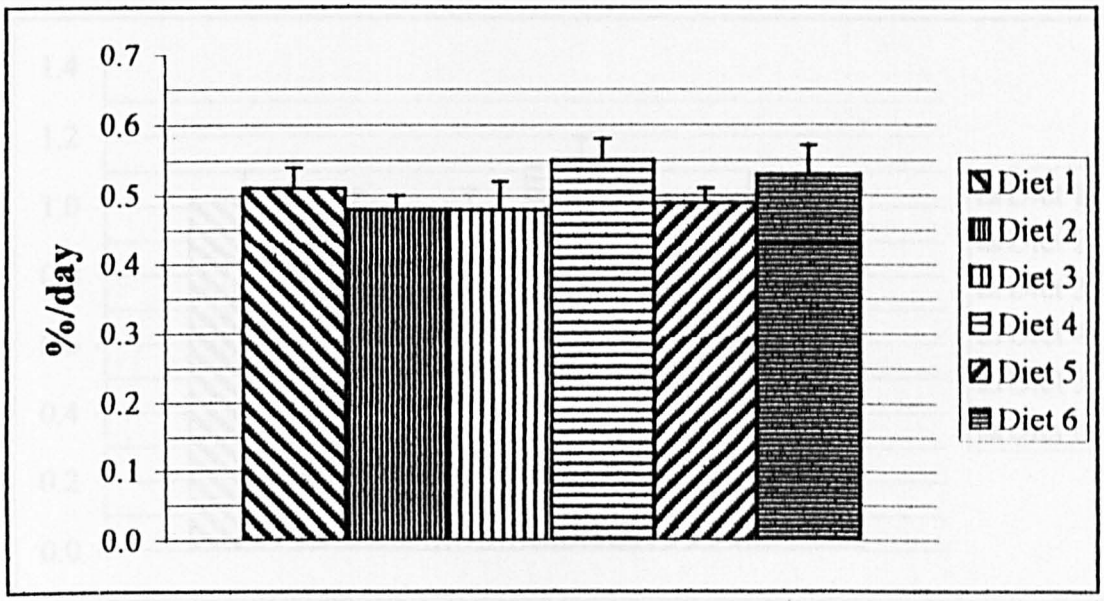


Figure 7.2 Assessment of growth and feed performance in Experiment 5: food conversion ratio. Bars indicate one standard deviation.

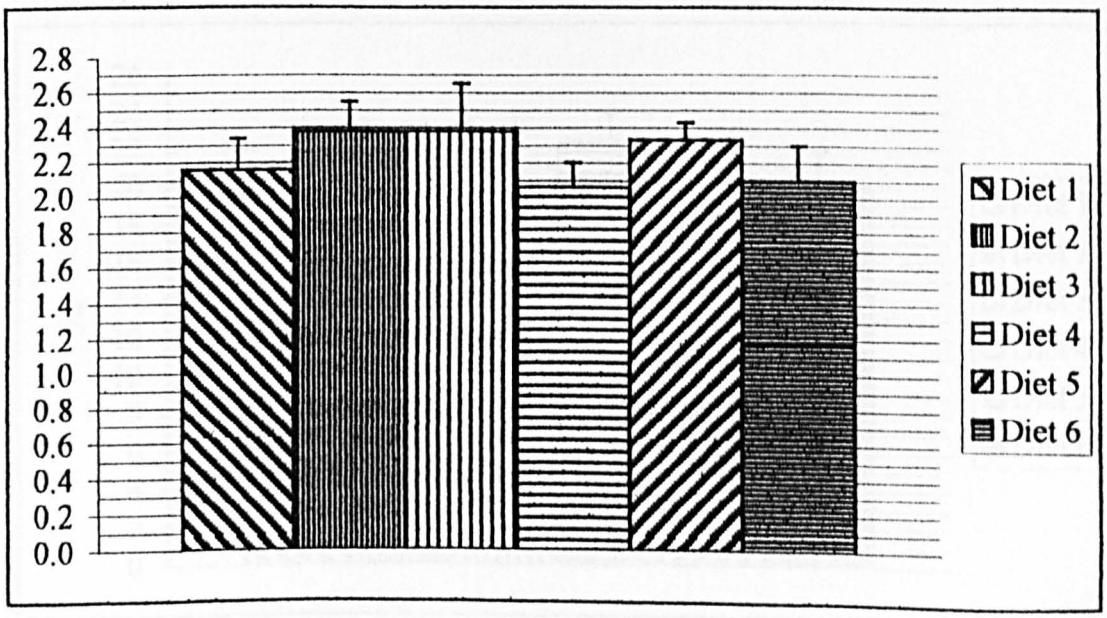


Figure 7.3 Assessment of growth and feed performance in Experiment 5: protein efficiency ratio. Bars indicate one standard deviation.

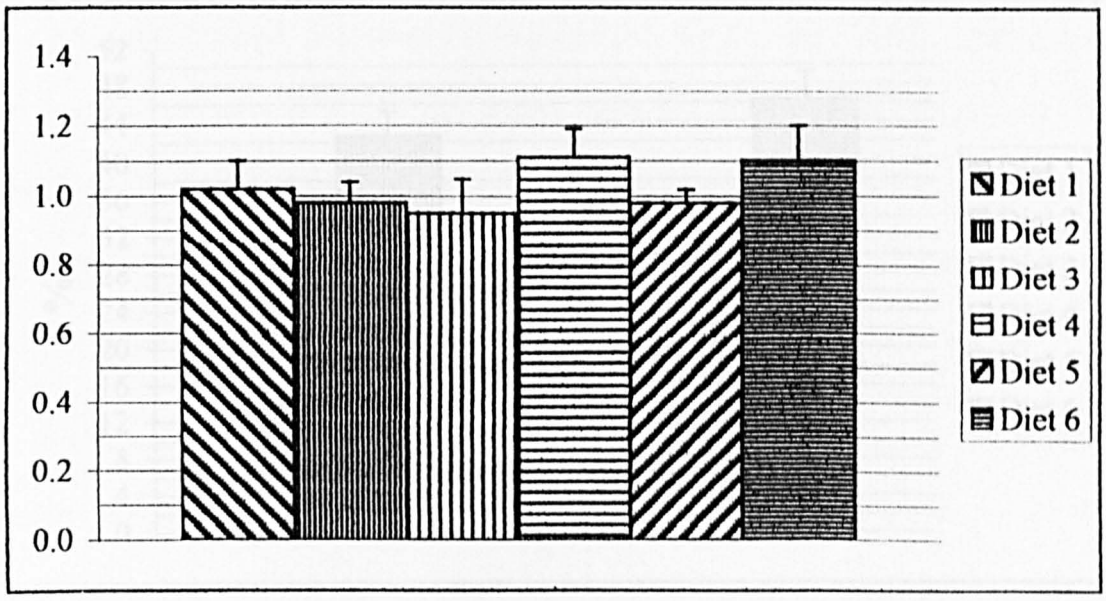


Figure 7.4 Assessment of growth and feed performance in Experiment 5: apparent net protein utilisation. Bars indicate one standard deviation.

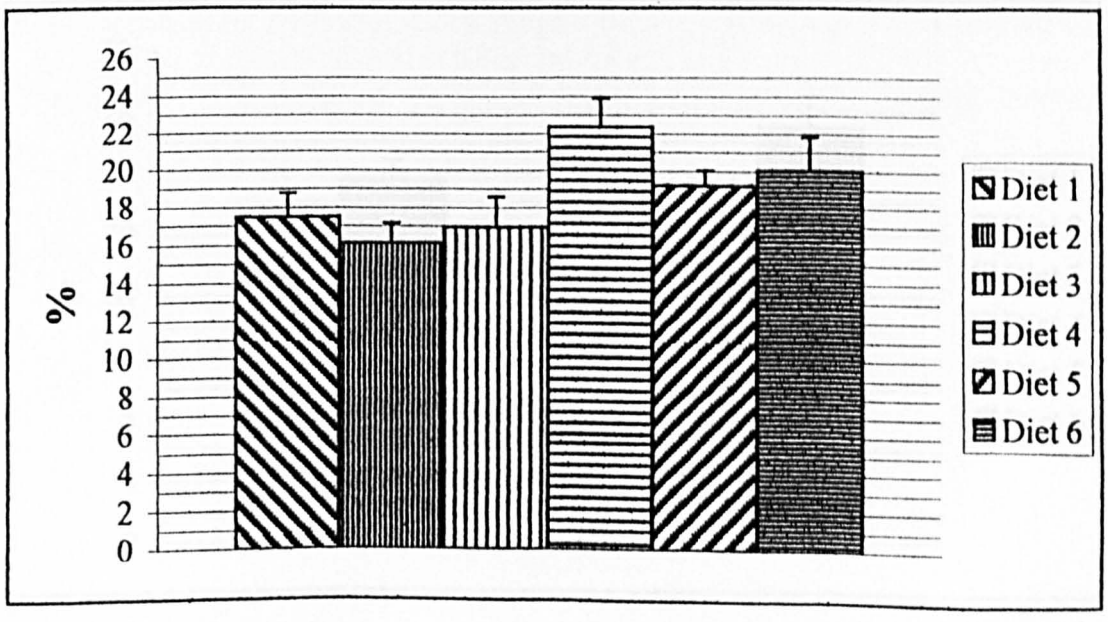


Figure 7.5 Assessment of growth and feed performance in Experiment 5: apparent net lipid utilisation. Bars indicate one standard deviation.

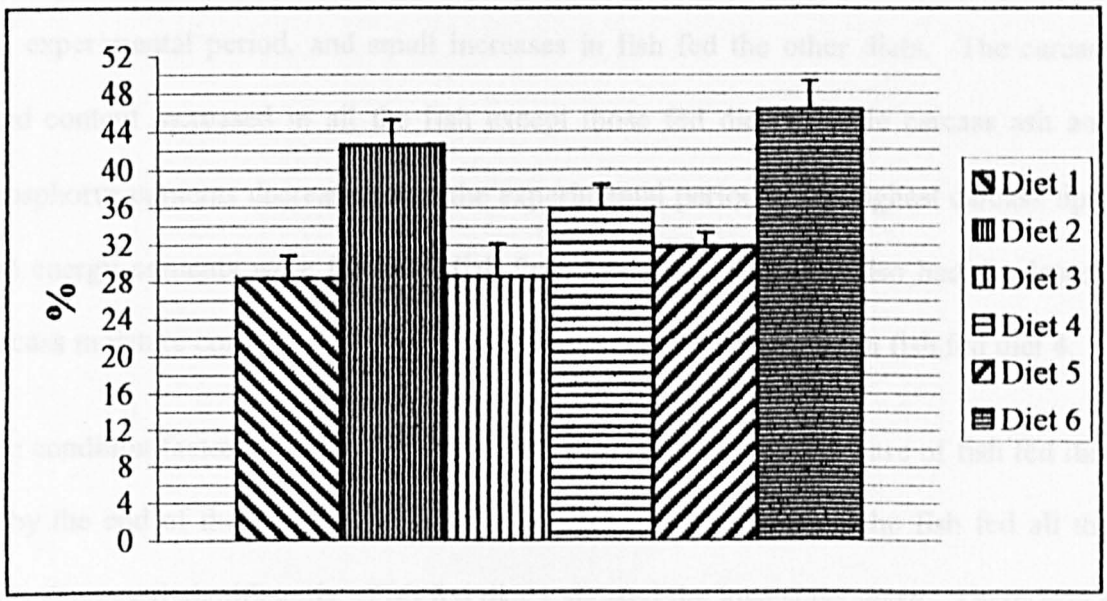
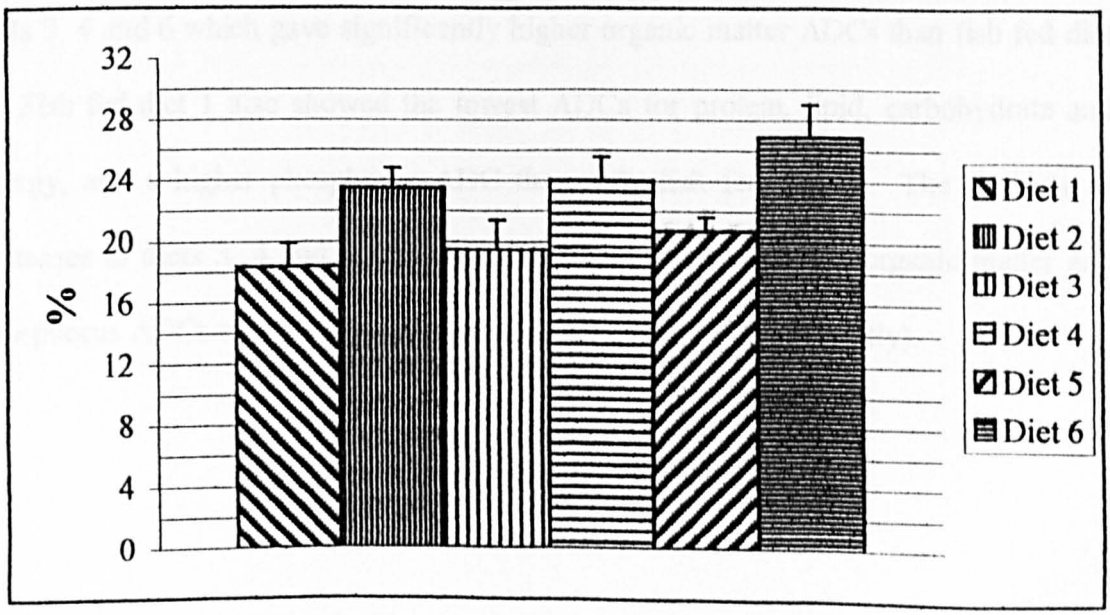


Figure 7.6 Assessment of growth and feed performance in Experiment 5: energy efficiency. Bars indicate one standard deviation.



7.3.2 CARCASS COMPOSITION, CONDITION FACTOR AND HEPATOSOMATIC INDEX (Table 7.6)

There was a small decrease in carcass moisture contents of fish fed diets 2 and 6 over the experimental period, and small increases in fish fed the other diets. The carcass lipid content increased in all the fish except those fed diet 1, while carcass ash and phosphorus contents decreased over the experimental period. The highest carcass lipid and energy contents were found in fish fed diets 2 and 6, which also had the lowest carcass moisture contents and the highest protein content was found in fish fed diet 4.

The condition factor of all the fish increased, significantly so in the case of fish fed diet 4, by the end of the experiment, and the hepatosomatic indices of the fish fed all the diets decreased significantly. Fish fed diet 1 showed the lowest decrease. There was a decrease in HSI going from fish fed diet 3 containing 0.33 g/kg protease to fish fed diet 6 containing 1.33 g/kg protease.

7.3.3 APPARENT DIGESTIBILITY COEFFICIENTS (Table 7.7)

The only significant differences obtained in these analysis was that found in fish fed diets 3, 4 and 6 which gave significantly higher organic matter ADCs than fish fed diet 1. Fish fed diet 1 also showed the lowest ADCs for protein, lipid, carbohydrate and energy, and a higher phosphorus ADC than only fish fed diet 5. The addition of proteases in diets 3, 4 and 6 increased fish protein, carbohydrate, organic matter and phosphorus ADCs over the unsupplemented diet 2 (but not significantly).

Table 7.6 Effect of dietary treatments on the body (whole) composition of fish in Experiment 5. Condition factor and hepatosomatic index are presented as means with the standard deviation in brackets. Means in a row followed by the same superscript are not significantly different ($P < 0.05$).

	Initial	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Pellet type		Extruded	Extruded	Extruded	Extruded	Extruded	Extruded
Fish meal inclusion (g/kg)		320	260	260	260	260	260
Soybean meal inclusion (g/kg)		220	320	320	320	320	320
Low pH protease (g/kg)		0	0	0.33	0.66	1.00	1.33
Moisture (g/100g)[1]	64.73	64.80	63.03	65.56	65.35	65.08	63.15
Protein (g/100g)[1]	16.33	16.64	16.38	16.84	17.70	17.36	17.01
Lipid (g/100g)[1]	14.07	13.38	16.16	14.23	14.37	14.35	16.07
Ash (g/100g)[1]	4.45	3.97	3.74	3.56	3.97	3.51	3.97
Phosphorus (g/100g)[1]	0.81	0.76	0.71	0.58	0.62	0.65	0.75
Energy content (kJ/g)[1]	9.85	9.41	10.32	9.48	9.85	9.81	10.21
Condition factor	1.45^{a} (0.12)	1.51^{ab} (0.09)	1.51^{ab} (0.08)	1.58^{ab} (0.10)	1.59^{b} (0.08)	1.49^{ab} (1.13)	1.55^{ab} (0.16)
Hepatosomatic index	1.90^{b} (0.33)	1.36^{a} (0.40)	1.60^{ab} (0.48)	1.69^{ab} (0.21)	1.67^{ab} (0.26)	1.60^{ab} (0.33)	1.42^{a} (0.26)

1. Values are averages of pooled carcass samples.

Table 7.7 Effect of dietary treatments on apparent digestibility coefficients (ADC) in Experiment 5 calculated using faeces collected during weeks 8 and 12. Data are presented as means with the standard deviation in brackets. Means in a row followed by the same superscript are not significantly different ($P < 0.05$).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Pellet type	Extruded	Extruded	Extruded	Extruded	Extruded	Extruded
Fish meal inclusion (g/kg)	320	260	260	260	260	260
Soybean meal inclusion (g/kg)	220	320	320	320	320	320
Low pH protease (g/kg)	0	0	0.33	0.66	1.00	1.33
Protein ADC (%)	87.83[a] (0.71)	89.33[a] (1.72)	90.90[a] (1.45)	90.26[a] (1.30)	88.37[a] (2.88)	90.91[a] (0.15)
Lipid ADC (%)	81.40[a] (4.68)	85.39[a] (4.10)	84.14[a] (5.72)	85.10[a] (4.66)	85.62[a] (7.54)	88.89[a] (2.76)
Carbohydrate ADC (%)	81.37[a] (1.74)	84.94[a] (2.87)	86.97[a] (3.83)	83.74[a] (0.88)	84.27[a] (1.44)	85.25[a] (1.60)
Energy ADC (%)[2]	85.60[a]	88.15[a]	88.63[a]	88.27[a]	87.38[a]	90.10[a]
Organic matter ADC (%)	78.13[a] (0.77)	81.30[ab] (2.54)	83.69[b] (1.87)	83.81[b] (0.99)	79.73[ab] (2.38)	82.91[b] (1.32)
Phosphorus ADC (%)	51.49[a] (3.12)	52.80[a] (3.67)	54.99[a] (9.11)	57.69[a] (6.02)	44.46[a] (3.93)	54.28[a] (4.96)

1 Energy calculated using the following values: protein, 23.4 kJ/g; lipid, 39.8 kJ/g; carbohydrate, 17.2 kJ/g.

7.4 DISCUSSION

No significant differences were found in SGRs, FCRs and PERs between the fish fed the different diets in this experiment. However, the results for fish fed diets 4 and 6, containing 0.66 g/kg and 1.33 g/kg low pH protease, appeared to be better than those given by fish fed the other diets.

There was a drop in performance as the level of the SBM went up from 220 g/kg (diet 1) to 320 g/kg (diet 2), consistent with what was been found in Experiment 1. A similar set of results were obtained in this trial to that obtained in Experiment 1 with regard to feeding the diets with a different SBM inclusion level, at least in terms of SGR, FCR, PER and ANPU, none of which were significant in both experiments. However, while in Experiment 1 the ANLU and EE of fish fed the 220 g/kg SBM diet were significantly higher than the ANLU and EE of fish fed the 320 g/kg SBM diet, in this experiment, they were significantly lower. The impact of SBM level on the performance of fish has already been seen in the discussion of Experiment 1.

The lack of improvement in performance of fish fed the 0.33 g/kg protease diet 3 over that of fish fed the unsupplemented diet 2 was probably due to the low inclusion level, which was insufficient to bring about changes in SGR and FCR.

A number of authors have reported increases in performance of animals when fed diets containing supplementary enzymes up to a certain inclusion level followed by a decrease in performance with a further increase in enzyme inclusion level, as seen in this experiment. Some of these same authors have also found that if the level of enzymes in the diets is increased even further a rise again in the performance of the experimental animals was seen after the drop, as also seen in this experiment.

Thus, in a trial reported by Collier and Hardy (1986b), feeding growing/finishing pigs diets to which the enzyme cocktail Enzyme FC2 (containing a neutral proteinase, α-amylase and β-glucanase) was added at levels of 0.25, 0.5 and 1.0 g/kg improved performances were obtained compared to pigs fed the unsupplemented diet. However, the performance increased up to the 0.5g/kg enzyme diet but was observed to have dropped again in pigs fed the 1.0 g/kg enzyme diet.

Bedford and Classen (1992a) fed broiler chickens a number of diets containing various combinations of rye and wheat to which 1, 2, 4, 8 and 16 g/kg of a Finnfeeds xylanase and β-glucanase product had been added. In all enzyme supplemented diets the SGR and FCR of the chickens was increased relative to the chickens fed the unsupplemented diets. However, the performance of the chickens depended on the level of rye/wheat in the diet. A number of different trends were seen in the results obtained by these authors. In some cases, performance increased up to one inclusion level beyond which no further improvement in performance of the chickens was observed (as had been seen in Experiment 4). In other cases performance of the chickens beyond the optimum inclusion level actually decreased, and in other cases beyond the optimum inclusion level the performance decreased and then rose again, as was seen in this experiment.

Robinson *et al.* (1996) fed 6.5g catfish, *I. punctatus*, diets to which 500, 1000, 2000 and 4000 Units of phytase/kg had been added. Both SGR and FCR of fish fed the experimental diets were better than fish fed the control diet. However, the results of both these parameters was better going from fish fed the diet containing 500 to fish fed the diet containing 1000 Units/kg, then performance dropped with fish fed the 2000 Units/kg diet and increased slightly again with fish fed the 4000 Units/kg diet.

A number of hypotheses can be put forward as to why the performance of fish fed these diets should follow such a pattern. One of these, explaining the initial drop, is that the

enzymes are present at a concentration at which they release nutrients at such a level as to allow bacteria to multiply and there is a consequential uptake of nutrients by bacteria to the detriment of the host animal. It would be interesting to test this hypothesis using antibiotics with the diets containing supplemental enzymes. Another hypothesis is that the high activity of the enzymes actually releases substances, such as antinutritional factors, which may affect digestion or have other effects on the physiology of the animal.

It is also possible that the activities of the enzymes release nutrients to such an extent that the digestive system or the whole physiology of the organism cannot cope with the immediate availability of the nutrient, for example, sugars in the case of some fish (Steffens, 1989; Tucker, 1992; Wilson, 1994; De Silva and Anderson, 1995). It could be that the actual digestion products of the supplemental enzyme, act as a suppresser or inhibitor of the endogenous enzymes.

The rise again in performance has been even more difficult to explain. One idea is that the enzymes release positive factors from the ingested food, although why it should occur at the higher inclusion level and not before is unclear. The high concentration of enzymes might be attacking the dead bacterial cell walls in the gut releasing substances which might be having a beneficial effect on the host organism in the long run. Again, it would be interesting to test the effect of using antibiotics in conjunction with the supplementary enzymes.

The results from such studies indicate the importance of enzyme dose, and that if the correct inclusion is not used the results obtained might be misleading and lead to wrong conclusions. There is still a great deal to be done.

The possibly negative effect of an incorrect dose of an enzyme could partially explain the results in Experiment 3 for fish fed diets with high pH protease alone and α-

galactosidase alone compared to the unsupplemented diet. Using combinations of enzymes complicates matters even further, since each enzyme would have its 'optimum' inclusion level and if not at the correct level could influence the overall effect seen of the fish fed the diet containing this combination of enzymes. The 'wrong' inclusion levels of the α-galactosidase might have been the reason why it had a negative effect on the performance of fish fed diets 4 and 5 in Experiment 4.

The results of this experiment indicate that the reaction of the fish to enzymes added to extruded fish feed was different to their reaction when enzymes were added to pressed feeds as seen in Experiment 4.

These results only go to show that further research is required to determine what is actually happening when supplementary enzymes are added to diets, and that dose response experiments should be given importance in this respect.

CHAPTER 8

LIVER HISTOLOGY

8.1 INTRODUCTION AND AIMS OF THIS INVESTIGATION

As has been reviewed extensively in Section 1.4.2, SBM (as well as many other plant ingredients) contain numerous antinutritional factors which not only reduce the utilisation of ingested food but may simultaneously cause changes in the digestive tract and associated organs. Such changes may vary from disruption of the brush border to degenerative changes in the liver and pancreas of terrestrial animals.

In the few studies carried out in fish has been found that diet composition can cause changes to one extent or another in the histology of the digestive tract (MacLeod, 1978; Burkhardt and Storch, 1989; Van den Ingh *et al.*, 1991, 1996; Murray *et al.*, 1996) and associated organs (Moscone-Bac, 1990; Margulies, 1993; Robaina *et al.*, 1995).

In order to investigate whether variations in dietary level of SBM or enzyme addition bring about any histological changes in the liver of gilthead sea bream, samples were taken from fish used in Experiments 1, 3, 4 and 5. In this analysis, the position of the nuclei in the hepatocytes and the presence of fat globules around pancreatic tissue was observed.

8.2 MATERIALS AND METHODS

The intestines and related tissues of fish sampled in the experiments 1, 3, 4 and 5 for tissue histology, including the liver, were placed into fixative for at least 72 hours. The fixative used was neutral phosphate buffered formalin (100 mL 41% formaldehyde, 900 mL distilled water, 4 g sodium dihydrogen phosphate monohydrate, 6.5 g disodium hydrogen phosphate anhydrous).

Tissues were processed in an automatic tissue processor (Shandon Citadel 1000), followed by embedding in paraffin wax (50°C)(Shandon Histocentre 2). Section cutting was carried out on a rotary microtome (Shandon M1) with a thickness setting of 4 μm. Staining was carried out using Haematoxylin (Harris) and Eosin. The slides were eventually viewed and photographed using a Zeiss Axiophot Photomicroscope.

Liver sections were processed and observations of the positions of the nuclei in the cells were made, with the following classification used: N for normal, indicating that the nuclei were randomly placed in all the cells of the tissue section, G when the nuclei of all the cells in the section were situated eccentrically as if organised in small groups, and R for regional, indicating that regions of the liver tissue had cells with grouped nuclei and other regions had a normal distribution of nuclei. The pancreatic tissue was divided into three groups depending on the relative size of the tissue present, either small, medium or large. The presence of fat globules around the pancreatic tissue was noted and the relative quantity present given a value ranging from 0 (none present) to 3.

Photographs were taken to illustrate these differences.

8.3 RESULTS (Table 8.1, Plates 8.1 to 8.4)

Table 8.1 summarises the results of the histological analysis indicating the organisation of the nuclei within the hepatocytes and the level of fat present around the pancreatic tissue in the liver. Plates 8.1 to 8.4 show the differences in nuclei distribution that were observed and the relative quantities of fat deposits seen around the different sizes of pancreatic tissue present in the liver sections taken from the experimental fish.

Table 8.1 Summary of results of histological examination of livers. The data presents the observed position of nuclei within cells and the relative amount of fat globules present (0, 1 to 3, indicating increasing size) and the size of hepatopancreatic tissue around which the fat globules were seen.

Experiment	Diet/ Day	Nuclei Position	Small Pancreatic tissue	Medium Pancreatic tissue	Large Pancreatic tissue
1	Day zero	Normal	0	0	0
1	1	Normal	0	1, 2	1, 2
1	2	Normal	0	0	0
1	3	Normal	0	0	1, 2, 3
1	4	Normal	0	1, 2, 3	1
1	5	Normal	0	0	0
1	6	Normal	0	3	3
1	7	Normal	0	1	1
3	Day zero	Grouped	0	1	0
3	1	Grouped	0	1	0
3	2	Grouped	0	0	1, 2, 3
3	3	Regional	0	1	1
3	4	Regional	0	0	3
3	5	Regional	0	0	0
3	6	Regional	0	3	3
3	7	Regional	0	0	0
4	Day zero	Regional	0	0	1, 2, 3
4	1	Regional	0	1, 2, 3	1, 2, 3
4	2	Grouped	0	1, 2, 3	1, 2, 3
4	3	Normal	0	1, 2	1, 2
4	4	Normal	0	1, 2, 3	0
4	5	Normal	0	1	0
4	6	Grouped	0	0	1
4	7	Regional	0	0	1, 2
5	Day zero	Grouped	1, 2	1, 2	1, 2
5	1	Grouped	0	0	0
5	2	Grouped	0	0	0
5	3	Grouped	1, 2, 3	1, 2, 3	1, 2, 3
5	4	Grouped	0	1, 2, 3	1, 2, 3
5	5	Grouped	0	1	0
5	6	Grouped	1, 2, 3	1, 2, 3	1, 2, 3

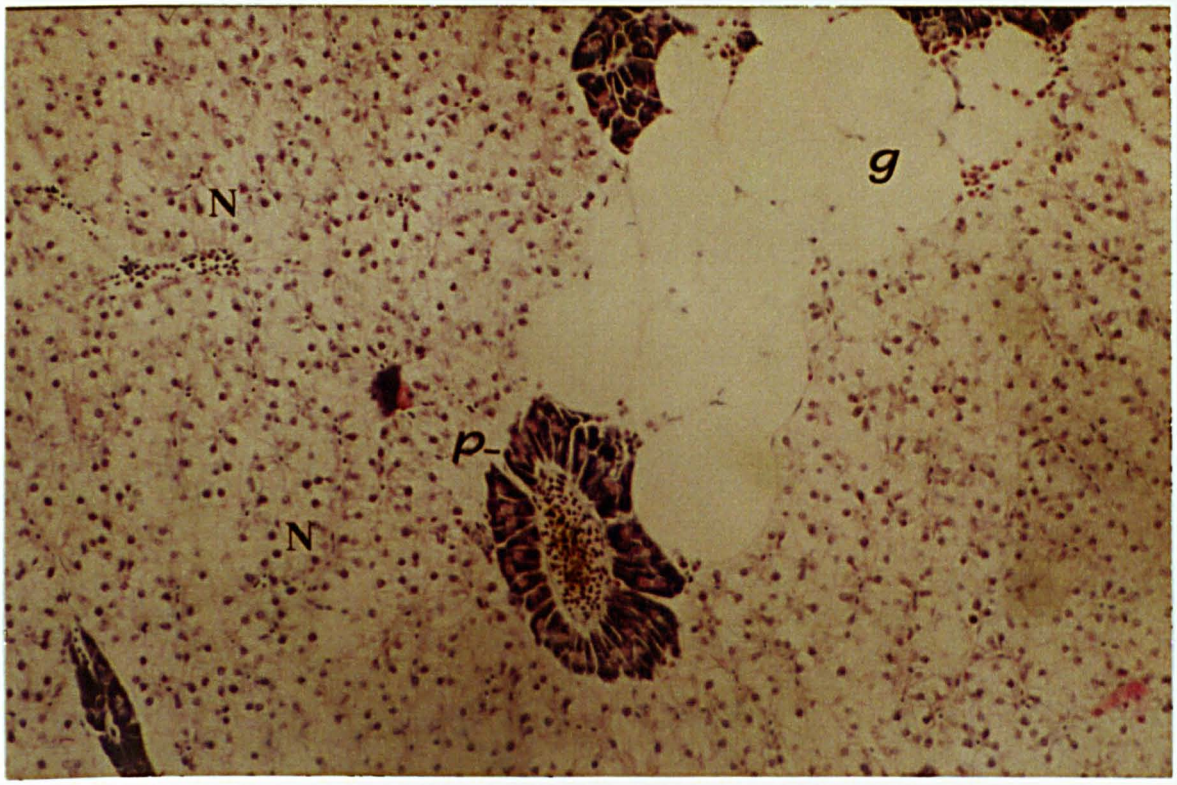


Plate 8.1 Photomicrograph of liver section at a magnification of x200 showing Normal (N) distribution of nuclei within the hepatocytes and the presence of fat globules (g) at a relative quantity of 3 around a piece of pancreatic tissue (p).

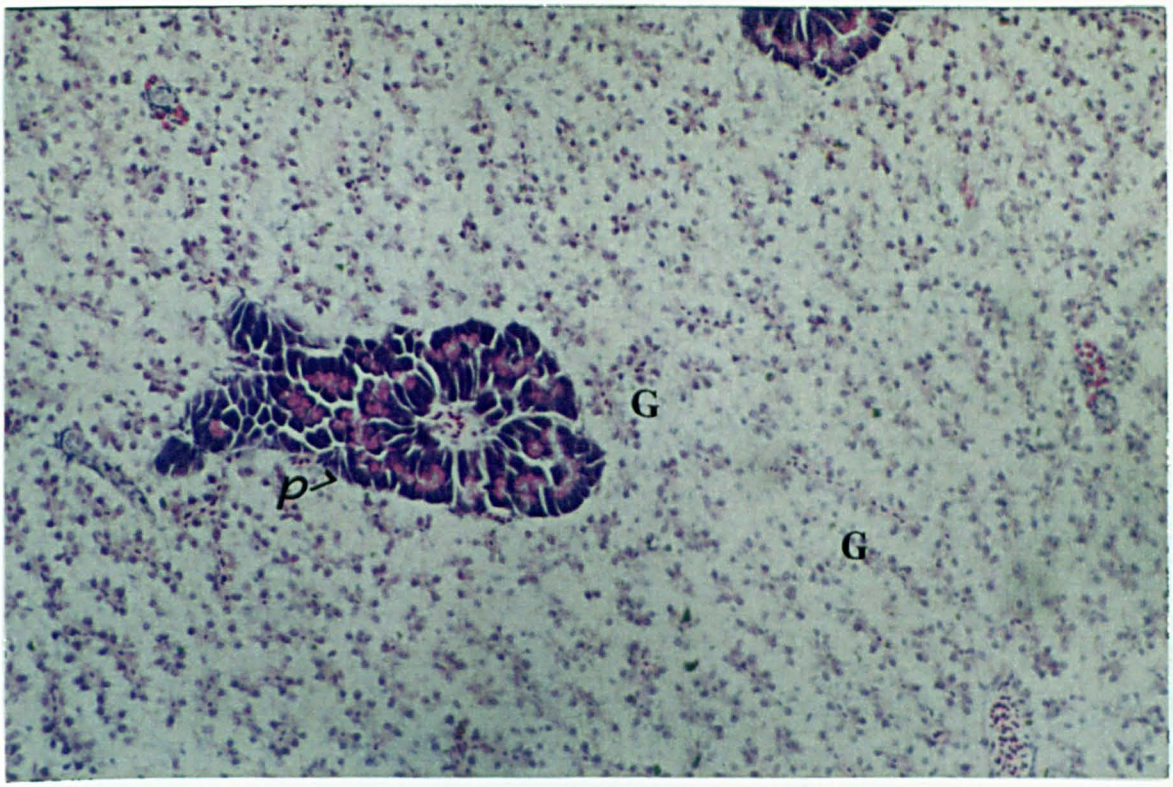


Plate 8.2 Photomicrograph of liver section at a magnification of x200 showing organised Groups (G) of nuclei within the hepatocytes. No fat globules are present around the small sized pancreatic tissue (p) seen here.

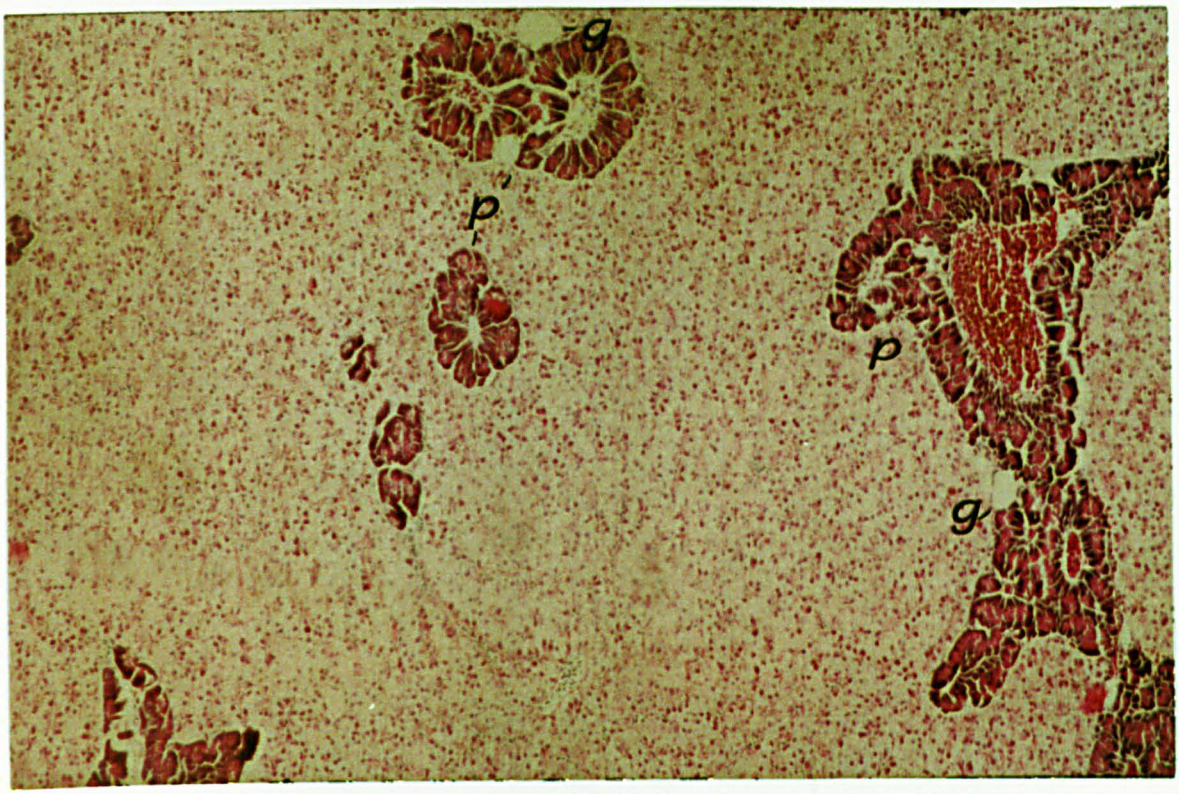


Plate 8.3 Photomicrograph of liver section at a magnification of x100 showing different sizes of pancreatic tissue (p) defined as small, medium and large. Fat globules (g) are present at a relative quantity of 1 around the medium and large sized pancreatic tissue.

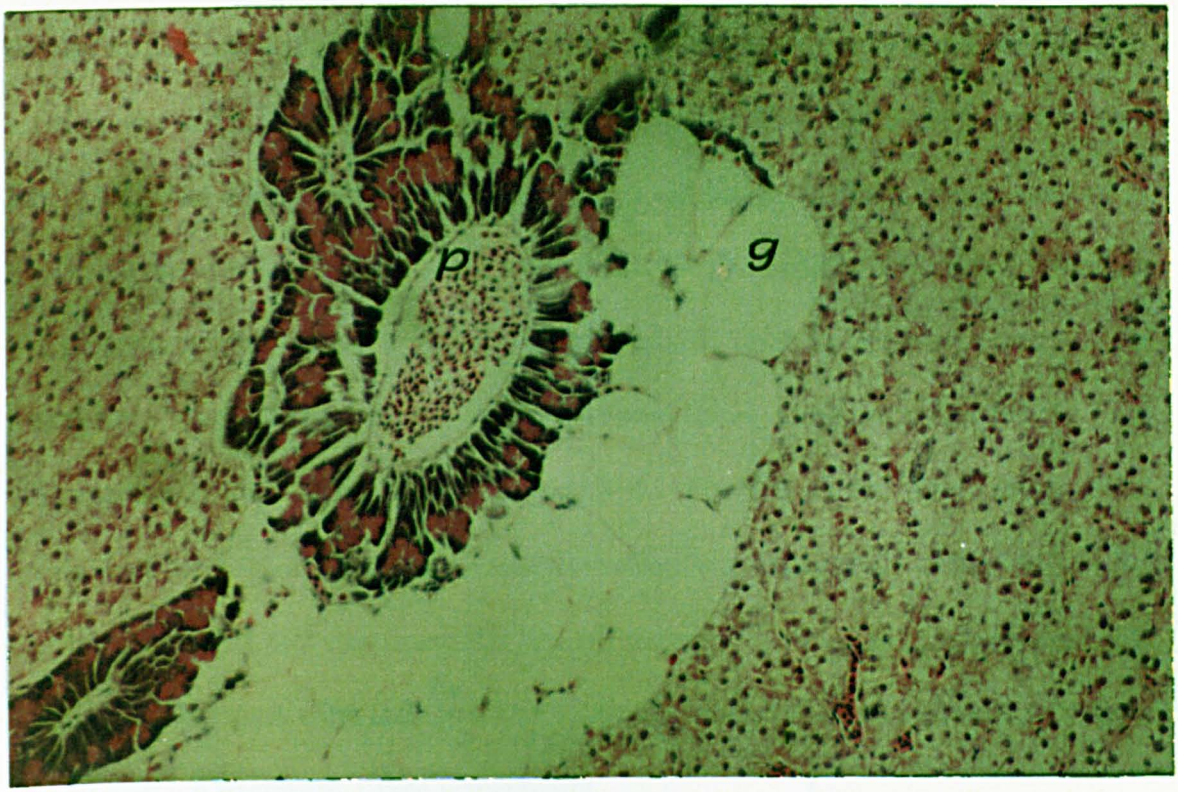


Plate 8.4 Photomicrograph of liver section at a magnification of x200 showing a large deposit of fat globules (g), at a relative quantity of 3, around a piece of medium-sized pancreatic tissue (p).

8.3.1 EXPERIMENT 1

No differences in nuclear position within the cells was observed for the fish fed any of the diets. Fat globules were observed around the medium and large pancreatic tissue areas, but none in the small pancreatic tissue. No fat globules were observed in fish at day zero. The livers of fish fed the 220 g/kg SBM diet 1 contained small and medium sized groups of fat, while the livers of fish fed the higher SBM diet 2 and 5 did not have any at all. The inclusion of enzymes at both of the higher soybean levels, resulted in the laying down of fat globules in the liver of fish fed these diets. With fish fed the low pH protease cocktail added to the 320 g/kg SBM diet (diet 3), the whole range of fat group sizes were observed around large pancreatic tissue, while with fish fed the high pH protease cocktail added to the 320 g/kg SBM diet (diet 4), the whole range of fat group sizes were this time observed around medium sized pancreatic tissue. In fish fed the 440 g/kg SBM diet containing the low pH protease and high pH protease cocktails (diets 6 and 7 respectively) large and small deposits were laid down around the medium and large-sized pancreatic tissue respectively.

8.3.2 EXPERIMENT 3

In this experiment, there were differences in the organisation of the nuclei in the cells of the fish livers. Fish at day zero and after feeding diets 1 and 2 had nuclei which were arranged in such a way that they seemed to be grouped and not in a randomised, unorganised way. In these fish this organisation was found all over the liver section. Fish fed the other diets (diets 3 to 7) also exhibited this organisation in the liver but only in part of the liver tissue and not all over the section.

No fat globules were found around the small pancreatic tissue patches in the livers of fish fed any of the diets. Addition of low pH protease to the unsupplemented diet 1 (diet 4) resulted in the presence of large amounts of fat globules around some large pancreatic tissue, but none around middle or small sized tissue, in fish fed this diet. In fish fed diet 3, containing the high pH protease, a small quantity of fat was also found around the middle and large sized pancreatic tissue. Supplementation of α-galactosidase alone (diet 5), and its combination with low pH protease (diet 7) actually reduced the amount of fat present in the livers of fish fed these diets, but its combination with the high pH protease in diet 6 resulted in the presence of large amounts of fat globules around both middle and large sized pancreatic tissue of fish. Feeding of the extruded diet 2 increased the level of fat globules deposited around large pancreatic tissue, although none was seen around the middle-sized tissue as compared to the livers of fish fed the pressed diet containing the same enzymes (diet 7).

8.3.3 EXPERIMENT 4

Quite a variation in nuclear organisation was found in the liver sections of fish from this experiment. While day zero fish showed a regional organisation of nuclei, as did fish fed diet 1, feeding of higher low pH protease levels, diets 2 and 3, resulted in a completely grouped organisation of nuclei in the liver and a normal random distribution of nuclei respectively. Addition of α-galactosidase to the pressed protease diets (4 and 5) reduced the level of organisation of the nuclei in fish fed these diets, as did addition of low pH protease to the extruded diet (diet 7).

Day zero fish had all sizes of fat globules around large pancreatic tissue only. Feeding the fish 0.5 and 1.0 g/kg low pH protease diets (diets 1 and 2) led to the presence of all

sizes of fat globules around middle and large sized pancreatic tissue. Feeding fish the 1.5 g/kg low pH protease diet 3 resulted in small and medium-sized fat globules around both medium and large pancreatic tissue. Addition of α-galactosidase (diets 4 and 5) eliminated the presence of fat globules around the large pancreatic tissue of fish fed these diets and only small fat globules were seen in the fish fed the 1 g/kg low pH protease cocktail diet (diet 5) in the middle-sized pancreatic tissue. In fish fed the unsupplemented extruded diet 6 only small groups of fat were seen around the large pancreatic tissue in the livers. The livers of fish fed the enzyme supplemented extruded diet 7 had small and medium sized fat deposits around the large pancreatic tissue.

8.3.4 EXPERIMENT 5

There were no differences in the organisation of the nuclei in the livers of fish taken from day zero or in the livers of fish after feeding of the experimental diets, all exhibiting grouped nuclei.

Day zero fish had small and medium sized fat deposits around all sizes of pancreatic tissue in their livers. No fat deposits were observed in the livers of fish fed the unsupplemented diets 1 and 2, and fish fed the 1g/kg low protease diet 5 only had small deposits around medium sized pancreatic tissue. On the other hand, addition of enzymes at other inclusion levels caused increases in the incidence of fat globules. Addition of both 0.33 g/kg and 1.33 g/kg protease to the 320 g/kg SBM diet (diets 3 and 6 respectively) resulted in the deposition of all sizes of fat deposits around all sizes of pancreatic tissue in fish fed these diets. In the case of fish fed the 0.66 g/kg protease diet 4, the effect was similar but with no deposit around small pancreatic tissue.

8.4 DISCUSSION

The histology carried out in this study was very limited due to time restrictions. In fact, slides of the stomach, pyloric caeca and intestines had been prepared, but insufficient time was available to carry out a proper study of these tissues. The histology carried out in this analysis was only limited to a small number of liver sections from fish fed each of the diets and did not cover analysis of whole liver sections.

In the analysis carried out in this trial with fish from Experiments 1, 3, 4 and 5, various amounts of fat globules were also seen around the pancreatic tissue in the livers. However, from the results of Experiment 1 and 5, there was no increase in the amount of fat globules when fish were fed diets containing higher amounts of SBM, and if anything there was actually a decrease when fish fed diet 1 in Experiment 1 are compared to fish fed diets 2 and 5. It should be noted that although such deposits were present, they were not present to any great extent in any of the liver tissues examined.

There was no apparent correlation between the inclusion of the enzymes on the presence or absence of these fat globules, nor any correlation between the presence of these fat globules and the carcass lipid content or hepatosomatic index of the fish fed a particular diet.

Another interesting observation which was made in this set of trials was the positioning of the nuclei in the hepatocytes. In some fish the nuclei were found more or less randomly distributed around the cells, while in others they were all eccentrically positioned, with the nuclei of adjacent cells seemingly 'grouped'. In yet other fish the nuclei in one part of the tissue examined were randomly positioned and in others they were 'grouped'. Again, no correlation between SBM level or enzyme inclusion and the distribution of nuclei was made out.

Although no relationships could be made out in this experiment between the lipid morphology observed and dietary SBM level or enzyme inclusion, other authors have observed differences in histology in both the liver and parts of the digestive tract.

When Robaina *et al.* (1995) fed 40 g gilthead sea bream, *S. aurata*, diets in which 0, 100, 200 and 300 g/kg SBM were included (770, 690, 610, 540 g/kg FM respectively), a greater number of lipid droplets around the pancreatic tissue were observed in the liver of fish fed the SBM diets and eccentric located nuclei were present when the level of SBM in the diet was equal to or higher than 200 g/kg.

Van den Ingh *et al.* (1991) fed Atlantic salmon, *S. salar*, a diet containing either herring FM and diets in which part of the herring fish meal had been replaced with either full fat SBM (300 g/kg, 350 g/kg FM) or soybean protein concentrate (280 g/kg SBM, 260 g/kg FM). The proximal intestine of fish fed the diet containing the soybean concentrate showed little differences compared to the fish fed the high FM diet, but in the distal intestine, the fish fed the diet containing full fat SBM showed marked changes in morphology compared to the fish fed the high FM diet.

Van den Ingh *et al.* (1996) fed Atlantic salmon, *S. salar*, a number of diets containing SBM heat treated to different degrees (260 g/kg SBM, 400 g/kg FM). All fish fed the SBM containing diets exhibited alternations in the distal intestine compared to fish fed a diet containing 730 g/kg FM.

The literature proposes conflicting views of what the position of nuclei in the cell should be. The photographs provided by Robaina *et al.* (1995) showed nuclei in the liver of sea bream in the 'grouped' position, which they took as 'normal'. According to Amin *et al.* (1992) the 'normal' teleost hepatocyte has a central nucleus. Margulies (1993) defined healthy first-feeding and late larval scombrid hepatocytes as those

containing distinct nuclei which were often displaced, and healthy late larval and early juvenile scombrid hepatocytes as having clear and laterally situated nuclei.

Unfortunately, is the analysis carried out in these experiments no time was available to determine whether the vacuoles in the hepatocytes were glycogen or lipid. Margulies (1993) defined healthy first-feeding and late larval scombrid hepatocytes as those containing abundant intracellular vacuoles and healthy late larval and early juvenile scombrid hepatocytes as having very large intracellular vacuoles, often occupying most of the intracellular area. Robaina *et al.* (1995) observed an increased deposition of lipid and decreased glycogen deposits in the liver of gilthead sea bream, *S. aurata*, with increased levels of SBM and found areas with high levels of hepatocyte vacuolisation and disorganisation in some samples from fish fed the 300 g/kg SBM diet. Hibiya (1982) stated that fairly large quantities of lipid and glycogen are observed in the cytoplasm. Ando *et al.* (1993) studied the livers of five species of fish (obtained from the local fish market) and found that the hepatocytes of each fish contained different amounts of intracellular lipid droplets.

The literature cited above and the results obtained in this experiment indicate that different fish exhibit different effects in relation to the presence of lipid, and unknown factors influence the normal structure of the liver.

CHAPTER 9

Relative activities of a number of digestive enzymes in the alimentary canal of the gilthead sea bream, *Sparus aurata*.

9.1 INTRODUCTION AND AIMS OF THIS INVESTIGATION

Although a large number of investigations have been carried out to determine the presence of particular enzymes in the guts of numerous fish (see Section 1.6), the number of investigations carried out which actually compare the activities of different enzymes in different parts of the gut is quite limited (Fish, 1960; Hsu and Wu, 1979; Buddington and Doroshov, 1986; Das *et al.*, 1987; Uys and Hecht, 1987; Fagbenro, 19909; Sabapathy and Teo, 1993; Chakrabarti *et al.*, 1995; Ojeda and Caceres, 1995). The results of these authors has shown that the enzymes of different fish have different relative activities depending on the part of the gut being studied.

Although a number of digestive enzymes have been identified as present in the digestive tract of gilthead sea bream (see Section 1.8.4), no investigation has been carried out to determine the relative activities of various digestive enzymes in the different sections of the digestive tract.

In this investigation, the first of its type in the gilthead sea bream, the digestive tract was divided into five parts, consisting of the stomach, phyloric caecae, and three equally sized portions of the intestine, and the relative activities of 6 enzymes, pepsin, trypsin, chymotrypsin, carboxypeptidases A and B and amylase determined.

9.2 MATERIALS AND METHODS

9.2.1 SAMPLE PREPARATION

The fish fed diet 1 in Experiment 5 were used in this analysis. Prior to sampling the fish were not fed for 24 hours. At each sampling 4 fish were killed using ice and dissected immediately. The different sections of the intestines of these fish were pooled for the analyses (see Figure 1.1). The stomachs, pyloric caecae and the three equally divided parts of the intestine of the four fish were separated, blotted, weighed and homogenised in aqueous suspension (1:10, wet weight/volume ice-cold distilled water) and the resulting solution was then centrifuged in a Hermle ZK510 centrifuge at 4000 revs/min (3750 x g) at 4°C for 30 minutes (Das *et al.*, 1987; Fagbenro, 1990; Sabapathy and Teo, 1995). The supernatants were separated and kept in a refrigerator (4°C) until used. Fresh samples of enzyme extracts were prepared daily. All analyses were carried out in triplicate. The methods used were standard analyses for each of the enzymes but which had not been optimised for the gilthead sea bream.

Preliminary tests were carried out for each of the enzymatic analysis during which various dilutions of the supernatents were tested to determine which gave the highest changes in absorbance in the given time within the absorbance range of the instrument. These properly diluted sample solutions were then used for the rest of the analysis.

9.2.2 PEPSIN

This analysis was carried out at 15, 20, 25 and 30°C, with the enzyme solutions being incubated at the appropriate temperature for 15 minutes prior to initiating the reaction in a digital water bath. The reaction was initiated by adding 0.5 mL of properly diluted enzyme solution to previously incubated 2.5 mL of 2% casein (Sigma C0376) in 0.06M

HCl, pH 1.8 (Rick and Fritsch, 1974; Hsu and Wu, 1979). After exactly 10 minutes incubation, the reaction was stopped by the addition of 5.0 mL 5% trichloroacetic acid. The solutions were left to stand for one hour and then centrifuged at 3000 revs/min (2100 x g) for 30 minutes. The absorbance of the supernatant was then taken at 280 nm (Perkin Elmer UV/VIS Spectrophotometer Lambda 2). Activity was expressed as change in absorbance/min/g tissue.

9.2.3 TRYPSIN

All solutions were incubated at 25°C. 0.2 mL properly diluted sample solution was added to 6 mL of 0.00104M N_{α}-p-toluenesulfonyl-L-arginine methyl ester (TAME, Sigma T4626) in tris buffer (1.47 g $CaCl_2.2H_2O$ dissolved in 200 mL 0.2M tris(hydroxymethyl)aminomethane diluted to 1 L, pH 8.1)(Hummel, 1959). The absorbance at 247 nm was recorded and taken again after 10 minutes exactly. Activity was expressed as the change in absorbance/min/g tissue.

9.2.4 CHYMOTRYPSIN

All solutions were incubated at 25°C. 0.2 mL properly diluted sample solution was added to 6 mL of 0.0005M N-benzoyl-L-tyrosine ethyl ester (BTEE, Sigma B6125) in tris buffer (10.55 g $CaCl_2.2H_2O$ dissolved in 250 ml 0.2M tris(hydroxymethyl)aminomethane, adjusted to pH 7.8, diluted to 1 L, and 432 mL methanol added)(Hummel, 1959). The absorbance at 254 nm was recorded and taken again after 10 minutes exactly. Activity was expressed as the change in absorbance/min/g tissue.

9.2.5 CARBOXYPEPTIDASE A

All solutions were incubated at 25°C. 0.2 mL properly diluted sample solution was added to 6 mL of 0.001M hippuryl-L-phenylalanine (Sigma H6875) in tris buffer (3.025 g tris(hydroxymethyl)aminomethane and 29.25 g NaCl in 1 L, pH 7.5)(Folk and Schirmer, 1963). The absorbance at 254 nm was taken immediately and after 10 minutes exactly. Activity was expressed as the change in absorbance/min/g tissue.

9.2.6 CARBOXYPEPTIDASE B

All solutions were incubated at 25°C. 0.2 mL properly diluted sample solution was added to 6 mL of 0.001M hippuryl-L-arginine (Sigma H6625) in tris buffer (3.025 g tris(hydroxymethyl)aminomethane and 5.85 g NaCl in 1 L, pH 7.6)(Folk *et al.*, 1960). The absorbance at 254 nm was recorded and taken again after 10 minutes exactly. Activity was expressed as the change in absorbance/min/g tissue.

9.2.7 AMYLASE

All solutions were incubated at 25°C. 1 mL of properly diluted enzyme was incubated for 3 minutes with 1 mL 1% starch (1 g soluble starch (Sigma S2630) and 0.035 g NaCl in 100 mL 0.02M Na_3PO_4, pH 6.9)(Berfeld, 1951). The reaction was stopped by addition of 2 mL 3,5-dinitrosalicylic acid reagent. The solution was then heated for 5 minutes in boiling water, cooled, and 20 mL distilled water added. The absorbance at 540 nm was read and a standard curve was established with maltose (Sigma M5885), to convert readings into milligrams of maltose. Activity was expressed as mg maltose liberated/min/g tissue.

9.2.8 STATISTICAL ANALYSIS

Data from the three replicates for each treatment were combined to provide the data for the statistical analysis. Homogeneity of variances between samples was tested using the Levene's test (Dixon *et al.*, 1988). Multiple comparisons between means were made using the Student-Newman-Keuls test. In the case where a homogeneity of variances was not found, the nonparametric Kruskal-Wallis test was performed. The significance levels of the tests was taken as 0.05.

9.3 RESULTS (Table 9.1, Figures 9.1, 9.2)

No pepsin activities were detected in the pyloric caecae or any part of the intestine at any temperature except for a comparatively low activity at 30°C. The activities of pepsin in the stomach were in fact significantly different from the pepsin activities of the other parts of the intestine, whatever the temperature. Pepsin activity of the stomach increased with temperature. The activity of the stomach pepsin more than doubled with a 10°C rise in temperature.

Trypsin activity at 25°C was the highest in the upper intestine, followed by the activity in the pyloric caecae, the former being significantly higher than the activity in the stomach, which was the lowest observed. On the other hand, chymotrypsin activity was highest in the pyloric caecae, which was double the activity of the next active regions, which were the middle and upper intestine. These latter two regions gave only slightly higher chymotrypsin activities than both the stomach and lower intestine.

The carboxypeptidase A activity of the upper intestine was higher than the activity in the pyloric caecae, which in turn had a higher activity than the middle intestine and

Table 9.1 Activity of some digestive enzymes in the gut of *Sparus aurata*. Data are presented as means with the standard deviation in brackets. Means in a row followed by the same superscript are not significantly different ($P < 0.05$).

	Stomach	Pyloric caeca	Upper Intestine	Middle Intestine	Lower Intestine
Pepsin (ΔAbs/min/g tissue) at 15°C	2.06^{b} (0.84)	0.00^{a} (0.00)	0.00^{a} (0.00)	0.00^{a} (0.00)	0.00^{a} (0.00)
Pepsin (ΔAbs/min/g tissue) at 20°C	3.46^{b} (1.14)	0.00^{a} (0.00)	0.00^{a} (0.00)	0.00^{a} (0.00)	0.00^{a} (0.00)
Pepsin (ΔAbs/min/g tissue) at 25°C	5.27^{b} (1.77)	0.00^{a} (0.00)	0.00^{a} (0.00)	0.00^{a} (0.00)	0.00^{a} (0.00)
Pepsin (ΔAbs/min/g tissue) at 30°C	7.87^{b} (3.06)	0.11^{a} (0.10)	0.06^{a} (0.08)	0.07^{a} (0.06)	0.08^{a} (0.08)
Trypsin (ΔAbs/min/g tissue) at 25°C	0.53^{a} (0.11)	3.35^{ab} (0.99)	3.97^{b} (0.61)	1.61^{ab} (0.16)	1.08^{ab} (0.13)
Chymotrypsin (ΔAbs/min/g tissue) at 25°C	2.00^{a} (0.35)	4.81^{a} (2.47)	2.27^{a} (0.43)	2.31^{a} (0.34)	1.97^{a} (0.12)
Carboxypeptidase A (ΔAbs/min/g tissue) at 25°C	0.49^{a} (0.20)	1.42^{ab} (0.59)	1.98^{b} (0.15)	0.75^{ab} (0.42)	0.51^{ab} (0.22)
Carboxypeptidase B (ΔAbs/min/g tissue) at 25°C	0.70^{ab} (0.44)	1.21^{b} (0.15)	0.74^{ab} (0.36)	0.51^{ab} (0.12)	0.35^{a} (0.28)
Amylase (mg maltose liberated/min/g tissue at 25°C	0.74^{a} (0.47)	3.58^{b} (0.49)	2.67^{b} (1.69)	3.38^{b} (1.79)	3.65^{b} (1.43)

Figure 9.1 Pepsin activity in different parts of the digestive tract of the gilthead sea bream at different incubation temperatures Bars indicate one standard deviation.

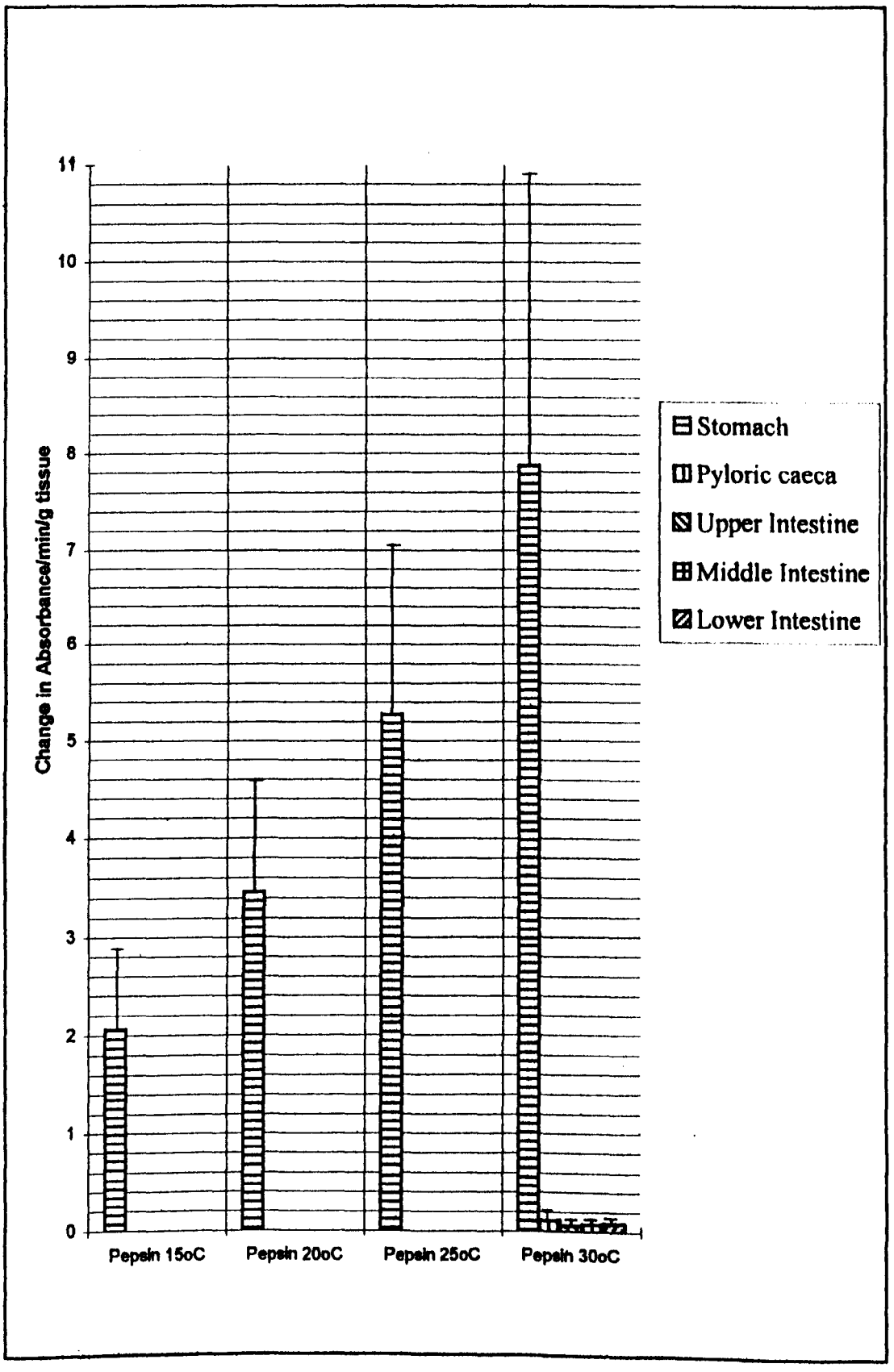
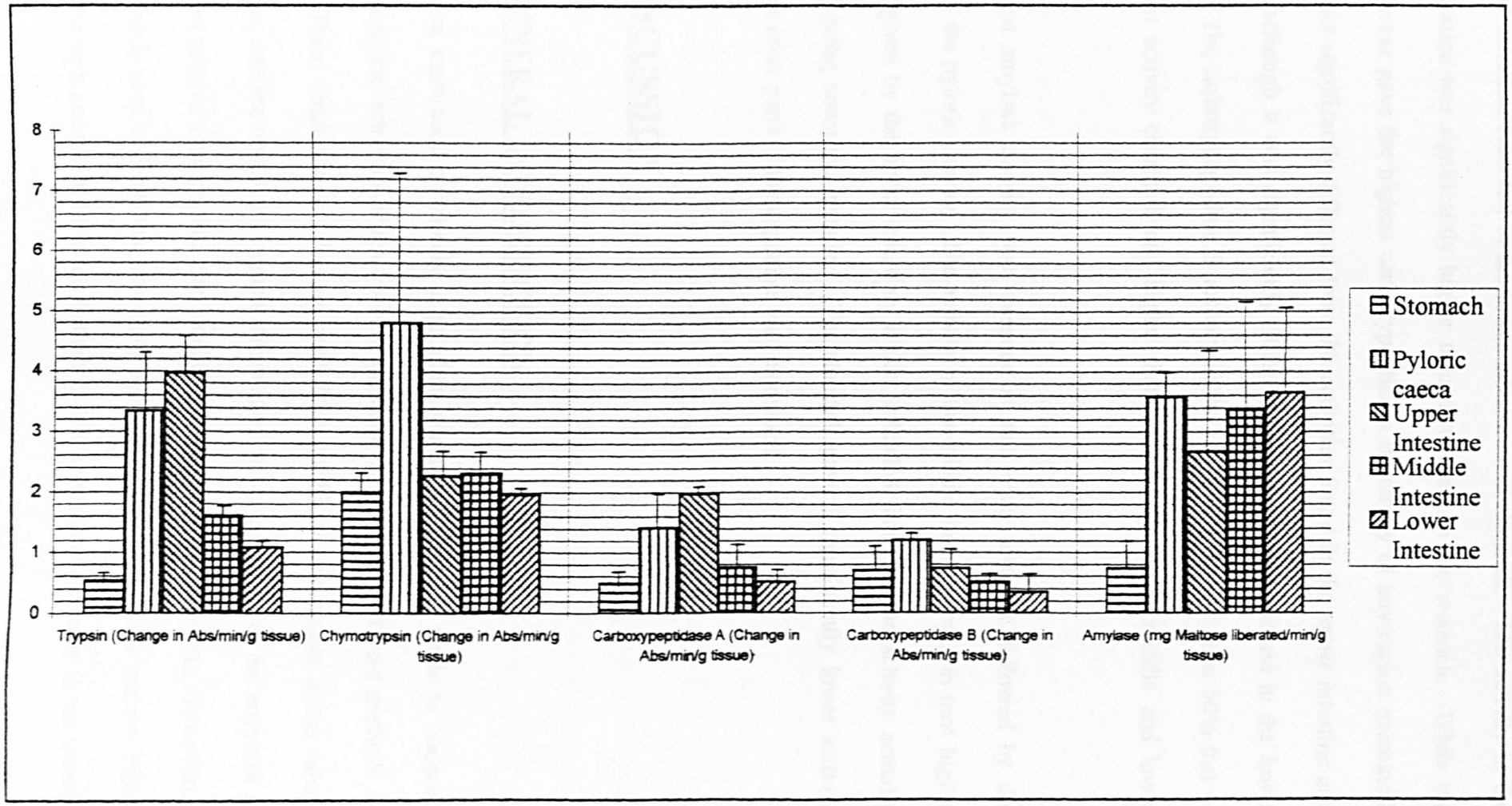


Figure 9.2 Trypsin, chymotrypsin, carboxypeptidase A and B, and amylase activities in different parts of the digestive tracts of the gilthead sea bream at 25°C. Bars indicate one standard deviation.



finally the activities recorded in the lower intestine and stomach. The activity in the upper intestine was significantly higher than that found in the stomach. While the pyloric caecae gave the highest carboxypeptidase B activity of any region examined, this was not significantly different from the activities found in the upper intestine and stomach, although it was significantly different from the activity found in the lower intestine. The carboxypeptidase B activity in the stomach was more than 50% that of the highest activity obtained, and higher than the activity in the middle and lower intestine.

The highest amylase activity was recorded in the lower intestine followed by the activity in the pyloric caecae. The activity in the middle intestine was in turn higher than that given by the upper intestine, which indicated that amylase activity actually increased going down the intestine. The stomach gave a significantly lower activity than all the other parts of the digestive tract examined.

9.4 DISCUSSION

9.4.1 GENERAL INTRODUCTION

As has been mentioned previously, a problem when an attempt is made to compare digestive enzyme activities arises because different authors use different methods of analysis. These variations in technique make it very difficult to compare actual values of activities, and the trends and comparative results in the activities of the enzymes in the different parts of the digestive tract shall be considered in the following discussion. That the trends seen in these analyses agree with those of some authors and not others confirms the high amount of variation in the relative activities of enzymes in the various

parts of the digestive tract of different fish. It is also evident from these results and those in the literature that certain enzymes are not necessarily found only in one particular part of the digestive tract but may be quite widely distributed. Clearly, different fish exhibit different enzyme profiles.

9.4.2 PEPSIN ACTIVITY

As would be expected from the general digestive physiology, the pepsin activity observed in the sea bream was limited to the stomach. A low pepsin activity was detected in the other parts of the intestine when the assay was carried out at 30°C, but this may just have been 'pepsin-like' activity rather than pepsin activity.

The lack or limited activity of pepsin in all other parts of the intestine other than the stomach was also found by Fish (1960) in perch, *P. fluviatilis* and *T. mossambica*, Buddington and Doroshov (1986) in the white sturgeon, *A. transmontanus*, and Chakrabarti *et al.* (1995) in *Channa striatus*. Hsu and Wu (1979) found the same trend in *Anguilla japonica*, *Channa maculatus* and *Clarias fuscus*.

However, a number of authors have found that other parts of the intestine showed a comparable pepsin activity to that found in the stomach. Fagbenro (1990) found that the duodenal part of the gut of *Clarias isheriensis* showed more than 65% of the activity found in the stomach. No pepsin activity was found in the rest of the gut. Sabapathy and Teo (1993) found pepsin activity in the oesophagus and intestine which was more than 66% and 25% respectively that of the activity in the stomach of sea bass, *L. calcarifer*.

On the other hand, Sabapathy and Teo (1993) found that the pepsin activity in the oesophagus, intestine and pyloric caecae of the rabbitfish, *S. canaliculatus* was actually higher than that of the stomach by 670, 175 and 58% respectively. This was also found

to be the case in *T. mossambica* by Hsu and Wu (1979) with the stomach pepsin activity only reaching 9% that found in the rest of the intestine. *Notopterus notopterus*, *Aristichthys nobilis*, *Labeo calbasu*, *Hypophthalmichthys molitrix*, *Labeo rohita*, *Oreochromis niloticus*, *Cyprinus carpio* and *Puntius javanicus* all had higher pepsin activities in the oesophagus or parts of the rest of the intestine (which was divided into three equal parts)(Chakrabarti *et al.*, 1995) than did the stomach.

9.4.3 TRYPSIN ACTIVITY

The highest trypsin activity found in the sea bream was in the upper part of the intestine followed by the activity found in the pyloric caecae, which was quite similar to that of the upper intestine. As expected, the lowest activity was found in the stomach, and the trypsin activity decreased along the length of the intestine.

The intestines of *Anguilla japonica*, *Channa maculatus* and *Tilapia mossambica* showed much higher activities than did the stomachs (Hsu and Wu, 1979), while the stomach of *Clarias fuscus* had 40% of the activity found in the intestines, and the pyloric caecae of *Channa maculatus* showed 60% of the activity found in the intestines. Both rabbitfish, *S. canaliculatus*, and sea bass, *L. calcarifer* showed much lower trypsin activities in the stomach than in the intestines (Sabapathy and Teo, 1993), but while in the rabbitfish the activity in the pyloric caecae was only slightly higher than that found in the stomach, in the sea bass it was half that found in the rest of the intestines. Practically no trypsin activity was found in the stomachs of white sturgeon, *A. transmontanus* (Buddington and Doroshov, 1986), *Clarias gariepinus* (Uys and Hecht, 1987) and *Clarias isherensis* (Fagbenro, 1990). While Fagbenro (1990) found a higher trypsin activity in the upper part of the intestine, Uys and Hecht (1987) found a higher

activity in the lower parts of the intestine, followed by the upper part of the intestine and then the pyloric caecae.

9.4.4 CHYMOTRYPSIN ACTIVITY

For this enzyme, the highest activity was detected in the pyloric caecae which was more than double that of the next highest active regions which were the upper and middle intestines. Both the stomach and the lower intestine showed activities which were only slightly lower than that of the upper and middle intestines.

Very little chymotrypsin activity was found in the stomachs of sea bass, *L. calcarifer* (Sabapathy and Teo, 1993), *C. gariepinus* (Uys and Hecht, 1987) and white sturgeon, *A. transmontanus*. Uys and Hecht (1987) found that chymotrypsin activity decreased going along the length of the intestine in *Clarias gariepinus*, with even less activity than in the lower part of the intestine found in the pyloric caecae. The rabbitfish, *S. canaliculatus*, had a higher chymotrypsin activity in the intestine which was more than double that in the pyloric caecae, which was 40% higher than that found in the stomach (Sabapathy and Teo, 1993). Higher activities than in the pyloric caecae and stomachs of *Anguilla japonica*, *Channa maculatus*, *Clarias fuscus*, *Tilapia mossambica* and *Ctenopharyngodon idellus* were also found in the intestines by Hsu and Wu (1979). On the other hand, in the sea bass, *L. calcarifer*, the chymotrypsin activity of the pyloric caecae was 33% higher than that found in the rest of the intestine (Sabapathy and Teo, 1993), and the lower part of the intestine of *Clarias isheriensis* was found to contain a higher chymotrypsin activity than the upper part of the intestine and stomach (Fagbenro, 1990).

9.4.5 CARBOXYPEPTIDASES A AND B ACTIVITIES

The highest activity of these enzymes was found in the upper intestine and pyloric caecae respectively. In the case of carboxypeptidase A, although the activity decreased along the length of the intestine, the activity found in the pyloric caecae was higher than that found in the other parts of the intestine, and the activity found in the stomach was almost equal to that found in the lower intestine. The activity of carboxypeptidase B also decreased along the length of the intestine, but the activity found in the stomach was almost as high as that found in the upper intestine and higher than that found in the other parts of the intestine.

The occurrence of both carboxypeptidases in the stomach of the sea bream was unlike what was found in the work of Buddington and Doroshov with white sturgeon, *A. transmontanus*, in which no carboxypeptidase A or B activities were found in the stomach of this fish. These authors found a higher carboxypeptidase A but lower carboxypeptidase B activities in the spiral valve than in the intestine (no further sub-divisions were made) of this fish.

9.4.6 AMYLASE ACTIVITY

The amylase activity was found to increase going down the intestine, with the highest activity being found in the lower intestine, slightly higher than the activity found in the pyloric caecae. Amylase activity was also found in the stomach though much lower than found in the upper intestine.

Uys and Hecht (1987) observed a decrease in activity in catfish, *C. gariepinus*, going down the intestine, although amylase activity in the pyloric caecae was the highest and very low activity was found in the stomach. Ojeda and Caceres (1995) also found a decrease in amylase activity going down the intestine and no activity in the stomach of

Aplodactylus punctatus. Fish (1960) found a slightly higher amylase activity in the stomach of *Tilapia mossambica* than the lower intestine but lower than the activity found in the upper intestine. Fagbenro (1990) found the highest amylase activity in the stomach of *Clarias isheriensis*, followed closely by the activity in the upper intestine. Sabapathy and Teo (1993) found a higher amylase activity in the intestine of rabbitfish, *S. canaliculatus* than in the pyloric caecae and stomach, but a slightly higher activity in the pyloric caecae than in the intestine of sea bass, *L. calcarifer* (no activity was detected in the stomach).

Das *et al.* (1987) also found a slightly higher amylase activity in the pyloric caecae of mullet, *L. parsia*, than in the upper intestine. Amylase activity in the stomach was found by these authors to be only slightly lower than that in the lower intestine. Similar amylase activities were found in the pyloric caecae, upper and lower intestine of the perch, P. *fluviatilis*, which were higher than the activity found in the stomach.

In their investigations of amylase activity in a number of fish Chakrabarti *et al.* (1995) found quite a mixed set of results. In *Channa striatus* the highest activity was detected in the lower part of the intestine, with activity decreasing going up the intestine, as found in the analysis carried out in this work. In *Notopterus notopterus*, *Aristichthys nobilis*, *Catla catla*, *Labeo calbasu*, *Cirrhinus mrigala* and *Puntius javanicus* the highest activity was found in the middle intestine. In *Oreochromis niloticus* and *Labeo rohita* the highest activity was found in the oesophagus and only in the *Hypophthalmichthys molitrix* and *Cyprinus carpio* was the highest activity found in the upper intestine. In quite a number of these fish the activity in the stomach was actually higher than that found in some of the other parts of the intestine.

CHAPTER 10

pH variation in the digestive tract of the gilthead sea bream, *Sparus aurata*, after being fed one or two meals.

10.1 INTRODUCTION AND AIMS OF THIS INVESTIGATION

As in the case of relative enzyme distributions in the guts of fish (Chapter 9), but more so, the number of studies carried out investigating the variations of pH in fish guts after feeding is very limited although pH optima of a number of digestive enzymes have been determined by some authors (see Section 1.6). No information was found in the literature describing studies carried out on the pH in various parts of the digestive tract of gilthead sea bream.

Analysis of pH and the variations in the various parts of the gut occurring after feeding is important when the activity of the digestive enzymes in the various parts of the digestive tract are considered. Enzymes to be added as supplements to feeds should be selected according to the conditions in the digestive tract of the target animal in order to maximise activity.

Knowledge of the pH variations existing in the guts of fish fed artificial feeds is important when supplementary enzymes are to be used. In this investigation the digestive tract of the gilthead sea bream was divided into four parts and the variation in pH followed in fish fed once or twice with a commercial extruded diet.

10.2 MATERIALS AND METHODS

96 fish were acclimated to a feeding rate of 1.3%, using diet 1 of Experiment 5, divided in two feeds at 0830 and 1430, and then starved for 48 hours prior to refeeding on the day the analysis was carried out. The fish were divided into two groups, the first group being killed (in groups of 6 fish) in lethal anaesthetic (0.6 mL/L 2-phenoxyethanol) at

two hourly intervals until 2030 without being given the second feed. The second group were given the first feed and also the second feed, after which 6 fish were killed at 2 hourly intervals in lethal anaesthetic (0.6 mL/L 2-phenoxyethanol) until 2030. Samples from both groups were taken again at 0830 of the second and third day, with an additional sample being taken from the second group at 1430 of the second day.

Sampled fish were immediately dissected. The intestine was divided into three equal parts, not including the rectum, using pieces of thin thread. An additional tie was made between the stomach and its point of attachment to the intestine. pH readings were taken from the stomach and the three intestinal regions. pH was measured using a Reagecon Series GC Glass pH Micro Combination Electrode (GCMF 11-100, 4 mm tip) on a Jenway pH meter at 21°C after calibration with standard buffer solutions. The pH probe was inserted into the middle of the section being studied and the stable reading recorded.

10.3 RESULTS (Table 10.1, Figures 10.1 to 10.4)

When fish were given only one feed the stomach pH decreased after feeding down to a low of 2.6 after 8 hours before rising and falling again. With two feedings, there was an increase in pH immediately upon feeding again with a decrease similar to that seen in the single fed fish to a minimum of 2.5 after 12 hours followed by a similar increase in pH and then a dip. The final recorded pH of the stomach of fish fed twice was 4.5, higher than the pH of the fish fed only once, which was 3.5.

In the upper intestine there was a slight decrease in pH by 2 hours after feeding. The pH then went up again to decrease slightly again at 8 hours before rising above pH 7 to 7.6 at the last reading taken. The second feeding delayed the 8 hour decrease recorded

Table 10.1 Variation in pHs with time along the digestive tract of *Sparus aurata* after feeding one or two meals. Data are presented as means with the standard deviation in brackets.

Hour	Fish fed at:	Average Fish weight (g)	Stomach	Upper Intestine	Middle Intestine	Lower Intestine
		pH				
0	0830	167.83 (22.86)	5.52 (0.09)	6.78 (0.36)	7.15 (0.41)	6.93 (0.24)
2	0830	158.00 (19.36)	4.68 (0.45)	6.47 (0.22)	7.13 (0.23)	7.07 (0.53)
4	0830	145.67 (10.69)	3.94 (0.47)	6.57 (0.47)	7.58 (0.36)	7.59 (0.40)
6	0830	158.83 (34.50)	3.03 (0.74)	6.62 (0.76)	7.59 (0.30)	7.80 (0.28)
8	0830	150.17 (30.01)	2.55 (0.41)	6.55 (0.67)	7.47 (0.38)	7.73 (0.29)
10	0830	150.83 (19.19)	4.90 (1.76	6.75 (0.42)	7.24 (0.23)	7.52 (0.33)
12	0830	152.17 (13.17)	5.08 (1.46)	7.06 (0.64)	7.73 (0.51)	7.71 (0.46)
24	0830	160.00 (22.30)	3.59 (1.64)	7.29 (0.46)	7.54 (0.23)	7.23 (0.26)
48	0830	152.67 (21.08)	3.48 (0.88)	7.52 (0.57)	7.87 (0.47)	7.59 (0.48)
Fish fed again						
6	0830, 1430	162.00 (24.73)	4.81 (0.88)	6.64 (0.23)	7.48 (0.18)	7.59 (0.39)
8	0830, 1430	150.67 (32.81)	4.34 (0.84)	6.63 (0.19)	7.22 (0.28)	7.64 (0.22)
10	0830, 1430	146.50 (19.52)	3.81 (0.84)	6.58 (0.13)	7.26 (0.28)	7.67 (0.28)
12	0830, 1430	146.67 (24.21)	2.53 (0.96)	6.53 (0.33)	7.57 (0.22)	7.92 (0.24)
24	0830, 1430	176.17 (34.54)	3.40 (1.66)	6.87 (0.31)	7.31 (0.31)	7.46 (0.38)
30	0830, 1430	142.00 (32.01)	4.64 (1.92)	7.76 (0.38)	8.06 (0.27)	7.55 (0.52)
48	0830, 1430	166.83 (21.61)	4.51 (1.46)	6.85 (0.07)	7.42 (0.39)	7.18 (0.51)

Figure 10.1 pH variation in the stomach of gilthead sea bream after one and two feeds. Two sets of readings are presented: the continuous line indicates the set of readings taken after fish were given the first feed; the broken line indicates the readings taken only after fish were given the second feed 6 hours after the first feed.

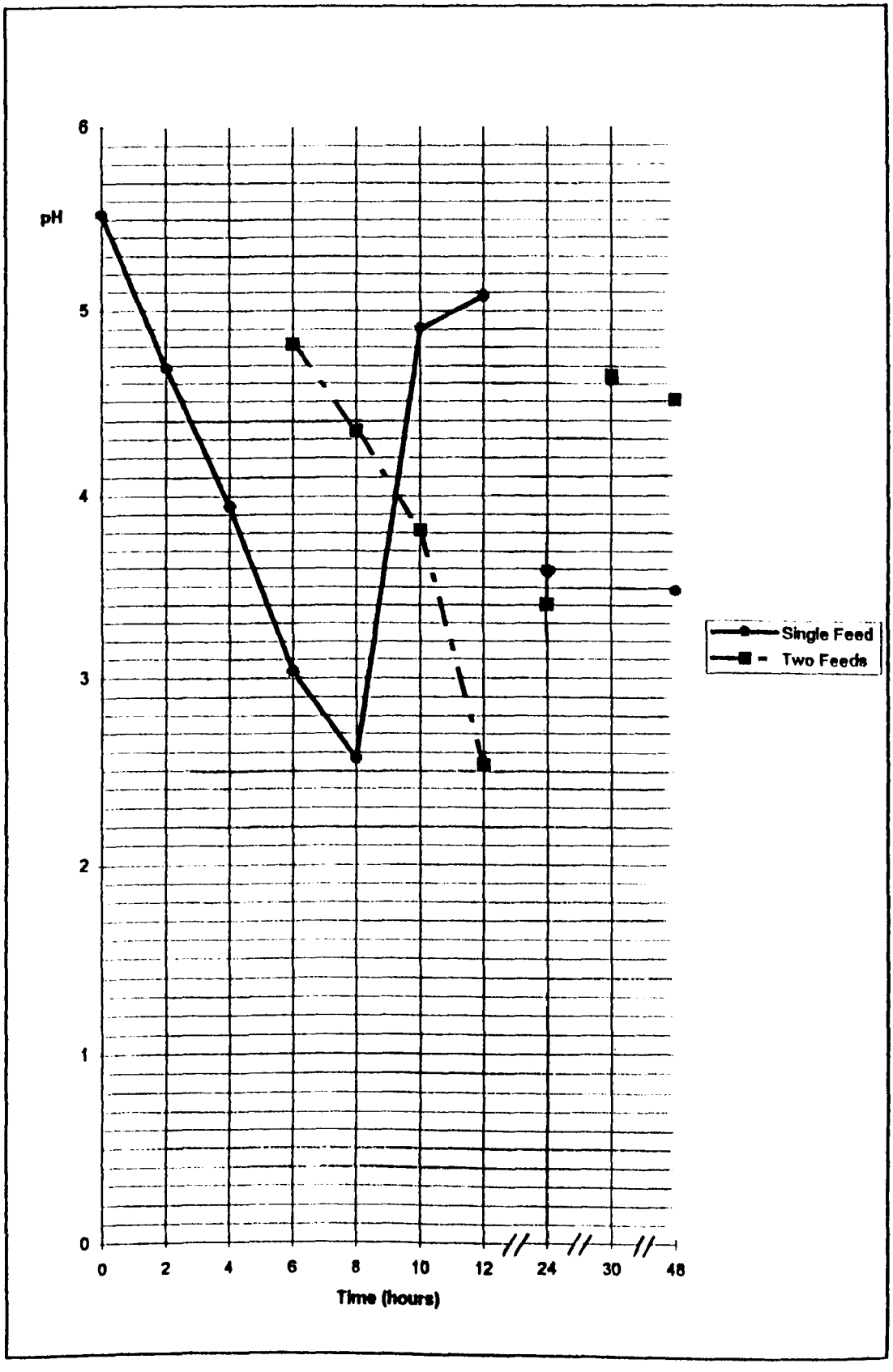


Figure 10.2 pH variation in the upper intestine of gilthead sea bream after one and two feeds. Two sets of readings are presented: the continuous line indicates the set of readings taken after fish were given the first feed; the broken line indicates the readings taken only after fish were given the second feed 6 hours after the first feed.

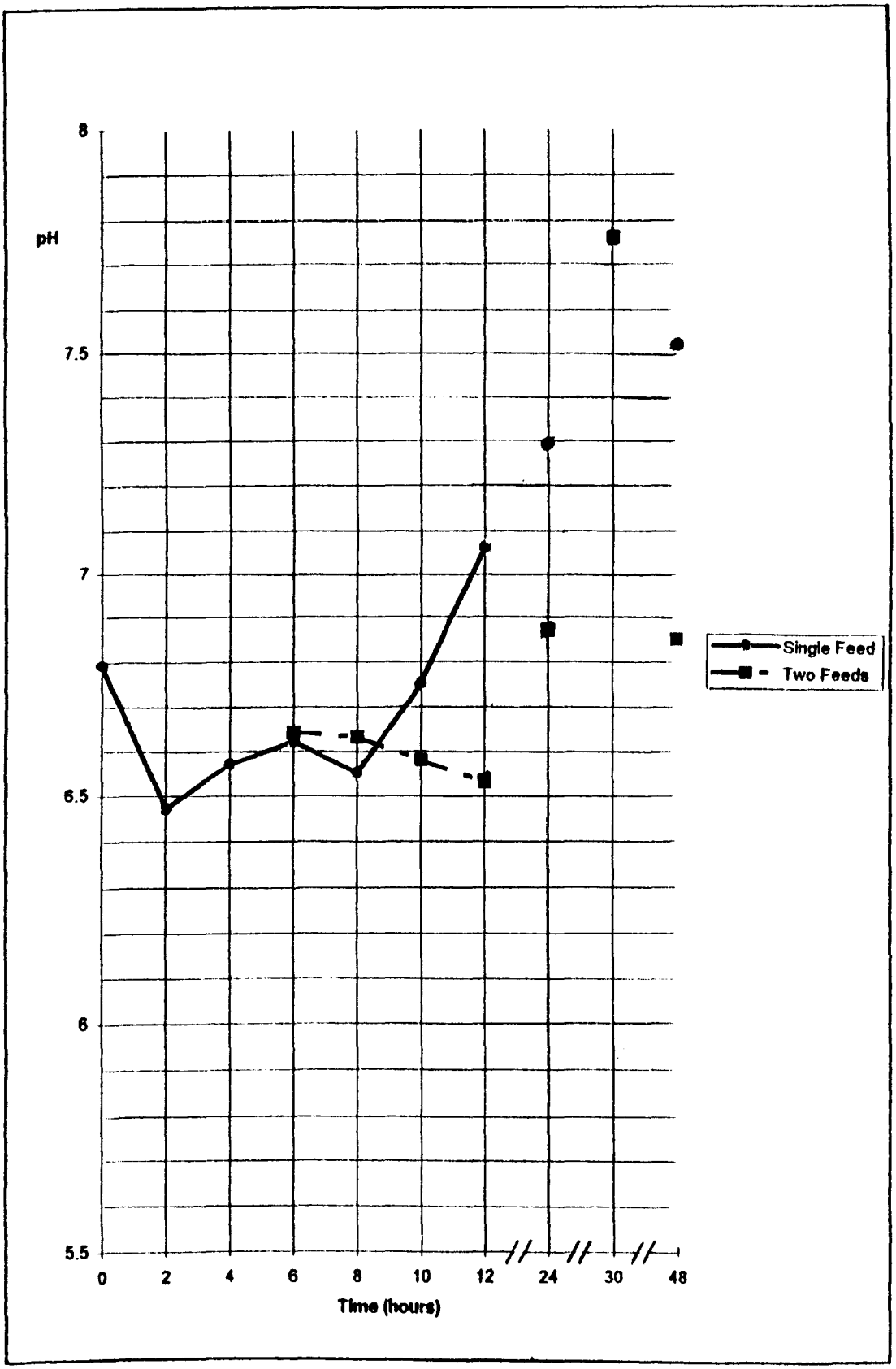


Figure 10.3 pH variation in the middle intestine of gilthead sea bream after one and two feeds. Two sets of readings are presented: the continuous line indicates the set of readings taken after fish were given the first feed; the broken line indicates the readings taken only after fish were given the second feed 6 hours after the first feed.

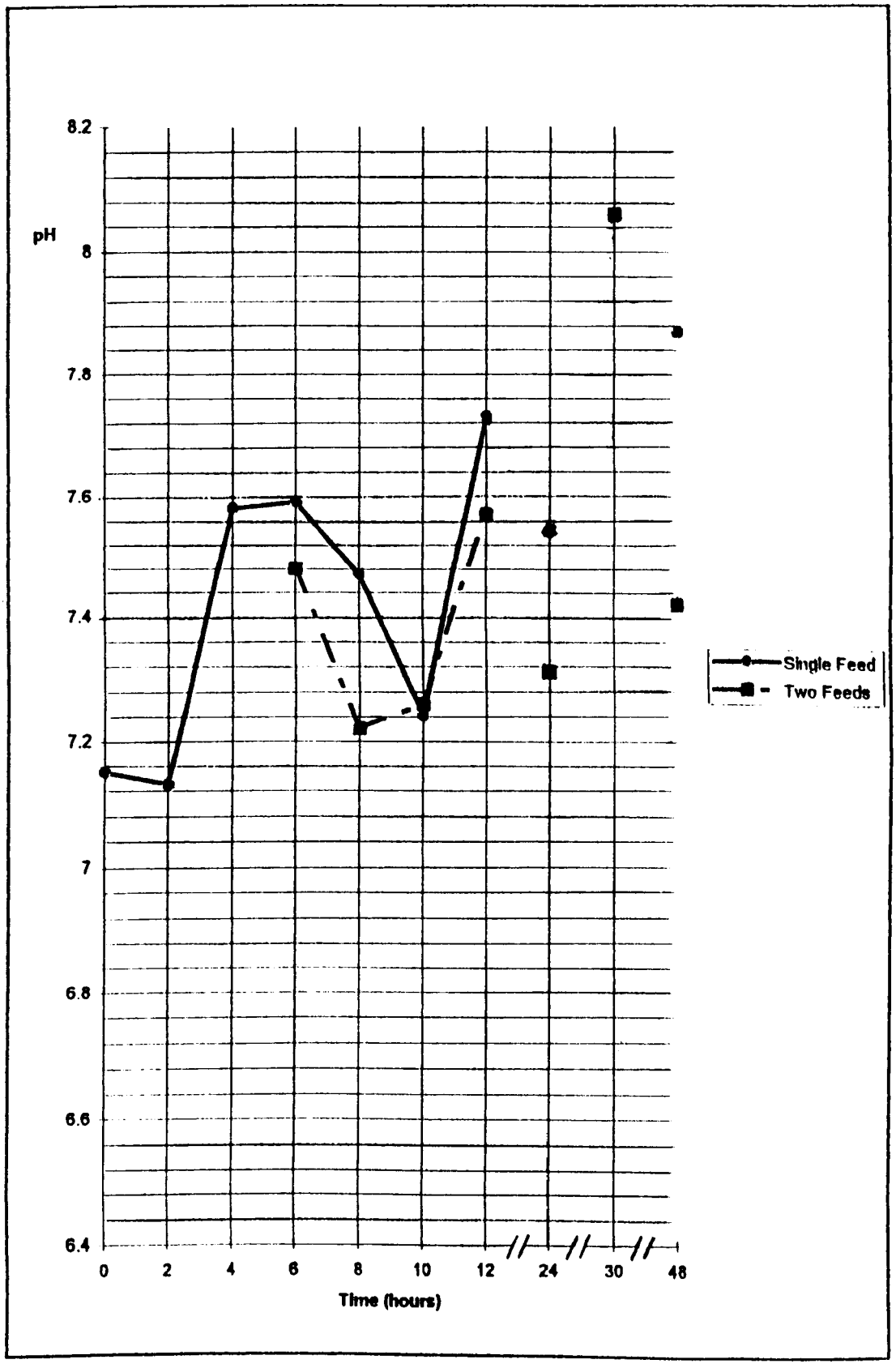
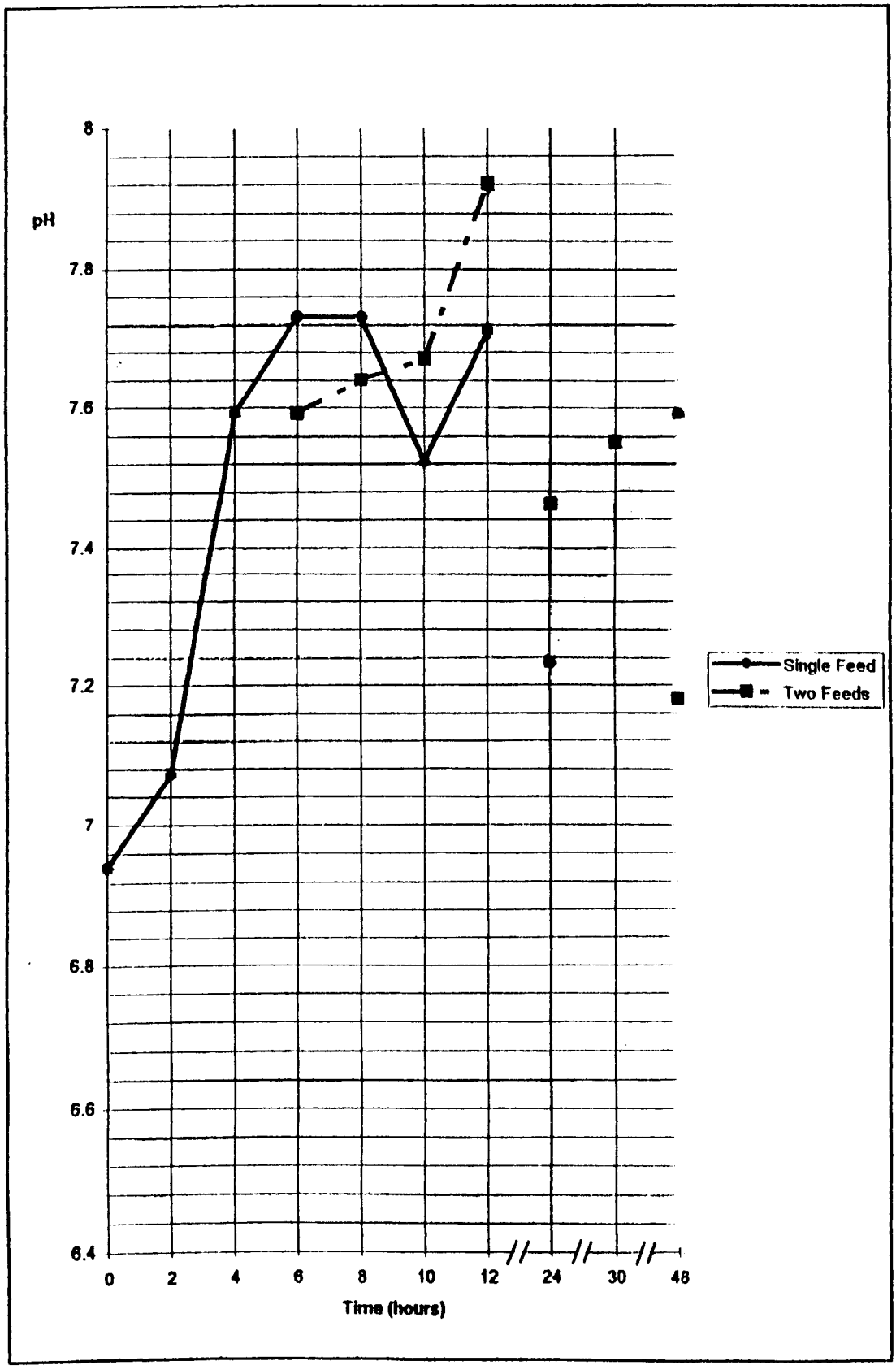


Figure 10.4 pH variation in the lower intestine of gilthead sea bream after one and two feeds. Two sets of readings are presented: the continuous line indicates the set of readings taken after fish were given the first feed; the broken line indicates the readings taken only after fish were given the second feed 6 hours after the first feed.



in the fish fed once to 12 hours before also experiencing a sharp increase in pH to reach a maximum recorded pH of 7.8 after 30 hours with a steep decrease to 6.8 again at 48 hours after first feeding. The lowest recorded pH was 6.5, 2 hours after first feeding.

As was seen in the upper intestine, the middle intestine showed a decrease after 2 hours and a subsequent increase in pH to decrease again by 10 hours. The pH then rose to 7.7 at 12 hours to decrease slightly and rise again to the highest level at 48 hours. In fish fed twice the same sequence of rise and fall as the single-fed fish was seen, with a decrease at 8 hours, a peak at 12 hours, another decrease and then a maximum recorded peak of 8.1 at 30 hours followed by a decrease by 48 hours. The lowest pH was 7.1, recorded 2 hours after first feeding.

Fish fed once showed a rapid increase in the lower intestine from the minimum pH value of 6.9 after feeding to 7.8 at 6 hours followed by a decrease, rise and another decrease and rise, the decreases recorded at 10 hours and 24 hours. The lower intestine of fish fed twice showed an increase after feeding to reach the maximum recorded value of 7.9 at 12 hours to decrease to 7.5 by 24 hours, rise slightly and decrease again by 48 hours.

10.4 DISCUSSION

10.4.1 STOMACH

The pH in the stomach of the sea bream ranged from 2.53 up to a high of 5.52 which was recorded immediately after feeding. The lower pH reached lay in the range 2.0 to 3.0 generally given as the optimum pH for pepsin (Moroshita *et al.*, 1964; Moriarty, 1973; Uys and Hecht, 1987; Munilla-Moran and Sabordo-Rey, 1996a), although the

time during which the pH in the stomach was actually within this range was quite limited.

After first feeding a gradual decrease in the pH was observed. This decrease occurred until the eighth hour after feeding after which the pH increased and decreased again by 24 hours after the initial feeding. A similar course of changes was seen when the fish were fed a second feed.

Norris *et al.* (1973) also observed a decrease in pH from 5.0 to 1.0 – 2.0 after one hour of feeding bluegill, *L. macrochiris*, and Moriarty (1973) saw a decrease from 7.0 to 1.4 – 2.0 by 6 hours after feeding Nile tilapia, *T. nilotica*. Maier and Tullis (1984) found that the pH in the stomach of *Oreochromis mossambicus* actually increased from an initial pH of 1.1 to 4.7 after 2 hours to fall to a pH of 1.2 again by 8 hours.

The rapid decrease seen after first feeding indicated a rapid secretion of HCl into the stomach. The low pH attained after 6 hours was diluted when the fish were fed a second time, with the pH rising to 4.81, after which further excretion again reduced the pH to 2.5 by the 12th hour of the trial. The absence or very limited amount of food in the stomach would explain the increase in pH seen after the initial decrease, due to no acid secretion, seen in the stomach. However, after these increases there were decreases again in pH, even though no food had been given. The fish had been acclimated to a certain feeding regime, and these decreases could be evidence that the fish executed a preparatory excretion of acid in anticipation to feeding.

10.4.2 INTESTINE

10.4.2.1 General Introduction

The variation in pH of the three parts of the intestine studied over a 24 hour period follow a pattern of ups and downs, and the following discussion shall attempt to explain

these changes in view of the movement of food along the digestive tract and secretions of the intestine.

First of all, it is well to note that there was a regular pattern in pH followed both by fish fed only one morning feed and fish fed two feeds, with the same trend in increases and decreases in pH.

The upper intestine had a pH range over the first 24 hours after feeding from a minimum of 6.47 to a high of 7.52, the middle intestine from 7.13 to 7.87, and the lower intestine from 6.93 to 7.92. These measured ranges of pHs are in fact slightly lower than the measured pH optima of trypsin and chymotrypsin (7.8 to 9.0)(Alliot *et al.*, 1974; Jany, 1976; Clark *et al.*, 1985; Uys and Hecht, 1987; Simpson *et al.*, 1989; Joakimsson and Nagayama, 1990; Sabapathy and Teo, 1995), leucine aminopeptidase (7.0 to 9.0)(Khablyuk and Proskuryakov, 1983; Clark *et al.*, 1987; Joakimsson and Nagayama, 1990; Sabapathy and Teo, 1995), amylase (6.4 to 8.0)(Moriarty, 1973; Fal'ge and Shpannkhof, 1976; Uys and Hecht, 1987; Ni *et al.*, 1992; Tang *et al.*, 1994; Munilla-Moran and Saborido-Rey, 1996b), and lipase (7.0 to 7.5)(Schlottke, 1938).

10.4.2.2 Upper Intestine

There was an initial reduction in the pH of the upper intestine immediately after feeding of both meals, followed by large increases in pH and a final decrease to a level similar to that seen before the first feeding. The initial reaction was different from that seen in *O. mossambicus* by Maier and Tullis (1984) who observed an initial increase before a decrease was observed by 4 hours after feeding and after which the pH increased again. The opposite observations are probably attributed to the same effect, namely the entry of acidic material into the upper intestine. In the case of the sea bream used in this investigation, there appeared to be an initial input of acidic material into the upper intestine earlier than seen by the other authors. The increase seen after this decrease

would have been due to the continued excretion of bicarbonate into the lumen, at sufficient concentration to neutralise the acid present in the material coming out of the stomach and increase the pH. The increased secretion even after no food was offered after 24 hours could again indicate an anticipatory response to feeding.

10.4.2.3 Middle Intestine

The observations of the pH in this part of the intestine consisted of a number of increases and decreases in pH as time passed. This was again also observed by Maier and Tullis (1984) in *O. mossambicus*.

The initial decrease seen in the observations of this trial is practically non existent, however there was a rapid increase in pH followed by a decrease which was probably caused by the arrival of food from the upper intestine. Such a decrease occurred as a result of a dilution effect brought about by the lower pH of the material in the upper intestine which had a pH of around 6.6 at the time when this decrease was observed. This decrease was observed in fish fed once and twice, and both were followed by another rapid increase in pH as bicarbonate was excreted. Unlike the continual increase in pH seen in the upper intestine, before a decrease in pH was again observed, a decrease was observed by the 24^{th} hour in the middle intestine, possibly as a result of the reduction or stoppage of bicarbonate excretion. That there was a subsequent increase in pH could again indicate an anticipatory action to feeding.

10.4.2.4 Lower Intestine

As seen in the above parts of the intestine, there were a sequence of increases and decreases in pH in this region of the intestine, as had also been observed in the *O. mossambicus* by Maier and Tullis (1984).

As seen in the middle intestine, there is an initial rapid increase in pH as soon as the fish are fed, followed by a decrease as material from the middle intestine arrives in the lower part of the intestine. The pH of the material in the middle intestine at the time the decrease in pH was observed in the lower intestine was 7.5 which was lower than that of the lower intestine. That this dilution effect occurs is shown by the fact that the decrease in pH seen in the middle intestine was higher than that seen in the lower intestine which corresponds with the fact that the differences in the pHs of the upper and middle intestine was larger than the differences between the middle and lower intestine.

As seen in the middle intestine there is another increase in pH after this decrease and then another decrease, again probably as a result of no further excretion of bicarbonate. As seen in the above regions there was also another rise in pH after 24 hours.

Reference to the pH optima of the enzymes used in the five experiments carried out on enzyme supplementation in Chapters 3 to 7 and the possible influence of the gut pH on their activity has already been made. It is well known that pH has an important influence on the level of activity of any enzymes. Hence, it is important that the enzymes that are to be chosen as supplements to feed should be chosen with the known conditions in which they are to work in mind, depending on the target species. To this end, further studies on pH variation should be carried out to clarify further the effects of different diets and feeding regimes, and a proper evaluation of this data should be made when the enzymes to be added to a feed are chosen.

CHAPTER 11

The effect of time of feeding, feeding rate and feeding frequency on gastric evacuation in the gilthead sea bream, *Sparus aurata.*

11.1 INTRODUCTION AND AIMS OF THESE INVESTIGATIONS

Numerous parameters, such as meal size and meal frequency, have been found to influence the gastric evacuation in fish (Section 1.7.1). These parameters affect the rate of movement of food from the stomach into the rest of the intestine and may affect digestion of ingested food.

Gastric evacuation studies have an application in the fish farming industry as a means of determining the factors which influence movement of food given to the fish in the stomach and other parts of the digestive tract. Such studies could be of use in determining feeding frequencies, feeding rates and the time to feed the fish when developing farm feed management strategies.

Only one paper was found in the literature relating to the studies of gastric evacuation in the gilthead sea bream determined following a single feed (Andrade *et al.*, 1996). In the series of studies carried out here the effects of time of feeding, feeding rates and feeding frequencies on the rate of gastric evacuation in gilthead sea bream fed pressed or extruded feeds were investigated. Coefficients of determination were calculated for the three most common models used to describe the data which was determined by serial slaughter (see Sections 1.7.2 and 1.7.3).

11.2 MATERIALS AND METHODS

A series of gastric evacuation trials were performed. The serial slaughter method was used in these trials (Brett and Higgs, 1970; De Silva and Owoyemi, 1983; Fletcher *et al.*, 1984; Atland and Barlaup, 1991). These trials were carried out in the same tank

system and with the same environmental conditions as the main experiments. The fish were acclimated to the tanks and to the particular feeding regime to be investigated. This acclimation period lasted at least 10 days, depending on the feeding behaviour of the fish, after which the fish were starved for 48 hours before the resumption of feeding as pre-determined. The fish were fed either the pressed diet 1 of Experiment 1, a commercial pressed Ewos diet (3 mm, 49.2% protein, 11.9% lipid) or the extruded diet 1 of Experiment 5.

Table 11.1 gives a list of the studies carried out and the pellet type and feeding regime used.

After feeding, 6 fish were killed by lethal anaesthetic (0.6 mL/L 2-phenoxyethanol) at the pre-determined times. The fish were then frozen at -20°C. Stomach contents were extracted while still frozen and dried at 105°C.

The dry stomach contents were calculated as a percentage of the fish weight.

Three evacuation models frequently used in the literature were fitted, using a non-linear least squares procedure, to each set of results obtained in the different treatments conducted:

linear : $W_t = W_0 - R_e t$,

square root: $\sqrt{W_t} = \sqrt{W_0} - R_e t$,

and exponential model: $W_t = W_0 e^{-Ret}$,

where W_t is the percentage dry weight of stomach content at time t (hours), W_0 is the initial percentage dry stomach weight and R_e is the instantaneous rate of gastric evacuation.

The adjusted non-linear coefficient of determinations were calculated for each model for each treatment to provide a measure of goodness of fit of the three models. The best model was found to be the exponential model, and the results of this analysis were used

Table 11.1 List of gastric evacuation trials carried out indicating pellet type and feeding regime used.

Trial	Pellet type	Feeding regime
1	Pressed	Fed to satiation, once a day in the morning.
2	Pressed	Fed to satiation, once a day in the afternoon.
3	Pressed	Fed 0.5% body weight/day, in one morning meal.
4	Pressed	Fed 1.0% body weight/day in one morning meal.
5	Pressed	Fed to satiation twice a day.
6	Pressed	Fed 1.6% body weight/day in two meals.
7	Pressed	Fed 1.6% body weight/day in three meals.
8	Extruded	Fed 0.4% body weight/day in one morning meal.
9	Extruded	Fed 0.8% body weight/day in one morning meal.
10	Extruded	Fed 1.3% body weight/day in two meals.
11	Extruded	Fed 1.3% body weight/day in three meals.

to determine the time taken for the stomach to empty by 25%, 50% and 75% of the original dry weight calculated.

11.3 RESULTS (Table 11.2)

Feeding fish only once in the morning to satiation (trial 1) gave a lower evacuation time than feeding to satiation in the evening (trial 2), with 50% evacuation occurring after about 4 hours and 10 hours respectively.

Doubling the feed quantity in a meal increased, but did not double, the evacuation time in both pressed (trials 3 and 4) and extruded fed fish (trials 8 and 9), although the actual quantity of contents evacuated per unit time was greater in the fish fed the larger quantity of food.

When feeding the fish two times a day in trials 6 and 10, the first meal was evacuated faster than the second meal although the same amount of food had been given in each meal; the differences observed in the case of feeding twice to satiation (trial 5) were minimal.

When the fish were fed three meals a day, the second meal was evacuated faster than the first meal, whilst the third meal took the longest to be evacuated. These results were found in both pressed and extruded-fed fish (trials 7 and 11).

Table 11.2 Results of Gastric Evacuation trials giving calculated time required for different proportions of the stomach contents to be evacuated.

Trial	Pellet type, feeding regime of trial	Av. Fish weight (g)[1]	Meal	Time (hours) for proportion of contents to evacuate		
				25%	50%	75%
1	Pressed, fed to satiation once a day, in morning	55.73 (9.68)	1	1.82	4.38	8.75
2	Pressed, fed to satiation once a day in afternoon	65.38 (10.73)	1	4.01	9.67	19.33
3	Pressed, fed 0.5% body weight/day in one morning meal	75.95 (10.18)	1	1.45	3.49	6.98
4	Pressed, fed 1.0% body weight/day in one morning meal	70.43 (11.74)	1	2.06	4.96	9.92
5	Pressed, fed to satiation twice a day	83.77 (16.86)	1	1.44	3.46	6.93
			2	1.46	3.51	7.01
6	Pressed, fed 1.6% body weight/day in two meals	86.97 (11.31)	1	1.09	2.63	5.25
			2	1.77	4.25	8.50
7	Pressed, fed 1.6% body weight/day in three meals	94.30 (6.73)	1	1.29	3.09	6.19
			2	1.25	3.01	6.03
			3	1.53	3.69	7.37
8	Extruded, fed 0.4% body weight/day in one morning meal	137.94 (24.59)	1	0.99	2.37	4.75
9	Extruded, fed 0.8% body weight/day in one morning meal	149.94 (24.69)	1	1.60	3.85	7.70
10	Extruded, fed 1.3% body weight/day in two meals	158.23 (32.19)	1	1.47	3.54	7.07
			2	1.61	3.87	7.74
11	Extruded, fed 1.3% body weight/day in three meals	149.90 (29.45)	1	0.89	2.15	4.30
			2	0.63	1.52	3.05
			3	1.79	4.30	8.61

1. Figures in brackets are standard deviations.

11.4 DISCUSSION

11.4.1 GENERAL INTRODUCTION

As has been pointed out in numerous instances in the discussions so far, the large variation in methodology used by experimenters greatly complicates making comparisons between similar experiments. The data obtained from gastric evacuation studies is another case in point, with similar experiments often giving contradictory results, such as in the determination of whether meal size and feeding frequency affect gastric evacuation. Even in experiments that have had similar conclusions, the way data is obtained and presented is complicating. Nonetheless, such research is relevant in order to understand and attempt to quantify the factors affecting the movement of food in the intestine of fish which could then have an affect on other processes occurring in the digestive tract.

11.4.2 TIME OF FEEDING

The trial carried out using satiation feeding in either the morning (Trial 1) or the evening (Trial 2) gave very interesting results. Feeding only in the afternoon more than doubled the time required to evacuate the same percentage of stomach contents compared to fish fed to satiation in the morning. Both sets of fish had consumed similar quantities of food, and if anything fish fed in the morning actually consumed more. No comparable experiment had been found in the literature. The longer time taken for evacuation in the fish fed in the afternoon, was longer than any recorded in the whole set of trials carried out, including those in which fish had also been fed in the afternoon. This could be an adaptation to the afternoon feeding regime related to the digestive physiology.

11.4.3 EFFECT OF MEAL SIZE

In these trials involving both pressed feeds (Trials 3 and 4) and extruded feeds (Trials 8 and 9) the amount of food fed was doubled in the second experiment. In all these experiments feeding was carried out in the morning only. In both these sets of experiments, the fish fed the higher amount of food did not take double the time to evacuate a given percentage of food as the fish fed the lower dose. In the case of the fish fed the pressed feeds, the time taken by fish fed the higher dose to evacuate a given percentage of food was 1.4 times that of fish fed the lower meal. In the case of the fish fed the extruded feeds, this difference was 1.6 times higher.

Although it took a longer time for the sea bream to completely evacuate the stomach when fed a larger meal, the results indicate that the actual rate of evacuation of material was actually higher than in the case of fish fed the smaller meal.

This agrees with the results obtained by Smith *et al.* (1989) with 30 to 70 g walleye pollack, *Theragra chalcogramma.* Increasing the food given from 0.5 to 1.0% body weight increased the time for gastric evacuation to occur by 1.6 times. When the fish were fed 2.5% body weight per day the time it took the fish to evacuate the same percentage of food was 2.7 times that taken to evacuate the smaller meal. This trial was carried out at 6°C.

Macpherson *et al.* (1989) found evidence confirming this trend (in trials conducted at 14°C). When they fed 200 g dogfish, *Scyliorhinus canicula*, 2 fish prey of the *Plesionika* species, the stomach still contained 69% of the food after 20 hours compared to 50% when the dogfish were fed only 1 prey item. This was also the case when the authors fed 1 or 2 *Pasiphaea sivado* fish to the dogfish, from 77% evacuation after 10 hours compared to only 52%. When these authors fed 1, 2 or 3 *Gadiculus argenteus argenteus* prey, differences in evacuation rates were again observed, where by 20 hours

the stomachs contained 39, 44 and 53% respectively of the food initially consumed. The differences were also seen when the authors fed dogfish 1, 2 or 3 *Sapietta* sp. prey. By 20 hours 57, 50 and 24% evacuation had been achieved respectively.

Swenson and Smith (1973) also found that an increase in meal size did not bring about a proportional increase in the rate of evacuation. These authors fed 178 to 381 mm walleye, *Stizostedion vitreum vitreum*, 1.1 to 1.9 g fathead minnows, *Pimephales promelas*. When the walleye ate from 0.1 to 10 mg/g fish, there was 60% evacuation after 8 hours, when they ate from 10 to 20 mg/g fish 55% and when they ate above 20 mg/g fish only 44% (at 14.5°C). When 3.1 to 5.0 g minnows were fed to the walleyes, the corresponding evacuations for the same quantities of food eaten were 46, 30 and 28%.

Flowerdew and Grove (1979) found that increasing the meal size of 60 to 700 g turbot, *Scophthalmus maximus*, from 1% body weight to 5% body weight increased the time for gastric evacuation by 180% at 8°C and by 110% at 19°C.

Grove *et al.* (1985) carried out a further series of gastric evacuation trials with turbot, *S. maximus*. When they fed 40 to 50 g turbot at 10°C meals of 0.32, 0.64 and 0.95 g the time for complete evacuation increased from 13.5 to 17.5 to 31.5 hours respectively. There was also an increase in gastric evacuation time when they fed 10 to 20 g turbot meals of 0.01 to 0.05, 0.05 to 0.1 and 0.1 to 0.2 g, this time from 5.0 to 6.7 to 8 hours respectively.

Jobling *et al.* (1977) working with 10 to 200g dab, *Limanda limanda*, found that increasing the feeding rate from 1% to 5% body weight resulted in a four fold increase in the time taken to evacuate a given percentage of food from the stomach (16.4°C).

Garber (1983) fed 50 and 80 g yellow perch, *Perca flavescens*, a 0.4 or 0.8% body weight meal. In the smaller fish fed the larger meal 52% of the food had been

evacuated by 6 hours compared to 59% in the fish fed the smaller diet (22°C). In the larger fish the fish fed the larger amount of food had evacuated only 43% compared to 55% by fish fed less food in the same time.

The above authors and others (Beamish, 1972 (largemouth bass, *Micropterus salmoides* Lacepede); Mills *et al.*, 1984 (yellow perch, *Perca flavescens*); Bromley, 1987 (turbot, *S. maximus*); Beyer *et al.*, 1988 (Northern squawfish, *Ptychocheilus oregonensis*); Ruggerone, 1989a (coho salmon, *O. kisutch*); Paul *et al.*, 1990 (Pacific cod, *Gadus macrocephalus*); Rogers and Burley, 1991 (smallmouth bass, *Micropterus dolomieui*); Dos Santos and Jobling, 1992 (cod, *Gadus morhua*); Sims *et al.*, 1996 (lesser spotted dogfish, *Scyliorhinus canicula*); Paakkonen and Marjomaki, 1997 (burbot (*Lota lota*)) agreed with the results obtained in this experiment with sea bream, but not the results obtained by Brett and Higgs (1970)(sockeye salmon, *O. nerka*), Elliott (1972, 1991)(brown trout, *S. trutta*), Persson (1979, 1981)(perch, *Perca fluviatilis*), Talbot *et al.* (1984)(Atlantic salmon, *S. salar*) and Bromley (1988)(whiting, *Merlangus merlangus*).

A number of the authors who found that meal size did affect gastric evacuation have argued that this may be due to negative feedback processes caused by the emptying of food into the upper intestine, such that the degree of stomach wall distension could initiate physiological mechanisms that alter the pattern of evacuation (Jobling, 1986; Beyer *et al.*, 1988; Ruggerone, 1989a; Sims *et al.*, 1996).

11.4.4 MULTIPLE MEALS

When the sea bream were fed to satiation twice a day (Trial 5), the gastric evacuation rates were equal for the two feeds. This was not the case when a fixed quantity of pressed or extruded food was given. In the case of when the fish were fed fixed

amounts of food twice a day (Trials 6 and 10), the second feed took longer for a given quantity of food to be evacuated from the stomach, although the difference was less in the fish fed the extruded feed.

The results obtained with the fish fed the pressed and extruded feeds at a fixed rate agree with the results obtained by Schade (1982)(common carp, *C. carpio*), Persson (1984)(roach, *Rutilus rutilus*; perch, *P. fluviatilis*), Rosch (1987), Fletcher *et al.* (1984)(dab, *Limanda limanda*), Talbot (1984)(Atlantic salmon, *S. salar*) and Elliot (1991)(brown trout, *S. trutta*). The results obtained when the fish were fed to satiation did not show any differences in gastric evacuation after each feed, as found also by Elliott (1972)(brown trout, *S. trutta*) and Sarokon (1975)(rainbow trout, *O. mykiss*).

Ruggerone (1989b) fed coho salmon, *O. kisutch*, one or two feeds (2 hours after first feed) at 10°C. After four hours from each feeding 70% of the first meal had been evacuated but only 40% of the second meal. When one feed alone was given to the fish, 64% of the stomach contents had been evacuated by 4 hours.

Noble (1973) found that when he fed 60 mm yellow perch, *Perca flavescens*, a single meal of daphnids, total gastric evacuation was achieved by 12 hours (15°C). However, when this initial meal was followed immediately by an excess of food, the first meal was evacuated in 6.1 hours. When this was repeated on 30 to 40 mm perch, total evacuation was achieved in only 1.5 hours instead of 6.5 hours when the initial meal was not followed by excess food (22°C).

When fish were fed three times a day (Trials 7 and 11), some changes were seen in the resultant gastric evacuations. In both types of feeds the gastric evacuation rate of the food after the last feed was longer than that of any of the previous feeds, and the evacuation rates after the second feed were less than had been the gastric evacuation after the first feed, unlike what had happened when fish were fed twice a day. This

result fits in with the results obtained by the above authors where a further meal increases the rate of evacuation of the previous meal.

In the gastric evacuation studies carried out by Andrade *et al.* (1996) 73 to 183g gilthead sea bream, *S. aurata*, were fed pellets at 1.6% body weight in one feed (23°C). Only 73% of the original meal remained after just 10 minutes, and 1% after 9 hours. The result obtained by these authors for gastric evacuation rate is much faster than anything obtained in the trials carried out in this study, with the fastest rate being obtained in Trial 11 using extruded feed which still took 40 minutes to evacuate 25% of the original meal from the stomach. The higher temperature during the trial of Andrade *et al.* could explain at least part of the higher evacuation rate obtained. However, not enough is known of the gastric evacuation in the sea bream to quantify this effect or the effect of temperature, fish weight and high feeding quantities.

CHAPTER 12

Effect of different feeding rates on individual feed consumption in a population of gilthead sea bream, *Sparus aurata*.

12.1 INTRODUCTION AND AIMS OF THESE INVESTIGATIONS

In any aquaculture venture, one of the main concerns is variability in feeding activity and growth of individual fish in a population of fish. All farmers appreciate that there are daily variations in behaviour and feeding activity in a population of fish and numerous studies have attempted to quantify and study the parameters affecting and impacts of such variations (e.g. Smagula and Adelman, 1982; Danzmann *et al.*, 1987; Boujard and Leatherland, 1992; Carter *et al.*, 1992; McCarthy *et al.*, 1993; Helland *et al.*, 1996).

These authors have found a close relationship between the amount of food consumed by an individual fish and its growth. It has also been found that as the amount of food offered to a population of fish decreases the level of hierarchy in the population increases, with the result that less individuals end up consuming most of the food.

The investigations which are described here are a first attempt at determining the individual feed consumption in a population of gilthead sea bream. Fish were fed at three different feeding rates, 0.5% and 1.0 body weight and to satiation, and data obtained after slaughter.

12.2 MATERIALS AND METHODS

In order to determine the variation in consumption of a population of fish, 40 fish were put into a tank (the same tanks used in the main experiments) and fed at 1% and 2% body weight/day and to satiation (determined as the point at which the fish did not consume offered pellets after 5 minutes) in two feeds at 0830 and 1600 for an

acclimation period. The same pressed feeds used in the gastric evacuation studies were used. The fish were then not fed for 48 hours prior to renewed feeding at 0830. After feeding, the fish were killed by lethal anaesthetic (0.6 mL/L 2-phenoxyethanol) and frozen. The stomach contents were removed while still frozen and dried at 105°C.

The dry stomach contents were expressed as a percentage of the fish weight.

12.3 RESULTS

In all three trials carried out, there was a very wide variation in stomach contents. When a particular range of fish weights is taken there was a large variation in the amount of food in the stomachs of the fish in this range, whichever trial was considered.

In the trial where fish were fed either a 0.5% body weight meal (average fish weight 90.90 g, standard deviation 20.51 g)(Figures 12.1 and 12.2), and where fish were fed a 1% body weight meal (average fish weight 91.93 g, standard deviation 21.65 g)(Figures 12.3 and 12.4) the frequencies of the percentages of food found in the stomachs of the fish describe distributions which approach a normal distribution. In both these trials, the distribution of percentages obtained had an overall range of 0.9%, the first from 0 to 0.9%, and the second from 0.4 to 1.3% of fish weight.

Figure 12.1 Variation in dry stomach contents expressed as a percentage of fish weight in a population of gilthead sea bream fed a meal of 0.5% body weight.

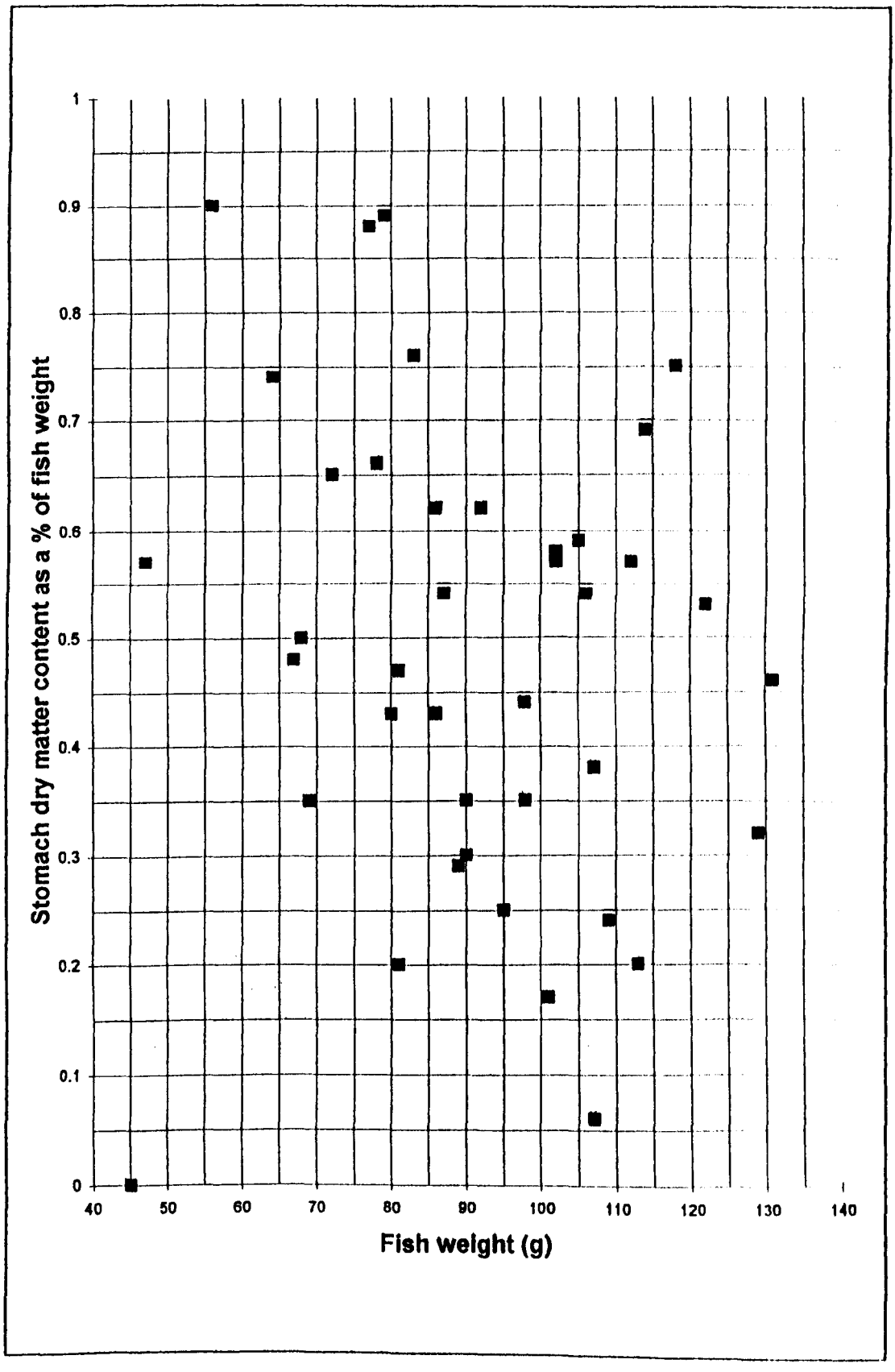


Figure 12.2 Graph showing distribution of dry stomach contents expressed as a percentage of fish weight in a population of gilthead sea bream fed a meal of 0.5% body weight.

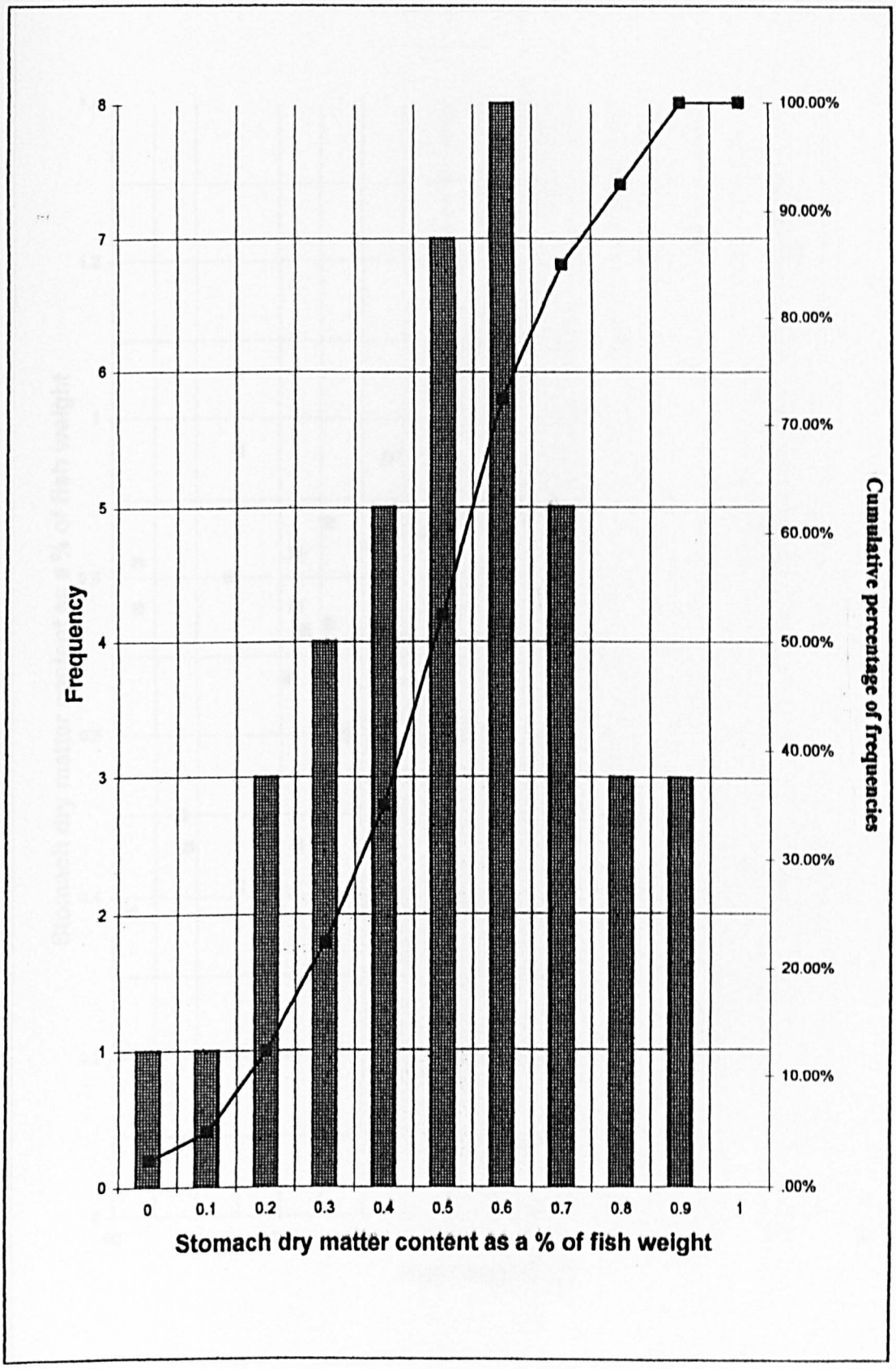


Figure 12.3 Variation in dry stomach contents expressed as a percentage of fish weight in a population of gilthead sea bream fed a meal of 1.0% body weight.

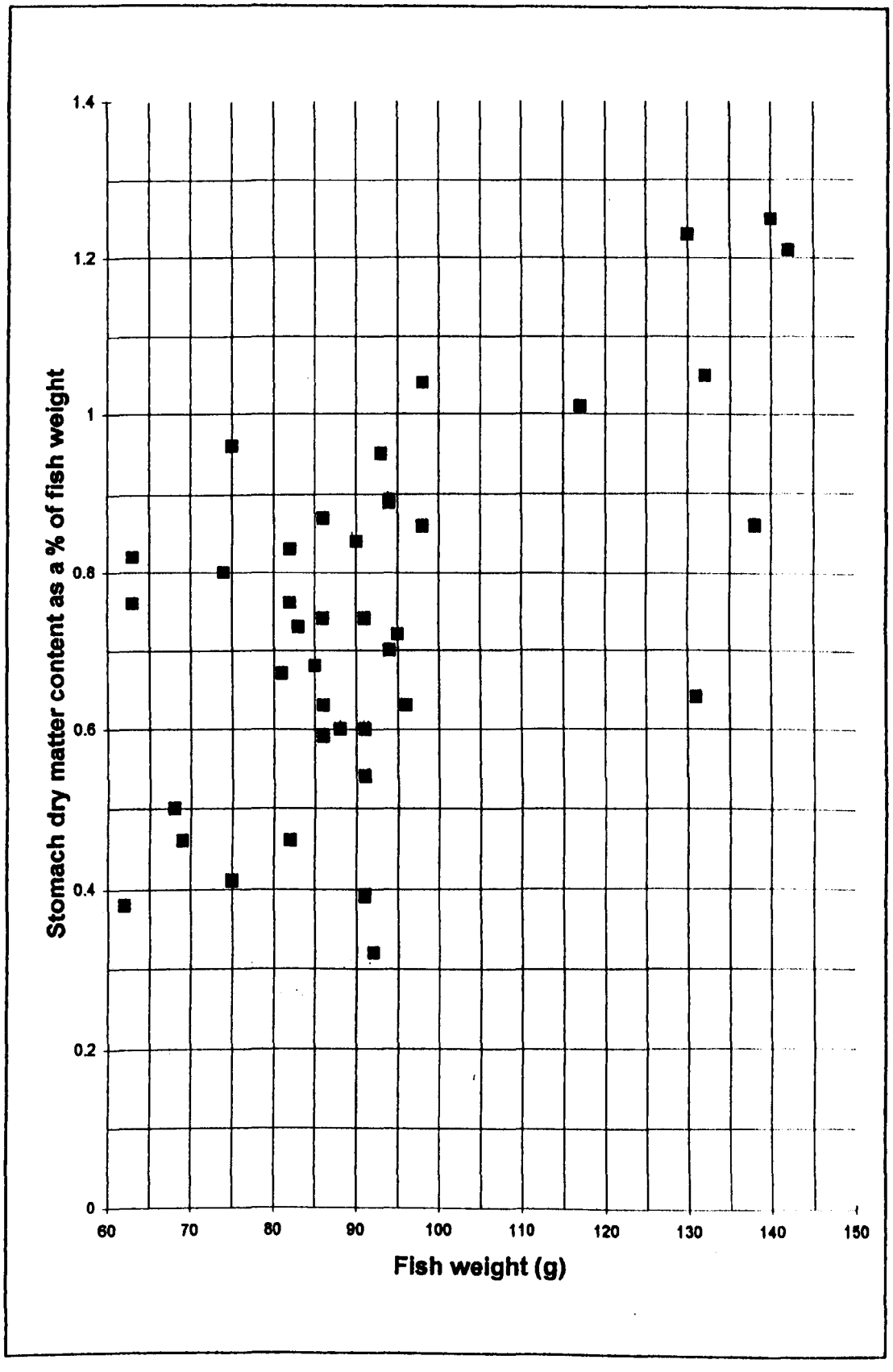
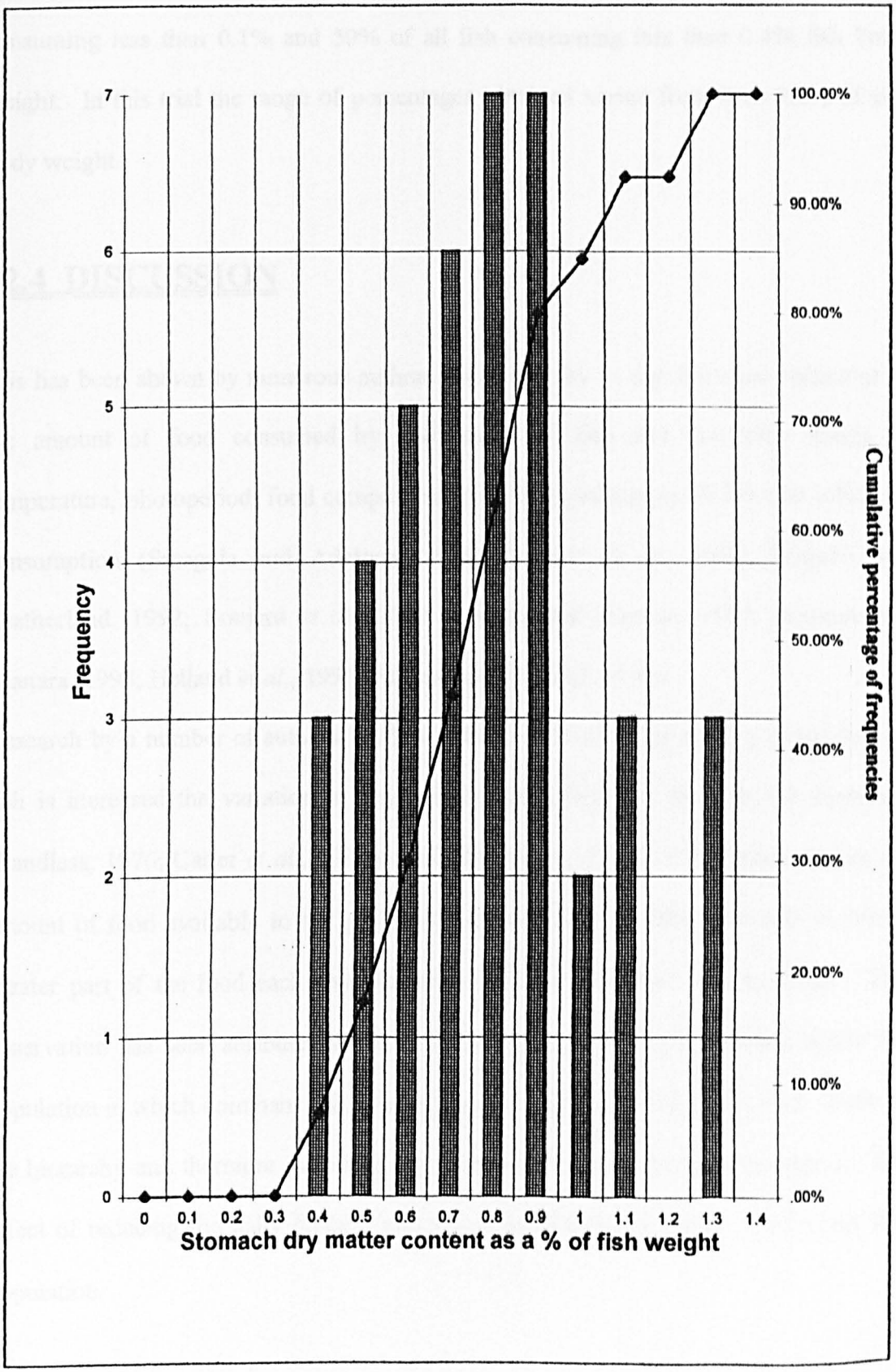


Figure 12.4 Graph showing distribution of dry stomach contents expressed as a percentage of fish weight in a population of gilthead sea bream fed a meal of 1.0% body weight.



In the case where the fish were fed to satiation (average weight 48.48 g, standard deviation of 2.05 g)(Figures 12.5 and 12.6), the distribution of frequencies of percentages does not approach a normal distribution, with 20% of all the fish consuming less than 0.1% and 50% of all fish consuming less than 0.4% fish body weight. In this trial the range of percentages obtained varied from 0 to 2.2% of fish body weight.

12.4 DISCUSSION

It is has been shown by numerous authors that from day to day there are variations in the amount of food consumed by populations of fish and that such things as temperature, photoperiod, food composition and other parameters all have an affect on consumption (Smagula and Adelman, 1982; Tackett *et al.*, 1988; Boujard and Leatherland, 1992; Boujard *et al.*, 1992; Alanara and Brannas, 1993; Brannas and Alanara, 1993; Helland *et al.*, 1996; Paspatis and Boujard, 1996).

Research by a number of authors has found that as the ration given to a population of fish is increased the variation in food consumption between the fish fed decreases (Landless, 1976; Carter *et al.*, 1992b; McCarthy *et al.*, 1992). This means that as the amount of food available to the population increased, more fish were able to take a greater part of the food each day than they would if less food was available. This observation has been attributed to the establishment of feeding hierarchies within the population in which dominant fish take up more food than do individuals lower down in the hierarchy and therefore have less day to day variation in food consumption. The effect of reducing the daily feeding ration was to increase hierarchy level in the fish population.

Figure 12.5 Variation in dry stomach contents expressed as a percentage of fish weight in a population of gilthead sea bream fed to satiation.

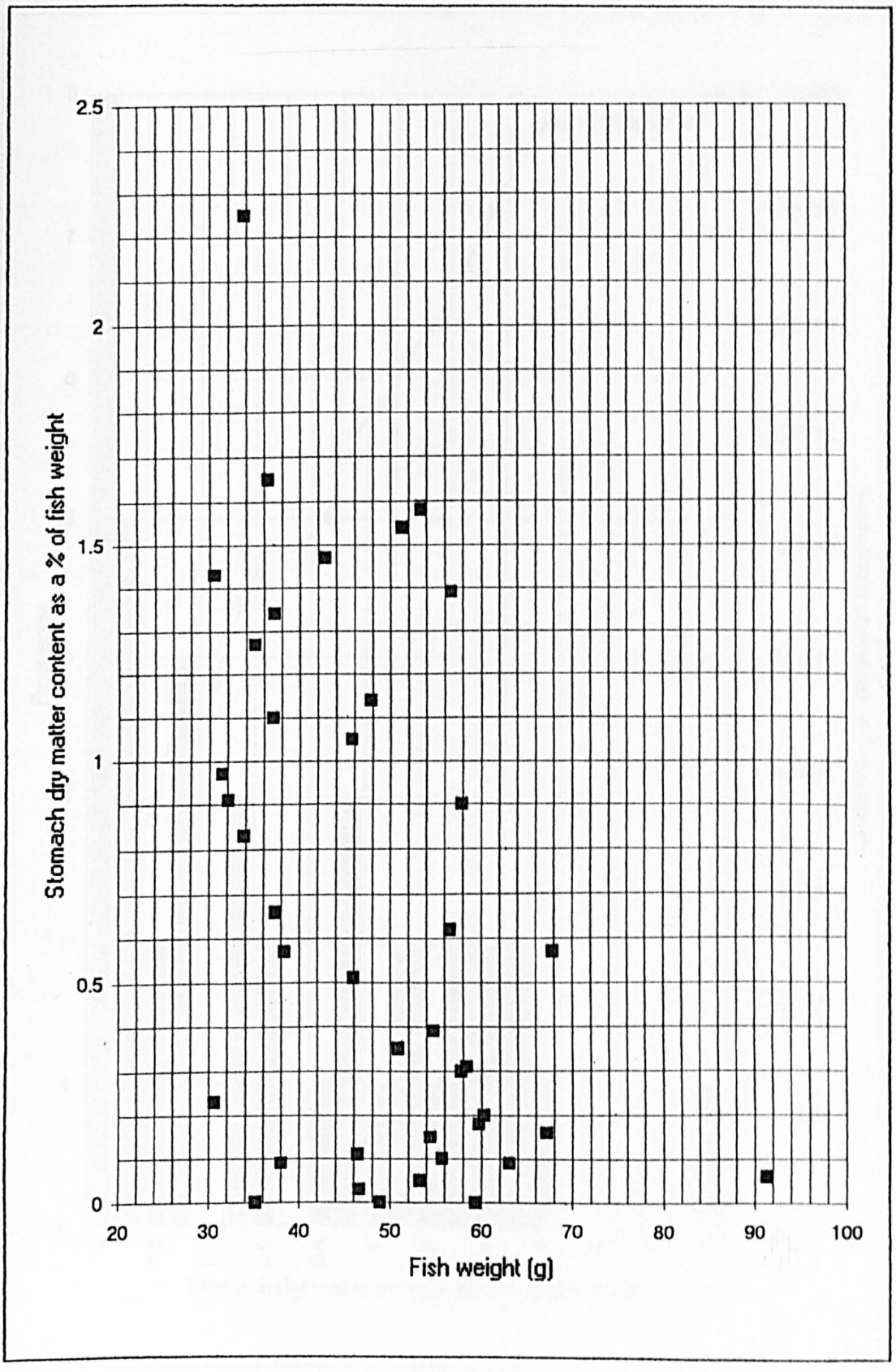
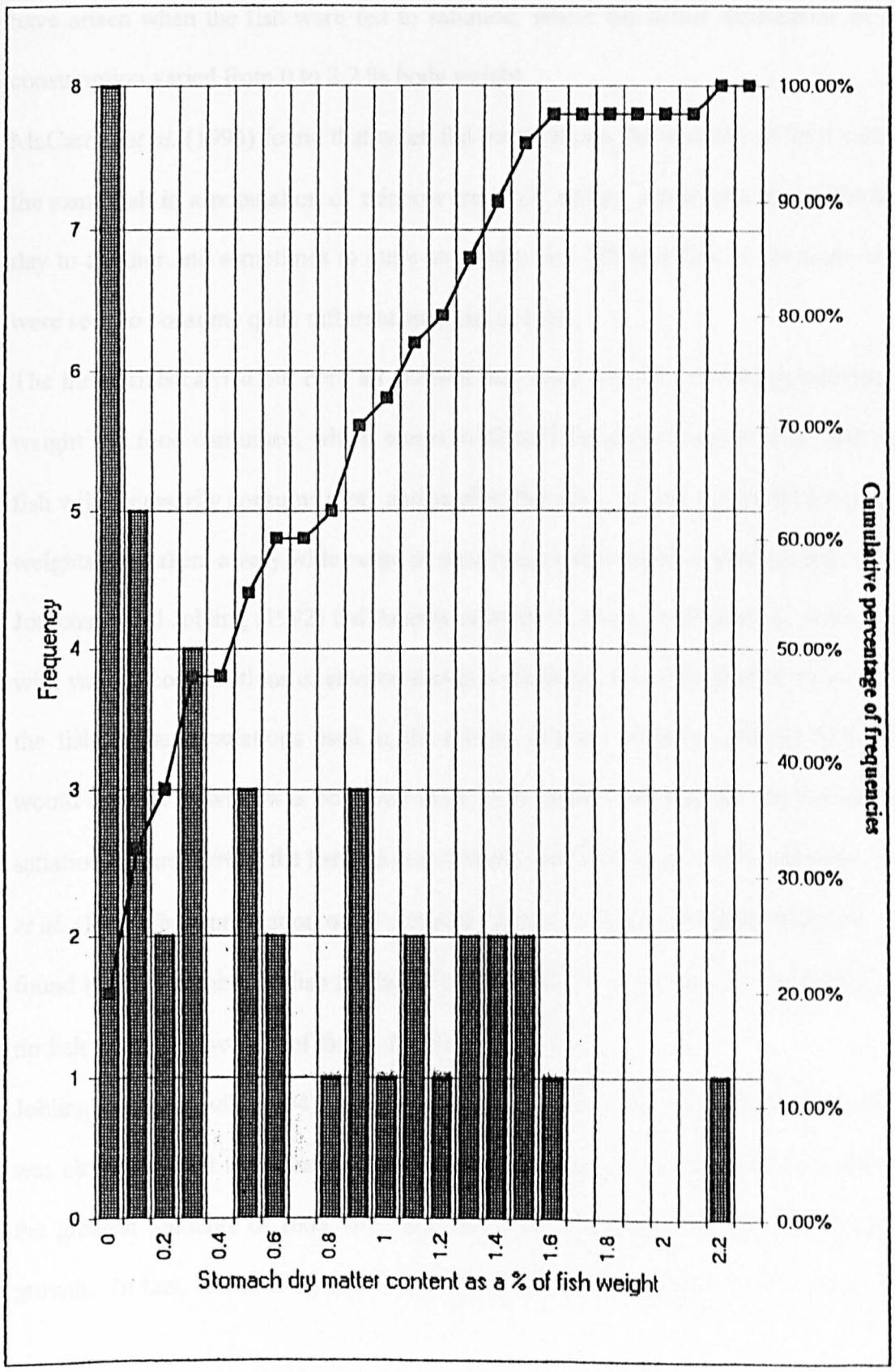


Figure 12.6 Graph showing distribution of dry stomach contents expressed as a percentage of fish weight in a population of gilthead sea bream fed to satiation.



This did not seem to happen in the case of the sea bream fed at 0.5 and 1.0% body weight per day, since the variation in food consumptions had the same extent of stomach content variation of 0.9% of the body weight. However, such a situation may have arisen when the fish were fed to satiation, where the actual distribution of food consumption varied from 0 to 2.2 % body weight.

McCarthy *et al.* (1993) found that when fed fixed rations, the quantity of food eaten by the same fish in a population of rainbow trout, *O. mykiss*, sometimes varied from one day to another and sometimes to quite an extent, and different fish of the same weight were seen to consume quite different amounts of food.

The three trials carried out here all showed that there was no correlation between fish weight and food consumed, which seems to discard the often imagined idea that larger fish will necessarily consume more and smaller fish less. In fact, if a particular range of weights was taken, a very wide range of percentages of stomach contents could be seen. Jorgensen and Jobling (1992) fed Atlantic salmon, *S. salar*, to satiation in various trials with various combinations of environmental conditions. It was found that up to 35% of the fish in the populations used in these trials did not consume offered food. This would agree with what was observed in the trial carried out with the sea bream fed to satiation, where 20% of the fish did not consume any food when fed to satiation. Kadri *et al.* (1997) fed a population of 16 Atlantic salmon, *S. salar*, at regular intervals. They found that the number of fish feeding at each particular feed varied considerably, with no fish feeding at over 20 of the 63 feeds conducted.

Jobling and Baardvik (1994) found that the growth of Arctic charr, *Salvelinus alpinus*, was closely related to the amount of food it consumed, so that the fish that consumed the greatest amounts of food were also those that displayed the most rapid rates of growth. In fact, Carter *et al.* (1992a, b, 1994) and McCarthy *et al.* (1993) have found

that up to 89% of the variation in growth rate between individual fish could be accounted for by differences in food consumption. Genetic and physiological variations play important roles in determining consumption and growth (Danzmann *et al.*, 1987; Torrissen, 1991; Hawkins, 1991).

The implication of varying daily food consumptions within populations of fish and its effect on growth is obviously of importance to fish farmers who attempt to use a feeding management which optimises food utilisation. As in the many other aspects of fish nutrition, more work in this area is required.

CHAPTER 13

SUMMARY AND CONCLUSIONS

The results of the series of experiments presented here are the first data on the use of enzymes in combination with SBM as a means of replacing FM in the diets of gilthead sea bream, *Sparus aurata.*

The reduction in performance seen in the first experiment when the SBM level in sea bream diets was increased from 220 to 320 g/kg at the expense of FM (from 320 to 260 g/kg) was alleviated when enzyme cocktails were added. In fact there was a significant improvement when 1 g/kg low pH active protease and 1 g/kg α-galactosidase together and of 1 g/kg high pH active protease and 1 g/kg α-galactosidase together were added to diets containing 320 g/kg SBM, but this was not repeated when the same enzyme combinations were added to diets containing 440 g/kg SBM. Fish fed supplemented 320 g/kg SBM diets not only performed better than fish fed the unsupplemented 320 g/kg SBM diet but also better than fish fed the diet containing higher FM levels (320 g/kg). The best results were given by fish fed the diet supplemented with low pH protease and α-galactosidase. Although subsequent experiments were carried out using diets with 320 g/kg SBM, the results obtained with the 440 g/kg SBM enzyme supplemented diets does not mean that such an inclusion level should be completely ignored but indicates that further studies are required before similar improvements can be obtained.

Unfortunately, Experiment 2 had to be abandoned due to the poor feeding behaviour of the normally very actively feeding sea bream, despite efforts to solve the feeding problem. No clear reason for this problem was established in the time available.

Experiment 3 suggested that the enzymes used in Experiment 1 had quite different effects on performance of sea bream when supplemented singly at a dose of 1.0 g/kg to the 320 g/kg SBM diet. No significant differences were found between the diets when SGR, FCR or PER were considered. Of all the pressed diets used, only fish fed the diet

with low pH protease alone showed any improvement (although non-significant) over fish fed the unsupplemented diet. Fish fed diets with either the high pH protease or the α-galactosidase alone appeared to show inferior performances compared to fish fed an unsupplemented diet. Using the combinations of enzymes as in Experiment 1, but this time with only 0.5 g/kg of each enzyme, did not improve performance compared to fish fed the unsupplemented diet.

In Experiment 4 there were no significant differences in performance of fish fed the pressed 320 g/kg SBM diets to which 0.5, 1.0 or 1.5 g/kg of the low pH protease had been added. When fish were fed the 0.5 and 1.0 g/kg low pH protease diets to which 1.0 g/kg α-galactosidase had also been added, fish performance (SGR, FCR and PER) appeared to be poorer (but not significantly so) than when fish were fed diets with protease alone. When sea bream were fed an extruded 320 g/kg SBM diet containing 1 g/kg of the low pH protease the results indicated a slight improvement in performance compared to the performance of fish fed an unsupplemented extruded diet, although the improvements in SGR, FCR and PER were not significant.

When, in Experiment 5, sea bream were fed 320 g/kg SBM extruded diets to which 0, 0.33, 0.66, 1.00 or 1.33 g/kg of the low pH protease had been added, a polynomial dose response was obtained, but again no significant differences were obtained between the SGRs, FCRs or PERs of fish fed the various diets. The results obtained for fish fed diets containing 0, 0.33 and 1.00 g/kg protease varied little. Fish fed diets to which 0.66 and 1.33 g/kg protease had been added gave similar SGRs, FCRs and PERs and although the performance appeared better than that of fish fed the other 320 g/kg SBM diets and the 320 g/kg FM, 220 g/kg FM diet, the differences were not significant.

Histological observations of liver sections taken from fish used in the above experiments did not show any relationships between the position of nuclei in

hepatocytes or the presence of fat globules around hepatopancreatic tissue to either SBM levels or enzyme inclusions in the diets, although a more detailed investigation could be carried out to provide a better overall picture of dietary effects.

When the results of Experiments 1, 3, 4 and 5 are analysed, at least in terms of SGR, FCR and PER, the only significant differences obtained were found in Experiment 1 when the two enzyme cocktails were used with 320 g/kg SBM diets. Such an improvement in performance was not seen in any of the subsequent trials either when combinations of enzymes were used or when the three enzymes were used alone, irrespective of inclusion level. Although numerous trends were detected in the effect of individual enzymes or their inclusion level, further studies would be required to confirm these trends and demonstrate significant differences.

It is possible that significant differences may have arisen if these experiments could have been carried out on a commercial scale and over longer periods of time. Even small (non-significant) differences obtained over a short period of time could be of importance if maintained over a full commercial cycle lasting up to 15 months. The practical implications of any positive results with the combination of lower FM containing diets and enzymes on a commercial scale might then be significant. Firstly, the price of diets containing reduced levels FM should be lower. Additionally, any improved performance may mean that not only are fish brought to market sooner, but this is achieved using a smaller quantity of food. Another effect of any improvements in food utilisation might be reduced pollution effects on the environment, an increasingly sensitive issue.

The processes underlying the effects of enzyme supplementation on growth and feed utilisation in fish are still largely unknown. Even in cases where the mode of action is known, as with the α-galactosidase, the results obtained do not show evidence of the

expected benefits to be gained by the breakdown of oligosaccharides. The mechanisms by which supplemental enzymes act on ingested food and their relationship with other physiological processes in the gut are yet to be studied in detail in poultry and pigs let alone in aquaculture species.

There is a significant lack of information on the physiological processes related to digestion and the digestive tract in gilthead sea bream.

The studies carried out on six enzyme activities and pH variations along the length of the intestine provided new information. The results showed that all the enzymes except pepsin are present, with different relative activities, in all parts of the digestive tract. Further work in this area should be carried out to investigate the activities of the various digestive enzymes at different time intervals after feeding. The study on pH variation after one or two feeds also provided useful information on the ranges of pH occurring in the different parts of the digestive tract which may have had a bearing on the activities of the enzymes used in the experiments carried out. In the first 24 hours after feeding, the lowest pH recorded in the stomach was 2.5 and the pH in the rest of the intestine varied between 6.5 and 7.7. However, further work is required to analyse the impact on pH of using different feeds, feeding rates and feeding frequencies.

The gastric evacuation studies carried out here were also the first investigating the effect of feeding rates, feeding frequencies and time of feeding on gastric evacuation in the gilthead sea bream. Doubling the feeding rate increased the time for a given percentage of food to be evacuated by only 1.4 and 1.6 times the time for an equivalent percentage of the smaller meal to be evacuated in fish fed pressed and extruded feeds respectively. When fish were fed multiple meals the evacuation rate of a meal was found to be increased by a subsequent meal. The time at which fish were fed was also found to influence the evacuation rate, with a morning meal being evacuated faster than

an evening meal. Such studies should be combined with other investigations such as growth trials, digestive enzyme activities and gut pH variations in order to determine how these parameters influence growth and feed utilisation in aquaculture species.

The results of trials investigating variation in consumptions in populations of sea bream agree with the work of other authors on the high level of variation in intake that occurs in a population of fish when given a meal. It was also observed that in a population fish of the same size sometimes consumed very different quantities of food. The importance of this to fish farms does not need further emphasis, but additional studies should be carried out, preferably making use of non-invasive methods of measuring feed intake.

The scope of much of the research taking place in the aquaculture industry is directed at improving the growth and utilisation of the feed given to aquaculture species. The numerous trials carried out by the poultry and pig industry, and a number of those carried out on aquaculture species show that use of supplementary enzymes does have the potential to alleviate the increasing FM supply problem for an ever expanding industry.

This work only considered two ingredients, SBM and FM, and only three types of enzymes. Although the early experiment showed that using enzymes could bring about the desired effect, this improvement was not consistently significant in the subsequent trials. More information is obviously required on the activities and mode of action and application of these and other enzymes, such as the effect of different inclusion levels. Other investigations with different combinations of ingredients need to be carried out too.

Due to constraints on both supply and cost, it would seem inevitable that FM levels in feeds for intensively culture species such as gilthead sea bream will fall in the future.

This will result in an increase in the level of plant proteins in fish feeds and it is obvious that considerable effort needs to be directed at improving the utilisation of these ingredients. One aspect that requires further evaluation in this respect is the use of feed enzyme supplements. This thesis has served to indicate some avenues for further study.

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