Digestibility of feed ingredients and nutrients in artificial diets for fish

Thesis submitted by
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All research work involving experimental animals described in this thesis was conducted within the guidelines of "The Australian Code of Practice for the care and use of Animals for Scientific Purposes". The research project undertaken received ethical clearance from the Deakin University Animal Ethics Committee (approval number A43/92).

In memory of the man

who taught to strive to achieve

Dr John Marcus Appleford (MBBS)

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Summary

A knowledge of the digestibility of dietary ingredients and nutrients is essential to the formulation of nutritionally adequate, cost-effective artificial diets. Digestibility coefficients for fish have generally been determined using a single inclusion level and time point. The aim of the present study was to determine the effects of inclusion level and time on the digestibility of cellulose, tuna oil, corn starch, and soybean meal.

Incorporation of cellulose into the diet of the carnivorous Murray cod, *Maccullochella peelii peelii*, enhanced digestibility at 10% inclusion, while inhibiting digestion at higher inclusion. Conversely cellulose appeared digestible at a nutritionally significant level for the omnivorous common carp, *Cyprinus carpio*. Decreased digestibility at higher inclusion levels suggesting a saturable mechanism. Antibiotics failed to inhibit cellulose digestibility, indicating a lack of involvement of intestinal micro-flora, however microbiological analysis suggested the antibiotic treatment employed did not effectively eliminate gut bacteria. Cellulose inclusion did not consistently affect dietary protein or ash digestibility, however lipid digestibility decreased linearly with the level of cellulose incorporation. Antibiotics did not affect dietary nutrient digestibility.

The digestibility of tuna oil at 10% and 15% inclusion indicated reduced digestibility with inclusion. However daily faecal analysis showed a continuing adaptation to the high lipid diet, with digestibility of tuna oil at the later time points similar at both inclusion levels. Tuna oil inclusion did not affect dietary protein or ash digestibility.

Raw corn starch digestibility for common carp was high, greater than 79%, at all inclusion levels. At restricted feeding starch digestibility was found to increase with inclusion up to 40%. At 20% inclusion digestibility of energy from starch reflected dry matter digestibility of the ingredient. Incorporation of 20% starch decreased protein digestibility, however this was not of a nutritionally significant level.

A negative effect of inclusion on the ground solvent-extracted soybean (SBM) digestibility was found, digestibility decreasing at greater than 20% inclusion. There was also a trend for protein digestibility to decrease with inclusion, however this was not significant. Dry matter, protein and energy digestibility for SBM by common carp was not affected by grinding, sifting or grinding the sifted middlings. Ground SBM inclusion did not affect dietary crude lipid digestibility. Soybean oil digestibility was extremely variable and was not affected by inclusion level.

Throughout the study digestibility varied with time, showing both adaptation over time and a cyclic daily variation. Analysis of daily faecal samples exemplified the degree to which these factors affect digestibility measurement.

Where measured, ash was significantly digestible, indicating ash should not be used as an endogenous digestibility marker.

The above results suggest digestibility varies with both ingredient inclusion level and time. Dietary ingredients may also adversely affect the digestibility of other dietary nutrients. When determining the digestibility of dietary ingredients, it would appear necessary to determine digestibility coefficients over the range of inclusion to be used and over time. Any adverse effects of the dietary ingredient on the digestibility of other dietary components also needs to be considered.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AcAla3 NA	acetyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanalide
AcAla3 - O - ME	acetyl-L-alanyl-L-alanyl-L-alanyl-methyl ester
ADC	apparent digestibility cooefficient
AIA	acid insoluble ash
Ala	alanine
Asp	asparagine
ATEE	acetyl-L-tyrosine ethyl ester
BAEE	N-benzoyl-L-arginine ethyl ester
BAPNA	N-benzoyl-DL-argininep-nitroanilide
BSDL	bile salt dependant lipase
BTEE	benzoyl-L-tyrosine ethyl ester
BTNA	benzoyl-L-tyrosine-p-nitroanilide
Ca	calcium
CPA	carboxypeptidase A
CPB	carboxypeptidase B
Cl	chlorine
Cr_2O_3	chromium oxide
Cu	copper
Da	dalton
DFSBM	defatted soybean meal
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetraacetic acid
EPU	equivalent pepsin units
Fe	iron
FFSBM	full fat soybean meal
g	gram
GPNA	glutyl-L-phenylalanine-p-nitroanalide
Hg	mercury
His	histidine
HRA	hydrolysis resistant ash
HROM	hydrolysis resistant organic matter
J	joule
l	litre
LAP	leucine aminopeptidase
Na	sodium
Phe	phenylalanine
PMB	p-hydroxymercuribenzoate

PMSF	phenyl methyl sulphonyl flouride
PP	di-isopropylphosphoflouridate
Pro	proline
PU	pepsin units
RIA	radioimmunoassay
Ser	serine
Suc (Ala)3 NA	succinyl-L-alanyl- -alanyl-L-alanyl-p-nitroanalide
Suc - AAPP - p - NA	succinyl-ala-ala-pro-phe-p-nitroanalide
SBTI	soybean trypsin inhibitor
Sn	tin
Solka floc	purified wood cellulose
TAME	tosyl-arginine ethyl ester
TLCK	tosyl-L-lysine chloromethyl ketone
TPCK	tosyl phenylalanine chloromethyl ketone
TU	trypsin units
TU_A	trypsin units (absorbance)
U	units
VFA	volatile fatty acid
VLDL	very low density lipoprotein

Glossary of Common and Scientific Names of Fishes

Common Name	Scientific Name
African bony tongue	*Heterotis niloticus*
African lungfish	*Protopterus aethiopicus*
Angler	*Lophiomus setigerus*
Artic charr	*Salvelinus alpinus*
Atlantic cod	*Gadus morhua*
Atlantic hagfish	*Myxine glutinosa*
Atlantic halibut	*Hippoglossus hippoglossus*
Atlantic salmon	*Salmo salar*
Bighead carp	*Aristinchthys nobilis*
Black sea anchovy	*Engraulis encrasicholus*
Black sea bream	*Acanthopagrus schlegelli*
Blue catfish	*Ictalurus furcatas*
Blue tilapia	*Oreochromis aureus*
Bluefin tuna	*Thannus thynnus*
Bonefish	*Carrasius auratus gibellio*
Brown trout	*Salmo trutta*
Burbot	*Lota lota*
Catla	*Catlacatla*
Capelin	*Mallotus villosus*
Channel catfish	*Ictalurus punctatus*
Chinook salmon	*Oncorhynchus tshawytscha*
Chum salmon	*Oncorhynchus keta*
Common bream	*Abramis brama*
Common carp	*Cyprinus carpio*
Cunner	*Tautogolubrus adspensus*
Deepwater dogfish	*Etmopterus spinax*
Dover sole	*Solea solea*
Dwarf African catfish	*Clarias isheriensii*
European perch	*Perca fluviatilis*
European sea bass	*Dicentrarchus labrax*
Goldfish	*Carrasius auratus*
Grass carp	*Ctenopharyngodon idella*
Green chromid	*Etroplus suratensis*
Greenland cod	*Gadus ogac*
Green snakehead	*Ophiocephalus (channa) punctatus*

Grenadier	*Coryphaenoides rupestris*
Herring	*Clupea harengus*
Hybrid striped bass	*Morone saxalatis X M. chrysops*
Japanese anchovy	*Engraulis japonica*
Japanese eel	*Anguilla japonica*
Lake trout	*Salvelinus namaycush*
Lake sturgeon	*Acipenser fulvescens*
Largemouth black bass	*Micropterus salmoides*
Longnosed gar	*Lepisosseus osseus*
Luderick	*Girella tricupsidata*
Mackerel	*Scomber japonicus*
Mahseer	*Tor khudree*
Matrinchã	*Brycon* c.f *melanopterus*
Menhaden	*Brevoorita tyrannus*
Milkfish	*Chanos chanos*
Mossambique tilapia	*Oreochromis mossambica*
Mrigal	*Cirrhinus mrigala*
Mullet	*Mugil cephalus*
Mummichog	*Fundulus heteroclitus*
Murray cod	*Maccullochella peelii peelii*
Nile tilapia	*Oreochromis niloticus*
Pacific salmon	*Oncorynchus spp.*
Pike	*Esox lucius*
Pike perch	*Stizotedison lucioperca*
Plaice	*Pleuronectes platessa*
Polar cod	*Boregadus saida*
Pompano	*Trachinotus carolinus*
Pumpkinseed sunfish	*Lepomis gibbosus*
Rabbitfish	*Siganus cannaliculatus*
Rainbow trout	*Oncorhynchus mykiss*
Red sea bream	*Pagrus major*
Roach	*Rutilius rutulus*
Rock bass	*Ampholites rupestris*
Rohu	*Labeo rohita*
Sabrefish	*Pelecus cultratis*
Sea bass	*Lates calcarifer*
Sea bream	*Sparus auratush*
Sharptoothed catfish	*Clarias gariepinus*

Sheatfish	*Silurus glanis*
Siberian sturgeon	*Acipensar baeri*
Silver bream	*Rhabdosagrus sarba*
Silver carp	*Hypohthalmichthys molitrix*
Sockeye salmon	*Oncorhynchus nerka*
Spiny dogfish	*Squalus acanthis*
Starry ray	*Raja radiata*
Striped bass	*Morone saxalatis*
Tambaqui	*Colossoma macropomum*
Tench	*Tinca tinca*
Turbot	*Scophthalamus maximus*
Walking catfish	*Clarias batrachus*
Walleye	*Stizostedion vitreum*
Whitefish	*Coregonus laveratus*
Whitefish hybrids	*Coregunus wartmanni X C. laveratus*
White sturgeon	*Acipensar transmontanus*
Yellow perch	*Perca flavescens*
Yellow tail	*Seriola quinqueradiata*

Chapter 1

Introduction

1.1 Introduction

The ancient proverb "Give a man a fish and he will live for a day, teach him to fish and he will have food for life" exemplifies the value placed on fish as a source of food from ancient times. This was true for most ancient cultures. Since before recorded history fish have been hunted, gathered and harvested by humans throughout the world, becoming the largest single source of animal protein. A high food value for fish has since been established with harvesting, trading, preserving and preparing of fish playing an important socioeconomic role in practically all civilisations.

1.1.1 Aquaculture - the Need and Advantages

Aquaculture is currently defined as:

> "...the farming of aquatic organisms including fish, crustaceans, molluscs and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators etc. Farming also implies individual or corporate ownership of the stock being cultivated generally for human consumption or use (FAO, 1990)"

As such aquaculture includes the stocking and management of natural waters through to production in controlled environment, self contained, intensive systems. The major aim of aquaculture, however, is protein production, with fish derived protein providing the major source of animal protein throughout the world. Recently fisheries production has approached, if not surpassed, the maximum sustainable yield resulting in a shortfall of fish for human consumption (Robinson, 1984). This combined with a continually increasing world population and the disproportionate increase in agricultural production as compared to population in many countries, has pushed towards increased aquaculture for the production of animal protein for human consumption (Robinson, 1984). Aquaculture is advantageous to wild fisheries in that it has the potential to allow more predictable quality and quantity of yield than conventional fisheries as it can be more effectively controlled. Harvesting is more efficient and somewhat guaranteed, animals can be provided as defined by the market requirements and manipulation of environmental conditions and stock genetics can maximise yields.

Aquaculture also demonstrates competitiveness against terrestrial agriculture. Fish convert food more efficiently than terrestrial animals and can utilise agricultural waste and by-products, thus placing less demand on the available nutrients throughout the

world and showing potential for reduced food costs. Land marginal in quality or unsuitable for agriculture may sustain successful fish farming ventures. Furthermore, in times when a greater emphasis is being placed on the nutritional values of foods, fish has significant advantages over lamb, beef and pork as a source of protein. Fish flesh is the cheapest and best source of animal protein with high quality, digestibility and utilisation for humans, and it is generally adequate in all the nutrients with the exception of calcium and vitamins A and C.

1.1.2 History of Aquaculture

Aquaculture has been used as a method of providing fish for thousands of years. Basic culture of carp is thought to have been practiced in China as early as 2000 B.C. Strong evidence also exists to suggest that before this, in ancient Babylon, active, well developed aquaculture endeavours flourished. The recognition of the potential nutritional, environmental and economic advantages of aquaculture has provided further pressure to develop commercially viable and profitable aquaculture enterprises throughout the world during the last 100 years.
Major scientific advances in the latter half of this century has allowed a large increase in fish hatcheries and commercial aquaculture. Finfish culture has extended to hundreds of different species and is currently responsible for the production of millions of metric tons of fish (approximately 12.7×10^6 metric tonnes worth US $25.5 million in 1989; FAO, 1993). Culture operations are becoming more refined and intensive with a shift from growing fish in open, earthen ponds and feeding on natural foods to holding them in artificial confinements and using supplemental feeding (FAO, 1993).

1.1.3 Artificial Diets and Aquaculture

The decreased dependency on natural food organisms associated with intensification of culture necessitates the development of nutritionally complete prepared feeds that will support the growth of the cultured species. High quality diets allow cultured organisms to attain their genetic potential for growth and reproduction, assist in reducing the onset of idiopathic disease and negate the effects of environmental stress. In order to produce such diets it is necessary to acquire a complete understanding of the principles underlying diet preparation. Of these principles, the nutritional requirements of a species and its ability to utilise dietary ingredients are fundamental.

However, only for channel catfish, *Ictalurus punctatus* (Wilson, 1991), common carp, *Cyprinus carpio* (Satoh, 1991) and rainbow trout, *Oncorhynchus mykiss* (Cho and Cowey, 1991) have the nutrient requirements been exhaustively researched. These

studies have revealed that fish, similar to terrestrial animals, require protein, lipids, minerals, vitamins and energy sources. The requirements for these nutrients differing between species and within a species at the different stages of its life cycle.

The concept for developing artificial diets for fish is, as for terrestrial animals, to nourish the animal to a desired level or form of productivity as profitably as possible. Since feed cost constitutes the highest recurring expense of intensive aquaculture, up to 70%, the cost of diets may determine the success of an operation and therefore reducing feed costs is a constant concern to most aquaculture endeavours. Utilising information of nutrient requirements allows diets to be formulated to meet the specific needs of species, thus avoiding loss in profitability associated with depressed growth rates due to undernourishment and the expense of over using expensive nutritional supplements. As more nutritional information becomes available artificial diets may be refined further resulting in more cost-effective feeds.

Besides the nutritional value of the food, other factors influence the cost-effectiveness of a diet. The effects of feeding on water quality and the loss of nutrients through leaching if feed is not eaten within a reasonable time must be considered. Therefore, water stability, feeding method and ration size are of concern to producers of artificial diets for fish to not only avoid waste of expensive ingredients, but also to reduce the risk of water quality problems.

1.2 Digestion

Digestion may be defined as processing ingested food material into molecules readily absorbable by the animal. Digestion in fish includes mechanical reduction of food particle size (trituration) by teeth, pharyngeal teeth or the gizzard, enzyme solubilization of organics, pH solubilization of inorganics and emulsification of lipids. Therefore, the ability to digest a particular feed depends on the presence of the appropriate anatomical apparatus for ingesting and breaking down the diet, as well as the presence and sufficient quantities of digestive enzymes and fluids to reduce it to the required molecular form. The anatomy of the digestive tract and its pattern of enzyme activities reflects the herbivorous, omnivorous or carnivorous nature of the species (Sabapathy and Teo, 1993). From this it can be seen that the study of digestibility and the activities of digestive enzymes are fundamental to the development of the technology to effectively produce balanced diets suitable for aquaculture.

1.2.1 Anatomy of the Digestive Tract

The morphology of the digestive tracts of various teleostean species has been reviewed by Kapoor *et al.* (1975), Ferraris and Ahearn (1984), Lovell (1988), Smith (1989) and Wee (1992).

Digestive tract gross anatomy in teleosts tends to be highly correlated to the natural food sources and feeding habits of the species. In general, carnivorous fish have developed prey capture mechanisms and a large stomach for storage of sizeable single meals. The proteinaceous meals are highly digestible, resulting in the evolution of a short, simple gut with a thick absorptive mucosa. Omnivorous and herbivorous species possess organs to mechanically break down plant cell walls and release their contents for digestion. Accompanying this is a long, thin gut to increase gut retention and maximise feed exposure to the digestive processes.

Histologically the intestinal wall of fish generally resembles that of higher vertebrates (Ferraris and Ahearn, 1984). A luminal layer of columnar cells with microvilli provides an absorptive epithelium which overlies the connective tissue of the submucosa. Surrounding this are the smooth muscle layers of the muscularis externa and an external serosal lining. However, the intestinal epithelium of fish does not generally form circular folds and villi, as in the mammalian intestine, but rather a series of longitudinal ridges (Ferraris and Ahearn, 1984). The extent of this folding varies with species and is thought to be dietary related. In carnivorous species the upper intestine exhibits extensive folding with a decrease in the lower intestine. The intestine of herbivores displays less mucosal folding than the carnivore gut, with no real variation between upper and lower gut segments (Ferraris and Ahearn, 1984) .

Ferraris and Ahearn (1984) and Buddington *et al.* (1987) suggested that both gut length (including the associated pyloric caeca) and mucosal folding are strategies for maximising absorption from differing food sources. Allocation of absorptive epithelium to a short gut with pyloric caeca and extensive mucosal folding compliments the digestion and absorption of a highly digestible diet, but is incompatible with fast transit diets of low digestibility.

As the current study involved the use of artificial diets composed of ingredients of homogeneous particle size, the digestion of these diets should not require any specific major anatomical structures. Therefore, the anatomy of the fish gastrointestinal tract will not be described in any further detail in this review. However, reference will be made to some adaptations in the relevant sections on nutrient digestion.

1.2.2 Digestive Fluids and Enzymes

Enzyme and pH solubilization and the emulsification of lipids requires the release of enzymes and fluids into the digestive tract, where the majority of digestion takes place. Allowing for the presence of agastric species of fish, the digestive enzymes and fluids in fish generally originate from four sources: gastric secretions, pancreatic secretions, bile and intestinal enzymes. The sources and contents of digestive secretions for teleosts have been reviewed by Smith (1989).

Digestive enzymes are found to vary in their action and are classified according to their effect as proteases (protein hydrolysis), lipases (lipid hydrolysis), esterases (ester hydrolysis) and carbohydrases (carbohydrate hydrolysis). The enzymes themselves are proteinaceous molecules with the ability to catalyse the hydrolytic reactions of digestion. These classes of enzymes have all been identified in the gastrointestinal tracts of teleosts, with both inter- and intra-species variation being found in the pattern of distribution and activities of enzymes.

Digestive enzymes have been reported in all sections of the teleostean gastrointestinal tract, including the rectum (Jany, 1976). There is, however, debate on the presence of digestive enzymes in the oesophagus, with many authors attributing oesophageal activity to be due to regurgitation, especially in agastric species such as carp. The presence of protease activity in the oesophagus of the milkfish, *Chanos chanos*, however has been harder to refute (Benitez and Tiro, 1982). Milkfish possess a spirally folded oesophagus, similar to the absorptive intestine of other species, which is accompanied by numerous mucosal glands. Ferraris *et al.* (1987) suggested these glands were involved in both mucus production and osmoregulation. Numerous other oesophageal enzyme activities, several also describing oesophageal glands, have been reported in gastric species, suggesting "true" oesophageal digestion. Anderson (1991) reported a large proportion of total protease activity in the oesophagus of the luderick, *Girella tricuspidata*, a gastric marine herbivore, while 25% of the acidic protease activity of the black sea anchovy, *Engraulis encrasicholus*, was also found in the oesophagus (Martinez and Serra, 1989). Peptic and tryptic activity in the oesophagus of the rabbitfish, *Siganus canaliculatus*, and peptic activity in the oesophagus of the seabass, *Lates calcarifer*, were described by Sabapathy and Teo (1993). Kapoor *et al.* (1975) also described the presence of pepsinogen secreting glands in the oesophagus of some species. Similarly Sarbahi (1951) discovered the presence of amylase, maltase and invertase in the oesophagus of the goldfish, *Carassius auratus*, which he believed may bear relation to his previous work where highly racemose glands were found in the mucosa of the oesophagus of the rohu, *Labeo rohita*, a species in the

same family as Cypridinae. Sarbahi (1951) also referenced work describing the continuation of gastric glands into the oesophagus of the sturgeon.

Less debate surrounds the presence of enzyme activity in the other regions of the gastrointestinal tract. Several enzymes have been reported in the stomach fluids of gastric fish. Pepsin being the best known gastric enzyme. Other gastric enzymes include chitinase, produced by the stomach mucosa, lipase and esterase which are thought to be involved in intracellular digestion in the gastric mucosa, and, cellulase, considered by some to be an exogenous enzyme from gut micro-flora or ingested feed items.

Intracellular and extracellular digestion also occur in the intestine of fish. Digestive enzymes and fluids for this region of the gastrointestinal tract originate from the hepatopancreas, intestinal wall, gut micro-flora and natural foods. Membrane bound enzymes include leucine amino peptidase, acid and alkaline phosphatases, amyloglucosidase and γ glutamyl transferase. The intracellular intestinal enzymes of fish have been little studied although dipeptidases and tripeptidases have been described. Extracellular enzymes in the intestinal lumen appear to be of pancreatic origin. These enzymes include proteases (trypsin, chymotrypsin, carboxypeptidase, elastase and collagenase), lipases, carboxyesterase and numerous carbohydrases. The presence of the above enzymes is well documented and has been reviewed by Smith (1989). A detailed review of the distribution and relative activities of these digestive enzymes will be given in the relevant sections on nutrient digestibility.

Other sources of digestive enzymes in teleosts, as with terrestrial animals, are the resident gut micro-flora and the exogenous enzymes commonly found in natural feeds. The involvement of micro-floral fermentation in fish digestive processes has been suggested in studies on cellulose digestibility (Stickney and Shumway, 1974; Das and Tripathi,1991). Recently, however, studies on marine herbivores have provided the strongest argument for a role of intestinal bacteria in digestion. Kandel *et al.* (1994) and Rimmer and Wiebe (1987) discovered unique, valved, caecum-like pouches near the posterior end of the intestine. These well vascularised organs, capable of containing up to 1.5 to 2 times the volume of the stomach suggested a fermentation chamber-type arrangement similar to that of terrestrial herbivores. Within these pouches the abundance and diversity of micro-organisms was greatest, as was the concentration of volatile fatty acids, an end product of fermentation.

The influence of feed associated exogenous enzymes has been reported by Lauff and Hofer (1984) in studies on larval whitefish hybrids, *Coregonus wartmann* X *C lavaretus*. Exogenous trypsin supplied approximately 80% of the tryptic activity at

first feeding, quickly declining to approximately 20%, a level similar to that of rainbow trout and roach, *Rutilus rutilus*. The proportions of exogenous chymotrypsin and aminopeptidases were similar to those of trypsin. Segner *et al.* (1989) described positive histochemical staining in zooplankton prey items in the foregut and stomach of whitefish, *Coregonus lavaretus*, larvae. Feed associated cellulase activity has also been reported, this activity attributed to bacteria colonising feed (Prejs and Blasczyk, 1977; Deegan *et al.*, 1990) and cellulase producing prey items (Niederholzer and Hofer, 1979; Lindsay and Harris, 1980).

The final source of digestive fluids is bile, a mixture of the metabolic products of the liver, including breakdown products of cholesterol and steroids, and decomposition products of haemoglobin (bilirubin and biliverdin). Bile is also the major pathway of excretion for foreign substances (xenobiotics) from the liver. The role of bile in fish digestion appears to be limited to the emulsification of fats by bile acids (bile salts) allowing the lipids to be acted upon by pancreatic lipases. This will be described in more detail in section 1.2.4 on lipid digestion.

1.2.3 Protein Digestion

To be of nutritional value, crude protein needs to be reduced to its component molecules and made available to the metabolic processes of the animal. The mechanisms of digestion are responsible for breaking down ingested protein molecules into amino acids, di-/tri-peptides and small polypeptides which are subsequently internalised via the mechanisms of absorption. Once within the organism these molecules may be digested further, utilised as building materials for tissue proteins or catabolised as a source of energy. Digestion and absorption in teleosts appears to be efficient and non-limiting with values being high for most dietary protein sources, even at elevated levels of inclusion. Any decreases in the efficiency of these processes may generally be attributed to reduced hydrolysis or absorption due to the presence of inhibitors.

The digestion of protein by fish has been reviewed by Ash (1985) and Smith (1989). In general, the physiological processes by which protein is hydrolysed and the types of enzymes involved are consistent in most monogastric vertebrates. Therefore, reviews of the processes of the digestion of protein will only really vary in the pattern of distribution and the activities of these digestive enzymes. In this discussion of the general mechanisms of protein digestion in teleosts emphasis will be placed on the variation in digestive enzymes.

1.2.3.1 Proteolytic Enzymes

Proteolytic enzymes - proteases - break down ingested protein into simpler molecules through hydrolysis of peptide bonds within the molecule. These enzymes are classified according to their site of hydrolysis with enzymes acting in the middle of the molecule termed endopeptidases and those hydrolysing terminal peptide links are referred to as exopeptidases. Both these enzyme groups are produced in zymogen forms (inactive precursors) which are activated at the site of digestion, thus lessening the risk of self digestion.

The susceptibility of peptide bonds to specific peptidases is governed by the nature of the chemical groups bordering the bond, with a bond readily hydrolysed by one protease being resistant to hydrolysis by others.

Endopeptidases are responsible for reducing ingested protein molecules to smaller polypeptides, the nature of which depend upon the type and activity of endopeptidases present. Generally, most normally occurring protein molecules are efficiently broken down to polypeptides by the three main endopeptidases, these being pepsin, chymotrypsin and trypsin. Pepsin hydrolyses bonds on the amino side of an aromatic radical, chymotrypsin reduces peptide links on the carboxyl side of an aromatic radical and trypsin acts on bonds adjacent to arginine and lysine molecules (De Silva and Anderson, 1995). Other protein substrates, however, require specialised endopeptidases such as the elastin-elastase system.

Exopeptidases are divided into carboxypeptidases, aminopeptidases and dipeptidases, again depending on the site of action. Carboxypeptidases remove the terminal amino acids at the free carboxyl end of the polypeptide, while aminopeptidase liberates amino acids at the free amino end of the chain (De Silva and Anderson, 1995). Dipeptidases are thought to be responsible for the intracellular digestion of dipeptides absorbed from the alimentary canal.

1.2.3.1.1 Pepsin

Pepsins are gastric endopeptidases formed by partial hydrolysis of the zymogen, pepsinogen. Activation occurs autocatylitically under acidic conditions. Early work on pepsin was performed on porcine molecules, multiple pepsins being reported (Fruton, 1971). These enzymes were approximately 34 kDa (pepsinogen 41 kDa) in size. Optimal pH was between 2 and 3.5, depending on the nature of the substrate, with irreversible denaturation occurring at pH greater than 7. Porcine pepsins have broad side chain specificity, with sensitive bonds generally present in dipeptidyl units

containing at least one hydrophobic amino acid residue such as phenylalanine, tyrosine, leucine or methionine, although a number of exceptions were noted (Fruton, 1971).

Pepsin activity has most commonly been determined by assaying for proteolytic activity, using a protein substrate, over the physiological pH range. A hydrolytic effect with optimum activity under acidic conditions (especially at pH 2 or 3) is termed "pepsin" or "pepsin-like" activity. Further identification of true pepsin activity involved inhibiting activity with pepstatin (a bovine pepsin inhibitor) and denaturation under alkaline conditions. Pepsins are irreversibly inactivated at elevated pH's, whereas other acidic proteases show reduced activity but are stable under alkaline conditions.

Investigations of teleostean pepsins have generally identified protease activity at a low pH as pepsin, activity being expressed in units with one unit of pepsin (PU) defined as the amount of pepsin catalysing the release of 1 μmole or 1 mg of tyrosine per minute under assay conditions. Some authors expressed pepsin activity as equivalent units (EPU) as compared to a commercially available standard. The above terms will be used in the following review.

Several workers reported changes in gastric protease activity with pH indicating a teleostean pepsin/HCl system. Gastric protease activity in sheatfish, *Silurus glanis*, decreased from 50 to 1.9 PU g^{-1} protein with a pH increase from 2 to 7.5 (Jónás *et al.*, 1983). Similar changes in activity were found for the European perch, *Perca fluviatilis*, and Mozambique tilapia, *Oreochromis mossambica*, by Fish (1960), with Bayliss (1935) describing gastric proteolytic activity in plaice, *Pleuronectes platessa*, to be optimal between pH 1.5 and 2.5, and lost above pH 5.5. Pepsin activity (PU mg^{-1} protein) in larval seabass increased from 3.8 at day 8 to 85.2 by day 30, with a corresponding decrease in the pH of the presumptive stomach from 7.7 to 3.2, suggesting the early development of a pepsin/HCl system (Walford and Lam, 1993).

Protease activity with an optimum pH of 2.8 was reported in the intestine and stomach of tilapia, however removal of gut contents isolated activity to gastric tissue, indicating a pepsin produced by the stomach mucosa (Nagase, 1964). Munilla-Moran and Stark (1990) found gastric protease activity with an optimum pH between 2 and 3 in the turbot, *Scophthalmus maximus*. Peptic activity (3.4 PU g^{-1} wet tissue weight) with an optimal pH of 3 was demonstrated in the stomach contents of the sharptoothed catfish, *Clarias gariepinus* (Uys and Hecht, 1987). Glass *et al.* (1987) described the main stomach protease activity in Atlantic halibut, *Hippoglossus hippoglossus*, acting at pH 1.8, with smaller amounts of another protease acting at pH 4.5. Reimer (1982) found

pepsin activity ranging between 4×10^{-4} and 80×10^{-4} EPU ml^{-1} in the gastric juice of the Amazon fish matrinchã, *Brycon* c.f. *melanopterus*. Rebhein *et al.* (1986) reported pepsin activity (PU mg^{-1} protein) of 7.3, 61.8 and 206.9 in the stomach tissue of the Antarctic fishes *Natothermia marmorata*, *Champsocephalus gunnai* and *Chaenocephalus asceratus* respectively, with a reduced activity in the stomach contents. White sturgeon, *Acipenser transmontanus*, displayed high gastric peptic activity, 25 PU mg^{-1} protein (Buddington and Doroshov, 1986). Acidic protease activity has also been described in the carnivorous freshwater perches, *Ambassis nama* and *Ambassis ranga* (Ray 1989), the dwarf African catfish, *Clarias isheriensii* (Fagbenro, 1990), the largemouth black bass, *Micropterus salmoides* (Sarbahi, 1951) and the herbivorous luderick (Anderson, 1991).

The level of pepsin activity has been related to both diet quality and quantity. Ananichev (1959) reported pepsin activity in burbot, *Lota lota*, pike perch, *Stizostedian luciperca* and common bream, *Abramis brama* to be greatest at periods of peak nutrition. However, Nagase (1964) found that the secretion of pepsin was independent of protein quality or quantity, and Kohla *et al.* (1992) reported pepsin activity in tambaqui, *Colossoma macropomum*, to be independent of dietary nutrient level but positively correlated with stomach content, suggesting a mechanical control of pepsin secretion.

Pepsin activity appears to reflect natural dietary preferences of the species, probably in relation to the dietary protein content. Sabapathy and Teo (1993) reported a higher pepsin activity for the carnivorous seabass than the herbivorous rabbitfish, and the herbivorous Dover sole, *Solea solea*, displayed approximately 5% the acidic protease activity of the carnivorous turbot and Atlantic halibut (Glass *et al.*, 1989). High peptic activity was also displayed by the white sturgeon (Buddington and Doroshov, 1986), and the larvae of rainbow trout and black sea bream, *Acanthopagrus schlegelli*, (Kawai and Ikeda, 1973a; 1973b) indicating their carnivorous nature. The slight acidic protease activity in the stomach of the herbivorous milkfish (Benitez and Tiro, 1982) and very low pepsin activity in carp larvae (Kawai and Ikeda, 1973b) suggesting a low dependence on pepsin predigestion in these herbivorous species.

Development of pepsin activity in larval fish described a late onset of pepsin-like activity in Dover sole (Clark *et al.*, 1986; Glass *et al.*, 1989), European seabass, *Dicentrarchus labrax* (Zambonino Infante and Cahu, 1994) and striped bass, *Morone saxatilis* (Baragi and Lovell, 1986). Delayed development of activity was ascribed to the poor development of the gastric glands during early larval stages in marine fish. A marked increase in peptic activity in the larvae of turbot (Munilla-Moran and Stark,

1989) and lake sturgeon, *Acipenser fulvescens* (Buddington, 1985), was concomitant with the commencement of exogenous feeding.

Several teleosts have been found to lack peptic activity, including the mullet, *Mugil cephalis* (Ishida, 1935) and goldfish (Sarbahi, 1951). Jany (1976) reported no proteolytic activity in the usual acid range for pepsin, but did find activity at pH 4.5 in the stomachless bonefish, *Carassius auratus gibelio*. Seiderer *et al.* (1987) also described little evidence of proteolytic activity in the stomach of the anchovy *Engraulis capensis*, however these assays were performed at pH 7.8 and therefore are not true indications of pepsin activity. This seems probable as Martinez and Serra (1989) subsequently found the black sea anchovy to exhibit a peak protease activity at pH 3. Further analysis revealed a specific acidic protease activity of 0.7 and 0.9 PU mg^{-1} protein in the oesophagus and stomach respectively.

Using immunoflouresence techniques, Reifel *et al.* (1985) identified pepsinogen in the gastric mucosa rock bass, *Ambloplites rupestris*, longnose gar, *Lepisoseus osseus*, pumpkinseed sunfish, *Lepomis gibbosus*, and yellow perch, *Perca flavescens*. Antibodies to both human pepsinogen I and II showed a localised reaction in the oxynticopeptic cells (similar to mammalian chief cells) of the gastric glands in the corpus stomach, suggesting these species possess two immunohistochemically distinct types of pepsinogen. This is in accordance with the identification of multiple pepsins/pepsinogens in other vertebrates (Fruton, 1971).

Pepsin has been isolated and characterised from the gastric mucosa of several species including Atlantic cod, *Gadus morhua* (Gildberg *et al.*, 1990), capelin, *Mallotus villosus* (Gildberg and Raa, 1983), chum salmon, *Oncorhynchus keta* (Sánchez-Chiang *et al.*, 1987), Greenland cod, *Gadus ogac* (Squires *et al.*, 1986a; 1986b), polar cod, *Boregadus saida* (Arunchalam and Haard, 1985), and rainbow trout (Twinning *et al.*, 1983). These molecules were generally found to be in the zymogen form, being activated at pH 2.0 with HCl. Identification of these gastric proteases as pepsin was based on optimum pH in the range of 2.5 to 3.2, indicating a preference for acidic conditions, and inhibition by pepstatin or irreversible inactivation in alkaline conditions. Affinities for a haemoglobin substrate were found to vary, however all enzymes showed high activities at low temperatures, suggesting an adaptation to the poikiliotherm environment. Ugolev and Kuz'mina (1993) showed the optimal temperature of pepsin in membrane enzyme preparations from burbot, pike, *Esox lucius*, and sheatfish to have an extremely broad peak (20°C to 60°C) with 40 to 70% of maximal activity retained at low temperatures (0°C). Molecular weights of the zymogen precursors were between 40.0 and 45.0 kDa while the activated pepsin

molecules displayed molecular weights from 23.0 to 38.0 kDa. The ranges of these molecular weights are generally in the range of those reported for human, bovine, porcine and chicken molecules (Fruton, 1971).

1.2.3.1.2 Trypsin

Trypsin is a proteolytic enzyme produced by the pancreatic cells of vertebrates in an inactive zymogen form, trypsinogen, and released into the intestine where it is activated, usually autocatalytically. In studies of terrestrial proteolytic enzymes many esterolytic and proteolytic enzymes exhibiting peak activity between pH 7-9 have been termed as trypsins or "trypsin-like" enzymes. For this reason Keil (1971) defined trypsins as enzymes of molecular weight 20.0-25.0 kDa, which preferentially hydrolyse peptic bonds involving the carboxyl group of basic amino acids, arginine and lysine and with a serine and a histidine residue in the catalytic mechanism. In order to fulfil this criteria it was necessary to identify the presence of serine (Ser 183) and histidine (His 46) residues within the active site through the use of inhibitors such as diisopropylphosphoflouridate (DPF), an organic phosphate that binds Ser 183, and tosyl-L-lysine chloromethyl ketone (TLCK) which irreversibly binds His 46. It was also necessary to display the specificity of the enzyme, generally by using arginine or lysine containing synthetic substrates in trypsin specific assays.

In the literature available on the proteolytic enzymes of fish, trypsin has received by far the most attention. However, similar to the terrestrial vertebrate studies many workers investigating teleostean trypsin have used general proteolytic assays (usually employing casein or haemoglobin as a substrate) and designated proteolytic activity with a peak in the pH range of 8 to 9.5 as trypsin or "trypsin-like" activity. In this review the term "trypsin-like activity" will be used for findings of studies using assays not specific for trypsin activity.

Intestinal trypsin-like activity has been reported in many species of fish including Atlantic salmon, *Salmo salar* (Usher *et al.*, 1990), dwarf African catfish (Fagbenro, 1990), *Engraulis capensis* (Seiderer *et al.*, 1987), the freshwater perches *Ambassis nama*, *Ambassis ranga* and *Colisa fasciata* (Ray, 1989), mahseer, *Tor khudree* (Bazaz and Keshavanath, 1993), Mozambique tilapia (Nagase, 1964) and mullet (Ishida, 1935). Rehbein *et al.* (1986) identified trypsin-like activity in the intestinal contents as well as the intestinal tissue of *Notothensia rosii marmorata*, *Notothenia neglecta* and *Champosecephalus gunnai*. Other studies assayed segments of gut and their associated glands separately, allowing a more detailed anatomical location of trypsin-like activity. Sarbahi (1951) reported trypsin-like activity in the hepatopancreas, intestinal bulb and the anterior and posterior intestine of the goldfish. That study also

identified trypsin-like activity in tissues from the pancreas, liver and pyloric caeca, and juices from the stomach, duodenum and small intestine of the largemouth black bass. Bayliss (1935) also found trypsin-like activity to be present in the mucous membrane of the intestine and the hepatopancreas of the plaice, with the majority in the hepatopancreas. Similarly, Das and Tripathi (1991) reported trypsin-like activity in the hepatopancreas and intestine of the grass carp, *Ctenopharyngodon idella*. However, the luderick only contained trypsin-like activity associated with the pyloric caeca (Anderson, 1991).

Trypsin-like activity has been identified in the larval stages of development of the black sea bream (Kawai and Ikeda, 1973a), common carp (Kawai and Ikeda, 1973b), rainbow trout (Kawai and Ikeda, 1973b) and the seabass (Walford and Lam, 1993).

Early synthetic substrates specific for the identification of trypsin activity were produced prior to much of the work on teleost proteases (Erlanger *et al.*, 1961). These substrates permitted the identification of trypsin activity by its preferential hydrolysis of peptic bonds containing the carboxyl group of basic amino acids, arginine or lysine. Assays using these substrates therefore provided specific trypsin activity. This activity was expressed as units mg^{-1} protein, with one unit of trypsin (TU) defined as the amount of trypsin catalysing the transformation of 1 μmole of substrate under assay conditions. However, the routine use of spectrophotometrical assays based on the substrates *N*-α-benzoyl-D,L-arginine *p*-nitroanilide (BAPNA), tosyl-arginine methyl ester (TAME) and *N*-benzoyl-L-arginine ethyl ester (BAEE) has led to the presentation of trypsin activities as change in absorbance per unit time per unit wet weight tissue or per ml extract, without conversion of values to trypsin units. Therefore, in the following the term TU_A will be defined as the amount of trypsin resulting in an increase in absorbance of 0.001 $minute^{-1}$ under assay conditions.

Using specific assays trypsin activity was identified in the Atlantic halibut (Glass *et al.*, 1989), Dover sole (Glass *et al.*, 1989), turbot (Glass *et al.*, 1989) and four species of cichlids (Sturmbauer *et al.*, 1992). Basal (starved) trypsin activity of 15.8 ± 7.8 TU ml^{-1} and regular feeding level of 446.4 ± 200.6 TU ml^{-1} were reported tambaqui (Kohla *et al.*, 1992), and matrinchã displayed trypsin levels from 280 to 530 TU ml^{-1} in the gut fluid depending on diet (Reimer, 1982). Tryptic activity was identified in the intestinal wall of the omnivorous carp, 190.4 TU_A mg^{-1} extract, herbivorous silver carp, *Hypophthalmichthys molitrix*, 331.0 TU_A mg^{-1} extract, and carnivorous sheatfish, 1514.0 TU_A mg^{-1} extract (Jónás *et al.*, 1993).

Again studies have been performed describing the distribution of tryptic activity within the alimentary tract and its associated glands. Bitterlich (1985) reported tryptic activity

in both silver carp and bighead carp, *Aristichthys nobilis*, that increased over the first three segments of the intestine and decreased rapidly thereafter. Trypsin activity in Atlantic halibut was greatest in the plyoric caeca and decreased through the foregut and midgut to be absent in the hindgut (Glass *et al*., 1987). Munilla-Moran and Stark (1990) described trypsin activity in the turbot to be constant throughout the intestine and the rectum, only decreasing in the hindgut and rectum following starvation. Similarly, white sturgeon had low tryptic activity in the stomach but uniform activity in the intestine and spiral valve (Buddington and Doroshov, 1986). Sabapathy and Teo (1993) identified tryptic activity in the oesophagus (3130 TU_A g^{-1} wet weight tissue), stomach (3530 TU_A g^{-1} wet weight tissue), intestine (2169 TU_A g^{-1} wet weight tissue) and pyloric caeca (4350 TU_A g^{-1} wet weight tissue) of the rabbitfish, while activity in the seabass was isolated to the intestine (1170 TU_A g^{-1} wet weight tissue) and the pyloric caeca (630 TU_A g^{-1} wet weight tissue). Milkfish contained intestinal (anterior 1760 TU mg^{-1} protein and posterior 4050 TU mg^{-1} protein) and pyloric caecal (5072 TU mg^{-1} protein) as well as pancreatic (5510 TU mg^{-1} protein) activity (Benitez and Tiro, 1982). Trypsin activity was mainly in the bulbous juice, hepatopancreas and rectum of the bonefish (Jany, 1976), and in the hepatopancreas and not the intestine of the Atlantic cod (Overnell, 1973). Eshel *et al.* (1994) found trypsin activity to be low in the pyloric caeca of European seabass and hybrid striped bass, *Morone chrysops* X *M. saxatilis*, activity increasing in the intestine of both species.

Larval studies using specific assays have identified trypsin activity in Artic charr, *Salvelinus alpinus* (Dabrowski *et al.*, 1992), whitefish hybrids, *Coregonus wartmanni* X *C. laveratus* (Lauff and Hofer, 1984), Dover sole (Clark *et al.*, 1986), lake sturgeon (Buddington, 1985), rainbow trout (Lauff and Hofer, 1984), roach (Lauff and Hofer, 1984), seabass (Walford and Lam, 1993), striped bass (Baragi and Lovell, 1986) and turbot (Munilla-Moran and Stark, 1989). Trypsin activity has also been identified in whitefish (Segner *et al.*, 1989) and seabream, *Sparus auratush* larvae (Sarasquette *et al.*, 1993) using histochemical techniques and radioimmunoassay (Hjelmeland *et al.*, 1988; Pedersen *et al.*, 1987; Pedersen *et al.*, 1990; Pedersen and Anderson, 1992; Ueberschair *et al.*, 1992) and fluorescence techniques (Ueberschair *et al.*, 1992) have been used to identify tryptic activity in larval herring, *Clupea harengus*.

Tryptic activity is generally depressed during yolk absorption and increases at first feeding. Larval seabream lacked trypsin activity until the commencement of exogenous feeding (Sarasquette *et al.*, 1993). Rainbow trout, black sea bream and carp all displayed low tryptic activity until yolk depletion (days 20, 3 and 30 respectively) after which activity increased with age, with 80 day old carp exhibiting

levels 25% that of adults (Kawai and Ikeda, 1973a; 1973b). Trypsin activity in larval Dover sole was present at day 24 (1760 TU_A mg^{-1} protein) and increased thereafter (Clark *et al.*, 1986). Similarly, Lauff and Hofer (1984) found trypsin activity increased with age in rainbow trout, whitefish hybrids and roach. Tryptic activity in larval seabass increased at first feeding from 1200 TU mg^{-1} protein (day 7) to 5000 TU mg^{-1} protein (day 17), however a decrease to 1300 TU mg^{-1} protein by day 30 was observed (Walford and Lam, 1993). Similarly development in trypsin activity was present at first feeding in striped bass larvae, activity increasing to day 12 and decreasing to day 16 (Baragi and Lovell, 1986). Activity subsequently increased to be above day 12 levels by day 25. Pedersen *et al.* (1987), measuring the total trypsin content of larval herring by radioimmunoassay (RIA), also showed a three phase development. Trypsin content increased from day 0 (100 ng $larvae^{-1}$) to day 4 (900 ng $larvae^{-1}$), when exogenous feeding commenced. Activity decreased to day 14, followed by an increase with age. Although this study showed an increased trypsin content during yolk absorption, which is contradictory to the other studies, the RIA used could not differentiate between trypsin and trypsinogen and therefore the observed increase may be due to trypsinogen synthesis and not trypsin activation. Increased reserves of trypsinogen would suggest a preparation for the commencement of exogenous feeding. Contradicting the general pattern of larval trypsin development described, trypsin activity in lake sturgeon remained low throughout both the yolk utilisation and larval feeding, only increasing post metamorphosis (Buddington, 1985).

The synthesis and activation of trypsin in fish appears to be similar to that of terrestrial vertebrates. Ueberschair *et al.* (1992), differentiating between trypsin and trypsinogen by fluorescence and RIA techniques, found the pancreas of larval herring contained predominantly inactive trypsinogen and the intestine activated trypsin. This work, supported by Munilla-Moran and Stark (1989) who suggested that trypsinogen is rapidly activated autocatylitically in the intestine of larval turbot, indicates that teleostean trypsin is synthesised in the zymogen form in the pancreas/hepatopancreas and released to the intestine where activation occurs. Although Sarbahi (1951) suggested trypsin secretion by both the hepatopancreas and intestinal mucosa of goldfish, it appears the later may have been due to trypsin bound to the glycocalyx of the enterocytes, as was indicated by Segner *et al.* (1989) for the intestine of larval whitefish.

The majority of studies on the distribution of tryptic activity throughout the intestine of fish indicate trypsin release into the intestine posterior to the gastro-intestinal junction, or its equivalent region in agastric species (Jany, 1976; Benitez and Tiro, 1982;

Bitterlich, 1985; Martinez and Serra, 1989; Segner *et al.*, 1989; Sarasquette, 1993). The occurrence of trypsin activity in the stomach and oesophagus of the rabbitfish (Sabapathy and Teo, 1993) appears to be the exception, although this activity may be due to regurgitation.

Evidence exists for the subsequent reabsorption of trypsin along the length of the gastrointestinal tract. Bitterlich (1985) suggested that decreased trypsin activity in the posterior 7 segments of silver and bighead carp intestines represented enzyme reabsorption (97.9 and 97.8% respectively). Similarly Sarasquette *et al.* (1993) observed a trypsin activity decreasing from the anterior to posterior of the larval seabream digestive tract, as did Glass *et al.* (1987) in Atlantic halibut. However the strongest evidence for this comes from work by Segner *et al.* (1989). Using histochemical techniques, tryptic activity was identified associated with the food bolus and brush border in the intestine of larval whitefish, showing greater staining intensity in the anterior half of the intestine. Immediately posterior to the ileorectal valve the tryptic activity of the bolus decreased to zero, but enterocyte activity was still present. In the rectal region all activity was lost. Contradicting the theory of trypsin reabsorption was the presence of trypsin activity in the posterior intestine of the goldfish (Sarbahi, 1951), increased tryptic activity in the posterior intestine of the milkfish (Benitez and Tiro, 1982) and significant trypsin activity in the rectum of the stomachless bonefish (Jany, 1976).

The rate of trypsin secretion appears to be related to both the quantity and quality of the food available. Highest trypsin secretion in burbot, pike perch and bream coincided with the periods of most intense nutrition (Ananichev, 1959), while trypsin activity in tambaqui was positively correlated with gut fullness (Kohla *et al.*, 1992) . Tryptic activity in cichlid species increased with increasing proportion of animal food items in the diets (Sturmbauer *et al.*, 1992). Further work on larval herring has shown trypsin secretion to be proportional to prey availability, density and consumption. Trypsin content was greater for feeding than non-feeding larvae (Ueberschair *et al.*, 1992), and the total trypsin content of the larvae increased with increased prey (copepod larvae) density, secretion of trypsin from the pancreas to the intestine being dependant on the number of ingested prey (Pedersen *et al.*, 1987). The stimulation for trypsin release to the intestine was later suggested to be mechanical when polystyrene spheres were found to stimulate trypsin secretion with the level dependent on the diameter of the spheres (Hjelmeland *et al.*, 1987; Pedersen and Andersen, 1992).

Several studies have implicated the quality of diet in trypsin secretion. Intestinal tryptic activity increased with the protein content of the diet for both larval (Kawai and

Ikeda, 1972; 1973a; Segner *et al.*, 1989; Dabrowski *et al.*, 1992) and adult fish (Nagase, 1964; Reimer, 1982; Kohla *et al.*, 1992). The trypsinogen content of the hepatopancreas of grass carp increased when animals were fed a high protein, low fibre diet (Das and Tripathi, 1991). Reimer (1982) described trypsin activity for a high protein diet nearly twice that of a high carbohydrate diet, with a mid-range activity for a diet high in fat, and Bazaz and Keshavanath (1993) observed an increase in trypsin activity with sardine oil supplementation of the diet. Zambonino-Infante and Cahu (1994) described enhanced trypsin secretion following synthetic essential amino acid substitution (5%) into a fishmeal based diet, suggesting free amino acids may also stimulate trypsin release in fish. These variations in trypsin activity with dietary nutrient levels suggest hormonal as well as mechanical control of trypsin release. Such control has been proposed to be due to the release of a trypsin binding peptide into the intestine (probably of pancreatic origin) which binds excess trypsin, unbound peptide feeding back to stimulate trypsin secretion (Hjelmeland *et al.*, 1988). Evidence for hormonal control was also provided by Bitterlich (1985) who found trypsin secretion increased to compensate for a sub-optimal pH in the intestine of silver carp.

Trypsin release has also been correlated to environmental stimuli by Dabrowski *et al.* (1992) who hypothesised that trypsin synthesis varied as the pancreas responded to photosynthetic and feedback stimulation resulting in cyclic growth patterns in larval Artic charr.

The isolation of trypsin has been of great industrial interest as 80% of the enzymes used as processing aids in the food industry are protein hydrolases. Fish trypsins were seen as being particularly advantageous and as such have been isolated and characterised in numerous species including the black sea anchovy (Martinez *et al.*, 1988), Atlantic cod (Overnell, 1973; Raae and Walker, 1989; Simpson *et al.*, 1990), capelin (Hjelmeland and Raa, 1982), the catfish, *Parasilurus asotus* (Yoshinaka *et al.*, 1984a; 1984b), chinook salmon, *Oncorhynchus tshawytscha* (Croston, 1960), chum salmon (Uchida *et al.*, 1984a; 1984b), common carp (Cohen *et al.*, 1981a; 1984b), cunner, *Tautogolubrus adspersus* (Simpson and Haard, 1987), Greenland cod (Simpson and Haard, 1984), Japanese anchovy, *Engraulis japonicus* (Heu *et al.*, 1995), mackerel, *Scomber japonicus* (Pyeun *et al.*, 1991; 1992), menhaden, *Brevoorita tyrannus* (Pyeun, 1990), mullet (Guizani *et al.*, 1991), Pacific salmon, *Oncorhynchus spp.* (Pivnenko *et al.*, 1989), rainbow trout (Kristjannsen, 1991) and the sardine, *Sardinops melanosticta* (Murakami and Noda, 1981).

Many of these species exhibited multiple isoenzymes of trypsins. Atlantic cod (Raae and Walther, 1989), black sea anchovy (Martinez *et al.*, 1988), capelin (Hjelmeland

and Raa, 1982), mackerel (Pyeun *et al.*, 1991, 1992), and menhaden (Pyeun *et al.*, 1990) displayed two isoenzymes, four trypsin molecules were described for carp (Cohen *et al.*, 1981a) and six anionic and one cationic trypsins were isolated in chum salmon (Uchida *et al.*, 1984a). Simpson and Haard (1987) found only one trypsin molecule in stomachless cunner, but also described a smaller enzyme with trypsin-like properties. Reeck *et al.* (1970) separated 3 trypsinogens from the pancreas of the African lungfish, *Protopterus aethiopicus*.

Using SDS polyacrylamide gel electrophoresis, the molecular weights of isolated trypsin molecules have been determined to range from 22.0 to 28.0 kDa. These molecular weights are similar to those described by Keil (1971) to represent true trypsins, although it appears that teleostean trypsins may be slightly larger than those of terrestrial vertebrates. Specific substrates and inhibitors were used to identify the remaining criteria of true trypsins as suggested by Keil (1971).

Optimal temperatures for trypsin activity were found to be between 40°C and 60°C, lower than those of mammalian trypsins, suggesting an adaptation to the poikiliotherm environment. Ugolev and Kuz'mina (1993) similarly found the optimal temperature of tryptic activity in a membrane enzyme preparation to be 50 to 60°C. Heat stability was lower than mammalian trypsins, with at least 50% activity generally lost at 50°C for 30 minutes. However, the trypsins isolated from the stomachless cunner were remarkably heat stable, retaining 50% activity at 100°C for 30 minutes (Simpson and Haard, 1987). Optimum pH was generally 7.5 to 10.0 using BAPNA as a substrate, while higher optimal pH's (9 to greater than 11+) were found when assaying with casein. Molecules were unstable in an acidic environment and most stable between pH 7 and 9. The amino acid composition of the molecules varied between species, but generally favoured a high acidic to basic amino acid ratio; a profile causing instability in acidic conditions. A decreased number of cysteine residues allowing the formation of fewer disulphide bonds may account for the reduced heat stability in these molecules.

The trypsin-like enzyme described by Simpson and Haard (1987) in the stomachless cunner was designated so on the basis of its ability to hydrolyse the synthetic substrates BAPNA and TAME. This molecule is smaller than conventional trypsins (14 kDa), has a low acidic to basic amino acid ratio resulting in an improved acid stability and was not inhibited by benzamidine. This shows the importance of fulfilling all three criteria suggested by Keil (1971). Similarly an alkaline protease was described in the pyloric caeca of the sardine which weakly hydrolysed a trypsin

specific substrate but was not inhibited by trypsin inhibitors (Murakami and Noda, 1981).

Krogdahl and Holm (1983) suggested a greater substrate affinity of teleostean trypsins than those of other vertebrates. Rainbow trout trypsin showed greater activity than trypsins of man, *Homo sapiens*, rat, *Rattus norwegicus*, pig, *Sus scropha*, cow, *Bos taurus*, chicken, *Gallus domesticus*, mink, *Mustela vison*, and fox, *Alopex lagopus*. These assays were all performed at 37°C, which was closer to the optimal temperature for trout trypsin than for the trypsins of the other species and therefore biased the result. Intra species variation in trypsin activity was also shown by Hofer *et al.* (1975) when the affinity of trypsins from agastric cyprinids showed a higher affinity for the BAPNA substrate than those of gastric species of fish and three species of reptiles at their temperature preferenda.

1.2.3.1.3 Chymotrypsin

The other major alkaline endopeptidase present in the intestine of vertebrates is chymotrypsin. Investigations of chymotrypsin have indicated a close relationship to trypsin in origin (Nilsson and Fänge, 1969; 1970). Bovine chymotrypsin, an alkaline endopeptidase, is synthesised in the pancreas as a catalytically inert zymogen, chymotrypsinogen. Carried to the intestine in the pancreatic juices, chymotrypsinogen is converted to the active form, chymotrypsin, through the action of proteolytic enzymes. Activated bovine chymotrypsin has a molecular weight of approximately 25 kDa and is composed of three peptide chains (Hess, 1971). The active site of bovine chymotrypsin requires an active serine residue (Ser 195) , thus classifying it as a serine protease, as well as asparagine (Asp 102) and histidine (His 57). Chymotrypsin primarily hydrolyses the amide bonds of peptides adjacent to the carboxyl group of the aromatic L-amino acid residues of tryptophan, tyrosine and phenylalanine. Hydrolysis also occurs adjacent to large hydrophobic amino acid residues such as leucine and methionine (Hess, 1971).

The analysis of chymotrypsin in teleosts has received considerably less attention than that of trypsin, mainly due to early difficulty in efficiently assaying for chymotryptic activity. The development of specific substrates, such as succinyl-ala-ala-pro-phe-*p*-nitroanilide (Suc-AAPP-p-NA), which were water soluble, stable at pH 8, and easily measured and recorded, provided the means for effectively assaying for chymotryptic activity (Del Mar *et al.*, 1979). This led to an increase in the investigation of chymotryptic activity and the development of other specific substrates such as glutyl-L-phenylalanine-p-nitroanilide (GPNA), acetyl-L-tyrosine ethyl ester (ATEE), benzoyl-L-tyrosine ethyl ester (BTEE) and N-benzoyl-L-tyrosine-*p*-nitroanilide (BTNA).

Using spectrophotometric assays based on these substrates, specific chymotrypsin activity has been determined and is generally expressed as units mg^{-1} protein, with one unit (U) of chymotrypsin defined as the amount of chymotrypsin catalysing an increase in absorbance of 0.001 $minute^{-1}$ under assay conditions. This definition will be used below unless otherwise stated.

General intestinal chymotryptic activity has been reported by in Atlantic halibut (Glass *et al.*, 1989), Dover sole (Glass *et al.*, 1989), dwarf African catfish (Fagbenro, 1990) and turbot (Glass *et al.*, 1989). Jónás *et al.* (1983), using a crude homogenate of the intestinal wall, found chymotryptic activity (U mg^{-1} wet tissue weight) in the sheatfish (360), silver carp (952) and common carp (1540).

Other studies on the activity of chymotrypsin in teleosts have reported the pattern of distribution throughout the intestine and its associated glands. Chymotryptic activity in the anterior intestine of the Atlantic hagfish, *Myxine glutinosa*, was equivalent to 15 μg bovine chymotrypsin g^{-1} wet weight tissue, with a decrease in activity through the midgut (80%) to the hindgut (65%) (Nilsson and Fänge, 1970). White sturgeon displayed uniform chymotrypsin activity in intestine and spiral valve (Buddington and Doroshov, 1986). Chymotryptic activity was reported in the intestine (330 U g^{-1} wet weight tissue) and the pyloric caeca (440 U g^{-1} wet weight tissue) of the seabass (Sabapathy and Teo, 1993) and in the juice of the pyloric caeca of the rainbow trout (Krogdahl and Hohm, 1983). Glass *et al.* (1987) found activity in the pyloric caeca and intestine of Atlantic halibut with highest activity in the midgut and uniform activity in the pyloric caeca, foregut and hindgut. Uniform chymotryptic activity was found throughout the intestine of turbot with a decrease to low levels in the rectum (Munilla-Moran and Stark, 1990). Starvation resulted in decreased chymotrypsin from the foregut to the hindgut. Martinez and Serra (1989) identified chymotrypsin activity in the intestine (0.54 μmol product min^{-1} mg^{-1} protein) and pyloric caeca (0.47 μmol product min^{-1} mg^{-1} protein) of the black sea anchovy, with lower activity present in the stomach (0.05 μmol product min^{-1} mg^{-1} protein) and oesophagus (0.04 μmol product min^{-1} mg^{-1} protein). A similar pattern was described for the rabbitfish, activity greatest in the intestine (3730 U g^{-1} wet weight tissue) and pyloric caeca (1680 U g^{-1} wet weight tissue), and lower in the oesophagus (620 U g^{-1} wet weight tissue) and stomach (1170 U g^{-1} wet weight tissue) (Sabapathy and Teo, 1993). Highest activity in sharptoothed catfish was in was found in the foregut (16000 U g^{-1} wet weight tissue) followed by decreasing activity along the intestine to the midgut (9000 U g^{-1} wet weight tissue) and hind gut (4000 U g^{-1} wet weight tissue), with minimal activity in the pancreas (1000 U g^{-1} wet weight tissue) (Uys and Hecht, 1987). The pancreatic activity was attributed to the presence of enzyme in the zymogen form. This

was supported by Jany (1976) who identified inactive chymotrypsin in the pancreas and active enzyme in the bulbous intestine of the stomachless bonefish. Similarly, Nilsson and Fänge (1969) only detected pancreatic activity in *Chimaera monstrosa* after activation with proteases, while intestinal juices contained only activated enzyme. Assaying activated samples, Overnell (1973) identified the majority of chymotryptic activity in the mesentery (including diffuse pancreatic tissue) and lower activity in the pyloric caeca of the Atlantic cod. Benitez and Tiro (1982) also reported the highest chymotrypsin activity in the pancreas (0.230 μmol product min^{-1} mg^{-1} protein) of the milkfish, with decreasing levels in the pyloric caeca (0.115 μmol product min^{-1} mg^{-1} protein), posterior intestine (0.104 μmol product min^{-1} mg^{-1} protein) and anterior intestine (0.042 μmol product min^{-1} mg^{-1} protein). European seabass and hybrid striped bass exhibited only low chymotryptic activity in the pyloric caeca and lower intestine (Eshel *et al.*, 1994).

The patterns of distribution of chymotrypsin described above tend to support a synthesis and secretion of teleostean chymotrypsin similar to that of the bovine enzyme with inactive chymotrypsinogen being synthesised in the pancreas and released into the intestine where activation occurs. A decrease in the activity of chymotrypsin along the length of the intestine, with the exception of the milkfish (Benitez and Tiro, 1982), would also suggest that chymotrypsin may also be reabsorbed through the intestinal mucosa as is thought to occur with other pancreatic enzymes. The recovery in the pancreas of labelled chymotrypsin injected into the intestine and the *in vitro* uptake of chymotrypsin by pancreatic cells provides further evidence for enzyme reabsorption (Diamond, 1978).

The development of chymotryptic activity in larval fish has also been investigated. Chymotryptic activity in larval Dover sole was found to increase rapidly from day 24 (11.3 U mg^{-1} protein) to day 200, with only a slight further increase to adult levels (42.0 U mg^{-1} protein) (Clark *et al.*, 1986). Lauf and Hofer (1984) found varying patterns of development in larval roach, rainbow trout and whitefish hybrids. Roach larvae showed negligible activity up to day 19 after which activity increased gradually with time to day 40 and then rapidly to day 50. Rainbow trout activity increased from first feeding to day 40, but after this returned to first feeding levels by day 80, and chymotryptic activity was low in whitefish hybrids at first feeding and showed no change up to day 150. Similar to rainbow trout, lake sturgeon larvae also showed an increase in chymotryptic activity at first feeding, reaching a peak during larval feeding and subsequently decreasing following metamorphosis (Buddington, 1985). Chymotrypsin activity in larval striped bass was present at first feeding and appeared to remain constant during development except for a possible slight decrease from day

12 to 16 (Baragi and Lovell, 1986). Dabrowski *et al.* (1992) also found low chymotryptic activity in Artic charr larvae which did not change with time, although there was a suggestion of a 3 week cycle of activity levels. These measurements were made as enzyme activity per unit body weight and therefore the relative weight of the intestine must be considered.

Chymotrypsin has been completely or partially isolated and purified from the viscera or pancreas of several teleostean fish species, including Atlantic cod (Raae and Walther, 1989; Ásgeirsson and Bjarnason, 1991), carp (Cohen *et al.*, 1981a; 1981b), chinook salmon (Croston, 1960), Japanese anchovy (Heu *et al.*, 1995), rainbow trout (Kristjanssen and Nielsen, 1992) and the sardine, *Sardinops melanosticta* (Murakami and Noda, 1981). In these studies, two isoenzymes have been reported for Atlantic cod and rainbow trout, while the common carp was thought to have three isoenzymes. The molecular weights of chymotrypsin for these species, as determined by SDS page, were similar to those of bovine chymotrypsin, varying between 25 and 29 kDa. Enzyme stability was affected by pH, with molecules unstable in an acid environment, stable between pH 5.5 and 9.0, and less stable at a pH greater than 10. This differs from bovine chymotrypsin which is acid stable. Optimal pH, however, was similar to the bovine enzyme with a broad range between pH 7 and 9. As with other teleostean enzymes, chymotrypsin appears adapted to a poikiliothermic environment with low optimal temperatures, between 45°C and 55°, and low heat stability. Inhibition of these enzymes by amino and peptide chloromethyl ketones and phenyl methyl sulphonyl fluoride (PMSF) indicates the presence of histidine and serine residues in their active sites, similar to bovine chymotrypsin.

1.2.3.1.4 Elastase

Elastase is a serine endopeptidase secreted from the pancreas in the zymogen form, proelastase. Intestinal activation occurs by the tryptic cleavage of a single peptide bond, similar to trypsin and chymotrypsin. The activated enzyme is a powerful protease, digesting a large variety of protein substrates, but differing from other serine endopeptidases by its ability to digest elastin and substrates with uncharged, non-aromatic side chains. It thus complements the action of trypsin and chymotrypsin (Hartley and Shotton, 1971).

Elastase activity was first reported when ox pancreatic extract and impure trypsin preparations were found to hydrolyse elastin, an insoluble fibrous protein of the connective tissue, to soluble peptides. This activity was attributed to trypsin until a separate distinct enzyme was isolated from porcine pancreas and termed elastase.

Since this discovery, elastase has been identified in all mammals investigated (Hartley and Shotton, 1971).

Initial elastase studies investigated the porcine enzyme. The characteristics of this enzyme have been reviewed by Hartley and Shotton (1971). Porcine elastase is composed of a single peptide chain of approximately 25 kDa. Molecule stability is pH and temperature dependant, being stable between pH 4 and 10 and below 50°C. Rapid autolysis occurs at room temperature and optimal pH (8.8). Inactivation due to brief exposure to acidic conditions is reversible, however prolonged exposure causes irreversible denaturation. Various ions also affect enzyme activity. Elastase is specifically inhibited by elastinal, however inhibition by soybean trypsin inhibitor (SBTI), TLCK and tosyl phenylalanine chloromethyl ketone (TPCK) is ineffective, indicating a catalytic site differing from the other serine proteases and allowing identification of true elastase activity. Studies using synthetic substrates indicated that residues on the N-terminal side of the peptide bond hydrolysed contribute to substrate specificity, with a strong preference for alanyl residues. Methyl side chains appeared important for the substrate orientation at the catalytic site, while the site of substrate binding was distant from that of the bond hydrolysed.

The most common method for measuring elastase activity involves determining the solubilisation of elastin per unit time by colorimetrically measuring dye release to solution from dyed elastin. Congo red elastin and orcein elastin were the most common substrates, however cross staining of proteins by congo red elastin resulted in the stain specific orcein elastin being preferred (Hartley and Shotton, 1971; Clark *et al.*, 1985). The non-linear nature of these assays makes the comparison of results from different workers difficult. Spectrophotometric assays have generally used synthetic ester substrates such as N-succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide ($SucAla_3NA$), N-acetyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide ($AcAla_3NA$) and N-acetyl-L-alanyl-L-alanyl-L-alanyl methyl ester ($AcAla_3$-O-Me). Discrepancies in characteristics reported for elastin using natural and synthetic substrates, particularly optimal pH, suggest that these assays may be measuring different enzyme activities (Clark *et al.*, 1985).

Studies identifying and detailing elastase activity in fish have generally used natural substrates, with few studies reporting activity against a synthetic substrate. Orcein elastin was the preferred natural substrate. Studies purifying and characterising elastase, however, generally used esters, $SucAla_3NA$ being the most popular. Due to the difficulties comparing elastase activities these activities determined for fish elastase will not be detailed in this review.

Moderate to high elastase activity has been reported in the gastrointestinal tract of the black sea anchovy (Martinez and Serra, 1989), Atlantic halibut (Glass *et al.*, 1987; 1989), *Chimaera monstrosa* (Nilson and Fänge, 1969), Dover sole (Clark *et al.*, 1985; 1986; Glass *et al.*, 1989), European seabass (Eshel *et al.*, 1994), hybrid striped bass (Eshel *et al.*, 1994), rabbitfish (Sabapathy and Teo, 1993) and turbot (Glass *et al.*, 1989). Distribution of elastase showed the majority of activity in the pyloric caeca and intestine, with little or no activity present in the other regions of the gut. Minimal activity was detected in the gut of seabass (Sabapathy and Teo, 1993). Low activity was found in the intestine of white sturgeon, activity increasing posteriorly to the spiral valve, while very slight gastric activity was present (Buddington and Doroshov, 1986). No elastase activity was found in the intestine of Atlantic hagfish (Nilsson and Fänge, 1970) or the stomachless bonefish (Jany, 1976).

Development of elastase in the larvae of Dover sole showed activity at day 24 which rapidly increased to 93% of adult levels by day 80. In all, an eight fold increase in activity was observed between day 24 and adult levels (Clark *et al.*, 1985).

Pancreatic elastase activity has been reported in the African lungfish (Reeck *et al.*, 1970), *Chimaera monstrosa* (Nilson and Fänge, 1969), and angler, *Lophiomus setigerus*, bluefin tuna, *Thannus thynnus*, carp, the catfish, *Parasilurius asotus*, Japanese eel, *Anguilla japonica*, European seabass, rainbow trout and yellow tail, *Seriola quinqueradiata* (Yoshinaka *et al.*, 1985b). Scrapings of the external surface of pyloric caeca from Atlantic halibut, where the diffuse pancreas is located, exhibited elastase activity indicating enzyme of pancreatic origin (Glass *et al.*, 1987). Enzyme activation was required prior to the detection of pancreatic activity suggesting that, as with the porcine form, elastase in fish is stored and secreted in the zymogen form.

Elastase has been isolated and purified from the pancreas of carp (Cohen *et al.*, 1981a), the intestine of Dover sole (Clark *et al.*, 1985) and the pyloric caeca of Atlantic cod (Raae and Walther, 1989; Gildberg and Øverbø, 1990) and rainbow trout (Bassiompierre *et al.*, 1993). These enzymes possessed characteristics similar to those of porcine elastase. Molecular weights were generally in the range of 25-28 kDa, although Dover sole elastase was reported to be only 19.5 kDa (Clark *et al.*, 1985). Such a discrepancy in molecular weight may be the result of the technique used. This is exemplified where the use of chromatography with Sephadex G200, the method used by Clark *et al.* (1985) in the study on Dover sole, estimated an Atlantic cod elastase to be 20 kDa, while SDS PAGE, the preferred technique in the majority of studies, estimated the molecular weight to be 27 kDa (Raae and Walther, 1989). Similar difficulties were experienced in determining optimal pH. Clark *et al.* (1985)

reported optimal pH to be approximately 9.5 using natural substrates and approximately 8.2 using synthetic esters, bringing into question the nature of the activities measured by the assays. Optimal pH between 7.6 to 8.0 were determined using synthetic esters for rainbow trout and Atlantic cod elastase (Raae and Walther, 1989; Bassiompierre *et al.*, 1993). Stability of fish elastase was generally greatest between pH 5 and 9, with rapid inactivation below pH 4. Optimal temperature was reported to be 45°C, with enzymes unstable at higher temperature. Inhibition of fish elastase by PMSF and elastinal showed an affiliation of these enzymes with the serine protease and elastase families. Decreased activity was also observed in the presence of the chymotrypsin inhibitor TPCK (Gildberg and Øverbø, 1990; Bassiompierre *et al.*, 1993), but this effect was attributed to enzyme denaturation by the solvent dimethylsulphoxide (DMSO) (Gildberg and Øverbø, 1990).

A pancreatic metalloenzyme similar to porcine and teleostean elastase has been isolated and characterised from the catfish, *Parasilurus asotus* (Yoshinaka *et al.*, 1984b; 1985a). The affinity of this enzyme for $SucAla_3NA$, elastin, casein and haemoglobin and its molecular weight (24 kDa), optimal temperature (40-45°C) and pH (7.5) were all similar to catfish and porcine elastase. However, the presence of zinc in the enzyme and differing amino acid composition and effects of inhibitors separate this enzyme from the pancreatic elastases and it is therefore termed a pancreatic metalloprotease. A similar enzyme has also been identified in bluefin tuna and yellowtail (Yoshinaka *et al.*, 1885b).

1.2.3.1.5 Carboxypeptidase A and B

As previously described, endopeptidases are responsible for the initial hydrolysis of proteins to peptides. Following this, the sequential cleavage of peptides by exopeptidases ensures the availability of free amino acids for absorption. However, in comparison to the attention paid to the major endopeptidases (pepsin, trypsin and chymotrypsin), research into the role of the exopeptidases, carboxypeptidases and aminopeptidases in teleost digestion has been limited.

Carboxypeptidases are exopeptidases synthesised by pancreatic acini in an inactive zymogen form, procarboxypeptidases, and secreted into the intestine where they are activated by trypsin. As with other proteolytic enzymes the majority of work has been performed on the carboxypeptidases of terrestrial vertebrates. This work resulted in two enzymes being described, carboxypeptidase A (CPA) and carboxypeptidase B (CPB). Bovine CPA and CPB have been found to be generally alike except for their substrate specificity. Both are metalloproteases composed of a single polypeptide chain approximately 34-35 kDa in size and containing a zinc atom. Providing the

terminal carboxylate group is free, both CPA and CPB remove the carboxyl-terminal amino acid from the peptide chain . However their action differs in that CPA preferentially acts upon aromatic and branched chain amino acids, while CPB acts on basic amino acid residues with the exception of histidine (Hartsuck and Lipscomb, 1971; Folk, 1971).

Specific substrates such as hippuryl-L-phenylalanine and hippuryl -L- arginine allowed developed of assays for CPA and CPB activity, providing for its ready determination. Activity quantified by these assays is generally expressed in units, with one unit (U) of activity being defined as the amount of enzyme liberating 1 μmol of product minute^{-1} under assay conditions. This definition will be used in the following text.

Although a paucity of information is available regarding the activities of carboxypeptidases in fish, the available literature indicates that the synthesis, secretion and activation is similar to that of bovine carboxypeptidases. Nilsson and Fänge (1969) identified CPB activity (CPA activity was not assayed) in the intestinal juice of *Chimaera monstrosa* . No pancreatic activity was found in this fish until activation of the extract by proteolytic enzymes, after which both CPA and CPB activities were measured. These authors also reported CPA activity in the intestinal extract of the hagfish. After extract activation by proteases, Reeck *et al.* (1970) identified CPA and CPB activity in the pancreatic extract of the African lungfish. Similarly, Overnell (1973) found the majority of CPA and CPB activity of Atlantic cod associated with the pyloric caecal mesenteries containing diffuse pancreatic tissues. Carboxypeptidase A and CPB peptidase activity (0.6 U mg^{-1} protein) and CPA esterase activity (0.8 U mg^{-1} protein) were also demonstrated in the pyloric caeca of the Atlantic cod (Overnell 1973). White sturgeon exhibited only low CPA and CPB activity in the intestine and spiral valve, with activity absent in the stomach (Buddington and Doroshov, 1986). Munilla-Moran and Stark (1990) described CPA and CPB activity in the turbot to be uniform along the length of the intestine with a decrease to low levels in the rectum. Starvation reduced the level, but not the pattern of activity. The black sea anchovy exhibited very low CPA and CPB activities in the pyloric caeca (0.03 and 0.02 U mg^{-1} protein respectively) and intestine (0.03 U mg^{-1} protein) (Martinez and Serra, 1989). Similarly, Eshel *et al.* (1994) found CPA and CPB activity in the pyloric caeca and intestine of the European seabass and the hybrid striped bass. Intestinal activity of both CPA and CPB was reported in Atlantic halibut, Dover sole and turbot (Glass *et al.*, 1987, 1989). From these studies it appears CPA and CPB are produced in the pancreas as zymogens and released into the intestine where activation occurs. Unfortunately the available data does not allow us to hypothesise about possible reabsorption of these enzymes.

The development of carboxypeptidase activity has been described for Dover sole (Clark *et al.*, 1986) . In this species CPA activity was found to increase slightly from day 29 (8.4 U mg^{-1} protein) to day 40, with a rapid two fold increase to day 200. Following this a slight increase to adult levels occurred (23 U mg^{-1} protein). Carboxypeptidase B activity was less than that of CPA and showed a gradual increase from day 24 (1.4 U mg^{-1} protein) to day 200, again with only a slight subsequent increase to adult levels (10.5 U mg^{-1} protein). Carboxypeptidase A activity in larval striped bass was identified at first feeding decreasing until day 16, after which it increased by day 25 to values greater than those at first feeding (Baragi and Lovell, 1986).

The isolation and purification of teleostean carboxypeptidases has been limited, but characteristics similar to those of the porcine enzymes have been shown. Yoshinaka *et al.* (1985a; 1985b) isolated CPA from the pancreas of the catfish *Parasilurus asotus*. This enzyme, following activation, was described as a zinc metalloprotease with a molecular weight approximately 34 kDa. Optimal pH was approximately 7.5 and enzyme stability pH dependant, being most stable at pH 6 and labile below pH 3. Enzyme hydrolysis was preferential towards carboxyl terminal aromatic and branched chain amino acids. Cohen *et al.* (1981a) identified both CPA and CPB pancreatic activity in the common carp, both enzymes present in their zymogen form and requiring activation prior to detection. Although CPA activity was lost during purification, CPB was isolated and found to be a single chain polypeptide approximately 34 kDa in size. This molecule was stable at pH 7-8, denatured slowly between pH 5-6 and 9-11, and rapidly below pH 5.

1.2.3.1.6 Aminopeptidases

The other exopeptidases are the N-terminal peptidases. These enzymes hydrolyse peptide bonds at or near the amino-terminus of the peptide chain, generally requiring a free α-amino group. Hydrolysis results mostly in free amino acids, although some dipeptides are released. Numerous distinguishable enzymes displaying the characteristics of N-terminal exopeptidases may be present in the same organism. Of these, leucine aminopeptidase (LAP), as observed in the extracts of swine intestinal mucosa and kidney, has been described as the "classic" N-terminal exopeptidase (De Lange and Smith, 1971).

Leucine aminopeptidase is classified by its rapid hydrolysis of leucyl compounds, although it also hydrolyses a range of compounds not containing leucyl residues. The porcine enzyme has a molecular weight of approximately 300 kDa and is composed of four subunits; the bovine molecule is thought to be composed of 10 subunits each of

molecular weight 32 kDa. Leucine aminopeptidase was thought to be a metalloenzyme containing magnesium or manganese, due to its strong activation by these ions, however studies on bovine LAP have revealed it to be a zinc-metalloenzyme (De Lange and Smith, 1971).

The non-specific nature of substrates for LAP assays, such as leucylnaphthylamide or leucyl peptides, combined with the considerable overlap in substrate specificities of the large number of N-terminal exopeptidases, has made identification of specific LAP activity difficult (De Lange and Smith, 1971). L-leucinamide, a substrate rapidly hydrolysed by LAP and resistance to hydrolysis by dipeptidases or other proteolytic enzymes, has increased the assay specificity. However, De Lange and Smith (1971) suggested activities still be simply termed aminopeptidases until highly purified enzyme preparations showing specificity similar to that of porcine LAP are isolated.

Work on fish aminopeptidases has generally reported the identification of LAP activity from crude extracts, although the term aminopeptidase activity would now seem more appropriate for these studies. Such a limitation was noted by Hirji and Courtney (1982) and Bouck (1979). Therefore, this review will use the general term aminopeptidase.

Intestinal aminopeptidase activity was reported in Atlantic halibut (Glass *et al.*, 1987; 1989), *Chimaera monstrosa* (Nilsson and Fänge, 1969), Atlantic hagfish (Nilsson and Fänge, 1970), and turbot (Glass *et al.*, 1989). Fraise *et al.* (1981) reported a uniform distribution of aminopeptidase activity along the entire gut in the catfish *Ameiurus nebulosus* and common carp. Aminopeptidase activity in white sturgeon was absent in the stomach and increased from the intestine to the posterior spiral valve (Buddington and Doroshov, 1986). Other studies have identified the majority of aminopeptidase activity in the pyloric caeca and intestine of fish. Eshel *et al.* (1994) described low aminopeptidase activity in the pyloric caeca and intestine of the European seabass and hybrid striped bass. Activity was uniform along the digestive tract of both species, although activity in the hybrid striped bass was slightly greater. Sabapathy and Teo (1993) described a distribution of aminopeptidase in seabass and rabbitfish with greatest activity in the intestine and pyloric caeca. Lower activity was also identified in the stomach of both species, while oesophageal and rectal activities were found in seabass. High levels of aminopeptidase activity were found in the pyloric caeca and intestine of the black sea anchovy (Martinez and Serra, 1989), and 95% of the aminopeptidase activity in rainbow trout (weight adjusted) was proximal to the last pyloric caecum (Plantikow and Plantikow, 1985). Turbot displayed greatest aminopeptidase activity to be in the intestine, particularly the foregut, with activity also

present in the stomach and rectum (Munilla-Moran and Stark, 1990). Similarly Dover sole, a species with a poorly defined stomach and no pyloric caeca, contained the large proportion of activity in the foregut and midgut, with lower levels in the stomach, hindgut and rectum. A 4-fold loss of activity in Dover sole mucosal extracts following sample centrifugation suggested that the enzymes were membrane bound (Clark *et al.*, 1987). This was supported in a study by Hendricks *et al.* (1990) in which high aminopeptidase activity was detected in Atlantic salmon brush border membrane preparations. Overnell (1973) described aminopeptidase activity in the intestinal pyloric caeca and hepatopancreas of Atlantic cod.

Histological studies describe a similar but more detailed distribution. Aminopeptidase activity in European perch was highest in the pyloric caeca and the anterior and middle intestine and decreased in the posterior intestine and rectum, although this may have been artefactual due to a decreased surface area in these regions (Hirji and Courtney, 1982). At a cellular level activity was restricted to the mucosa, specifically the brush border and adjacent lumen and the supranuclear cytoplasm of the columnar epithelial cells. Goblet cells, infranuclear cytoplasm and basement membrane showed no staining. Activity was also absent in the oesophagus, stomach and pancreas. Bouck (1979) described aminopeptidase activity in the epithelial cells of the pyloric caeca, midgut and hindgut of yearling and adult rainbow trout. Staining was most intense in the apical regions of the cells, with the most active cells in the hindgut region. No activity was present in the pancreas.

The enzyme distribution described suggests the final stages of peptide digestion takes place in the lumen, on the plasma membranes of the microvilli and in the supranuclear cytoplasm. High levels of aminopeptidase activity in the intestine and pyloric caeca reflect the roles of these organs in absorption and therefore the final stages of protein digestion.

Development of aminopeptidase activity in juvenile fish has been described using both crude extracts and histological techniques. Assays of crude extract showed the presence of low aminopeptidase activity in 24 day old Dover sole, activity quickly increasing 3-fold to a peak at day 200 (Clark *et al.*, 1986). Aminopeptidase activity present in 7 day old European seabass, increased over the next 7 days and subsequently returned to day 7 values (Cahu and Zambonino Infante, 1994). Aminopeptidase was identified in roach, whitefish hybrids and rainbow trout larvae in the first two weeks post feeding (Lauff and Hofer, 1984). Larval roach activity was higher than the other species and showed a rapid increase after day 19. However,

whitefish hybrid and rainbow trout larval aminopeptidase activity remained low to day 120 (Lauff and Hofer, 1984).

Histological techniques presented a more detailed appraisal of the early development of aminopeptidase activity. Day old larval turbot exhibited aminopeptidase activity in the presumptive posterior intestine, the only region with a differentiated epithelium (Cousin *et al.*, 1987). By day 2, this distal activity had increased and activity had developed in the presumptive anterior and middle intestine; lumen distension on day 3 allowed demonstration that the activity was associated with epithelial cells. Activity remained weak caudally and strong distally during gastrointestinal tract morphogenesis, with the pyloric caeca developing activity comparable with that of the anterior intestine by day 20. No gastric aminopeptidase activity was observed. Whitefish larvae displayed low aminopeptidase activity in the first third of the intestine with intensity gradually increasing distally to the rectum, after which it decreased (Segner *et al.*, 1989). Intestinal staining was mostly associated with the enterocyte brush border and was partly in the adjacent cytoplasm, with staining in the rectum mostly associated with the supranuclear cytoplasm. The intestine of the sharptoothed catfish stained positively for aminopeptidase activity at first feeding, with only a slight increase to feeding day 10. Staining, again associated with the enterocyte brush border, was strongest caudally and decreased distally to be absent in the rectum (Verreth *et al.*, 1992). Positive aminopeptidase staining was found in the ciliated border of the intestinal epithelial cells of 2 to 30 day old walleyes, *Stizostedion vitreum* (Mitchell *et al.*, 1986). Aminopeptidase activity associated with the brush border of the intestine of larval milkfish appeared late in the larval stage, after day 21 (Ferraris *et al.*, 1987).

Aminopeptidase activity appears to be influenced by dietary and environmental factors. Plantikow and Plantikow (1985) found aminopeptidase activity to vary with feed quality and quantity, temperature and osmolarity. Segner *et al.* (1989) also described an effect of diet on the aminopeptidase activity in larval whitefish, which was later supported by Rösch and Segner (1990) when activity varied in animals fed one of four artificial diets or a natural diet. However, it was uncertain whether these differences reflected the nutrient content of the diet or its digestibility. Dietary preference was suggested as the reason for higher activity in the carnivorous seabass than the herbivorous rabbitfish (Sabapathy and Teo, 1993). Aminopeptidase staining in starved turbot larvae was observed along the intestine at day 5, but weakened from day 5 to 10 (Cousin *et al.*, 1987). Similarly adult turbot aminopeptidase activity was found to be greatly reduced (8% of fed animals) following two months starvation (Cousin *et al.*, 1987). Aminopeptidase activity in larval European seabass was

affected by the age of weaning onto artificial diets, however it was suggested this may have been due to a malnutrition caused by early weaning (Cahu and Zambonino Infante, 1994).

Although aminopeptidases from fish have not been isolated, work performed on crude extracts has suggested enzyme characteristics similar to those described for terrestrial vertebrates. Enzyme inhibition by o-phenanthroline (Martinez and Serra, 1989) and ethylenediaminetetraacetic acid (EDTA) (Clark *et al.*, 1987) indicated the presence of metalloproteases, while magnesium (Clark *et al.*, 1987) and manganese (Nilsson and Fänge, 1969; Clark *et al.*, 1987) induced activation of aminopeptidase activity was also described. An optimal pH of approximately 8 was also similar to that of the porcine and bovine enzymes (Overnell, 1973; Clark *et al.*, 1987; Sabapathy and Teo, 1995). However, molecular weight, approximated to be 170 kDa for Atlantic cod aminopeptidase (Overnell, 1973), was smaller than that of porcine and bovine molecules. Optimal activity occurred at 60°C (Sabapathy and Teo, 1995). Inhibition of enzyme activity by PMSF and TPCK implicated histidine in the active site (Clark *et al.*, 1987; Sabapathy and Teo, 1995).

1.2.3.1.7 Dipeptidases

The final class of enzymes involved in protein digestion to be discussed are the intestinal peptide hydrolases termed dipeptidases. Dipeptidases are N-terminal exopeptidases thought to possess broad specificities which overlap considerably. These enzymes act upon dipeptides released by earlier proteolysis either immediately prior to uptake of free amino acids or after dipeptides have been absorbed into the enterocyte cytoplasm. Therefore, dipeptidases are located on the membrane of intestinal microvilli or in the cytoplasm of enterocytes.

Work performed on the dipeptidases of terrestrial vertebrates provides the majority of our knowledge of these enzyme. Early studies generally looked at the activity of crude extracts using a series of dipeptide substrates, making it impossible to elucidate whether activities measured with different substrates were due to a single enzyme or the action of multiple enzymes. Debate exists as to the number of dipeptidases present in the intestine and the range of specificity of each enzyme. Studies using electrophoretically homogeneous enzymes suggested a limited number of dipeptidases with broad specificities rather than a large number of dipeptidases with narrow specificity. Das and Radhakrishan (1973) reported the majority of theoretically possible dipeptides were hydrolysed by one versatile "master" dipeptidase present in monkey small intestine, suggesting that differing dipeptides were all substrates for the same enzyme and that varying activities reflect the affinities for these substrates.

Piggott and Fottrell (1975) agreed that peptide hydrolases hydrolyse a wide variety of peptides, although they suggested that some degree of specificity existed with each enzyme and the enzymes acted in a complementary fashion.

As with the other classes of exopeptidases the literature available on the dipeptidases of fish is sparse. Most investigations have determined the activities of crude extracts, thereby indicating only the ability to hydrolyse the substrates and not the hydrolases present.

Using glycyl-leucine, glycyl-phenylalanine and phenylalanyl-glycine as substrates, Ash (1979, 1980) investigated the dipeptidase activity in the stomach, pyloric caeca, caecal intestine, post caecal intestine and large intestine of brown trout, *Salmo trutta*, and rainbow trout. Substrate hydrolysis occurred in all sections of the gastrointestinal tract of both species, with activity greatest towards glycyl-leucine, moderate for glycyl-phenylalanine and lowest for phenylalanyl-glycine. Activity also varied along the gut, being highest in the pyloric caeca and large intestine, intermediate in the caecal and post caecal intestines, with only trace activity present in the stomach. Use of *p*-hydroxymercuribenzoate (PMB), a specific inhibitor of cytoplasmic dipeptidases, revealed that the distribution of activity was substrate dependant. The majority of glycyl-leucine and glycyl-phenylalanine hydrolysis (> 77%) was associated with cytoplasmic enzymes, while phenylalanyl-glycine hydrolysis tended to be bound to the brush border. This proportion of cytoplasmic activity is similar to that shown for glycyl-leucine by monkey intestine where 95% of activity was PMB inhibited (Das and Radhakrishan, 1973). Gastric activity for all three substrates in brown and rainbow trout was not inhibited by PMB, indicating activity in the brush border. The low level of gastric activity and its resistance to inhibition by PMB suggested this activity may be due to sample contamination. Glycyl-glycine hydrolysing activity has also been identified in the intestine of rainbow trout, with hydrolysis attributed to both brush border (31%) and cytoplasmic enzymes (69%) (Böge *et al.*, 1981).

Intestinal extract of Atlantic hagfish and *Chimaera monstrosa* also exhibited dipeptidase activity, with a lack of activity in the digestive fluid suggesting intracellular or membrane bound enzymes (Nilsson and Fänge, 1969; 1970). Sastry (1977) identified dipeptidase activity in the intestine and liver of the omnivorous walking catfish, *Clarias batrachus*, herbivorous carp, *Cirrhinus reba*, and carnivorous green snakehead, *Ophiocephalus* (*Channa*) *punctatus*, with dipeptide hydrolysis also occurring in the pyloric caeca of green snakehead and the pancreas of the walking catfish. Activity was greatest in the intestine and appeared to reflect dietary preference, being higher in the carnivore and omnivore than the herbivore. Kuz'mina (1978) and

Ugolev and Kuz'mina (1994) also described intestinal dipeptidase activity in several species of fish.

1.2.3.2 Processes of Protein Digestion

The process of protein digestion in terrestrial vertebrates generally has a gastric and intestinal phase. Fish, however, are divided into two classes on the basis of the presence or absence of a morphologically and physiologically discernible stomach. Stomachless (agastric) fish therefore lack preliminary acidic or peptic secretions and digestion is a totally alkaline event (eg Cyprinidae).

1.2.3.2.1 Gastric Phase of Protein Digestion

In gastric species of fish, as in most terrestrial animals, proteolysis is initiated in the stomach through the coordinated secretion of pepsin and hydrochloric acid (gastric acid). The degree of hydrolysis of peptide bonds occurring within the stomach depends upon the rate of gastric secretion, the activity of pepsin, the retention time within the stomach and the penetration of the ingested material by the gastric fluids and enzymes. However, the role of the gastric phase in protein digestion of fish is a controversial topic with contrasting opinions as to whether digestion in the stomach liberates free amino acids, makes peptide bonds more susceptible to hydrolysis within the intestine or whether the stomach simply acts as a reservoir controlling the flow of ingested materials into the intestine.

Bayliss (1935) found that proteolytic activity in the presence of gastric mucosa of plaice failed to break down proteins beyond the polypeptide stage, suggesting a true endopeptidase activity. The presence of pepsin under acidic conditions during rainbow trout protein digestion hydrolysed some insoluble proteins, however it did not lead to the liberation of free amino acids (Grabner and Hofer, 1989). These authors suggested gastric predigestion provides only minor advantages to the digestion of protein, advantages that may be easily compensated for by intestinal digestion.

A lack of impairment of protein digestion in agastric species and the heavy dependence upon tryptic rather than peptic enzymes in juvenile fish have been used to support the idea of a non-essential gastric predigestion. However, agastric species and larval fish appear to have developed adaptations to overcome a lack of gastric digestion. Hofer *et al.* (1975) described a greater affinity of trypsins from four stomachless Cyprinidae (*Rhodeus amarus*, tench, *Tinca tinca*, goldfish and common carp) for BAPNA than trypsins of gastric species (rainbow trout, brown trout and European perch), suggesting an adaptation of the agastric trypsin to digesting native proteins as opposed

to the acid denatured proteins of gastric species. It was also suggested that the pinocytotic absorption and intracellular digestion of intact proteins by agastric species and larval fish may provide a mechanism of overcoming incomplete protein digestion by allowing the use of polypeptides. However, the demonstration of similar pinocytic protein absorption in the juveniles and adults of gastric species precludes this argument.

Therefore, it would appear that the gastric phase of protein digestion results in the initial hydrolysis of polypeptides to smaller molecules that may be more efficiently digested in the intestine, without the release of free amino acids. This predigestion, although advantageous to gastric species, appears to have been compensated for in agastric species and larval fish.

1.2.3.2.2 Intestinal Phase of Protein Digestion

In teleosts, intestinal digestion includes activity within the pyloric caeca as no enzyme secretion occurs within this organ, but rather enzymes present are thought to be from the small intestine. Luminal enzymes of the small intestine and pyloric caeca include the endopeptidases trypsin, chymotrypsin, and elastase, and the exopeptic carboxypeptidases. Intestinal and pyloric caecal proteolytic enzymes are also associated with the enterocytes comprising the extracellular, brush border bound or cytoplasmic aminopeptidases and peptide hydrolases. The actions of which appears to be at the intermediate and terminal stages of protein digestion and may be a link in the mechanisms of absorption.

Therefore the intestinal phase of protein digestion in fish, as in terrestrial vertebrates, is capable of completely hydrolysing ingested proteins to free amino acids or small peptides suitable for absorption. Although it must be noted that fish are capable of absorbing intact polypeptides in the mid- and hind-gut via pinocytosis, some of which are subsequently digested intracellularly (refer section 1.3.1.3).

1.2.4 Lipid Digestion

Lipid is an important dietary nutrient to fish, supplying the fatty acids used as energy, precursors for hormone synthesis and structural elements to maintain the functional integrity of cell membranes. However, despite the importance of lipid in fish nutrition, lipid digestion is poorly understood. Similar to terrestrial vertebrates the majority of lipid in fish diets is generally supplied as triglyceride, although in marine species wax esters, which are used for lipid storage by some invertebrates, also comprise a significant proportion of the dietary lipid intake.

The general process of lipid digestion in fish appears to occur via similar mechanisms to those employed by the other vertebrates, involving the actions of bile and lipid hydrolases (lipases). Bile in fish, as with other vertebrates, is produced by the liver, stored in the gall bladder and enters the anterior intestine and pyloric caeca by way of the bile duct. As the bile contains little or no lipolytic activity (Bayliss, 1935; Lie and Lambertsen, 1985), the major role of bile in lipid digestion is the emulsification of lipids, thereby decreasing lipid droplet size. Increased surface area to volume ratio enhancing the action of pancreatic lipases, which act at the water-lipid interface due to their hydrophilic nature. Lipases hydrolyse the constituent fats and esters producing smaller lipid molecules and free fatty acids. However, differing to proteases and carbohydrases, lipases also catalyse the synthesis of fats or other esters, making lipid hydrolysis a reversible process. Accumulation of digestion products in the region of lipid digestion blocks further hydrolysis through the attainment of equilibrium. Reaction products are therefore rapidly removed from the site of hydrolysis by their inclusion into micelles formed by bile salts and the intestinal lipid contents are thus divided between the oil phase and the micellar form. The resulting mixed micelles subsequently transport the products of lipid digestion to the enterocyte membrane where absorption occurs. The efficiency of this process in fish is high, with most fats being readily digestible (Cowey and Sargent, 1977; Lie and Lambertsen, 1991), although the reaction rate is considerably slower than that of mammals (Smith, 1989; Ijima *et al.*, 1990).

The diverse nature of lipases and difficulty in analysing the products of digestion has resulted in fish lipases being less well studied than the proteases. Many investigators, using crude extracts and assays for general lipase activity, simply identify the ability to hydrolyse dietary lipid without characterising the enzymes involved. These studies have described lipolytic activity in a number of species of fish with the majority of lipase activity occurring in the intestine and pancreas, with occasional gastric lipolytic activity. These data suggest that, as in higher vertebrates, intestinal lipase synthesis occurs in the pancreas and it is secreted to the intestine where activation and digestion occur.

Lipolytic activity in Mozambique tilapia occurred in the stomach and intestine, with activity marginally higher in the stomach (Nagase, 1964). Gastric lipase activity similar to intestinal levels was identified in white sturgeon, although activity in both the intestine and stomach was low (Buddington and Doroshov, 1986). Ishida (1935) and Bayliss (1935) described a lack of gastric lipolytic activity in the mullet and plaice respectively. Plaice also displayed a high bile activated lipolytic activity (optimum pH 7.5 to 8.0) in the intestinal mucous membrane and liver (Bayliss, 1935). Intestinal

lipolytic activity was also demonstrated in the matrinchã (Reimer, 1982) and luderick (Anderson, 1991). Milkfish were found to possess the majority of lipase activity in the intestine, with greater activity in the anterior than the posterior intestine (Borlongan, 1990). Substantial lipase activity was also observed in the pyloric caeca and pancreas, and slight, but significant activity was found in the oesophagus. Activity in the pancreas and pyloric caeca was also quite high in grass carp (Hickling, 1966; Das and Tripathi, 1991) and the freshwater perches *Colisa fasciata*, *Ambassis ranga* and *Ambassis nama* (Ray, 1989) displayed lipase activity associated with the hepatopancreas and intestine, with activity in the hepatopancreas apparently greater. Similarly, lipase activity was identified in the liver, pancreas and intestine of mahseer (Bazaz and Keshavanath, 1993). Sarbahi (1951) identified lipase activity in the hepatopancreas and juices of the intestinal bulb and anterior intestine of the goldfish, and the duodenum and small intestine of largemouth black bass.

These studies indicated that dietary quality and quantity effected the level of lipolytic activity. Reimer (1982), Borlongan (1990) and Bazaz and Keshavanath (1993) described an increase in lipase activity with the level of lipid in the diet. Ananichev (1959) reported increased lipase activity at periods of peak nutrition, and Das and Tripathi (1991) found activity enhanced by feeding a combination of natural and artificial diets. However, Anderson (1991) found lipase activity to be inconsistent, attributing this to the fact that lipid was not a major nutrient source for the herbivorous luderick.

Development of lipolytic activity in fish has also been described. Lipase activity was absent in turbot during the larval stage, appearing in the musculature of the oesophagus at day 15, followed by activity in the muscularis of the stomach and pharynx. Between days 20 and 40 activity developed in the apex of the enterocytes of the pyloric caeca and intestine and at day 40 activity was identified in pancreatic tissue. However, no activity was identified in the intestinal lumen (Cousin *et al.*, 1987). These results differ from those of Buddington (1985) who described lipase activity in the chyme of lake sturgeon to increase rapidly at onset of exogenous feeding, being highest during the larval feeding stage before decreasing to low levels after metamorphosis. The appearance of lipid droplets in the enterocytes of juvenile fish at first feeding (Rösch and Segner, 1990; Deplano *et al.*, 1991) indicates the early development of lipid digestion and absorption, as was evident in lake sturgeon.

The previous work identified the presence and distribution of lipase activity but did not attempt to characterise the enzymes involved. Characterisation of enzyme activity is important for determining the best lipid source to supply in diets. Although the

dynamics of lipid digestion in mammals is well understood, the knowledge of lipid dynamics in fish is still poor. As was noted by Sargent *et al.* (1989), a lack of discrete pancreatic tissue in many species of fish has made the investigation of the dynamics of lipid digestion difficult, obscuring the differentiation between pancreatic lipases to be released for intestinal lipid digestion and hepatic lipases involved in the metabolism and mobilisation of absorbed lipids. Comparison of the early studies on lipid digestion was also complicated by the investigation of a number of very different species and tissues, the methods of tissue and enzyme preparation, and the varying assays employed.

In mammals pancreatic lipases hydrolyse triglycerides cleaving fatty acid residues from the 1- and 3- positions on the glycerol molecule, resulting in the production of free fatty acids and 2-monoglycerides. These enzymes preferentially remove short chain, saturated fatty acids. Pancreatic lipase activity is dependent upon colipase, a polypeptide which restores lipid activity inhibited by bile salts and protects against enzyme denaturation. Pancreatic bile salt dependent lipases (BSDL's) are enzymes with broad substrate specificity responsible for cholesterol and fatty acid ester hydrolysis. An additional physiological role for BSDL's is thought to be the hydrolysis of ester bonds resistant to the pancreatic lipase, such as those incorporating long chain polyunsaturated fatty acids.

The available information on the dynamics of lipid nutrition in fish has generally been supplied by investigations of the effects of crude extracts on lipid sources *in vitro* or the analysis of feeds and gut contents *in vivo*. These studies do not allow the effects of individual enzymes to be elucidated, nor the identification of enzymes hydrolysing intermediate products or with overlapping substrate specificities. However, this holistic approach provides an insight into the end products of lipid digestion and allows the specificities of the overall process of lipid digestion to be determined.

Lipid digestion in fish has been reviewed by Cowey and Sargeant (1977), Leger (1985), and Sargent *et al.* (1989). These authors described a process of lipid digestion involving two types of lipases. These were a "true" colipase-dependent pancreatic lipase and a non-specific BSDL, with the latter being at lower concentrations than the "true" lipase (Sargent *et al.*, 1989). Both enzymes were Ca^{2+}-dependent and stimulated by bile salts, although only the non-specific enzyme was dependent upon the presence of bile salts (Leger, 1985; Sargent *et al.*, 1989). The enzymes displayed complementary activities with non-specific BSDL having enhanced activity at high bile salt concentrations, while the activity of the colipase-dependent enzyme was increased at low bile salt concentrations (Leger, 1985).

Hydrolysis of triglyceride by this dual enzyme system results in the cleavage of fatty acids from all three positions on the glycerol backbone of triglyceride, releasing free fatty acids and glycerol (Cowey and Sargeant, 1977; Leger, 1985; Sargent *et al.*, 1989). Non-specific BSDL thought to be responsible for the intermediate hydrolysis of 2-monoglyceride (Sargent *et al.*, 1989). Although lipid digestion in fish showed no specificity for the site of the fatty acid residue on the glycerol molecule, lipases preferentially hydrolysed polyunsaturated fatty acids regardless of their position, while saturated acids and very long chain monoenoic acids were least digestible (Lie and Lambertsen, 1985; Lie *et al.*, 1987; Lie and Lambertsen, 1991; Koven *et al.*, 1994). Digestion was bile salt activated but not dependent, with greatest activity in the anterior intestine (Lie and Lambertsen, 1985).

Wax ester hydrolysis by fish was slower than triglyceride hydrolysis (Cowey and Sargeant, 1977; Lie and Lambertsen, 1985; Sargent *et al.*, 1989; Lie and Lambertsen, 1991). The reduced rate of hydrolysis of wax esters is probably due to the higher melting point (Lie and Lambertsen, 1985) and the extremely hydrophobic nature of these molecules, making them difficult to emulsify and therefore less accessible to the action of digestive lipases. No specific wax ester lipase has been identified in fish and it is thought that the hydrolysis of these molecules is catalysed by the non-specific BSDL (Leger, 1985; Lie and Lambertsen, 1985). Hydrolysis results in the formation of fatty alcohols and free fatty acids (Cowey and Sargeant, 1977; Sargent *et al.*, 1989). Again lipolytic activity was preferential towards wax esters containing polyunsaturated fatty acids (Lie and Lambertsen, 1985; 1991)

Although bile salt-dependent sterol esterase activity has been identified in fish, hydrolysis releasing sterols and free fatty acids (Sargent *et al.*, 1989), phospholipid digestion has not been reported in fish (Sargent *et al.*, 1989).

The digestion of dietary lipids determined using crude extracts suggested digestive lipolytic activity was non-specific to the position of fatty acids on the glycerol molecule. However, recent work by Gjellesvik *et al.* (1989) investigating a partially purified triglyceride lipase from the pyloric caeca of Atlantic cod produced different results. This enzyme was 1-,3-specific for triglyceride hydrolysis, resulting in free fatty acids and 2-monoglyceride as end products of digestion. An absolute bile salt dependence was exhibited for the hydrolysis of insoluble substrates, this dependence being less pronounced for soluble substrates, and activity was not dependent upon colipase. It was suggested in this study that the primary digestive lipase in cod is a BSDL with positional specificity. A later study, in which cod BSDL was purified to homology, showed BSDL to be the only enzyme with lypolytic activity in extracts

from the pyloric caeca, indicating this enzyme was solely responsible for lipid digestion (Gjellesvik *et al.*, 1992). The purified enzyme again displayed 1-,3-positional specificity, preferentially hydrolysing the bonds associated with long chain polyunsaturated fatty acids which are abundant in the cold water marine environment (Gjellesvik, 1991; Gjellesvik *et al.*, 1992).

1.2.4 Carbohydrate Digestion

Carbohydrates are naturally occurring organic molecules composed of six carbon atoms, or multiples thereof, combined with hydrogen and oxygen in the proportions of water. The basic carbohydrate molecules are simple sugars (monosaccharides) such as glucose, galactose and fructose. Monosaccharides are chemically bonded to form polypeptides, classified as di-, tri-, oligo- and poly-saccharides depending on the number of monosaccharide groups comprising them. Nutritionally carbohydrates serve solely as energy sources, having no essential component and therefore no absolute dietary requirement. However, the other dietary nutrients utilised as energy sources, lipid and protein, have absolute dietary requirements and these nutrients may be spared from catabolism for energy by the provision of carbohydrate in the diet.

The carbohydrates most commonly occurring in the natural diets of fish are the polysaccharides starch, chitin and cellulose. Starch is also the carbohydrate additive generally used in artificial diets for fish. To be of nutritional value, these polysaccharides must be reduced to simple sugars which are then absorbed. Hydrolysis is a stepwise process, similar in format for all three polysaccharides although requiring different enzymes and being performed to varying degrees of success depending on both the carbohydrate source and the species concerned.

1.2.4.1 Starch Digestion

Starch, a storage molecule synthesised by plants, is a polymer of D-glucose units linked by α-1,4-glucosidic bonds (amylose) with some α-1,6-glucosidic linked branched chains (amylopectin). In terrestrial vertebrates, starch digestion is initiated through the action of α-amylase, an endoenzyme which cleaves the α-1,4-glucosidic bonds of the amylose component forming di-, tri- or hexa-saccharides. The glucose bound by α-1,6-linkage to the branch of amylopectin is split by isomaltase (oligo-α-1,6-glucosidase) and the remaining amylose hydrolysed by α-amylase. The resulting reducing sugars are subsequently hydrolysed by α-glucosidases releasing monosaccharides.

Starch digestion appears to follow the same mechanisms in fish. Clark *et al.* (1984) described starch digestion by Dover sole where initial hydrolysis released a homologous series of reducing sugars, indicating random hydrolysis by an endoenzymic α-amylase, followed by hydrolysis of the lower maltosaccharides by α-glucosidase. The release of glucose from maltose and maltotriose substrates suggesting strong activity of the α-1,4- and α-1,6-glucosidases, maltase and isomaltase. Differing optimal pH of α-amylase and α-glucosidase activity suggest the presence of different protein molecules, while curves depicting the rate of glucose release during starch digestion also suggest an endoenzyme acting with an oligosaccharidase. Glass *et al.* (1987) also described hydrolysis of starch by α-amylase and hydrolysis of the digestion products by the disaccharidases maltase and isomaltase in Atlantic halibut.

The majority of studies investigating starch digestion by fish have therefore centered around the carbohydrases α-amylase and maltase (α-1,4-glucosidase), with some work performed on isomaltase (α-1,6-glucosidase).

1.2.4.1.1 Amylase

The endoenzymatic digestion of starch by α-amylase produces reducing sugars which are further hydrolysed to monosaccharides. It serves a similar role in the hydrolysis of glycogen, the major storage carbohydrate of animals. In mammals there are thought to be three sites of production of α-amylase - salivary glands, exocrine pancreas and the brush border of intestinal enterocytes. Hydrolysis of starch begins in the buccal region and continues in the stomach through slight acid hydrolysis, but the bulk of digestion occurs in the intestine as a result of the action of pancreatic amylase.

The distribution of α-amylase activity has been extensively studied in fish. Activity has been determined using crude extracts, as α-amylase has not been purified from fish due to enzyme denaturation during purification. Assays for measuring amylase activity have used both natural (starch and glycogen) and synthetic (p-nitrophenyl-α-D-meltaheptaoside, p-nitrophenyl-α-glucoside) substrates. The variety of assay methods used and the corresponding multitude of expressions of activity make comparisons of results from different studies difficult. This difficulty is further exacerbated by the variations of assay techniques and acclimatisation temperatures employed by differing laboratories. Therefore, the general distributions of amylase activity in the digestive tracts of species will be discussed, with interspecies variation from the same study being identified.

General intestinal amylolytic activity has been described for tambaqui (Kohla *et al.*, 1992), matrinchã (Reimer, 1982) and four species of cichlids (Sturmbrauer *et al.*, 1992). Studies detailing α-amylase distribution in the gastrointestinal tract and its associated glands in fish have been performed using a variety of species. The results show a similar trend with the majority of activity located in the intestine, pyloric caeca and pancreas/hepatopancreas. Intestinal α-amylase activity in the Dover sole was greatest in the foregut and decreased along the intestine (Clark *et al.*, 1984). Similarly, α-amylase activity in silver and bighead carp increased over the anterior three segments of the intestine, followed by a rapid decrease in activity over the final seven segments (Bitterlich, 1985). Chakrabarti *et al.* (1995) found α-amylase activity to be greatest in the midgut and decrease in the hindgut of bighead carp, catla, *Catlacatla*, and mrigal, *Cirrhinusmrigala*. Silver carp, Nile tilapia, *Oreochromis niloticus* and rohu exhibited uniform activity along the intestine (Chakrabarti *et al.*, 1995). Strong foregut activity decreasing to the hindgut was identified in the common carp (Chakrabarti *et al.*, 1995), Mozambique tilapia (Fish, 1960; Nagase, 1964) and the sharptoothed catfish (Uys and Hecht, 1985). Mozambique tilapia (Fish, 1960; Nagase, 1964) and the sharptoothed catfish (Uys and Hecht, 1987) also displayed high pancreatic activity. Activity in the grass carp (Hickling, 1966; Das and Tripathi, 1991) and goldfish (Sarbahi, 1951) was also present in the hepatopancreas and intestine, decreasing along the length of the intestine in grass carp (Hickling, 1966), and the majority of α-amylase activity was associated with the pancreas of Atlantic cod (Overnell, 1973) and European perch (Fish, 1960). Atlantic halibut displayed activity primarily in the pyloric caeca and diffuse pancreas, although intestinal activity was still high (Glass *et al.*, 1987), and milkfish displayed high amylolytic activity in the pancreas, pyloric caeca and intestine (Chiu and Benitez, 1981). Greatest activity was identified in the intestine and pyloric caeca in *Engraulis capensis* (Seiderer *et al.*, 1987), African bony tongue, *Heterotis niloticus* (Ugwamba, 1993), luderick (Anderson, 1991), rabbitfish (Sabapathy and Teo, 1993) and turbot (Munilla-Moran and Stark, 1990). Intestinal activity in the African bony tongue (Ugwamba, 1993) and turbot (Munilla-Moran and Stark, 1990) decreased along its length, however in the turbot activity increased in the rectum to values found in the foregut (Munilla-Moran and Stark, 1990). Luderick also displayed high amylase activity in the rectum (Anderson, 1991). Seabass (Sabapathy and Teo, 1993) and plaice (Bayliss, 1935) displayed only very low amylolytic activities restricted to the intestine and pyloric caeca. Very low α-amylase activity was also found in the intestine and spiral valve of white sturgeon (Buddington and Doroshov, 1986).

A 50% loss of amylase activity in Dover sole following centrifugation, suggested that in fish the intestinal membrane may bind amylase or contribute to its action (Clark *et al.*, 1984).

Amylolytic activity has also been reported in the oesophagus and stomach of bighead carp (Chakrabarti *et al.*, 1995), common carp (Chakrabarti *et al.*, 1995), *Engraulis capensis* (Seiderer *et al.*, 1987), Mozambique tilapia (Nagase, 1964), mrigal (Chakrabarti *et al.*, 1995), Nile tilapia (Chakrabarti *et al.*, 1995), rabbitfish (Sabapathy and Teo, 1993), rohu (Chakrabarti *et al.*, 1995), and silver carp (Chakrabarti *et al.*, 1995), while gastric activity alone was identified in Atlantic halibut (Glass *et al.*, 1987), Dover sole (Clark *et al.*, 1984), African bony tongue (Ugwamba, 1993), white sturgeon (Buddington and Doroshov, 1986), mullet (Ishida, 1935) and the sharptoothed catfish (Uys and Hecht, 1987). The oesophageal activities were generally very low, being attributed to the regurgitation of intestinal enzymes (Nagase, 1964).

The distribution of α-amylase activity detailed above describes a pattern expected for pancreatic enzyme synthesis with release into the pyloric caeca and/or anterior intestine. The majority of starch hydrolysis therefore occurs in the anterior intestine and the pyloric caeca. Pancreatic release of α-amylase was further supported by changes in amylolytic activities in response to feeding. In carp, hepatopancreatic α-amylase levels decreased and intestinal activity increased following feeding, although there was a 5 to 7.5 hour delay before maximal intestinal activity was reached. A second feeding resulted in elevated activity in the posterior intestine, suggesting amylase secreted into the anterior intestine was pushed back with the food bolus by the freshly ingested feed while reserves in the hepatopancreas were insufficient to return the anterior intestinal activity to pre-second feed levels (Onishi *et al.*, 1976). Similarly, pancreatic activity was high and intestinal activity low in larval turbot prior to feeding, while 4 to 5 hours post feeding activity was low in the pancreas and high in the intestine (Cousin *et al.*, 1987). Maintenance of pancreatic activity and the decrease in intestinal activity during starvation of turbot larvae also indicated pancreatic synthesis and release in response to feeding (Cousin *et al.*, 1987).

Decreasing amylolytic activity along the length of the intestine in fish has been attributed to reabsorption of α-amylase by the intestinal mucosa (Bitterlich, 1985). Diamond (1978) also suggested reabsorption of intact α-amylase activity in fish. However, high rectal amylolytic activities displayed in luderick (Anderson, 1991) and turbot (Munilla-Moran and Stark, 1990) do not support this hypothesis.

Studies on the development of amylolytic activity in larval fish indicated early α-amylase synthesis and secretion, although the pattern of development appears to vary between species. Amylolytic activity in larval turbot was identified in the pancreas by day 3 , the lumen of the anterior and middle intestine by day 4 and throughout the intestine and pyloric caeca by day 5. Activity was greatest in the anterior regions of the gut. High levels of activity was present in the pancreas throughout the larval period (Cousin *et al.*, 1987). Activity was identified in black sea bream (Kawai and Ikeda, 1973a), rainbow trout larvae (Kawai and Ikeda, 1973b) and Artic charr alevins (Dabrowski *et al.*, 1992), increasing with time during early development. Carp α-amylase activity was present 7-10 days post hatching, increasing at day 30 and reaching half adult values by day 120. Larvae of lake sturgeon and striped bass showed a three phase development of amylolytic activity (Buddington, 1985; Baragi and Lovell, 1986). Low intestinal α-amylase activity in lake sturgeon prior to first feeding increased to reach peak levels during the larval feeding phase and subsequently decreased and remained low after metamorphosis (Buddington, 1985). Striped bass larvae displayed high activity by day 4 (60% day 30 values), which remained constant until day 12, after which it decreased to day 16 and then again increased to day 32 (Baragi and Lovell, 1986).

Amylolytic activity appears to be highly correlated with the natural feeding preferences of the species. Ugolev and Kuz'mina (1994) found α-amylase activity to increase from typical predators to predator-facultative benthos or planktophages to typical benthophages and phytophages. Sarbahi (1951) found amylase activity throughout the tissues and juices of the digestive tract of the omnivorous goldfish, but could not find any activity in the carnivorous largemouth black bass. Similarly Fish (1960), using washed mucosal extracts, identified diffuse amylase activity in the intestine and pancreas of herbivorous Mozambique tilapia, while only pancreatic activity was identified in the carnivorous European perch. Sabapathy and Teo (1993) reported higher α-amylase activity in the herbivorous rabbitfish than the carnivorous seabass.

Apart from the interspecies variations in α-amylase activity due to the carnivorous or omnivorous nature of the animals, intraspecies variations in amylolytic activity appears correlated with food quality and quantity, and environmental stimuli . Reimer (1982) found α-amylase activity in matrinchã increased in response to high starch diets, but not diets with elevated levels of protein or fat. Nagase (1964) also described an increased α-amylase activity in Mozambique tilapia in response to a carbohydrate rich diet, while carp increased α-amylase activity within a week in response to elevated dietary carbohydrate levels (Kawai and Ikeda, 1972). Grass carp fed a combination of artificial and natural diet displayed greater amylolytic activity compared to animals fed

only natural diet (Das and Tripathi, 1991). The activity of α-amylase in carp was greater for diets containing starch or lactose than diets supplemented with maltose or sucrose (Kawai and Ikeda, 1972). Diet had no effect on the development of amylase activity in larval black sea bream (Kawai and Ikeda, 1973a), carp (Kawai and Ikeda, 1973a) or striped bass (Baragi and Lovell, 1986).

Amylolytic activity towards different substrates was also found to vary. In both rabbitfish and seabass activity measured using a glycogen substrate was much lower than that measured using starch, suggesting a lesser role for glycogen as an energy source in these species (Sabapathy and Teo, 1993). Similarly, Ishida (1935) reported lower glycogenase than α-amylase activity in the stomach of mullet. Although Seiderer *et al.* (1987) reported high carbohydrase activity against oyster glycogen in *Engraulis capensis*.

A relationship between α-amylase levels and diet availability also exists. Ananichev (1959) found amylolytic activity to increase at periods of peak nutrition. Kohla *et al.* (1992) reported a positive correlation between α-amylase activity and gut fullness in tambaqui. In this species amylolytic activity declined quickly during starvation and remained low until refeeding after which activity overshot control values by 110 to 150%. Two months starvation greatly reduced gastric and rectal α-amylase activity in turbot, while only causing a slight decrease in intestinal values (Munilla-Moran and Stark, 1990). However, food availability did not effect α-amylase activity in the luderick (Anderson, 1991).

These studies indicate the presence of a feedback system stimulating or inhibiting amylase secretion, a hypothesis supported by the ability of fish to increase α-amylase secretion to overcome unfavourable intestinal conditions. In carp α-amylase is inhibited by wheat amylase inhibitor (Hofer and Sturmbauer, 1985; Natarjaran *et al.*, 1992), although an increase in α-amylase secretion resulted in the maintenance of the intestinal activity (Sturmbauer and Hofer, 1986; Natajaran *et al.*, 1988). Similarly, reduced α-amylase activity in the intestine of silver carp due to non-optimal gut pH was compensated for by increased enzyme secretion (Bitterlich, 1985).

An influence of environmental factors on amylolytic activity has been suggested in several studies. Secretion of α-amylase by rudd and roach was shown to be dependent on temperature, which also correlated to natural food availability, with a different mechanism responsible for each species (Hofer, 1979). Rudd displayed an inflexible annual rhythm of α-amylase secretion unable to be adapted to artificial temperature changes, suggesting a response to photoperiod, while roach altered secretion in response to direct temperature changes. Dabrowski *et al.* (1992)

suggested a three week rhythm in α-amylase secretion by Artic charr alevins, possibly related to a lunar cycle.

Although isolation and purification of α-amylase from the alimentary tract of fish has not been performed, studies using crude extracts and starch substrates have provided information on the properties of this enzyme. Optimal pH for amylolytic activity has been reported in the range of 6.2 to 8.5 (Bayliss, 1935; Nagase, 1964; Overnell, 1973; Chiu and Benitez, 1981; Clark *et al.*, 1984; Bitterlich, 1985; Glass *et al.*, 1987; Ugwamba, 1993; Sabapathy and Teo, 1994), although values seem to centre around a pH of between 6.5 and 7.0. Optimal temperature of α-amylase was found to be 50°C for milkfish α-amylase (Chiu and Benitez, 1981), 40°C in common bream (Ugolev and Kuz'mina, 1994), 30°C in pike perch, burbot and sabrefish, *Pelecus cultratis* (Ugolev and Kuz'mina, 1994), and 25°C in the rabbitfish (Sabapathy and Teo, 1994). Activity was also effected by the presence of ions. Bayliss (1935) described amylase activation by sodium chloride in silver and bighead carp, while the presence of Ca^{2+} has been reported to increase amylase activity up to 10 fold in a variety of species (Clark *et al.*, 1984; Glass *et al.*, 1987; Munilla-Moran and Stark, 1990).

1.2.4.1.2 Maltase

An exo- α-1,4-glucosidase, maltase hydrolyses the α-1,4-glucosidic linkage binding the two glucose residues in maltose, a disaccharide formed naturally during the hydrolysis of starch. In mammals, maltase is bound to the brush border of the intestinal enterocytes, releasing glucose for absorption. However, as with exopeptidases, the literature available on the exoenzymes, or disaccharidases, involved in carbohydrate digestion by fish is limited.

As expected from the distribution of maltase in mammals, studies on fish have suggested a limited maltase distribution compared to α-amylase. Maltase is generally restricted to the pyloric caeca and intestine. Although no maltase activity was reported in the carnivorous largemouth black bass (Sarbahi, 1951), high levels of intestinal activity were reported in Dover sole (Clark *et al.*, 1984), carp (Kawai and Ikeda, 1973b) and African bony tongue (Ugwamba, 1993). Maltase activity has also been described in the pyloric caeca and intestine of the Atlantic halibut, (Glass *et al.*, 1987), goldfish (Sarbahi, 1951), milkfish (Chiu and Benitez, 1981), rabbitfish (Sabapathy and Teo, 1993), rainbow trout (Buddington and Diamond, 1987) and seabass (Sabapathy and Teo, 1993). Activity in the rabbitfish and seabass was, however, very low (Sabapathy and Teo, 1993). The relative activity of maltase varied along the intestine, increasing distally in the milkfish (Chiu and Benitez, 1981), remaining constant in the Atlantic halibut (Glass *et al.*, 1987) and decreasing distally

in the rainbow trout (Buddington and Diamond, 1987). Histologically whitefish larvae displayed activity which increased in the posterior portion of the intestine and was limited to the brush border (Segner *et al.*, 1989). Maltase activity was also reported in the tissue of the hepatopancreas and oesophagus of the goldfish (Sarbahi, 1951), although no activity was identified in the oesophagus, stomach or liver of rabbitfish and seabass (Sabapathy and Teo, 1993).

The synergistic action of α-amylase and maltase during starch digestion would suggest a synchronous development of these enzymes during larval stages. Such a development of activity has been described. Maltase activity was present in rainbow trout at day 20, increasing to juvenile levels by day 40 (Kawai and Ikeda, 1973a). Activity in carp larvae was identified between days 7 and 10 post hatching, remaining constant until day 30 and subsequently increasing to half of adult levels by day 120 (Kawai and Ikeda, 1973b).

An influence of diet on maltase activity has also been reported. Activity in carp increased in response to a high carbohydrate diet, adapting within a week (Kawai and Ikeda, 1973b). Diet also appeared to affect the level of activity in whitefish larvae, with activity higher in larvae fed a dry diet compared to those feeding on zooplankton (Segner *et al.*, 1989).

Again no work has been performed using the purified maltase and therefore information on the characteristics of this enzyme in fish is lacking. However, Ugolev and Kuz'mina (1994) showed that the optimal temperature of maltase in burbot was 50°C while other species tested, including predators, predators-facultative benthophages, benthophager and planktophages all displayed optimal activity at 60°C.

1.2.4.1.3 Isomaltase

Also termed oligo-1,6-glucosidase and limiting dextrinase, isomaltase is a disaccharidase which hydrolyses the α-1,6 linkages binding glucose molecules in isomaltose, a product of starch digestion by α-amylase. Information on isomaltase in fish is restricted to identification of isomaltase activity in the intestine of Atlantic halibut (Glass *et al.*, 1987) and Dover sole (Clark *et al.*, 1984), although the activity found in Dover sole was only slight.

1.2.4.1.4 Other Endo-carbohydrases

Another carbohydrate storage molecule in plants commonly consumed by herbivorous fish is lamarinose, a water soluble polymer of D-glucose found in greatest quantities in the brown seaweeds belonging to the family Laminaria (Whistler and Smart, 1953).

While lamarinose-like carbohydrates (crysolaminarin) are the major storage product found in some diatoms (Seiderer *et al.*, 1987, Sturmbauer *et al.*, 1992). Lamarinase catalyses the hydrolysis of both these molecules.

Lamarinase activity has been identified throughout the digestive tract in the herbivorous rabbitfish (Sabapathy and Teo, 1993) and the opportunistic feeding *Engraulis capensis* (Seiderer *et al.*, 1987), with activity generally lower in the oesophagus and stomach than the intestine. Sabapathy and Teo (1993) suggested lamarinase may be produced endogenously in herbivorous species and released from feed items in carnivores. Laminarase activity was also described in the anterior intestine of the detritivorous cichlids *Tropheus moorii* and *Petrechromis orthograthus* (Sturmbrauer *et al.*, 1992). Sturmbrauer *et al.* (1992) suggested lamarinase activity developed as an evolutionary response to dietary changes.

1.2.4.1.5 Other Exo-carbohydrases (disaccharidases)

Activity of several other disaccharidases has been reported in the intestines of fish.

Trehalose, (α-D-glucosido)-α-D glucoside, is a non-reducing disaccharide storage molecule occurring in yeasts, fungi and seaweeds. It is hydrolysed by the specific enzyme trehalase to produce two molecules of glucose. Trehalase activity has been identified in milkfish (Chiu and Benitez, 1981), rabbitfish (Sabapathy and Teo, 1993), rainbow trout (Buddington and Diamond, 1987) and seabass (Sabapathy and Teo, 1993). This activity is most likely released from feed items, particularly in the carnivorous rainbow trout and seabass.

Enzymatic degradation of sucrose has been described in Atlantic halibut (Glass *et al.* (1987), Dover sole (Clark *et al.*, 1984), goldfish (Sarbahi, 1951), milkfish (Chiu and Benitez, 1981), mullet (Ishida, 1935) and rabbitfish (Sabapathy and Teo, 1993), although it was absent African bony tongue (Ugwamba, 1993) and largemouth black bass (Sarbahi, 1951). Ugolev and Kuz'mina found sucrase activity in a variety of species to increase with dietary preference from typical predators to typical benthophages and planktophages. Similarly, degradation of lactose occurred in Atlantic halibut (Glass *et al.*, 1987), but not in milkfish (Chiu and Benitez. 1981), goldfish (Sarbahi, 1951), African bony tongue (Ugwamba, 1993) or largemouth black bass (Sarbahi, 1951). Sucrase and lactase activities were generally very low, and probably due to non-specific maltase and isomaltase activity. This explanation appears plausible when it is considered that sucrose, a disaccharide composed of glucose and fructose, exists mainly in sugar cane and other terrestrial plants, while lactose (galactosylglucose) occurs naturally in mammalian milk.

1.2.4.2 Chitin Digestion

Chitin is the second most abundant organic molecule on earth, being particularly plentiful in the marine environment. It is an insoluble polysaccharide occurring as a long, unbranched polymer of N-acetyl-D-glucosamine (an aminosugar) units bound by β-1,4-linkages. This structure provides properties beneficial to a structural and support molecule, and chitin is the major exoskeletal component in the arthropods. However, the distribution of chitin extends much wider, providing a structural constituent of the coelenterate perisarc, annelid setae, mollusc shells and even selected fungi.

The abundance of chitin in aquatic systems gives it special importance in fish nutrition where chitin may provide a major energy source following enzymatic degradation (Danulat, 1986a). The biological digestion of chitin is performed by a system of extracellular enzymes which consecutively hydrolyse the polymer to its constituent monomer. Degradation is initiated by the endocarbohydrase chitinase which hydrolyses native chitin, tetramers and higher oligosaccharides to the di- and tri-saccharides (chitobiose and chitotriose respectively). Subsequent hydrolysis to the monosaccharide N-acetyl-D-glucosamine, which is absorbed in the intestine, is catalysed by the disaccharidase chitobiase.

1.2.4.2.1 Chitinase

Chitinase, an endocarbohydrase, is also categorised as endo-β-N-acetyl-glucosaminidase or chitodextrinase. Chitinase activity has been described in a number of species of fish. Lindsay (1984b) identified chitinase activity in the stomach or foregut (anterior 1/3 intestine) of 29 species of Northern European marine fish. Although a 1000 fold range of interspecies variance was found, intraspecies variation was only 4 fold suggesting the presence of a characteristic chitinase level for each species. Fänge *et al.* (1979) examined chitinase activity in the stomach mucosa and exocrine pancreas (when discrete) of six marine fish including a cyclostome (Atlantic hagfish), a holocephalan (*Chimaera monstrosa*), a teleost, (grenadier, *Coryphaenoides rupestris*) and three elasmobranches (spiny dogfish, *Squalus acanthias*, deepwater dogfish *Etmopterus spinax* and starry ray, *Raja radiata*). Activity was high in the stomach mucosa and low in the pancreas of gastric species and high in the pancreas of agastric animals, with the exception of the cyclostome which showed little or no chitinase activity. High chitinase activity was also found in the gastric mucosa of rainbow trout, with the majority of activity in the mucosa of the cardiac stomach (Lindsay, 1984b). Rehbein *et al.* (1986) identified greatest chitinase activity in the stomach tissue of four species of fish which consumed Antarctic krill. Activity

decreased in the stomach contents, intestinal contents and intestinal tissue. High chitinase activity was identified in the stomach contents, gastric mucosa and intestinal contents of the Atlantic cod, activity was lower in the intestinal mucosa and pyloric caeca, the latter included the diffuse pancreas in the associated mesentery (Danulat and Kausch, 1984; Danulat, 1986a; 1986b). Sabapathy and Teo (1993) described chitinase activity in the stomach and intestine of seabass, activity of the gastric enzyme being greater and displaying activity under both acidic (pH 3.0) and alkaline (pH 9.0) conditions. Intestinal activity was only observed in an alkaline environment. Similarly, Clark *et al.* (1984) described chitinase activity in the intestine of Dover sole under alkaline conditions. Low chitinase activity was found in the oesophagus, stomach and intestine of *Engraulis capensis* (Seiderer *et al.*, 1987), and Glass *et al.* (1987) described only trace amounts of chitinase activity in the stomach, pyloric caeca and intestine of Atlantic halibut.

Danulat and Kausch (1984) suggested Atlantic cod chitinase was of both exogenous and endogenous origin. Chitinolytic bacteria accounted for between 1.2 and 23.5% of the total gut bacteria. However, higher chitinase activities in the gastric mucosa than the stomach contents suggested a primarily endogenous chitinase production (Fänge *et al.*, 1979; Danulat and Kausch, 1984). A reduced role of bacteria in chitinase activity was also indicated in Atlantic cod by Danulat (1986a) when no correlation was found between chitinase activity and the number of chitinolytic bacteria, which comprised 48.8 and 33.5% of the intestinal and gastric bacteria respectively. The persistence of chitinase activity following elimination of the gut micro-flora with antibiotics in Atlantic cod (Danulat, 1986a) and rainbow trout (Lindsay *et al.*, 1984) also indicated chitinolytic bacteria did not contribute greatly to enzyme activity. Fish fed diets containing chitinolytic bacteria also failed to show significantly altered chitinase activity, levels being the same as diets containing antibiotics (Lindsay *et al.*, 1984).

These studies indicate chitinase activity to be broadly distributed throughout the species of fish investigated, displaying characteristic levels for each species although varying greatly between species. The gastric mucosa is the main chitinase secreting tissue of fish with well defined stomachs, enzyme secretion by the pancreas and other intestinal digestive glands compensating in agastric species.

Factors controlling the level of chitinase activity appear uncertain. Lindsay (1984b) found no correlation between the level of chitinase activity and the amount of chitin consumed. Rainbow trout gastric chitinase activity was unaffected by the addition of 4 to 25% of precipitated chitin to the diets (Lindsay *et al.*, 1984). However, chitinase activity in Atlantic cod increased dramatically in response to a diet of whole

crustaceans, particularly in the pyloric caeca, although activity in fish fed a diet of crustacean shells was low considering it contained the highest level of chitin (Danulat, 1986b). These studies suggest chitinase secretion may be stimulated by dietary components other than chitin. The low chitinase activity at high chitin concentrations may be due to the adsorption of chitinase by chitin, as was shown in rainbow trout (Lindsay, 1984a).

A correlation does exist, however, between the level of chitinase activity and the extent to which species disrupt their prey (Lindsay, 1984b). Fish breaking up prey with pharyngeal teeth or a gizzard displayed low chitinolytic activity, while fish gulping intact prey had elevated levels. These data indicate that the primary function of chitinase is the disruption of the chitinous exoskeleton of the prey. The low chitinase activity, its early presence in the digestive tract and the active digestion of chitin from feed zooplankton in the oesophagus and stomach of *Engraulis capensis* also suggested a primary role of chitinase associated with the initial disruption of the ingested crustaceans (Seiderer *et al.*, 1987).

The role of chitin digestion in providing nutrients to the fish appears limited. Danulat (1986b) found crustacean shells alone provided better condition factor and liver lipid in Atlantic cod than starved animals, suggesting that dietary chitin may be of some nutritional value. However, rainbow trout fed diets with 4, 10 and 25% precipitated chitin replacing starch displayed reduced growth rates, indicating chitin was a poor energy source. The inability of the relatively high levels of chitinase in rainbow trout to significantly digest dietary chitin when included at 10 and 30% further supported this finding (Lindsay *et al.*, 1984). The replacement of starch with 10% N-acetyl glucosamine did not compromise growth, suggesting the ability of fish to utilise the amino sugar as an energy source provided chitin can be efficiently digested (Lindsay *et al.*, 1984).

Kono *et al.* (1987) isolated and characterised chitinase from red sea bream, *Pagrus major*. This enzyme was found to have a molecular weight of 46 kDa using SDS page, an optimal pH of 5.5 and optimal temperature of 50°C. It was inhibited strongly by Fe^{2+} and Sn^{2+}, and moderately by Hg^{2+}, while Cu^{2+} provided slight activation. The end products from the hydrolysis of chitin by the purified enzyme were N-acetyl glucosamine and N,N'-diacetylchitobiose. Optimal pH has also been reported for chitinase from several other species based on activities from crude extracts. The optimal pH of gastric and intestinal chitinase from Dover sole was 4.5 to 5.1 and 5.1 to 5.5 respectively (Danulat and Kausch, 1984), and for the chitinases from the gastric mucosa of spiny dogfish and grenadier were 1.6 to 3.6 and 1.25 respectively (Fänge

et al., 1979). However, chitinase from the pancreas of the stomachless *Chimaera monstrosa* was found to have a high activity at pH of around 8-10, with a weaker peak in activity at pH 3.0 (Fänge *et al.*, 1979). Seabass also displayed chitinase activity at both acidic and alkaline pH (Sabapathy and Teo, 1993), while the optimal pH of Dover sole intestinal chitinase was found to be alkaline, pH 9.5 (Clark *et al.*, 1984).

1.2.4.2.2 Chitobiase

Chitobiase, or exo-N-acetyl β-D-glucosaminidase, is a disaccharidase which hydrolyses chitobiose and chitotriose, releasing the monosaccharide N-acetyl-D-glucosamine. As with other disaccharidases the literature available on the activity of this enzyme in fish is limited.

Chitobiase activity has been identified in several species of fish. Lindsay (1984b), investigating 29 species of Northern European marine fish, described chitobiase activity in the stomach or anterior intestine of all species. Fänge *et al.* (1979) found high chitobiase activity in the intestine of Atlantic hagfish, *Chimaera monstrosa*, Grenadier, spiny dogfish, deepwater dogfish and starry ray. High chitobiase activity was also identified in the intestinal mucosa of rainbow trout (Lindsay *et al.*, 1984), with moderate activity present in the intestine of Dover sole (Clark *et al.*, 1984), while Atlantic halibut displayed high chitobiase activity in the intestine, stomach and pyloric caeca (Glass *et al.*, 1987). Danulat (1986b) described chitobiase activity to be negligible in the stomach tissue and moderate in the pyloric caeca and intestinal tissue and contents of the Atlantic cod.

The functional interaction between chitinase and chitobiase would suggest a correlation between the activities of these two enzymes. However, chitobiase activity showed no correlation with the activity of chitinase in the 29 marine species studied by Lindsay (1984b). High chitobiase activity was associated with low chitinase activity in the foregut of agastric species in that study suggesting a role for chitobiase in cleaving molecules, other than chitin, containing N-acetyl glucosamine dimers.

Chitobiase activity in rainbow trout (Lindsay *et al.*, 1984) and Atlantic cod (Danulat, 1986b) was not effected by the dietary level of chitin. Although N-acetyl glucosamine did result in an increase in chitobiase activity (Lindsay *et al.*, 1984).

Few studies have attempted to characterise the chitobiase enzyme found in fish. Investigations using crude extracts reported optimal pH to be in the range of 4.25 to 5.0 (Fänge *et al.*, 1979; Clark *et al.*, 1984).

1.2.4.3 Cellulose Digestion

Native cellulose is a single polymer of cellobiose residues. A disaccharide of glucose, cellobiose differs only in the nature of the glycosidic linkages from maltose. Cellobiose, a β-glucoside, containing β-1,4-glucosidic linkages. On average native cellulose contains up to 10,000 β-1,4-linked glucose monomers, this long chain polymer structure conveying to cellulose its characteristic properties of high tensile strength and insolubility in almost all solvents. However, treatment with strong acids or alkali and even mechanical grinding results in the alteration of the physical structure of cellulose, in extremes breaking and shortening the polymer chains. These shortened (denatured) chains resemble native cellulose chemically, being insoluble glucose polymers, but they are thought to be more susceptible to enzymatic decomposition.

The production of purified celluloses often involves extreme conditions. For example the purification of wood cellulose (Solka floc) causes extensive degradation of the glucose polymers, resulting in Solka floc being more likely to be attacked by digestive enzymes (Halliwell, 1959). The increased susceptibility to decomposition associated with purification has also been observed when celluloses freed of lignin and other matter became almost completely digestible (Van Soest, 1973). Therefore, purified celluloses, such as the types commonly used in nutritional experiments, may be more likely to be digested than the native undegraded celluloses.

Initial digestion of cellulose is performed by cellulases which catalyse the hydrolysis of cellulose to the cellobiose dimer. The resulting disaccharide is subsequently attacked by cellobiase, a β-glucosidase, which hydrolyses the β-1,4-glucosidic bond releasing β-glucose.

1.2.4.3.1 Cellulase

The decomposition of native cellulose to the soluble cellobiose dimer is thought to have two distinct steps. Firstly native cellulose is degraded by an enzyme, cellulase, to shorter linear polymer chains, similar to the effect caused during cellulose purification. Subsequently, these chains are hydrolysed by a second enzyme, β-1,4-polyglucosidase, to produce small soluble molecules. This later enzyme is thought to act on the same bonds as those found in the soluble carboxymethylcellulose (Halliwell, 1959). Thus decomposition of native cellulose requires two enzymes. However it does not appear that all "cellulolytic" organisms possess both enzymes. Although some organisms have been found to render native cellulose soluble,

extracts from some organisms which readily hydrolyse soluble derivatives of cellulose (for example carboxymethylcellulose) have little or no effect upon insoluble or native forms (Halliwell, 1959). It has therefore been proposed that the term cellulolytic be restricted to organisms capable of decomposing native cellulose.

Similarly the differentiation between the digestion of native and degraded celluloses extends to the description of digestive enzymes. Many studies investigating enzymes with cellulolytic properties report the production of reducing sugars or the reduction in the viscosity of soluble derivatives of cellulose (Halliwell, 1959). As described earlier however, such enzymes may be ineffective against native cellulose. The more accurate assay for the determination of true "cellulase" would appear to be the conversion of insoluble cellulose into soluble fragments, as this seems to be the limiting step for many organisms. Often "cellulases" identified by their action on soluble cellulose derivatives have not been tested for hydrolysis of insoluble cellulose. It has therefore been suggested that these enzymes be termed "carboxymethylcellulases" and enzymes causing the solubilisation of native cellulose be referred to as true "cellulases" (Halliwell, 1959).

Assays based on the reduction of soluble cellulose derivatives such as carboxymethylcellulose, have been used in the majority of literature available on "cellulase" activity in the gastrointestinal tract of fish. The problems associated with such assays, as outlined previously, were raised by Niederholzer and Hofer (1979) and should be kept in mind. However, for simplicity and to keep in common with the majority of published literature, a distinction between assay methods will not be made in this review and the term "cellulase" will be applied to activity against both soluble and insoluble assay substrates.

Work performed on cellulase activity in fish was generally based on the knowledge of cellulose digestion in herbivorous terrestrial vertebrates such as the ruminants. These animals lack endogenous cellulase activity, but have developed specialised structures in their digestive tracts to provide conditions favouring the production of large quantities of cellulase producing bacteria and the process of fermentation. Therefore, when investigating cellulase activity in other phyla, many investigations determine the presence of cellulolytic micro-organisms in the digestive tract and attribute cellulase production to these symbiotic organisms. Such was the case for invertebrates until the work of Yokoe and Yasumasu (1964). Assaying 74 mostly herbivorous or omnivorous species these authors identified endogenous cellulase in protozoans, coelenterates, annelids, molluscs and arthropods. Suggesting that although cellulolytic micro-organisms were present in these species, there was also

endogenous cellulase activity and this correlated with animal phylogeny. However, no endogenous cellulase activity was reported in species of fish assayed during this investigation.

Since the work of Yokoe and Yasumasu (1964) many studies have been performed determining cellulase activity in the alimentary tract of fish. These studies have provided conflicting information, with intra- and inter-species variation reported. This variability not only relates to the presence or absence of cellulase activity, but also to the suggested sources of activity.

Several broad studies have been performed to identify cellulase activity in fish, its pattern of distribution and the source of this activity. These studies have produced differing, but plausible, hypotheses on the source of teleostean cellulase activity. Stickney and Shumway (1974) assayed 148 elasmobranches and teleosts representing 62 species. Of these species, 16 estuarine species and the channel catfish exhibited cellulase activity. The classification of these species provided no phylogenetic evidence of cellulase activity. Activity was identified in both gastric and agastric species throughout the Teleostei, with variable results obtained for fishes within the same family. There was also no correlation between reported feed habits of the fish and high and low cellulase activities, although actual gut content analyses were not performed. Such analyses may have proved beneficial as the estuarine species exhibiting cellulase activity all inhabited waters with a high proportion of plant detritus, suggesting an environmental or feeding link. Contradicting this, however, was the occurrence of cellulase activity in the digestive tract of channel catfish which were not exposed to cellulase activity or detritus for at least a year, being raised in an intensive indoor commercial culture system and fed on a cellulase-free artificial diet. Streptomycin treatment of these fish removed cellulase activity, while it remained in starved, untreated fish. These findings suggested that cellulase activity was provided by resident gut micro-flora and not produced endogenously or supplied with the feed. Unfortunately no micro-floral quantification was performed in the study.

Investigating cellulase activity in freshwater fishes, Prejs and Blasczyk (1977) assayed the anterior and posterior intestines of five cyprinids and the stomach of a salmonoid. The gut contents of each animal were determined. Cellulase activity was found to vary greatly within species, between species and along the length of the gastrointestinal tract. However, the presence and level of the cellulase activity generally correlated with the amount of highly processed plant detritus in the gut. These authors suggested that detritus was intensively colonised by cellulose

degrading bacteria prior to ingestion by the fish, and also proposed, that cellulase activity was of microbial origin. However Prejs and Blaszcyk (1977) differed from Stickney and Shumway (1974) by suggesting that the bacteria were associated with ingested feed and were not resident gut flora. Prejs and Blaszcyk (1977) also found contradictory evidence in the presence of cellulase activity comparable with that of detritus feeding animals in a detritus-free carp intestine, suggesting activity other than that derived from feed associated bacteria. Again gut micro-flora were not quantified in this study, while the detritus was not assayed for cellulase activity. Such information would have added to the completeness of the study.

Lindsay and Harris (1980) assayed cellulase activity in wild caught marine and freshwater fish, as well as farmed fish, representing 42 species from 12 orders. Activity was identified in at least one animal from 37 of the species studied. As in the study by Prejs and Blaszcyk (1977), cellulase activity varied both within and between species and along the length of the gut. Based on gut contents analysis, the species studied were grouped by dietary preference into omnivores, piscivores and invertevores (a classification used by the authors to describe fish feeding almost exclusively on invertebrates). Most omnivorous (72%) and piscivorous fish (86%) displayed nil or slight cellulase activity, while the invertevores displayed moderate to high levels of activity. Examination of feed items revealed cellulase activity in crustaceans, gastropods and oligochaetes, while plants and artificial diets displayed no activity. Detritus was not assayed. Cellulase activity appeared to originate from the ingestion of invertebrates containing cellulase or cellulolytic micro-flora. Therefore, it was suggested cellulase activity was acquired with invertebrate but not detrital feed items. This hypothesis supported by the work of Yokoe and Yasumasu (1964), but conflicted with the study of Prejs and Blaszcyk (1977) in which cellulase activity was absent in the stomach of the *Coregonus albula* which contained predominantly Crustacea.

The work of Stickney and Shumway (1974), Prejs and Blaszcyk (1977) and Lindsay and Harris (1980) presented three hypotheses for the origin of cellulase activity in fish. Since these experiments several studies have investigated cellulase activity in fish species, generally as part of broader enzyme studies. Results from these investigations vary greatly, providing evidence both for and against all three hypotheses.

Low levels of cellulolytic activity identified in the roach and rudd (Niederholzer and Hofer 1979), with a five fold increase in fish feeding on zooplankton and arthropods compared with those consuming plants and detritus supports the hypothesis of

Lindsay and Harris (1980). Although Chakrabarti *et al.* (1995) identified cellulase activity throughout the gut of bighead carp consuming 60:40 plant:animal feed, a lack of cellulase activity in carnivores (*Chana striatus*, *Notopterus notoptera* and Nile tilapia), omnivores (catla, common carp, *Labeo rohu*, mrigal, rohu and silver carp) and a herbivore (*Puntius javanious*) in the same pond make it difficult to suggest an exogenous cellulase source. The absence of cellulase activity in *Engraulis capensis*, an open water species feeding actively on crustaceans (Seiderer *et al.*, 1987), suggests cellulase activity may be limited to estuarine and coastal species where cellulose is likely to be encountered.

Cellulolytic micro-flora were identified in the gut of Mozambique tilapia (Dyundik *et al.*, 1991), and Lésel *et al.* (1986) related cellulase activity in the goldfish and grass carp to the presence of cellulolytic gut micro-flora. Analysis of the gut micro-flora of menhaden identified cellulase-active colonies of yeasts (Deegan *et al.*, 1990) known to have a significant role in detritus formation, indicating yeasts introduced with detritus became resident gut micro-flora and provided cellulase activity. However, the absence of cellulase activity in silver carp and bighead carp, species displaying a predominance of detritus in their feeds, suggested not all plant detritus provide cellulolytic bacteria (Bitterlich, 1985). Similarly a lack of cellulase activity in the coastal and estuarine inhabiting herbivorous luderick despite the presence of sizeable micro-flora populations, indicated that access to cellulose and a resident gut micro-flora did not necessarily confer cellulase activity (Anderson, 1991). Cellulase activity was also found to be lacking in Atlantic halibut (Glass *et al.*, 1987), Dover sole (Clark *et al.*, 1984) and milkfish (Chiu and Benitez, 1981).

Das and Tripathi (1991) identified cellulase activity associated with both the gut and hepatopancreas of grass carp. In that study, animals reared indoors and fed cellulase-free diets exhibited high levels of cellulase activity, suggesting cellulase activity was due to a feed independent persistent gut micro-flora. Tetramycin treatment reduced cellulase activity, although there was some residual activity. Combined with the presence of activity in the hepatopancreas, it was concluded that cellulase may also be synthesised endogenously. Without quantification of gut bacteria it is difficult to ascertain whether residual activity was due to endogenous production or gut flora remaining after antibiotic treatment.

Most studies described above lack quantification of at least one variable, making it difficult to draw rigorous conclusions. However, the available information indicates that cellulase activity in fish is not uniformly or discretely apportioned. The variation in the presence of cellulase activity both within species and between related species

indicates exogenous cellulase activity, from an origin not universally accessible nor simply classified. As with terrestrial vertebrates no conclusive evidence exists to suggest endogenous teleostean cellulase.

Attempts to identify the source of cellulase activity have produced various hypotheses which are not mutually exclusive. To say any one of these describes the source of cellulase activity for fish would be presumptuous. Combining the three hypotheses proposed by Stickney and Shumway (1974), Prejs and Blaszcyk (1977) and Lindsay and Harris (1980) may give the best description. It appears cellulase activity is introduced from the external environment, taking one of several forms. Activity may be produced by cellulolytic bacteria that persist in the gut, becoming part of a resident gut micro-flora. Alternatively, cellulolytic bacteria may intensively colonise feed items, for example detritus, and have only a transient presence in the intestine. Finally, cellulase present within a prey item (eg. invertebrates) may be released during its digestion. Cellulase activity in a fish may result from any or all of the above, depending on the life history of the animal. By using combinations of the suggested hypotheses much of the inter- and intra-species variation may be explained.

Multiple origin of cellulase was suggested by Moerland (1985) to explain cellulase activity in the mummichog, *Fundulus heteroclitus*. In that study cellulase activity in laboratory acclimatised populations persisted over time, being unrelated to recent dietary history and closely associated with the gut lining. However, higher activity was detected in field collected animals, and gut content cellulase activity was only identified in autumn when the detritus pool was enriched with frost killed plants undergoing the initial stages of micro-floral degradation. It was hypothesised a resident gut micro-flora produced basal cellulase levels, with production enhanced by feed associated micro-flora in wild caught animals.

From the above studies it appears long term detailed investigations of fish populations, measuring all potential exogenous and endogenous cellulase producing parameters may give the best idea of the sources of cellulase activity in any particular species.

1.2.4.3.2 Cellobiase

A paucity of information is available on cellobiase activity in fish. Cellobiase activity was described by Clark *et al.* (1984) and Glass *et al.* (1987) in Dover sole and Atlantic halibut respectively, but both studies identifyied only trace activity in the intestine.

1.2.4.3.3 Teleostean Anatomical Modifications for Fermentative Digestion

As with terrestrial species, it appears cellulose digestion in fish may be dependent upon cellulolytic bacteria and the process of fermentation. The development of specialised organs by terrestrial herbivores to facilitate bacterial proliferation and fermentative digestion, hence improving the utilisation of cellulose rich feeds, was mentioned above (section 1.2.4.3.1). The absence of such organs in fish suggested fermentation may not digest cellulose at a nutritionally significant level. That is, if fish utilise fermentation for the digestion of a major dietary component they would have also developed an anatomical fermenting chamber.

Rimmer and Wiebe (1987) first described such a fermenting chamber in fish. Working on two species of herbivores, *Kyphosus cornelii* and *K. sydneyanus*, these authors identified well vascularised, valved, caecum-like pouches at the posterior end of the intestine capable of containing up to 1.5 to 2.0 times the volume of the stomach. These structures possessed the anatomical complexity to support microbial fermentation. The idea of an anatomical fermenting chamber was supported by the presence within the organs of a denser, more complex micro-flora population and high concentrations of volatile fatty acids (VFA's), the end product of fermentative digestion. This finding was a major step towards confirming the role of micro-flora in the digestive processes of fish. Recently, Kandel *et al.* (1994) also reported the presence of VFA's in the hindguts of four herbivorous fishes (*Cebidichthys violaceus*, *Medialuna californiensis*, *Kyphosus bigibbus* and K. *vaigiensis*). Apart from *C. violaceus*, all species showed a full array of VFA's and again displayed a posterior chamber of a blind caecum set off from the rest of the intestine by a sphincter. The wide range of VFA's, high microbial diversity and specialised intestinal morphology indicate that these three species have relatively well developed fermentation systems.

1.3 Absorption

After digestive enzymes and fluids have reduced ingested food materials to smaller molecules, the products of digestion are transported into the circulatory or lymphatic systems of the animal so they may be metabolised. The mechanisms allowing these ions and molecules to traverse the gastrointestinal tract membranes constitute the process of absorption. Apart from ingested nutrients, mucous and digestive enzymes and juices released by the animal itself are also absorbed.

The large diversity in the nature of ions and molecules released by digestion, or by the animal for digestion, resulted in a complexity of different absorptive mechanisms.

These mechanisms, broadly classified as simple diffusion, facilitated diffusion and active transport, are common to most animals with only minor variations.

Simple diffusion, the movement of molecules from regions of high concentration to low concentration due to the kinetic energy of the molecule, requires no input of energy. Facilitated diffusion again relies on the kinetic energy of the molecules for energy, however carrier molecules are required to permit the passage of absorbed molecules and ions down the concentration gradient and across the cell membranes. Active transport, as opposed to the mechanisms of diffusion, moves molecules against the concentration gradient to regions of higher concentration. Active transport therefore requires the input of exogenous energy, generally supplied by the transepithelial sodium gradient, and the use of carrier molecules.

1.3.1 Protein Absorption

Proteins may be absorbed as free amino acids, peptides or whole proteins. All potentially contribute to the total α-amino-nitrogen absorption in teleosts, however the relative contribution and hence nutritional significance of peptide and intact protein absorption remain subject to debate. The majority of protein absorption is thought to occur in the anterior regions of the gut, with some amino acid absorption through the stomach, when present, and hindgut.

1.3.1.1 Amino Acid Absorption

In higher vertebrates free amino acid absorption occurs predominantly in the small intestine (Buddington and Diamond, 1987). Amino acids, co-transported across the brush border with sodium and utilising the transapical sodium concentration gradient, accumulate higher intracellular amino acid concentrations than those in the lumen and blood. Amino acids subsequently cross the basolateral membrane and enter the blood via facilitated diffusion down the established concentration gradient. Therefore sodium-dependent carrier molecules, low apical membrane amino acid permeability and minimal intracellular nutrient metabolism are required to obtain the intracellular concentration of amino acids needed for absorption. Each transport mechanism displays saturation and generally accepts a limited number of substrates. Amino acids within each group show varying affinities for the carrier mechanism. Although it appears some amino acids may be transported by more than one system.

The absorption of free amino acids in fish has been reviewed by Ferraris and Ahearn (1984) and Ash (1985). Although these reviews are dated, more recent studies agree

with the mechanisms of amino acid transport described by these authors (Buddington *et al.*, 1987; Lorenzo *et al.*, 1989; Sun and Farmanfarmaian, 1992; Cozzi *et al.*, 1993).

Variation in amino acid flux with substrate concentration in the intestine of fish indicates two or more simultaneous processes of amino acid absorption. Including at least one saturable mechanism plus non-saturable movement. The saturable component of amino acid transport in fish is apparently associated with absorption at lowest substrate concentrations, ensuring uptake of less common amino acids through high affinity processes, while non-saturable absorption allows the uptake of substrates present at high concentrations. Lower maximal amino acid uptake by herbivorous compared with omnivorous and carnivorous species, suggested an adaptation of these processes to natural intestinal amino acid concentrations. Buddington *et al.* (1987) has shown these differences to be genetic and not phenotypic adaptations to the natural diet, with maximal amino acid transport not adapting when herbivores are fed high protein diets.

Characterisation of the saturable component of amino acid uptake indicated the presence of sodium-dependent amino acid transport systems in fish. Transepithelial fluxes revealed active amino acid transport, with decreased transport associated with low luminal Na^+ concentrations suggesting sodium dependence. Total sodium dependence was generally not shown. Marked hyperpolarisation of the electrically positive serosa of freshwater species and depolarisation of the negative serosa of marine species in the presence of amino acids also supported sodium dependant uptake.

Amino acid carrier mechanisms in fish also displayed structural specificities. Competitive inhibition amongst amino acids and between sugars and amino acids suggesting mechanisms shared by several amino acids and/or sugars. Metabolic inhibitors also decreased amino acid absorption. From this data it appears that, as in higher vertebrates, co-transport of amino acids and sodium occurs in fish. In contrast to higher vertebrates transport however, Bogé and Rigal (1981) suggested amino acid uptake mechanisms in fish had a chloride requirement. Although Cl^- did not appear to energise the system, as was found for Na^+.

Amino acid transport systems not exhibiting saturation appears widespread in fish. This non-saturable component of amino acid influx may result from either simple diffusion or mechanisms not displaying saturation within the concentration ranges utilised. However, lack of identification of carrier molecules led to the concept of non-saturable, Na^+-independent, simple diffusion-like absorbance of amino acids in the upper intestine of fish.

Fish also exhibit amino acid absorption in the lower intestine, a characteristic again not shared by higher vertebrates. Hindgut amino acid absorption, generally restricted to carnivorous and omnivorous species, is thought to compensate for gut length. It is carrier mediated in nature, possibly having a scavenging role. However, Cozzi *et al.* (1993) showed L-methionine transport to be smaller in the upper intestine than the lower intestine of the gilthead bream, suggesting that transport of amino acids is realised all along the intestinal tract.

Differences in the amino acid absorption mechanisms exist between freshwater and marine fish. Freshwater species, as with higher vertebrates, accumulate intracellular concentrations of amino acids greater than those found in the gut lumen and blood, an ability not shared by marine species (Ferraris and Ahearn, 1984). This inability to concentrate amino acids may be due to either high brush border membrane permeability, substantial intracellular nutrient metabolism or elevated rates of efflux over the basolateral membrane. Non-saturable transport also varies being lower in marine species than freshwater species (Ferraris and Ahearn, 1984). This difference has been linked to the fatty acid composition of the brush border membrane. Changes in fatty acid composition with adaptation to seawater increase membrane permeability, again implicating membrane permeability in the differences in amino acid absorption between marine and freshwater fish (Ferraris and Ahearn, 1984).

Alteration in amino acid absorption in response to stimuli has been reported to occur via a diphasic response (Sun and Farmanfarmaian, 1992). Sun and Farmanfarmaian (1992) found increased amino acid absorption stimulated by growth hormone was mediated initially by increased carrier mechanism numbers per cell, followed by a general increase in the intestinal mass. Correlation between growth hormone secretion and nutritional status, and increased intestinal mass shown to occur in vertebrates in response to dietary regime, suggests this diphasic response may also be applicable to differing nutritional states.

1.3.1.2 Dipeptide Absorption

Vertebrate mechanisms of dipeptide absorption are less well characterised than those for free amino acids, but they are thought to be saturable, with competition shown between peptides. Peptide absorption in fish is still uncertain, with very limited available information. However, distribution of dipeptidase activity (refer section 1.2.3.1.8) and dipeptide absorption (Bogé *et al.*, 1981; Buddington and Diamond, 1987) suggests the presence of absorption mechanisms similar to their counterparts in mammalian gastrointestinal tracts (Ash, 1985).

1.3.1.3 Intact Protein Absorption

Uptake of whole proteins by fish occurs via pinocytosis into cytoplasmic vacuoles in the intestinal epithelial cells of the mid- and hind-gut (Stroband and van der Veen, 1981; Ash, 1985). This absorption of intact protein was initially thought to be an adaptation by larval and agastric fish to overcome the lack of gastric predigestion, however this theory has lost support with the subsequent identification of macromolecular absorption in gastric fish. Although the nutritional significance of macromolecular protein absorption is unconfirmed, Ash (1985) proposed several nutritional advantages of absorption of intact protein. In larval fish, which are agastric, intact protein absorption would be advantageous as protein may reach the posterior intestine rapidly and in a partially digested state. In adult fish it may serve as a "standby function" to compensate for periods when protease secretion is low and protein enters the intestine (for example post starvation), while whole protein absorption may be of nutritional advantage by conserving protein and energy through the reabsorption of hydrolysis resistant digestive enzymes.

Smith (1989) hypothesised that once absorbed, protein macromolecules appear to enter one of two distinct protein pathways depending on whether they are to be used as nutrient or antigen. Nutrient molecules are incorporated into larger vacuoles within the intestinal cells and slowly digested. Antigen protein is released into the intercellular space between adjacent epithelial cells, where the lymphoid cells are located, and an immune response may be initiated. However, the identification of orally administered ACTH in the blood of chinook salmon, and the biological response produced by this hormone, suggested that absorbed polypeptides of dietary origin may also be transported directly to the circulatory system without loss of biological function (McLean *et al.*, 1990). This is further supported by literature describing the reabsorption of digestive enzymes (Diamond, 1978).

1.3.2 Lipid Absorption

Absorption of lipid encompasses the mechanisms from the passage of fatty acids across the brush border of the intestinal enterocyte to their presence in the blood or lymph of the animal. In fish, lipid absorption is relatively poorly studied compared to the study of protein absorption. However, over the past 20 years increased attention has been paid to the absorption and transport of lipid in fish. These studies, reviewed by Cowey and Sargeant (1977), Leger (1985), Sargent *et al.* (1989), Sheridan (1988) and Smith (1989) indicate that the mechanisms of lipid digestion in fish are similar to those of mammals.

Mammalian lipid absorption has been well documented, occurring via two mechanisms. Short chain fatty acids (<10 carbon atoms), due to their appreciable water solubility, diffuse directly from the lumen of the intestine into the enterocytes and thence into the blood. Long chain fatty acids (>10 carbon units), 2-monoglyceride and cholesterol, being hydrophobic, are taken up by bile salt micelles, forming mixed micelles. These mixed micelles transport insoluble lipids to the intestinal epithelial cells where, upon contact, the lipids diffuse through the luminal epithelial membrane and into the enterocytes. Within the enterocyte, triglycerides and phospholipid are resynthesised, while cholesterol is re-esterified with long chain fatty acids. Triglycerides, phospholipid and cholesterol are subsequently incorporated into chylomicrons and very low density lipoproteins (VLDL's), which are released from the enterocyte into the lymph. These molecules are then transported in the lymph vessels to the thoracic duct where they enter the circulatory system (Waldeck, 1983).

In fish, the end products of lipid digestion (mostly free fatty acids, mono- and di-glycerides, glycerol and fatty alcohols) are predominantly absorbed across the epithelium of the pyloric caeca and anterior intestine, with absorption extending to the distal intestine when high lipid diets are ingested. The lipophilic nature of these molecules allows them to penetrate the enterocyte brush border by diffusion. Once within the enterocyte, fatty acids either remain free or are re-esterified with glycerol and mono-/di-glycerides to form triglycerides (Lie and Lambertsen, 1991). Fatty alcohols are mostly oxidised to free fatty acids and re-esterified to form triglyceride (Lie and Lambertsen, 1991) with some re-esterified to form wax esters. Triglyceride is therefore the major component of transported lipid (Lie and Lambertsen, 1991). Sterols and cholesterol, if present, are re-esterified before release from the cell.

A two step model has been described for the absorption of lipid in fish, comprising a slow and a fast component (Sheridan, 1988). The fast component, a free fatty acid delivery system, consists of plasma soluble short chain free fatty acids and longer chain free fatty acids thought to be bound to carrier molecules. This system results in the appearance of free fatty acids in the blood stream shortly after lipid digestion. The slower component represents a triglyceride delivery system. In this system the triglycerides resulting from the re-esterification of free fatty acids are aggregated into large lipoprotein complexes, similar to the mammalian system, forming chylomicron-like and VLDL-like particles. Ijima *et al.* (1990) suggested that when digestion is slow, as occurs at low temperatures, high density lipoprotein-like and low density lipoprotein-like particles may become the main transporters of lipid. The transport particles are subsequently passed into the lymph system and are transported via the lymph and blood.

Formation of transport particles within the enterocyte provides a rate limiting step for lipid absorption. Lipid droplets containing esterified lipids in the enterocyte cytoplasm are thought to represent storage pools arising when the transport particle synthesis pathway, and thus the triglyceride delivery system, is overloaded. The formation of lipid droplets occurs in most fish larvae upon the commencement of lipid absorption, when the enterocytes lack the cellular maturity to adequately perform lipoprotein synthesis (Deplano *et al.*, 1991). The formation of lipid droplets may be dependent on the nature of the lipid supplied (Rösch and Segner, 1990).

1.3.3 Carbohydrate Absorption

As described previously carbohydrates are hydrolysed releasing monosaccharides (refer section 1.2.4). It is these simple sugars which are subsequently absorbed across the intestinal and pyloric caecal epithelium and therefore carbohydrate absorption may be addressed as the transport of sugars. The majority of work having been performed on glucose.

The intestinal transport of sugars has been reviewed by Ferraris and Ahearn (1984). From this review it appears that the transport of organic solutes, amino acids and sugars, occurs via similar mechanisms, in some cases even sharing common transport pathways. Therefore as described in section 1.3.1.1 for amino acids, the absorption of sugars in fish intestine occurs via two or more processes occurring simultaneously, involving saturable and non-saturable components. Again saturable uptake appears associated with absorption at lower substrate concentrations, while non-saturable absorption allows the absorption of substrates at high concentration. At least one saturable mechanism is a sodium dependent, carrier mediated mechanism, with carriers showing nutrient structural specificity.

The characteristics of sugar uptake mechanisms and their distribution are similar to those described for amino acids, with the exception of chlorine dependence in the marine herbivore *Boops salpa* (Bogé and Rigal, 1981). Differences in the methods of absorption are quantitative in nature and not qualitative. For example, in contrast to amino acid absorption, maximal sugar uptake was lower in omnivorous and carnivorous than herbivorous species, suggesting adaptation to carbohydrate content naturally present in the diet. These differences were shown to be genetic adaptations to the natural diet (Buddington *et al.*, 1987). The variation in monosaccharide uptake between herbivorous, omnivorous and carnivorous species was greater than amino acids. Buddington *et al.* (1987) used the essentiality of nutrients to explain this difference. Sugars, being non-essential nutrients, have no dietary requirement and are used solely to supply energy. Carnivores, obtaining their energy requirements

predominantly from a high protein diet, consume little carbohydrate and therefore dedicate limited biochemical apparatus to absorbing sugars. Some amino acids, on the other hand, are essential and so have a dietary requirement. This requirement varies little between species and is satisfied by a similar daily protein intake, resulting in the daily absorption of amino acids being only slightly lower in herbivores than carnivores. However, due to the low protein and high carbohydrate content of the diet, herbivorous fish consume large quantities of carbohydrates which are digested and absorbed.

1.4 Regulation of Gastrointestinal Tract Activity

The volume of digestive juices and the rate at which the gut fills and empties are important factors in the processes of digestion. Gastrointestinal motility and digestive fluid and enzyme secretion are therefore integrally involved in the regulation of digestion. Factors affecting these processes alter the ability of an animal to digest and absorb feed items.

1.4.1 Digestive Fluid and Enzyme Secretion

Although the activities of digestive enzymes have been detailed in many species (section 1.2) little is known about the mechanisms and regulatory processes involved with the secretion of digestive juices and enzymes in fish. However, as in terrestrial animals, secretion appears to generally correspond with food ingestion and passage through the alimentary canal. A coordinated enzyme and acid or alkaline fluid secretion is achieved through the stimulation of cells in the immediate vicinity by the food itself and the initiation of nervous and/or hormonal mechanisms. The nervous stimuli responsible for secretion and activity of muscles responsible for bolus movement through the alimentary canal involve the autonomic nervous system, and this movement is therefore involuntary (Holmgren *et al.*, 1983; Smith, 1989).

1.4.2 Gastric and Gut Evacuation Times

Gut transit time and gut and gastric evacuation times describe the rate at which the gut fills and empties. These values therefore indicate the time available for digestion and are generally used to quantify the rate of digestion (Smith, 1989).

The rate at which the gut fills is a function of both feeding frequency and gastric emptying. Feeding frequency depends on stomach fullness and is therefore performed at intervals determined by the rate of gastric emptying. Appetite returns when stomach content decreases (Holmgren *et al.*, 1983; Smith, 1989). Fullness of the anterior segment of the intestine is the stimulus for agastric species (Holmgren *et al.*, 1983).

This whole process is thought to be governed by the energy content of the diet. Fish feeding to satisfy their energy requirements, monitor energy intake and in turn regulate stomach emptying and feeding frequency to ensure energy demands are met (Holmgren *et al.*, 1983; Smith, 1989). Both gastric emptying and feeding frequency increase with decreasing dietary energy (Holmgren *et al.*, 1983).

Time taken for gastric emptying is also influenced by a delay between food ingestion and the commencement of emptying (Rösch, 1987). This delay appearing to be dependant upon temperature and possibly meal type (Holmgren *et al.*, 1983; De Silva and Anderson, 1995).

1.4.3 Nervous Innervation of the Gastrointestinal Tract

Secretion into the gut or movement of muscles responsible for food passage through the gastrointestinal tract are involuntary mechanisms initiated or transmitted by the autonomic nervous system. The autonomic nervous system in fish has been reviewed by Holmgren *et al.* (1983). In teleosts this system has been divided into three sections, these being cranial, spinal and enteric. The cranial and spinal sections are the equivalent of the mammalian parasympathetic and sympathetic nervous systems respectively, supplying the extrinsic nervous stimulation to the gastrointestinal tract. The enteric section lies between the muscle layers of the gastrointestinal tract. Together these systems regulate the activity of the gut of teleosts with the extrinsic nerves (cranial and spinal) exhibiting their effect by modifying the otherwise autonomous actions of the enteric system.

In teleosts extrinsic input to the gut from the cranial system is thought to be limited to vagal gastric nerve fibres that are responsible for controlling muscle tone. Inhibitory fibres relax the stomach during ingestion of meals, while excitatory fibres increase tone, possibly to the extent of regurgitation of ingested noxious substances. The spinal section of the autonomic nervous system also supplies the stomach (if present) and is the major supply of nervous stimulation to the teleost intestine. Sympathetic innervation results primarily in the cessation of peristalsis and myogenic activity, and stimulation of the tunica media of the blood vessels causes vasoconstriction and decreased blood flow. Circulating adrenalin, a sympathetic neurotransmitter, induces a similar affect.

Enteric control of the gut involves both the enteric nervous system (myenteric plexuses) and neurotransmitter release from mucosal cells themselves. Myenteric plexuses receive inhibitory or stimulatory extrinsic nerve input and innervate muscle, glands and probably each other. Mucosal cells exhibit endocrine and paracrine

functions, releasing numerous polypeptides which affect both nearby cells as well as distant segments of the gut. Some polypeptides may also be secreted into the lumen of the gut, stimulating acid or enzyme production directly and suggesting a possible exocrine function.

1.4.3 Factors Affecting Gastrointestinal Tract Activity

In aquaculture nutrition, it is important to understand the digestive physiology of the cultured species and its responses to both environmental and physiological parameters. Such knowledge will allow regulation of feed ingredient usage, feeding regimes and husbandry techniques, in a way suitable to the species and the stage of development, ensuring optimal productivity through the optimisation of digestion.

Gastrointestinal tract activity, both motility and secretion, are effected by a number of environmental and physiological parameters. These are listed below with little accompanying detail, however it should be noted that many of these parameters are manifested through alteration in the activity of the digestive enzymes. These effects have been previously detailed in section 1.2.

(i) *Meal Size* The rate of gastric emptying and digestion is thought to be affected by meal size. An increased meal size per unit body weight increases digestion time, but not necessarily proportionally (Bromley, 1987; 1988; Smith, 1989; Ruggerone, 1989, De Silva and Anderson, 1995). In contradiction however, it has also been reported that larger meal size did not decrease the rate of digestion (De Silva and Anderson, 1995).

(ii) *Temperature* The physiological processes of most poikiliothermal animals are extremely susceptible to changes in ambient temperature. As such, all aspects of teleost digestion appear to be affected by temperature fluctuations (Smith, 1989). The manifestation of temperature effects, however, seems to be varied. A greater efficiency of digestion at lower temperatures is displayed by some species, while others digest more efficiently at higher temperatures (Smith, 1989). These temperature dependent variations in digestion are thought to be largely mediated by alteration of digestive enzyme activity (Smith, 1989).

(iii) *Food Type* Food type affects both the rate and efficiency of digestion. Alteration of gastric emptying and gut transit time by meal types (eg. excessive fat) result in varied digestion rate (Storebakken, 1985; Bromley, 1987; Smith, 1989, De Silva and Anderson, 1995), while ingestion of food items that are difficult to digest alters digestive efficiency (eg. exoskeletons).

(iv) *Stress* Catecholamine secretion followed by increased circulating levels of corticosteroids describes a typical response to stress by teleosts. Adrenalin, a catecholamine, inhibits peristalsis, myogenic activity and blood flow (Holmgren *et al.*, 1983). Increased circulating adrenalin and corticosteroid levels may lead to gut stasis, slowing digestive functions (Holmgren *et al.*, 1983; Smith, 1989). Decreased gut motility also leads to static, but still active, digestive enzymes that unless moved through the gut may begin to digest the gut wall itself (Smith, 1989).

Apart from those factors already mentioned day length, parasitic infection, reproductive status and age may also influence the digestive process (Smith, 1989). Stocking density, apart from producing stress may also effect digestion through a hierarchal effect (Smith, 1989).

1.5 Quantifying the Processes Of Digestion

Although chemical analysis of a diet may indicate a nutritionally adequate feed, this information serves little purpose unless it is accompanied by data on the proportion of nutrients made available to the animal through digestion and absorption. The quantification of these physiological processes is provided by calculating the digestibility of a feedstuff. Digestibility coefficients provide a relative measure of the extent to which ingested food, feed ingredients and their nutrient components have been digested and absorbed by the animal and are usually expressed as a percent of the whole. Providing all biotic and abiotic effects are minimal, digestibility coefficients may be used as determinants of the biological availability of nutrients in diets, thereby indicating the "true" potential of a diet for growth and allowing formulation of diets so as to satisfy both nutrient and economic requirements.

1.5.1 Methods of Measuring Diet Digestibility

A vital element in studying fish nutrition is the accurate, dependable measurement of the digestibility. Digestibility determination is based upon measuring faecal losses as an indication of the nutrients absorbed by the animal. Digestibility coefficients calculated as the percentage difference between the amount of feed, ingredient or nutrient ingested and the amount recovered in the faeces. Calculating digestibility coefficients therefore requires a knowledge of the proportion of ingested feed that is defaecated and the chemical analysis of both feeds and collected faeces.

Two general methods are employed for determining digestibility coefficients, requiring either collection of total faecal matter produced after consumption of a known amount of feed (direct measurement) or measurement of the concentration of an inert marker in

the feed and faeces (indirect measurement). Each of these methods allows the quantification of multiple nutrients simultaneously, using relatively small numbers of animals and is cost and time effective. However, although both potentially produce acceptable results, each has inherent problems.

1.5.1.1 Direct Measurement

Direct measurement calculates diet digestibility by determining the faecal matter voided from a measured amount of ingested feed or nutrient, the ratio providing feed or nutrient digestibility. Therefore, accurate calculation of feed intake and quantitative collection of the faeces emitted corresponding to that ingested food are required. Both measurements are difficult to perform in fish studies, subjecting this method of determining digestibility coefficients to many errors. The major source of error in direct measurement of digestibility are associated with the quantitative collection of all faeces produced and leaching of nutrients from the feed and faeces to the environment.

These problems may be overcome by collecting faeces manually upon defecation via immediate pipetting (refer section 1.5.4.2), which is extremely labour intensive and time consuming and probably stressful to the fish. Using decantation (Hajen *et al.*, 1993a) and automatic continual filtration (De la Noüe and Choubert, 1986; Choubert *et al.*, 1982), quantitative collection of faeces with minimal nutrient loss through leaching has been performed (refer section 1.5.4.2) although requiring expensive apparatus. De La Noüe and Choubert (1986) demonstrated accurate determination of digestibility using the direct method for diets which were solid and produced well formed faeces, however fragile diets resulted in the loss of material and erroneous calculations.

Even with complete faecal collection it is essential to ensure that the collected faecal samples are not contaminated with uneaten or regurgitated feed or other animal products. Hajen *et al.* (1993a) reporting up to 8.9% of the total weekly dry weight "faecal" collection was attributable to scales, necessitating the removal of these prior to analysis.

Total faecal collection from fish is therefore at best laborious and time consuming or expensive, and in some circumstances impossible. For this reason most workers employ indirect digestibility calculation.

1.5.1.2 Indirect Measurement

Indirect digestibility measurement is the most common method of determining digestibility coefficients in fish. Measurements are made by choosing a natural marker or adding a marker to the diet which is neither digested nor absorbed, or

digested/absorbed at a known rate. The increase in marker concentration through the digestive tract is then followed as digestible components of the diet are absorbed. Analysis of feed and faecal samples allow digestibility coefficients to be calculated from the concentration differences of the marker. Therefore only samples of food and faeces need to be collected, obviating the problems associated with the quantitative collection of feed and faeces.

Several problems also exist with measuring digestibility indirectly. As with direct measurement, the loss of nutrients through leaching to the water is of concern. It is also necessary to ensure that a representative faecal sample be obtained, since a proportion and not the total faeces voided is collected. There is also an assumption that the quality of faeces collected does not change with time. However, the major criticism levelled at the use of indirect calculation of digestibility is associated with the choice of marker.

Markers must be indigestible or only slightly digestible and move along the gut at the same rate as the rest of food material and recovery of the marker should preferably be quantified. This procedure should be followed for all studies as a reliable marker for one species may not work with another species or accuracy may vary with the diet used. Dietary components used as markers may be internal (endogenous) or external (introduced). Internal markers occur naturally in the diet, while external markers are distributed in small quantities throughout the diet during manufacture.

1.5.1.2.1 External Markers

The successful use of external markers in nutritional studies on domestic terrestrial animals resulted in fish nutritionists initially choosing this type of marker for the determination of digestibility coefficients for fish. Apart from being indigestible and not moving differentially along the gut, external markers must not influence the physiology of digestion of the experimental animal nor exhibit any toxic effects. As such almost any indigestible, inert material has the potential to be used as an exogenous marker providing it is easily quantified in the feed and faeces.

Of the markers used in terrestrial studies, chromium oxide (Cr_2O_3) became most commonly used in fish digestibility studies, appearing an adequate external marker in fish nutritional studies. De la Noüe and Choubert (1982) showed quantitative collection of faeces resulted in complete recovery of Cr_2O_3, while digestibility coefficients determined using Cr_2O_3 were comparable with calculations via the direct method (De la Noüe and Choubert, 1982; Hajen *et al.*, 1993a). However, concerns were raised as to the validity of this substance as a marker in fish. Bowen (1978) and

Lorico-Querijero and Chiu (1989) suggested that differential movement of Cr_2O_3 occurred in the gastrointestinal tract of Mozambique tilapia and Nile tilapia respectively. A solution, partially at least, for the differential rate of movement of the marker through the gut can be obtained by pooling faecal material over several days (De Silva, 1985). Pooling of faecal samples has the additional advantage of decreasing variance in the measurement due to daily variations in digestibility (De la Noüe *et al.*, 1980; De Silva and Perera, 1983; 1984; Ferraris *et al.*, 1986; De Silva *et al.*, 1990). Incomplete faecal recovery of Cr_2O_3 and the difficulty in determining the concentration of chromium in samples has been recognised in work on terrestrial vertebrates. Recently Saha and Gilbreath (1993) suggested a modified method of calculating digestibility using the Cr_2O_3 indicator. This method utilised correction factors for faecal recovery and the analytical recovery of chromium using total faecal collection and samples prepared of known concentration respectively.

While investigating the effect of chromium on carbohydrate utilisation, Shiau and Chen (1993) found that Cr_2O_3 significantly increased the efficiency of utilisation of dietary glucose, suggesting Cr_2O_3 may influence animal physiology. Chromium is known to act as a co-factor for insulin and to be part of an organic glucose tolerance factor and could therefore alter the natural responses to dietary carbohydrates if included as a marker. Dry matter, protein, lipid and carbohydrate digestibility coefficients for tilapia hybrids, *Oreochromis niloticus* X *O. aureus*, were significantly lower for diets containing 2% chromium oxide compared to those containing 0.5% chromium oxide (Shiau and Liang, 1995). Chromium oxide also affects the faecal lipid composition and intestinal micro-flora of the Artic charr (Ringø, 1993). Differences due to the presence Cr_2O_3 attributed to Cr_2O_3 affecting the digestive and/or absorptive processes in fish and the growth and attachment ability of the micro-flora.

Apart from the differential movement and possible physiological effects of Cr_2O_3, the potential technical errors during quantification and physical risks involved in analysis suggested it would be useful to consider other markers in digestibility studies (De Silva, 1985). Tacon and Rodrigues (1984) incorporated polyethylene and acid washed sand into diets at 0.5, 1.0 and 2.0% as external markers, with disappointing results. Apart from the 1.0% polyethylene inclusion level all digestibility coefficients produced significantly lower digestibility values with larger variation than those determined using Cr_2O_3. Lower digestibility coefficients suggest that the marker moves through the gastrointestinal tract slower than the digesta. Lied *et al.* (1982), however, had more success in finding an alternative external marker, showing titanium (IV) oxide to be an equivalent marker to Cr_2O_3 for protein digestibility studies. This

marker was chosen because it was easily measured in the Kjeldahl digests, simplifying analysis.

Another potential external marker is metallic iron powder, which showed no differential movement in studies of food consumption, gastric evacuation and assimilation efficiencies in Atlantic salmon (Talbot and Higgins, 1983). Buddington and Diamond (1987) used glass beads in studies of digesta in rainbow trout. No attempt has yet been made to ascertain the usefulness of these markers for digestibility studies.

1.5.1.2.2 Internal Markers

Difficulty incorporating external markers into commercial feeds and natural diets, and debate over the validity of external markers, led researchers to investigate the use of markers inherent to fish feeds and natural feeds. Several natural dietary components have since been studied as potential reference compounds for digestibility trials in fish. The value of possible internal markers are generally determined by comparison with the digestibility coefficients calculated using an external marker (Cr_2O_3 mostly) or the quantitative recovery of the marker.

Crude fibre and hydrolysis resistant organic matter (HROM) are two dietary components studied for use as internal markers. These components basically refer to the same group of materials. Cellulose and lignin comprise the majority of crude fibre, while the major cantons of HROM are cellulose and chitin, when present. De Silva and Perera (1983) and De Silva (1985) found the quantitative recovery of both crude fibre and HROM from the macrophyte *Hydrilla verticellata* not significantly different to 100%, suggesting appropriate internal markers. Buddington (1980) also reported no significant HROM assimilation by rainbow trout and three species of tilapia, although high variability was present. In that study HROM was incorporated into diets via the addition of chitin and cellulose. No statistical difference was observed between digestibility coefficients estimated using crude fibre and Cr_2O_3 for blue tilapia, *Oreochromis aureus*, (De Silva *et al.*, 1990) and rainbow trout (Tacon and Rodrigues, 1984). However, HROM was slightly, but significantly, digested by Mozambique tilapia (Bowen, 1981). Varying success has therefore been experienced when using crude fibre and HROM as indicator of digestibility.

Debate over the ability of fish to digest both cellulose and chitin, with cellulase and chitinase activity both identified in fish (refer section 1.2.4.3), also created uncertainty over using crude fibre and HROM as digestibility indicators. The acid insoluble mineral component of the diet is considered a preferred indicator.

Hydrolysis resistant ash (HRA), also termed acid insoluble ash (AIA), refers to the mineral ash resistant to acid hydrolysis. De Silva and Perera (1983) and De Silva (1985), investigating digestibility in the green chromid, *Etroplus suratensis*, reported a highly variable recovery of HRA in faeces, always exceeding 100%, indicating that it was an inappropriate marker. However, the HRA content of the macrophyte used, *Hydilla verticellata*, was low and also highly variable, probably contributing to high experimental error and the uncertainty of digestibility calculations. However, Atkinson *et al.* (1984) suggested AIA to be an adequate indicator in rainbow trout diets, obtaining values comparable although slightly higher than those calculated using Cr_2O_3. Addition of celcite, a natural component of AIA, to the diets improved calculation precision without affecting digestibility. Celcite addition thereby overcame the high variability associated with low AIA content in formulated diets. Atkinson *et al.* (1984) concluded that AIA, with or without celcite, was an easily measured and comparatively inexpensive alternative to Cr_2O_3.

Several other indigenous markers have been investigated. Hickling (1966) used ash and silica as markers in natural diets for grass carp. De Silva *et al* (1984) showed ash to be concentrated at the same rate as HROM, crude fibre and HRA by wild Mozambique tilapia, suggesting the use of this dietary component as an internal marker under special circumstance. However, Bowen (1981) found ash to be significantly digested by Mozambique tilapia, and Buddington (1980) found significant ash assimilation by rainbow trout and three species of tilapia to be 44.02% and 44.71% respectively, indicating ash was an inappropriate marker for these species. Bjorndal (1985) reported ash digestibility by various marine herbivores. Also noted in this review were two factors complicating the use of ash as an internal marker. Firstly ash can be dissolved and lost in either liquid or gas phase during gut passage. Secondly, accuracy of ash samples may be comprised by contamination or selective feeding. Although this later difficulty is more relevant to studying herbivores in ecosystems than in tank held animals.

The use of ions as internal markers for determining digestibility coefficients in Atlantic cod was investigated by Lied *et al.* (1982). Calcium and zinc produced similar digestibility values as titanium (IV) oxide provided stomach content levels and not feed levels were used. The difference was attributed to the ingestion of seawater. Further trials suggested calcium and zinc could be used as indicators to accurately determine protein digestibility in wild feeding cod. Iron, however, was an ineffective marker (Lied *et al.*, 1982).

1.5.2 Apparent Versus True Digestibility Coefficients

Expressed correctly as percentages, the digestibility coefficients measured above are known as "apparent digestibility coefficients." The term "apparent" reflects the contamination of faeces with endogenous materials, affecting digestibility calculation by the addition of nutrients. Therefore "true digestibility coefficients" must allow for the introduction of biotic and abiotic factors to the faeces by the animal.

The major source of introduced material in faeces arises from biotic factors such as the micro-flora of the gastrointestinal tract. Micro-flora presents a major problem in the faeces of domesticated terrestrial animals as gut colonisation is quite extensive. Pig nutritionists overcame micro-floral problems by collecting faeces via terminal ileum cannulation, recording "ileal digestibility" values (Batterham, 1991; Baterham *et al.*, 1990a; 1990b; 1990c; 1993). Faecal samples removed after absorption is finished, yet while the effect from gut micro-flora is still negligible.

Although micro-floral effects are less in fish, biotic factors are still present. Decreased protein digestibility at low dietary concentrations has been attributed to an increased contribution by digestive enzymes to faecal protein (Smith and Lovell, 1973; De Silva and Perera, 1984; Lorico-Querijero and Chiu, 1989), demonstrating the usefulness of true digestibility calculation if easily performed (Lorico-Querijero and Chiu, 1989).

Aquaculture nutritionists quantify the introduction of endogenous products by feeding fish diets lacking the nutrient of interest and analysing faeces to determine the extent the nutrient is incorporated during gut transit. Correction factors are subsequently determined and true digestibility coefficients calculated (Page and Andrews, 1973; Lorico-Querijero and Chiu, 1989). Using this method Lorico-Querijero and Chiu (1989) found up to 14% difference between apparent and true digestibility coefficients in the posterior intestine of Nile tilapia. However, it is generally accepted in fish nutrition that apparent digestibility coefficients are accurate enough for most nutritional studies.

1.5.3 Methods of Measuring Ingredient Digestibility

The methodology described above determines the digestibility of a complete diet and the nutrients from that diet. However, it is often important to determine digestibility coefficients of individual ingredients used to formulate diets. As such, the concept of digestibility studies has further developed by specifying the nutrients absorbed from individual dietary ingredients.

Although feeding dietary ingredients as a diet proved successful in rats (Hansen *et al.*, 1991), fish accept few potential ingredients for aquaculture diets when fed individually. Ingredient digestibility was, therefore, initially calculated after force feeding individual ingredients to the animal. This method ignored the effects of feed palatability and the alterations to the animals behaviour, and thereby does not provide a natural physiological response. These difficulties were overcome by the incorporation of test ingredients into reference diets. Comparison of test diet and reference diet digestibility allowed the calculation of ingredient digestibility (Cho *et al.*, 1982). This method permitted ingredient digestibility to be determined in nutritionally balanced diets consumed naturally by the fish. However, it must be assumed ingredients tested do not alter reference diet digestibility.

In early studies a 30% inclusion level of test ingredients into reference diets was used (Cho *et al.*, 1982; Wilson and Poe, 1985). However, ingredients of different origin may alter reference diet digestibility when included at elevated levels, and therefore the appropriate inclusion level of test ingredients should be determined. De Silva *et al.* (1990) investigated the preferred level of inclusion of leaf meal. Leaf meal, being of plant origin, had higher levels of ash and fibre than animal by-products, possibly altering reference diet digestibility. Based on the observations that digestibility co-efficients decreased with increasing ingredient incorporation, variance in calculated digestibility decreased as the level of test ingredient increased and the main point of inflection of curves depicting the relationship of total dry matter and protein digestibility, these authors suggested that an inclusion level of 15 or 20% was more desirable for estimating the digestibility of leaf meal. It was emphasised, however, that this level was pertinent for leaf meal and probably similar products, but should not be generalised to all ingredient measurements without further investigation. To date little further investigation of inclusion level has been performed.

Digestibility coefficients calculated for individual ingredients using this method appear additive, allowing diet formulation on the basis of the digestibility of its constituent ingredients. That is diet formulation on the "available" nutrients and not chemical composition. This was supported by Wilson and Poe (1985) who reported apparent digestibility coefficients for ingredients determined for channel catfish provided reliable protein and energy digestibility coefficients for formulated diets. Calculated and determined digestibility coefficients of diets also showed good agreement in studies on rats (Hansen *et al.*, 1991).

1.5.4 Methods of Faecal Collection

As the measurement of digestibility coefficients requires the analysis of faeces, it is essential collected faeces are representative of naturally defecated material. The major difficulty associated with collecting faecal matter from fish is the loss of nutrients from faeces through leaching. The aquatic environment lends itself to the enhancement of nutrient loss through dissolution into surrounding water. Loss of water soluble nutrients has the potential to significantly affect digestibility calculations (Windell *et al.*, 1978b; Smith *et al.*, 1980). Lied *et al.* (1982) reported water insoluble protein accounted for only 35-47% and 27% of the total dry matter protein in the intestine and rectum of Atlantic cod, and Smith *et al.* (1980) reported over half the faecal nitrogen in the liquid fraction of faeces collected in a metabolic chamber. Similarly, Windell *et al.* (1978b) found dry matter, protein and lipid digestibility estimates to increase by 11.5, 10.0 and 3.7% respectively following 1 hour in water. Post defecation loss of water soluble nitrogenous compounds therefore causing overestimation of both diet and protein digestibility.

Determination of digestibility coefficients by the direct method also requires the quantitative collection of faeces, posing an additional problem to faecal collection methodologies apart from reducing the effects of leaching. In order to overcome these problems several methods of faeces sampling have been devised. These methods are divided into two general categories, direct sampling and non-direct faecal collection.

1.5.4.1 Direct Faecal Sampling

Removal of faeces from the gastrointestinal tract prior to natural defecation is termed direct sampling. Direct sampling methods completely avoid the effects of leaching on faecal samples. Techniques include dissection, stripping and anal suction. However, faecal samples may not represent naturally defecated material due to the stress of the frequent handling and anaesthesia involved and the potential contamination with endogenous products.

Austreng (1978) suggested manual stripping of faeces from rainbow trout as the most convenient and accurate way of collecting faecal samples, faeces collected from the rectum by stripping from the ventral fins to the anus as protein absorption continues into the rectum. Although this method has been used in many studies, doubt has since been cast over its accuracy. Initially, contamination of the faeces with non-faecal waste nitrogen was of concern. Austreng (1978) noted this problem, suggesting it could be ignored as 80% of the non-faecal nitrogen was excreted through the gills of rainbow trout (Smith, 1971) and therefore errors due to urine contamination of would

be insignificant. Spyridakis *et al.* (1989a) described a method in which the application of ventral pressure to remove the urinal and genital products was followed by lateral pressure over the last 3 cm in European seabass to separate stripped faeces from urine. However, these authors found stripped faeces to be contaminated with sexual products, mucous, blood and other endogenous products resulting in underestimation of digestibility coefficients, especially of lipids. Stripping was also considered to cause an underestimation of digestibility through contamination with endogenous products in chinook salmon and rainbow trout (Windell *et al.*, 1978a; Hajen *et al.* 1993a).

Anal suction removes faeces by insertion of a cannula into the anus of an anaesthetised fish and applying slight suction. Collecting faeces from the posterior region of the intestine. Although Windell *et al.* (1978a) found anal suction to give reliable digestibility values, Spyridakis *et al.* (1989a) reported anal suction to contaminate samples with endogenous products resulting in the underestimation of digestibility.

Collection of faecal samples by dissection avoids the contamination of faecal samples with endogenous products as is the case with stripping and anal suction. Faeces removed by dissection are generally taken from the terminal region of the intestine to allow maximal protein absorption in the posterior intestine and rectum (Windell *et al.*, 1978a; Austreng, 1978; Spyridakis *et al.*, 1989a), although Lied *et al.* (1982) suggested that samples should be taken from the posterior intestine excluding the rectum. However, dissection still appears to underestimate digestion due to incomplete protein absorption and stress due to anaesthesia and handling (Spyridakis *et al.*, 1989a; Hajen *et al.*, 1993a). Dissection also sacrifices animals for only small quantities of faeces. Thus large numbers of animals are required to ensure adequate sample for analyses is obtained and to overcome inter-animal variation.

As can be seen from the above, all three methods of direct sampling have several inherent problems, resulting in faecal samples not representative of naturally defecated material. All methods tend to be extremely stressful due to anaesthesia and handling, generally resulting in sudden defaecation and an acceleration of the gut transit time. Premature removal of faeces also reduces endocytotic ingestion of intact protein in the posterior gut by enterocytes. Stripping and anal suction contaminate faecal samples with sexual products, mucus and blood, all resulting in underestimation of digestibility (Spyridakis *et al.*, 1989; Hajen *et al.*, 1993), and dissection results in the collection of extremely small amounts of faeces per animal.

1.5.4.2 Non-direct Faecal Sampling

The problems associated with the methods of direct sampling may be avoided by collecting faeces released into the water. Such methods of collecting naturally defecated faeces are termed non-direct faecal sampling. During non-direct sampling, fish are able to live normally in culture conditions with little disturbance until the end of the experiment. Non-direct sampling also permits relatively large numbers of fish to be included in experimental treatments, eliminating effects of inter-animal variation and allowing easy procurement of adequate faeces for analysis.

The composition of non-directly collected faeces depends on both the speed and method of collection. Leaching is dependent on the length of time faeces are in the water prior to collection, with many water soluble products leaching within the first 5 minutes, and crumbing of the faeces increases nutrient leaching (Cho *et al.*, 1982; Anderson, 1988). De la Noüe and Choubert (1986) suggested adding binders to diets to decrease the crumbing of faecal pellets. Thus non-direct methods of faecal collection must remove faeces from the water as soon as possible without interfering with the integrity of the pellet.

Siphoning is probably the simplest and most economical method of non-direct faeces collection, although it is also the most susceptible to the effects of leaching. Faecal material settled on the bottom of tanks is collected at predetermined periods by simple siphoning. Faeces generally remain in running water for extended periods of time. Although such a method would suggest large effects due to leaching, Anderson (1988) found nutrient composition of faecal pellets netted immediately upon defaecation and placed in aquaria showed no significant leaching effects compared to samples collected by siphoning. These data suggested leaching of nutrients may be minimal if faecal pellet integrity is maintained, although elevated protein-nitrogen content at 24 hours was present which was attributed to the action of bacteria. Kürzinger *et al.* (1986) also found good agreement between digestibility measurements based on 24 hour faecal collection and the results of dissection from the intestine, with exception of high protein diets. De Silva and Perera (1983) reported digestibility coefficients calculated from faeces collected in the morning before feeding and from faeces collected during the day showed no consistently higher or lower values, concluding leaching had no significant effect on digestibility estimations for siphoned faeces. However, this was later qualified by De Silva *et al.* (1990) suggesting that leaching stabilises after one hour and all values would be reasonably similar if faeces were in the water for greater than one hour. Smith and Lovell (1973) described an increase in digestibility coefficients measured using siphoning, attributing these to the effects of leaching.

Immediate pipetting collects faeces upon release into the water. Faeces removed by immediate pippetting prior to the effects of leaching probably best represents the natural faecal composition. However this method requires continual monitoring of tanks and is extremely labour intensive and time consuming, not being appropriate for large studies.

Smith (1971) and Smith *et al.* (1980) collected faeces in a metabolic chamber. This method allowed separation of excretions and quantitative collection of each excretion. However the chamber appeared extremely stressful to the animals with restriction of swimming movements causing fish to struggle in the chamber, force feeding resulting in regurgitation of food and animals being abraded by the securing diaphragm. It is therefore unlikely faeces produced by animals in this system would be representative of naturally defaecated material. The use of metabolic chambers was also restricted by the fact that each chamber could hold just one fish, reducing faecal collection and increasing errors due to variation between animals.

Faecal collecting via decantation, the Guelph system, was suggested by Cho *et al.* (1982). Faeces removed from the tank by the water flow are collected into a static container at the base of a settling column, where they remained until collection. Values similar to dissection and stripping were achieved using this system, suggesting minimal nutrient loss due to leaching (Cho *et al.*, 1982). Hilton and Slinger (1986) found digestibility coefficients measured using the Geulph system to be slightly greater than those calculated using dissected faeces, although these differences may have arisen from the different size classes of experimental fish used and not the effect of leaching. However, Spyridakis *et al.* (1989a) found leaching due to the time lapse before faeces reached the collection point led to overestimation of digestibility. A similar difficulty in using the Guelph system was encountered by Hajen *et al.* (1993a) and was attributed it to incomplete entry of faeces into the collection column. This problem could be corrected by alteration to the flow dynamics of the system.

Another method of faecal collection is automatic continuous filtration. Faeces removed from aquaria by high water flow (for example 4 litres/minute in a 60 litre aquaria) falls onto fine mesh screens which separate faeces from water as they move linearly. Faeces was subsequently ejected into pans where it is frozen within 30 seconds. Continual filtration generally removes faeces from the water within 6 to 17 seconds (De la Noüe and Choubert, 1986), thus minimising leaching. This method avoided the problems of direct sampling, was more labour efficient than immediate collection while resulting in minimal loss of nutrients through leaching (Spyridakis *et al.*, 1989a). However, similar to decantation, care must be taken to ensure rapid and complete

removal of faeces from the aquaria with adequate water flow and system dynamics. Another difficulty with continual filtration was faeces fragmentation by drainage water. This was overcome by lowering the height of the outlet above the screens (Choubert *et al.*, 1982) and the addition of binders to feeds to ensure the production of non-fragile, well formed faeces (De la Noüe and Choubert, 1986; Spyridakis *et al.*, 1989a). However collection of fragile diets remains a problem with this system.

The methods of non-direct faeces collection mentioned above all have advantages and disadvantages. The choice of collection method depends on the extent of digestibility work to be performed in the laboratory, the degree of accuracy required in digestibility calculations and whether the expense associated with automated collection systems can be justified. After comparing the methods of faecal sampling, both direct and indirect, Spyridakis *et al.* (1989a) suggested continual filtration as the preferred technique for digestibility trials, citing several advantages. Fish remained undisturbed throughout the entire period of the trial. Faeces were quickly removed from the water and frozen, minimising the effects of both leaching and bacterial populations. Whilst being automated, continual filtration presented highly reproducible results. Continual filtration may also allow accurate quantification of total faeces production for use in direct digestibility determinations (Choubert *et al.*, 1982; De la Noüe and Choubert, 1986), although this is limited to diets producing sturdy faecal pellets. However, the apparatus required for this method is expensive making it only appropriate only if extensive work on digestibility coefficients was to be performed. For elementary digestibility work the use of standard aquaria and siphoning appears acceptable provided food particles are not mixed with faeces, fish do not re-ingest voided faeces and faecal samples are collected at predetermined intervals after feeding with care taken to minimise the breaking of faecal strands (De Silva *et al.*, 1990).

From this it can be seen many problems exist with faecal collection in aquatic environments. Unless methods of faecal sampling are improved, digestibility coefficients determined from faecal samples will always be used with a fair degree of uncertainty. Therefore, much work seems necessary to achieve the accurate and reliable measurement of digestibility coefficients for fish.

1.5.5 *In vitro* Assessment of Digestibility

The methods of quantifying digestibility described above have all addressed *in vivo* measurement of digestion. However attempts have been made by nutritionists to develop rapid and reliable *in vitro* methods of quantifying digestibility. As protein is a key component in diets much of the effort has been focussed upon developing *in vitro* methods of quantifying protein digestibility. Such methodology involves the

introduction of the protein of interest into a system containing various proteolytic enzymes. These systems may be one step, utilising three or four intestinal enzymes, or two step, in which gastric and one or more intestinal proteases are included. This technique for quantifying protein digestibility *in vitro* has been developed studying terrestrial animals, however recently it has been adapted to salmonid nutrition (Dimes and Haard, 1994; Dimes *et al.*, 1994a; 1994b).

1.6 The Common Carp, *Cyprinus carpio*

The common carp, *Cyprinus carpio*, belongs to the order Cypriniformes and family Cyprinidae. A freshwater fish, common carp has a scaleless head, a body covered with varying degrees of heavy, curved scales and possesses an unusually long dorsal fin compared to other cyprinidae with 17 to 22 branched rays. The third spines of the dorsal and anal fins are strongly serrated making them difficult to remove from netting. At each corner of a slightly protrusive mouth are long fleshy barbels with shorter ones on the upper lip, giving a readily distinguishable appearance. These are thought to act as sense organs when feeding. The mouth itself possesses no teeth, however pharyngeal teeth are present. The common carp is a stomachless, or agastric, fish. Several subspecies of the common carp are known but as the fish are geologically young (no older than the Pleistocene), little difference is observed.

1.6.1 Natural Habitat

Carp are native to Japan, China and Central Asia. However, after human transportation, they are now found across the globe and are believed to be the most wide spread freshwater fish. By 1860 carp had been raised in most if not all European countries. In North America carp have become widespread in lakes since their introduction in the mid-nineteenth century, while carp were first released in Australia in Victoria in 1907-08 (Barnham, 1991), infesting several major river systems. Between 1914 and 1957 common carp were introduced to every South-east Asian country. Recently carp have been introduced to Africa and Latin America for the purpose of aquaculture.

Carp prefer to inhabit shallow sunny waters with a muddy bottom and an abundance of aquatic plants, avoiding clear swift flowing or cold waterways. They are generally active during the warmer months and are relatively dormant in winter. Carp are extremely hardy animals tolerating turbid water conditions and high water temperatures, and the capacity for anaerobic metabolism is thought to aid carp in tolerating low dissolved oxygen concentrations. It is little wonder then that carp have adapted so well to many different environments throughout the world.

1.6.2 Natural Feeding

In the wild, juvenile carp feed mainly on zooplankton, later in life feeding directly on benthic invertebrates. Other foods consumed in nature by carp include algae, small fish, earthworms and some terrestrial invertebrates. Various kinds of detritus, particularly decaying plant matter also provide a source of nutrition. Adult carp are efficient bottom feeders, sucking up the substrate, ingesting food items and expelling non-food particles, generally adding to the turbidity of the water as they feed.

1.6.3 Aquaculture of the Common Carp

The hardiness of the common carp complemented by their non-fastidious feeding habit means this species is very successful in aquaculture. Farming is thought to have begun many centuries ago, with early references to pond culture in China dating back to 2000 BC. Aristotle also made reference to carp, it therefore being likely that Romans and Greeks fattened carps in ponds. Carp culture in Austria dates back as far as 1227, and most European countries farmed carp by the end of the nineteenth century. Carp production was dominated by the USSR in the 1980's, contributing more than 60% of production, with Japan and Indonesia being the other two major producers. The most widely culture fish in the world today, an estimated 200,000 tonnes of carp is produced annually (Barnham, 1991).

1.6.4 Nutritional Requirements of the Common Carp

The need to intensify the culturing procedures employed for carp led to much scientific study of their nutrition, with most work being conducted on small fish with practical diets (Satoh, 1991; National Academy Press, 1993).

Using qualitative and quantitative analysis carp have been found to require the same essential amino acids as other fish, these being arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Studies on the level of protein required to provide adequate amounts of these amino acids and sufficient digestible energy to produce optimal growth demonstrated a requirement of 30-38% in a practical diet.

Fish, like terrestrial animals, utilise lipids for energy, cellular structure, membrane integrity and as a carrier for fat-soluble vitamins. Unlike terrestrial animals, however, the tissues of fish contain high amounts of n-3 highly unsaturated fatty acids. Using young carp it has been concluded that an essential fatty acid requirement exists for 18:2n-6 and 18:3n-3, with optimum growth obtained at a 1% inclusion level for both.

Carp seem to tolerate levels of good quality lipid from 5 to 25% in their diets, although visceral fat deposits increased markedly with increasing dietary lipid levels due to the additional energy in the diets. The energy provided in a diet through lipid and carbohydrate can replace the energy otherwise provided by the catabolism of protein, thus exhibiting a protein-sparing effect. The optimum digestible energy:protein ratio for growth of carp has been found to be 97 to 116.

Carbohydrates may be utilised for energy for immediate use or stored as glycogen in the liver and muscle by fish. The digestibility of starch is believed to vary greatly between fish species with omnivorous fish, such as carp, having a relatively high utilisation. As protein, lipid and carbohydrate are all energy sources for fish it is difficult to place an optimum level of inclusion of carbohydrate in artificial diets for common carp. However, a value of between 30 and 40% seems to be generally accepted as optimal.

Vitamins and minerals are also essential nutrients for growth in fish. Common carp have been shown to require two fat-soluble (vitamins A and E) and eight water soluble vitamins (thiamine, riboflavin, pyridoxine, pantothenate, niacin, biotin, choline and inositol) through qualitative and quantitative studies. Magnesium, phosphorous, copper, cobalt, iron, manganese and zinc are thought to be required by carp.

1.6.5 Common Carp as Experimental Animals

Common carp were chosen as experimental animals for this study because they are ideally suited for studies on digestibility. Digestibility trials require relatively large numbers of animals per trial to ensure ample collection of faeces for analysis. Carp populations are extensive throughout Victoria, Australia, and therefore carp are readily available in large numbers at low cost from wild stocks, thus ensuring animal numbers would be adequate throughout the trials. Carp are hardy animals, adapting quickly to alterations in their environmental parameters. They are easily transported to, and held in, scientific aquaria at high stocking densities, allowing large numbers of fish to be transported and maintained with minimal risk of mortalities. The non-fastidious feeding habits of carp allow them to accept a wide range artificial diets. As the manipulations of the experimental diets in this study required the inclusion of large quantities of a single dietary ingredient which may reduce the palatability of the diet, this was an essential feature to allow sufficient feeding on all diets. Finally the nutritional requirements and digestive processes of carp have been well documented and provided ample information for comparison of data and to assist in the explanation of said data.

1.7 Aims of the Present Study

Although fish appear to have the apparatus capable of digesting most dietary ingredients the extent to which digestion occurs needs to be quantified. The accurate determination of digestibility coefficients being of primary importance to the formulation of nutritionally balanced and cost-effective diets, as well the precise formulation of experimental diets to be used in nutritional studies. However to date the evaluation of digestibility coefficients for fish is restricted, providing data for dietary ingredients incorporated at a single level of inclusion and at a single time point. Such methods do not investigate changes in the digestibility of dietary ingredients with the level of inclusion and time. It is necessary to determine the digestibility of ingredients over the range of inclusion levels used in diets and the effects that ingredient inclusion at these levels have on other dietary nutrients. It is also necessary to determine any change in digestibility over time and with the adaptation of animals to the diet. These factors must be quantified in order to accurately formulate diets. No other studies have done so.

The aims of the present work were therefore to:

(i) determine the digestibility of four common dietary ingredients - an inert filler (cellulose), a plant protein source (soybean meal), a lipid source (tuna oil) and a carbohydrate source (raw corn starch) - and where possible to determine how these dietary ingredients affect the digestibility of other dietary nutrients,

(ii) determine the effects of the level of inclusion and time on the measurement of digestibility coefficients, and

(iii) formulate an improved methodology for the determination of digestibility coefficients.

Chapter 2

Materials and Methods

2.1 Introduction

Methods described in this chapter are those commonly used throughout the study. Included are descriptions of collection, maintenance and handling of animals, proximate analysis, faecal collection, diet preparation and the measurement of digestibility and growth. Other methods used to obtain data peculiar to a particular chapter have been described within that chapter.

Unless otherwise stated chemicals described in this chapter are analytical reagent grade obtained from either BDH Chemicals Australia Pty Ltd (Kilsyth, Victoria, Australia) or Ajax Chemicals (Auburn, NSW, Australia) depending on price and availability. All solutions were prepared using either deionised water (dH_2O) (Millipore, Milford, Massachusetts, USA) or Milli Q water (ddH_2O) (Millipore, Milford, Massachusetts, USA).

2.2 Animals

2.2.1 Fish Capture

Common carp, *Cyprinus carpio*, ranging in weight from 10-200g were netted, by a professional fisherman, using winged fyke nets and placed in large plastic containers holding freshwater. Animals were held in these containers for the duration of net clearing (maximum 2-3 hours). Regular water changes were employed to maintain adequate dissolved oxygen as artificial aeration was not possible. Netting was performed overnight and fish collected in the early morning to minimise stress associated with heat and confinement during capture.

Animals were collected from Reedy Lake and Lake Connewarre, Victoria, Australia.

2.2.2 Post-Capture Treatment

Fish were transferred into 50 litre foam containers containing freshwater taken from the lake and cooled with ice to approximately 8°C. Lowered water temperature had an anaesthetic effect, with fish entering stages 3-4 of anaesthesia (Hikasa *et al.*, 1986). Fish were then transported by road as soon as possible following capture to the aquarium room at Deakin University, Geelong, Victoria, Australia. No aeration was supplied during transportation.

Fish were maintained at Deakin University in either 70 litre aquaria or a 200 litre holding tank. The aquaria and holding tank were initially maintained as static tanks with 25% water exchange daily. Malachite green (Cat. No. 34054, BDH Chemicals

Ltd, Poole, England) and salt (Cheetam Salt Pty Ltd, Geelong, Victoria, Australia) were added at concentrations of 1 ppm and 2 ppt respectively, as a routine prophylactic treatment aimed at eliminating protozoan infections for five days. Following treatment, the malachite green and salt were thoroughly rinsed to waste and the aquaria and holding tanks placed on a continuous flow of recirculated freshwater at approximately 600 and 1500 ml.minute^{-1} respectively. Each tank was provided with additional aeration by a Model 100 "The Pump" aerator pump (MAS Imports, Coburg, Victoria, Australia) through air stones.

Water temperature was generally maintained at approximately 20°C, but varied from 17 to 22°C with seasonal temperature fluctuations and aquarium room usage. Overhead fluorescent lighting was set to provide a 12 hour light/12 hour dark cycle.

2.2.3 Water Quality

Removal of particulate matter by sand filtration (Quiptron Model S609, Quiptron Pool Products, Noble Park, Victoria, Australia) and ammonia and nitrite by biological filtration were employed as means of maintaining water quality. Ammonia (Aquaquant 14400, E. Merck, Darmstadt, Germany), nitrites (Aquaquant 14408, E. Merck, Darmstadt, Germany), nitrates (Low Range Nitrite Test Kit Model N1-14, Cat. No. 14161-00, Hach, Loveland, Colorado, USA), chlorine (Aquaquant 14434, E. Merck, Darmstadt, Germany) and pH (H1 8520 Laboratory Micro-Processor pH Meter, Hanna Instruments, Woonsocket, USA) levels were monitored regularly. Maximum acceptable levels of ammonia, nitrates, nitrates and chlorine were set at 0.2, 0.05, 0.1 and 0.05 ppm respectively, and a pH range of 6.8 to 7.2 was deemed satisfactory.

Water quality generally remained within the desired parameters, however some fluctuations did occur. Ammonia, nitrites and nitrates, when high, were removed by flushing the system with good quality water. Lime was used as a buffer to regulate pH levels. Chlorine levels did not exceed the acceptable maximum.

2.3 Anaesthesia and Handling

To minimise the stress associated with weighing, carp were anaesthetised in a 70 litre tank containing a 0.3 mM benzocaine (ethyl-p-aminobenzoate, E1501, Sigma Chemical Company, St Louis, Minnesota, USA). Used at this concentration for this species, benzocaine was thought to be a safe and effective anaesthetic, allowing rapid recovery (Mattson and Riple, 1989). However, smaller carp showed some susceptibility to the anaesthetic and it was therefore necessary to prevent these animals from reaching stage 5 (Hikasa *et al.*, 1986) of anaesthesia.

The appropriate weight of benzocaine was pre-dissolved in a minimal amount of absolute ethanol and injected by syringe below the surface of the water. This method ensured complete and rapid dissolution of the benzocaine. Fish were anaesthetised by immersion in the benzocaine solution and removed upon reaching stages 4 or 5 of anaesthesia (Hikasa *et al.*, 1986) depending on fish size. Fish were placed in freshwater, weighed and returned to their aquaria. Full recovery generally occurred within 10 minutes.

2.4 Faecal Collection

Faeces were collected daily from aquaria by siphoning. Water flow usually concentrated fecula pellets into a single corner, facilitating collection. Samples were siphoned with a rubber hose of approximately 5 mm internal diameter into a 400 ml beaker and subsequently transferred into 75 ml specimen containers. Excess water was aspirated using Pasteur pipettes and the faeces air dried to a constant weight at 50°C to avoid loss of volatile substances. All care was taken to not disturb the constitution of the pellets during collection.

2.5 Proximate Analysis

Dried diet and faecal samples were stored dry at room temperature prior to proximate analysis.

2.5.1 Gross Energy

Energy was determined by bomb calorimetry using either a Ballistic Bomb Calorimeter (Gallenkamp, England) or a Parr Semimicro Calorimeter (Parr Instrument Co., Moline, Illinios, USA) depending on availability.

(i) Ballistic Bomb Calorimeter - samples (100-600 mg) were weighed into metal crucibles and ignited in the presence of excess oxygen (Industrial Grade, Class 020, CIG., St Leonards, NSW, Australia). Heat generated was recorded using an Omniscribe chart recorder (Houston Instruments, Austin, Texas, USA). Prior to sample oxidisation the bomb was allowed to reach equilibrium. Two pieces of ignition wick and an empty crucible served as a blank, while the bomb calorimeter was calibrated using a powdered sucrose standard (energy content 16.7 kJ/g).

(ii) Parr Semimicro Calorimeter - samples (50 to 220 mg) were pelleted, weighed placed on metal crucible and ignited in the presence of excess oxygen (Industrial Grade, Class 020, CIG, Chatswood, NSW, Australia). Heat generated was measured using a Series 4500 Microscribe strip chart recorder (Houston Instruments, Austin,

Texas, USA). Prior to sample oxidation the bomb was allowed to reach equilibrium. An alloy wire of known calorific value (0.0096 J/mm) was used as an ignition fuse. The bomb calorimeter was calibrated using pelleted benzoic acid (energy content 26.6 kJ/g).

2.5.2 Total Crude Protein

Total crude protein was determined from total nitrogen using the Kjeldahl method. Total nitrogen was determined using an automatic distillation and titration or a colorimetric technique, depending on the number of samples to be analysed as the colorimetric method allows a more rapid throughput of samples than the distillation and titration method.

Samples (50-550 mg) to be analysed by automatic distillation and titration were digested using 20 ml concentrated sulfuric acid and 4 g of a 10:1 (w:w) anhydrous sodium sulphate:cupric sulphate pentahydrate catalyst mixture heated at 380°C in a Gerhardt Kjeldatherm (Gerhardt, Bonn, Germany) until a colour change to green was observed. Noxious vapours from the process of digestion were removed using a Gherhardt Turbosog (Gerhardt, Bonn, Germany). Digested samples were cooled, excess sodium hydroxide added and the solution titrated against a known concentration of hydrochloric acid to determine the nitrogen content calculated on a Gherhardt Vapodest (Gerhardt, Bonn, Germany). Hydrochloric acid was standardised by titration against a sodium carbonate solution using a methyl orange indicator.

Colorimetric determination of total nitrogen utilised a wet oxidation of the sample with sulphuric acid and hydrogen peroxide, using a selenium catalyst. Lithium sulphate was added to the digestion mixture to raise the temperature of digestion. Samples (50 - 320 mg) were digested in 4.4 ml of digestion mixture (350 ml H_2O_2, 0.42 g selenium powder, 420 ml H_2SO_4) at 365°C until the mixture was clear, approximately 150 minutes. The digest was cooled to room temperature, made up to 75 ml with deionised water and mixed well by inversion. Standards (0, 10, 20, 30, 40, 50 and 60 μg nitrogen ml^{-1}) were prepared by dissolving ammonium sulphate in deionised water. Nitrogen was determined colorimetrically by the salicylate-hypochlorite method of Baethgen and Alley (1989). Standards and samples (0.1 ml) were transferred to a 20 ml glass vial. Five ml of reagent 1 (34g sodium salicylate, 25 g sodium citrate, 25 g sodium tartrate and 0.12 g sodium nitroprusside made up to 1 litre with deionised water) was added, samples mixed well and left for 15 minute. Five ml of reagent 2 (30g sodium hydroxide and 10 ml sodium hypochlorite made up to 1 litre with deionised water) was added, samples mixed well and left for 1 hour prior to reading absorbance at 655 nm on a Milton Roy Spectronic 1201 (Milton Roy Co., USA).

Nitrogen concentration ($\mu g\ ml^{-1}$) was determined against the standard curve and subsequently converted to percent nitrogen.

Crude protein was calculated as measured nitrogen multiplied by 6.25.

2.5.3 Total Lipid

Lipid content was determined using an adaptation of the method described by Folch *et al.* (1959) or by gravimetric determination depending on the amount of sample available.

In the adaptation of the Folch method, samples (0.2 - 5.0 g) were homogenised and weighed into conical flasks. A volume (ml) of approximately 10-20 times the weight of sample of 2:1 (v:v) chloroform:methanol was added to the flasks. Flasks were agitated vigorously and allowed to sit overnight. The extract was filtered through fat-free filter paper into a pre-rinsed separating funnel, filtrate volume measured and 20% of the volume of deionised H_2O (Millipore, Milford, Massachusetts, USA) added. Separating funnels were agitated, pressure released and allowed to sit overnight. The layer containing chloroform/methanol solvent mixture and lipids was decanted off and the volume measured. Glass vials dried at 50°C overnight were pre-weighed and 1.00 or 2.00 ml of the decant pipetted into them and allowed to dry overnight at room temperature. The weight of dried lipid was measured and percentage lipid calculated.

In the gravimetric method, dried samples (10 - 600 mg) were weighed into a dry pre-weighed vial. Lipid was removed from the samples by suspending them in 5 ml of 2:1 (v:v) chloroform:methanol mixture, extracting lipid by vortexing, sonicating for 15 minutes, centrifuging at 5000 r.p.m. for 10 minutes and removing the organic layer. Samples were resuspended, vortexed and centrifuged twice more to ensure complete extraction of lipid. Following lipid extraction samples were dried overnight and reweighed. Weight of lipid was measured as the difference between initial and final sample weight, and percent lipid calculated.

2.5.4 Chromium Oxide

Chromium oxide (Cr_2O_3) content was determined by atomic absorption spectrophotometry or colorimetric spectrophotometry as described by Arthur (1970) and Furukawa and Tsukahara (1966) respectively. Methods were changed during the study due to limited resources and the expense of atomic absorption spectrophotometry. Although slightly quicker the atomic absorption spectrophotometry did not provide any greater accuracy nor reproducibility and was

therefore discontinued for the more economical colorimetric method. The method used for each experiment will be detailed in the particular chapter.

Samples (150-700 mg) containing 1-8 mg Cr_2O_3 were digested using 10 ml of a 5:3 (v:v) mixture of 70% nitric acid and 70% perchloric acid. Pre-digestion took place for approximately 1 hour followed by boiling on a hotplate/stirrer (Hotplate magnetic stirrer, Analite, Australia) until an irreversible colour change from green to yellow, orange or red (depending on the chromium content) occurred indicating complete oxidation to dichromate ions. After cooling to room temperature digests were diluted using double deionised H_2O (Millipore, Milford, Massachusetts, USA) in volumetric glassware to a final volume dependent on sample type and weight. Calcium as calcium phosphate was added to atomic absorption spectrophotometry standards and unknowns at 200 ppm to overcome interference due to ions.

Diluted samples were either:

(i) aspirated in an atomic absorption spectrophotometer (Model IL 951 AA/AE Spectrophotometer, Instrumentation Laboratory Inc., Wilingham, USA) at 425.4 nm, band width 0.5, using a nitrous oxide (Industrial Grade, Linde Gas Pty Ltd, Thomastown, Victoria, Australia)/acetylene (Industrial Grade, Gas Code 040, CIG, St Leonards, NSW, Australia) flame (acetylene flow 6 $l.minute^{-1}$) or

(ii) transferred to a cuvette and the optical density read at 350 nm against ddH_2O (Millipore, Milford, Massachusetts, USA) on a Hitachi U-3200 Recording Spectrophotometer (Hitachi Ltd, Tokyo, Japan) or a Milton Roy Spectronic 1201 (Milton Roy Co., USA).

Chromium concentration was determined by plotting atomic absorption or optical densities respectively on standard curves.

2.5.5 Moisture

Moisture content was determined by weight loss. Samples were dried to constant weight at 50°C, avoiding the loss of volatile substances. Weight difference between wet and dried samples was used to calculate percentage water.

2.5.6 Total Fibre

Total dietary fibre was determined by weight following enzymatic degradation of non-fibre dietary components. Scintered glass filters were thoroughly cleaned and heated

to 525°C for 1 hour, followed by soaking and rinsing in dH_2O. Following air drying at room temperature, 0.5 g of celite was weighed onto the filters and the filters and celite dried to constant weight at 130°C. Filters and celite were cooled to room temperature and stored in a desiccator until use. Approximately 1 g of dried sample was weighed accurately to three decimal places into a 400 ml stoppered conical flask. Fifty ml of phosphate buffer (pH 6.0) and 0.2 ml of termamyl solution (heat stable α-amylase) were added and the flasks incubated at 60°C in a water bath for 15 minutes, with gentle aggitation of the solution at 5 minute intervals. Digests were cooled to room temperature, pH adjusted to 7.50 with 0.17N NaOH and 5 mg of amyloglucosidase added. Samples were incubated at 60°C for 30 minutes under constant gentle agitation. Following cooling to room temperature, pH was adjusted to 4.50 with 0.20N H_3PO_4, 0.3 ml of amyloglucosidase added and flasks constantly agitated at 60°C for 30 minutes. Whilst still held at 60°C 280 ml of 95% ethanol which had been preheated to 60°C was added. Digests were removed from the water bath and a precipitate allowed to form upon cooling to room temperature for 60 minutes. Scintered filters containing celite were weighed and the celite distributed into a uniform bed using 78% ethanol and gentle suction. Whilst maintaining suction the digest precipitate was quantitatively transferred to the sintered glass filters and rinsed with three washes of 78% ethanol (20 ml), 2 washes of 95% ethanol (10 ml) and 2 washes of acetone (10 ml). Scintered glass filters with the washed residue were dried at 70°C overnight, cooled in a dessicator and reweighed. Weight of precipitate was calculated. Protein content of the precipitate was determined using the Kjeldahl method (refer section 2.5.2), weight of protein subtracted from weight of precipitate and percent dietary fibre determined.

2.5.7 Nitrogen Free Extract

Nitrogen free extract (NFE) was determined by difference. Dry matter percentages of protein, lipid, ash and Cr_2O_3 were subtracted from 100% to provide a value (% dry matter) for NFE.

2.6 Apparent Digestibility Coefficients

Apparent digestibility coefficients (ADC's) were determined for feeds, feed ingredients and nutrients using an indirect method. Test ingredients were substituted into a reference diet at known levels of inclusion and the digestibilities of these experimental diets compared to that of the reference diet (Wilson and Poe, 1985, De Silva *et al.*, 1990). Chromium oxide (Cr_2O_3) was used as an inert marker. This marker has been used in previous digestibility studies on common carp (Takeuchi *et al.*, 1979, Kurzinger *et al.*, 1986; Degani and Revach,1991), tilapiines (Lorico-Querijero and

Chiu, 1989; De Silva *et al.*, 1990), Artic char (Ringo, 1991), rainbow trout (Takeuchi *et al.*, 1979, Oliva-Teles and Kaushik,1990) and channel catfish (Wilson and Poe, 1985). It is assumed that Cr_2O_3 does not affect the physiological processes of digestion, moves through the gastrointestinal tract at the same rate as feed ingredients and is not absorbed/secreted by the animal. Pooling of faeces over several days was generally used in this study as a means of obtaining sufficient faeces for analysis and overcoming any possible variability in diet digestibility (De Silva *et al.*, 1985).

Chromium oxide (Aldrich Chemical Company Incorporated, Milwaukee, USA) was incorporated into feeds (0.8-1.0% dry weight ingredients) and analysed in both feeds and faeces. ADC's were calculated using the ratio of tracer in feed and faeces according to the following formulae:

Equation 2.1:

$$\text{ADC diet (\%)} = 100\text{-}100\left(\frac{\%Cr_2O_3 \text{ in the diet}}{\%Cr_2O_3 \text{ in the faeces}}\right)$$

Equation 2.2:

ADC Ingredient (%) =

$$\left(\frac{100}{X}\right)\left(\text{ADC test diet} - \frac{100 - X}{100}\,\text{ADC reference diet}\right)$$

Equation 2.3:

ADC Nutrient (%) =

$$100\text{-}100\left(\frac{\%Cr_2O_3 \text{ in the diet}}{\%Cr_2O_3 \text{ in the faeces}}\right)\left(\frac{\%\text{nutrient in the faeces}}{\%\text{nutrient in the diet}}\right)$$

Equation 2.4:

ADC nutrient ingredient (%)=

$$\left(\frac{100}{X}\right)\left(\text{ADC nutrient test diet} - \frac{100 - X}{100}\,\text{ADC nutrient reference diet}\right)$$

- where X refers to the level of inclusion of the test ingredient in the test diets (%).

2.7 Diet Preparation

Diets were formulated on a percentage dry weight of ingredients basis. Dry ingredients were mixed thoroughly, followed by the addition of the tuna oil. Water was added to the dry ingredient mix (beginning at approximately 30% total moisture) until pellets extruded, generally at 33-40% total moisture. Pellets were extruded using

a Hobart #12 chopper attachment (Hobart Corporation, Troy, Ohio), die hole diameter of 1/8", powered by a Model A120 Hobart Mixer (Hobart Corporation, Troy, Ohio). Following processing, diets were air dried at 50°C, placed in plastic bags and stored at -18°C.

Proximate analysis of all ingredients used in diet preparation is given in Table 2.1. Vitamins and minerals were supplied to all diets in a commercially available trout premix (Colbourn-Dawes, Wagga Wagga, NSW, Australia).

2.8 Weight Measurement

Length and weight measurements were made using a perspex weigh tray with an attached perspex ruler (mm increments) set on an analytical balance (A&D Company Limited, Japan). Anaesthetised fish, with excess water removed with a damp bench pad (avoiding disturbance of the mucous coat of the fish), were positioned on the pre-moistened weigh tray and and weights measured to the nearest 0.01 g.

Table 2.1 Proximal composition as determined by analysis of feed ingredients used in the preparation of experimental diets.

Ingredient	Proximate composition				
	Protein[a]	Lipid[a]	Ash[a]	Energy[b]	Moisture
Fish meal[1]	83.6	10.7	19.2	20.9	13.5
Casein[2]	87.0	1.0	1.7	22.8	9.0
Ox liver[3]	57.2			27.9	69.0
Gelatin[4]	88.0	0.0		15.0	6.6
Ground soybean meal[1]	39.9	3.31	7.8	19.1	8.4
Sifted soybean meal[1]	46.8			18.8	8.8
Ground soybean meal middlings[1]	44.5			19.2	8.3
Semolina[5]	11.4	1.6	0.5		14.1
Wheat gluten[6]	74.0	1.0	1.0		8.0
Starch (corn flour)[7]	0.4		<0.1	19.0	8.8
Cellulose (BW40 Solka floc)[8]	0.3	1.2		18.5	3.3
Tuna oil[9]	0.2	100.0	<0.1	42.3	0.0

a values are percentage dry weight, [b] values are kJ/g dry weight

Source of ingredients:

1 Metropolitan Commodities Pty Ltd, Port Melbourne, Victoria, Australia
2 Bonlac Food Ltd, Melbourne, Victoria, Australia
3 Minced ox liver (M.C. Herds, Geelong, Victoria, Australia)
4 Davis Gelatine Australia Company, Melbourne, Victoria, Australia
5 NB Loves Mills Ltd, Melbourne, Victoria, Australia
6 Bunge Bioproducts Pty Ltd, Altona North, Victoria, Australia
7 Goodman Fielder Mills, NSW, Australia
8 James River Corporation, Berlin, New Hampshire
9 Juro Pty Ltd, Victoria, Australia

Chapter 3

Quantification of Cellulose Digestibility

3.1 Introduction

Native or alpha cellulose is a water insoluble complex carbohydrate produced by plants as a structural component of the rigid cell wall. Comprising between 20 and 50% of the dry weight of vegetable matter (Van Soest, 1973), cellulose is the most abundant of all the naturally occurring organic substances. As a readily available natural polymer, cellulose was seen to have immense industrial importance, including in the feed industry.

Initially fish nutritionists thought cellulose to be indigestible by teleosts and placed it with other supposedly indigestible complex carbohydrates, such as lignin, under the generic name of fibre. Cellulose, in fact, provides the major component of crude fibre and generally accounts for variations in the crude fibre fractions (Van Soest, 1973). The ready availability of cellulose combined with the fact that it was thought to be not only indigestible, but also physiologically inert, led to it being used as an inert filler in experimental diets in fish nutritional studies. However, despite its relative abundance and common use in feeds for animals, the fermentation of cellulose and its nutritional value to animals is far from completely understood. Two question in particular are of considerable immediate importance for fish nutrition. Is cellulose indigestible? And, if cellulose is in fact indigestible, what are the physiological effects of including cellulose in experimental diets?

3.1.1 The Physiological Effect of Cellulose

Studies into the physiological effects of cellulose incorporation into experimental diets for teleosts have generally assumed cellulose to be indigestible. Changes in growth rates and carcass composition were used in these studies to indicate the physiological effects of cellulose. The results from these studies have been conflicting, with reports of changes in growth produced by increased cellulose levels in diets varying from little or no effect (Bromley and Adkins, 1984; Szlaminska *et al.*, 1991) to slight positive effects at low inclusion levels (Dioundick and Stom, 1990) or conversely negative effects at all inclusion levels (Leary and Lovell, 1975; Hilton *et al.*, 1983; Anderson *et al*, 1984; Qadri and Jameel, 1989). Little effort has been made to measure the digestibility of either cellulose or the experimental diets. Therefore, with the exception of some general conclusions, advancement in the clarification of the physiological effects of cellulose have been limited.

The variation in the results produced by studies on the physiological effect of cellulose may be at least partly due to the methods of formulating experimental diets.

Techniques used for diet formulation can be grouped into three categories, these being the replacement of other carbohydrate sources with cellulose, replacing experimental control diets proportionally with cellulose, and incorporating an alternative "inert" ingredient into a reference diet and replacing this substance with cellulose. The results obtained using each of these methods are discussed below.

Studies involving the replacement of alternative carbohydrate sources by cellulose in experimental diets are generally designed to test cellulose as an alternative source of carbohydrate. The substitution of starch with cellulose alters nutrient ratios and the total digestible energy of diets, making generalisations about the effects of cellulose based on growth performance difficult. However, authors of these studies often draw conclusions about the effect of varying cellulose level in experimental diets. Growth of rainbow trout was enhanced at low levels of incorporation of cellulose, up to 28%, at the expense of dextrin with a decrease in growth at higher inclusion levels (Buhler and Halver, 1961). Similarly, Dioundick and Stom (1990) demonstrated that diets containing 2.5 and 5.0% cellulose provided better growth than cellulose free or 10% cellulose diets for the Mozambique tilapia. It was suggested that some supplemental fibre promotes growth by facilitating the effective passage of food through the gut, thus increasing diet digestibility and growth (Buhler and Halver, 1961; Dioundick and Stom, 1990). Higher levels of cellulose inclusion, and the reciprocal decrease in starch, were thought to reduce the digestible energy of the diet, thereby retarding growth (Dioundick and Stom, 1990). Dioundick and Stom (1990) concluded that "the absence of cellulose in diets for juvenile tilapia is just as undesirable as its surplus". The above studies demonstrate the ineffectiveness of cellulose as a carbohydrate source, however they suggest a positive effect of cellulose on the physiology of the fish when included at low levels.

Davies (1985) found that up to 20% cellulose included at the expense of dietary starch in experimental diets for trout had no detrimental effects on the growth performance and utilisation of dietary nutrients, suggesting it was possible to consider α-cellulose as an inert bulking agent for experimental diets. However, if cellulose was inert then specific growth rate should decrease with cellulose inclusion at the same feed consumption. The fact that neither specific growth rate nor feed intake were altered significantly with the inclusion of cellulose indicates that cellulose must in some way be adding to the nutritional status of the fish or increasing diet utilisation.

The second method of diet formulation incorporates cellulose into experimental diets at the expense of a basal diet mix, thus replacing the basal diet as a whole. This

method maintains the ratios of digestible nutrients in the diet, however it results in a "dilution" of the basal diet, decreasing the total amount of nutrients and energy consumed per unit weight of ingested feed.

Using this method Bromley and Adkins (1984) found that the growth of rainbow trout was not altered by cellulose levels up to 30%, however at higher levels (40 and 50%) growth retardation occurred. Analysis of feeding rates revealed that animals receiving lower levels of cellulose (less than 30%) compensated by increasing feed ingestion, stabilising nutrient intake and therefore growth. Increased feed consumption would be expected as fish feed to satisfy their energy requirement and diluted feeds would contain less metabolisable energy. Accompanying the increased feed intake was increased stomach volume. However, at higher levels of cellulose inclusion, even with enlarged stomachs, elevated feeding could not maintain nutrient intake, and growth rates decreased. Similarly, Szlaminska *et al.* (1991) found that cellulose levels up to 35% had no effect on the growth rate of larval carp after the first week, suggesting an adaptation to the experimental diets possibly due to an increased feed ingestion. These studies suggest that cellulose itself had no effect on the physiological processes of the fish and may be an acceptable filler, provided adequate nutrient levels were attainable. Feeding rates would need to be monitored to accurately determine this nutrient intake. Bromley and Adkins (1984) further qualified this by suggesting that fish with smaller stomachs may have difficulty consuming adequate quantities of feed and that cellulose inclusion levels and nutrient ingestion should be monitored more carefully in such species.

Also working on rainbow trout, Hilton *et al.* (1983) attempted to further isolate the physiological effect of cellulose. In that study, feed rates were adjusted to ensure equivalent levels of nutrients were ingested by each experimental group. The *ad libitum* feeding level of the group supplied the basal diet was determined and the feeding rates adjusted for the two test groups (10 and 20% cellulose inclusion) in an attempt to provide the same nutrient intake level assuming that cellulose provided no additional nutrient. It was found that fish on the test diets could not consume their ration, even though an increased stomach volume or distensibility was observed, and growth was correspondingly reduced. This experiment was similar to the work described by Bromley and Adkins (1984). In a second feeding experiment, they measured the *ad libitum* feeding rate of the fish offered the 20% cellulose test diet and adjusted feeding levels for the remaining two groups. In this experiment, all groups consumed the allotted feed and therefore similar nutrient levels. However, the high cellulose supplementation still reduced growth rates, suggesting that cellulose *per se* caused depression of the growth response of rainbow trout. A

reduced gastric emptying time following cellulose incorporation into the diets was also observed (Hilton *et al.*, 1983). Decreasing gastric emptying time permits increased ingestion of low energy diets, although also potentially decreasing diet digestibility.

Test diets formulated by the replacement of a practical type diet with cellulose were also employed by Leary and Lovell (1975) who found decreased growth rates for channel catfish at inclusion levels of cellulose from 2 to 16%. These authors attributed the reduced growth rates to a decreased nutrient digestibility caused by the physical obstruction of digestive enzymes by cellulose. Such a response to cellulose was reported by Smith (1971) when inclusion of 50% cellulose in experimental diets resulted in a significant decrease in the digestibility of the protein component of the diet. Similarly, Kawai and Ikeda (1973a) demonstrated that the inclusion of cellulose (up to 60%) at the expense of fishmeal in experimental diets for juvenile rainbow trout decreased intestinal activity of the digestive enzymes pepsin, trypsin, amylase and maltase. Whether this decrease was due to a chelating effect or a decrease in stimulation of secretion is unclear. Whatever the mechanism, digestive potential was decreased. Leary and Lovell (1975) concluded that cellulose inclusion was not beneficial to practical type diets, although in purified diets it may slow gut passage through the intestine thus increasing digestibility and growth, as was indicated by Dioundick and Stom (1990).

Work by Anderson *et al.* (1984) and Qadri and Jameel (1989) on Nile and Mozambique tilapia respectively, used diet formulations designed to eliminate the effects of varying feeding levels and nutrient intake. In those studies polypropylene powder was added to the control diets at an inclusion level of 40%. Polypropylene powder has no nutritional value and is thought to produce little or no physiological response. Cellulose was subsequently incorporated into the experimental diets up to a level of 40% at the expense of the polypropylene powder, thus replacing one "inert" ingredient with another, and maintaining previous nutrient ratios and nutrient intake per unit feed consumed. Feeding rates were also controlled so that altered nutrient intake could not occur through preferential feeding. Results from these studies showed decreased growth rate with increasing cellulose inclusion. Food conversion efficiency, net protein retention, carcass fat and condition factor were all found to be reduced by the inclusion of cellulose into the diets. These results indicate that for Nile and Mozambique tilapia, cellulose does exhibit a negative effect on the physiology of the animal. The means by which this effect is exerted has not been determined.

From these studies it appears that cellulose may produce a physiological effect, although the nature of this effect and its extent still remains uncertain and requires further study. As such it would appear unwise to use cellulose as a bulking agent in experimental diets without first determining its effects in the species to be studied, thus ensuring that the changes observed are due to the intended dietary manipulation and not a compounding effect from cellulose. The experiments described above also demonstrate the need to carefully design experimental protocols to isolate the effects of elevated levels of cellulose in the diet.

3.1.2 Digestibility of Cellulose

In order for an ingredient to be an acceptable filler it must not contribute to the nutritional value of the diet, that is it must be completely indigestible. The question of cellulose digestibility by teleosts, as with its physiological effects, appears to be subject to much debate, with several schools of thought and no definite answers.

3.1.2.1 Structure and Enzymic Decomposition of Cellulose

Cellulose is an insoluble long chain polysaccharide comprised of cellobiose residues (refer section 1.2.4.3). Initial digestion of cellulose is performed by cellulases which catalyse the hydrolysis of cellulose to the cellobiose dimer. The resulting disaccharide is subsequently attacked by cellobiase, a β-glucosidase, which hydrolyses the β-1,4-glucosidic bond releasing β-glucose (refer section 1.2.4.3).

Digestion of a dietary ingredient requires the presence of the appropriate digestive apparatus. Generally, studies pertaining to cellulose digestibility in fish have been directed towards this digestive apparatus and not the actual quantification of cellulose digestion from natural or experimental diets. As such, the presence or absence of cellulase activity in the alimentary tracts of fish has received the majority of attention from investigators. Cellulase and cellobiose activity in fish have been previously reviewed in detail in section 1.2.4.3. The possession of anatomical fermentative chambers, similar to the structures present in terrestrial ruminants, used by fish for cellulose digestion have also been discussed in section 1.2.4.3.3.

3.1.2.2 Studies Quantifying Cellulose Digestibility in Teleosts

The majority of work to date has identified cellulase activity within the gastrointestinal tract of fish, but has not determined the extent of cellulose digestion. The levels of cellulase described may merely disrupt plant cell walls, releasing cell contents for digestion, and acting as an accompaniment to trituration and acid lysis. Such a function was suggested for the low levels of cellulase measured in roach and

rudd (Niederholzer and Hofer, 1979). Alternatively, fish may be capable of digesting cellulose to an extent enabling it to be utilised as a nutrient source, either at low levels or to provide substantial nutritional value. This relies on the assumption that the enzyme measured is in fact capable of digesting the type of cellulose present in the diet (refer section 1.2.4.3.1). While some studies, as discussed previously, used growth rates to indicate the utilisation of cellulose as a nutrient source, and others measured stable isotope incorporation (Deegan *et al.*, 1990), the actual quantification of cellulose digestion needs to be performed.

The quantification of cellulose digestibility has been sparse. Hilton *et al.* (1983), while looking at the effect of cellulose on the growth of rainbow trout, measured dry matter digestibility coefficients of the experimental diets. As expected cellulose decreased diet digestibility, however large errors prohibited conclusions regarding digestibility of the cellulose component of the diet.

Bergot (1981) quantified the digestibility of purified wood cellulose by rainbow trout and common carp. Digestibility coefficients combined with low levels of intestinal VFA's indicated that cellulose was indigestible by both species. However, Shcherbina and Kazlauskene (1971) reported carp to digest up to 50% of cellulose from soya and castor cakes. Finding peaks in digestion in both the anterior and posterior intestine, cellulose digestion was attributed to endogenous and bacterial cellulase respectively. These authors also found wheat cellulose to be indigestible, indicating digestibility to vary with the cellulose source, a proposition also made by Van Soest (1973). Further contradiction is found in the data of Smith (1971) who reported that α-cellulose incorporated at 50% into experimental diets for rainbow trout was 13.7% digestible. In that study the digestibility of cellulose was not significantly different to that found for raw starch included at a similar level.

3.1.3 Aims of the Present Study

From the above, it appears cellulose may not be an inert filler, exerting physiological effects and being digestible by fish. Discussion of the physiological effects of cellulose often implicates alterations to diet digestibility, either positively or negatively. Therefore, to clarify the potential of cellulose as an inert filler, the effects of graded levels of cellulose on diet digestibility were investigated. Solka floc, a commonly used wood cellulose filler, was incorporated at varying levels into experimental diets for the carnivorous Murray cod, *Maccullochella peelii peelii*, and the omnivorous common carp. Digestibility of the cellulose, experimental diets and where possible other dietary components were measured to determine the effects of cellulose inclusion on digestibility for species of differing dietary preference.

3.2 Materials and Methods.

Four experiments were performed to determine cellulose digestibility. Initially experiments were conducted for Murray cod (Trial 1) and common carp (Trials 2 and 3) to identify the effects of cellulose on digestibility for a carnivorous and an omnivorous species. Results obtained from the common carp cellulose digestibility trial led to a fourth experiment being performed in which antibiotics were incorporated into the diets for carp (Trial 4).

3.2.1 Experimental Diets

The test ingredient in the present trial was BW 40 Solka floc, a powdered wood cellulose produced by James River Corporation, Berlin, New Hampshire. The proximate analyses of BW40 Solka floc are detailed in Table 2.1.

Trial 1:

As Murray cod are carnivorous fish an ox liver-fishmeal based reference diet was chosen to determine cellulose digestibility for this species. Test diets were formulated by replacing the reference diet on a dry weight basis with purified wood cellulose at 10%, 20% and 30% inclusion levels. The formulation of diets used in Trial 1 are detailed in Table 3.1.

Trial 2:

To determine cellulose digestibility in common carp, a casein-based reference diet was used (Mackie and Mitchell, 1985). Cellulose was incorporated into the test diet at 20% inclusion level on a dry weight basis. Formulation of the diets used in Trial 2 are detailed in Table 3.2.

Trial 3:

The formulation of diets used to determine the digestibility of graded levels of cellulose are detailed in Table 3.3. A casein-based reference diet was again used, however the level of fibre in the reference diet was reduced compared to Trial 2 in order to facilitate the manufacture of the high cellulose diets. Cellulose was incorporated into the reference diet at 10%, 20%, 30% and 40% inclusion levels.

Table 3.1 Formulation (percent dry matter) of the experimental diets used in the Murray cod cellulose digestibility trial (Trial 1).

Dietary Components	Diet Type			
	Reference	10% cellulose	20% cellulose	30% cellulose
Ox liver	32.0	28.8	25.6	22.4
Fishmeal	62.0	55.8	49.6	43.4
Cellulose	0.0	10.0	20.0	30.0
Tuna oil	3.0	2.7	2.4	2.1
Gelatine	2.0	1.8	1.6	1.4
Vitamin/mineral premix	1.0	0.9	0.8	0.7
Cr_2O_3	1.0	1.0	1.0	1.0

Table 3.2 Formulation (percent dry matter) and proximate composition determined by analysis (percent dry matter) of the experimental diets used in the initial cellulose digestibility trial for common carp (Trial 2).

Dietary Components	Diets	
	Reference	20% Cellulose
Casein	60.0	48.0
Wheat gluten	5.0	4.0
Corn flour	10.0	8.0
Tuna oil	9.0	7.2
α-Cellulose	10.0	28.0
Vitamin & mineral premix	5.0	4.0
Cr_2O_3	1.0	0.8
Proximate Composition		
Moisture[1]	36.3	40.2
Crude protein	59.0	47.4
Crude lipid	8.7	6.1
Crude fibre	18.6	34.4
Ash	5.4	4.5
Carbohydrate	7.2	6.7
Gross energy (kJ/g)	23.4	21.8
Cr_2O_3	1.1	0.9

[1] Expressed as a proportion of the diet as fed.

Table 3.3 Formulation (percent dry matter) and proximate composition determined by analysis (percent dry matter) of the experimental diets used in the graded cellulose digestibility trial for common carp (Trial 3).

Dietary Components	Diets				
	Reference	10% Cellulose	20% Cellulose	30% Cellulose	40% Cellulose
Casein	64.0	57.6	51.2	44.8	38.4
Corn flour	20.0	18.0	16.0	14.0	12.0
Tuna oil	10.0	9.0	8.0	7.0	6.0
α-Cellulose	0.0	10.0	20.0	30.0	40.0
Vitamin & mineral premix	5.0	4.5	4.0	3.5	3.0
Cr_2O_3	1.0	1.0	1.0	1.0	1.0
Proximate Composition					
Crude protein	59.3	54.3	48.1	42.0	36.9
Ash	4.4	4.4	3.9	3.6	3.1
Cr_2O_3	0.9	0.9	0.9	0.8	0.9

Trial 4.

The formulation of diets used to determine the effect of antibiotics on cellulose digestibility are detailed in Table 3.4. The reference diet chosen for the experiment was casein based, however due to the high digestibility of the reference diet used in Trial 3 (refer Section 3.3) it was decided to incorporate more indigestible fibre into the reference diet to ensure differential movement of marker and faeces did not occur (Cho, personal communication). Due to the increased level of fibre in the reference diet, incorporation of the test ingredient was limited to 10%, 20% and 30% inclusion levels. Two batches of each formulation were mixed. One acted as the control and the antibiotics chloramphenicol (Parke-Davis Company, Australia) and streptomycin (Cat. No. S-6501, Sigma Chemical Company, St Louis, Minnesota, USA) were added at 300 mg/kg to the other batch prior to pelleting (refer section 3.2.3).

Stickney and Shumway (1974) added streptomycin (200 mg l^{-1}) to the aquarium water in order to eliminate gut micro-flora. Analysis of the gut micro-flora of six control and six animals treated using this methodology showed no reduction in the bacterial populations of the gastrointestinal tract of common carp as a result of this treatment. It was therefore decided to add antibiotics to the experimental diets. Streptomycin and chloramphenicol, being broad-spectrum antibiotics, were added to the experimental diets at a concentration of 300 mg kg^{-1} of diet, a dose several fold in excess of the concentration used in the prevention of fish diseases (Austin, 1985).

All diets were formulated and prepared as described in section 2.7.

3.2.2 Gastrointestinal Micro-Flora Quantification

The effectiveness of antibiotic treatment was determined by quantifying the numbers of intestinal gut micro-flora. Carp were netted and immediately sacrificed using cervical dislocation. All following procedures were performed aseptically. The abdominal cavity was opened and the gastrointestinal tract exposed. The gut was isolated via ligation with sterile surgical thread. The gut was divided into the foregut (anterior one-third) and hindgut (posterior two-thirds) using a double ligation, and was subsequently removed to a sterile dissection board. The intestine was severed between the two ties dividing foregut from hindgut. Gut contents were stripped from each intestinal segment and discarded. Each segment was subsequently flushed with 3 ml of 0.9% saline solution into a sterile container.

Table 3.4 Formulation (percent dry matter) and proximate composition determined by analysis (percent dry matter) of the experimental diets used in graded cellulose with antibiotics digestibility trial for common carp (Trial 4).

Dietary Components	Diets							
	Reference	10% Cellulose	20% Cellulose	30% Cellulose	Reference	10% Cellulose	20% Cellulose	30% Cellulose
Casein	60.0	55.0	50.0	45.0	60.0	55.0	50.0	45.0
Semolina	5.0	4.5	4.0	3.5	5.0	4.5	4.0	3.5
Wheat gluten	5.0	4.5	4.0	3.5	5.0	4.5	4.0	3.5
Corn flour	10.0	9.0	8.0	7.0	10.0	9.0	8.0	7.0
Tuna oil	5.0	4.5	4.0	3.5	5.0	4.5	4.0	3.5
α-Cellulose	10.0	19.0	28.0	37.0	10.0	19.0	28.0	37.0
Vitamin & mineral premix	4.0	3.6	3.2	2.8	4.0	3.6	3.2	2.8
Cr_2O_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Antibiotics	-	-	-	-	+	+	+	+
Proximate Composition								
Crude protein	46.3	40.8	35.4	30.8	48.5	43.1	35.3	32.4
Crude lipid	7.7	6.4	5.5	4.9	7.7	6.4	5.5	4.7
Ash	3.5	3.4	3.3	2.8	4.3	4.1	3.1	2.7
NFE	41.6	48.5	54.8	60.6	38.4	45.3	55.2	59.2
Gross energy (kJ/g)	18.5	17.5	18.1	17.4	18.6	18.0	18.2	17.9
Cr_2O_3	0.9	0.9	1.0	0.9	1.0	1.1	1.0	1.0

Gut micro-flora were plated onto nutrient agar plates at dilutions of 10^0 and 10^2. Duplicate plates were then exposed to aerobic or anaerobic conditions and were stored at 20°C. Plates were inspected daily and colonies were counted after 10 days of incubation. The concentrations of the bacterial populations were expressed as the number of colonies per ml of saline.

3.2.3 Experimental Animals, Feeding and Faecal Collection

Common carp were obtained as described in Chapter 2. Murray cod juveniles were purchased from a commercial supplier (Mr Barry Doyle) or a government fish hatchery (Snob's Creek Fish Hatchery, Victorian Department of Conservation and Environment), both located at Alexandra, Victoria, Australia. Fish were maintained, anaesthetised and weighed as described in Chapter 2. In all trials, faeces were collected by siphoning as described in Section 2.4.

Trial 1:

Due to the cannibalistic nature of Murray cod when stressed, and the limited number of fish available preventing fish to be stocked at sufficiently high levels, fish were held at stocking densities pre-determined by the behaviour of the individual animals in order to prevent predation. The stocking densities and total biomass, which varied in each tank, are detailed in Table 3.5. The experimental diets were fed to duplicate tanks of juvenile Murray cod as detailed in Table 3.5. Fish were fed to satiety twice daily and feeding continued for 10 days. After 10 days animals fed the 30% cellulose inclusion level test diet appeared unsettled and the trial was stopped before cannibalistic behaviour commenced.

Faeces were not collected for the first 5 days following the commencement of feeding in Trial 1 to allow the animals to completely void the previous diet from their digestive tract and to adapt to the experimental diets. Faeces were collected for the 5 subsequent days.

Trial 2:

Common carp of mean weight 79.5g (range 17.9 to 202.6g, standard deviation 41.6g) were stocked at 10 fish per 70 litre aquaria. The reference and test diets were fed to four replicate tanks at an initial feeding rate of 0.5% body weight.wet weight diet.day^{-1} using a single daily feeding regime. As fish adapted to the diets, the

Table 3.5 Summary of stocking densities and diet distribution in the Murray cod cellulose digestibility trial (Trial 1).

Diet Type	Biomass (g)	Number of Fish	Mean Weight (g)	Standard Deviation
reference diet	125.8	19	6.6	2.4
10% cellulose	104.3	17	6.1	2.0
20% cellulose	90.8	16	5.7	2.2
30% cellulose	65.2	16	4.1	1.4
reference diet	118.0	28	4.2	1.5
10% cellulose	127.1	28	4.5	1.6
20% cellulose	71.1	19	3.7	1.0
30% cellulose	104.0	29	3.6	1.3

feeding rate was increased to 2% body weight.wet weight diet.day^{-1} at day 5. Experimental diets were fed for 32 days.

Faeces collection commenced on day 2 of Trial 2 and continued until day 32. On days 6, 7, 13, 20, 24, 26 and 27 faeces were not collected due to a lack of faeces voided during the collection period.

Trial 3:

Each experimental diet was supplied to four replicate tanks of common carp. Fish weighing 45.5g (range. 7.0 to 94.0g, s.d. 18.9g) stocked at 15 fish per 70 litre aquaria. Animals were fed at a rate of 2% body weight.wet weight diet.day^{-1} , over three feedings at approximately 1 hour intervals. This feeding regime was used to ensure all food was consumed. Feeding continued for 18 days.

Animals were fed the experimental diets for 6 days prior to the commencement of faeces collection. Following this faeces were collected in two five day blocks, days 7 to 11 and days 14 to 18.

Trial 4:

Each experimental diet was fed to four replicate tanks. Common carp of mean weight 49.0g (range 13.8 to 115.7g, standard deviation 20.5g) were stocked at a density of 15 animals per 70 litre aquarium. Fish were initially fed 1% body weight.wet weight diet. day^{-1}, which was increased to 2% body weight.wet weight diet.day^{-1} once adaptation to the experimental diets had occurred and prior to the commencement of faecal collection. Feeding was performed over four feedings at approximately hourly intervals and was continued for 23 days.

Faecal collection commenced 7 days after diets were initially offered to the fish. Faeces were collected from day 7 to day 23 with the exclusion of days 9, 11, 17 and 18, on which no faeces were voided during the collection period.

At the completion of the digestibility trial the gut micro-flora was quantified in 8 animals (mean weight 109.2 ± 24.6g) fed diets not supplemented with antibiotics and 8 animals (mean weight 118.1 ± 20.3g) fed antibiotic supplemented diets.

3.2.4 Diet and Faeces Analysis

Proximate analyses of diets and faeces, and the ADC's of diets, ingredients and nutrients were determined as described in Chapter 2.

Trial 1:

Faeces were pooled per dietary treatment over the duration of faecal collection in order to obtain enough faeces for replicated analysis. As only limited faeces were available for proximate analysis, only Cr_2O_3 levels were determined in both experimental diets and pooled faeces.

Trial 2:

The experimental diets used in Trial 2 were analysed for moisture, protein, lipid, fibre, ash, carbohydrate, energy and Cr_2O_3 (Table 3.2).

Faeces collected for each dietary treatment were pooled per day. Subsequently three consecutive days of faeces were pooled for each dietary treatment to obtain adequate faeces for analyses, this pooling was based around days 3, 9, 21 and 29. Faeces were analysed for Cr_2O_3, energy and protein.

Trial 3:

The contents of protein, ash and energy were determined for the experimental diets used in Trial 3 (Table 3.3).

Dried faeces were pooled per treatment replicate over each five day collection period, days 7 to 11 and days 14 to 18. Pooled faeces were subsequently analysed for Cr_2O_3, protein and ash.

Trial 4:

The diets used in Trial 4 were analysed for protein, lipid, ash, NFE, energy and Cr_2O_3 (Table 3.4).

Faeces were pooled per experimental replicate over the duration of the collection period and were also analysed for Cr_2O_3, amino acids, ash, energy, NFE, lipid and protein.

All ADC values were calculated using the ratio of marker in feed and faeces according to the method described in section 2.6.

3.2.5 Statistical Analysis

Data were analysed using regression analysis, t-test of slopes and repeated measures and one-way analysis of variance as indicated (Zar, 1984; Ott, 1988). *Post hoc* analyses were performed using Scheffe's or Tukey's test. Analyses were performed using StatView 512+™ statistical analysis software (Brain Power Inc., California, USA) and SPSS (SPSS Incorporated 1990, Michigan, Illinios, USA).

3.3 Results

3.3.1 Diet Digestibility

Trial 1:

Regression analysis of the apparent digestibility co-efficients (ADC's) of diets for Trial 1 revealed a significant negative correlation between diet digestibility and the level of inclusion of cellulose (ADC = 72.15-1.14.inclusion level; $r^2 = 0.950$, $p < 0.0001$) (Figure 3.1). The slope of this line was significantly lower ($p < 0.001$) than that of a line representing zero digestibility of cellulose (ADC = 72.10-0.72.inclusion level), indicating that the incorporation of cellulose reduced whole diet digestibility (Figure 3.1).

Trial 2:

The ADC's of the reference and test diets for Trial 2 are shown in Figure 3.2. Values for each time point are single replicates due to the pooling of faeces. Digestibility of both the reference and test diets displayed variation with time, values ranging from between 75.1% and 79.9% to between 60.4% and 68.5% for the reference and test diets respectively.

The mean ADC's for the experimental diets measured at the four time points are detailed in Table 3.6. Inclusion of 20% cellulose into the reference diet significantly ($p < 0.05$, one way analysis of variance) decreased whole diet digestibility from 77.7% to 64.4%.

Trial 3:

The ADC's of the experimental diets used in Trial 3 are shown in Figure 3.3. Regression analysis demonstrated a significant negative regression ($p < 0.001$) between diet digestibility and inclusion level of cellulose for both samples pooled from days 7 to 11 and from days 14 to 18 (ADC = 90.25 - 0.52.inclusion level; $r^2=0.929$; $p < 0.001$ and ADC = 91.56 - 0.63.inclusion level; $r^2=0.938$; $p < 0.001$ respectively). The slope of these lines were shown to be significantly greater than that expected for zero digestibility ($p < 0.001$) and significantly lower than that expected for complete digestibility ($p < 0.001$), indicating that the cellulose component of the diet was partially digested by common carp. The slopes of these regressions were also found to be significantly different ($p < 0.05$) to each other indicating an effect of time on diet digestibility.

Figure 3.1

Apparent digestibility coefficients (%) of the experimental diets (◆) for Murray cod (Trial 1) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 2), ADC = 72.15 - 1.14.inclusion level ($r^2 = 0.950$). A line depicting the apparent digestibility coefficients expected if cellulose was indigestible is also shown (□), ADC = 72.10 - 0.72.inclusion level.

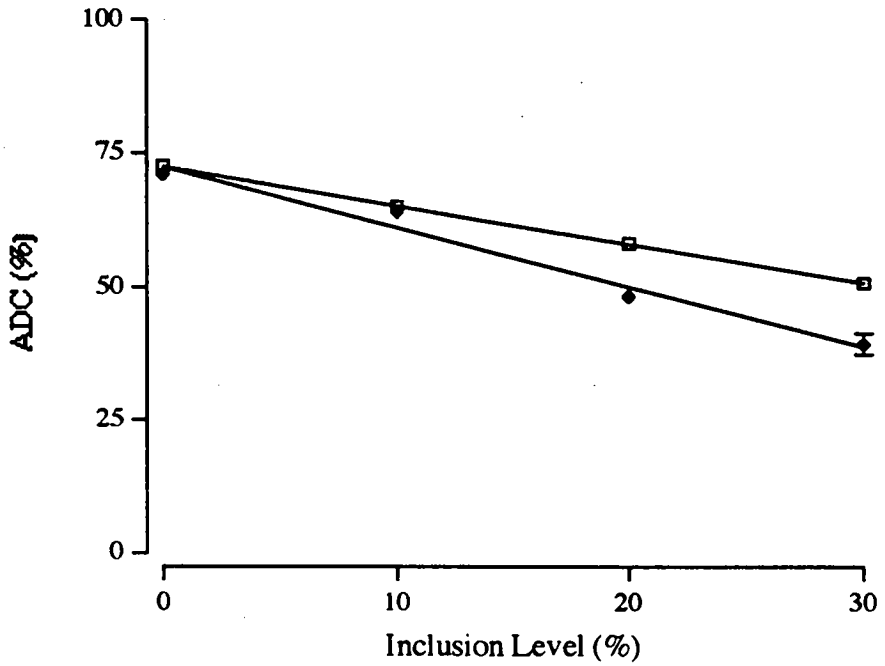

100
75
50
25
0
ADC (%)
0
10
20
30
Inclusion Level (%)

Figure 3.2
Apparent digestibility coefficients (%) of the reference diet (⊡), 20% cellulose inclusion test diet (▩) and cellulose (■) versus time (days) in Trial 2. Each value represents a single data point.

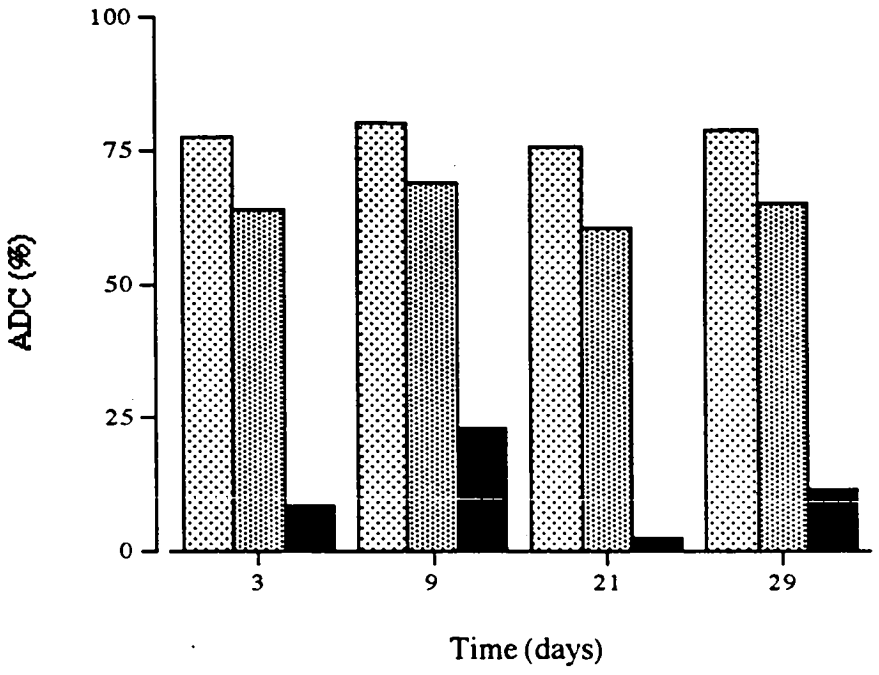
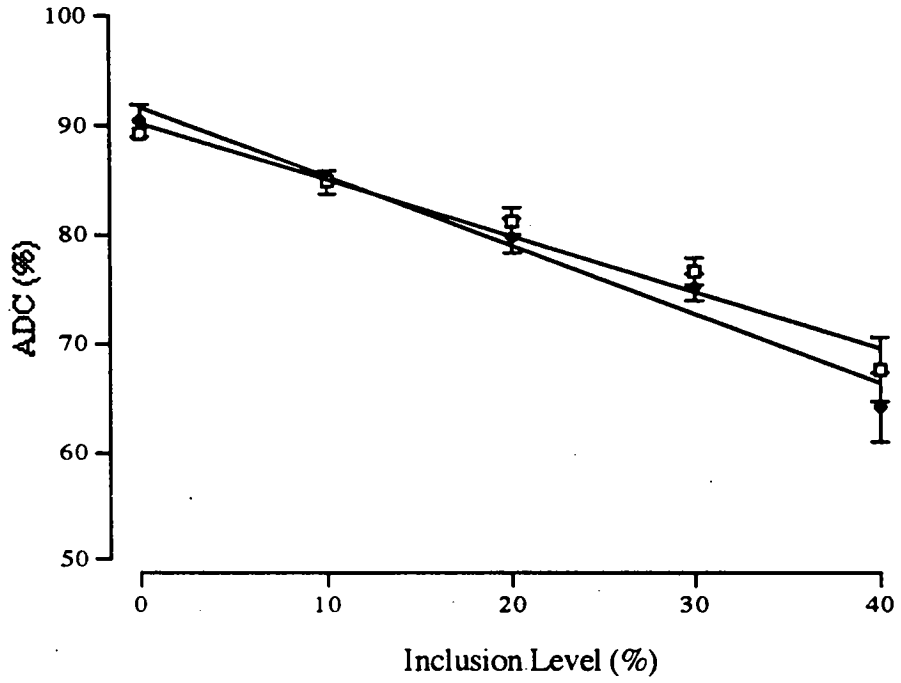

100
75
50
25
0
ADC (%)
3
9
21
29
Time (days)

Table 3.6 Mean values for the apparent digestibility coefficients measured over four time points in Trial 2. Values are mean ± standard deviation (n = 4 for dry matter and crude protein, n = 3 for energy). *** Protein digestibility was not determined for cellulose.

	ADC (%)		
	Dry matter	Crude protein	Crude Energy
Reference diet	77.7 ± 2.0	98.9 ± 0.6	86.2 ± 1.5
Test diet	64.4 ± 3.3	98.5 ± 0.3	77.8 ± 3.7
Cellulose	11.1 ± 8.8	***	39.1 ± 17.8

Figure 3.3
Apparent digestibility coefficients (%) of the experimental diets over days 7 to 11 (□) and days 14 to 18 (◆) for common carp (Trial 3) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 3). Regression analysis provided the models described by the formula ADC = 90.25 - 0.52.inclusion level (r^2 = 0.929) and ADC = 91.56 - 0.63.inclusion level (r^2 = 0.938) for days 7 to 11 and days 14 to 18 respectively.


100
90
80
70
60
50
ADC (%)
0
10
20
30
40
Inclusion Level (%)

Trial 4.

Figures 3.4 shows the ADC's of experimental diets with and without antibiotic supplementation respectively. Whole diet digestibility was significantly reduced by the inclusion of cellulose at all levels both with (ADC = 83.22 - 0.64.inclusion level; $r^2 = 0.984$; $p < 0.001$) and without antibiotic supplementation (ADC = 82.69 - 0.62.inclusion level; $r^2 = 0.962$; $p < 0.001$). The slopes of these regressions were significantly less than that of a line representing zero digestibility ($p < 0.05$) and significantly greater than that of a line representing complete digestibility ($p < 0.05$) indicating that the cellulose component of these diets was partially digested.

The digestibility of the reference and 30% cellulose diets were not significantly affected by the inclusion of antibiotics ($p > 0.05$, one way analysis of variance). The ADC of the 10% cellulose test diet containing antibiotics (74.3 ± 0.5 %) was significantly lower ($p < 0.05$) than that without (77.0 ± 0.5 %). The 20% cellulose test diets (70.9 ± 0.19% supplemented with antibiotics displayed significantly higher ($p < 0.05$, one way analysis of variance) ADC's than the unsupplemented diets (64.2 ± 0.7%).

3.3.2 Ingredient Digestibility

Trial 1:

Ingredient ADC's for Trial 1 are shown in Figure 3.5. Cellulose digestibility was slightly positive at an inclusion level of 10% (13.7 ± 11.7%) and subsequently decreased significantly ($p < 0.05$) to be negative at the 20% (-21.8 ± 6.6%) and 30% (-17.7 ± 12.2%) inclusion levels.

Trial 2:

Cellulose digestibility coefficients for the Trial 2 are shown in Figure 3.2. As faeces were pooled over the experimental replicates and time, each data point represents a single value. Ingredient digestibility again showed variation with time, with ADC's ranging between 2.0% and 22.9% for the four time points measured. The mean ADC's of cellulose at 20% inclusion measured at the four time points was 11.1 ± 8.8% (Table 3.6). This value indicates cellulose may be digestible by common carp.

Figure 3.4
Apparent digestibility coefficients (%) of the experimental diets with (◆) and without (□) antibiotic supplementation for common carp (Trial 4) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 4). Regression analysis provided the models described by the formulae ADC = 83.22 - 0.64.inclusion level (r^2 = 0.984) and ADC = 82.69 - 0.62.inclusion level (r^2 = 0.962) for diets supplemented and not supplemented with antibiotics respectively.

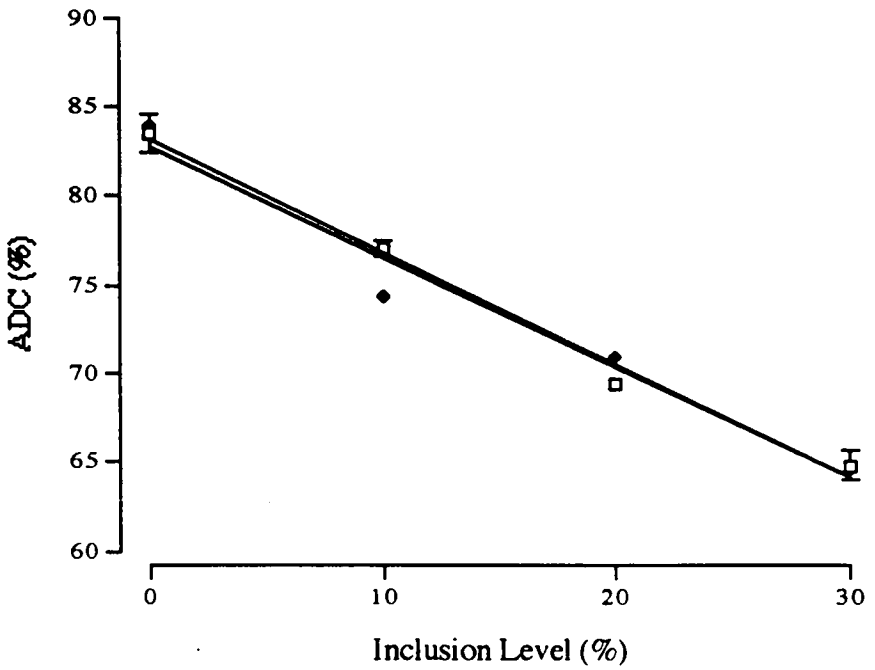
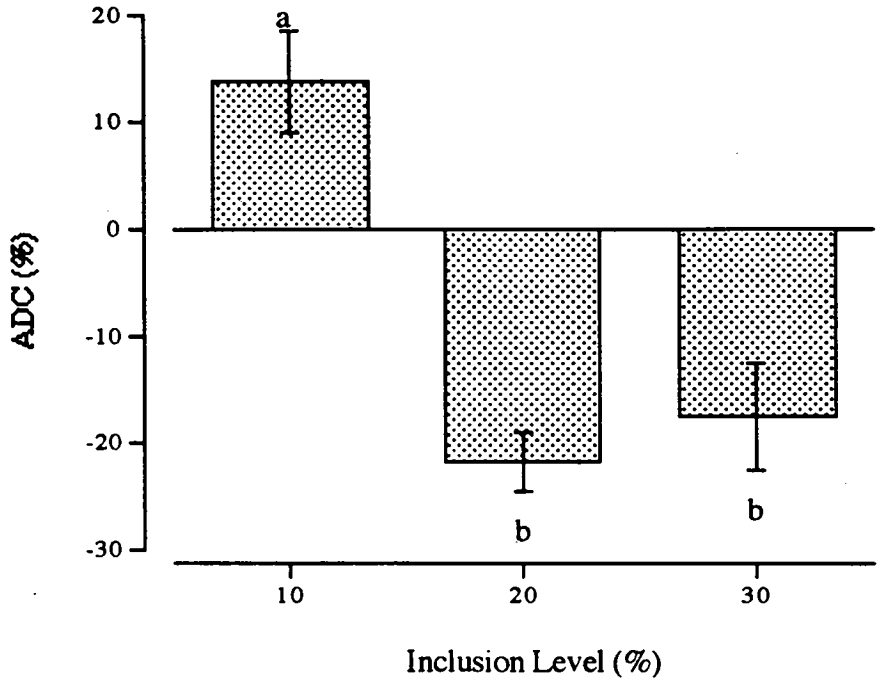

90
85
80
75
70
65
60
ADC (%)
0
10
20
30
Inclusion Level (%)

Figure 3.5

Apparent digestibility coefficients (%) of cellulose for Murray cod (Trial 1) versus inclusion level (%). Values are mean ± standard deviation (n = 2). Values with the same superscript are not significantly different at $p < 0.05$.


20
10
0
-10
-20
-30
ADC (%)
a
b
b
10
20
30
Inclusion Level (%)

Trial 3:

The ADC's of cellulose for days 7 to 11 and days 14 to 18 are shown in Figures 3.6 and 3.7 respectively. Repeated measures analysis showed the digestibility of cellulose by the carp was significantly effected by both inclusion level and time ($p = 0.0031$ and $p = 0.0108$ respectively). For days 7 to 11 cellulose digestibility was 42.7 ± 11.7%, 48.5 ± 6.4% and 46.4 ± 3.9% for 10, 20 and 30% inclusion levels respectively, while it decreased to 34.4 ± 7.4% at the 40% inclusion level (values are mean ± standard deviation). The decrease in digestibility at the 40% inclusion level, however, was not significant since it was also highly variable. For days 14 to 18 cellulose digestibility was found to be lower than that calculated for days 7 to 11, being 39.7 ± 3.9%, 36.5 ± 8.4%, 39.0 ± 8.8% and 23.7 ± 7.9% for the 10, 20, 30 and 40% inclusion levels respectively. The cellulose digestibility at the 40% inclusion level was significantly lower ($p<0.05$) than the values for both the 10 and 30% inclusion levels. The decrease in cellulose digestibility from days 7 to 11 to days 14 to 18 supports the observed difference in the slopes of the regressions for whole diet digestibility (Figure 3.3).

The effect of decreasing cellulose digestibility at higher inclusion levels is more clearly seen if the regression of whole diet digestibility versus cellulose inclusion level excludes the 40% diet (Figure 3.8). Exclusion of this value improves the fit of both regression lines, from $r^2=0.929$ to $r^2=0.958$ (ADC = 89.22 - 0.42.inclusion level, $p < 0.001$) for days 7 to 11 and from $r^2=0.938$ to $r^2 = 0.981$ (ADC = 90.39-0.52.inclusion level, $p < 0.001$) for days 14 to 18 (Figure 3.8). In both cases, ADC values for the 40% diets fall well below the value predicted by the regression models.

Trial 4:

ADC's of cellulose for Trial 4 are shown in Figure 3.9. Ingredient digestibility in diets without antibiotic supplementation ranged between 13.4 and 21.2%. Two way analysis of variance showed a significant effect ($p < 0.001$) of cellulose inclusion level, the addition of antibiotics and an interaction between the two. *Post hoc* analysis by Tukey's test revealed that cellulose digestibility was not significantly different ($p > 0.05$) at the three inclusion levels of cellulose in diets without antibiotic supplementation (18.6 ± 4.8%, 13.3 ± 1.4%, 21.2 ± 2.8%). ADC's for cellulose incorporated at 10% into a reference diet containing antibiotics was found to be negative (-12.8 ± 5.0%), representing a negative effect of cellulose on the whole diet

Figure 3.6
Apparent digestibility coefficients (%) of cellulose over days 7 to 11 for common carp (Trial 3) versus inclusion level (%). Values are mean ± standard deviation (n = 3).

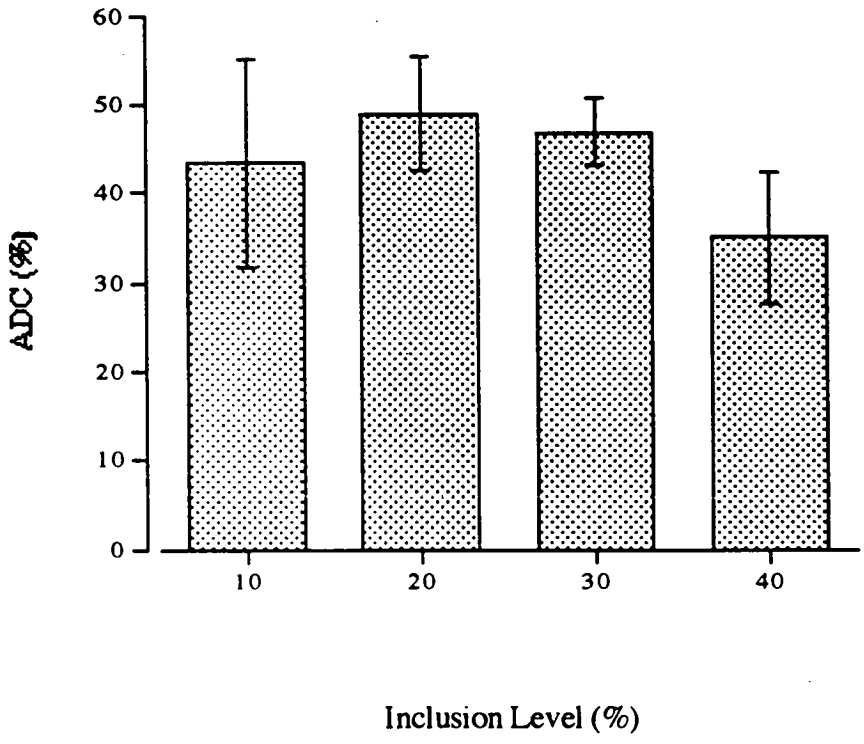

60
50
40
30
20
10
0
ADC (%)
10
20
30
40
Inclusion Level (%)

Figure 3.7

Apparent digestibility coefficients (%) of cellulose over days 14 to 18 for common carp (Trial 3) versus inclusion level (%). Values are mean ± standard deviation (n = 3). Values with the same superscript are not significantly different at $p < 0.05$.

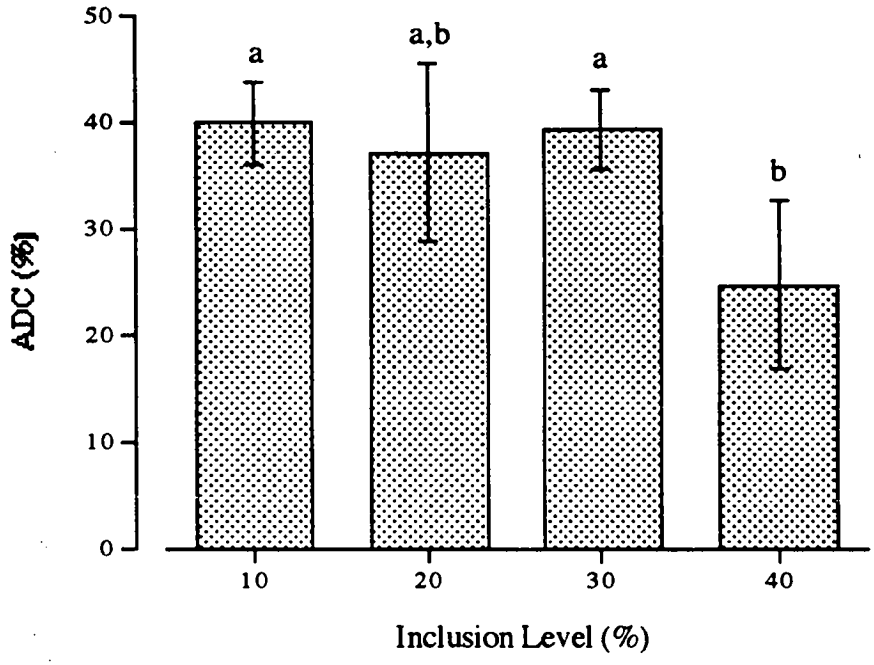

50
40
30
20
10
0
ADC (%)
a
a,b
a
b
10
20
30
40
Inclusion Level (%)

Figure 3.8
Apparent digestibility coefficients (%) of the experimental diets over days 7 to 11 (□) and days 14 to 18 (◆) for common carp (Trial 3) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 3). Regression models were derived excluding the 40% inclusion value and are represented by the formulae ADC = 89.22 - 0.49.inclusion level ($r^2 = 0.958$) and ADC = 90.39 - 0.52.inclusion level ($r^2 = 0.981$) for days 7 to 11 and days 14 to 18 respectively.

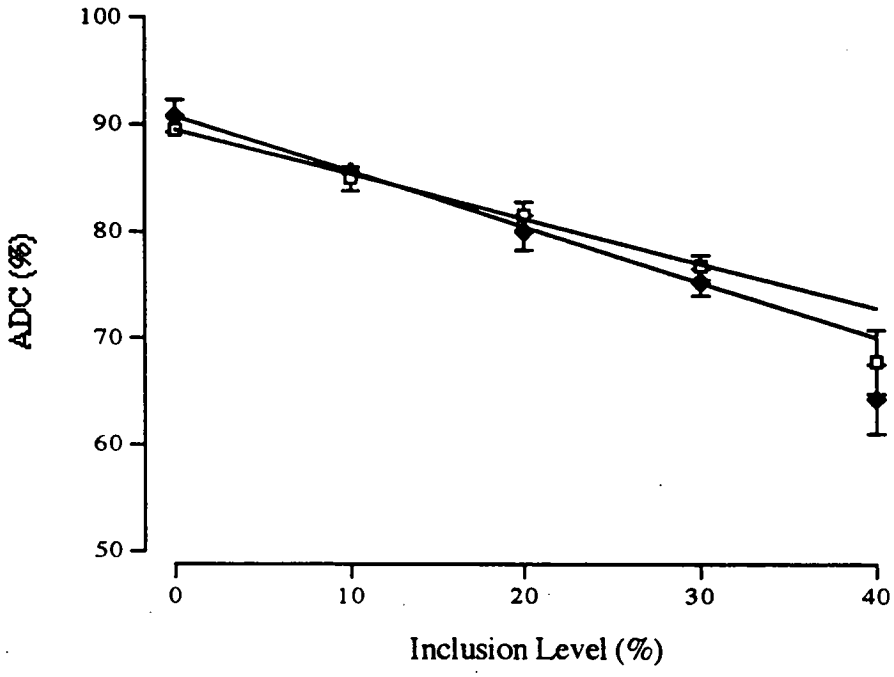
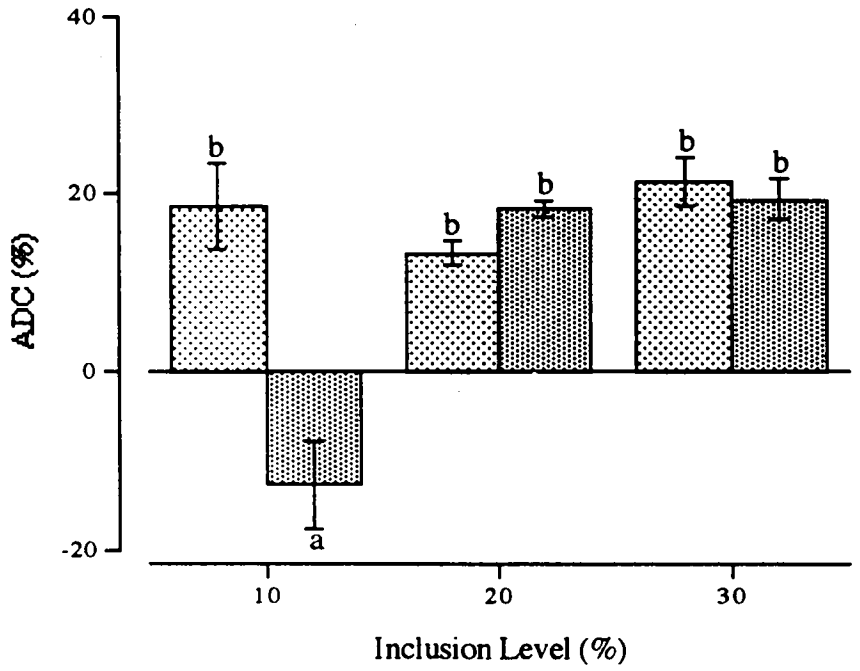

100
90
80
70
60
50
ADC (%)
0
10
20
30
40
Inclusion Level (%)

Figure 3.9

Apparent digestibility coefficients (%) of cellulose in experimental diets unsupplemented (⊡) and supplemented (▩) with antibiotics for common carp (Trial 4) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 4). Values with the same superscript are not significantly different.


40
20
0
-20
ADC (%)
b
a
b
b
b
b
10
20
30
Inclusion Level (%)

digestibility. Tukey's test indicated that ADC of cellulose in this diet was significantly lower than the ADC's of cellulose included at 20% (18.4 ± 0.9%, $p < 0.001$) and 30% (19.2 ± 2.2%, $p < 0.001$) in an antibiotic supplemented reference diet. Digestibility of cellulose incorporated at 20% and 30% into diets supplemented with antibiotics was not significantly different ($p > 0.05$). ADC's of cellulose at a 10% inclusion level were significantly different ($p < 0.001$) in diets with and without antibiotic supplementation. ADC's of cellulose at 20% and 30% inclusion levels in antibiotic supplemented diets were not significantly different ($p > 0.05$) to ADC's for cellulose incorporated into the corresponding antibiotic-free diets at 20% and 30% inclusion levels.

3.3.3 Protein Digestibility

Trial 2:

ADC's of dietary protein for the reference and test diets used in Trial 2 are shown in Figure 3.10. As faeces were pooled over the experimental replicates and time, each data point represents a single value. Protein digestibility was high, greater than 98.2% for all diets and tended to be uniform throughout the trial.

The mean ADC's of protein from the experimental diets measured at the four time points are detailed in Table 3.6. One way analysis of variance showed there was no significant effect ($p < 0.05$) of including 20% cellulose into the diet on dietary protein digestibility.

Trial 3:

Analysis of dietary protein digestibility in Trial 3 showed a high protein digestibility (greater than 97%) for all diets. ADC's of protein from the experimental diets are shown in Figures 3.11 and 3.12. Using repeated measures analysis, inclusion level and time were again found to have a significant effect on digestibility ($p = 0.0001$ and $p = 0.0052$ respectively). ADC's of protein increased significantly with inclusion of cellulose above 10% for days 7 to 11 ($p < 0.05$, Scheffé's test) (Figure 3.11) and above 20% for days 14 to 18 ($p < 0.05$, Scheffé's test) (Figure 3.12) compared to the reference diet. Protein digestibility decreased ($p < 0.05$, Scheffé's test) from days 7 to 11 to days 14 to 18, following a similar pattern to the whole diet and cellulose digestibility coefficients.

Figure 3.10
Apparent digestibility coefficients (%) of crude protein from the reference diet (⊡)and 20% inclusion test diet (▩) versus time (days) in Trial 2. Each value represents a single data point.

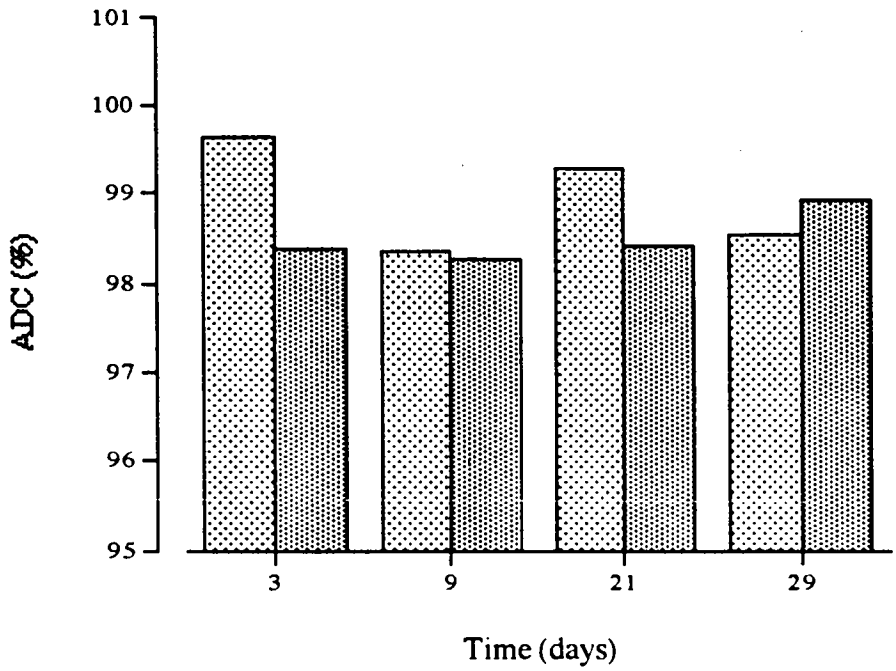

101
100
99
98
97
96
95
ADC (%)
3
9
21
29
Time (days)

Figure 3.11
Apparent digestibility coefficients (%) of crude protein from the experimental diets over days 7 to 11 for common carp (Trial 3) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 3). Values with the same superscript are not significantly different.

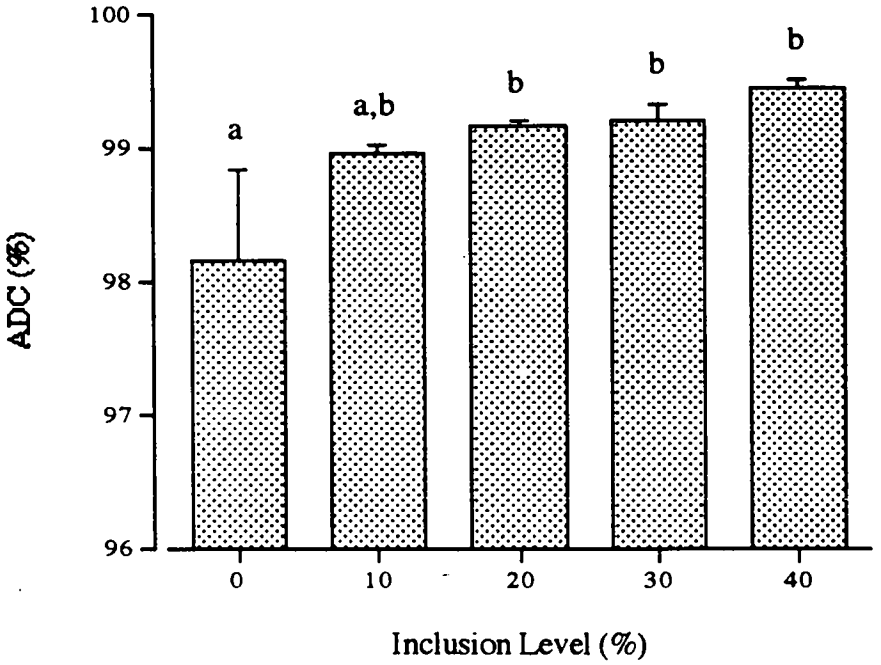
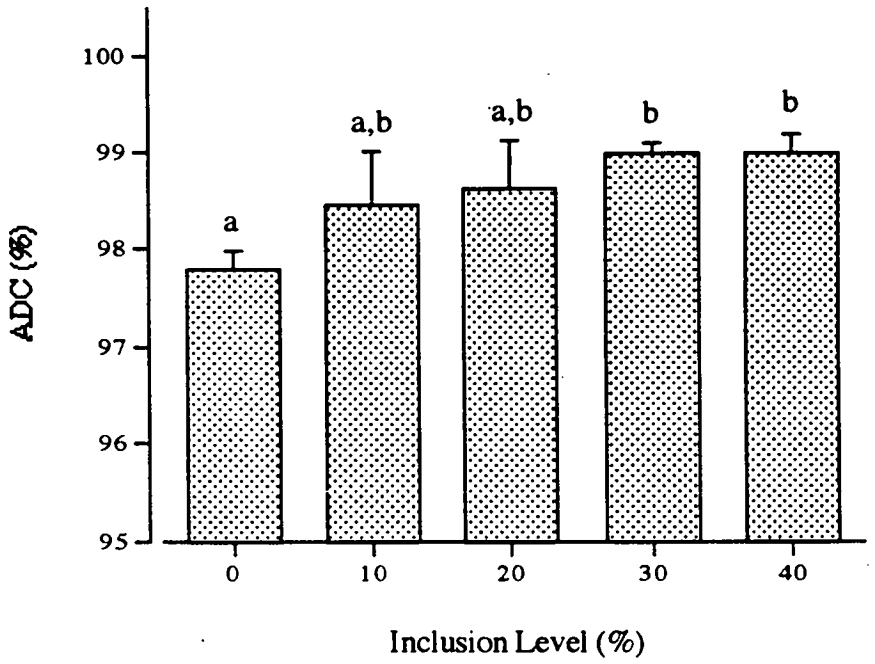

100
99
98
97
96
ADC (%)
a
a,b
b
b
b
0
10
20
30
40
Inclusion Level (%)

Figure 3.12

Apparent digestibility coefficients (%) of crude protein from the experimental diets over days 14 to 18 for common carp (Trial 3) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 3). Values with the same superscript are not significantly different.


100
99
98
97
96
95
ADC (%)
a
a,b
a,b
b
b
0
10
20
30
40
Inclusion Level (%)

Trial 4:

ADC's of dietary protein for the reference and test diets used in Trial 4 are shown in Figure 3.13. Protein digestibility was high, greater than 98.9%, for all diets. Two way analysis of variance found that there was no significant effect ($p > 0.05$) of cellulose incorporation on protein digestibility of the experimental diets. The inclusion of antibiotics into the experimental diets did not significantly affect protein digestibility ($p > 0.05$, two way analysis of variance).

3.3.4 Lipid Digestibility

Trial 4:

The ADC's of lipid for the experimental diets used in Trial 4 are shown in Figure 3.14. Lipid digestibility for both unsupplemented diets and diets supplemented with antibiotics showed a significant negative regression with the inclusion level of cellulose, described by the formulae ADC = 84.83 - 0.80.inclusion level ($r^2 = 0.735$; $p < 0.001$) and ADC = 87.66 - 0.83.inclusion level ($r^2 = 0.916$; $p < 0.001$) respectively. In diets not supplemented with antibiotics lipid digestibility decreased from 84.0 ± 4.2% in the reference diet to 60.6 ± 8.5% in the 30% inclusion level diet, while in antibiotic supplemented diets lipid digestibility was 86.9 ± 1.5% to 62.0 ± 5.5% in the reference and 30% inclusion diets respectively. Inclusion of antibiotics into the experimental diets did not significantly effect lipid digestibility ($p > 0.05$, one way analysis of variance).

3.3.5 Ash Digestibility

Trial 3:

ADC's of ash in the experimental diets used in Trial 3 are shown in Figure 3.15a and 3.15b. Ash digestibility varied between 22.5 ± 1.0% (reference diet) and 38.6 ± 6.5% (20% inclusion diet) for days 7 to 11 and between 25.9 ± 12.2% (reference diet) and 46.6 ± 2.9% (20% inclusion diet) for days 14 to 18. Analysis of variance showed a significant effect of cellulose inclusion on ash digestibility for both days 7 to 11 ($p = 0.047$) and days 14 to 18 ($p = 0.0065$). For days 7 to 11, ash digestibility increased at lower inclusion levels of cellulose and was significantly greater ($p < 0.05$, Scheffé's test) in the 20% inclusion diet than in the reference diet (Figure 13.15a). Ash digestibility subsequently decreased to 27.1 ± 5.6% at 40% inclusion. For days 14 to 18, cellulose digestibility was significantly greater ($p < 0.05$, Scheffé's test) compared to the reference diet at 10%, 20% and 30% inclusion of

Figure 3.13

Apparent digestibility coefficients (%) of crude protein from the experimental diets supplemented (⊡) and unsupplemented (▦) with antibiotics for common carp (Trial 4) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 4).

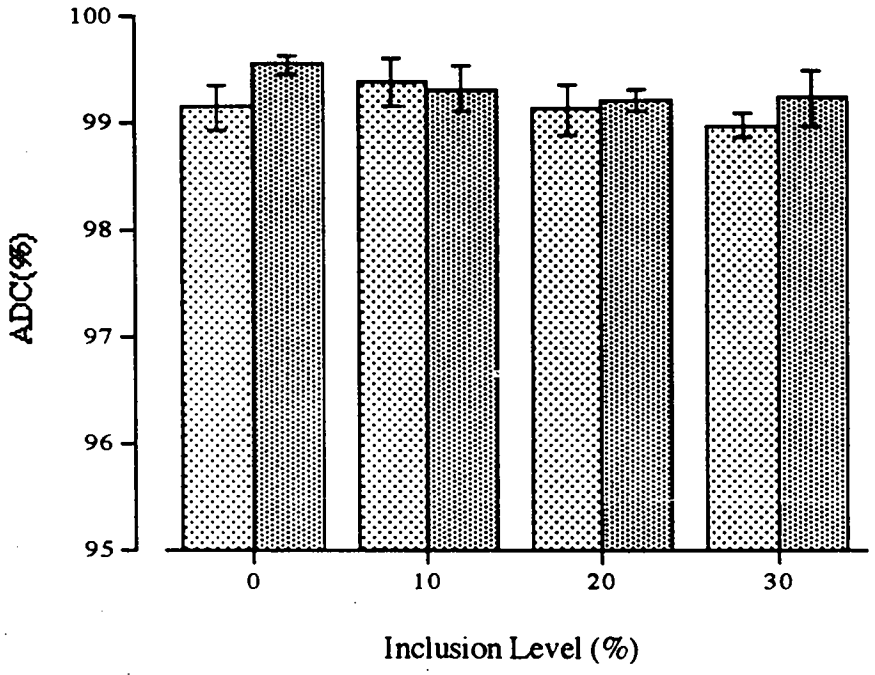

100
99
98
97
96
95
ADC(%)
0
10
20
30
Inclusion Level (%)

Figure 3.14

Apparent digestibility coefficients (%) of crude lipid from the experimental diets supplemented (◆) and unsupplemented (□) with antibiotics for common carp (Trial 4) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 4). Regression analysis provided the models described by the formulae ADC = 87.66 - 0.83.inclusion level (r^2 = 0.916) and ADC = 84.83 - 0.80x (r^2 = 0.735) for diets with and without antibiotic supplementation respectively.

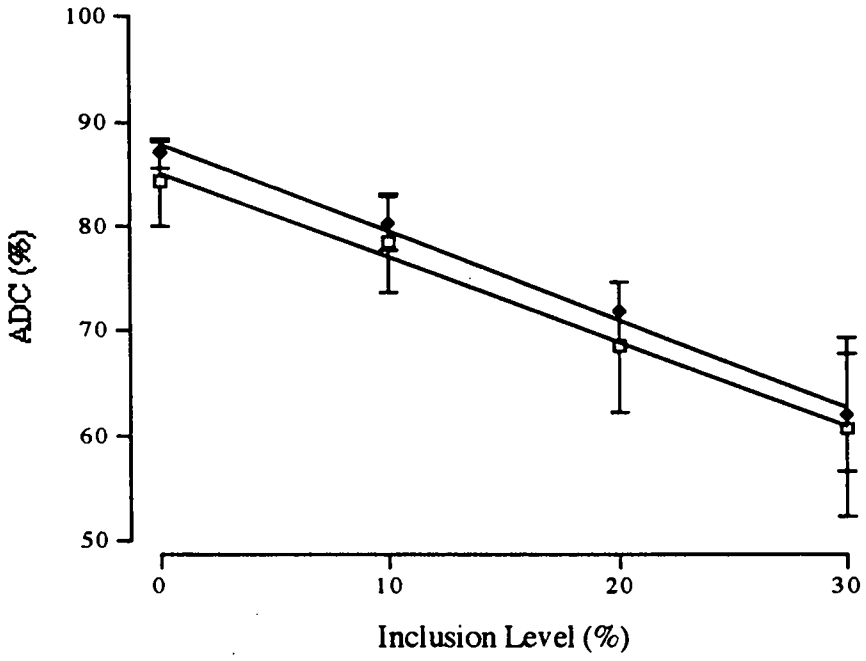

100
90
80
70
60
50
ADC (%)
0
10
20
30
Inclusion Level (%)

Figure 3.15a

Apparent digestibility coefficients (%) of ash from the experimental diets over days 7 to 11 for common carp (Trial 3) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 3). Values with the same superscript are not significantly different.

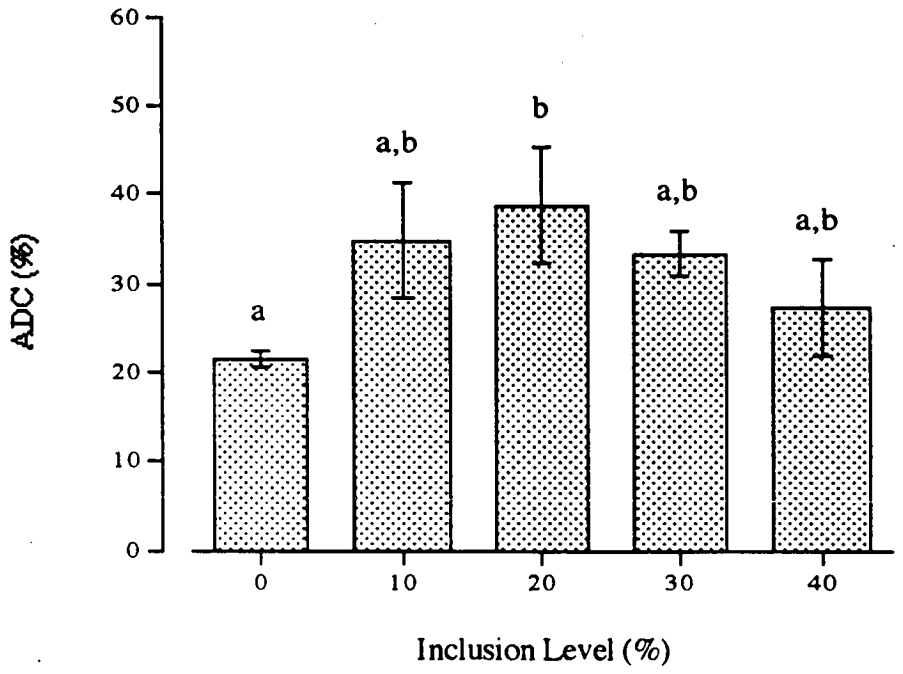
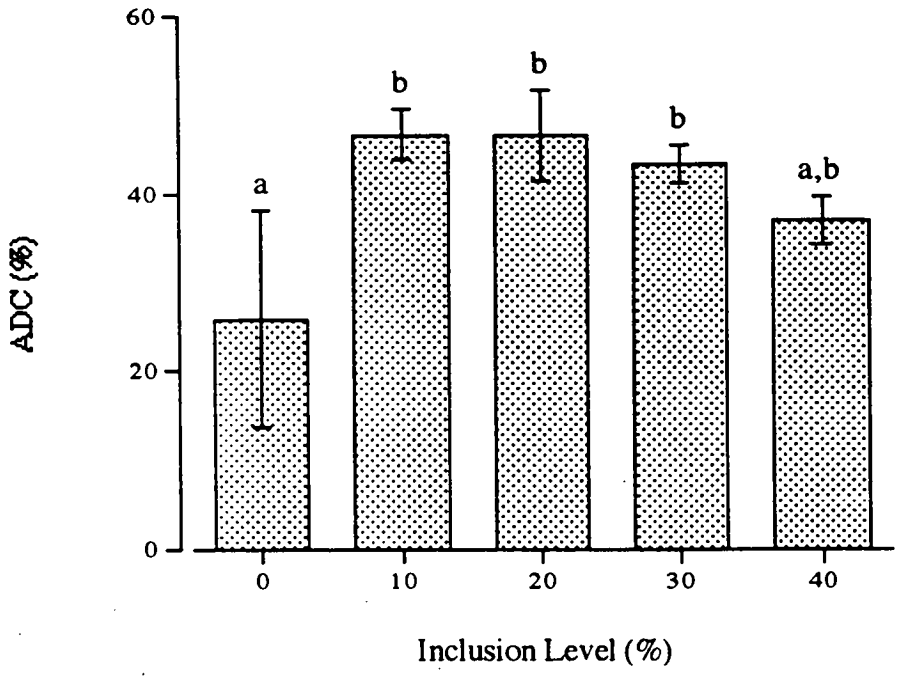

60
50
40
30
20
10
0
ADC (%)
a
a,b
b
a,b
a,b
0
10
20
30
40
Inclusion Level (%)

Figure 3.15b

Apparent digestibility coefficients (%) of ash from the experimental diets over days 14 to 18 for common carp (Trial 3) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 3). Values with the same superscript are not significantly different.


60
40
20
0
ADC (%)
a
b
b
b
a,b
0
10
20
30
40
Inclusion Level (%)

cellulose (Figure 13.15b). The effect of time on ash digestibility was not determined due to a lack of sample resulting in an inability to assay a number of repeated sampling points.

Trial 4:

Ash digestibility values for the experimental diets used in Trial 4 are shown in Figure 3.16. ADC's for ash varied between 21.5 ± 4.9% (10% diet) and 30.1 ± 1.5% (20% diet) for diets not supplemented with antibiotics and between 20.0 ± 8.1% (20% diet) and 34.4 ± 13.0% (10% diet) for diets with antibiotic supplementation. Two way analysis of variance showed no significant effect of cellulose inclusion level ($p = 0.8248$) or antibiotics ($p = 0.5569$) on ash digestibility.

3.3.6 Energy Digestibility

Trial 2:

ADC's for energy of the experimental diets and test ingredient in Trial 2 are shown in Figure 3.17. As faeces were pooled over the experimental replicates and time, each data point represents a single value. Digestibility of dietary energy showed some variation with time, with values ranging from 84.6% (day 9) to 87.3% (day 29) for the reference diet and 74.3% (day 9) to 81.78% (day 29) for the test diet. Crude energy digestibility appeared to decrease as a result of the incorporation of cellulose into the experimental diet and increase with time during the trial.

The mean ADC's for crude energy from the experimental diets measured at the four time points are detailed in Table 3.6. Crude energy digestibility from the reference diet, 86.2 ± 1.5%, was significantly higher ($p < 0.05$, one way analysis of variance) than that for the 20% inclusion diet, 77.8 ± 3.7%.

Digestibility of energy from cellulose ranged from 24.9% (day 21) to 59.1% (day 29). The mean ADC's for crude energy from cellulose at 20% inclusion measured at the four time points was 39.1 ± 17.8% (Table 3.6).

Trial 4:

ADC's for the crude energy of the experimental diets in Trial 4 are shown in Figure 3.18. Crude energy digestibility for diets both supplemented and unsupplemented with antibiotics showed a significant negative regression with the inclusion level of cellulose, described by the formulae ADC = 87.91 - 0.58.inclusion level ($r^2 = 0.965$, $p < 0.001$) and ADC = 87.61 - 0.56.inclusion level ($r^2 = 0.990$, $p < 0.001$)

Figure 3.16

Apparent digestibility coefficients (%) of ash from the experimental diets with (▩) and without (□) antibiotic supplementation for common carp (Trial 4) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 4).

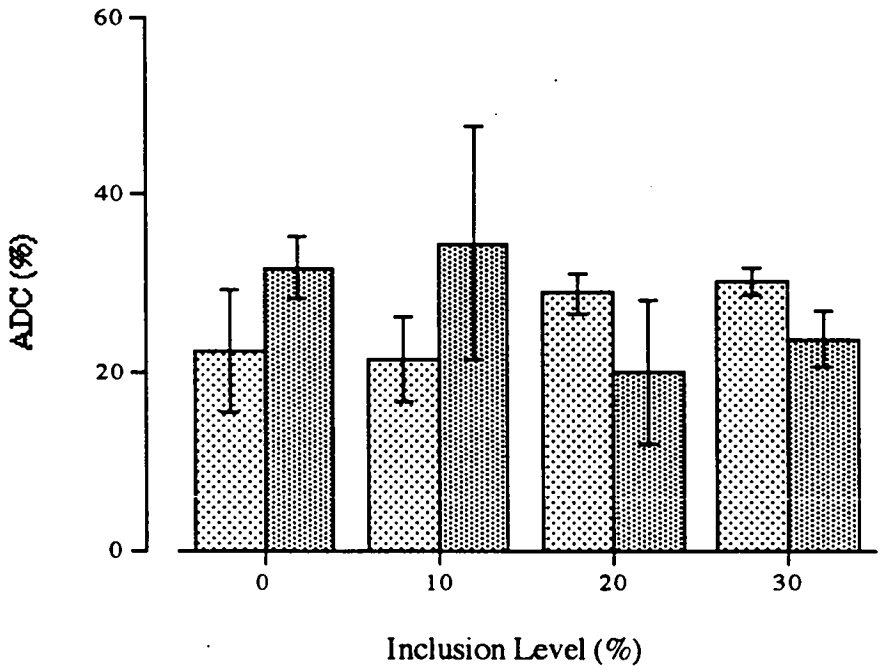

60
40
20
0
ADC (%)
0
10
20
30
Inclusion Level (%)

Figure 3.17
Apparent digestibility coefficients (%) of crude energy from the reference diet (⊡), 20% inclusion diet (▩) and cellulose (■) for common carp (Trial 2) versus time (days).
Each value represents a single replicate.

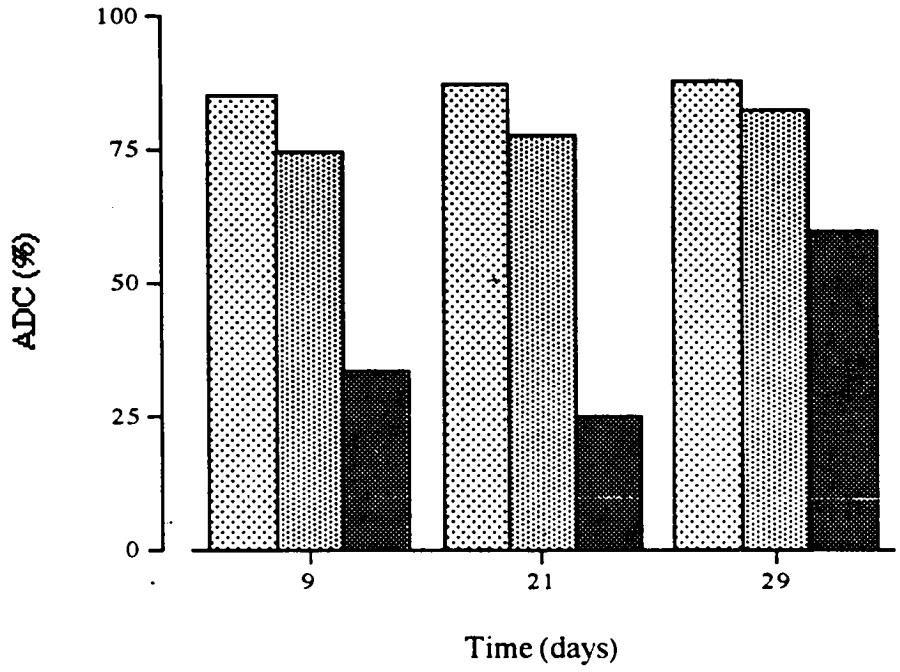
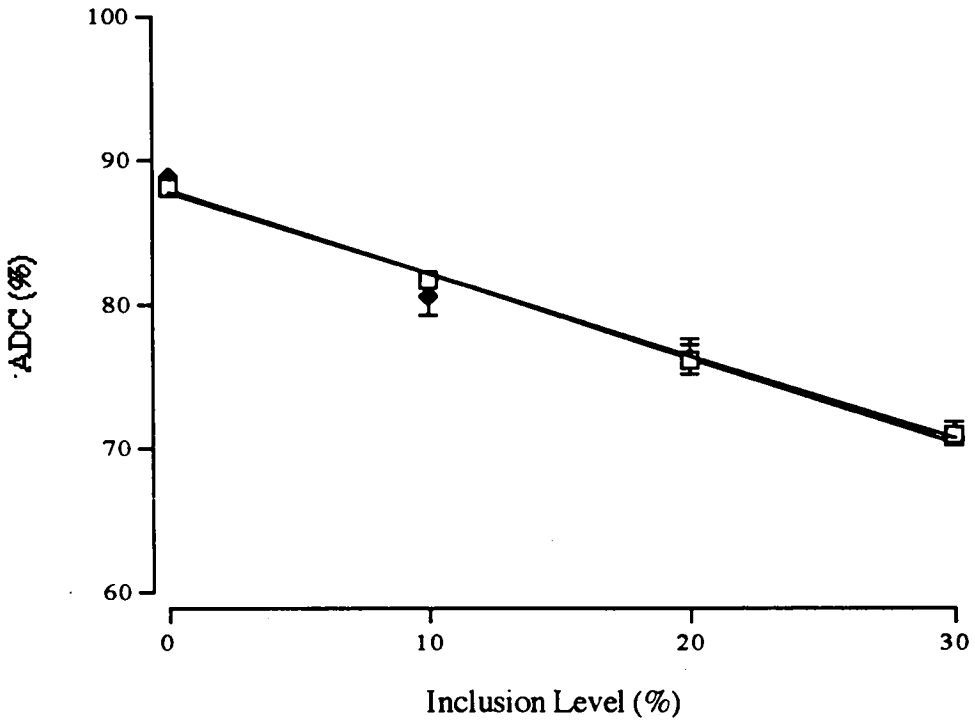

100
75
50
25
0
ADC (%)
9
21
29
Time (days)

Figure 3.18

Apparent digestibility coefficients (%) of crude energy from the experimental diets with (◆) and without (□) antibiotic supplementation for common carp (Trial 4) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 4). Regression analysis provided models described by the formulae ADC = 87.91 - 0.58.inclusion level ($r^2 = 0.965$, $p < 0.001$) and ADC = 87.61 - 0.56.inclusion level ($r^2 = 0.990$, $p < 0.001$) for diets with and without antibiotic supplementation respectively.


100
90
80
70
60
ADC (%)
0
10
20
30
Inclusion Level (%)

respectively. In diets supplemented with antibiotics crude energy digestibility decreased from 88.9 ± 0.2% in the reference diet to 70.8 ± 0.4% in the 30% inclusion level diet, while in the unsupplemented diets crude energy digestibility decreased from 88.1 ± 0.6% to 71.0 ± 1.0% in the reference and 30% inclusion level diets respectively. The inclusion of antibiotics into the experimental diets did not significantly ($p > 0.05$, one way analysis of variance) affect energy digestibility.

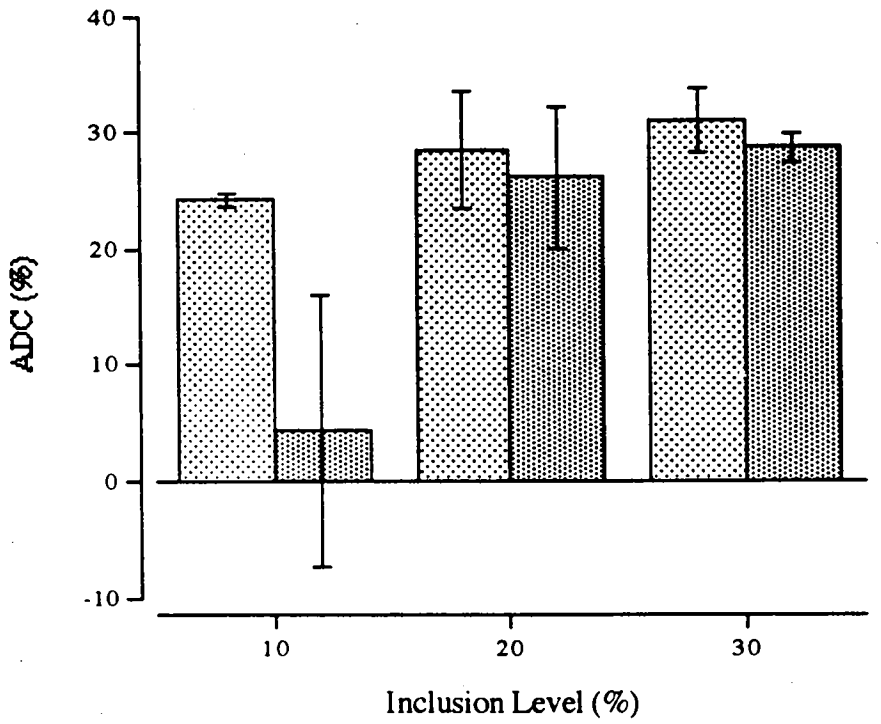
The digestibility of crude energy from cellulose for Trial 4 is shown in Figure 3.19. Two way analysis of variance showed a significant effect of cellulose inclusion level ($p < 0.001$), the addition of antibiotics ($p < 0.01$) and an interaction between the two ($p < 0.05$). *Post hoc* analysis by Scheffé's test revealed that the ADC's for crude energy from cellulose were not significantly different ($p > 0.05$) in diets without antibiotic supplementation (24.2 ± 0.6%, 28.3 ± 5.0%, 30.9 ± 2.8%). Digestibility of crude energy from cellulose incorporated at 10% into a reference diet containing antibiotics (4.3 ± 11.7%) was found to be significantly lower ($p < 0.05$) than the ADC's of crude energy from cellulose included at 20% (26.1 ± 6.1%) and 30% (28.6 ± 1.3%) in an antibiotic supplemented reference diet. Digestibility of crude energy from cellulose included at 20% and 30% into diets supplemented with antibiotics was not significantly different ($p > 0.05$). ADC's of crude energy from cellulose at a 10% inclusion level were significantly different ($p < 0.05$) in diets with and without antibiotic supplementation. Digestibility of crude energy from cellulose at 20% and 30% inclusion levels in antibiotic supplemented diets was not significantly different ($p > 0.05$) to ADC's of crude energy from cellulose incorporated at 20% and 30% into diets not supplemented with antibiotics.

3.3.7 Effects of Antibiotic Treatment on Gut Micro-Flora

Analysis of the gut micro-flora from fish used in Trial 2 showed bacteria numbers in the hindgut were greater than foregut populations and that there was no significant decrease ($p > 0.05$, Student's t test) in the number of bacteria following the consumption of diets containing antibiotics. Numbers of bacteria in the foregut were 18.8 ± 38.7 ml of saline^{-1} in untreated and 11.25 ± 27.8 ml of saline^{-1} in antibiotic treated animals. Hindgut populations were 1345.1 ± 1245.6 ml of saline^{-1} and 1608 ± 1504.7 ml of saline^{-1} in the untreated and treated animals respectively.

Figure 3.19

Apparent digestibility coefficients (%) of crude energy from cellulose incorporated into experimental diets unsupplemented (□) and supplemented (▦) with antibiotics for common carp (Trial 4) versus inclusion of cellulose (%). Values are mean ± standard deviation (n = 4). Values with the same superscript are not significantly different.


40
30
20
10
0
-10
ADC (%)
10
20
30
Inclusion Level (%)

3.4 Discussion

In order to be an inert filler a dietary ingredient must be indigestible and must not affect the digestibility of other dietary nutrient or ingredients. As cellulose is commonly used as an inert filler in both experimental and commercial diets used for fish it is necessary to determine the digestibility of cellulose and its effect on the digestibility of other dietary ingredients and nutrients. These experiments have determined the effect of including cellulose at varying levels on digestibility for the carnivorous Murray cod and the omnivorous common carp.

It is apparent that including cellulose in the diet of the carnivorous Murray cod results in a reduction in whole diet digestibility to a greater extent than could be explained by the substitution of an inert filler. When substituting an inert filler into a reference diet with a digestibility of 72%, the filler would be expected to reduce diet digestibility by 7.2% for each 10% of inclusion. In the present study, cellulose appeared to be decreasing diet digestibility by about 11% on average for each 10% substituted into the reference diet. Such a negative effect of cellulose is not surprising when it is considered that the Murray cod is a carnivorous species at all stages of its life cycle. Exposure to dietary cellulose in the natural environment would be negligible.

Negative effects of cellulose inclusion into experimental diets on growth rates of fish have also been previously reported (Leary and Lovell, 1975; Hilton *et al.*, 1983; Anderson *et al.*, 1984; Bromley and Adkins, 1984; Qadri and Jameel, 1989). In those studies, the reduced growth rate was attributed to decreased nutrient intake (Bromley and Adkins, 1984), physical blocking of digestive enzymes thereby limiting enzyme access to the components of the diet (Leary and Lovell, 1975) and a decreased gut retention time due to the elevated levels of fibre present in the cellulose supplemented diets (Hilton *et al.*, 1983). In the present study, the digestibility of diets into which cellulose has been included indicates that cellulose had a negative effect on the digestive physiology of the fish. In this case reduction in food intake is not a viable explanation. Such an effect may have been mediated by a decrease in gut retention time decreasing the time available for digestion or by blocking digestive enzymes. A negative digestibility of cellulose has previously been reported for white sturgeon (Herold *et al.*, 1995). In that study cellulose included at 27% was reported to be -10% digestible, a value similar to that found in the present study. However, Herold *et al.* (1995) suggested the negative digestibility was due to experimental error or the excretion of endogenous products.

While whole diet digestibility showed a negative effect of cellulose, ingredient digestibility coefficients suggested the inhibitory effect of cellulose may be a function of inclusion level. Cellulose ADC's were positive at the 10% inclusion level suggesting that this species can compensate for the increased dietary cellulose level (up to 10%) and low levels of cellulose in the diets of Murray cod may be acceptable if not beneficial.

Limited faecal collection prevented further analysis which would have determined whether the apparent cellulose digestibility at low levels indicated a true cellulose digestibility or reflected an increased absorption of other dietary components. It may be that the apparent digestibility of cellulose at low levels by the Murray cod was an artefact. Although cellulose has been reported to be digestible by omnivorous fish (Shcherbina and Kazlauskene, 1971; Dyundik *et al.*, 1991), studies reporting cellulose digestibility by carnivorous species are lacking. Cellulase activity has been reported in carnivorous fish (Neiderholzer and Hofer, 1979; Lindsay and Harris, 1980) but was attributed to ingestion of cellulase containing invertebrates. As the fish used in this trial had been held within an aquarium room and fed artificial diets for at least 6 months prior to the experiment, it is unlikely that cellulase activity derived from their wild diet would be present in their gut. It has been reported that the presence of cellulose at low levels in an experimental diet increases growth performance by enhancing diet digestibility (Leary and Lovell, 1975; Dioundick and Stom, 1990). Dioundick and Stom (1990) reported that the replacement of starch with cellulose in experimental diets for juvenile tilapia increased growth at low levels of incorporation (2.5-5%) and subsequently decreased growth at higher levels (10%), leading to the conclusion that the lack of cellulose in diets was just as undesirable as its surplus. That study did not measure cellulose digestibility, but the results obtained would be comparable to alterations in digestibility as were observed in the present study. It must be added that increase growth at low cellulose levels has generally applied to purified diets, and in practical type diets cellulose has been found to decrease growth (Leary and Lovell, 1975). In the present study, higher inclusion levels resulted in negative ADC's for cellulose indicating that elevated levels of cellulose had an adverse effect on the digestive physiology of the fish. Therefore, inclusion of cellulose at greater than 10% results in inhibitory or negative effect on the digestive processes of Murray cod

In the initial trial cellulose appeared to be digestible by common carp when included in the reference diet at 20%. The degree of cellulose digestibility was generally low and appeared to vary with time. A detailed study to determine the extent of cellulose digestibility by common carp was therefore undertaken.

The ADC's of diets by common carp in the graded cellulose digestibility trial suggested an ability to digest cellulose at a level that would be of nutritional significance to the species, and that this digestibility varies with time. The slopes of the regression lines through the ADC's of the diets for both days 7 to 11 and days 14 to 18 were greater than that expected for zero and less than that expected for complete digestibility, indicating that the cellulose component of the diet was partially digested. Ingredient ADC's indicated carp digested dietary cellulose at between 20 and 50% depending on the level of inclusion. Ingredient digestibility was generally higher for inclusion levels up to 30%, ranging from approximately 42% to 49% for days 7 to 11 and 36% to 40% for days 14 to 18. However, for both days 7 to 11 and days 14 to 18 the digestibility of cellulose was found to decrease when included at 40%. Due to large variance this decrease was only significant for days 14 to 18. Such a decrease suggests a saturation of the mechanism of cellulose digestion and absorption. When the 40% inclusion level ADC value was removed from the regression analysis of diet digestibility for both days 7 to 11 and days 14 to 18, it was found that cellulose inclusion into test diets resulted in a relatively constant decrease in whole diet digestibility up to an inclusion level of 30%. The slope of the regression analyses excluding the 40% inclusion level were also in better agreement with the values obtained from the ingredient ADC's calculations.

Data from Trial 4 also indicated that cellulose was digestible by common carp, although the level of digestibility in this trial was lower than that reported in Trial 3. The extent of cellulose digestibility in this trial was not effected by the level of inclusion of antibiotics.

The levels of cellulose digestibility reported in the present study are similar to those found by Shcherbina and Kazlauskene (1971). These authors reported that up to 50% of the cellulose was absorbed from experimental diets, although this digestibility was dependent on the source of the cellulose. Cellulose supplied from soya cake and castor cake was found to be significantly digestible while cellulose supplied by wheat was indigestible, perhaps reflecting the effects of processing on the dietary cellulose. In the present study, a purified wood cellulose (BW40 Solka floc) was used. Halliwell (1959) suggested processing of wood cellulose to remove impurities resulted in the denaturation of the cellulose molecules, thereby making them more vulnerable to attack by digestive enzymes. However, Bergot (1981) found that purified wood cellulose incorporated into experimental diets was indigestible by carp, seemingly refuting that argument and contradicting the findings of this study. Studies of cellulase activity also do not support a high cellulose digestibility by carp. Several studies have reported low levels or the absence of

cellulase in carp (Yokoe and Yasumasu, 1964; Prejs and Blaszcyk, 1977; Lindsay and Harris, 1980).

In Trial 3 both diet and ingredient digestibility significantly decreased from days 7 to 11 to days 14 to 18. The ADC's of dietary protein were also found to decrease with time, a trend that existed for all parameters measured and probably reflects an adaptation to the experimental diets by the carp.

Stickney and Shumway (1974) showed that streptomycin treatment removed cellulase activity from the gastrointestinal tract of channel catfish while it remained in starved and untreated fish, thus suggesting that cellulase was produced by resident gut micro-flora. In the present study the inclusion of antibiotics into the experimental diets had no significant effect on the digestibility of cellulose by common carp. However the data from the quantification of gut micro-flora populations showed that the incorporation of streptomycin and chloramphenicol into the experimental diets at 300 mg kg^{-1} was not sufficient to significantly reduce micro-flora populations in the present trial. Therefore definite conclusions regarding the mechanism of cellulose degradation in the gastrointestinal tract of common carp cannot be drawn.

A marked reduction in digestibility of the 10% inclusion test diet containing antibiotics was observed during Trial 4. Such a large decrease in digestibility is aberrant, particularly when compared to the normality of digestibility in animals receiving the other antibiotic containing diets. It is possible that the 10% inclusion test diet supplemented with antibiotics may have inadvertently received higher doses of the antibiotics. Chloramphenicol is known to have anaemic and haemorrhagic effects in humans when used at high concentration for prolonged periods (Nahata, 1991), and Danulant (1986a) noticed toxic effects of chloramphenicol on Atlantic cod. Soivio and Koskela (1992) also reported decreased digestibility in fish treated with antibiotics.

The digestibility of dietary protein by common carp in the present study was high, greater than 97% for all diets used in Trial 2, Trial 3 and Trial 4, reflecting the purified nature of the casein-based reference diet. The high digestibility of protein from a casein-based diet in the present study is in accordance with the digestibility of casein based diets previously reported (Kitamikado *et al.*, 1964; Halver, 1989).

Dietary protein digestibility was found to increase with the inclusion level of cellulose in Trial 3. The increase in protein digestibility was significant at higher inclusion levels, although they were not sufficient to explain the increased diet digestibility, indicating that cellulose itself was being digested. DeSilva *et al.* (1990)

previously noted a trend for decreasing protein digestibility with increasing crude fibre in experimental diets (crude fibre being cellulose and lignin) in blue tilapia. In Trials 2 and 4 of the present study protein digestibility did not appear to change with increasing cellulose, and thus increasing crude fibre. It has been suggested that cellulose incorporation into purified diets slows the passage of the diet through the gastrointestinal tract thereby increasing the time available for digestion (Leary and Lovell, 1975; Dioundick and Stom, 1990). Increased protein digestibility may also have resulted from an increased ratio of protease to dietary protein. This occurs as a result of the cellulose diluting protein in the diet while still supplying the necessary bulk to stimulate protease secretion at similar levels.

The digestibility of dietary crude lipid in the reference diet for Trial 4 was 84.0%. This value approximates the digestibility of fish oils previously reported for carp (Takeuchi *et al.*, 1979; Chu *et al.*, 1991). However, the inclusion of cellulose into the experimental diets appeared to have a significant negative effect on lipid digestibility with crude lipid digestibility decreasing linearly with the inclusion of cellulose into the reference diet. Lipid digestibility decreased by greater than 20% with the inclusion of 30% cellulose. Inclusion of cellulose into experimental diets has been shown not to affect lipid digestibility (Steffens *et al.*, 1989; Herold *et al.*, 1995) , although elevated levels of natural plant fibre adversely affected lipid digestibility (Steffens *et al.*, 1989). Fagbreno (1992) also found that plant fibre, in the form of cocoa husk, decreased lipid digestibility for dwarf African catfish. Crude starch, which is poorly digestible by fish, has also been shown to reduce lipid digestibility (Medalé *et al.*, 1991). To date a hypothesis regarding the reduction in lipid digestibility due to the inclusion of poorly digested ingredients has not been suggested, however such an effect may be due to physical blocking of the digestive processes.

Ash digestibility in Trials 3 and 4 varied between 20% and 40%. These values are in the range of ash digestibility previously reported for fish (Buddington, 1980; Bjorndal, 1985). In Trial 3 ash digestibility was found to increase significantly up to 20% inclusion followed by a subsequent decrease at 40% inclusion of cellulose, indicating an effect of cellulose on ash digestibility. However, a similar effect was not seen during Trial 4, where no consistent effect of cellulose on ash digestibility was observed.

The digestibility of crude energy from the experimental diets and cellulose was only determined in Trials 2 and 4. Crude energy digestibility from the experimental diets was found to decrease with the incorporation of cellulose, showing a significant

negative regression with cellulose inclusion level. Such a decrease in crude energy digestibility from the experimental diets would appear to be due to the low digestibility of cellulose. The digestibility of crude energy from cellulose was generally found to be consistent with the dry matter digestibility of cellulose. As cellulose is a purified complex carbohydrate it would be expected that crude energy digestibility would reflect ingredient dry matter digestibility. Crude energy digestibility from cellulose did not appear to be affected by the level of incorporation of cellulose into the reference diet or the inclusion of antibiotics into the experimental diets. The digestibility of energy from cellulose incorporated at 10% into a reference diet containing antibiotics was significantly reduced, reflecting the very low cellulose digestibility for that diet.

The inclusion of antibiotics into the experimental diets did not appear to effect digestibility for common carp at the concentrations used. There was no significant change in the digestibility of dietary lipid, protein, ash or energy due to the incorporation of antibiotics into the experimental diets. Although, as previously discussed, the digestibility of the 10% inclusion test diet was reduced and may be related to an overdose of antibiotics. Antibiotics have previously been reported to have an adverse effect on digestibility. Soivio and Koskela (1992) found that a 20% decrease in faecal micro-flora of rainbow trout due to antibiotics was associated with a decrease in the ADC's of energy, protein and fat of between 8% and 11%, with carbohydrate digestibility decreasing by 3%. Assuming that the decrease in nutrient digestibility was directly attributable to a decrease in the gut micro-flora and that gut micro-flora populations in the present study did not appear to be effected by the addition of antibiotics, it is not surprising to find no effect of antibiotics on nutrient digestibility.

It is clear from this study that cellulose is not an inert filler. Not only does it appear to be digestible by the omnivorous common carp, but it also appears to affect the digestion of other dietary nutrients in both the carnivorous Murray cod and the common carp.

Chapter 4

Factors Affecting Soybean Meal Digestibility

4.1 Introduction

On the basis of its high biological value, fishmeal has traditionally been the preferred source of protein in both aquatic and terrestrial animal feeds. However, the increased use of artificial diets has placed greater demands on fishmeal production, resulting in a rise in cost of high quality fishmeal. Increased demand has also lead to uncertain availability of high quality fishmeal and doubt over the ability of fishmeal production to meet future increases in demand (De Silva and Anderson, 1995). Thus the partial or complete replacement of fishmeal with low cost, readily available protein sources of consistent quality has the potential to decrease feed costs and enhance aquaculture profitability. Fishmeal replacement may also allow farmers to formulate their own diets using ingredients produced "on-farm". In developing countries, this is particular advantageous as the cost and availability of commercial pellets are constraints on supplemental feeding (Wee and Shu, 1989). For these reasons, the use of alternative animal and vegetable protein sources in aquaculture diets has been extensively investigated. However, the variety of methods and sources used in these studies have resulted in a range of often conflicting results.

4.1.1. Soybean Meal as an Alternative Protein Source

The use of soybean meal (SBM) as an alternative protein source in aquaculture feeds has been well researched. Cost, market availability and nutritional value indicate that soybeans (*Glycine max*) are amongst the best plant protein sources for supplementing aquaculture feeds. Soybeans are less expensive than animal protein sources, in particular fishmeal, and are readily available throughout the world. Soybeans have a high protein content with an amino acid profile favourable for meeting the essential amino acid (EAA) requirements of aquatic species and contain a high level of oil providing a source of essential fatty acids and digestible energy. However, soybeans contain a range of anti-nutritional factors and other toxic substances which adversely effect the growth and health of animals.

4.1.2. Nutritional Composition of Soybean Meal.

As with other crops, the chemical composition of soybeans varies throughout the world depending on the variety of plant and the conditions under which the crop is grown. Variation in the composition of the commercial product, SBM, may also result from the processing methods employed. Proximate analyses of SBMs used in aquaculture studies are detailed in Table 4.1. Major differences exists in the chemical composition of meals depending on whether they are full fat SBM (FFSBM) or if the majority of lipid is removed via solvent extraction (DFSBM) (Table 4.1). The use of

various portions of the seed (dehulled or grits) also causes variation in proximate analysis. Although the type and extent of heat treatment employed had little effect on the crude composition of the meal, the processing of soybeans does alter the composition of the final product. Data in Table 4.1 demonstrates that the proximal composition of SBM processed in a similar manner is still quite varied.

4.1.2.1. Protein and Amino Acid Content of Soybean Meal.

Protein is the major nutrient of SBM, with values ranging from 39.77% wet weight in FFSBM to 49.72% wet weight in DFSBM (Table 4.1). Higher protein contents are found in solvent extracted soybean grits and dehulled meals, being 52.4% and 49.7% wet weight respectively. A high protein content is important for protein sources used in aquaculture diets as fish have a high dietary requirement for protein (Table 4.2).

Although the protein content of soybeans is high, crude protein content alone does not ensure an adequate protein source. The amino acid profile of the protein must be able to meet the EAA requirements of the animal. The EAA profile of SBM (Table 4.3) is amongst the best reported for plant protein sources for meeting the EAA requirements of fish (Table 4.4). Comparison of Tables 4.3 and 4.4 shows that SBM contains adequate amounts of the majority of EAA for teleosts. As is the case with most plant protein sources, SBM is deficient in the sulphur amino acids (methionine and cysteine), with threonine and tryptophan the next potentially limiting amino acids. Improved growth performance following supplementation of SBM with synthetic amino acids has supported the limiting effect of these amino acids (Rumsey and Ketola, 1975; Dabrowska and Wojno, 1977; Abel *et al.*, 1984; Shiau *et al.*, 1987). Although the crude lysine content of soybeans is adequate to meet the requirements of fish (Tables 4.3 and 4.4), available lysine has been shown to be deficient following heat treatment of the meal to destroy anti-nutritional factors (Dabrowski and Kozak, 1979; Viola *et al.*, 1981/2,1983, 1992).

4.1.2.2. Lipid and Fatty Acid Content of Soybean Meal.

Full fat SBM has a high lipid content, up to approximately 20% wet weight (Table 4.1), and therefore provides an excellent source of dietary lipid. Triglycerides form the major fraction of this lipid, approximately 96%, with phospholipids, commonly grouped under the term "soybean lecithin", contributing 1-3% of the lipid fraction

Table 4.1 Proximate composition of SBM's used in fish nutritional studies (FFSBM - Full fat SBM, [a] - % dry weight).

Authors	Processing Method	Dry Matter	Protein	NFE	Lipid	Fibre	Ash
Shiau *et al.* (1987)	Solvent extracted	87.7	41.5	34.9	1.1	4.6	5.6
Reigh & Ellis (1992)	Solvent extracted	86.05	43.92		1.1	6.8	6.8
Haniffa & Selvon (1992)		100.0	46.6		9.6		5.3
de la Peña *et al.* (1987)	FFSBM heated	89.9	46.8		14.1	6.5	6.4
Wilson et al. (1981)	Solvent extracted		49.8[a]				
Anderson *et al.* (1992)	Solvent extracted	88.5	49.7		2.3		6.1
	Solvent extracted	90.4	47.2		2.5		6.3
Hossain *et al.* (1992)	FFSBM	90.0	44.5	15.4	19.8	6.0	4.3
Chu *et al.* (1991)	Solvent extracted	88.9	38.8		1.7	5.8	5.4
Lorico-Querijero & Chui (1989)	Solvent extracted	89.3	40.3	36.2	1.4	5.8	5.8
Abel *et al.* (1984)	FFSBM	93.5	40.8	24.0	13.6	4.6	10.8
	FFSBM gentle thermal heat	93.7	41.5	23.7	13.8	3.9	10.8
	FFSBM intense thermal heat	93.6	41.4	22.8	13.9	4.5	11.0
	FFSBM gentle hydrothermal heat	93.6	41.1	24.9	13.6	3.3	11.0
	FFSBM intense hydrothermal heat	93.6	44.2	24.2	13.8	3.4	11.0
Shiau *et al.* (1990)	FFSBM	88.5	40.2		19.2	2.6	4.4
	Solvent extracted	86.2	42.2		4.8	4.6	6.0
Jackson *et al.* (1982)	Solvent extracted prepressed	88.1	42.6	32.1	1.1	6.0	6.3
Fowler and Banks (1976)	Solvent extracted toasted grits	91.2	52.4		1.5		5.8
Lim and Akiyama (1992)	FFSBM heat treated	90.0	38.0		18.0	5.0	4.6
	Solvent extracted	89.0	44.6		1.4	6.2	6.5
	Solvent extracted dehulled	90.0	49.7		0.9	3.4	5.8

Table 4.2. Estimated protein requirements (%) of fish. Table modified from Wilson and Halver (1986).

Species	Age	Protein Sources (%)	Estimated Requirements
Brown Trout (*Salmo trutta*)	Fry	cas, fm, fpc	48-53[a]
Channel Catfish (*Ictalurus punctatus*)	Juvenile 2nd & 3rd years	whole egg cm, sbm, fm, wm	32-36 24[b]
Chinook salmon (*Oncorhynchus tshawytscha*)	Juvenile	cas, gelatin, amino acids	40
Coho salmon (*Oncorhynchus kisutch*)	Juvenile	cas	40
Common carp (*Cyprinus carpio*)	Juvenile	cas	31 or 38
Estuary Groper (*Epinephelus salmoides*)	Juvenile	tuna muscle meal	40-50
Gilthead bream (*Chrysophrys aurata*)	Juvenile	cas, fpc, amino acids	40
Grass carp (*Ctenopharyngodon idella*)	Fry	cas	41-43
Grey mullet (*Mugil capito*)	Fry	cas, fm, amino acids	24[c]
Japanese eel (*Anguilla japonica*)	Juvenile	cas, amino acids	44.5
Japanese parrot fish (*Opelegnathus fasciatus*)	Juvenile	cas	45[d]
Largemouth bass (*Micropterus salmoides*)	Juvenile	cas, fpc	40
Milkfish (*Chanos chanos*)	Fry	cas	40
Plaice (*Pleuronectes platessa*)	Juvenile	cod muscle	50
Puffer fish (*Fugu rubripes*)	Juvenile	cas	50
Rainbow trout (*Salmo gairdneri*)	Fingerling	fm, cas	40[e]
Red drum (*Sciaenops ocellatus*)	Juvenile	red drum muscle	40[f]
Red sea bream (*Chrysophrys major*)	Juvenile	cas	55
Pearl gourami (*Trichogaster leerii*)	Fingerling	fm, wm, cm	26-36[g]
Smallmouth bass (*Micropterus dolomieui*)	Juvenile	cas, fpc	45
Snakehead (*Channa micropeltes*)	Juvenile	cas, fpc	52

Table 4.2 (cont.)

Species	Age	Protein Sources (%)	Estimated Requirements
Sockeye salmon (*Oncorhynchus nerka*)	Juvenile	cas, gelatin, amino acids	45
Striped bass (Morone saxatilis)	Juvenile	fm, soy proteinate	47
Tawes (*Puntius gonionotus*)	Fingerling	fm, blood meal, cas	35[h]
Tilapia			
(*Oreochromis aurea*)	Juvenile	cas, egg albumin	34
(*Oreochromis niloticus*)	Fry	fm, wm, alfm,	40[i]
	Juvenile	corn	30
	young	cas	30[i]
(*Oreochromis mossambica*)	Juvenile	fm, wm, alfm,	40
(*Tilapia zilli*)	Juvenile	corn	32
		fm	
		cas	
Walking catfish	Fry	fm, sbm, broken	30[j]
(*Clarias batrachus*)	Juvenile	rice	40[k]
		gel, cas	
White sturgeon (*Acipenser transmontanus*)	Juvenile	cas, wheat gluten, egg white	40[l]
Yellow tail (*Seriola quinqueradiata*)	Juvenile	sand eel & fish meal	55

cas: casein, cm: corn meal, fm: fishmeal, fpc: fish protein conceantrate, sbm: soybean meal, wm: wheat meal.

[a] Arzel et al. (1995), [b] Li and Lovell (1992), [c] Papaparaskera-Papoutsoglou and Alexis (1986), [d] Ikeda *et al.* (1988), [e] Satia (1974), [f] Serrano *et al.* (1992), [g] Degani and Gur (1992), [h] Wee and Ngamsnae (1987), [i] Siddiqui *et al.* (1988), [j] Chuapoehuk (1987), [k] Khan and Jafri (1990), [l] Moore et al. (1988)

Table 4.3 Essential amino acid profile (grams/100 grams protein) of SBM's used in fish nutritional studies (FFSBM - full fat SBM, Solv. Ext. - solvent extracted SBM).

Authors	Shiau *et al.* (1987)	Reigh & Ellis (1992)	Wilson *et al.* (1981)	Anderson *et al.* (1992)		Chu *et al.* (1991)	Shiau *et al.* (1989)		Jackson *et al.* (1982)	Cowey *et al.* (1971)	Lim and Akiyama (1992)	
Processing Method	Solv. Ext.	Solv. Ext.	Solv. Ext.	Solv. Ext.	Solv. Ext.	Solv. Ext.	FFSBM	Solv. Ext.	Solv. Ext. [a]	Solv. Ext.	FFSBM heat treated	Solv. Ext. [b]
Arginine	7.80	6.05	4.71	7.47	7.57	3.07	7.27	7.08	6.9	7.3	7.4	7.4
Histidine	3.08	2.98	2.75	3.38	3.22	1.91	3.91	2.58	2.5	2.7	2.7	2.5
Isoleucine	4.20	4.85	4.25	4.98	5.07	1.77	5.29	5.26	4.2	5.0	5.7	5.0
Leucine	8.30	6.83	7.97	7.83	7.65	4.08	8.47	8.93	6.6	7.6	6.8	7.5
Lysine	6.43	6.19	8.48	6.23	6.15	2.68	5.46	5.77	5.9	6.6	6.3	6.4
Methionine	0.68	1.59	1.72	1.42	1.95	0.96	1.02	1.55	1.2	1.3	1.4	1.4
(+ Cysteine)	(1.45)	(3.05)		(2.44)	(3.54)					(2.5)	(2.8)	(2.9)
Phenylalanine	5.72	4.35	4.67	5.16	4.95	2.39	5.22	5.19	4.8	5.3	5.5	4.9
(+ Tyrosine)	(8.82)	(6.83)	(8.60)	(8.72)	(8.41)	(4.19)	(7.29)	(6.91)	(8.4)	(9.4)	(8.7)	(8.3)
Threonine	4.02	3.76	4.41	4.27	4.22	2.00	2.89	2.26	3.9	4.4	4.4	3.9
Tryptophan		1.39		0.12	0.5					1.3	1.4	1.4
Valine	4.19	5.40	4.27	5.52	5.55	1.94	5.62	5.85	4.7	5.2	5.3	5.1

[a] prepressed, [b] dehulled.

Table 4.4. Estimated essential amino acid requirements (g/100 grams protein) of fish.

Amino acid	*Cyprinus carpio* [a]	*Salmo giadneri* [a]	*Oreochromis niloticus* [b]	*Catla catla* [c]	*Ictalurus punctatus*	*Chanos chanos* [h]	*Salvelinus namaycush*[i]	*Oncorhynchus keta*	*Oncorhynchus tshawtyscha*
Arginine	3.8	3.5	4.20	4.80		5.25			5.9[l]
Histidine	1.4	1.6	1.72	2.45		2.00		1.6[j]	
Isoleucine	2.3	2.4	3.11	2.35	2.96[c]	4.00	1.54-2.06		
Leucine	4.1	4.4	3.39	3.70	2.58[c]	5.11	2.74-3.66		
Lysine	5.3	5.3	2.15	6.23	5.1[d]	4.00		4.8[j]	5.0[m]
Methionine	1.6	1.8	2.68 [1]	3.55	2.34[e]	2.50			
(+ Cystiene)	(2.4)	(2.7)	(3.21)			(3.25)			
Phenylalanine	2.9	3.1	3.75 [2]	3.70 [3]		4.22 [4]			
(+ Tyrosine)	(4.9)	(5.2)	(5.54)		(5.0[f])	(5.22)			
Threonine	3.3	3.4	3.75	4.95	2.21[g]	4.50		3.0[j]	2.25[m]
Tryptophan	0.6	0.5	1.00	0.95	0.5[g]	0.60		0.73[k]	
Valine	2.9	3.1	2.80	3.55	1.54[c]	3.55	1.77-2.23		

[a] Ogino (1980), [b] Santiago and Lovell (1988), [c] Wilson *et al.* (1980), [d] Wilson *et al.* (1977), [e] Harding *et al.* (1977), [f] Robinson *et al.* (1980) [g] Wilson *et al.* (1985), [h] Borlongan and Coloso (1993), [i] Hughes *et al.* (1983), [j] Akiyama *et al.* (1985a), [k] Akiyama *et al.* (1985b),[l] Klein and Halver (1970),[m] Halver *et al.* (1958).

[1] with 0.15% cysteine present, [2] with 0.5% tyrosine present, [3] with 1.0% tyrosine present, [4] with 0.45% tyrosine present.

(Lim and Akiyama, 1992). The fatty acid profile of the triglycerides is dominated by polyunsaturated fatty acids. Linoleic acid (18:2n-6) is the predominant polyunsaturated fatty acid, although linolenic acid (18:3n-3) is also present at relatively high levels (Lim and Akiyama, 1992). Both these fatty acids are considered to be essential dietary elements for fish (Cowey and Sargent, 1977; Sargent *et al.*, 1989). The nutritional value of these polyunsaturated fatty acids is aided by the presence of natural anti-oxidants which prevent their oxidation (Lim and Akiyama, 1992). This natural protection is further enhanced by the destruction of lipoxidases during processing, ensuring soybean oil is relatively stable to oxidation (Abel *et al.*, 1984).

Apart from supplying essential fatty acids, soybean oil also provides a source of highly digestible energy that could be used for metabolism, thus sparing the catabolism of protein. Such a protein sparing effect by dietary lipids in fish nutrition (Millikin, 1983; Beamish and Medland, 1986; Tabachek, 1986; De Silva *et al.*, 1991) plays a major role in the formulation of cost effective feeds.

However, despite the nutritional value of soybean oil many nutritionists choose to use DFSBM, where the lipid level is greatly reduced (<1.0% wet weight) (Table 4.1). Diets are subsequently supplemented with oils from other sources or with reduced levels of soybean oil, allowing greater control of the lipid, fatty acid and digestible energy levels of the diets.

4.1.2.3. Carbohydrate Content of Soybean Meal.

Carbohydrates comprise approximately 30% of SBM on a wet weight basis (Table 4.1) but, the majority of this carbohydrate is considered to be unavailable to fish. The insoluble carbohydrate fraction consists predominantly of the high molecular weight cellulose, hemicellulose and pectins (Viola *et al.*, 1981/2), all of which are generally considered indigestible or at best partially digestible by fish (refer section 1.2.4.3). Starch, which has been shown to be digestible by fish (Chiou and Ogino, 1975; Bergot and Breque, 1983; Hemré *et al.*, 1989; Pfeffer *et al.*, 1991), contributes less than 1% of the polysaccharide fraction (Lim and Akiyama, 1992). The soluble carbohydrates account for approximately 10% of soybean carbohydrate (Lim and Akiyama, 1992). These carbohydrates are divided between sucrose, which is digestible by fish (refer 1.2.4.1.5), and raffinose and stachyose which are thought to be indigestible by fish. Therefore, although the carbohydrate content of SBM as determined by chemical analysis may be high, the actual nutritional value of the carbohydrate to fish is extremely limited.

4.1.2.4. Vitamin and Mineral Content of Soybean Meal.

Although SBM contains adequate amounts of water soluble vitamins, several fat-soluble vitamins are absent (Lim and Akiyama, 1992). In contrast, the crude levels of all essential minerals are adequate, although the majority of the phosphorus is present in the form of phytic acid and is therefore unavailable to fish. Phytic acid also chelates other di- and tri-valent metal ions rendering them unavailable (Lim and Akiyama, 1992). Although the lack of fat-soluble vitamins and the unavailability of minerals would restrict growth, these inadequacies are generally overcome by the routine addition of a vitamin/mineral premix to artificial diets, alleviating the need for vitamins and minerals to be provided by the protein source.

4.1.2.5. Anti-Nutritional Factors of Soybean Meal.

Although SBM, and in particularly FFSBM, is capable of contributing significantly to the nutritional quality of aquaculture diets, raw SBM contains numerous anti-nutritional factors. Anti-nutritional factors are heat labile, proteinaceous molecules which, after amino acid imbalance, are considered the main limiting factor in the use of plant protein sources in artificial diets. Tacon and Jackson (1985) named 12 such molecules found in raw soybeans, these being protease inhibitors, phytohaemagglutinins, glucosinolates, phytic acid, saponins, oestrogenic factor, flatulence factor, anti-vitamin A, anti-vitamin B_{12}, anti-vitamin D, anti-vitamin E and a possible mycotoxin. Several of these factors and their actions are reviewed in greater detail by Liener (1980).

The protease inhibitors present in SBM, generally referred to as soybean trypsin inhibitors (SBTI), are the SBM anti-nutritional factors most commonly studied by aquaculture nutritionists. Soybean trypsin inhibitors account for approximately 6.0% of the total protein of SBM and are thought to cause growth retardation and pancreatic hypertrophy (Lim and Akiyama, 1992). Channel catfish fingerlings fed diets with SBTI levels greater than 1.9 TIU (trypsin inhibition units) mg^{-1} showed reduced growth and poor feed efficiency (Robinson *et al.*, 1981). Fish fed higher protein concentrations appeared to better tolerate the SBTI than those fed less protein, channel catfish fingerlings exhibiting peak growth at 3.2mg SBTI mg^{-1} and 2.2mg SBTI mg^{-1} with 35% and 25% dietary protein respectively (Wilson and Poe, 1985). Common carp grew poorly if residual SBTI activity was above 5.4% of the activity in raw SBM (Viola *et al.*, 1983). Elevated levels of SBTI also resulted in decreased weight gain in Atlantic salmon (Olli *et al.*, 1994). Growth in Nile tilapia was adequate at dietary levels of less than 0.09% SBTI (Wee and Shu, 1989).

A decrease in enzyme activity has been associated with poor growth performance at high levels of SBTI. Trypsin activity decreased with increased levels of SBTI in rainbow trout, although compensation for small amounts of trypsin inhibitor (< 0.37%) appeared possible (Krogdahl *et al.*, 1994). Elevated levels of SBTI also reduced intestinal tryptic activity in Atlantic salmon, although an increase in trypsin activity in the pyloric caeca homogenate again suggested increased trypsin secretion compensated for the effect of SBTI (Olli *et al.*, 1994). Using an *in vivo* assay, Dabrowski *et al.* (1989) found decreased chymotryptic activity in rainbow trout, although faecal levels of tryptic activity were increased. The increase in faecal tryptic activity was attributed to the release of trypsin from the trypsin-SBTI complex in the hindgut. No correlation was found between the poor performance of channel catfish fingerlings fed SBTI and the activities of trypsin, chymotrypsin, lipase or amylase (Robinson *et al.*, 1981).

Nutrient digestibility was also affected by SBTI. Increasing SBTI levels (0.00 to 0.11%) resulted in decreased protein digestion (93% to 70%) in rainbow trout (Krogdahl *et al.*, 1994), and reduced protein and fat digestibility in Atlantic salmon (Olli *et al.*, 1994). Amino acid digestibility also decreased with increased SBTI levels (Dabrowski *et al.*, 1989; Krogdahl *et al.*, 1994). Individual amino acid digestion generally paralleled the protein digestibility, with the exception of cysteine where uptake was reduced to an even greater extent (Krogdahl *et al.*, 1994).

Mammalian studies have suggested elevated levels of SBTI cause increased secretion of pancreatic enzymes to compensate for diminished enzyme activities in the intestine leading to pancreatic hypertrophy (Lim and Akiyama, 1992). Although no morphological changes in pancreatic tissue were found in channel catfish fingerlings fed high levels of SBTI (Robinson *et al.*, 1981), the data suggest the potential for pancreatic hypertrophy does occur in teleosts. The ability of fish to compensate for low levels of SBTI (Robinson *et al.*, 1981; Viola *et al.*, 1983; Wilson and Poe, 1985; Krogdahl *et al.*, 1994) suggests increased enzyme secretion. Krogdahl *et al.* (1994) further proposed that high amounts of faecal cysteine leading to lower apparent digestibility co-efficients may have been the result of increased release of cysteine-rich proteolytic enzymes. Such increases in pancreatic activity of fish fed diets containing SBTI may lead to pancreatic hypertrophy.

4.1.3. Heat Treatment of Soybean Meal

To limit the effects of antinutritional factors, heat treatment is applied to SBM during processing (Liener, 1980). Several different types of heat treatment have been employed in laboratory or commercial production including boiling (Wee and Shu,

1989), toasting, micronising, autoclaving, jetsploding, dry extrusion and wet extrusion (Smith *et al.*, 1980; Lim and Akiyama, 1992). Adequate heat treatment effectively destroys the anti-nutritional factors in SBM, but the type of heat and exposure time are important in producing a high quality product. Ineffective inactivation of the anti-nutritional factors results from inadequate heating, while excessive heating results in the loss of available lysine due to Maillard reactions (Erbersdobler, 1989). Boiling FFSBM in water for 1 hour inactivated 80% of the SBTI (Wee and Shu, 1989). Heating in the presence of water (90-95°C for 15 or 30 minutes) more effectively denatured SBTI than heating in the absence of water (118°C for 30 or 150 seconds), with activity reduced to 5.4% or less with both hydrothermal treatments, but only the more intense thermal treatment (Abel *et al.*, 1984). No reduction in available lysine was found with either thermal or hydrothermal heating. Soybean meal moistened to 17% and heated at 105°C had SBTI activity reduced to 5.4% after 30 minutes, with complete loss of activity by 120 minutes (Viola *et al.*, 1983). However, heating resulted in a decrease in available lysine from 6.0% crude protein to 5.45% following 30 minutes of heating with a further decrease to 4.1% crude protein after 240 minutes.

4.1.4. Use of Soybean Meal in Artificial Diets for Aquaculture.

The inclusion of SBM into artificial diets has been extensively studied. In general, fish fail to thrive when fed diets containing raw or inadequately heated SBM but the evidence is contradictory.

Replacement of groundnut cake by raw SBM resulted in depressed growth and increased mortalities of sharptoothed catfish fingerlings at replacement levels from 25% to 100% (Balogun and Ologhobo, 1989). Growth and appetite were reduced in Nile tilapia fingerlings when untreated FFSBM was incorporated into the diets (Wee and Shu, 1989). Reduced growth rates, food conversion efficiencies and nutrient retentions were also observed in channel catfish fingerlings (Robinson *et al.*, 1981) and juvenile (Abel *et al.*, 1984) and adult (Viola *et al.*, 1983) common carp when inadequately treated SBM was incorporated into experimental diets. Growth in juvenile grass carp decreased with increased SBM levels even though dietary crude protein was greater, suggesting incomplete destruction of anti-nutritional factors (Dabrowski and Kozak, 1979).

The use of adequately treated SBM has provided varied results. The majority of these studies have determined fishmeal replacement in common carp, catfish, tilapia and the salmonids. Replacement of 50% of fishmeal protein with SBM resulted in only 60-65% of control growth for common carp (Abel *et al.*, 1984). Common carp fed diets

with 40%, 80% or 100% of the fishmeal protein replaced by SBM protein also showed decreased growth. Addition of 5% oil to 40% replacement diets restored growth to control values, while supplementation with 10% oil plus lysine (0.4 or 0.5%) restored control growth rates at 80% and complete replacement levels (Viola *et al.*, 1981/2). Similarly, carp fed heat treated SBM displayed acceptable growth rates, but growth significantly improved by lysine supplementation (Viola *et al.*, 1983; 1992). These studies indicated that available energy and lysine were the limiting nutrients in SBM for common carp. Murai *et al.* (1986) found diets containing 75% fishmeal protein replaced with SBM protein could achieve 90% of control diet growth if the amino acid profile was supplemented to control levels. Prior to supplementation, however, only approximately 60% of control growth was attained. The addition of methanol to diets to improve palatability of SBM had no significant effect suggesting that common carp may not be susceptible to appetite suppressing factors of SBM (Murai *et al.*, 1986).

Replacement of fishmeal with SBM in diets for channel catfish fingerlings reduced growth and food conversion efficiencies at all inclusion levels (Andrews and Page, 1974). Growth was not enhanced by amino acid supplementation, although addition of the non-lipid residue of fishmeal improved growth in all diets, suggesting the presence of a growth factor in the protein fraction of fishmeal. Similarly, Mohsen and Lovell (1990) found SBM to be an inadequate replacement for animal proteins (fishmeal and meat and bone meal) in diets for channel catfish fingerlings. Growth in blue catfish, *Ictalurus furcatus*, also decreased at ratios of SBM:fishmeal protein greater than 48:13 (Webster *et al.*, 1992). In contrast, Robinson and Li (1994) showed that SBM could completely replace SBM in diets for adult channel catfish; the differences between these studies were attributed to feeding regimes and the size of the animals. Webster *et al.* (1992) found that SBM in combination with distillers grain could adequately replace fishmeal in diets for juvenile channel catfish.

Shiau *et al.* (1987, 1990) replaced 30% of fishmeal protein with SBM protein in juvenile hybrid tilapia (*Oreochromis niloticus* X *O. aureus*) diets at crude protein levels of 24% (suboptimal) or 32% (optimal). Diets containing 32% protein produced significantly reduced growth rates which were reversible by supplementation with methionine (0.26%). However, at a crude protein level of 24% replacement of fishmeal with SBM alone caused no significant decrease in growth rate, indicating that at suboptimal protein levels, SBM could adequately replace up to 30% of fishmeal protein in diets for juvenile hybrid tilapia. Blue tilapia growth was not significantly affected by the complete replacement of fishmeal with SBM in diets with 36% crude protein, but at lower protein concentrations (15%, 22% and 29%) growth and food

conversion improved with the level of fishmeal in the diet (Davis and Stickney, 1978). Soybean meal, supplemented with methionine and threonine, replaced 25% of fishmeal protein in diets for mature Mozambique tilapia without significantly affecting growth, although higher levels of inclusion resulted in reduced growth (Jackson *et al.*, 1982) and food wastage at higher inclusions of SBM indicated potential palatability problems.

In diets for yearling rainbow trout, replacement of 39% of fishmeal protein with SBM resulted in decreased growth, food conversion and nutrient retention (Dabrowska and Wojno, 1977). Supplementation with cysteine (1%) and tryptophan (0.5%) produced growth comparable to fishmeal, while lysine and arginine supplementation had no effect. In a similar study, rainbow trout fingerlings only grew 47% in 6 weeks when fed diets with SBM as the sole protein source, and growth rates were significantly improved by amino acid supplementation (Rumsey and Ketola, 1975). Reinitz (1980) reported acceptable, but inferior growth rates for rainbow trout juveniles when fishmeal was completely replaced by SBM. Growth rates decreased with the increasing inclusion of SBM. In contrast, Cho *et al.* (1974) found replacing approximately 50% of fishmeal protein by SBM protein did not adversely affect growth rate, feed efficiency or mortality in juvenile rainbow trout.

Plaice exhibited depressed growth in response to diets containing 45% crude protein as SBM when compared to a fishmeal based diet (Cowey *et al.*, 1971). Growth and protein and fat deposition in chinook salmon also decreased when soybean grits replaced fishmeal in the diet (Fowler and Banks, 1976). Juvenile red drum showed equivalent growth and better food conversion efficiency when 50% of fishmeal protein was replaced with SBM (Reigh and Ellis, 1992). However, inclusion of SBM at a 75% replacement level caused decreased growth, with complete replacement resulting in reduced consumption, weight loss and death. El-Sayed (1994) showed replacing 25% of fishmeal protein with dehulled, solvent extracted SBM caused no significant decrease in growth, food conversion efficiency or protein efficiency in silver seabream, *Rhabdosargus sarba*, fingerlings although higher inclusion levels (50%, 75% and 100%) caused growth retardation. Similarly, 25% replacement of fishmeal protein with SBM did not effect the growth of juvenile milkfish (de la Peña, 1987).

4.1.3. Aims of the Present Study

The contradictions found in these studies are likely to be related to the variations in the SBM employed and a poor understanding of the influence of the form and inclusion level of SBM on digestive physiology. However it is clear that SBM will play an important role in future aquaculture diets. In order to formulate the best quality diets, a

knowledge of the digestibility of the nutrients present in SBM and of factors influencing that digestibility are required. The aims of the present study were:

(a) to determine the digestibility of ground and sifted SBM and SBM midlings,

(b) to determine the effects of inclusion level on the digestibility of SBM, and

(c) to determine the effects of time on SBM digestibility

4.2 Materials and Methods

In the present study two trials were performed to determine the digestibility of SBM, and the effects of time, processing methods and inclusion levels on SBM digestibility. The initial trial (Trial 1) was conducted to evaluate the digestibility of SBM at a single inclusion level, 20%, and the effect of processing on digestibility. A second trial (Trial 2) was conducted to assess the effect of inclusion level on the digestibility of ground SBM.

4.2.1 Experimental Diets

Solvent extracted crushed SBM (Metropolitan Commodities Pty Ltd, Melbourne, Victoria, Australia) was used as the source of SBM in the present study. The crushed SBM was either ground using a commercial grinder (ground SBM) or sifted through a 1.0mm sieve (sifted SBM). After removal of the fines by sifting the remaining crushed SBM was ground (ground SBM middlings). Ground SBM, sifted SBM and ground SBM middlings were subsequently used as the experimental ingredients. The proximate analyses of the experimental ingredients are detailed in Table 2.1.

Trial 1:

Digestibility was measured using a casein-based reference diet (Mackie and Mitchell, 1985). Ground and sifted SBM and ground SBM middlings were incorporated into the reference diet at 20% on a dry weight basis. The formulation of the experimental diets used in Trial 1 are detailed in Table 4.5. As the reference diet used in the experiment for the ground SBM experiment was made in a different batch to that used in the sifted SBM and ground SBM middlings experiments, and since diets of the same formulation may have a different proximate analyses due to variations in diet preparation technique, all diets were analysed individually. The proximal analyses of all diets are presented in Table 4.5. The composition of the appropriate reference diet was used for calculation of the digestibility coefficients.

Trial 2:

A casein-based reference diet was also used to determine the digestibility of graded levels of ground SBM. Ground SBM was incorporated into the reference diet at 10%, 20%, 30% and 40%. The formulation of the experimental diets for Trial 2 are

Table 4.5 Formulation (percent dry matter) and proximate composition determined by analyses (percent dry matter) of the experimental diets used in the Trial 1. As the reference diets for the study for ground SBM was produced separately to the reference diet for the other test ingredients the values for proximal composition of this diet are shown in parentheses.

Dietary Components	Diets			
	Reference	Sifted SBM	Ground SBM	Ground SBM middlings
Casein	60.0	48.0	48.0	48.0
Wheat gluten	5.0	4.0	4.0	4.0
Sifted SBM	0.0	20.0	0.0	0.0
Ground SBM	0.0	0.0	20.0	0.0
Ground SBM middlings	0.0	0.0	0.0	20.0
Corn flour	10.0	8.0	8.0	8.0
Tuna oil	9.0	7.2	7.2	7.2
α-Cellulose	10.0	8.0	8.0	8.0
Vitamin & mineral premix	5.0	4.0	4.0	4.0
Cr_2O_3	1.0	0.8	0.8	0.8
Proximate Composition				
Dry matter	68.19 (63.73)	66.76	66.19	65.77
Crude protein	58.22 (59.03)	54.62	56.24	58.33
Crude lipid	(8.72)		7.22	
Ash	(5.36)		5.42	
Crude fibre	(18.60)		22.93	
Carbohydrate	(7.21)		7.25	
Gross energy (kJ/g)	24.78 (23.41)	22.41	22.85	20.93
Cr_2O_3	0.90 (1.08)	0.74	0.94	0.76

detailed in Table 4.6.

All diets were formulated and prepared according to the methods described in Section 2.7.

4.2.2 Experimental Animals, Feeding and Faeces Collection

The common carp used in this experiment were collected, maintained and handled as described in Chapter 2.

Trial 1:

Trial 1 was conducted as two consecutive experiments, the first experiment determined the digestibility of ground SBM and the later experiment measured the digestibility of sifted SBM and ground SBM middlings. Thus two different groups of experimental animals were used. In the first experiment, ground SBM digestibility, common carp of mean weight 90.6g (range 17.3 to 202.6g, standard deviation 38.6g) were stocked at 10 fish per 70 litre aquaria. Each experimental diet was fed to four aquaria of fish. Initially the feeding rate was 0.5% body weight.wet weight diet.day^{-1}, with subsequent increases to 1.0% and 2.0% body weight.wet weight diet.day^{-1} as fish adapted to the diets. Feeding was performed using a single daily feeding regime and continued for 32 days.

Faeces collection commenced on day 2 of the ground SBM experiment and continued until day 32. On days 6, 7, 13, 20, 24, 26 and 27, faeces were not collected due to a lack of faeces voided during the collection period.

Common carp of mean weight 40.2g (range 14.8 to 87.7g, standard deviation 16.3g) were stocked at 15 fish per 70 litre aquaria for the sifted SBM and ground SBM middlings experiment. Each experimental diet was fed to triplicate tanks of fish at a feeding rate of 2% body weight.wet weight diet.day^{-1}. A single daily feeding regime was employed and feeding continued for 29 days.

In the sifted SBM and ground SBM middlings experiment faeces collection commenced on day 4 and continued until day 29. Faeces were collected daily with the exception of days 7, 14, 15, 23, 24 and 28 when little or no faeces was voided during the collection period.

Table 4.6 Formulation (percent dry matter) and proximal composition determined by analyses (percent dry matter) of the experimental diets used in Trial 2.

Dietary Components	Diets				
	Reference	10% Ground SBM	20% Ground SBM	30% Ground SBM	40% Ground SBM
Casein	60.0	54.0	48.0	42.0	36.0
Ground SBM	0.0	10.0	20.0	30.0	40.0
Wheat gluten	5.0	4.5	4.0	3.5	3.0
Corn flour	10.0	9.0	8.0	7.0	6.0
Tuna oil	9.0	8.1	7.2	6.3	5.6
α-Cellulose	10.0	9.0	8.0	7.0	6.0
Vitamin & mineral premix	5.0	4.5	4.0	3.5	3.0
Cr_2O_3	1.0	1.0	1.0	1.0	1.0
Proximate Composition					
Crude protein	58.90	55.00	53.22	51.81	50.16
Crude lipid	8.84	7.65	6.10	6.08	5.39
Ash	4.14	4.19	5.10	5.25	6.16
NFE	27.15	32.2	34.62	35.86	37.31
Energy (kJ/g)	19.2	19.5	19.0	18.7	18.1
Cr_2O_3	0.97	0.96	0.96	1.00	0.98

Trial 2:

Common carp of mean weight 45.8g (range 13.8 to 103.1g, standard deviation 19.3g) were stocked at 15 fish per 70 litre aquaria for Trial 2. Each experimental diet was fed to triplicate tanks at a feeding rate of 2% body weight.wet weight diet.day^{-1}. Daily rations were fed in four approximately equal rations at 1 hour intervals, ensuring all feed was ingested. Feeding continued for 24 days.

In the Trial 2 faeces collection commenced on day 9 to allow adaptation to the experimental diets. Faeces were subsequently collected daily until day 24, with the exception of days 11 and 18 when insufficient faeces was voided during the collection period to warrant siphoning tanks.

4.2.3 Diet and Faeces Analysis

Experimental diets and faeces were dried to a constant weight at 50°C and ground prior to analysis. Analyses were performed as described in Chapter 2.

Trial 1:

Insufficient faeces were collected each day to provide complete analysis. Therefore, faeces collected for each dietary treatment in the ground SBM digestibility experiment were pooled per day. Subsequently three consecutive days of faeces were pooled for each dietary treatment to obtain adequate faeces for analyses, this pooling was based around days 3, 9, 21 and 29. Pooled faeces were subsequently analysed for Cr_2O_3, energy and protein. All diets used in the ground SBM digestibility experiment were analysed for Cr_2O_3, ash, carbohydrate, energy, fibre, lipid and protein. The measured proximate analyses of the experimental diets are shown in Table 4.5.

Collected faeces for the sifted SBM and ground SBM middlings digestibility experiments were pooled for each dietary treatment per day, with subsequent pooling over consecutive days. Apart from the faeces pooled over days 8, 9, 10 and 11, faecal pooling was performed over 3 consecutive days based around days 17, 21 and 27. Faeces were analysed for protein, energy, lipid and Cr_2O_3. The experimental diets were analysed for protein, energy, lipid and Cr_2O_3. The measured proximate composition for these diets are shown in Table 4.5.

Trial 2:

The experimental diets used in Trial 2 were analysed for protein, lipid, ash, NFE, energy, and Cr_2O_3. The proximate analyses of the experimental diets are shown in Table 4.6.

Faeces for Trial 2 were pooled over days 9 to 17 and days 19 to 24. Pooled faeces were subsequently analysed for protein, lipid, ash, NFE, energy, and Cr_2O_3.

ADC values were calculated using the ratio of marker in feed and faeces according to the formulae described in section 2.6.

4.2.4 Statistical Analysis

Data were analysed using repeated measures and one-way analysis of variance as indicated (Zar, 1984; Ott, 1988). *Post hoc* analyses were performed using Scheffé's test. Analyses were performed using StatView 512+™ statistical analysis software (Brain Power Inc., California, USA) and SPSS (SPSS Incorporated 1990, Michigan, Illinios, USA).

4.3 Results

4.3.1 Digestibility of the Experimental Diets

Trial 1:

The ADC's of the reference diets and each of the test diets used in Trial 1 varied markedly with time (Figure 4.1 and Figure 4.2). Values for each time point are single replicates due to the pooling of faeces. Values ranged from 75.1% to 79.9% for the ground SBM reference diet (Figure 4.1), 66.1% to 72.5% for the ground SBM test diet (Figure 4.1), 79.8% to 87.25% for the sifted SBM/ground SBM middlings reference diet (Figure 4.2), 79.28% to 83.9% for the sifted SBM test diet (Figure 4.2) and 71.69% to 82.3% for the ground SBM middlings test diet (Figure 4.2). The changes in reference diet and test diet digestibility was generally in harmony. However, at day 29 for the ground SBM test diet (Figure 4.1) and day 21 for the ground SBM middlings test diet (Figure 4.2) the decrease in digestibility was excessive when compared to the other changes for that time point. Such non-harmonious and excessive changes in digestibility resulted in the calculation of apparently spurious ingredient ADC's for the test ingredient (refer Section 4.3.2).

Mean values for the whole diet ADC's over the four sampling times measured are detailed in Table 4.7. One-way analysis of variance showed a significant difference ($p < 0.001$) in the digestibility of the experimental diets. The inclusion of ground SBM into the reference diet significantly ($p < 0.05$, Scheffé's test) decreased diet digestibility, while the inclusion of sifted SBM and ground SBM middlings slightly, but not significantly ($p > 0.05$, Scheffé's test), reduced diet digestibility. The ground SBM test diet was significantly ($p < 0.05$, Scheffé's test) less digestible (69.6 ± 2.6%) than the sifted SBM (81.2 ± 1.9%) and ground SBM middlings (78.6 ± 4.9%) test diets. Digestibility of the two reference diets used in Trial 1 were not significantly different ($p > 0.05$, Scheffé's test).

Trial 2:

Repeated measures analysis of variance found whole diet digestibility to be significantly effected by the level of inclusion of SBM ($p < 0.001$) but not time ($p > 0.05$). The interaction between inclusion and time was also not significant ($p > 0.05$) and data were therefore pooled over time as shown in Figure 4.3. *Post hoc* analysis using Scheffé's test showed whole diet digestibility was significantly lower ($p < 0.05$) for the 40% inclusion test diet (78.8 ± 0.2%) than for the reference diet (83.0 ± 1.0%)

Figure 4.1

Apparent digestibility coefficients (%) versus time (days) of the ground SBM reference diet (⊡), 20% inclusion ground SBM test diet (▩) and ground SBM (■) in Trial 1. Each point represents a single replicate.

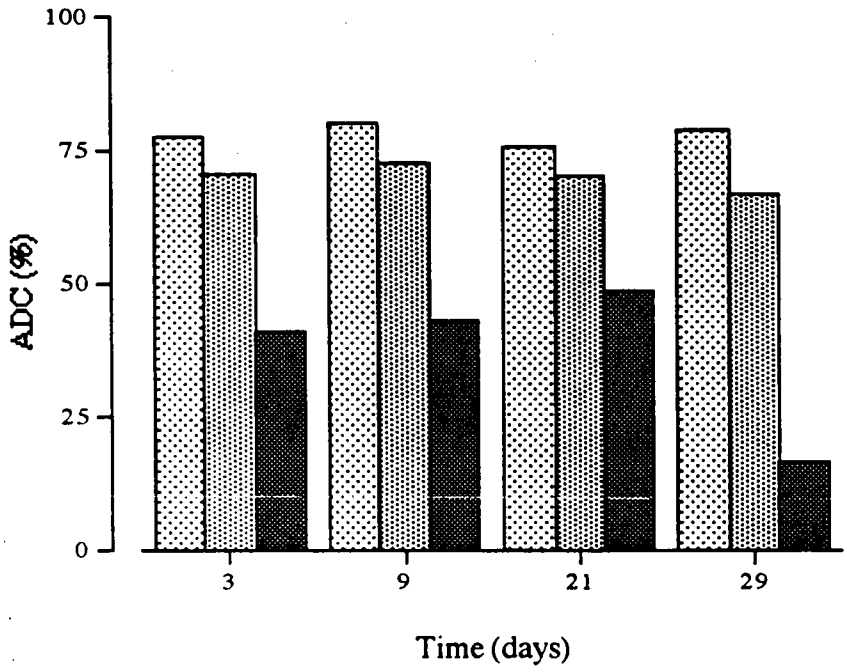

100
75
50
25
0
ADC (%)
3
9
21
29
Time (days)

Figure 4.2
Apparent digestibility coefficients (%) versus time (days) of the reference diet (⊡) and the 20% inclusion sifted SBM (▦) and ground SBM middlings (▩) test diets in Trial 1. Each point represents a single replicate.

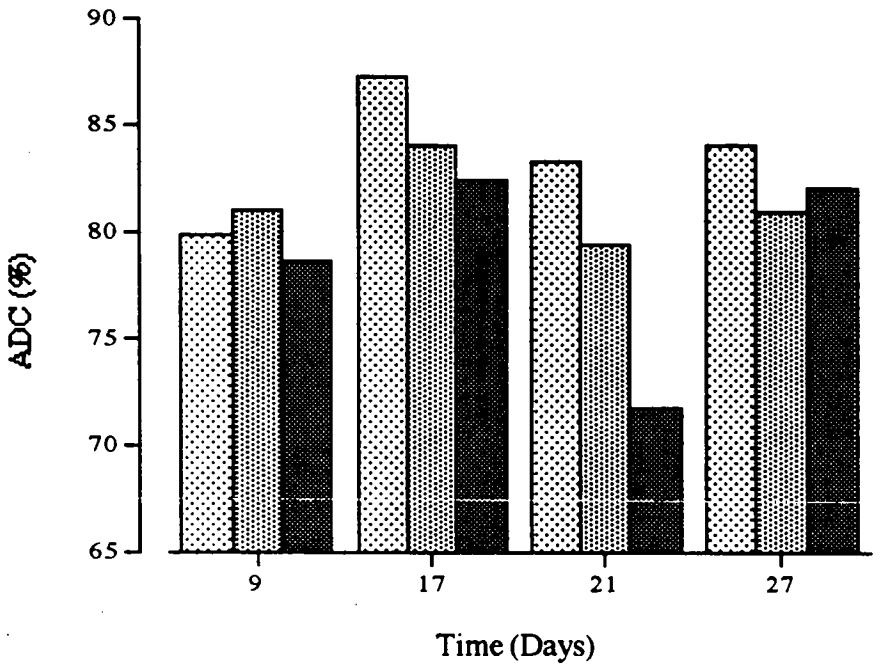
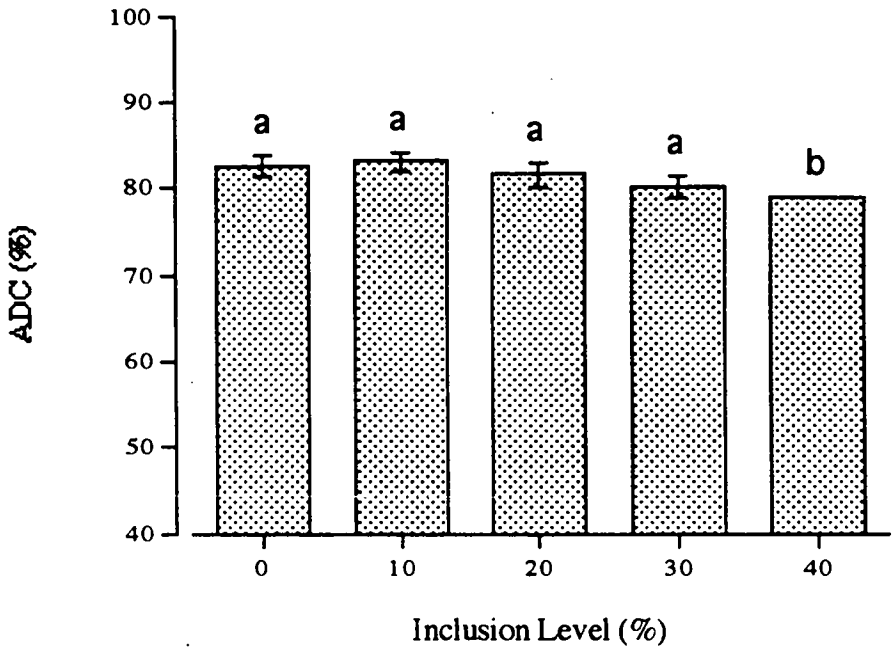

90
85
80
75
70
65
ADC (%)
9
17
21
27
Time (Days)

Table 4.7 Apparent digestibility coefficients (%) of diets, ingredients and nutrients averaged over the sampled time points for Trial 1 (ref - reference diet, GSBM - ground SBM, SSBM - sifted SBM, GSBMM - ground SBM middlings). Values are mean ± standard deviation (n = 4). Values with the same superscript are not significantly different.

	ADC Diet/Ingredient	ADC Protein	ADC Energy
Diet			
ref GSBM	77.7 ± 2.0[a]	99.1 ± 0.4	86.2 ± 1.5[a]
20% GSBM	69.6 ± 2.6[b]	97.1 ± 0.7	85.0 ± 2.2[ab]
ref SSBM/GSBMM	83.6 ± 3.1[a]	99.6 ± 0.3	89.2 ± 1.2[b]
20% SSBM	81.2 ± 1.9[a]	97.9 ± 0.6	86.7 ± 2.1[ab]
20% GSBMM	81.0 ± 2.1[a]	98.2 ± 1.0	86.5 ± 1.7[ab]
Ingredient			
GSBM	43.9 ± 3.7	89.5 ± 4.2	80.1 ± 14.6
SSBM	71.9 ± 9.6	91.0 ± 2.7	75.8 ± 7.6
GSBMM	70.1 ± 6.4	93.1 ± 4.0	77.3 ± 5.4

Figure 4.3

Apparent digestibility coefficients (%) of the experimental diets versus inclusion level (%) in Trial 2. Values (mean ± standard deviation, n = 6) are pooled over time. Values with the same superscript are not significantly different at $p < 0.05$.


100
90
80
70
60
50
40
ADC (%)
a
a
a
a
b
0
10
20
30
40
Inclusion Level (%)

and the 10% (81.5 ± 1.3%), 20% (80.1 ± 1.2%) and 30% (78.8 ± 0.2%) inclusion test diets.

4.3.2 Digestibility of the Test Ingredients

Trial 1:

ADC's for ground SBM (Figure 4.1), sifted SBM (Figure 4.4) and ground SBM middlings (Figure 4.4) again showed marked variation with time. Values for each time point are single replicates due to the pooling of faeces. Values ranged from 16.2% to 48.1% for ground SBM (Figure 4.1), 63.5% to 85.6% for sifted SBM (Figure 4.4) and 25.5% to 74.0% for ground SBM middlings (Figure 4.4). The ADC values for ground SBM and ground SBM middlings at days 29 and 21 respectively appear to be spurious, resulting from the non-harmonious variation in the digestibility of the reference and test diets. Therefore, these values have not been used in later calculations.

The mean values for ingredient digestibility over the time points measured are detailed in Table 4.7. A significant effect of processing method on SBM digestibility was found ($p < 0.01$, one-way analysis of variance). The mean ADC for ground SBM (43.9 ± 3.7%) was significantly lower than that of sifted SBM (71.9 ± 9.6) and ground SBM middlings (70.1 ± 6.4) ($p < 0.05$, Scheffé's test). There was no significant difference ($p > 0.05$, Scheffé's test) between the digestibility of sifted SBM and ground SBM middlings.

Trial 2:

Repeated measures analysis of variance showed a significant effect of inclusion level ($p < 0.05$) but not time ($p > 0.05$) on the digestibility of ground SBM. The interaction between inclusion level and time was also not significant ($p > 0.05$) and the data were therefore pooled over time as shown in Figures 4.5. Subsequent *post hoc* analysis using Scheffé's test revealed that digestibility of ground SBM at the 10% inclusion (86.3 ± 6.7%) was significantly higher ($p < 0.05$) than at 30% (74.4 ± 4.3%) and 40% inclusion (73.1 ± 1.2%).

Figure 4.4
Apparent digestibility coefficients (%) versus time (days) of sifted SBM (⊡) and ground SBM middlings (▩) in Trial 1. Each point represents a single replicate.

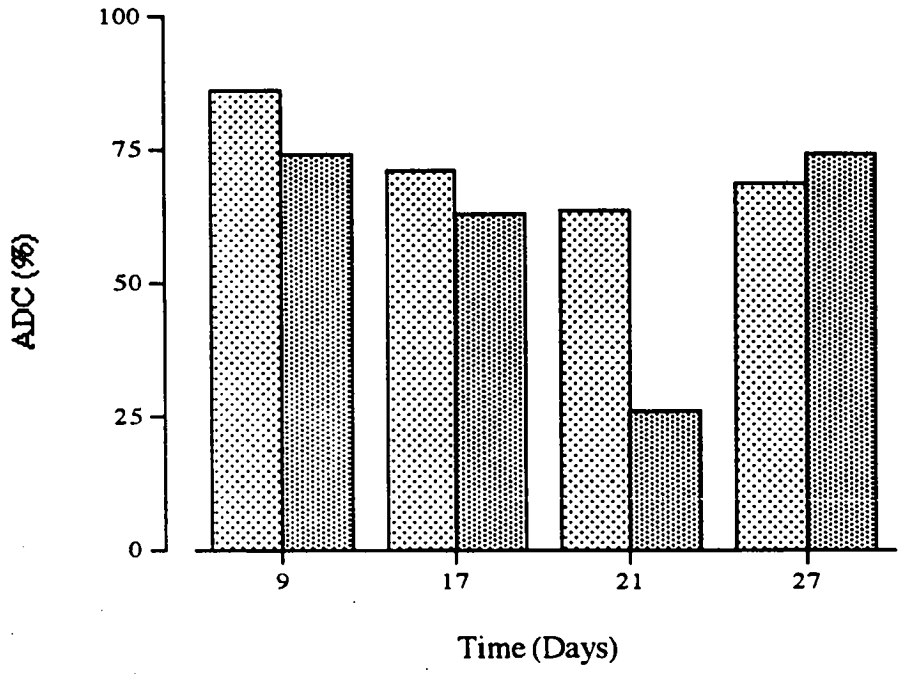
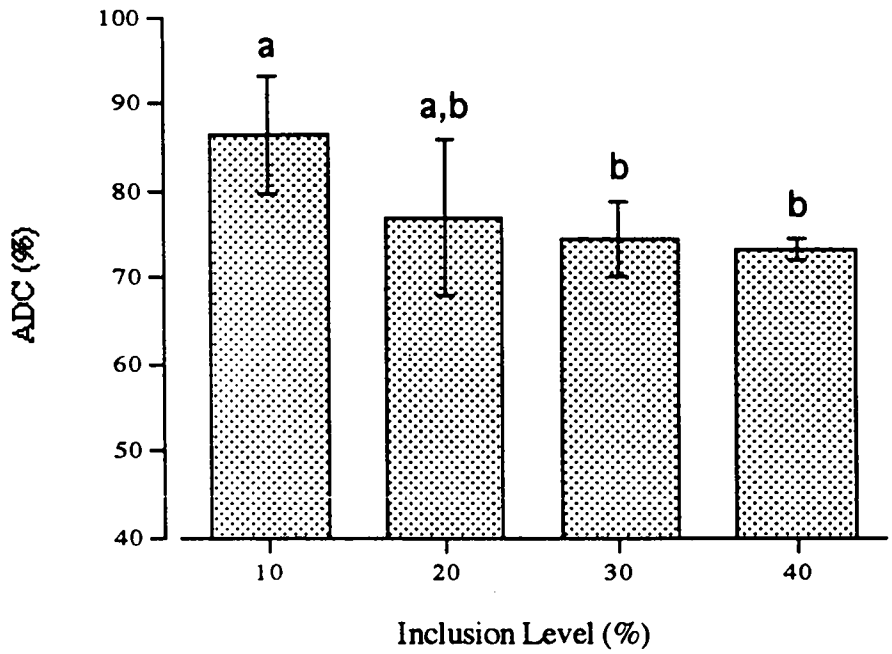

100
75
50
25
0
ADC (%)
9
17
21
27
Time (Days)

Figure 4.5

Apparent digestibility coefficients (%) of ground SBM versus inclusion level (%) in Trial 2. Values (mean ± standard deviation, n = 6) are pooled over time. Values with the same superscript are not significantly different at $p < 0.05$.


100
90
80
70
60
50
40
ADC (%)
a
a,b
b
b
10
20
30
40
Inclusion Level (%)

4.3.3 Digestibility of Protein in the Experimental Diets

Trial 1:

ADC's for crude protein from the experimental diets used in Trial 1 are shown in Figures 4.6 and 4.7. Values for each time point are single replicates due to the pooling of faeces. The digestibility of dietary protein was high, greater than 98% for the reference diets and greater than 96% for the test diets. Protein digestibility was found to vary considerably with time for all diets. The high values for dietary crude protein digestibility meant the variations were small with the ground SBM middlings test diet showing the largest overall variation in protein digestibility, 96.8% to 99.2%.

The mean values for dietary crude protein digestibility over the four time points for each experimental diet are detailed in Table 4.7. From this table it can be seen that the ADC's of protein from the ground SBM (97.1 ± 0.7%), sifted SBM (97.9 ± 0.6%) and ground SBM middlings (98.2 ± 1.0%) test diets were not significantly different ($p > 0.05$, one-way analysis of variance) to those of the relevant reference diets (99.1 ± 0.4% and 99.6 ± 0.3%). The digestibility of protein from the reference diets was not significantly different ($p > 0.05$, one-way analysis of variance).

Trial 2:

ADC's for protein in the reference and test diets in Trial 2 are shown in Figure 4.8. Protein digestibility of the diets was high, being greater than 98% for all experimental diets. Repeated measures analysis of variance showed a significant effect of both inclusion ($p < 0.001$) and time ($p < 0.05$) on dietary protein digestibility. *Post hoc* analysis using Scheffé's test found protein digestibility on days 8 to 17 was significantly higher ($p < 0.05$) for the reference diet (99.3 ± 0.1%) and the 10% inclusion test diet (99.6 ± 0.2%) than for the 30% (98.6 ± 0.0%) and 40% (98.1 ± 0.2%) inclusion test diets (Figure 4.8). ADC for protein from the 10% inclusion test diet was also significantly higher ($p < 0.05$) than for the 20% inclusion test diet (98.8 ± 0.4%). On days 19 to 24 protein digestibility for the reference diet (99.6 ± 0.1%) and the 10% (99.5 ± 0.3%) and 20% (99.1 ± 0.2%) inclusion test diets was significantly higher ($p < 0.05$) than for the 40% inclusion test diet (98.1 ± 0.2%), and the ADC for protein from the 30% inclusion level test diet (98.7 ± 0.3%) was significantly lower ($p < 0.05$) than that of the reference and 10% inclusion test diets (Figure 4.8). Although the digestibility of protein

Figure 4.6

Apparent digestibility coefficients (%) versus time (days) for crude protein from the ground SBM reference diet (⊡), 20% inclusion ground SBM test diet (▩) and ground SBM (■) in Trial 1. Each point represents a single replicate.

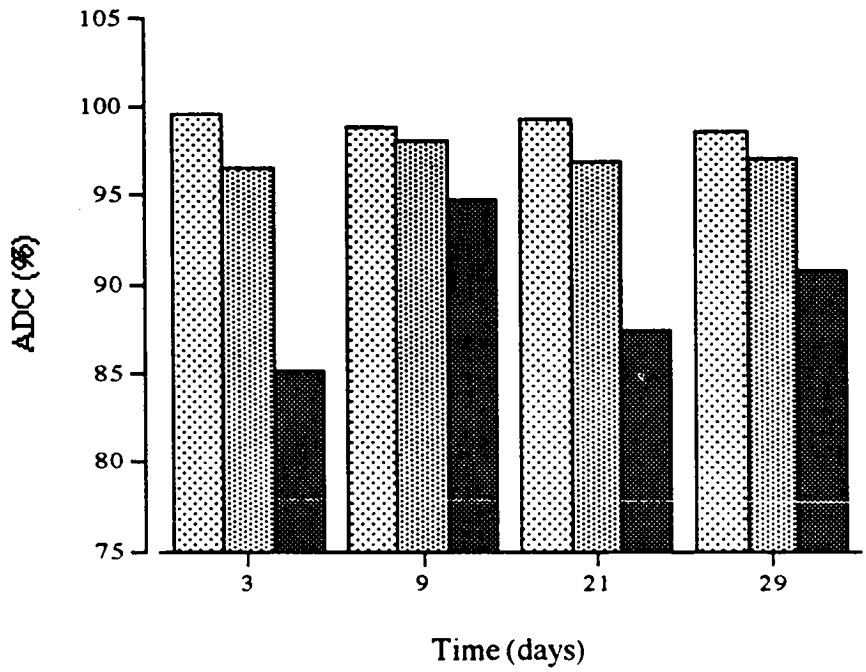

105
100
95
90
85
80
75
ADC (%)
3
9
21
29
Time (days)

Figure 4.7

Apparent digestibility coefficients (%) versus time (days) for crude protein from the reference diet (⊡) and the 20% inclusion sifted SBM (▦) and ground SBM middlings (▩) test diets in Trial 1. Each point represents a single replicate.

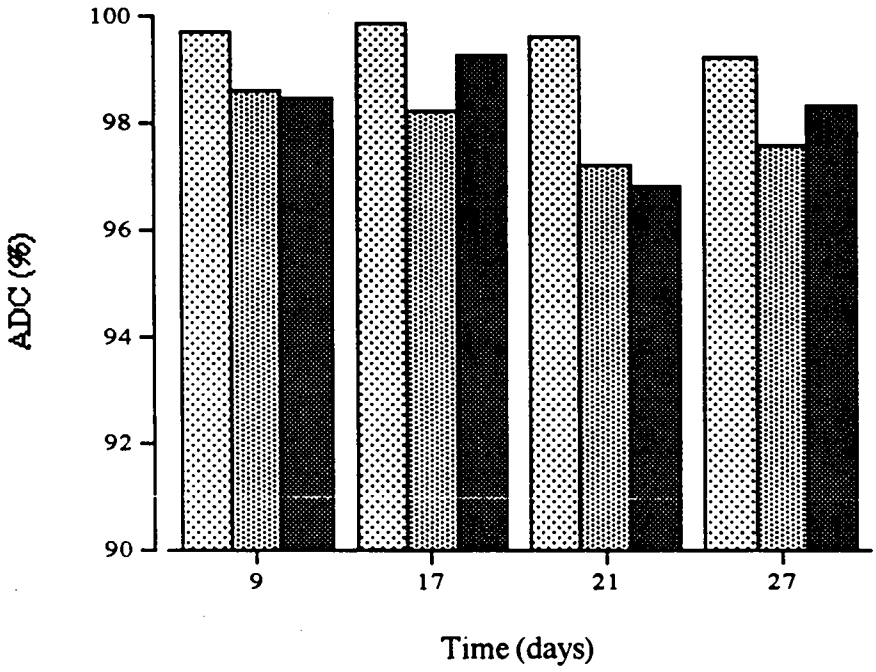
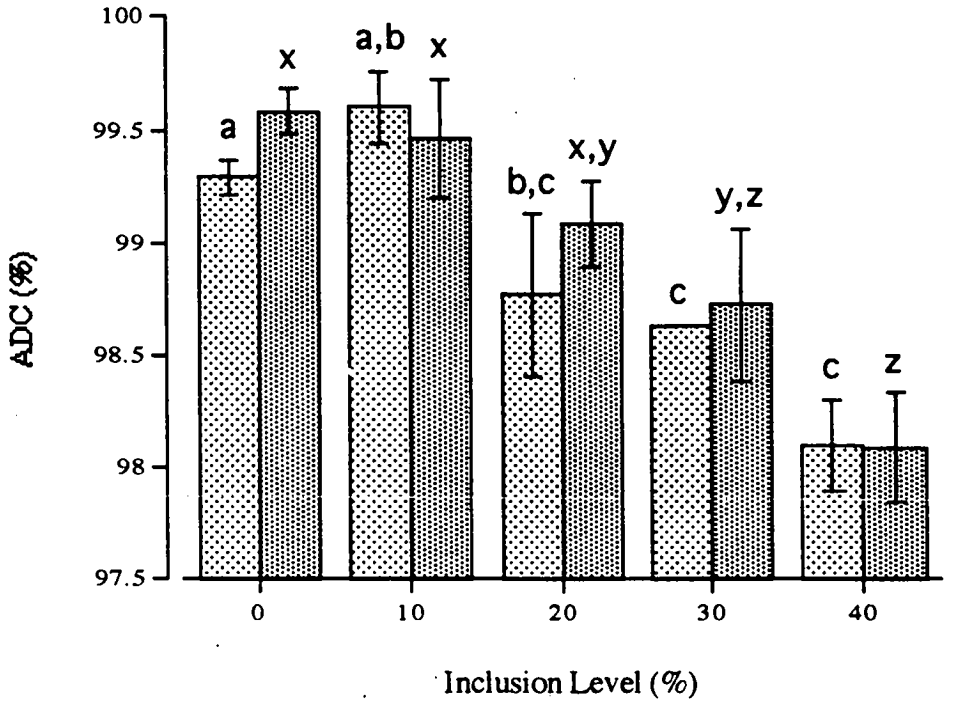

100
98
96
94
92
90
ADC (%)
9
17
21
27
Time (days)

Figure 4.8

Apparent digestibility coefficients (%) of crude protein from the experimental diets measured over days 8 to 17 (⊡) and days 19 to 24 (▦) versus inclusion level (%) in Trial 2. Values are mean ± standard deviation (n = 3). Values with the same superscript within a collection period are not significantly different at $p < 0.05$.


100
99.5
99
98.5
98
97.5
ADC (%)
a
x
a,b
x
b,c
x,y
c
y,z
c
z
0
10
20
30
40
Inclusion Level (%)

from the experimental diets was found to be effected by time using repeated measures analysis of variance, *post hoc* analysis using Scheffé's test failed to show a significant difference ($p > 0.05$) in the digestibility of dietary protein from the respective diets measured at each time point.

4.3.4 Digestibility of Protein in the Test Ingredient

Trial 1:

Variation in the ADC's for crude protein from ground SBM (Figure 4.6), sifted SBM (Figure 4.9) and ground SBM middlings (Figure 4.9) was again evident. Values for each time point are single replicates due to the pooling of faeces. Values ranged from 85.1% to 94.7% for ground SBM, 87.6% to 94.1% for sifted SBM and 87.6% to 96.8% for ground SBM middlings.

The mean ADC's for crude protein in the test ingredients over the four time points measured are detailed in Table 4.7. From this table it can be seen that there is no significant difference ($p > 0.05$, one-way analysis of variance) in the average ADC's for from ground SBM (89.5 ± 4.2%), sifted SBM (91.0 ± 2.7%) and ground SBM middlings (93.1 ± 3.9%).

Trial 2:

ADC's for crude protein from ground SBM are shown in Figure 4.10. Protein digestibility was high at all inclusion levels, being greater than 95%. Repeated measures analysis of variance found no significant effect of inclusion level ($p > 0.05$) nor time ($p > 0.05$) on the digestibility of protein from ground SBM.

4.3.5 Digestibility of Lipid in the Diets

Trial 2:

ADC's of crude lipid from the experimental diets used in the Trial 2 are shown in Figure 4.11. Dietary lipid digestibility varied from 62.5 ± 1.1% (40% inclusion) to 70.8 ± 4.8% (30% inclusion) for days 8 to 17 and from 68.3 ± 0.9% (40% inclusion) to 77.4 ± 0.4% (reference diet) for days 19 to 24, however repeated measures analysis of variance showed no significant effect of inclusion level on dietary lipid digestibility ($p > 0.05$).

Figure 4.9
Apparent digestibility coefficients (%) versus time (days) of crude protein from sifted SBM (⊡) and ground SBM middlings (▦) in Trial 1. Each point represents a single replicate.

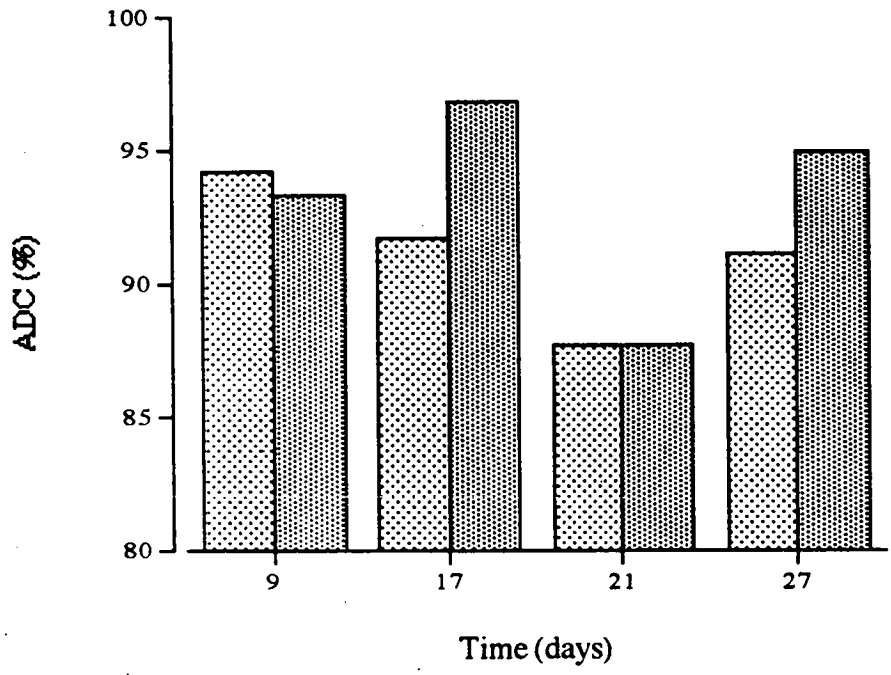

100
95
90
85
80
ADC (%)
9
17
21
27
Time (days)

Figure 4.10

Apparent digestibility coefficients (%) of crude protein from ground SBM measured over days 8 to 17 (□) and days 19 to 24 (▩) versus inclusion level (%) in Trial 2. Values are mean ± standard deviation (n = 3).

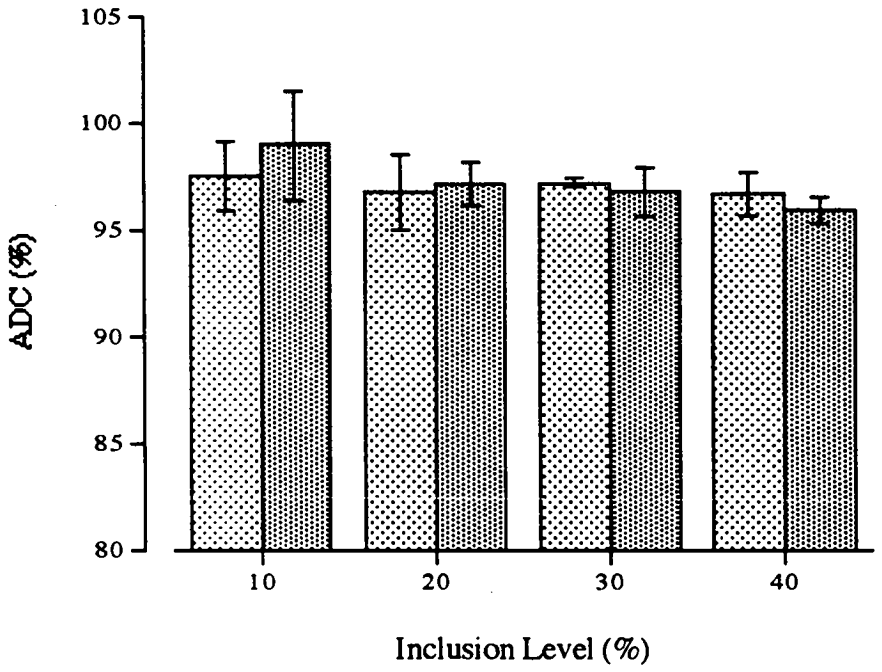
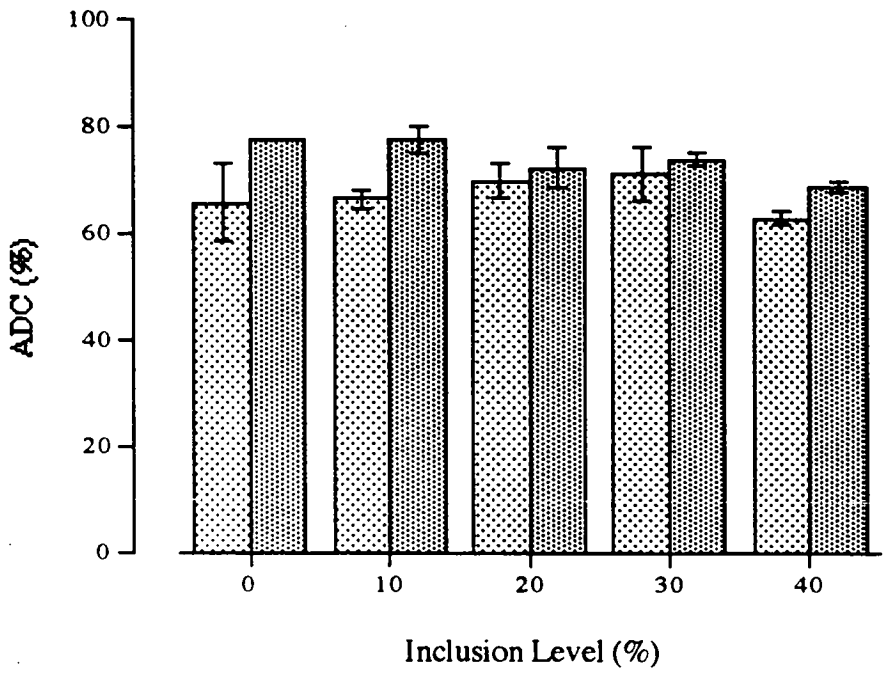

105
100
95
90
85
80
ADC (%)
10
20
30
40
Inclusion Level (%)

Figure 4.11

Apparent digestibility coefficients (%) of crude lipid from the experimental diets measured over days 8 to 17 (⊡) and days 19 to 24 (▦) versus inclusion level (%) in Trial 2. Values are mean ± standard deviation (n = 3).


100
80
60
40
20
0
ADC (%)
0
10
20
30
40
Inclusion Level (%)

Repeated measures analysis of variance revealed a significant effect ($p < 0.001$) of time on dietary lipid digestibility. The interaction between inclusion and time was not significant ($p > 0.05$) and therefore the data were pooled over inclusion level. Subsequent *post hoc* analysis using Scheffé's test showed lipid digestibility to be significantly higher ($p < 0.05$) over days 19 to 24 (74.1 ± 4.0%) than over days 8 to 17 (66.9 ± 4.6).

4.3.6 Digestibility of Lipid in the Test Ingredients

Trial 2:

ADC's for lipid from ground SBM in Trial 2 are shown in Figure 4.12. Lipid digestibility for ground SBM was highly variable, particularly at the lower levels of inclusion. Ingredient lipid digestibility tended to decrease at the 40% inclusion level (58.0 ± 2.6%) compared to the 10% (71.8 ± 16.0%), 20% (85.1 ± 15.6%) and 30% (83.3 ± 16.0%) inclusion levels for days 8 to 17. ADC's for lipid from ground SBM on days 19 to 24 showed a similar trend with the exception of the 20% inclusion level value (46.8 ± 19.0%). Digestibility of lipid from ground SBM also tended to be higher for days 8 to 17 than days 19 to 24 for each inclusion level. However, repeated measures analysis of variance showed no significant effect of either inclusion level ($p > 0.05$) or time ($p > 0.05$) on the digestibility of lipid from ground SBM.

4.3.7 Digestibility of Ash in the Diets

Trial 2:

ADC's for ash from the reference and test diets used in Trial 2 are shown in Figure 4.13. Lack of sample at the higher inclusion levels meant that the 30% and 40% test diets digestibility values for days 8 to 17 represent a single experimental replicate. Although ash digestibility showed an increasing trend with inclusion level (Figure 4.13), repeated measures analysis of variance showed that there was no significant effect ($p > 0.05$) of inclusion level nor time on the digestibility of ash from the experimental diets.

Figure 4.12

Apparent digestibility coefficients (%) of crude lipid from ground SBM measured over days 8 to 17 (⊡) and days 19 to 24 (▩) versus inclusion level (%) in Trial 2. Values are mean ± standard deviation (n = 3).

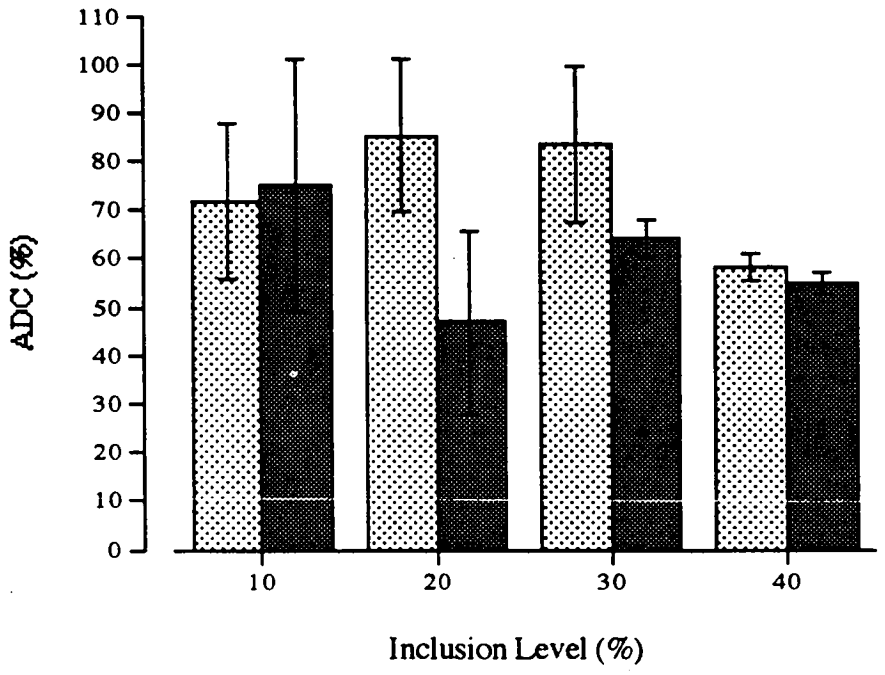
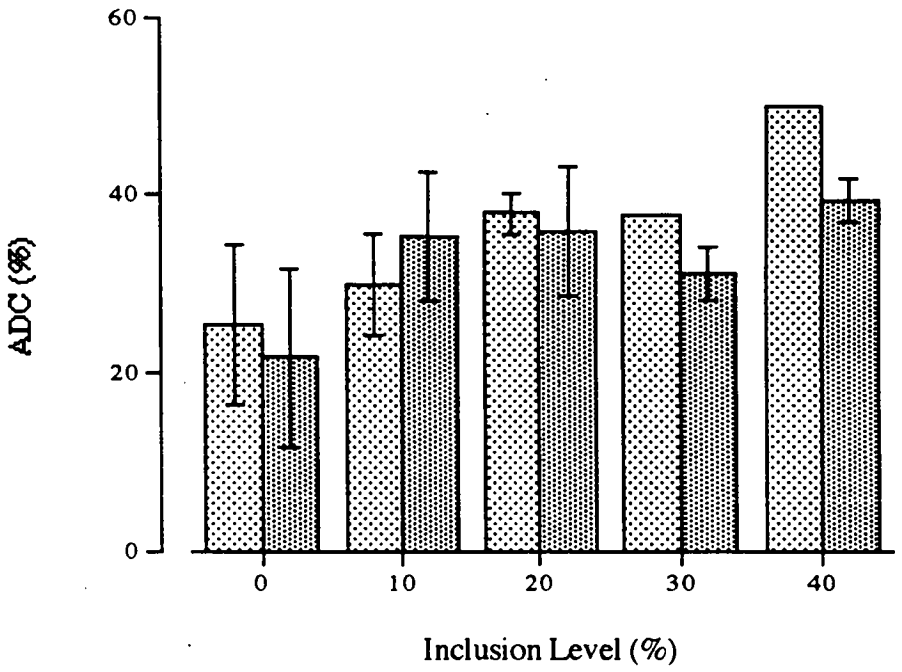

ADC (%)
110
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
Inclusion Level (%)

Figure 4.13

Apparent digestibility coefficients (%) of ash from the experimental diets measured over days 8 to 17 (□) and days 19 to 24 (▩) versus inclusion level (%) in Trial 2. Values are mean ± standard deviation (n = 3, except for the 30% and 40% inclusion diets at days 8 to 17 where n = 1).


60
40
20
0
ADC (%)
0
10
20
30
40
Inclusion Level (%)

4.3.8 Digestibility of Energy in the Experimental Diets

Trial 1:

The ADC's for crude energy from the experimental diets used in Trial 1 are shown in Figures 4.14 and 4.15. Values for each time point are single replicates due to the pooling of faeces. Crude energy digestibility in the experimental diets was found to vary with time, with the sifted SBM test diet showing the largest range of values, 83.5% to 88.2 % (Figure 4.15).

The mean values for the digestibility of crude energy from the experimental diets used in Trial 1 are detailed in Table 4.7. One-way analysis of variance showed the inclusion of 20% of ground SBM (85.0 ± 2.2%), sifted SBM (86.5 ± 2.1%) or ground SBM middlings (86.7 ± 1.7%) did not significantly effect ($p > 0.05$) the digestibility of energy from the relevant reference diet (86.2 ± 1.5% and 89.1 ± 1.3% respectively). Crude energy digestibility was also not significantly different ($p < 0.05$) for the two reference diets.

Trial 2:

ADC's for crude energy from the experimental diets used in Trial 2 are shown in Figure 4.16. Repeated measures analysis of variance found no significant effect of inclusion level ($p > 0.05$) nor time ($p > 0.05$) on the digestibility of dietary crude energy.

4.3.9 Digestibility of Energy in the Test Ingredients

Trial 1:

ADC's for energy from ground SBM, sifted SBM and ground SBM middlings are shown in Figures 4.14 and 4.17. Values for each time point are single replicates due to the pooling of faeces. Again marked variation in the digestibility of energy from the test ingredients was apparent, with values ranging from 63.4% to 90.6% for ground SBM, 65.0% to 82.9% for sifted SBM and 69.8% to 82.2% for ground SBM middlings.

The average of the ADC's for crude energy measured at the four time points for each test ingredient are shown if Table 4.7. There was no significant difference ($p < 0.05$, one-way analysis of variance) in the values for crude energy digestibility for ground SBM (80.1 ± 14.6%), sifted SBM (75.8 ± 7.6%) and ground SBM middlings (77.3 ± 5.4%).

Figure 4.14

Apparent digestibility coefficients (%) versus time (days) for crude energy from the ground SBM reference diet (⊡), 20% inclusion ground SBM test diet (▩) and ground SBM (■) in Trial 1. Each point represents a single replicate.

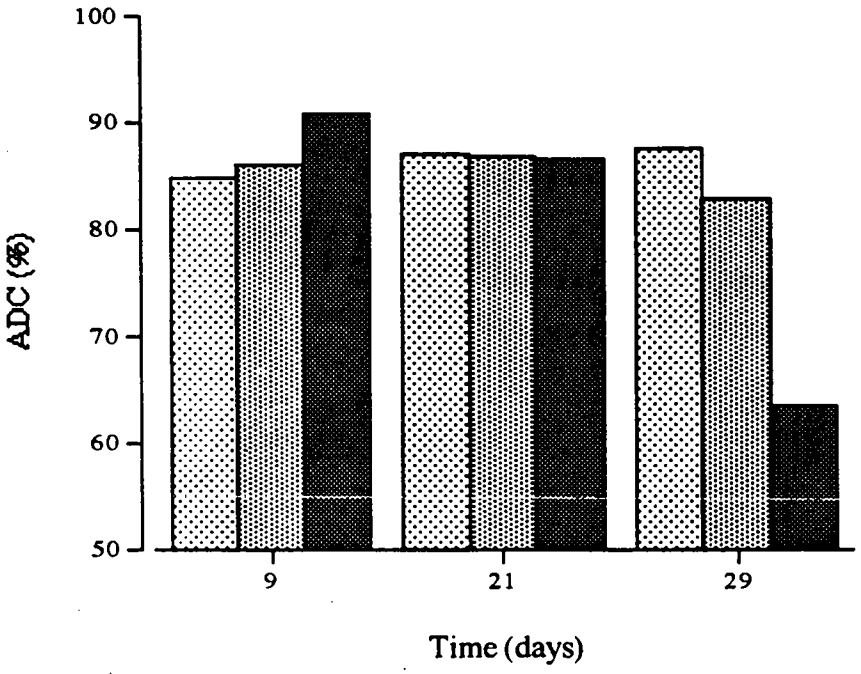

100
90
80
70
60
50
ADC (%)
9
21
29
Time (days)

Figure 4.15

Apparent digestibility coefficients (%) versus time (days) for crude energy from the reference diet (⊡) and the 20% inclusion sifted SBM (▦) and ground SBM middlings (▩) test diets in Trial 1. Each point represents a single replicate.

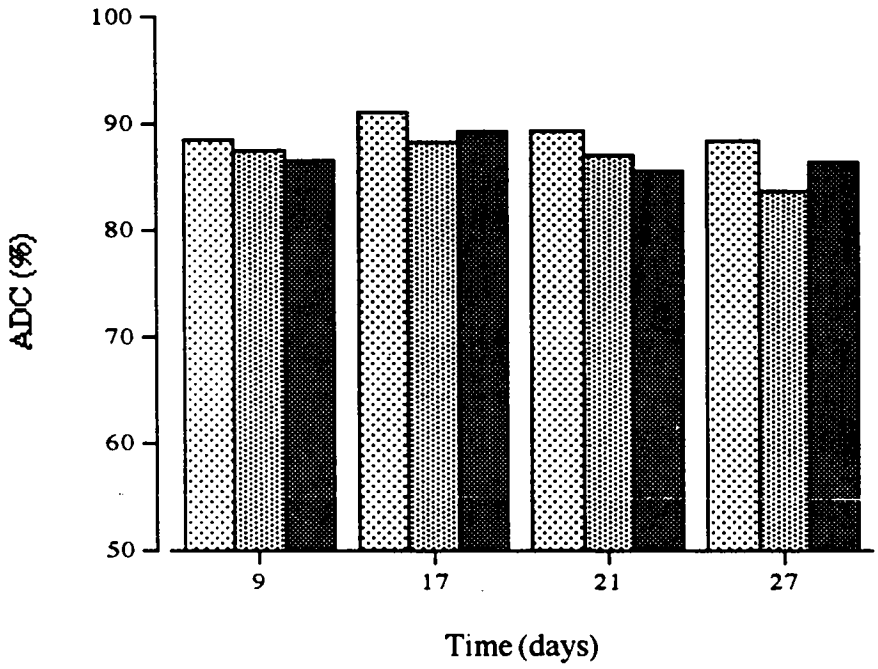

100
90
80
70
60
50
ADC (%)
9
17
21
27
Time (days)

Figure 4.16

Apparent digestibility coefficients (%) of crude energy from the experimental diets measured over days 8 to 17 (⊡) and days 19 to 24 (▦) versus inclusion level (%) in Trial 2. Values are mean ± standard deviation (n = 3).

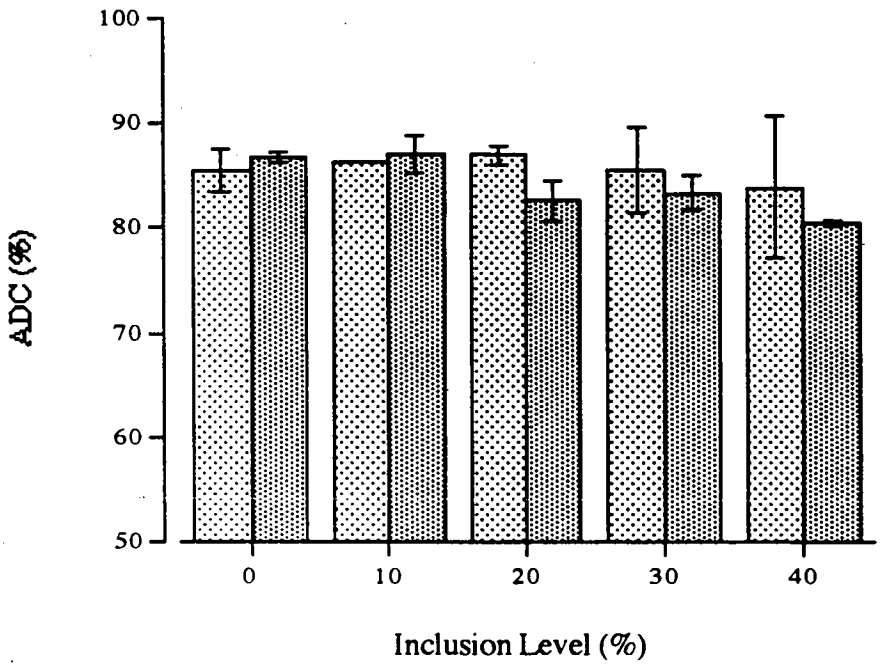
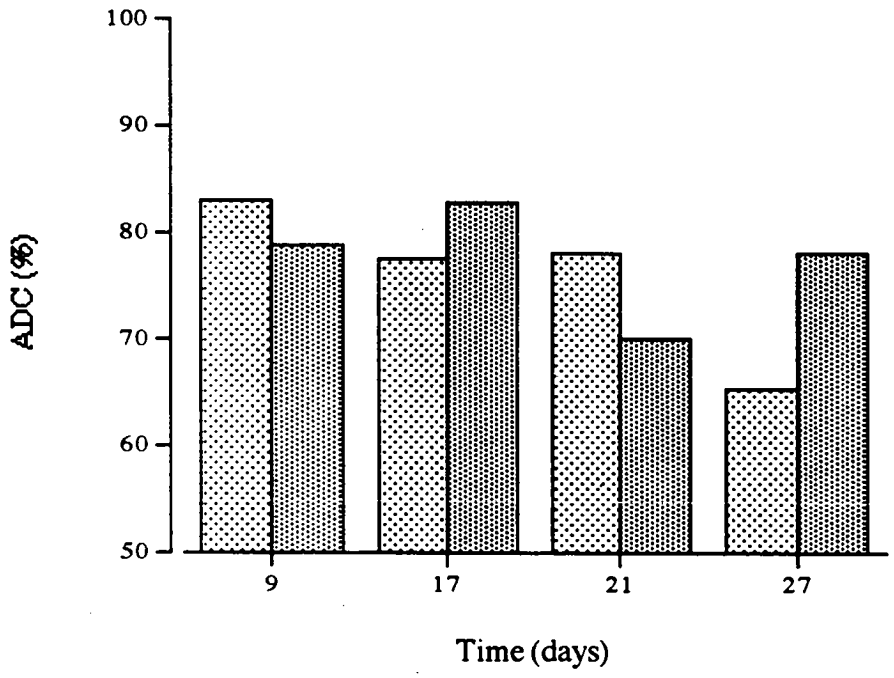

100
90
80
70
60
50
ADC (%)
0
10
20
30
40
Inclusion Level (%)

Figure 4.17
Apparent digestibility coefficients (%) versus time (days) of crude energy from sifted SBM (⚀) and ground SBM middlings (⚅) in Trial 1. Each point represents a single replicate.

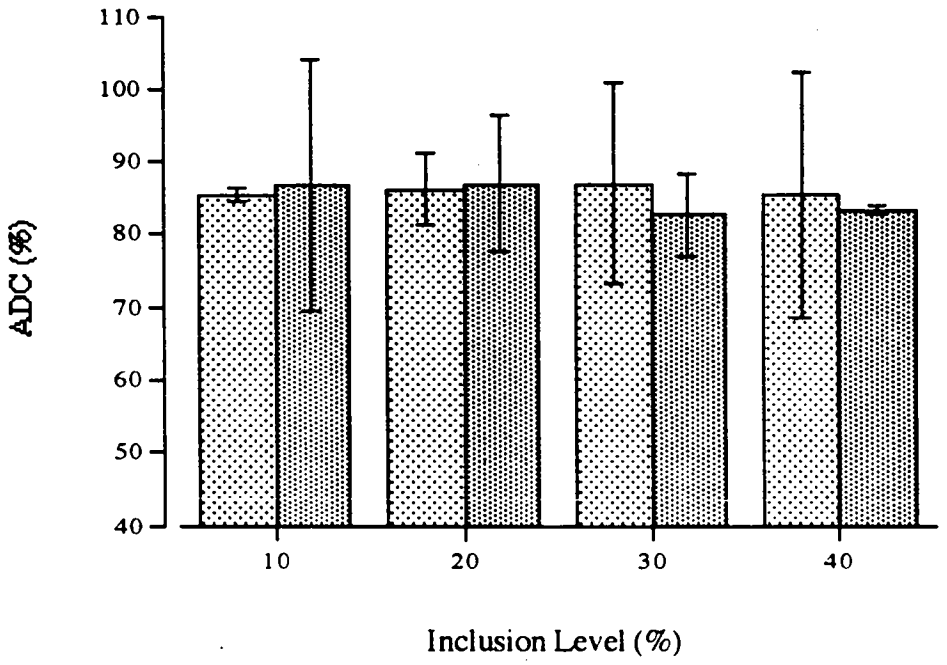

100
90
80
70
60
50
ADC (%)
9
17
21
27
Time (days)

Trial 2:

ADC's for crude energy from ground SBM in Trial 2 are shown in Figure 4.18. Repeated measures analysis showed a significant effect of time ($p < 0.05$) but not inclusion level ($p > 0.05$) on the digestibility of crude from ground SBM. The interaction between inclusion level and time was also not significant (> 0.05) and the data were therefore pooled over inclusion level. Subsequent *post hoc* analysis using Scheffé's test revealed that digestibility of crude energy from SBM was significantly higher ($p < 0.05$) over days 8 to 17 (88.4 ± 9.46%) than days 19 to 24 (77.1 ± 12.4%).

Figure 4.18

Apparent digestibility coefficients (%) of crude energy from ground SBM measured over days 8 to 17 (⊡) and days 19 to 24 (▩) versus inclusion level (%) in Trial 2. Values are mean ± standard deviation (n = 3).


110
100
90
80
70
60
50
40
ADC (%)
10
20
30
40
Inclusion Level (%)

4.4 Discussion

If SBM is to be used as a replacement for fishmeal in artificial diets for aquaculture it is necessary to accurately determine its digestibility in order to maintain the required digestible protein level and amino acid ratios. If the digestibility of SBM varies with the method of processing, the level of inclusion or time then these factors need to be quantified. In the present study the effects processing, inclusion level and time on the digestibility of SBM for common carp have been determined.

The portion of a plant used as a dietary ingredient has previously been shown to effect the digestibility of dietary nutrients (Olli *et al.*, 1992; Arnesen and Krogdahl, 1993). In the present study, Trial 1 investigated the digestibility of crushed SBM that had subsequently been finely ground or sifted. The digestibility of the by-product of the sifting process, the middlings, was also investigated. The effects of different processing methods on the digestibility of SBM has previously been determined for rainbow trout (Olli *et al.*, 1992). The results from Trial 1 showed that the digestibility of ground SBM meal was significantly lower, 43.9%, than that of sifted SBM, 71.9%, and ground SBM middlings, 70.1%. Although it appears low, the digestibility of ground SBM closely approximated the dry matter digestibility of solvent extracted SBM (45.3%) determined by Chu *et al.* (1991) for common carp. The dry matter digestibility of SBM for Atlantic salmon was found to be approximately 60%, and as Atlantic salmon are carnivorous it might be expected that the digestibility of SBM by an omnivorous fish would be greater than this, or at the least, similar. The ADC's determined for sifted SBM and ground SBM middlings appear to support this hypothesis.

The significantly lower digestibility of ground SBM relative to sifted SBM and SBM middlings is difficult to explain. Sifting crushed SBM to produce sifted fines and middlings appeared to differentiate the meal, with the sifted portion containing predominantly cotyledon and the middlings predominantly hull, while the ground SBM maintained a normal contribution of both parts of the seed. It has been found that the distribution of inhibitors in soybeans is not uniform, with the concentration of protease inhibitors two fold greater in the hull than in the cotyledon (Liener, 1980). Therefore, it may be expected that the digestibility of ground SBM middlings would be less than that of sifted SBM, with ground SBM meal having a midpoint value. As this pattern of digestibility was not observed, the partitioning of the constituents of the SBM due to the method of processing would not appear to be responsible for the observed differences in digestibility. A possible explanation for the low digestibility of ground SBM may be the adaptation of the animals to the experimental diets and

conditions. Animals used in the ground SBM digestibility experiment were more recently collected from the wild and thus had less time to adapt to the aquarium conditions and to feeding on artificial diets. Such a reason appears to be supported by the digestibility of SBM at 20% inclusion during Trial 2 of 76.9%. In this later trial, all animals had been in captivity for greater than 12 months. The similarity in digestibility of sifted SBM and ground SBM middlings seen in the present study reflects the observation that SBM hulls only caused a minor decrease in protein digestibility for rainbow trout (Olli *et al.*, 1992).

The method of processing did not significantly affect the digestibility of protein or energy. Protein digestibility ranged from 85.1% to 96.8% over the duration of the trial with mean values for ground SBM, sifted SBM and ground SBM middlings of 89.5%, 91.0% and 93.1% respectively. These values are slightly higher than the 80.5% and 83.7% determined by Chu *et al.* (1991) and Atack *et al.* (1979) respectively for the common carp, although it is similar to the protein digestibility reported for rainbow trout (Watanabe and Pongmaneerat, 1993), and channel catfish (Wilson and Poe, 1985). Other studies have reported ADC's for SBM protein ranging from 70% to 85% (Smith *et al.*, 1980; Ferraris *et al.*, 1986; Lorico-Querijero and Chiu, 1989; Anderson *et al.*, 1992; Hossain *et al.*, 1992; Hajen *et al.*, 1993b). The digestibility of crude energy was 80.1%, 75.8% and 77.3% for ground SBM, sifted SBM and ground SBM middlings respectively. These values are again slightly higher than the 64.7% reported by Chu *et al.* (1991) for carp, although they are within the range of values reported for other species, 65% to 80% (Smith *et al.*, 1980; Wilson and Poe, 1985; Hajen *et al.*, 1993b; Watanabe and Pongmaneerat, 1993).

The effect of inclusion level on the digestibility of ground SBM and its constituent nutrients was determined in Trial 2. In this trial, dry matter digestibility of ground SBM ranged from 86.3% at 10% inclusion to 73.1% at 40% inclusion. These values were again higher than those previously reported for carp (Chu *et al.*, 1991) and chinook salmon (Hajen *et al.*, 1993b). In view of the omnivorous nature of common carp, it is not unexpected that the dry matter digestibility would be in the range observed. Ground SBM digestibility was found to be significantly reduced at greater than 20% inclusion. The decrease in the digestibility of ground SBM may have resulted from the anti-nutritional factors, in particular protease inhibitors. The action of protease inhibitors would suggest a linear decrease in digestibility with the incorporation of SBM. Although the decrease in digestibility was not linear with the incorporation of SBM into the diet, the possibility of increased release of intestinal proteases may have masked the effect of anti-nutritional factors at low levels, 20% inclusion at days 8 to 17, after which a decrease in digestibility was observed. The

ability of fish to compensate for low levels of protease inhibitor has been demonstrated previously (Robinson *et al.*, 1981; Viola *et al.*, 1983; Wilson and Poe, 1985; Krogdahl *et al.*, 1994). The increase in the amount of insoluble carbohydrate in the experimental diet with the inclusion of SBM may also have adversely affected SBM digestibility (De Silva and Anderson, 1995).

The crude protein from the reference diets in Trial 2 was greater than 98% digestible by common carp in all cases. High digestibility of protein from casein-based diets has previously been reported (Kitamikado *et al.*, 1964; Halver, 1989). The digestibility of protein from the experimental diets decreased with the inclusion of SBM.

The digestibility of protein from ground SBM determined in Trial 2 was also high, greater than 95% at all levels of inclusion. Wilson and Poe (1985) reported apparent digestibility of SBM protein to be 95% and 97% for channel catfish from extruded and pelleted diets respectively, however as mentioned previously, reported ADC's for SBM protein have generally been much lower (Smith *et al.*, 1980; Ferraris *et al.*, 1986; Lorico-Querijero and Chiu, 1989; Anderson *et al.*, 1992; Hossain *et al.*, 1992; Hajen *et al.*, 1993b). As faeces were collected by siphoning in the present study, it might be expected that the protein digestibility would be over estimated due to the leaching of water soluble protein (refer section 1.5). Such an effect can be large and may explain the high digestibility of protein reported in the present study.

The digestibility of crude lipid from the experimental diets in Trial 2 ranged from 62.5% to 77.4%, with the digestibility of dietary lipid being significantly greater over days 19 to 24 than days 8 to 17. Dietary lipid digestibility in Trial 2 was lower than that generally reported for fish oil based diets (refer Section 5.1.1.1) and for a mixture of fish oil and SBM oil (5:2) by rainbow trout (Ellis and Smith, 1984). Although lower than previous studies, the ADC's for dietary lipid reported for Trial 2 are consistent with the values determined for tuna oil digestibility by common carp in the present study (refer Chapter 5). The increase in dietary lipid digestibility with time would appear to reflect an adaptation of the digestive physiology of the animals to the experimental diets. A similar increase in tuna oil digestibility with time was found in Chapter 7.

The ADC's of SBM oil by common carp in the present study were highly variable, reflecting the low level of crude lipid in solvent extracted SBM (3.3%). Soybean meal oil digestibility was found to range from 58% to 85% for days 8 to 17 and from 47% to 75% for days 19 to 24, however there was no significant effect of either inclusion level or time on digestibility. Again the digestibility of SBM oil was low compared

with values previously reported for grass carp and Mozambique tilapia, 99% and 93% respectively (Law *et al.*, 1986; Hossain *et al.*, 1992).

The low level of lipid digestibility, both from the experimental diets and from SBM, may be due to the high levels of complex carbohydrate present in the diets. The reference diets contained 10% α-cellulose while the inclusion of SBM into the diets would introduce further indigestible complex carbohydrate into the experimental diets (refer Section 4.1.2.3). It has previously been reported that high levels of complex carbohydrate may adversely affect lipid digestibility by fish (Steffens *et al.*, 1989; Medalé *et al.*, 1991; Fagberno, 1992). A similar effect was also reported in Chapter 3 of the present study.

The mean ADC's for the ash component of the experimental diets over the inclusion levels used in Trial 2 were 36.1% and 32.6% for days 8 to 17 and days 19 to 24 respectively. There was a trend for ash digestibility to increase with the level of inclusion of SBM, however large variance and a lack of replication at the higher inclusion levels prevented this effect from being statistically significant. The ADC's for dietary ash determined are in good agreement with the values for ash digestibility previously reported for fish (Budddington, 1990; Bjorndal, 1985).

Dietary crude energy digestibility in Trial 2 was not affected by the level of inclusion of SBM, although crude energy digestibility was found to decrease with time being significantly lower over days 19 to 24 than days 8 to 17. A consistent digestibility of SBM energy up to 40% inclusion supports the work of Anderson *et al.* (1991) who found the digestibility of crude energy from SBM was not affected by inclusion level up to 60%, although 100% substitution resulted in a significant decrease in energy digestibility. Anderson *et al.* (1991) also found an effect of time on the digestibility of crude energy from SBM, however in that study an increase in crude energy digestibility with an increase in the adaptation period to the diet (1 week versus 15 weeks). Although the change in the digestibility of SBM energy in these studies was not consistent, it would appear that time can significantly affect digestibility.

The digestibility of the experimental diets, test ingredients and dietary and ingredient nutrients in Trial 1 all showed a tendency to vary with time. This variation appeared to be cyclic and showed no real trend for an increase or decrease in digestibility throughout the trial. A similar temporal variation in digestibility has previously been reported by De Silva and Perera (1983; 1984). Pooling of faeces over a greater number of consecutive days in Trial 2 appeared to mask much of the temporal variation observed in Trial 1. However, the digestibility of dietary lipid and SBM crude energy were both found to change with time in Trail 2. It appears that the effect of time on

of time on digestibility observed in the present study consisted of a short term (daily) variation and a long term component. The daily variation in digestibility would appear to be due to a rhythmic cycle in the digestive physiology of the animal, while the long term changes in digestibility of the diets reflect adaptations in the digestive physiology of the animals to the experimental diets. Such changes in digestibility with time indicate the need to rigorously assess the effect of time on digestibility before values are used for the formulation of experimental or commercial diets.

Chapter 5

Factors Effecting Tuna Oil Digestibility

5.1 Introduction

As in higher vertebrates, fish require dietary lipid to provide fatty acids for energy, as precursors for hormone synthesis and as essential components in cell membranes. In artificial diets for fish, this lipid is predominantly supplied in the form of triglycerides. The processes by which these triglycerides are digested and absorbed are detailed in Sections 1.2.4 and 1.3.2 respectively. In brief, polyunsaturated fatty acids are preferentially hydrolysed from the triglyceride molecules, while very long chain monoenoic and saturated fatty acids are least digested. The resultant products of digestion, free fatty acids, mono- and di-glycerides and glycerol, are readily absorbed. Since fish oils contain an abundance of unsaturated fatty acids (Austreng *et al.*, 1979; Takeuchi *et al.*, 1979; Ellis and Smith, 1984), these oils would be expected to be highly digestible. However, little attention has been paid to accurately determining the digestibility of different lipid sources in fish; Phillips and Brockway (in Austreng *et al.*, 1979) suggested that a universal digestibility coefficient of 85% be used when calculating the calorific value for diets.

5.1.1 Determining the Digestibility of Lipid Sources

The majority of studies determining the digestibility of lipid have looked at the digestibility of the lipid fraction of a dietary ingredient or digestibility of the lipid from a whole diet (refer Section 5.1.2.1). To the authors knowledge, only Austreng *et al.* (1979, 1980) have investigated the digestibility of a purified lipid source by including it at a known level (15%) into a reference diet. The adverse effect of elevated levels of dietary lipid on the pelleting properties of diets and the digestive physiology of fish suggests that further work towards developing a methodology for the accurate measurement of the digestibility of purified lipid sources be performed.

5.1.2 Factors Affecting Lipid Digestibility

As a result of the occasional nature of such investigations limited information is available on lipid digestibility in fish, with most of the available data from work performed on rainbow trout. From these studies, it has been found that lipids are generally well digested, with digestibility influenced by the source and nature of the lipid, water temperature, animal size, levels of dietary components (including lipid) and feeding rates.

5.1.2.1 Effect of Lipid Source

The digestibility of dietary lipid by fish has been found to vary markedly depending on the source of the lipid. One characteristic affecting lipid digestibility appears to be the melting point of the fat used (Table 5.1). Rainbow trout (80g) adequately digested (85 to 90%) soybean oil, cod liver oil and crude capelin oil, but lipid digestibility was depressed when the melting point of hydrogenated fish oil exceeded 10°C (Austreng *et al.*, 1979, 1980). Takeuchi *et al.* (1979) found hydrogenated fish oil with a melting point of 53°C to be poorly digested by rainbow trout (54 to 75g), compared to pollock liver oil (Table 5.1). Table 5.1 shows that total lipid digestion by common carp decreases with increasing melting point of the lipid source (Takeuchi *et al.*, 1979). Kirchgessner *et al.* (1986) feeding carp (850g) diets ranging in crude fat from 5 to 20% and utilising a very heterogeneous group of lipid sources including animal fats (hardened sea animal fat, tallow and lard) and vegetable oils (soybean, sunflower, palm, peanut and coconut), found average lipid digestibility to be 83%, ranging from 75 to 95%. Mixtures containing lard or beef tallow displayed below average lipid digestibility, possibly due to the hardness of these fats (Kirchgessner *et al.*, 1986).

In the above studies, natural fish oils were well digested by rainbow trout and carp (Table 5.2), a trend that seems consistent in fish. Digestibility of anchovy, herring, capelin and brown scrapie oils at dietary inclusion levels from 9.5 to 43% by adult (1000g) rainbow trout ranged from 83.6 to 89.0%, while mixtures of fish oil and soybean oil (5:2) were similarly digestible, 86.6 to 90.8% (Ellis and Smith, 1984). Fish oil, mainly capelin oil, at a 23% inclusion level was 93.5% digestible by 240g rainbow trout (Storebakken and Austreng, 1987), and capelin oil was 89.9% and 98.4% digestible by 48g and 162g rainbow trout at a dietary level of 13.6% (Storebakken, 1985). The digestibility of lipid in a diet containing 8.4% herring oil was 97% for 196g rainbow trout, with digestibility of lipid in a commercial diet at 10.9% being 83.9% (Windell *et al.*, 1978a). Rainbow trout of 150g exhibited lipid digestibility of 92% for diets containing predominantly fish oils (Hilton and Slinger, 1986). Average digestibility of lipid from a commercial diet by rainbow trout of three size classes (18, 207 and 586g) and at three temperatures (7, 11 and 15°C) was 89% (Windell *et al.*, 1978b). Atlantic cod exhibited a lipid digestibility of 85% for a diet containing lipid from squid mantle and capelin oil (Hemre *et al.*, 1989). Lipid from a diet containing fish oils supplemented with 1% soybean lecithin (phospholipid) was 96 to 97% digestible by European sea bass (Spyridakis *et al.*, 1989a; 1989b) and fish oil at a 12.5% inclusion level was 90% digestible by Siberian sturgeon, *Acipenser beari*

Table 5.1 Effect of melting point, animal size and water temperature on lipid digestibility in rainbow trout and common carp. Apparent digestibility coefficients are expressed as percent (%) and animal weights and water temperature are in parentheses.

Lipid Source	Melting Point (°C)	Rainbow Trout[a] (80g) (3°C)	Rainbow Trout[a] (80g) (11°C)	Rainbow Trout[a] (54-75g) (11°C)	Common Carp[b] (32-36g) (12°C)	Common Carp[b] (32-36g) (24°C)	Common Carp[b] (15-17g) (27.5C)
Soybean oil	-8	87.9	89.2				
cod liver oil	-3	90.6	87.2				
crude capelin oil	10	85.8	84.8				
hydrogenated capelin	21	74.6	75.2				
oil	33	68.8	69.5				
	41	46.4	49.0				
pollock liver oil				96.0	91.2	88.7	89.2
beef tallow					72.3	89.7	81.5
hydrogenated							81.5
fish oil	38				72.2	82.9	71.9
	45				37.1	59.9	62.9
	53			14.5	34.1	31.5	39.3

[a] Austreng *et al.* (1979), [b] Takeuchi *et al.* (1979)

Table 5.2 Apparent digestibility coefficients (%) of fish oils for rainbow trout and common carp.

Lipid Source	Common Name	Weight (g)	Digestibility (%)	Reference
Cod liver oil	rainbow trout	80	87.2 - 90.6	Austreng *et al.* (1979)
Pollock Liver Oil	rainbow trout	54-75	96.0	Takeuchi *et al.* (1979)
	common carp	15-17	89.2	Takeuchi *et al.* (1979)
	common carp	32-36	88.7 - 91.2	Takeuchi *et al.* (1979)
Capelin oil	rainbow trout	48	89.9	Storebakken (1985)
	rainbow trout	80	84.5 - 85.8	Austreng *et al.* (1979)
	rainbow trout	162	98.4	Storebakken (1985)
	rainbow trout	1000	89.8	Ellis and Smith (1984)
Herring oil	rainbow trout	196	97.0	Windell *et al.* (1978a)
	rainbow trout	1000	86.3	Ellis and Smith (1984)
Anchovy	rainbow trout	1000	88.6	Ellis and Smith (1984)
Brown scrapie oil	rainbow trout	1000	87.6	Ellis and Smith (1984)

(Médale *et al.*, 1991). A diet containing predominantly fish oils with a dietary lipid level of 4.55%, exhibited 86.3% lipid digestibility for 300g carp (Chu *et al.*, 1991). Fish oil at a 12.1% inclusion level was 90.0% digestible by Mozambique tilapia (Hossain *et al.*, 1992). Diets containing fish silage, HCOOH- and H_2SO_4-treated, supplemented with cod liver and soybean oils displayed 89.3% and 90.7% lipid digestibility respectively for Mozambique tilapia (Hossain *et al.*, 1992). Digestibility of lipid from capelin roe was as high as 98.4% for Artic charr (Ringø, 1991). An unspecified animal oil containing four long chain unsaturated acids was 93% digestible by 34g Nile tilapia (Hanley, 1987). Pompano, *Trachinotus carolinus*, of 150 to 450g exhibited a reduced ability to digest menhaden oil at an 8% dietary lipid level, with a digestibility coefficient of only 68% (Williams *et al.*, 1985). Low digestibility was attributed to a short gut transit time, although fish were sampled by catheter one third of the distance from the rectum to the stomach, probably collecting faecal samples prior to the completion of digestion and absorption. Austreng (1978) reported only 75% lipid digestibility in the posterior intestine of rainbow trout which increased to 90% by the posterior rectum. Williams *et al.* (1985) also multiple-sampled the fish following anaesthesia, potentially biasing the resultant digestibility coefficients.

Digestibility of alternative lipid sources by fish has been found to vary markedly. Digestibility coefficients of lipid for grass carp was high for fishmeal oils (100%), copra cake oil (100%), soybean oil (98.8%) and napier grass (93.8%), while digestibility was greatly reduced for lipid from rice bran (73.4%), carpet grass (40.1%) and maize (19.5%) (Law *et al.*, 1986). The digestibility of lipid from mustard oil cake and soybean meal for Mozambique tilapia was 90.4% and 93.2% respectively, while lipid from diets containing linseed and sesame supplemented with cod liver and soybean oils was 90.9% and 87.2% digestible respectively (Hossain *et al.*, 1992). Similarly, Mozambique tilapia exhibited high lipid digestibility coefficients for diets containing silkworm pupae, cod liver oil and soybean oil, approximately 94% (Hossain *et al.*, 1992). Canola oil was found to be 92% digestible by 125g rainbow trout (Hilton and Slinger, 1986). The digestibility of lipid from a diet containing palm oil was 86.2% for dwarf African catfish fingerlings (Fagbemo, 1992). Dietary lipid composed of equal portions of cod liver oil, corn oil and lard was found to be approximately 95% digestible by white sturgeon (Herold *et al.*, 1995). Average digestibility of lipid from an aquatic macrophyte, *Hydrilla verticellata*, containing 11.25% lipid, was 67.2 % for the green chromid (De Silva and Perera, 1983), however *Hydrilla verticellata* containing 6.5% lipid had a lipid digestibility of only 42.8% for rohu (Ray and Das, 1994). Lipid digestibility from the aquatic macrophyte *Salvinia cuculata* (2.5% lipid) for the rohu was also low, 21.4%, while the

digestibility of lipid from *Lemna polyrhiza* (4.6% lipid), *Eichhornia crassipes* (5.8% lipid), *Pistia stratiotes* (2.5% lipid) and *Nymphoides cristatum* (3.9% lipid) was high, ranging from 85.4 to 95.2% (Ray and Das, 1994). Digestibility of lipid occurring in natural diets ranging from herbivorous to carnivorous and containing lipid levels from 5.9% to 9.8% by Mozambique tilapia was found to be between 13.6% to 59.2%, with no direct relationship of digestibility to the type of material in the diet (De Silva and Perera, 1984).

5.1.2.2 Effect of Water Temperature and Salinity on Digestibility

No effect of temperature on lipid digestibility was found for 80g rainbow trout kept at 3°C or 11°C, refer Table 5.1 (Austreng *et al.*, 1980). Similarly temperature did not affect the efficiency of lipid digestion in 207g and 586g rainbow trout held at 7°C, 11°C or 15°C, however small trout (18g) kept at 7°C had depressed lipid digestibility (80%) compared to animals maintained at 11°C or 15°C (85% and 86% respectively) (Windell *et al.*, 1979b). Takeuchi *et al.* (1979) reported no definite effect of temperature on lipid digestibility for common carp, although digestibility of beef tallow and hydrogenated fish oils with melting points of 38°C and 45°C did appear higher for fish held at 24°C compared to those at 12°C (Table 5.1). An increase in lipid digestibility with increasing temperature for carp has been reported in a review by Steffens (1989).

The digestibility of lipid has been found to be affected by the water salinity. Ringø (1991) found a significant difference in the digestibility of lipid from capelin roe (98.45% and 94.3%) and a commercial aquaculture feed (87.6% and 80.65%) for freshwater reared and saltwater reared Artic charr respectively.

5.1.2.3 Effect of Animal Size

No significant effect of fish size on lipid digestibility has been reported. Lipid digestibility was not significantly different in 19g, 207g and 586g rainbow trout, although there was a trend for more efficient lipid absorption in larger fish, with digestibility coefficients of 85 to 86%, 85 to 89% and 91 to 93% respectively (Windell *et al.*, 1978b). Storebakken (1985) found lipid digestibility to be lower in 48g (89.9%) than 162g (98.4%) rainbow trout. These values were not significantly different, however, due to large variance. The efficiency of lipid digestion from a number of sources was not different in carp weighing 15g to 17g and 32g to 36g (Table 5.1), although these experiments were conducted at different temperatures, 27.5°C and 24°C respectively (Takeuchi *et al.*, 1979).

5.1.2.4 Effect of Dietary Lipid Level

Elevation of dietary lipid level, 6.7% to 18.0%, by treating commercial pellets with olive oil had no effect on lipid digestibility for 40g rainbow trout (de la Higuera *et al.*, 1977). In Siberian sturgeon, an increased in dietary lipid from 12.5% to 21.8% resulted in depressed lipid digestibility, 89.7% to 68.3% (Médale *et al.*, 1991). However, this decrease in lipid digestibility may be due to a concomitant increase in the level of crude starch in the diet, as dry matter digestibility of the diets was also found to decrease, 61.9% to 40.5%.

5.1.2.5 Effects of Non-Lipid Dietary Components

The introduction of binders to improve diet stability is used in the production of aquaculture diets, however these substances may have an effect on nutrient digestibility. The commonly used binders sodium alginate and guar gum were reported to reduce lipid digestibility for both 48g and 162g rainbow trout, although these differences were again not significant due to large variance in the ADC's for the control diet (Storebakken, 1985). Storebakken and Austreng (1987) later found that several sodium alginates decreased lipid digestibility slightly, but significantly, when introduced at a 5% inclusion level into diets for 240g rainbow trout. These effects appeared to be related to the viscosity and not the gelling strength of the binder. European sea bass also exhibited a reduction in lipid digestibility from 97% to 86% when sodium alginate was included in diets at 15% (Spyridakis *et al.*, 1989b).

The use of poorly digested dietary components may also interfere with lipid digestibility. Steffens (1989) reported that the addition of cellulose did not affect lipid digestibility, although the introduction of natural plant fibre impaired lipid uptake, with lipid digestibility being 92% for wheat and 52% for wheat bran. Lipid digestibility for dwarf African catfish was found to decrease from 86.2% to 78.3% following the addition of 15% of a highly fibrous cocoa-pod husk meal to the diet. Further increases in the level of cocoa-pod husk meal to 30% and 45% resulted in only slight reductions in lipid digestibility to 76.0 and 75.7% respectively (Fagbemo, 1992). The digestibility of lipid for Siberian sturgeon was found to decrease with elevated lipid levels. The introduction of crude starch into these diets resulted in decreased dry matter digestibility of the diet, 61.9% to 49.3%, and therefore may also have resulted in the decrease in lipid digestibility (Médale *et al.*, 1991). However, lipid digestibility for Atlantic cod was not affected by the level of inclusion of gelatinised starch up to 30% (Hemre *et al.*, 1989). The low digestibility of fish oil for pompano was attributed to a short gut transit time (Williams *et al.*, 1985), but amongst other

confounding effects, 10% cellulose had been added to the diet, increasing the level of fibre and possibly decreasing lipid digestibility.

The inclusion of chromic oxide into diets for Artic charr resulted in an increased lipid content and altered fatty acid composition of the faeces (Ringø, 1993). Accompanying the increased faecal lipid content was a depression in the numbers of gastrointestinal bacteria flora. In view of this, it was suggested that chromic oxide may affect the digestion and absorption processes of the fish directly or may alter the gut micro flora which subsequently affects the lipid composition of the faeces.

5.1.2.6 Effect of Other Factors on Lipid Digestibility

Although the quantity of food ingested may affect the efficiency of digestion (section 1.4.3), no effect of ration size (0.4 to 1.6 body weight day^{-1}) on lipid digestibility (93 to 94%) was found in 194g rainbow trout (Windell *et al.*, 1978b). However, Steffens (1989) reported that increasing ration size resulted in decreasing lipid digestibility in carp.

Storebakken and Austreng (1987) reported lipid digestibility to increase significantly over the period of three consecutive trials, 90.7, 92.3 and 93.8%, for 240g rainbow trout. This effect was attributed by the authors to changes in fish weight and water temperature throughout the trials. However since lipid comprised 23% of the dry weight of the diet, digestibility may have increased due to the physiological adaptation of the animals to the elevated levels of dietary lipid. Thus there may be an effect of time upon lipid digestibility.

5.1.3 Fatty Acid Digestibility

The digestibility of fatty acids from soybean and fish oils for rainbow trout (80g) showed that the absorption of saturated fatty acids decreased with increasing chain length up to C_{18} (Table 5.3), and saturated fatty acids of longer chain length, up to C_{22}, exhibited enhanced digestibility (Austreng *et al.*, 1979; 1980). Monoenoic fatty acids displayed higher digestibility coefficients than saturated fatty acids of the same chain length, and digestibility generally increased when chain length exceeded C_{18} (Table 5.3). Polyunsaturated fatty acids were highly digestible, 18:3, 20:5 and 22:6 all having digestibility coefficients of 100% (Table 5.3). However, Ellis and Smith (1984) reported the monoenoic and saturated fatty acids from soybean and fish oils to be fairly uniformly digested by adult rainbow trout (Table 5.3), although uptake of the polyunsaturated fatty acids 20:5 and 22:6 was still generally very high (up to 100%).

Table 5.3 Apparent digestibility coefficients (%) of fatty acids for rainbow trout.

	Soybean oil[a]		Cod liver oil[a]		Crude capelin oil[a]		Hydrogenated capelin oil (Melting point)[a]						Soybean & fish oils[b]	Brown scrapie oil[b]
							21°C		33°C		41°C			
Temperature	3°C	11°C	3°C	11°C	3°C	11°C	3°C	11°C	3°C	11°C	3°C	11°C		
Fatty acids														
14:0	-	-	91.3	90.4	85.1	92.7	70.1	79.7	61.0	56.4	40.9	46.0	95	98
16:0	79.4	81.3	82.9	80.0	80.0	78.2	57.7	62.8	51.7	45.7	36.4	41.1	97	86
16:1	-	-	94.0	87.6	86.9	85.1	84.4	82.6	80.0	81.2	59.0	60.4	92	92
18:0	77.4	79.5	80.1	73.6	62.8	55.6	46.0	46.4	46.0	39.4	28.8	33.0	-	-
18:1	87.2	87.8	89.7	85.8	84.5	77.6	76.2	73.7	72.0	73.2	52.6	54.9	93	88
18:2+20:0	96.6	97.3	74.3	59.5	70.8	40.3	45.2	18.5	50.0	61.0	31.1	32.5	-	-
18:3	100.0	100.0	-	-	-	-	-	-	-	-	-	-	-	-
20:1	-	-	93.7	92.8	88.9	94.3	79.0	82.8	73.5	78.3	49.8	52.9	97	95
20:5	-	-	100.0	100.0	100.0	100.0	-	-	-	-	-	-	98	92
22:0	-	-	100.0	100.0	-	-	76.2	79.9	67.7	70.8	35.9	37.0	-	-
22:1	-	-	96.5	97.6	91.3	98.0	83.1	88.6	80.1	83.9	31.1	32.9	97	84
22:6	-	-	100.0	100.0	100.0	100.0	-	-	-	-	-	-	100	94

[a] 80g rainbow trout (Austreng *et al.*, 1979), [b] 1000g rainbow trout (Ellis and Smith, 1984)

Artic charr displayed both patterns of fatty acid digestibility depending on the source of the lipid (Ringo *et al.*, 1991). Fatty acids from a commercial pellet showed decreasing absorption of saturated fatty acids with increasing chain length and monoenoic fatty acids were more digestible than saturated fatty acids of the same length. However, monoenoic and saturated fatty acids from capelin roe displayed fairly uniform digestibility. No effect of temperature on the digestibility of fatty acids was shown for rainbow trout (80g) acclimatised to 3°C or 11°C (Austreng *et al.*, 1979). Fatty acid digestibility was, however affected by the melting point of the lipid source (Table 5.3). The effect was slight for mild hydration (melting point 21°C), becoming increasingly severe with the degree of hydration (melting points 33°C and 41°C). The effect of hydration on fatty acid digestibility was uniform for both saturated and unsaturated molecules (Table 5.3).

Artic charr displayed a decrease in fatty acid uptake when reared in saltwater as opposed to freshwater (Ringø, 1991). Significant decreases in the ADC's for 14:0, 16:0, 18:0, 18:1, 20:1 and 21:6 fatty acids were observed for lipid from capelin oil, while the digestibility of 16:0, 18:0, 16:1, 18:2, 20:5 and 22:6 fatty acids decrease significantly for lipid from a commercial aquaculture diet.

5.1.4 Aims of the Present Study

Fish oils are commonly used to provide essential fatty acids and energy to commercial and experimental diets for fish. From the above it can be seen that the digestibility of these lipid sources by fish is quite variable and dependant on a number of factors. In order to accurately formulate diets it is essential to have a detailed knowledge of the digestibility of all ingredients. The present work was undertaken to quantify the digestibility of tuna oil for common carp. In order to achieve this experiments were performed to:

(a) develop an experimental protocol to accurately determine the digestibility of tuna oil by common carp,
(b) quantify the digestibility of tuna oil at two inclusion levels, and,
(c) determine the effects of time on tuna oil digestibility.

5.2 Materials and Methods

The investigation of lipid digestibility for common carp was performed in two experiments. Due to the difficulty involved in pelleting diets containing an elevated lipid content a pilot study (Trial 1) was undertaken in order to develop an experimental protocol for quantifying the digestibility of purified lipid sources, obtain preliminary data on the digestibility of tuna oil for common carp and to determine the effects of inclusion level and time on the measured digestibility coefficient. Following this, a detailed study (Trial 2) using fewer experimental diets and an increased number of animals and replicates was performed to rigorously determine the factors affecting tuna oil digestibility for common carp.

5.2.1 Experimental Diets

The test ingredient in the present study was commercially available tuna oil (Juro Pty Ltd, Victoria, Australia). The proximate analysis of the experimental ingredient are detailed in Table 2.1.

Trial 1:

The formulation of the experimental diets for Trial 1 are detailed in Table 5.4. To determine the digestibility of tuna oil for common carp, a casein-based reference diet was used (Mackie and Mitchell, 1985). Tuna oil was incorporated into the casein-based reference diet at 5%, 10%, 15% and 20%, resulting in total lipid in the experimental diets ranging from 10.2% diet to 25.4% in the reference diet and 20% inclusion diet respectively (Table 5.4). This range of test ingredient inclusion levels was less than the 30% generally used, however a 15% inclusion level has been previously used in lipid digestibility trials (Austreng *et al.*, 1979; 1980).

Trial 2:

The formulation of the experimental diets for Trial 2 are detailed in Table 5.5. The formulation of the reference diet used in Trial 2 was altered so as to incorporate higher levels of indigestible material, facilitating the uniform movement of marker and dietary components through the gastrointestinal tract (Cho, personal communication). Results from Trial 1 indicated that 10% and 15% inclusion of the test ingredient were most appropriate when determining the digestibility of a purified lipid source (refer Section

Table 5.4 Formulation (percent dry matter) and analysed proximate composition as determined by analysis (percent dry matter) of the experimental diets used in Trial 1.

Dietary Components	Diets				
	Reference	5% Tuna Oil	10% Tuna Oil	15% Tuna Oil	20% Tuna Oil
Casein	60.00	57.00	54.00	51.00	48.00
Wheat gluten	5.00	4.75	4.50	4.25	4.00
Corn flour	10.00	9.50	9.00	8.50	8.00
Tuna oil	9.00	13.55	18.1	22.65	27.2
α-Cellulose	10.00	9.50	9.00	8.50	8.00
Vitamin & mineral premix	5.00	4.75	4.50	4.25	4.00
Cr_2O_3	1.00	0.95	0.90	0.85	0.80
Proximate composition					
Crude protein	58.77	54.98	52.18	49.12*	46.05
Crude lipid	10.23	14.12	18.57	22.60	25.39
Ash	4.25	4.06	3.90	3.62*	3.33
NFE	25.97	26.01	24.57	23.94	24.53
Energy (kJ/g)	23.2	23.3	24.0	26.3	26.8
Cr_2O_3	0.88	0.83	0.78	0.72	0.70

* denotes a midpoint value calculated from those values determined for the immediately preceding and following inclusion due to lack of sample.

Table 5.5 Formulation (percent dry matter) and proximate composition as determined by analysis (percent dry matter) of the experimental diets used in Trial 2.

Dietary Components	Diets		
	Reference	10% Tuna Oil	15% Tuna Oil
Casein	50.00	45.00	42.50
Wheat gluten	10.00	9.00	8.50
Corn flour	10.00	9.00	8.50
Tuna oil	4.00	13.60	18.40
α-Cellulose	20.00	18.00	17.00
Vitamin & mineral premix	5.00	4.50	4.25
Cr_2O_3	1.00	1.00	1.00
Proximate composition			
Crude protein	47.95	41.07	38.71
Crude lipid	5.46	14.43	19.91
Ash	3.85	3.76	3.62
NFE	41.80	39.81	36.77
Energy (kJ/g)	17.6	19.07	18.73
Cr_2O_3	0.94	0.93	0.99

5.4), agreeing with the 15% inclusion used by Austreng *et al.* (1979; 1980). Therefore, tuna oil was incorporated into the casein-based reference diet at 10% and 15%, resulting in total lipid in the experimental diets ranging from 5.5% to 19.9% in the reference diet and 15% inclusion diet respectively (Table 5.5).

All diets were formulated and prepared according to the methodology described in Section 2.7.

5.2.2 Experimental Animals, Feeding and Faeces Collection

Common carp used in these experiments were obtained, maintained and handled as described in Sections 2.2, 2.3 and 2.8 respectively. Faeces were collected daily from aquaria by siphoning as described in Section 2.4.

Trial 1:

Common carp of mean weight 46.7g (range 13.1 to 94.0g, standard deviation 19.8g) were stocked at 16 fish per 70 litre aquaria for Trial 1. Each experimental diet was fed to triplicate tanks at a feeding rate of 2% body weight.wet weight diet.day^{-1}. Feeding was performed using a single daily feeding regime and continued for 27 days.

During Trial 1 faecal collection commenced on day 4 and continued until day 27. Faeces were not collected on days 7, 14, 15, 23 and 24 due to a lack of faeces during the collection period.

Trial 2:

In Trial 2 common carp of mean weight 52.0g (range 14.4 to 115.7g, standard deviation 21.2g) were stocked at 15 fish per 70 litre aquaria. Each experimental diet was fed to five replicate tanks of experimental fish at an initial feeding rate of 1% body weight.wet weight diet.day^{-1}. After fish were observed to be consuming the entire feed allocation, feeding rate was increased to 2% body weight.wet weight diet.day^{-1}. The daily feed allocation was divided into four equal rations which were fed at one hour intervals, ensuring all feed was ingested. Experimental diets were fed for 17 days.

In Trial 2 the faeces were not collected over the initial 7 days in order to allow for adaptation to the experimental diet. Subsequently faeces were collected daily from day 8 to day 17.

5.2.3 Diet and Faeces Analysis

Experimental diets and faeces were dried to a constant weight at 50°C and ground prior to analysis. The experimental diets were analysed for Cr_2O_3, ash, energy, lipid, NFE and protein in both trials. The proximate analyses of the experimental diets used in Trial 1 and Trial 2 are shown in Tables 5.4 and Table 5.5 respectively.

Trial 1:

In Trial 1 the faeces from the three replicates for each treatment tanks were pooled per day. Faeces were subsequently further pooled per treatment over three consecutive days to acquire adequate faeces for proximate analysis, with the exception of day 12 when adequate quantities of faeces for analyses were produced without the need for pooling. Pooling of faeces was based around days 5, 9, 12, 17, 19, 21 and 26. Faeces pooled around days 9, 17, 21 and 26 were analysed for Cr_2O_3, energy, lipid and protein as described in Chapter 2. Ash content of faeces was determined for all experimental groups on faeces pooled around days 9 and 17. Ash content for faeces pooled around day 21 was only determined for the reference, 5% inclusion and 10% inclusion treatments, while for faeces pooled around day 26 ash content was determined for the reference, 10% inclusion and 15% inclusion treatments. Faeces pooled around days 5, 12, and 19 were only analysed for chromium oxide.

Trial 2:

In Trial 2 faeces were pooled over 10 consecutive days, days 8 to 17, for each experimental replicate. Faeces were analysed for Cr_2O_3, ash, energy, lipid, NFE and protein.

All proximate composition analyses were performed as described in Section 2.5.

Apparent digestibility coefficients were determined for diets, ingredients, nutrients and energy using chromium oxide as an inert marker. The ADC's were calculated using the ratio of marker in feed and faeces according to the formulae described in Section 2.6.

5.2.4 Statistical Analysis

Data were analysed using regression analysis, t-test of slopes and repeated measures and one-way analysis of variance as indicated (Zar, 1984; Ott, 1988). *Post hoc* analyses were performed using Scheffe's test. Analyses were performed using

StatView 512+™ statistical analysis software (Brain Power Inc., California, USA) and SPSS (SPSS Incorporated 1990, Michigan, Illinios, USA).

5.3 Results

5.3.1 Digestibility of the Experimental Diets

Trial 1:

The ADC's of the reference diet and test diets in Trial 1 are shown in Figure 5.1. Marked variation with time was observed in ADC's for both the reference diet and test diets, and led to a problem in interpreting the outcome of the experiment. The ADC of the reference diet and 5% inclusion test diet did not change in harmony; the ADC of the test diet for days 9 and 12 actually being greater than those of the reference diet (Figure 5.1). As a result unacceptable values (less than 0%) for the digestibility of the tuna oil were obtained for these days. These problems are not apparent when the changes in ADC's were harmonious (15% inclusion , days 9 to 26, Figure 5.1).

Whole diet digestibility appears to increase with time over the duration of the trial (Figure 5.1).

Trial 2:

The whole diet ADC's for Trial 2 are shown in Figure 5.2. Analysis of variance indicated a highly significant effect ($p < 0.001$) of tuna oil inclusion on whole diet digestibility. Scheffé's test showed the incorporation of 10% tuna oil into the reference diet did not significantly affect whole diet digestibility ($p > 0.05$), with ADC's being 76.9 ± 1.3% and 77.5 ± 0.5% for the reference diet and 10% inclusion test diet respectively. Inclusion of 15% tuna oil into the reference diet caused a significant decrease ($p < 0.05$) in whole diet digestibility (74.3 ± 0.4%) compared to that of both the reference diet or the 10% inclusion test diet.

5.3.2 Digestibility of the Test Ingredient

Trial 1:

The ADC's for tuna oil in Trial 1 are detailed in Table 5.6. As a result of the variations in the digestibility of the reference diet and test diets detailed above (section 5.3.1), the ADC's obtained using a 5% inclusion are spurious with several unacceptable values being present; -37.5%, 114.0% and -2.4% at days 5, 9 and 26 respectively. The incongruity in the variation of ADC's for reference diet and test diets mentioned previously corresponds with these values. It was concluded that 5% inclusion was

Figure 5.1

Apparent digestibility coefficients (%) versus time (days) for the reference diet (□) and the 5% (◆), 10% (○), 15% (▲) and 20% (■) inclusion test diets used in Trial 1. Each value represents a single replicate.

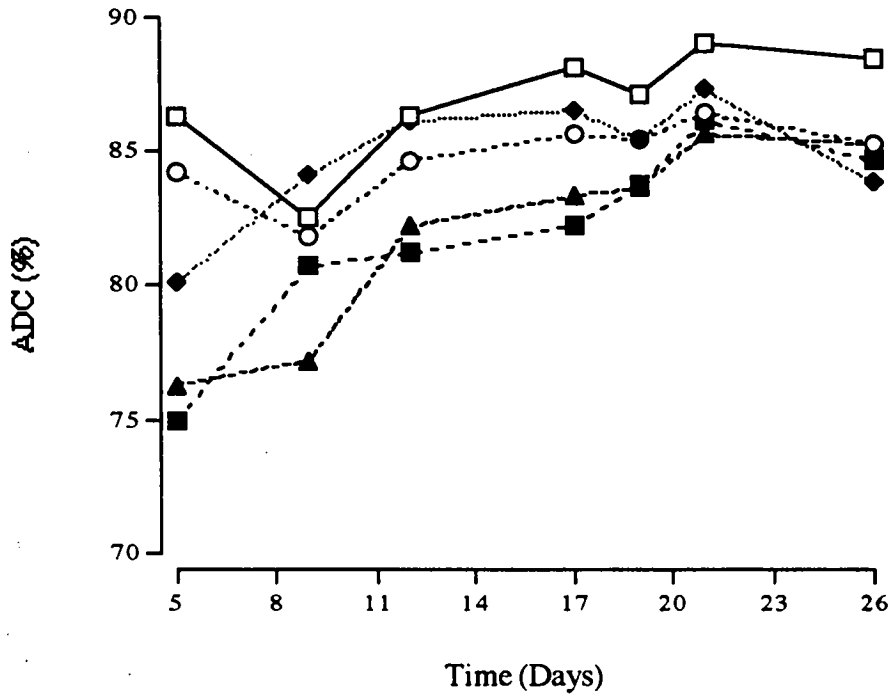
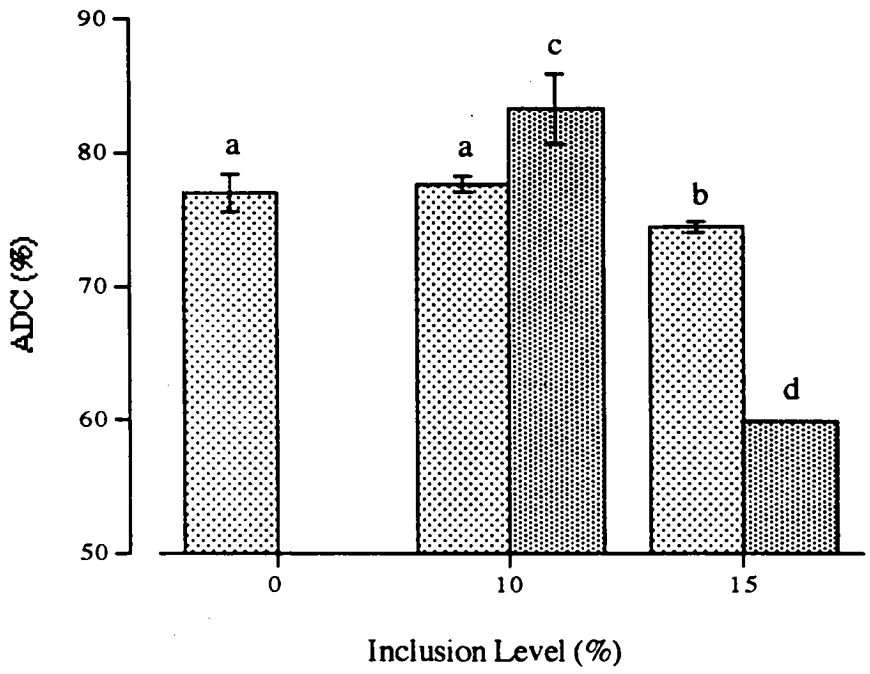

90
85
80
75
70
ADC (%)
5
8
11
14
17
20
23
26
Time (Days)

Table 5.6 Apparent digestibility coefficients (%) for tuna oil as calculated using 5, 10, 15 and 20% inclusion levels and at all time points sampled. Values are mean ± standard deviation.

Day	ADC's Tuna Oil			
	5% Tuna Oil	10% Tuna Oil	15% Tuna Oil	20% Tuna Oil
5	-37.5	66.8	16.7	30.0
9	114.0	76.0	47.4	73.8
12	83.1	69.5	58.9	61.1
17	56.8	63.6	56.4	58.5
19	52.0	69.7	63.7	70.2
21	53.9	62.9	66.0	74.6
26	-2.4	53.3	67.3	69.4

Figure 5.2

Apparent digestibility coefficients (%) of the reference and test diets (⊡) and the test ingredient (▩) versus inclusion (%) in Trial 2. Values are mean ± standard deviation (n = 5). Diet or ingredient ADC's with the same superscript are not significantly different at $p < 0.05$.


90
80
70
60
50
ADC (%)
a
a
c
b
d
0
10
15
Inclusion Level (%)

inadequate to provide meaningful data and so for the remainder of this section, values for the 5% inclusion test diet have not been included.

The day 5 ADC's showed that a measure of uncertainty also existed for digestibility coefficients determined at early time points (Table 5.6). Only one value, 66.8% calculated using the 10% inclusion test diet, appeared appropriate when compared to all other values. Other ADC's for day 5 indicate that the digestibility of the test ingredient is underestimated, being -37.5%, 16.7% and 30.0% for the 5%, 15% and 20% inclusion test diets respectively. Again, values obtained at day 5 will not be incorporated into data used to derive general conclusions.

Though variation was present, the ADC's for tuna oil at the remaining levels of inclusion at the time points measured appeared to be reproducible.

Initial analysis performed on the faeces pooled around days 9, 12, 21 and 26 suggested that digestibility may be enhanced at the 20% inclusion level (Figure 5.3). Analysis of further sampling points revealed a temporal periodicity associated with the ADC's for the test ingredient at the various inclusion levels (Figure 5.4). Mean ADC's for each inclusion level were calculated from the values obtained over the duration of the experiment (Figure 5.5). There was no significant effect of inclusion level on ingredient digestibility ($p > 0.05$, analysis of variance showed no significant difference).

Trial 2:

The ADC's for tuna oil in Trial 2 are shown in Figure 5.2. The digestibility of tuna oil was found to be significantly affected ($p < 0.05$, Student's t-test) by the level of tuna oil incorporation, being higher at 10% inclusion (83.1 ± 5.4%) than at 15% inclusion (59.8 ± 2.6%).

5.3.3 Digestibility of Energy in the Experimental Diets

Trial 1:

The digestibility of crude energy from the experimental diets used in Trial 1 are shown in Figure 5.6. ADC's for crude energy in the experimental diets are mean values calculated from the multiple sampling time points. Energy digestibility was high, ranging from 90.9% ± 2.1% in the reference diet to 87.7 ± 1.7% in the 15% inclusion test diet.

Figure 5.3
Apparent digestibility coefficients (%) for tuna oil versus inclusion (%) in samples pooled around day 21 of Trial 1. Each value represents a single replicate..

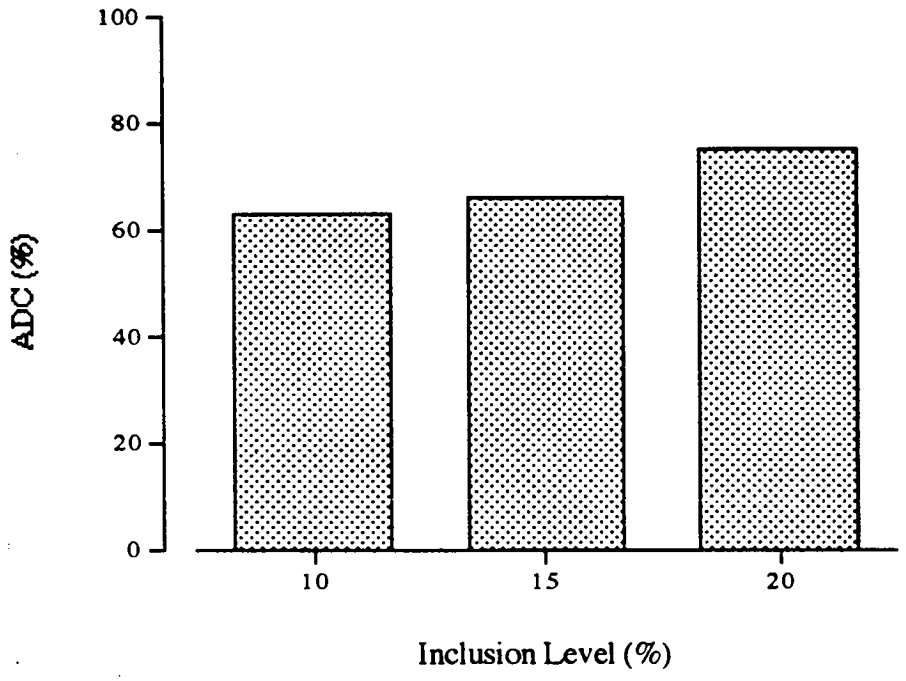

100
80
60
40
20
0
ADC (%)
10
15
20
Inclusion Level (%)

Figure 5.4
Apparent digestibility coefficients (%) for tuna oil included at 20% versus time (days) in Trial 1. Each value represents a single replicate.

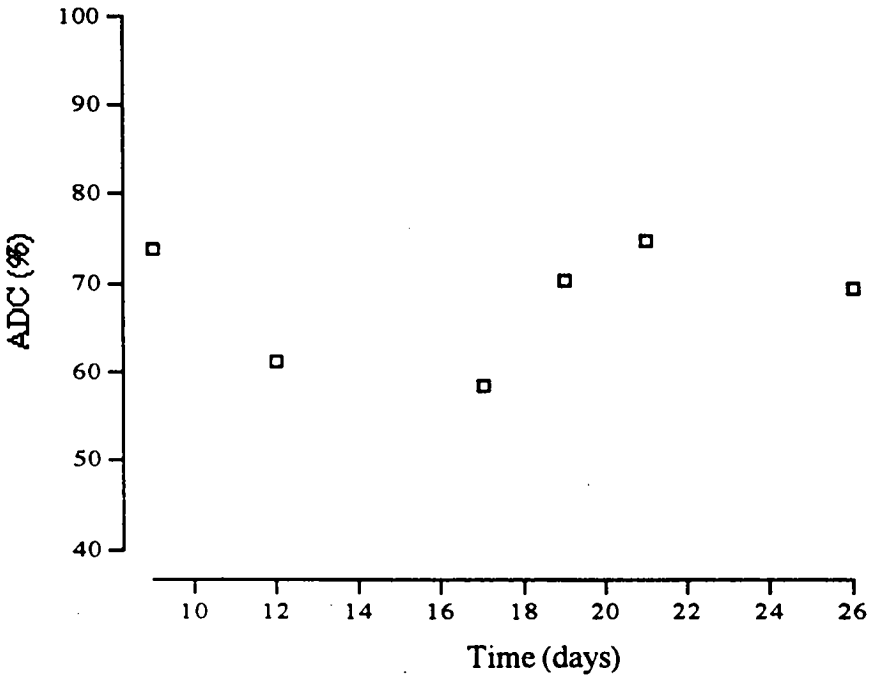

100
90
80
70
60
50
40
ADC (%)
10
12
14
16
18
20
22
24
26
Time (days)

Figure 5.5
Apparent digestibility coefficients (%) for tuna oil versus inclusion level (%) in Trial 1. Values are mean values calculated from all sampling times ± standard deviation (n = 6).

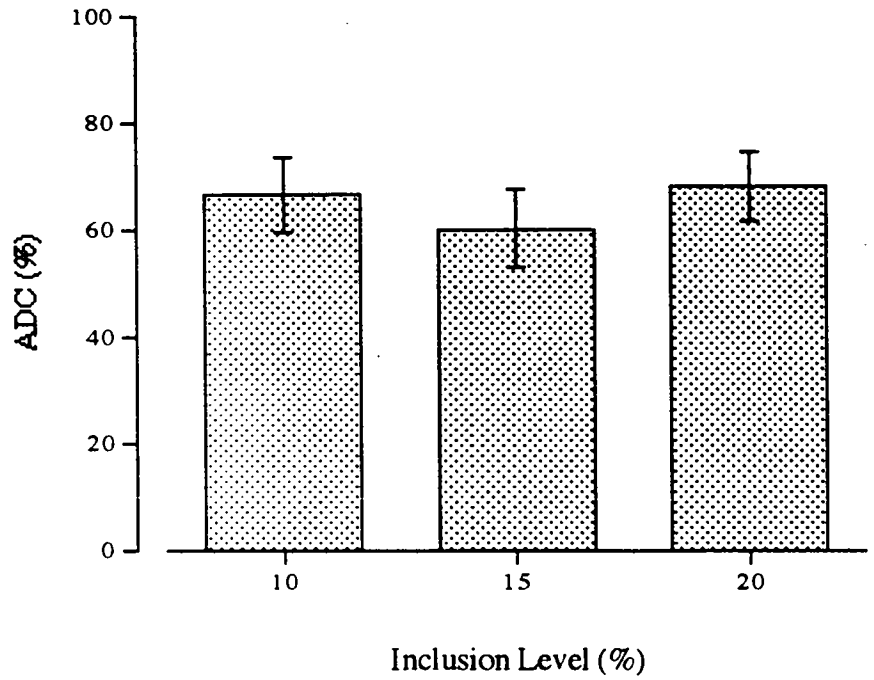
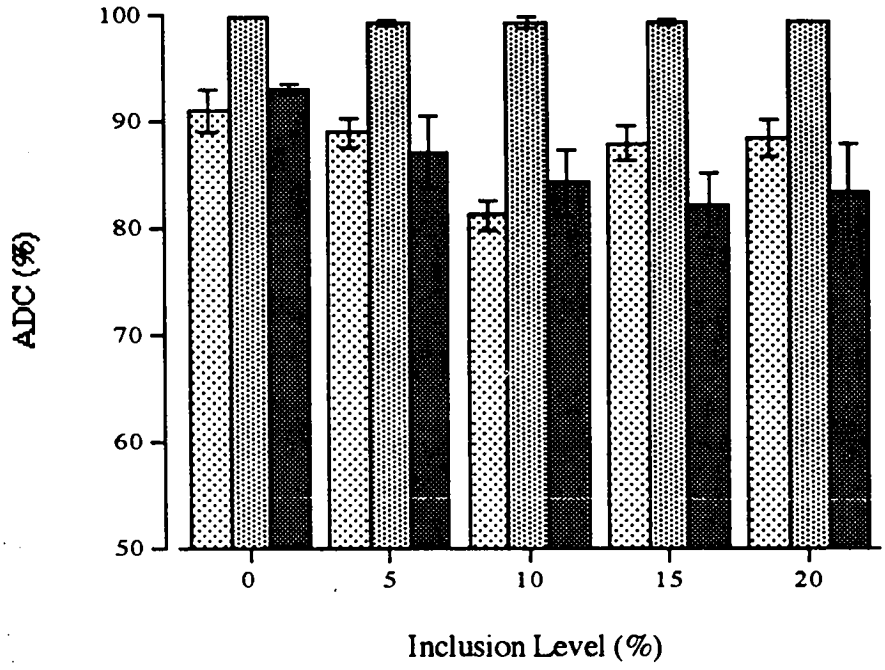

100
80
60
40
20
0
ADC (%)
10
15
20
Inclusion Level (%)

Figure 5.6

Apparent digestibility coefficients (%) for crude energy (⊡), protein (▩) and lipid (■) from the experimental diets used in Trial 1. Values are mean values calculated from all sampling times ± standard deviation (n = 4).


100
90
80
70
60
50
ADC (%)
0
5
10
15
20
Inclusion Level (%)

Analysis of variance showed no significant effect ($p > 0.05$) of the level of tuna oil incorporation on dietary crude energy digestibility.

Trial 2:

ADC's of crude energy in the experimental diets used in Trial 2 are shown in Figure 5.7. Analysis of variance indicated a significant effect ($p < 0.001$) of tuna oil inclusion on dietary crude energy digestibility. *Post hoc* analysis using Scheffé's test showed the incorporation of 10% tuna oil did not significantly effect ($p > 0.05$) dietary crude energy digestibility, with ADC's being 80.7 ± 1.0% and 80.2 ± 0.7% for the reference diet and the 10% inclusion test diet respectively. Inclusion of 15% tuna oil resulted in a significant decrease ($p < 0.05$, Scheffé's test) in dietary crude energy digestibility (73.4 ± 1.9%) compared to the reference diet and 10% inclusion test diet.

5.3.4 Digestibility of Energy in the Test Ingredient

Trial 1:

ADC's for crude energy in tuna oil in Trial 1, calculated as mean values of the ADC's obtained at the time points measured, are shown in Figure 5.8. The values for energy digestibility were not significantly different ($p > 0.05$, analysis of variance) from each other showing no effect of inclusion on energy digestibility. Two way analysis of variance showed the ADC's for energy were also not significantly different ($p > 0.05$) from the ADC's obtained for the test ingredient (Figure 5.5).

Trial 2:

The digestibility coefficients for energy from tuna oil in Trial 2 are shown in Figure 5.9. The digestibility of crude energy from tuna oil was found to be significantly higher ($p < 0.0001$, Student's t-test) at 10% inclusion (75.6 ± 6.5%) than at a 15% inclusion (38.0 ± 4.3%). Two-way analysis of variance showed the ADC's for energy from tuna oil were significantly different ($p < 0.001$) to ingredient digestibility. *Post hoc* analysis using Scheffé's test, showed no significant difference ($p > 0.05$) at a 10% inclusion level, however at a 15% inclusion ingredient digestibility was significantly higher ($p < 0.05$) than energy digestibility.

Figure 5.7

Apparent digestibility coefficients (%) for crude energy from the experimental diets used in Trial 2. Values are mean ± standard deviation (n = 5). Values with the same superscript are not significantly different at $p < 0.05$.

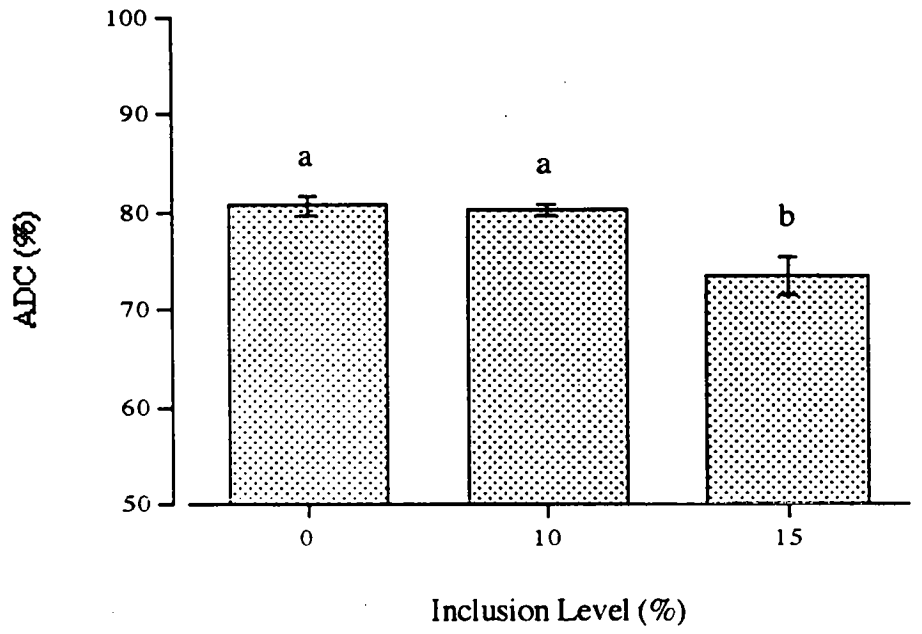

100
90
80
70
60
50
ADC (%)
a
a
b
0
10
15
Inclusion Level (%)

Figure 5.8

Apparent digestibility coefficients (%) for crude energy from tuna oil versus inclusion (%) in Trial 1. Values are mean values calculated from all sampling times ± standard deviation (n = 4).

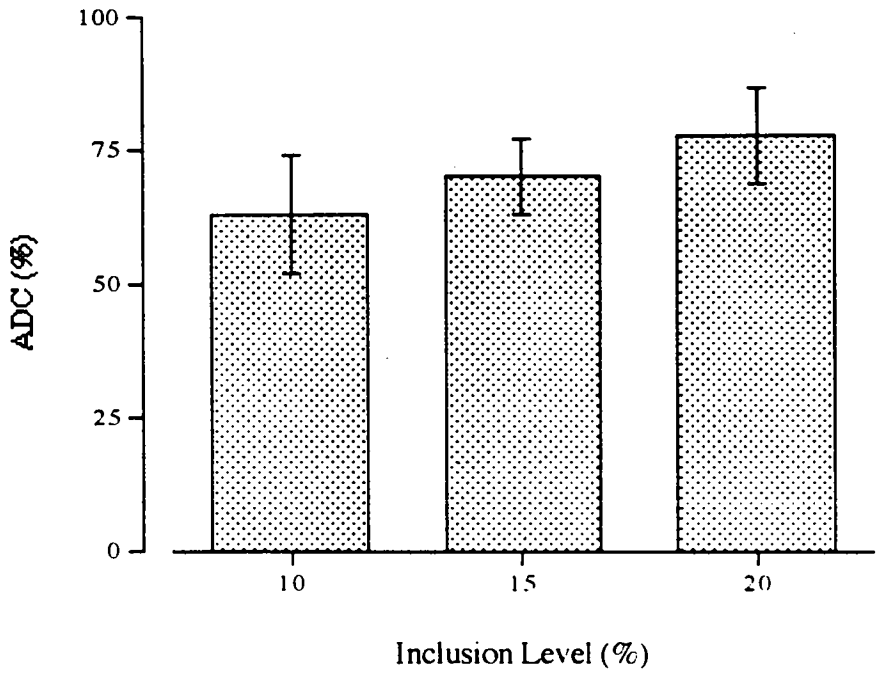
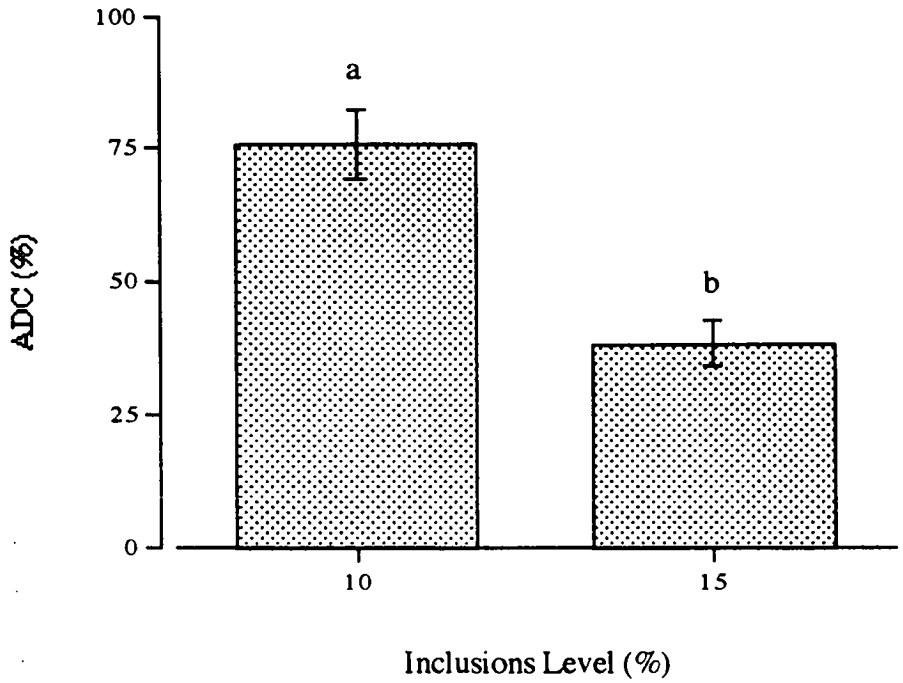

100
75
50
25
0
ADC (%)
10
15
20
Inclusion Level (%)

Figure 5.9

Apparent digestibility coefficients (%) for crude energy from tuna oil versus inclusion (%) in Trial 2. Values are mean ± standard deviation (n = 5). Values with the same superscript are not significantly different at $p < 0.05$.


100
75
50
25
0
ADC (%)
a
b
10
15
Inclusions Level (%)

5.3.5 Digestibility of Protein in the Experimental Diets

Trial 1:

ADC's of protein in the reference diet and test diets used in Trial 1 are shown in Figure 5.6. These values are mean values of the ADC's measured for each time point. Values for the digestibility of protein from the experimental diets were high, greater than 99.0%. Analysis of variance showed whole diet protein digestibility was not significantly affected ($p > 0.05$) by inclusion of tuna oil into the reference diet.

Trial 2:

ADC's for protein from the experimental diet used in Trial 2 are shown in Figure 5.10. Protein digestibility in Trial 2 was again high, greater than 99.0%. Analysis of variance again showed that the incorporation of tuna oil into the reference diet did not significantly ($p > 0.05$) affect whole diet protein digestibility.

5.3.6 Digestibility of Lipid in the Experimental Diets

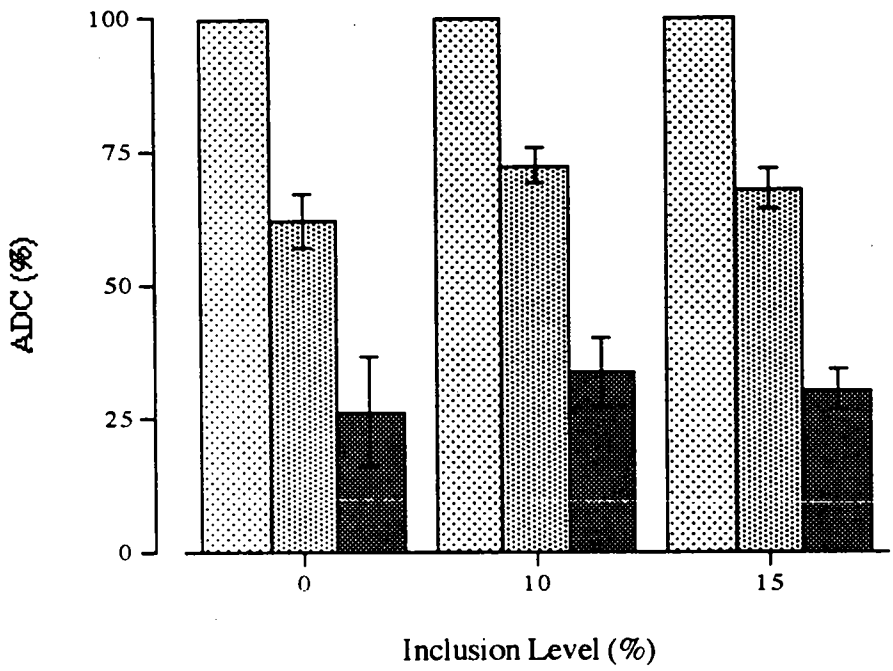
Trial 1:

ADC's for lipid from the experimental diets used in Trial 1 are shown in Figure 5.6. Values are means of the ADC's for each time point measured. Analysis of variance showed that dietary lipid digestibility was significantly effected by the inclusion of tuna oil into the experimental diet. Analysis with Scheffé's test revealed that lipid digestibility for the reference diet (92.9 ± 0.6%) was significantly higher ($p < 0.05$) than for the 10% (84.05 ± 3.4%), 15% (84.1 ± 3.1%) or 20% (83.2 ± 4.5%) inclusion level test diets. Digestibility of dietary lipid did not vary significantly ($p > 0.05$) between the test diets.

Trial 2:

The ADC's of lipid from the experimental diets used in Trial 2 are shown in Figure 5.10. Analysis of variance found a significant effect ($p < 0.01$) of the level of tuna oil inclusion on dietary lipid digestibility. Subsequent analysis with Scheffé's test revealed lipid digestibility was significantly higher ($p < 0.05$) in the 10% inclusion diet (71.9 ± 3.3%) than the reference diet (61.6 ± 5.0%). Digestibility of lipid from the 15% inclusion level diet (67.5 ± 4.0) was not significantly different ($p > 0.05$) to that of either the reference or 10% inclusion level diet.

Figure 5.10
Apparent digestibility coefficients (%) for dietary crude protein (⊡), lipid (▩) and ash (■)versus inclusion (%) in Trial 2. Values are mean ± standard deviation (n = 5).


100
75
50
25
0
ADC (%)
0
10
15
Inclusion Level (%)

5.3.7 Digestibility of Ash in the Experimental Diets

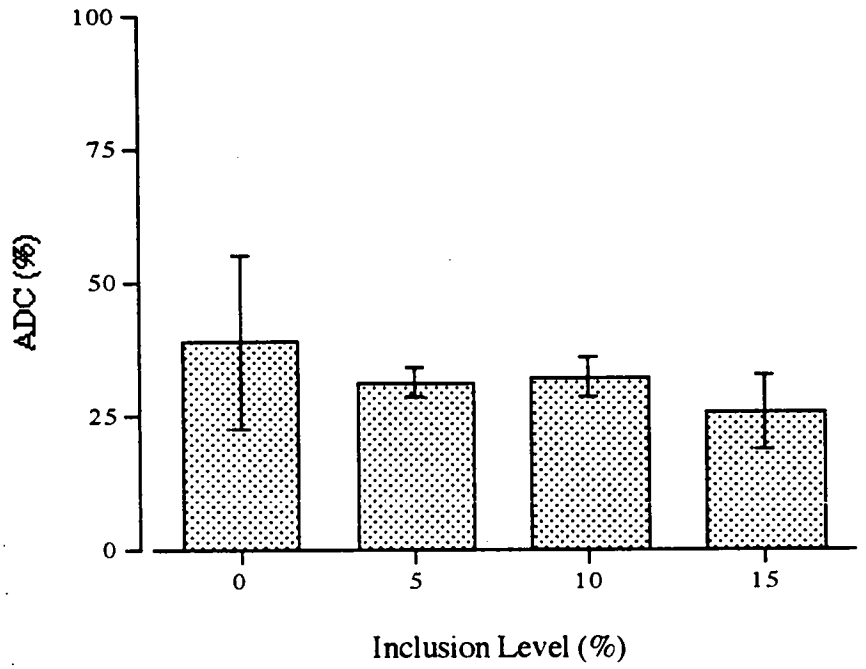
Trial 1:

ADC's for ash from the experimental diets in Trial 1 are shown in Figure 5.11. These values are again mean values of the ADC's at each time point measured. Ash digestibility varied between 25.1 ± 7.0% (15% inclusion diet) and 38.5 ± 16.2% (reference diet), with analysis of variance finding no significant affect ($p > 0.05$) of tuna oil inclusion on dietary ash digestibility.

Trial 2:

ADC's for ash from the experimental diets used in Trial 2 are shown in Figure 5.10. Ash digestibility ranged from 25.9 ± 10.1% (reference diet) to 33.2 ± 6.3% (10% inclusion diet). Analysis of variance again found no significant effect ($p > 0.05$) of the level of inclusion of tuna oil on the digestibility of the ash fraction of the diet.

Figure 5.11
Apparent digestibility coefficients (%) for dietary ash versus inclusion (%) in Trial 1.
Values are mean values calculated from all sampling times ± standard deviation (n = 4).


100
75
50
25
0
ADC (%)
0
5
10
15
Inclusion Level (%)

5.4 Discussion

It is necessary to determine the digestibility of lipid sources in diets for fish for a number of reasons. In formulating commercial diets, it is necessary to determine lipid digestibility so that the appropriate energy balance can be maintained. In formulating experimental diets, it is necessary to know the outcome of replacing one energy source with another in order to design meaningful experiments to examine fish nutrition. If lipid digestibility varies with the level of inclusion or time then these factors must be quantified. No other studies have done so.

Varying levels of tuna oil were included into a reference diets following standard protocols in an attempt quantify the digestibility of a purified lipid source. Subsequently the digestibility of tuna oil for common carp at 10% and 15% inclusion and the effects of the level of inclusion of tuna oil and time were determined.

It was found that the digestibility of both the reference diet and test diets varied with time, up to 5% between consecutive time points. Such variation in digestibility has previously been reported by De Silva and Perera (1983; 1984). The variation observed in the present study was not as substantial as that found by De Silva and Perera (1983; 1984), however these authors performed analysis on daily, unpooled faecal samples. The variation in ADC's reported by De Silva and Perera (1983; 1984) appeared to follow a two day high/two day low cycle. If common carp in the present study follow a similar rhythm, the pooling of faeces over 3 days would decrease the degree of variation, generally grouping either two highs and a low or two lows and a high, but it would not completely alleviate the variation.

This variation in the physiology of digestion has significant ramifications regarding the methodology used to accurately determine the digestibility of a test ingredient and implications about what digestibility of food ingredients really means with regard to provision of nutrients to an animal. In this experiment it was found that the ADC's for the reference diet and 5% test diet did not vary in harmony, resulting in the ADC's of the diets diverging at times and crossing at others. As a result inexplicable values for the digestibility of the test ingredient were calculated. When ADC's of the 5% inclusion test diet was higher than those of the reference diet a value of greater than 100%, or greater than complete digestion, was calculated for the test ingredient digestibility. Alternatively, at other time points, the ADC's of reference diet and 5% inclusion test diet diverged markedly, by more than 5%. A test diet having only a 5% inclusion of the test ingredient was required to account for a digestibility difference greater than itself, leading to the calculation of negative values for ingredient

digestibility. The calculation of ADC's for test ingredients amplifies the inherent variability in the biology of the fish and the chemical analysis of the samples, leading to large errors in the ADC's calculated. Therefore it was concluded the use of 5% inclusion to determine the digestibility of a test ingredient was inappropriate and so were not incorporated into later calculations.

Variability in ADC's of the reference diet and test diets also resulted in what appeared to be an underestimation of the ADC's calculated at the earliest (day 5) time point. The only test diet that varied in apparent harmony with the reference diet was the 10% diet, with the ADC of 66.8% calculated being more closely related to other values determined for this diet. The harmonious variation in digestibility between the 10% test diet and the reference diet is not considered a property of the level of inclusion, but rather a fortunate outcome of the present experiment. The uncertainty in determining ADC's for tuna oil early in the trial may be related to the adaptation of the digestive physiology of the animals to the test diets. This adaptation period appears particularly important when using high lipid diets that could be termed to be "physiologically extreme". It was therefore decided that the earliest time point should not be used in the determination of ADC's and hence the day 5 values were also excluded from the final calculation of test ingredient digestibility.

The ADC's calculated for the remaining diets and time points appeared to be reasonably consistent resulting in reproducible values for the digestibility of the test ingredient. As such these values were used when determining the ADC's of tuna oil.

Initial analysis suggested that digestibility of tuna oil may have been enhanced at the 20% inclusion. However, further analysis revealed that a periodicity or cycling effect is associated with the ADC's for tuna oil at the various levels of inclusion. The increase in tuna oil digestibility with inclusion was apparently an artefact caused by the coincidence of high and low values in the cycles for each test diet. Such cycling of digestibility makes it difficult to calculate the daily ADC's for ingredient digestibility. Such analysis may not only give an inaccurate measurement of ADC for the test ingredient, but may also create an impression of an effect due to inclusion level that may be artefactual. It is thus necessary to demonstrate ADC's to be reproducible over time before they should be accepted. The physiological implications of cycles in digestibility are examined further in Chapter 7.

An attempt was made to overcome the temporal variation in ADC's for the test ingredient during Trial 1 by the use of mean values of the ADC's determined at all time points measured for each level of incorporation of the test ingredient into the reference diet, resulting in mean ADC's of 65.8 ± 7.8%, 60.0 ± 7.4% and 68.8 ± 6.7% at 10%,

15% and 20% inclusion respectively. These values for tuna oil digestibility are low compared to the values previously found for other lipid sources for carp (Takeuchi *et al.*, 1979; Chu *et al.*, 1991) and other species (Windell *et al.*, 1978a; 1978b; Austreng *et al.*, 1979; Austreng *et al.*, 1980; Ellis and Smith, 1984; Storebaken and Austreng, 1987; Spyridakis *et al.*, 1989; Médale *et al.*, 1991; Hossain *et al.*, 1992). The ADC's in the present study reflect better the digestibility of hydrogenated fish oils (Austreng *et al.*, 1979; Takeuchi *et al.*, 1979; Austreng *et al.*, 1980), although menhaden oil digestibility for pompano was also found to be only 68% (Williams *et al.*, 1985). A general trend was observed for the digestibility of the experimental diets and tuna oil to increase over the duration of Trial 1. This may indicate that the physiology of the experimental animals, which were collected from the wild, may still have been adapting to the ingestion of artificial diets, thus resulting in the low digestibility coefficients.

In Trial 2 the dry matter digestibility of tuna oil at 15% inclusion (59.8 ± 2.6%) was similar to that determined in Trial 1. However the digestibility of tuna oil determined at 10% inclusion was notably higher in Trial 2 (83.1 ± 5.4%) than in Trial 1. The ADC for tuna oil at 10% inclusion better approximates those values for lipid digestibility found in the literature for common carp (Takeuchi *et al.*, 1979; Chu *et al.*, 1991) and other species (Windell *et al.*, 1978a; 1978b; Austreng *et al.*, 1979: 1980; Ellis and Smith, 1984; Hossain *et al.*, 1992). The reason for the greater tuna oil digestibility at 10% inclusion in Trial 2 is not clear, although it may reflect the better adaptation of the experimental animals to digesting artificial diets. The continuing low digestibility of tuna oil at 15% inclusion indicates that the additional increase in tuna oil either requires further physiological adaptation or that 15% inclusion has surpassed the maximal lipid digestion rate of the animal. The results from daily analysis of digestibility indicate a prolonged adaptation of experimental animals to experimental diets containing high levels of lipid incorporation (refer Chapter 6).

The digestibility of crude lipid from the experimental diets used in Trial 1, 83% to 93%, was in the reported range for common carp (Takeuchi *et al.*, 1979; Chu *et al.*, 1991) and other species (Windell *et al.*, 1978a; 1978b; Austreng *et al.*, 1979; Austreng *et al.*, 1980; Ellis and Smith, 1984; Storebaken and Austreng, 1987; Spyridakis *et al.*, 1989; Médale *et al.*, 1991; Hossain *et al.*, 1992). Lipid digestibility from the reference diet was significantly higher than for the test diets, with lipid digestibility from the test diets being uniform at all levels of inclusion. It would therefore seem unlikely that the reduced digestibility of lipid from the test diets compared to the reference diet was due to overloading of the lipid digestion system, as further increases in lipid content would result in a further reduction in digestibility.

Dietary lipid digestibility in Trial 2 was lower than that in Trial 1, being approximately 66%. This difference may reflect the higher levels of complex carbohydrate, cellulose and corn starch, present in the reference diet used in Trial 2. Cellulose and starch have previously been suggested as inhibitors of lipid digestion (Steffens, 1989; Médale *et al.*, 1991), and a decrease in lipid digestibility with the inclusion of cellulose into experimental diets has been shown in the present study (refer Chapter 3). Lipid digestibility increased significantly with 10% inclusion of tuna oil, while the inclusion of a further 5% of tuna oil resulted in a subsequent reduction in dietary lipid digestibility.

The results from Trial 1 showed no significant effect of inclusion level on dietary lipid digestibility. This is in accordance with the study of de la Higuera *et al.* (1977) who found no effect of inclusion on dietary lipid digestibility for rainbow trout. However lipid digestibility by juvenile Siberian sturgeon was found to decrease from 89.7% at a 12.5% lipid level to 68.3% at a 21.8% level (Medale *et al.*, 1991). It should be noted that this study determined the digestibility of total lipid from the diet which included lipid from both fishmeal and fish oil, and that the diet containing elevated lipid levels also possessed a high level of poorly digestible starch which may have interfered with lipid digestion (refer section 5.2.5). Trial 2, however, showed a decrease in lipid digestibility with an increase lipid inclusion from 10% to 15%, similar to that observed by Médale *et al.* (1991). The appearance of an effect of inclusion level on ingredient digestibility in Trial 2 reflects increase in lipid digestibility at 10% inclusion . Whether this is a true effect of inclusion surpassing the lipid digestive capabilities of the animal or an effect of physiological adaptation to the experimental diets remains to be determined.

Analysis of the digestibility of crude energy from the tuna oil in Trial 1 showed no effect of inclusion when mean values over time were used. Analysis of variance revealed that the digestibility of energy of the test ingredient was not significantly different to the ADC of the test ingredient itself. In Trial 2 the digestibility of energy from tuna oil was significantly lower at 15% inclusion than at 10% inclusion. Energy digestibility at 10% inclusion was again not significantly different to dry matter digestibility, however at 15% inclusion the digestibility of energy from tuna oil was significantly lower than dry matter digestibility. It would therefore appear that for purified lipid sources, it may not be acceptable to use the ingredient digestibility as an indicator of energy digestibility at higher levels of inclusion. However more work is required in this area before such a conclusion could be used universally.

It is important when developing experimental diets to ensure that the addition of an ingredient does not alter the digestibility values of other dietary components. The protein portion of the experimental diets used in the present study was supplied by the reference diet and therefore any change in dietary protein digestibility would be due to the addition of tuna oil to the diets. The major protein source used in the present study was casein. Protein digestibility in the experimental diets was found to be high, greater than 99%, for both trials. The high values for protein digestibility in the present study are in accordance with the values reported by Kitamikado *et al.* (1964) and Halver (1989) for casein-based diets and with the data obtained for reference diets described in earlier chapters. Protein digestibility was not significantly affected by the inclusion of tuna oil. This is in agreement with the study of Kitamikado *et al.* (1964) who found protein digestibility for common carp fed a casein-based diet was not affected by the addition of 30% olive oil to the diet. There was also no effect of dietary lipid inclusion on the digestibility of protein from silk-worm pupa for common carp (Kitamikado *et al.*, 1964). Similarly studies on Siberian sturgeon (Médale *et al.*, 1991), rainbow trout (de la Higuera *et al.*, 1977) and channel catfish (Page and Andrews, 1973) showed no effect of lipid on protein digestibility.

The digestibility of ash, although highly variable, was similar in both trials, ranging from approximately 25% to 38%. Digestibility of ash for fish has previously been reported by Buddington (1980) and Bjorndal (1985). The values for ash digestibility found in the present trial are consistent with those reported by those authors. Ash digestibility was not affected by the inclusion of tuna oil into the experimental diets.

Chapter 6

Starch Digestibility

6.1 Introduction

Carbohydrates serve solely as energy sources. In aquaculture diets they are utilised to limit, as far as possible, the catabolism of other dietary nutrients for energy, being a much less expensive alternative to protein and lipid as an energy source. As such, carbohydrate has no essential component and therefore no absolute dietary requirement. Of the potential sources of carbohydrate for use in the production of commercial diets, starch is the most commonly utilised.

6.1.1 Starch Digestibility for Fish

The mechanism of starch digestion has been detailed in section 1.2.4.1. Although possessing the necessary digestive apparatus, fish are generally considered to digest starch poorly. Compared to the digestibility of protein and lipid, starch digestibility in fish is low with digestibility coefficients below 60% commonly reported (Inaba *et al.*, 1963; Smith, 1971; Chiou and Ogino, 1975; Bergot and Breque, 1983; Hemre *et al.*, 1989; Pfeffer *et al.*, 1991). However, the digestibility of starch for fish is not uniform being affected by the technological treatment of the starch, the species of fish, the inclusion level of starch in the diet and the feeding regime employed.

6.1.2.1 Effect of Treatment on Starch Digestibility

One of the major factors affecting the digestibility of starch is the technological treatment of the starch prior to incorporation into the experimental diets. Carbohydrate digestibility has been found to vary inversely to the molecular complexity of the carbohydrate (Smith, 1971; Hung and Storebakken, 1994). Native, or raw, starch generally has a lower digestibility than cooked, or gelatinised, starch. Maize starch digestibility for rainbow trout increased following gelatinisation (Bergot and Breque 1983; Pfeffer *et al.*, 1991; Kim and Kaushik, 1992), as did the digestibility of potato starch (Chiou and Ogino, 1975), wheat starch (Inaba *et al.*, 1963; Brauge *et al.*, 1994) and SBM starch (Pongmaneerat and Watanabe, 1993). Increased maize starch digestibility was found to correspond with an increase in the susceptibility of the starch to degradation by amyloglucosidase (Pfeffer *et al.*, 1991). Chu *et al.* (1991) also found the digestibility of corn starch for carp to be improved by gelatinisation. Forneris *et al.* (1993) found the digestibility of cereals by carp was related to the different gelation levels and/or the processing method employed, with ADC's decreasing from puffed rice to flaked maize, precooked maize and raw maize. Fermentation also resulted in an increase in digestibility of the SBM carbohydrate

(Shimeno *et al.*, 1993). However, Arnesen and Krogdahl (1993) found pre-extrusion did not enhance wheat starch digestibility for Atlantic salmon.

The digestibility of starch has also been found to vary with the source. Chu *et al.* (1991) found digestibility of starch from corn and wheat to be higher than that of rapeseed and SBM starch. Arnesen and Krogdahl (1993) found the digestibility of wheat after-meal starch (the layer beneath the bran removed during processing) to be more highly digestible than crude or pre-extruded wheat starch for Atlantic salmon. Wheat and bread bran were found to be better carbohydrate sources for the European eel than soluble starch, sorghum or potato starch (Degani *et al.*, 1986). The variation in these digestibilities with the source of starch was again thought to be due to the levels of highly complex starch present (Arnesen and Krogdahl, 1993).

6.1.2.2 Effect of Species on Starch Digestibility

Starch digestibility coefficients for rainbow trout, carp, Atlantic cod, Atlantic salmon and white sturgeon are summarised in Table 6.1. The diversity of values reflecting the varying sources, inclusion levels and technological treatments of the starch. From Table 6.1 it can be seen that the digestibility of starch is generally higher in the omnivorous common carp than the carnivorous rainbow trout, Atlantic salmon and Atlantic cod. Raw starch digestibility was markedly higher for carp, 50% to 98%, than for rainbow trout, 22% to 54%, Atlantic salmon, 34% to 73%, and white sturgeon, 32%. The digestibility of cooked starch for carp was again high, rainbow trout appearing to have an intermediate ability to digest cooked starch, with cooked and extruded starch poorly digested by Atlantic salmon and Atlantic cod respectively. Improved starch digestibility by the omnivorous species may be explained by the increase in the intestinal α-amylase activity as the proportion of plantaceous material in the natural diet increases (refer section 1.2.4.1.1). The longer gut length of herbivorous and omnivorous species (refer section 1.2.1) also allows a greater time for the α-amylase to digest the hydrolysis resistant starch.

6.1.2.3 Effect of Inclusion Level on Starch Digestibility

Increasing the inclusion level of starch in experimental diets has generally been found to have an adverse effect on starch digestibility (Table 6.1). Spannhof and Plantikow (1983) showed that the absorption of starch for rainbow trout fed at a restricted rate initially increased in proportion to the amount of starch ingested. Absorption of starch then reached a maximum which varied depending on the quality of the starch, equivalent to a 20% dietary inclusion level for raw cereal or potato starch, and

Table 6.1 Starch digestibility coefficients for rainbow trout, carp, Atlantic cod, Atlantic salmon and white sturgeon.

Common name	Starch Source	Processing Method	Inclusion Level	ADC (%)	Author/s
Rainbow trout	corn	raw	30%	38.1 - 54.5%	Bergot and Breque (1975)
		cooked	30%	86.5 - 90.0%	
	corn	raw	50%	24.0%	Smith (1971)
		cooked	50%	51.6%	
	maize	raw	29%	22 - 45%	Pfeffer *et al.* (1991)
		extruded	29%	58 - 76%	
	maize	raw	30%	31.0 - 63.4%	Aguiree *et al.* (1995)
		cooked	30%	63.9 - 84.7%	
	wheat	raw	37.4%	22.0%	Inaba *et al.* (1963)
		cooked	40.2%	48.2%	
			24.8%	83.3%	
			11.5%	90.0%	
Common carp	potato	raw	49.2%	50%	Chiou and Ogino (1975)
			33.5%	52%	
			14.0%	60%	
		cooked	48.0%	84%	
			34.4%	85%	
			19.1%	85%	
	corn	raw	30%	98.0%	Chu *et al.* (1991)
		cooked	30%	112.5%	
Atlantic cod	potato	cooked	12%	40%	Hemre *et al.* (1989)
			7%	33%	
			3%	26%	

Table 6.1 cont.

Common name	Starch Source	Processing Method	Inclusion Level	ADC (%)	Author/s
Atlantic salmon	wheat	raw	45%	34%	Arnesen and Krogdahl (1993)
			30%	73%	
			15%	50%	
		extruded	45%	28%	
			30%	41%	
			15%	48%	
White sturgeon	corn	raw	27%	32%	Herold *et al.* (1995)

subsequently decreased as the proportion of starch in the diet further increased. Inaba *et al.* (1963) showed the digestibility of cooked potato starch for rainbow trout to decrease from 90% to 48.2% with an increase in inclusion level from 11.5% to 48.2%. Raw potato starch digestibility for carp was found to decrease from 60% to 50% with an increase in inclusion level from 14.0% to 49.2%, however inclusion level did not affect the digestibility of cooked starch (Chiou and Ogino, 1975). Pre-cooked potato starch digestibility for Atlantic cod decreased linearly from 40% to 26% with an increase in inclusion level from 3% to 12% (Hemre *et al.*, 1989). The effect of inclusion level on starch digestibility for Atlantic salmon was found to vary with the source of dietary starch (Arnesen and Krogdahl, 1993). Digestibility of wheat after meal starch was found to increase with inclusion level from 60% at 15% inclusion level to 95% at a 45% inclusion level, however no effect of inclusion level was seen with either crude wheat starch or pre-cooked wheat starch.

The general trend for starch digestibility to decrease with the inclusion level in the diet has been attributed to a negative feedback of starch on α-amylase. Wheat has been shown to contain an albumin which inhibits the action of α-amylase in carp and tilapia (Hofer and Sturmbauer, 1985; Sturmbauer and Hofer, 1986; Natarajan *et al.*, 1988; Natarajan *et al.*, 1992). Potato starch has been shown to adsorb α-amylase in the chyme of rainbow trout, decreasing activity by up to 70% (Spannhof and Plantikow, 1983). Spannhof and Plantikow (1983) also found that increasing levels of starch increased the rate of passage, up to two times, through the gut of rainbow trout. The resultant decrease in time available for starch digestion and the absorption of the products of hydrolysis may also be involved in the reduction in starch digestibility. Increased gut transit rates would also be expected to affect the digestibility of other dietary nutrients. Kitamikado (1964) found crude protein digestibility to decrease from 81% to 74% as starch inclusion increased from 10% to 60%. However, Smith (1971) found protein digestibility to be unaffected by the inclusion of raw or cooked starch into the diets of rainbow trout. An increased inclusion level of starch also did not affect dietary protein or fat digestibility for Atlantic cod (Hemre *et al.*, 1989).

6.1.2.4 Effect of Feeding Regime on Starch Digestibility

The rate at which diets containing starch are supplied to the experimental animals has also been found to affect starch digestibility, with restrictive feeding generally enhancing starch digestibility. Digestibility of starch for rainbow trout was found to be higher when fed at 0.5% body weight.wet weight diet.day^{-1} than at 1.0% body weight.wet weight diet.day^{-1} (Bergot and Breque, 1983). Similarly, Pfeffer *et al.* (1991) found digestibility of starch to be lower for rainbow trout fed to satiety as

opposed to those fed at 1.0% body weight.wet weight diet.day^{-1}, and Windell *et al.* (1978b) found a significant decrease in starch digestibility with an increase in feeding from 0.8% to 1.6% body weight.wet weight diet.day^{-1}. As starch digestibility varied with the feeding rate, Bergot and Breque (1983) suggested that it may be better to define the digestibility of starch at a specific intake level per 100g body weight.day^{-1}.

The decrease in starch digestibility with the increase in feeding rate suggests that the intestinal α-amylase may become overloaded with starch at high ingestion rates. This was supported by Andersen *et al.* (1984) who attributed a plateau in growth rates at elevated level of starch to a limit on the digestibility of starch by Nile tilapia.

Improved growth by rainbow trout fed a daily ration of 2.0% body weight.day^{-1} continuously rather than in individual meals, suggested that continuous feeding of a restricted ration may prevent the overloading of the intestinal α-amylase (Hung and Storebakken, 1994). Therefore it appears that not only is the amount of starch ingested per day important, but also the feeding regime employed.

6.1.3 Aims of the Present Study

Starch plays an important role in the formulation of commercial and experimental diets. A detailed knowledge of the digestibility of starch is therefore required for the effective formulation of aquaculture diets. It can be seen from the above that the digestibility of starch is affected by the inclusion level, feeding regime, source of starch, technological treatment of the starch and the species of fish. Thus starch digestibility must be species specific and take into account the factors which may affect digestibility. The aim of the present study were:

(a) to determine the digestibility of starch for the common carp,
(b) to determine the effects of inclusion level on starch digestibility, and
(c) to determine the effects of time on starch digestibility.

6.2 **Materials and Methods**

In the present study two feeding trials were conducted to determine the digestibility of starch and the effects of time and inclusion level on starch digestibility for common carp. An initial digestibility experiment investigated the digestibility of starch incorporated into a reference diet at a 20% inclusion level (Trial 1). Subsequently a second experiment was conducted to determine the effect of inclusion level on starch digestibility (Trail 2). Both experiments investigated the effect of time on starch digestibility.

6.2.1 Experimental Diets

The experimental ingredient used in this study was starch (corn flour) (Goodman Fielder Mills, NSW, Australia). The proximate analysis of the starch used is detailed in Table 2.1.

Trial 1:

To determine the digestibility of starch at a single inclusion level, a casein-based reference diet was used (Mackie and Mitchell, 1985). Starch was incorporated into the reference diet at a rate of 20% inclusion on a dry weight basis, resulting in a total of 28% starch (dry weight) in the test diet. The formulation of the reference diet and test diet for Trial 1 are detailed in Table 6.2.

Trial 2:

To determine the digestibility of starch at a graded series of inclusion levels, a casein-based reference diet was again used. Starch was incorporated into the reference diet at 10%, 20%, 30% and 40% inclusion levels on a dry weight basis, giving final levels of starch in the test diets of 19% to 46%. The formulation of the experimental diets for Trial 2 are detailed in Table 6.3.

All diets were formulated and prepared according to the methodology described in Section 2.6.

6.2.2 Experimental Animals, Feeding and Faeces Collection

The common carp used in this experiment were collected, maintained and handled as described in Chapter 2. All experimental animals were allocated randomly to aquaria. Faeces were collected by siphoning as described in Section 2.4.

Table 6.2 Formulation (percent dry matter) and proximate composition determined by analysis (% dry matter) of the experimental diets used in the Trial 1

Dietary	Diets	
Components	Reference	20% Starch
Casein	60.0	48.0
Wheat gluten	5.0	4.0
Corn flour	10.0	28.0
Tuna oil	9.0	7.2
α-Cellulose	10.0	8.0
Vitamin & mineral premix	5.0	4.0
Cr_2O_3	1.0	0.8
Proximate Analysis		
Dry matter	63.73	63.10
Crude protein	59.03	48.84
Crude lipid	8.72	6.23
Crude fibre	18.60	15.20
Ash	5.36	4.87
NFE	7.21	23.92
Gross energy (kJ/g)	23.41	21.91
Cr_2O_3	1.08	0.94

Table 6.3 Formulation (percent dry matter) and proximal composition determined by analysis (% dry matter) of the experimental diets used in Trial 2.

Dietary Components	Diets				
	Reference	10% Starch	20% Starch	30% Starch	40% Starch
Casein	60.0	54.0	48.0	42.0	36.0
Corn flour	10.0	19.0	28.0	37.0	46.0
Tuna oil	9.0	8.1	7.2	6.3	5.4
α-Cellulose	15.0	13.5	12.0	10.5	9.0
Vitamin & mineral premix	5.0	4.5	4.0	3.5	3.0
Cr_2O_3	1.0	1.0	1.0	1.0	1.0
Analysed Cr_2O_3	1.00	0.97	0.96	0.95	0.86

Trial 1:

Eighty common carp of mean weight 93.3g (range 27.3 to 202.6g, standard deviation 37.6g) were stocked at 10 fish per 70 litre aquaria. The reference diet and test diet were fed to four replicate tanks of fish at a feeding rate of 2.0% body weight.wet weight diet.day^{-1} using a single daily feeding regime. Prior to the commencement of the experiment, fish were fed a casein-based reference diet. Experimental diets were fed for 32 days.

Faeces collection commenced on day 2 of Trial 1 and continued until day 32. On days 6, 7, 13, 20, 24, 26, 27 faeces were not collected due to a lack of faeces voided during the collection period.

Trial 2:

Three hundred common carp of mean weight 43.6g (range 7.0 to 87.5g, standard deviation. 18.2g) were stocked at 15 fish per 70 litre aquaria. Each experimental diet was fed to four replicate aquaria of fish. The feeding rate was 2.0% body weight.wet weight diet.day^{-1}. The daily ration was fed over four approximately equal feeds at hourly intervals to ensure all food was consumed. Feeding was continued for 17 days.

Animals in Trial 2 were fed the experimental diets for 5 days prior to the commencement of faeces collection to allow the animals to completely void the previous diet from their digestive tract and to adapt to the experiment diets. Faeces were subsequently collected in two five day blocks, days 6-10 and days 13-17.

6.2.3 Diet and Faeces Analysis

Proximate analyses of diets and faeces, and the digestibility of diets, ingredients and nutrients were determined as described in Chapter 2.

Trial 1:

The experimental diets for Trial 1 were analysed for moisture, ash, fibre, gross energy, lipid, protein, NFE and Cr_2O_3. The measured proximate composition of the experimental diets are detailed in Table 6.2.

Faeces collected for each dietary treatment were pooled per day. Subsequently three consecutive days of faeces were pooled for each dietary treatment to obtain adequate

faeces for analyses, pooling based around days 3, 9, 21 and 29. Faeces were analysed for Cr_2O_3, energy and protein.

Trial 2:

In Trial 2 the Cr_2O_3 content of the experimental diets was measured and values are shown in Table 6.3.

Dried faeces were pooled per replicate over each five day collection period, days 6-10 and 13-17. Pooled faeces were again only analysed for Cr_2O_3.

6.2.4 Statistical Analysis

Data were analysed using repeated measures and one-way analysis of variance as indicated (Zar, 1984; Ott, 1988). *Post hoc* analysis was performed using Scheffe's test. Analyses were performed using StatView 512+™ statistical analysis software (Brain Power Inc., California, USA).

6.3 **Results**

6.3.1 Digestibility of the Experimental Diets

Trial 1:

The ADC's for the reference diet and 20% inclusion level test diet are shown in Figure 6.1. As faeces were pooled over replicate tanks and time, each value is a single replicate. Figure 6.1 shows whole diet ADC's for the reference and test diets to vary with time, with values ranging from 75.1% (day 21) to 79.9% (day 9) and 75.2% (day 29) to 85.0% (day 9) for the reference and test diets respectively. Although the digestibility of the test diet appeared to decrease with time, the digestibility of the reference diet showed no such trend.

The mean dry matter ADC's over the four time points measured are shown in Table 6.4. Using one-way analysis of variance, no significant difference ($p > 0.05$) was found between the mean dry matter ADC's for the reference diet and the test diet.

Trial 2:

The ADC's for the experimental diets used in Trial 2 are shown in Figure 6.2. Repeated measures analysis of variance revealed a significant effect of starch inclusion level ($p < 0.001$) and time ($p < 0.01$) on whole diet digestibility. *Post hoc* analysis using Scheffé's test found whole diet digestibility for the reference diet (83.5 ± 1.4%) and 10% test diet (83.1 ± 1.5%) to be significantly lower ($p < 0.05$) than that of the 40% test diet (86.7 ± 0.7%) on days 6 to 10. ADC for the 10% test diet was also significantly lower than that of the 30% test diet (86.3 ± 1.4%) on days 6 to 10. On days 13 to 17 the digestibility of the 40% test diet (89.6 ± 1.6%) was significantly different to that of the reference diet (84.6 ± 0.8%) and the 10% (84.0 ± 1.0%), 20% (86.0 ± 1.3%) and 30% (85.7 ± 1.7%) test diets. Analysis with Scheffé's test failed to show a significant difference ($p > 0.05$) between ADC's measured for each diet at the two time points.

6.3.2 Digestibility of the Test Ingredient

Trial 1:

The ADC's of starch for the preliminary trial are shown in Figure 6.3. Each value is a single replicate due to the pooling of faeces. The digestibility of starch appeared to vary with time (Figure 6.3). Starch digestibility was high, 96.7% and 105.5% on days 3 and 9 respectively, after which digestibility appeared to decrease, with values

Figure 6.1
Apparent digestibility coefficients (%) versus time (days) of the reference diet (⊡) and the 20% inclusion level test diet (▩) used in Trial 1. Each point represents a single replicate.

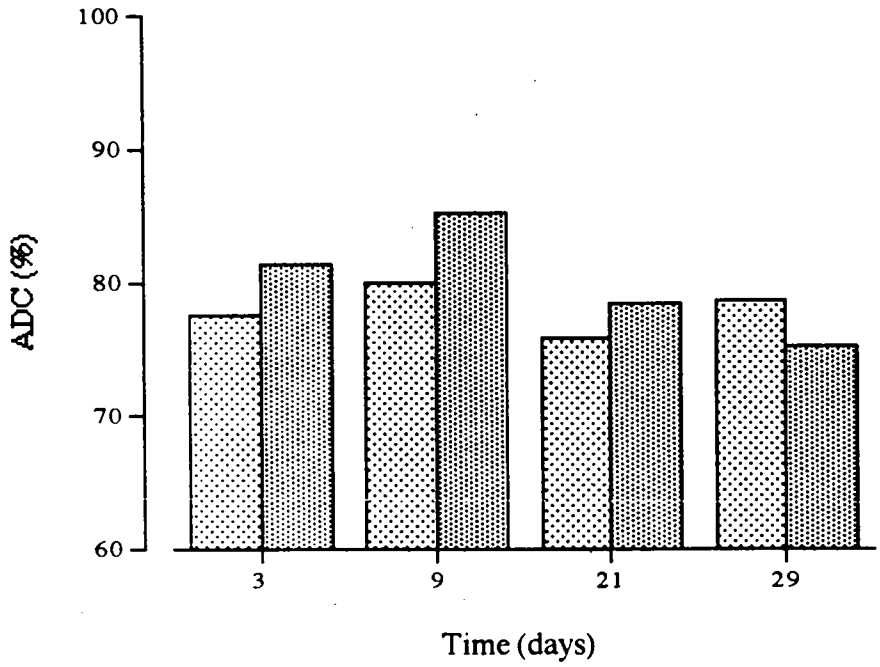

100
90
80
70
60
ADC (%)
3
9
21
29
Time (days)

Table 6.4 Dry matter, protein and energy apparent digestibility coefficients (mean ± standard deviation; n = 4 for dry matter and protein, n = 3 for energy) for Trial 1.

	ADC's (%)		
	Dry Matter	Protein	Energy
Reference diet	77.72 ± 2.04	99.06 ± 0.44	86.22 ± 1.46
Test Diet	79.94 ± 4.22	98.72 ± 0.68	88.54 ± 0.96
Starch	88.78 ± 18.33	***	97.79 ± 10.56

*** The ADC values of protein in corn starch were not calculated as the protein content of the ingredient was negligible.

Figure 6.2

Apparent digestibility coefficients (%) of the experimental diets used in Trial 2 measured over days 6 to 10 (⊡) and days 13 to 17 (▦) versus inclusion level of starch (%). Values are mean ± standard deviation (n=4).

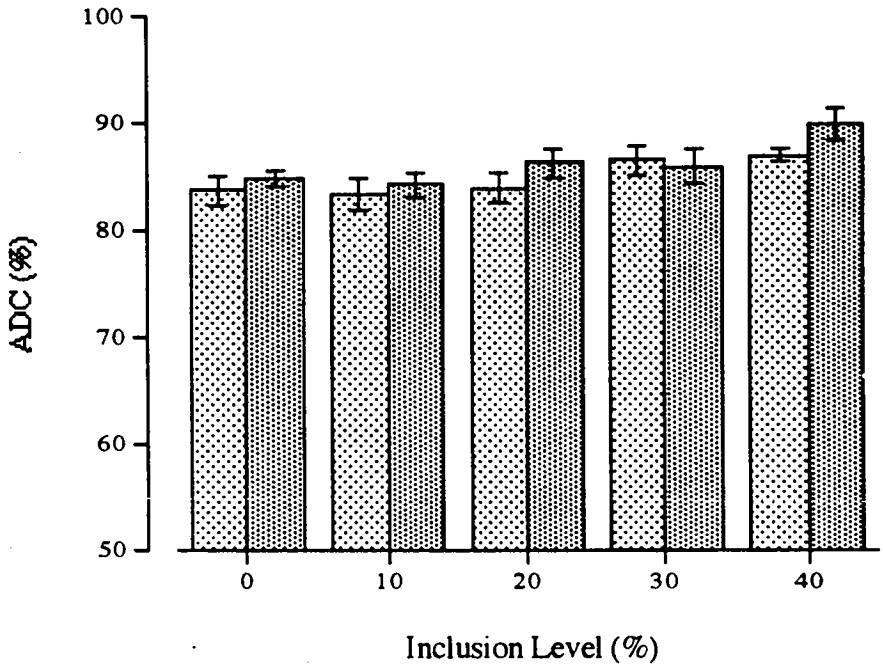
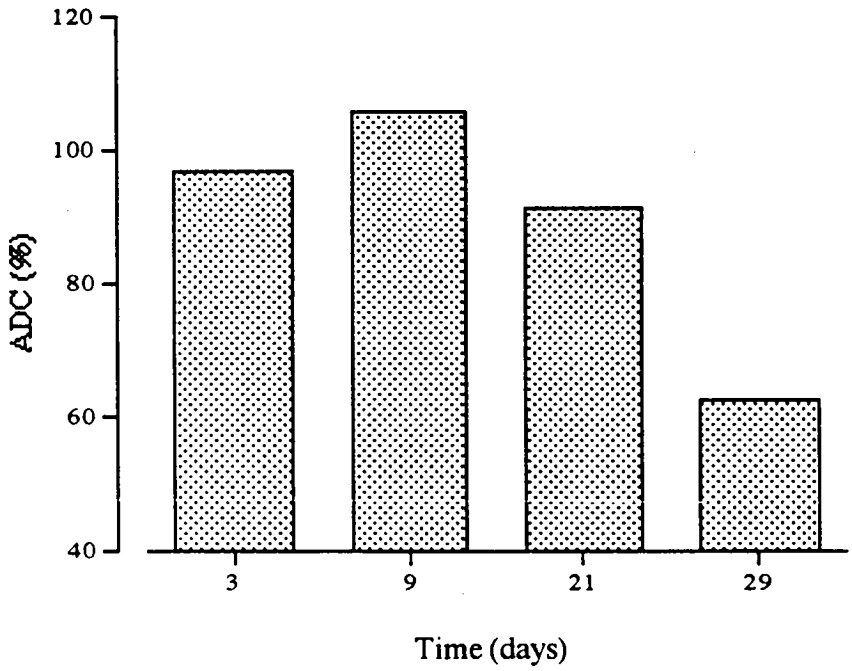

100
90
80
70
60
50
ADC (%)
0
10
20
30
40
Inclusion Level (%)

Figure 6.3
Apparent digestibility coefficients (%) of starch at a 20% inclusion level versus time in Trial 1. Each point represents a single replicate.


120
100
80
60
40
ADC (%)
3
9
21
29
Time (days)

on days 21 and 29 being 90.9% and 62.0% respectively.

The mean of the ADC's for starch at a 20% inclusion level measured at the four time points was 88.8 ± 18.3% (Table 6.4). The large standard deviation is due to the considerable reduction in digestibility with time.

Trial 2:

ADC's for starch included in the reference diet at graded levels are shown in Figure 6.4. Repeated measures analysis of variance revealed a significant effect of inclusion level ($p < 0.001$), but not time ($p > 0.05$), on the digestibility of starch. The interaction between inclusion level and time was also not significant ($p > 0.05$) and the data were therefore pooled over time. Subsequent *post hoc* analysis using Scheffe's test revealed that the digestibility of starch at 10% inclusion (77.3 ± 10.9 was significantly lower ($p < 0.05$) than that of starch at 30% (90.4 ± 5.4) and 40% inclusion (94.2 ± 4.1).

6.3.3 Digestibility of Protein in the Experimental Diets

Trial 1:

The ADC's for the protein portion of the experimental diets used in Trial 1 are shown in Figure 6.5. Values for each time point are single replicates due to the pooling of faeces. Protein digestibility was high for both experimental diets, being greater than 98.0%. ADC's for protein were high and protein digestibility between the time points for the experimental diets varied, with ADC's ranging from 98.6% (day 29) to 99.6% (day 3) and 99.54 (day 3) to 98.0% (day 29) for the reference and test diets respectively (Figure 6.5). Changes in protein digestibility for the reference diet were inconsistent, although the ADC for protein from the test diet appeared to decrease with time over the duration of the trial.

The mean ADC's for protein from the experimental diets measured at the four time points are shown in Table 6.4. No significant difference ($p > 0.05$, one-way analysis of variance) was found between the mean digestibility of dietary protein for the reference diet (99.1 ± 0.4%) and the test diet (98.7 ± 0.7%).

Figure 6.4

Apparent digestibility coefficients (%) for starch versus inclusion level (%) in Trial 2. Values (mean ± standard deviation, n=4) are pooled over time. Values with the same superscript are not significantly different.

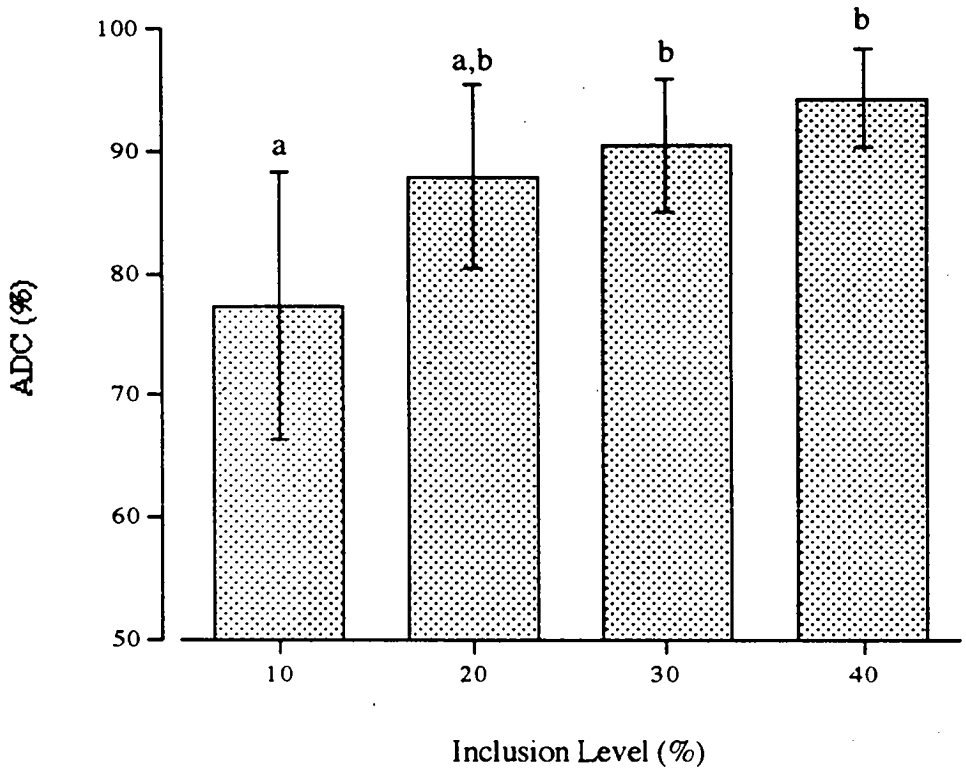
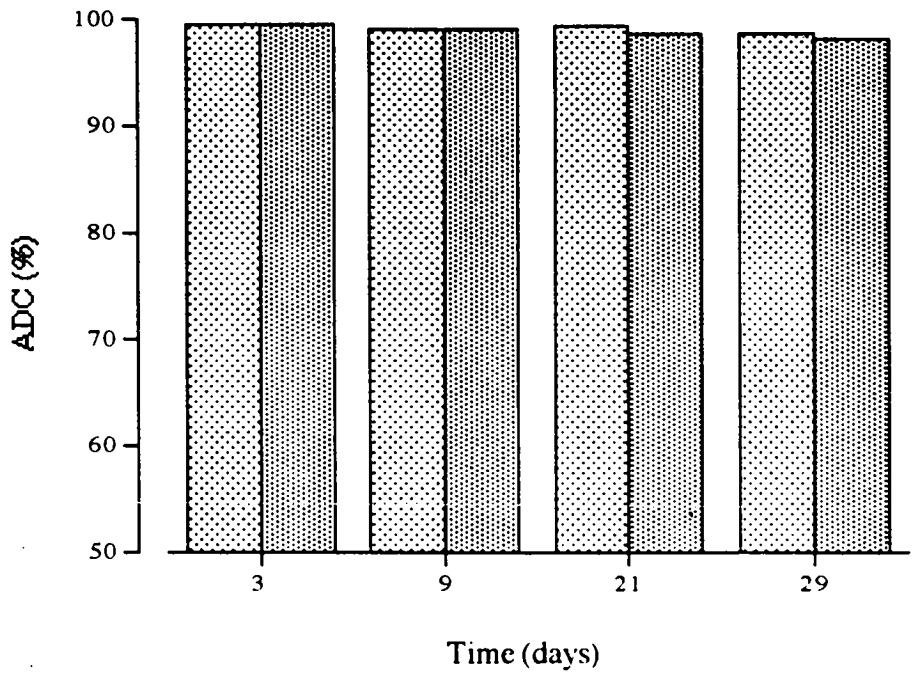

100
90
80
70
60
50
ADC (%)
a
a,b
b
b
10
20
30
40
Inclusion Level (%)

Figure 6.5

Apparent digestibility coefficients (%) of the protein component of the reference diet (⊡) and the 20% inclusion level test diet (▩) used in Trial 1 versus time (days). Each point represents a single replicate.


100
90
80
70
60
50
ADC (%)
3
9
21
29
Time (days)

6.3.4 Digestibility of Energy in the Experimental Diets

Trial 1:

The ADC's of energy in the test and reference diet used in Trial 1 are shown in Figure 6.6. Values at each time point represent a single replicate due to the pooling of faeces. The digestibility of energy from the experimental diets only varied slightly with time, with values ranging from 84.6% (day 9) to 87.3% (day 29) and 87.9% (day 21) to 89.6% (day 3) for the reference and test diets respectively (Figure 6.6).

The mean of the ADC's for energy from the experimental diets measured at each time point are detailed in Table 6.4. No significant difference ($p > 0.05$, one-way analysis of variance) was found between the mean digestibility of energy from the reference diet (86.2 ± 1.5%) and test diet (88.5 ± 1.0%).

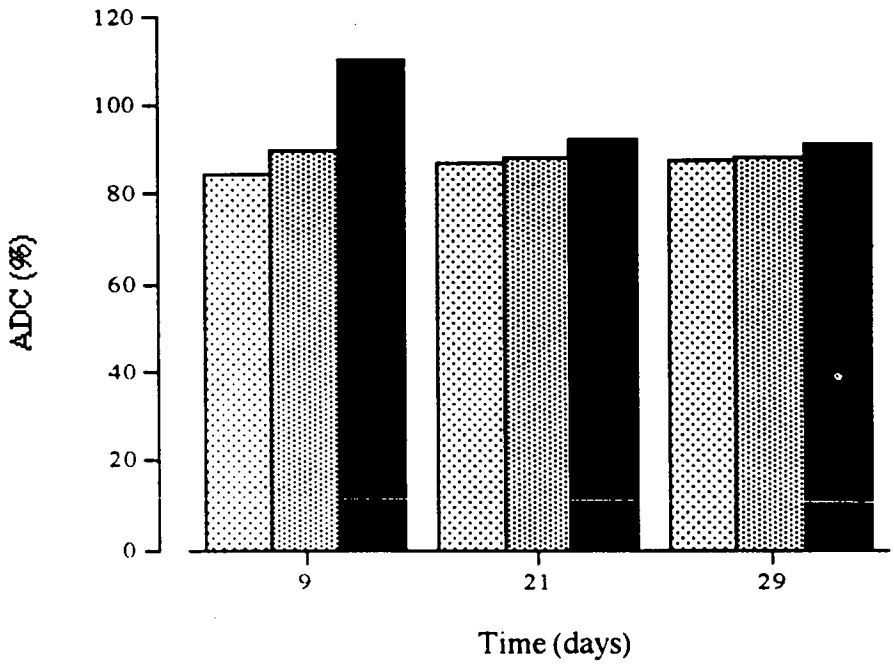
6.3.5 Digestibility of Energy in the Test Ingredient

Trial 1:

The ADC's of energy from starch included in the reference diet at a 20% are shown in Figure 6.6. Values for each time point represent a single replicate due to the pooling of faeces. Figure 6.6 shows the digestibility of energy from starch varied markedly with time, ranging 110.0% at day 9 to 91.0% at day 29.

The mean of the ADC's for energy measured at each time point was high, 97.8% (Table 6.4), being elevated by the day 9 value.

Figure 6.6
Apparent digestibility coefficients (%) of the crude energy from the reference diet (⊡), the 20% inclusion level test diet (▦) and starch (■) in Trial 1 versus time (days). Each point represents a single replicate.


120
100
80
60
40
20
0
ADC (%)
9
21
29
Time (days)

6.4 Discussion

The present study has determined the digestibility of starch for the common carp and the effects of time and inclusion level upon starch digestibility in this species.

The digestibility of starch for common carp was found to vary with the level of inclusion. In the initial trial the mean digestibility of starch was found to be 88.8%, which was consistent with the ADC's of 83.7% and 86.0%, for days 6 to 10 and days 13 to 17 respectively, determined in the second trial. The digestibility of starch in the graded inclusion level study ranged from approximately 78.5% at 10% inclusion (days 13 to 17) to 97.0% at 40% inclusion (days 13 to 17). Chu *et al.* (1991) found the dry matter digestibility of corn starch for common carp at 30% inclusion to be 70.3%, a value somewhat lower than the 92.6% and 88.2% measured for 30% inclusion in the present trial. However, the digestibility of the carbohydrate portion of the corn starch in the study of Chu *et al.* (1991) was found to be 98.0%, which is in better agreement with the corn starch digestibility coefficients measured in the present study. Since corn starch contains a low level of non-carbohydrate material, the large difference between the dry matter and carbohydrate digestibility coefficients observed by Chu *et al.* (1991) indicates that the inclusion of 30% corn starch adversely affected the digestibility of other dietary ingredients. This results in the apparent dry matter digestibility of corn starch being reduced. Chiou and Ogino (1975) reported lower digestibility of starch for common carp than the values obtained in this study, ranging from 50% to 60% at inclusion levels ranging from 14.0% to 49.2% (Table 6.1). However, the test ingredient used in that study was potato starch.

The digestibility of raw starch for common carp reported in the present study was higher than the literature values for rainbow trout (Inaba *et al.*, 1963; Smith, 1971; Bergot and Breque, 1975; Pfeffer *et al.*, 1991), Atlantic salmon (Arnesen and Krogdahl, 1993) and white sturgeon (Herold *et al.*, 1995). The omnivorous nature of the common carp, and the resultant increased natural dependence upon dietary complex carbohydrate for energy, would suggest that the carp are better adapted anatomically and physiologically to digest raw starch than the carnivorous rainbow trout, Atlantic salmon or white sturgeon.

In rainbow trout it has been reported that feeding rates for diets affects the digestibility of the starch component of the diet, with digestibility greater at restricted feeding levels (Bergot and Breque, 1983; Pfeffer *et al.*, 1991). Bergot and Breque (1983) found raw corn starch digestibility to decrease from 54.5% to 38.1% with an increase in feeding rate from 0.5% to 1.0% body weight.wet weight diet.day^{-1}. Similarly Pfeffer *et al.*

(1991) found the digestibility of raw maize starch to decrease from 45% to 22% with a change from restricted (1% body weight.wet weight diet.day^{-1}) to satiety feeding. The high digestibility coefficients in the present study suggest that a feeding rate of 2% body weight.wet weight diet.day^{-1} did not adversely affect the digestibility of raw corn starch for common carp. The ability of carp to adequately digest starch at this feeding rate may again reflect the natural dietary preference of this species.

Similarly the digestibility of starch at the 20% inclusion level was not affected by the feeding regime employed. Starch digestibility for fish fed 2% body weight.wet weight diet.day^{-1} in a single feeding regime, 88.8% (Trial 1), was similar to the digestibility of starch for fish fed an equivalent ration spread over four individual feeds, approximately 85% (Trial 2). Indicating that feeding the 20% inclusion level test diet at a rate of 2% body weight.wet weight diet.day^{-1} did not exceed the digestive capabilities of the common carp.

The digestibility of starch in the present study was found to increase with the level of inclusion from approximately 78% at a 10% inclusion level to greater than 90% at a 40% inclusion level. The trend in previous studies has been that the digestion of starch decreases with the level of inclusion in the diet (Inaba *et al.*, 1963; Chiou and Ogino, 1975; Spannhof and Plantikow, 1983; Hemre *et al.*, 1989). In those studies, the digestibility of starch for carp (Chiou and Ogino, 1975) was decreased to a lesser extent than for rainbow trout (Inaba *et al.*; 1963) or Atlantic cod (Hemre *et al.*, 1989). The decrease in digestibility of starch has been attributed to the presence of α-amylase inhibitors in the starch (Hofer and Sturmbauer, 1985; Sturmbauer and Hofer, 1986; Natarajan *et al.*, 1988; Natarajan *et al.*, 1992), the adsorption of α-amylase by starch (Spannhof and Plantikow, 1983) and a decrease in gut transit time (Spannhof and Plantikow, 1983). However, carp have been shown to be capable of increasing the secretion of α-amylase several fold to maintain α-amylase activity in the presence of inhibitors (Sturmbauer and Hofer, 1986) and α-amylase activity has been shown to be increased in carp (Kawai and Ikeda, 1972) and in other species (Nagase, 1964; Kawai and Ikeda, 1972; Reimer, 1982) in response to high carbohydrate diets. Common carp also possess a longer relative gut length than the rainbow trout used by Spannhof and Plantikow (1983). Spannhof and Plantikow (1983) found that when the daily intake of starch per gram body weight was considered, and not simply the inclusion level of starch in the diet, the amount of starch absorbed by rainbow trout increased initially (up 0.4 g starch per 100 g body weight.day^{-1}). Starch absorption subsequently plateaued and then decreased with further increases in the daily intake of dietary starch. In the present study common carp were fed at a restricted rate of 2% body weight.wet weight diet.day^{-1}, resulting in a daily starch intake of 0.92 g per 100

g body weight.day^{-1} for the 40% inclusion level test diet. Considering the natural omnivorous diet of common carp, as opposed to the carnivorous diet of rainbow trout, it is not unreasonable to suggest that the common carp is able to accommodate a higher daily consumption of starch prior to overloading the intestinal α-amylase system. The activity of α-amylase in the fish intestine has also been shown to increase in response to high carbohydrate diets (Nagase, 1964; Kawai and Ikeda, 1972; Reimer, 1982), suggesting a positive feedback system.

In the initial trial (Trial 1), the digestibility of starch appeared to decrease with time. This decrease in starch digestibility was accompanied by a decrease in whole diet and dietary protein digestibility suggesting that there was an effect on the general digestive physiology of the fish and that this was not restricted to the carbohydrase system. The decrease in digestibility may reflect a natural variation in digestibility of the reference and test diets as reported by De Silva and Perera (1983; 1984) and in Chapter 7 of this study and not be a result of adaptation to the experimental diets. This hypothesis is supported by the observation that starch digestibility was not affected by time for any inclusion level during Trial 2.

Energy from corn starch was highly digestible for common carp at a 20% inclusion level, being greater than 90% digestible (mean 97.8%) at all time points measured. At day 9 the digestibility of energy from starch was spuriously high, 110%. At day 21 and day 29 energy digestibility was 92.4% and 91.0% respectively, similar to the mean value for starch digestibility for the trial. The digestibility of energy from raw corn starch for common carp in the present study was higher than that reported by Chu *et al.* (1991). Those authors found energy digestibility to be 62.1% at 30% inclusion, a value similar to the dry matter digestibility of the starch. It would therefore seem probable that the digestibility of starch in the present study was approximated better by the day 21 and 29 values and that the value at day 9 was aberrant. In the absence of replication it is not possible to interpret this result conclusively.

The digestibility of protein in the present study is similar to values reported by Kitamikado *et al.* (1964) and Halver (1989) for a casein-based diet and Rychly and Spannhof (1979) for a casein/gelatin-based diet for rainbow trout. The digestibility of the protein component of the experimental diets appeared to be reduced over time at a 20% inclusion level, decreasing from 99.5% on day 3 to 98.0% on day 29. Values for dietary protein digestibility at days 21 and 29 were markedly lower for the test diet than the reference diet, suggesting a negative effect of starch on protein digestibility. However, the decrease in protein digestibility in the present study was not as marked as that observed for rainbow trout. Digestibility of protein from a casein based diet for

rainbow trout has been shown to be effected by the addition of starch to the diet, with protein digestibility decreasing from 96% to 79% with an increase in dietary protein from 10% to 80% (Kitamikado *et al.*, 1964). Kitamikado *et al.* (1964) also found the digestibility of protein from a white fishmeal based diet to be reduced by the increased inclusion of dietary starch. However, Herold *et al.* (1995) found protein digestibility for white sturgeon to be unaffected by the inclusion 27% raw corn starch in a casein-based diet. In view of the minimal size of the change in protein digestibility observed in the present study, the nutritional significance to the animal is also likely to be minimal. The change may be explained by the proteinaceous α-amylase being unavailable for resorption as it is bound to the undigested starch, thus increasing the faecal protein content (Spannhof and Plantikow, 1983).

Chapter 7

Temporal Variability in Digestibility

7.1 Introduction

The internal physiological processes of fish, as with those of terrestrial vertebrates, have been shown to have an inherent cyclic rhythm. Although this work has generally investigated the diurnal rhythms of plasma and tissue metabolites and plasma hormones (Ali, 1993), it is not incomprehensible that such a cyclic rhythm may also exist in the processes of digestion. Temporal variation in digestive enzyme secretion has been noted by investigators, however these cycles have involved a long term rhythm. Dabrowski *et al.* (1992) suggested that digestive enzyme secretion by larval Artic charr exhibited a three weekly rhythm that appeared related to the lunar cycle, and Hofer (1979) found rudd and roach to display annual rhythms in enzyme secretion related to photoperiod and water temperature respectively.

The presence of a rhythm in the digestive processes of fish should result in a cycling of the digestibility coefficients measured during nutritional trials. Studies investigating digestibility in fish generally pool faeces from individual fish or groups of fish over a number of consecutive days in order to obtain adequate quantities for analyses. As such, digestibility coefficients are measured at one time point and any temporal variation in digestibility would not normally be detected. In order to investigate daily variability in digestibility, it is necessary to collect sufficient quantities of faeces for digestibility marker and nutrient analyses from each experimental replicate on separate days. Such studies have been previously performed (de la Noüe *et al.*, 1980; De Silva and Perera, 1983; De Silva and Perera 1984; Ferraris *et al.*, 1986).

7.1.1 Temporal Variation in Digestibility Coefficients in Fish

Several studies have shown temporal variation in digestibility. Investigating the period of adaptation to experimental diets, Ferraris *et al.* (1986) found variability in the dry matter digestibility of fishmeal for milkfish. Digestibility coefficients measured at five time points over the first 28 days after the initial offering of the diet ranged from 72% to 80% (mean 76%). Similarly de la Noüe *et al.* (1980) found the dry matter, energy, protein, starch and canthaxanthine digestibility for rainbow trout to show daily variability. Although the extent to which the digestibility coefficients varied depended on the nutrient measured.

Protein digestibility from an aquatic macrophyte, *Hydrilla verticella*, for the green chromid was found to vary from 51.2% to 81.8% (De Silva and Perera, 1983). The variability in digestibility coefficients appeared to follow an ill-defined pattern where a day or two of high digestibility was followed by a day or two of low digestibility. No

correlation existed between digestibility and either food intake or the weight of faeces collected, and as the same feed was supplied throughout the trial variation could not be correlated to diet quality. Accompanying the daily variation in protein digestibility was a trend for an overall increase in protein digestibility throughout the duration of the experimental trial, indicating an adaptation of the digestive physiology of the animal to the diet.

Nile tilapia fed fishmeal-dextrin based artificial diets also displayed a daily variability in total diet and dietary protein digestibility (De Silva and Perera, 1984). Variation in digestibility again appeared to follow a rhythm of a day or two of high digestibility followed by a day or two of low digestibility, although this rhythmicity was not always clear. The variations in both total diet and dietary protein digestibility between consecutive days were often large, being up to 27.3% and 23.0% respectively.

As variation in digestibility had been shown for different species utilising very different diets and a wide range of digestibility markers, De Silva and Perera (1984) suggested that daily rhythmicity in digestibility was a true phenomenon and not an artefact due to methodology. It would therefore appear that the utilisation of diets and dietary nutrients varies with the internal state of the animal, resulting in the presence of a rhythm in digestibility.

7.1.2 Commercial Application of Variability of Digestibility

If the digestive capabilities of a cultured species exhibit a regular rhythm then it should be possible to develop a feeding methodology to suit this cycle and therefore reduce feeding costs. Feeding alternate diets containing high and low protein levels is one such mixed feeding schedule which has been shown to be effective in both laboratory and semi-intensive pond culture systems (De Silva and Anderson, 1995). Nile tilapia fry fed a high protein diet alternated with a low protein diet, for a varying number of days, grew at a similar rate to animals receiving only the high protein diet, with adoption of the best feeding schedule resulting in approximately 30% savings on feed costs (De Silva and Anderson, 1995). Utilisation of mixed feeding schedules in laboratory trials on catla and rohu also indicated a mixed feeding schedule would result in significant reduction in feed costs (De Silva and Anderson, 1995). Application of these feeding schedules to Indian major carp polyculture practices resulted in no adverse effects on either grow out time nor the performance of the targeted species, whilst there was up to 30% savings in feed costs (De Silva and Anderson, 1995).

7.1.3 Aims of the Present Study

Despite the potential savings in feed costs, few studies have investigated the daily variation in diet, ingredient and nutrient digestibility in aquaculture animals. Since such a rhythmicity would have significant ramifications for measuring digestibility, it was decided to perform a detailed study to look of the daily digestibility of diets, ingredients and dietary nutrients. The effect of inclusion levels of dietary ingredients on the daily digestibility of the ingredient and diet was also examined. The comparison of digestibility coefficients determined from daily faeces analysis and that of pooled faeces was also performed to identify the effects of faeces pooling on the accuracy of digestibility determination.

7.2 Materials and Methods

Three feeding trials were performed in order to investigate daily variability in digestion for common carp. These trials investigated the digestibility of diets containing graded levels of cellulose, SBM and tuna oil.

7.2.1 Experimental Diets

The diets used in the present study are the same as those used in previous experiments. The formulation and proximate composition of the diets for the graded cellulose, SBM and tuna oil digestibility trials are detailed in Tables 3.4, 4.6 and 5.5 respectively. All digestibility trials utilised casein-based reference diets (Mackie and Mitchell, 1985), although formulation of these diets varied slightly in order to facilitate the inclusion of the test ingredient. The level of test ingredient included into the reference diet also varied with cellulose incorporated into the reference diet at 10%, 20% and 30% inclusion, SBM included at 10%, 20%, 30% and 40% inclusion, and tuna oil was included at 10% and 15% inclusion.

7.2.2 Experimental Animals, Feeding and Faeces Collection

Common carp were collected, maintained, anaesthetised and weighed as described in Chapter 2. Animals in each digestibility trial were randomly allocated to tanks. Faeces were collected from aquaria daily by siphoning as described in section 2.4.

Cellulose digestibility trial:

Four hundred and eighty common carp of mean weight 49.0g (range 13.8 to 115.7g, standard deviation 20.5g) were stocked at 15 animals per 70 litre aquarium. Each experimental diet was fed to four replicate tanks. Fish were fed 1% body weight.wet weight diet.day^{-1} using a feeding regime where approximately 25% of the daily ration was delivered in each of four feedings at approximately hourly intervals to ensure all feed was consumed. Feeding was increased to 2% body weight.wet weight diet.day^{-1} as fish became adapted to the experimental diets. Feeding continued for 23 days.

In the cellulose digestibility trial a 5 day period for the adaptation of the experimental animals to the experimental diets was allowed prior to the commencement of faecal collection. Faeces were subsequently collected from days 7 to day 23 with the exclusion of days 9, 11, 17 and 18, on which no faeces was voided during the collection period.

SBM digestibility trial:

Three hundred common carp of mean weight 45.8g (range 13.8 to 103.1g, standard deviation 19.3g) were stocked at 15 fish per 70 litre aquarium. Each experimental diet was fed to four replicate aquaria of experimental animals. Fish were initially fed 1% body weight.wet weight diet.day^{-1}, which was increased to 2% body weight.wet weight diet.day^{-1} once adaptation to the experimental diets had occurred. Feeding was performed over four feedings at approximately hourly intervals to ensure complete ingestion of the entire feed ration. Feeding continued for 23 days.

Faeces collection in the SBM digestibility trial commenced on day 9, allowing an 8 day period for the experimental animals to adapt to the experimental diets. Faeces were subsequently collected daily until day 24, excluding days 11 and 18 when insufficient faeces was voided during the collection period.

Tuna oil digestibility trial:

Two hundred and twenty five common carp of mean weight 52.0g (range 14.4 to 115.7g, standard deviation 21.2g) were stocked at 15 fish per 70 litre aquaria. Each experimental diet was fed to five aquaria of experimental fish at an initial feeding rate of 1% body weight.wet weight diet.day^{-1}, with a subsequent increase to 2% body weight.wet weight diet.day^{-1} as animals adapted to the experimental diets. The daily feed allocation was divided into four equal rations which were fed at one hour intervals, ensuring all feed was ingested. Experimental diets were fed for 17 days.

In the tuna oil digestibility trial faeces were not collected over the initial 7 days in order to allow for adaptation to the experimental diets. Faeces were subsequently collected daily from day 8 to day 17.

7.2.3 Diet and Faeces Analysis

The diets used in all digestibility trials were analysed for protein, lipid, ash NFE, energy, and Cr_2O_3. The proximate analysis of the experimental diets for the cellulose, SBM and tuna oil digestibility trials are detailed in Tables 3.4, 4.6 and 5.5 respectively.

Initially faeces were analysed on a daily basis. When sufficient faeces was available, faeces from each day of the three digestibility trials was analysed for Cr_2O_3. Analysis of dietary nutrients on a daily basis was limited to the determination of protein and lipid contents in faecal samples from the tuna oil digestibility trial due to

the availability of faeces. Following analysis of daily Cr_2O_3 and nutrient contents, faeces from each digestibility trial were pooled per replicate and analysed for Cr_2O_3.

ADC values were calculated using the ratio of marker in feed and faeces according to the method described in section 2.6.

"Expected" ADC's served as a prediction of digestibility values for pooled faeces. "Expected" ADC's were determined by multiplying the daily ADC's by the fraction of the total faeces represented by the faeces collected on the particular day to attain the "Expected Daily ADC (%)", as described in the Equation 7.1, and summing the resultant values over the faecal collection period for each replicate.

Equation 7.1:

$$\text{Expected daily ADC } (\%) = \text{daily ADC}_{Aa} \times \left(\frac{\text{daily faecal collection } Aa}{\text{total faeces collection } A} \right)$$

where daily ADC $_{Aa}$ = ADC for replicate 'A' on day 'a';
daily faecal collection $_{Aa}$ = dry weight faeces collected for replicate 'A' on day 'a'; and
total faeces collection $_{A}$ = total dry weight faeces collected for replicate 'A'.

7.3 Results

7.3.1 Digestibility of the Experimental Diets

Cellulose digestibility trial:

The daily ADC's for the experimental diets used in the cellulose digestibility trial are shown in Figures 7.1a and 7.1b. Daily variation existed in the daily digestibility of the whole diet (Figures 7.1a and 7.1b). ADC's for diets not supplemented with antibiotics ranged from 82.4 ± 2.3% (day 19) to 85.4 ± 1.4% (day 14) (mean 84.1 ± 0.8%) for the reference diet, 74.8 ± 1.8% (day 8) to 78.3 ± 1.2% (day 14) (mean 76.4 ± 0.5%) for 10% inclusion test diet, 64.8 ± 1.8% (day 10) to 70.6% (day 14) (mean 68.2 ± 0.8%) for the 20% inclusion test diet and 52.8 ± 2.6% (day 7) to 63.3 ± 1.3% (day 19) (mean 60.6 ± 1.9%) for the 30% inclusion test diet. ADC's for diets supplemented with antibiotics ranged from 81.9 ± 2.5% (day 12) to 84.3 ± 0.5% (day 14) (mean 83.5 ± 0.4%) for the reference diet , 70.5 ± 1.3% (day 8) to 74.5 ± 0.3% (day 16) (mean 72.4 ± 0.7%) for the 10% inclusion test diet, 66.0 ± 2.6% (day 8) to 71.6% ± 2.3% (day 23) (mean 68.3 ± 0.7%) for the 20% inclusion test diet and 57.6 ± 3.6% (day 7) to 65.6 ± 3.8% (day 22) (mean 61.3 ± 1.7) for the 30% inclusion test diet.

Variation in digestibility between consecutive days appeared to be larger at the higher inclusion of cellulose into the experimental diets. The largest variations between consecutive days for diets with and without antibiotics respectively were 1.8% (days 19 and 24) (mean 0.7 ± 0.5%) and 2.0% (days 12 and 23) (mean 0.8 ± 0.7%) for the reference diets, 1.6% (days 13 and 24) (mean 0.8 ± 0.5%) and 2.8% (days 15 and 16) (mean 1.4 ± 0.9%) for the 10% inclusion test diets, 2.1% (days 20 and 21) (mean 1.4 ± 0.6%) and 3.0% (days 20 and 21) (mean 1.4 ± 1.0%) for the 20% inclusion test diets and 4.7% (days 7 and 8) (mean 1.6 ± 1.4%) and 3.1% (days 21 and 22) (mean 1.0 ± 0.9%) for the 30% inclusion test diets (Figure 7.1a and 7.1b).

The experimental diets showed an overall increase in digestibility throughout the experimental period (Figures 7.1a and 7.1b). ADC's for all diets, with the exception of the reference diet not supplemented with antibiotics, showed a tendency to be greater after day 12 than prior to day 12. This increase was greatest for diets incorporating 30% cellulose, with the ADC's for the 30% cellulose diet without antibiotics increasing from 52.8 ± 2.6% (day 7) to 62.3 ± 0.7% (day 23)

Figure 7.1a

Daily apparent digestibility coefficients (%) versus time (days) of the experimental diets not supplemented with antibiotics used in graded cellulose digestibility trial. Values are mean ± standard deviation (n = 4). (□ reference diet, ◆ 10% inclusion diet, ○ 20% inclusion diet, ▲ 30% inclusion diet).

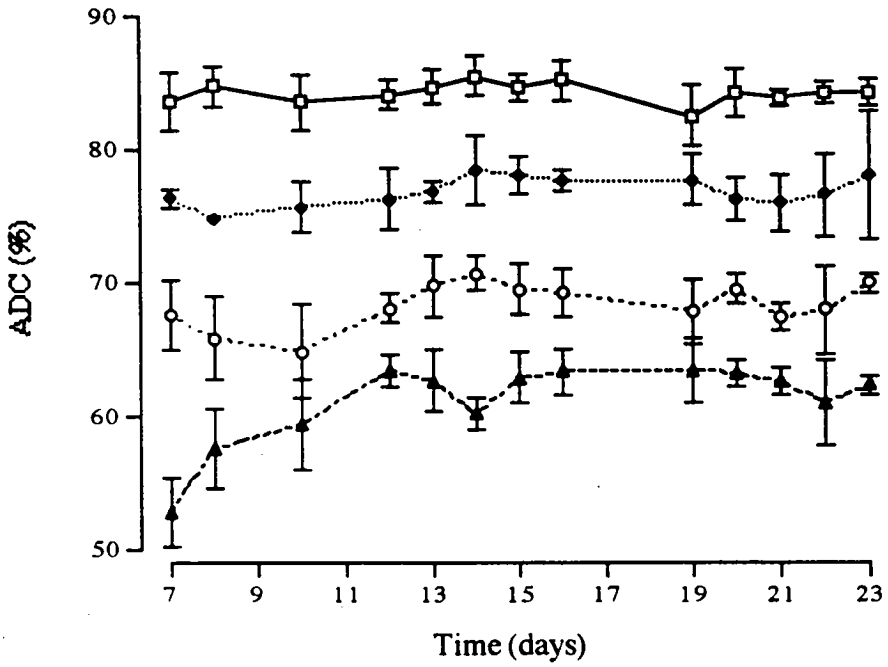
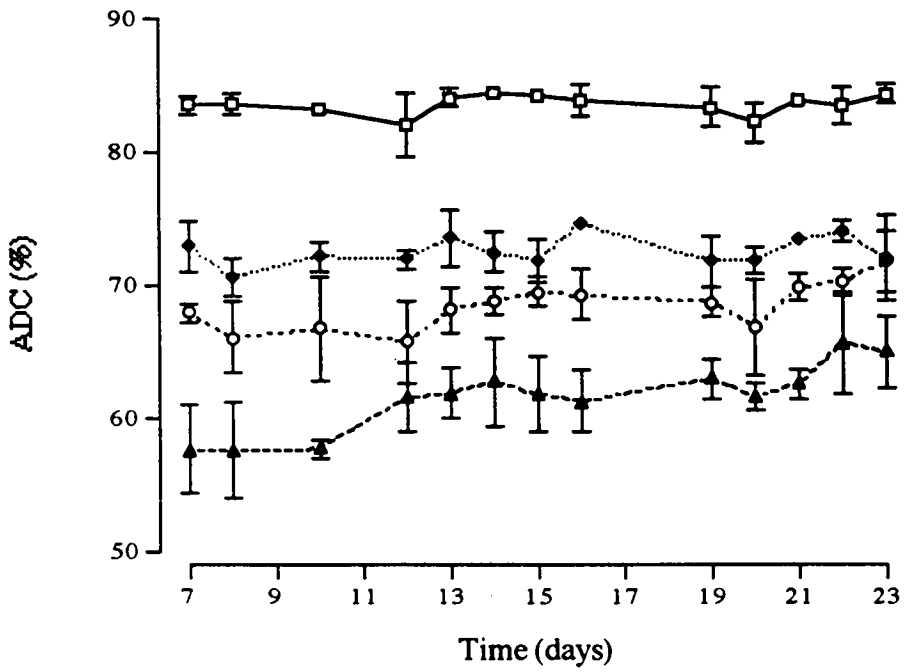

90
80
70
60
50
ADC (%)
7
9
11
13
15
17
19
21
23
Time (days)

Figure 7.1b

Daily apparent digestibility coefficients (%) versus time (days) of the experimental diets containing antibiotics used in graded cellulose digestibility trial. Values are mean ± standard deviation (n = 4). (□ reference diet, ◆ 10% inclusion diet, O 20% inclusion diet, ▲ 30% inclusion diet).


90
80
70
60
50
ADC (%)
7
9
11
13
15
17
19
21
23
Time (days)

SBM digestibility trial:

The daily ADC's for the experimental diets used in the ground SBM digestibility trial are shown in Figures 7.2a, 7.2b, 7.2c and 7.2d. All diets exhibited daily variation in digestibility. ADC's ranged from 78.5 ± 0.3% (day 8) to 83.5 ± 0.3% (day 24) (mean 82.3 ± 1.3%) for the reference diet and 81.4 ± 0.9% (day 13) to 83.8 ± 0.3% (day 12) (mean 82.9 ± 0.8%) for the 10% inclusion test diet, 74.2 ± 1.2% (day 8) to 83.5 ± 1.1% (day 20) (mean 81.3 ± 2.3%) for the 20% inclusion test diet, 73.04 ± 0.7% (day 8) to 81.8 ± 0.8% (day 20) (mean 78.6 ± 2.4%) for the 30% inclusion test diet and 69.6 ± 1.4% (day 10) to 80.5 ± 1.2% (day 16) (mean 76.3 ± 3.0) for the 40% inclusion test diet. Variability appeared to increase with the level of inclusion of the test ingredient with the highest variability between consecutive days being 3.5% (between days 8 and 9) (mean 0.7 ± 1.0%) for the reference diet and 2.4% (between days 12 and 13) (mean 0.7 ± 0.8%) for the 10% inclusion test diet, 6.8% (between days 8 and 9) (mean 1.8 ± 1.9%) for the 20% inclusion test diet, 5.4% (between days 8 and 9) (mean 3.4 ± 2.1%) for the 30% inclusion test diet and 6.3% (between days 9 and 10) (mean 1.9 ± 1.6%) for the 40% inclusion test diet.

The ADC's for the reference and the 10% and 20% inclusion test diets increased markedly from day 8 to day 9, after which digestibility values appeared to plateau (Figures 7.2a and 7.2b). ADC's for the 30% and 40% inclusion test diets appeared to increase gradually over the duration of the experimental period (Figures 7.2c and 7.2d).

Tuna oil digestibility trial:

The daily ADC's for the experimental diets used in the tuna oil digestibility trial are shown in Figures 7.3. The experimental diets again exhibited daily variation in digestibility (Figures 7.3). Digestibility values ranged from 72.4 ± 2.5% (day 9) to 77.0 ± 1.7% (day 16) (mean 74.6 ± 1.9%) for the reference diet and 71.8 ± 1.5% (day 9) to 77.9 ± 1.0% (day 16) (mean 75.3 ± 1.7%) for the 10% inclusion test diet and 68.2 ± 0.7% (day 9) to 76.5 ± 0.8% (day 17) (mean 72.8 ± 2.7%) for the 15% inclusion test diet. Fluctuations in digestibility between consecutive days were uniform over the three experimental diets with the largest variations between consecutive days for each diet being 4.5% (between days 14 and 15) (mean 1.5 ± 1.4%) for the reference diet and 4.3% (between days 9 and 10) (mean 1.8 ± 1.1%) for the 10% inclusion test diet and 3.8% (between days 9 and 10) (mean 1.6 ± 1.1%) for the 15% inclusion test diet.

Figure 7.2a
Daily apparent digestibility coefficients (%) of the reference diet (□) and 10% inclusion diet (◆) used in graded SBM digestibility trial versus time (days). Values are mean ± standard deviation (n = 3).

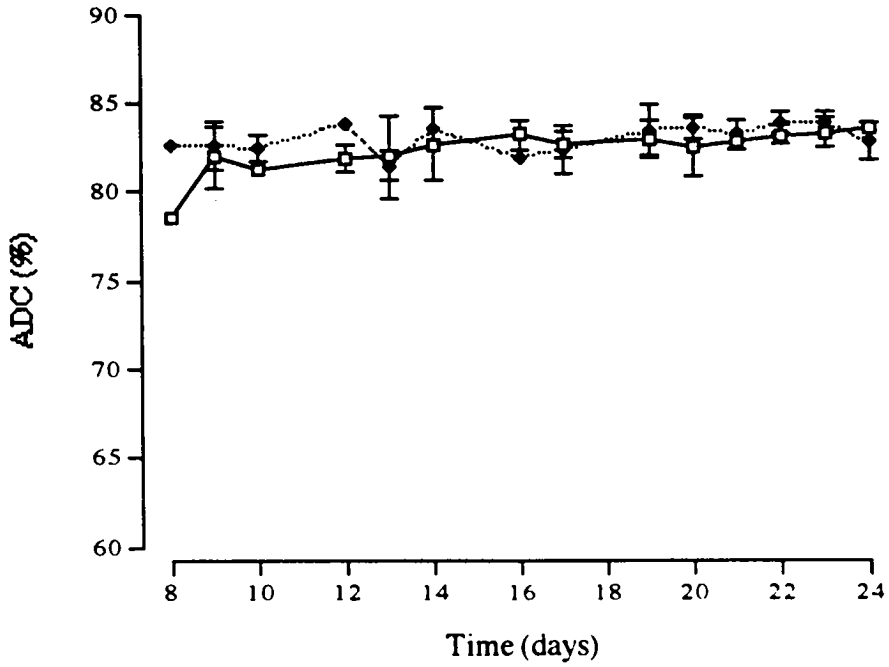

90
85
80
75
70
65
60
ADC (%)
8
10
12
14
16
18
20
22
24
Time (days)

Figure 7.2b
Daily apparent digestibility coefficients (%) of the reference diet (□) and 20% inclusion diet (◆) used in graded SBM digestibility trial versus time (days). Values are mean ± standard deviation (n = 3).

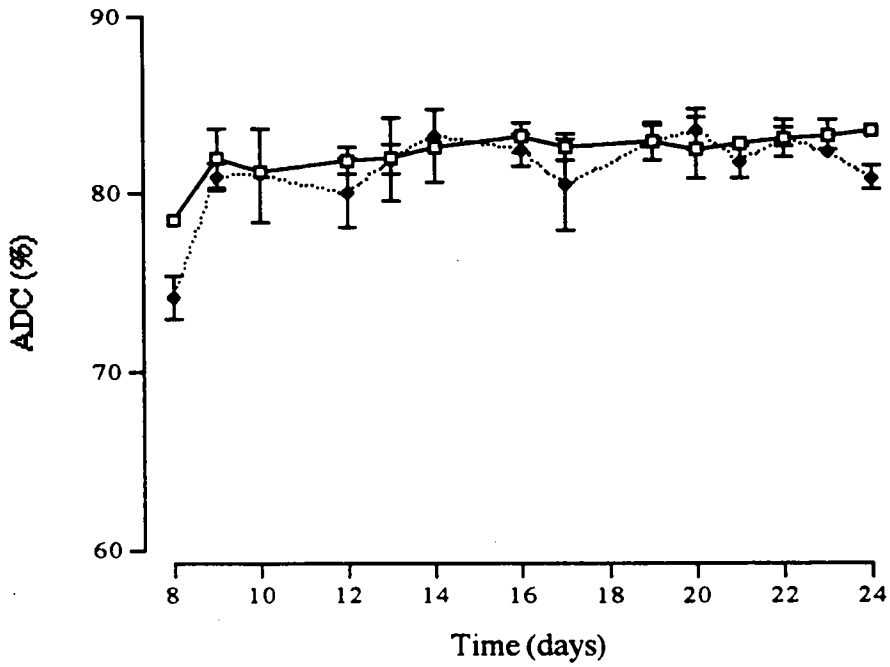

90
80
70
60
ADC (%)
8
10
12
14
16
18
20
22
24
Time (days)

Figure 7.2c
Daily apparent digestibility coefficients (%) of the reference diet (□) and 30% inclusion diet (◆) used in graded SBM digestibility trial versus time (days). Values are mean ± standard deviation (n = 3).

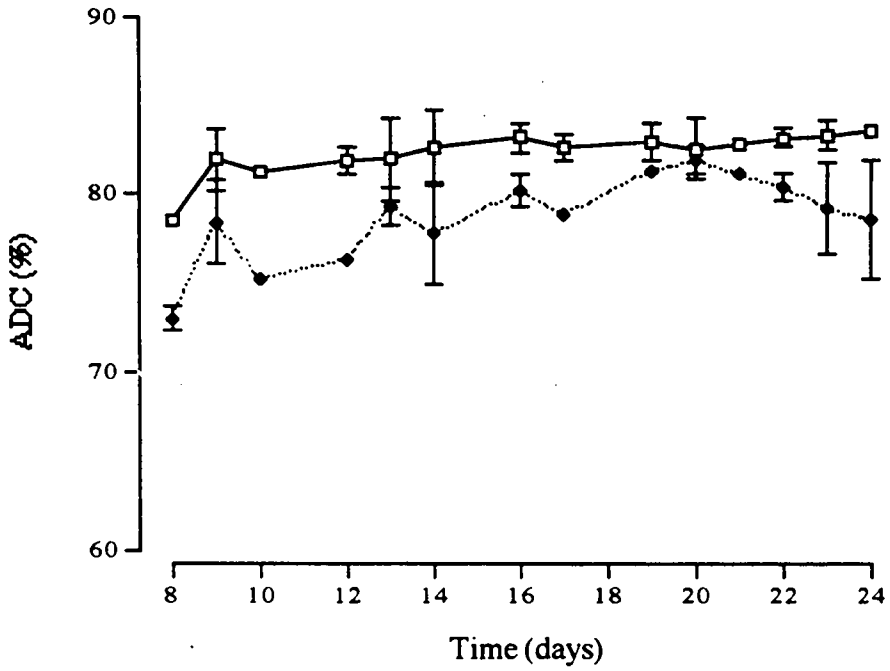

90
80
70
60
ADC (%)
8
10
12
14
16
18
20
22
24
Time (days)

Figure 7.2d
Daily apparent digestibility coefficients (%) of the reference diet (□) and 40% inclusion diet (◆) used in graded SBM digestibility trial versus time (days). Values are mean ± standard deviation (n = 3).

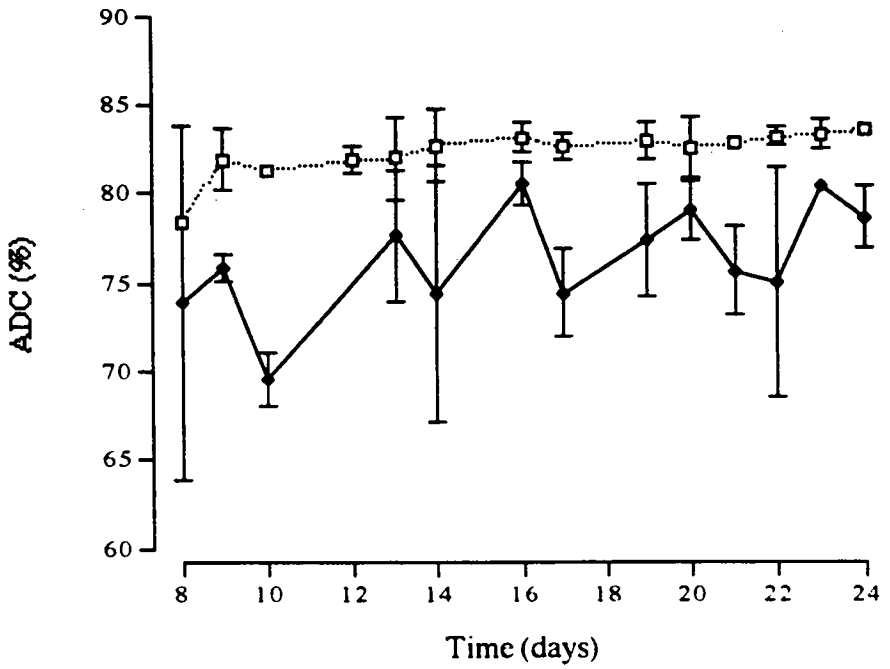
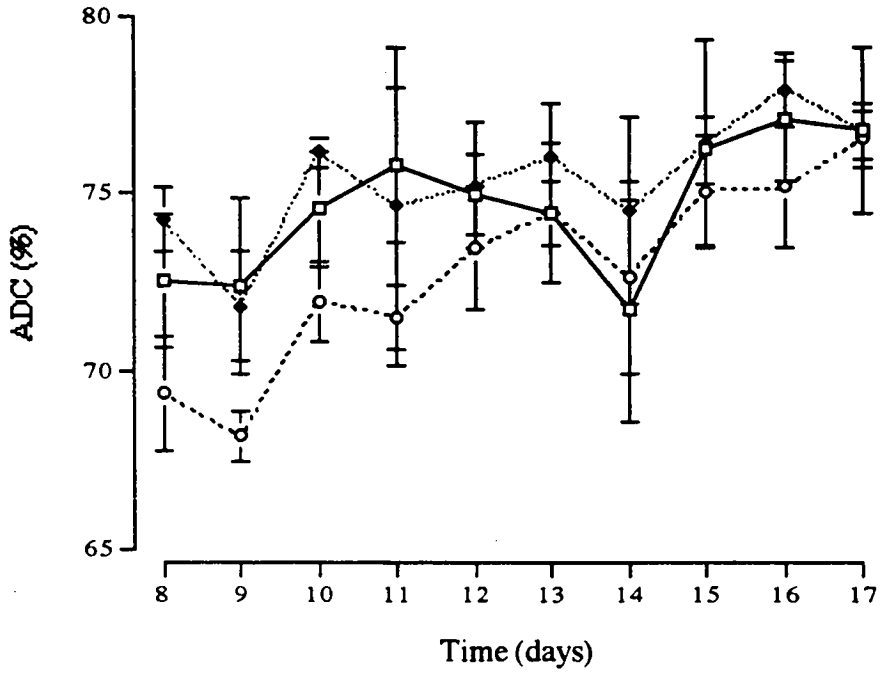

90
85
80
75
70
65
60
ADC (%)
8
10
12
14
16
18
20
22
24
Time (days)

Figure 7.3
Daily apparent digestibility coefficients (%) of the reference diet (□), 10% inclusion diet (◆) and 15% inclusion diet (○) used in graded tuna oil digestibility trial versus time (days). Values are mean ± standard deviation (n = 5).


80
75
70
65
ADC (%)
8
9
10
11
12
13
14
15
16
17
Time (days)

Associated with the daily variation in digestibility was a trend for the whole diet digestibility of the experimental diets to increase with time throughout the digestibility trial. The ADC's at day 17 for the reference diet, 10% inclusion test diet and 15% inclusion test diet (76.7 ± 2.3%, 76.7 ± 0.8%, and 76.5 ± 0.8% respectively) were all higher than the day 8 ADC's (72.6 ± 1.9%, 74.2 ± 0.9% and 69.4 ± 1.6% respectively). The increase in diet digestibility over the duration of the digestibility trial was not uniform for all three experimental diets. Over the initial time points of the digestibility trial (days 8, 9 and 10) digestibility of the 15% inclusion test diet appeared lower than the reference diet and the 10% inclusion test diet (Figure 7.3). By the end of the digestibility trial (day 17) the digestibility of the 15% inclusion test diet had increased to be similar to the ADC's of the reference diet and 10% inclusion test diet (Figure 7.3).

7.3.2 Digestibility of the Test Ingredient

Cellulose digestibility trial:

The daily ADC's of cellulose incorporated at graded levels into reference diets supplemented and unsupplemented with antibiotics are shown in Figures 7.4a and 7.4b respectively. Marked variation in digestibility was evident at all inclusion levels. The ADC's of cellulose in test diets not supplemented with antibiotics ranged from -14.0 ± 18.2% (day 8) to 34.1 ± 8.7% (day 19) (mean 11.1 ± 9.8%) at 10% inclusion, -13.2 ± 2.5% (day 8) to 12.4 ± 23.6% (day 23) (mean 5.7 ± 5.2%) at 20% inclusion and -19.0 ± 8.8% (day 7) to 18.9 ± 7.8% (day 19) (mean 6.1 ± 4.4%) at 30% inclusion. The ADC's for cellulose in test diets supplemented with antibiotics ranged from -46.0 ± 13.6% (day 8) to -8.4 ± 3.1% (day 16) (mean -11.7 ± 10.8%) at 10% inclusion, -4.0 ± 13.2% (day 8) to 21.4 ± 11.4% (day 23) (mean 4.5 ± 2.7%) at 20% inclusion and -3.0 ± 12.0% (day 8) to 24.1 ± 12.8% (day 22) (mean 3.1 ± 3.4%) at 30% inclusion.

Variation in digestibility of cellulose between consecutive days was greatest at 10% inclusion, decreasing at 20% inclusion which was similar to 30% inclusion. The largest daily variation in cellulose digestibility in diets supplemented and unsupplemented with antibiotics respectively were 30.2% (days 19 and 20) (mean 11.1 ± 9.8) and 31.2% (days 15 and 16) (mean 11.7 ± 10.9) at 10% inclusion, 16.8% (days 7 and 8) (mean 5.7 ± 5.2%) and 9.3% (days 7 and 8) (mean 4.5 ± 2.7%) at 20% inclusion, and 13.1% (days 7 and 8) (mean 6.1 ± 4.4%) and 11.50% (days 21 and 22) (mean 3.1 ± 3.4%) at 30% inclusion.

Figure 7.4a
Daily apparent digestibility coefficients (%) of cellulose included at 10% (□), 20% (◆) and 30% (○) into the reference diet in the absence of antibiotics versus time (days). Values are mean ± standard deviation (n = 4).

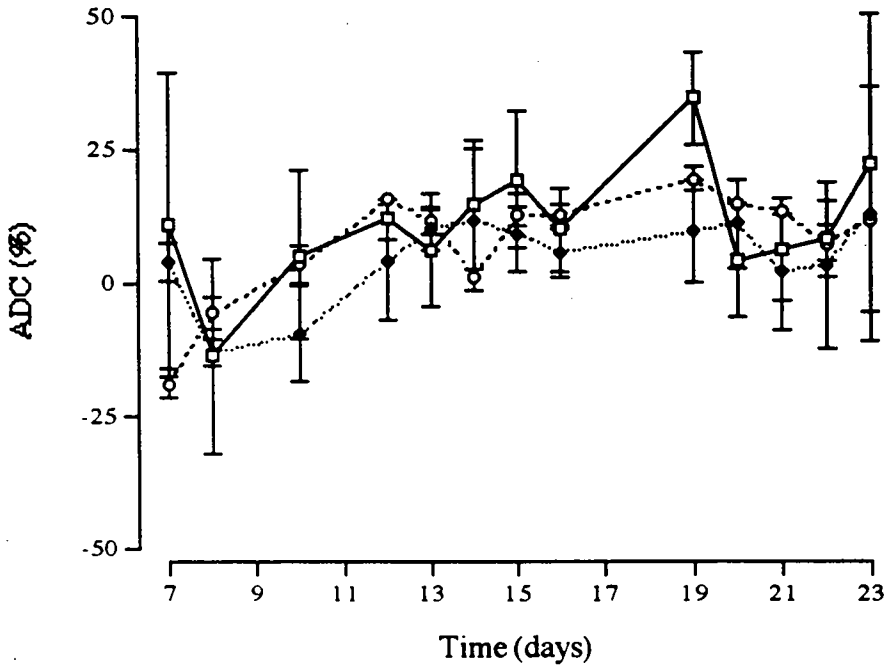
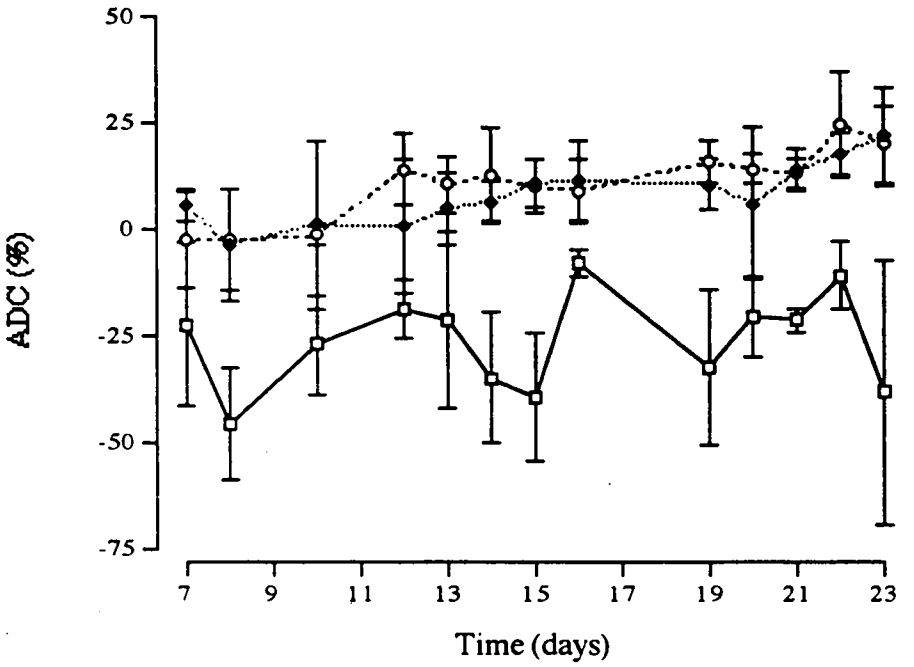

50
25
0
-25
-50
ADC (%)
7
9
11
13
15
17
19
21
23
Time (days)

Figure 7.4b
Daily apparent digestibility coefficients (%) of cellulose included at 10% (□), 20% (◆), and 30% (○) into the reference diet containing antibiotics versus time (days). Values are mean ± standard deviation (n = 4).


50
25
0
-25
-50
-75
ADC (%)
7
9
11
13
15
17
19
21
23
Time (days)

The digestibility of cellulose in all diets, with the exception of the 10% inclusion test diet supplemented with antibiotics, increased over the duration of the experiment (Figures 7.4a and 7.4b). ADC's for cellulose in all diets, with the exception of the 10% inclusion test diet supplemented with antibiotics, were initially low with values being negative over days 7 to 10. Subsequently cellulose digestibility increased with the majority of values greater than 10% over days 12 to 23. The digestibility of cellulose at 10% inclusion in the presence of antibiotics was negative throughout the digestibility trial and did not tend to increase during the experimental period.

SBM digestibility trial:

The daily ADC's for ground SBM are shown in Figure 7.5a and 7.5b. The digestibility of ground SBM varied markedly throughout the duration of the digestibility trial, values ranging from 70.6 ± 3.4% (day 16) to 118.8 ± 1.1% (day 8) (mean 88.5 ± 11.9) at 10% inclusion, 57.0 ± 5.9 (day 8) to 87.6 ± 5.7% (day 20) (mean 77.5 ± 7.8) at 20% inclusion, 60.4 ± 2.2% (day 8) to 77.0 ± 0.5% (day 21) (mean 70.2 ± 6.1%) at 30% inclusion and 51.9 ± 3.4% (day 10) to 77.2 ± 2.8% (day 20) (mean 68.4 ± 6.4%) at 40% inclusion. The magnitude of the daily variation appeared larger at 10% inclusion , with variability lowest at 30% inclusion. The greatest variation in ADC's between consecutive days were 34.6%, 20.1%, 10.0% and 14.7% at 10%, 20%, 30% and 40% inclusion respectively. The mean value of the daily variations in ADC's follows a similar pattern to that of the maximal variation, being 11.9 ± 1.1%, 7.7 ± 4.9%, 5.4 ± 3.1% and 8.0 ± 5.4% at the 10%, 20%, 30% and 40% inclusion respectively.

The digestibility of ground SBM at 10% inclusion decreased over the duration of the digestibility trial, with initial values ranging from 88.4 ± 14.1% to 118.8 ± 1.1% (days 8 to 12) decreasing to 75.0 ± 10.6% to 89.1 ± 6.2% late in the experiment (days 21 to 24) (Figure 7.5). The digestibility of ground SBM at 20%, 30% and 40% inclusion remained constant throughout the digestibility trial.

Tuna oil digestibility trial:

The daily ADC's for tuna oil are shown in Figure 7.6. Tuna oil digestibility varied markedly over the duration of the digestibility trial with values ranging from 72.5 ± 9.2% (day 9) to 89.8 ± 15.3% (day 13) (mean 82.2 ± 6.7%) at 10% inclusion and 44.2 ± 4.8% (day 9) to 78.0 ± 18.1% (day 14) (mean 62.6 ± 12.0%) at 15% inclusion. Daily variation in the digestibility of tuna oil was marked, ranging as high

Figure 7.5a
Daily apparent digestibility coefficients (%) of SBM included at 10% (□) and 30% (◆) into the reference diet versus time (days). Values are mean ± standard deviation (n = 3).

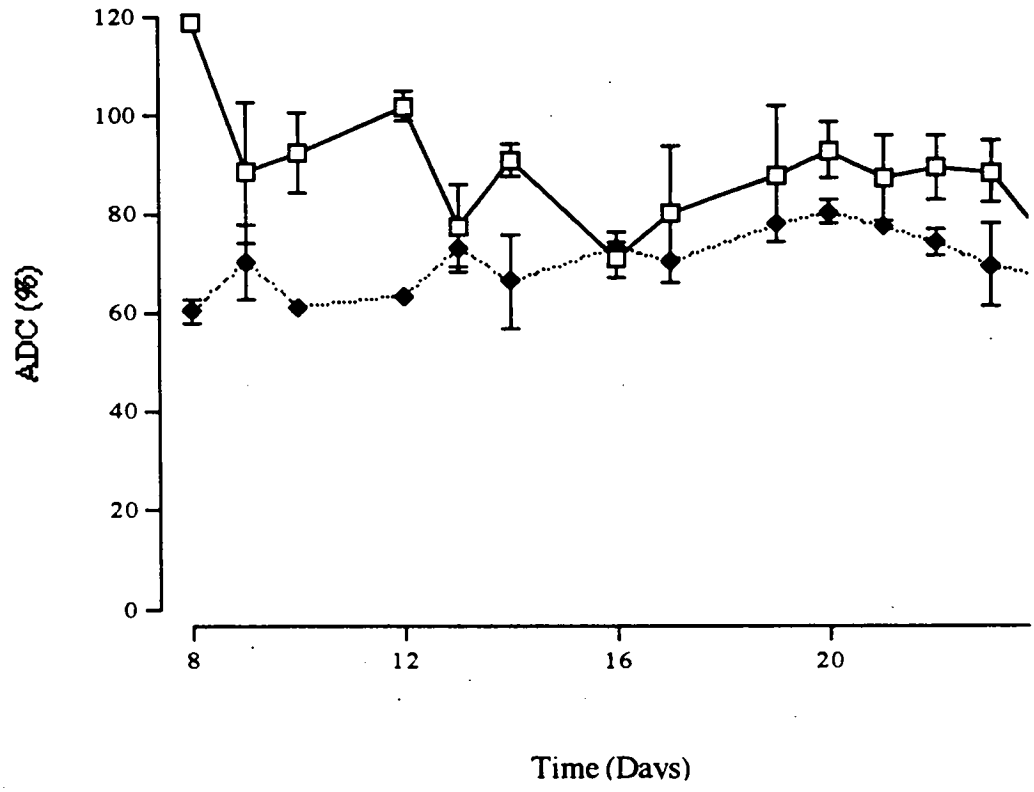

120
100
80
60
40
20
0
ADC (%)
8
12
16
20
Time (Davs)

Figure 7.5b

Daily apparent digestibility coefficients (%) of SBM included at 20% (O), and 40% (▲) into the reference diet versus time (days). Values are mean ± standard deviation (n = 3).

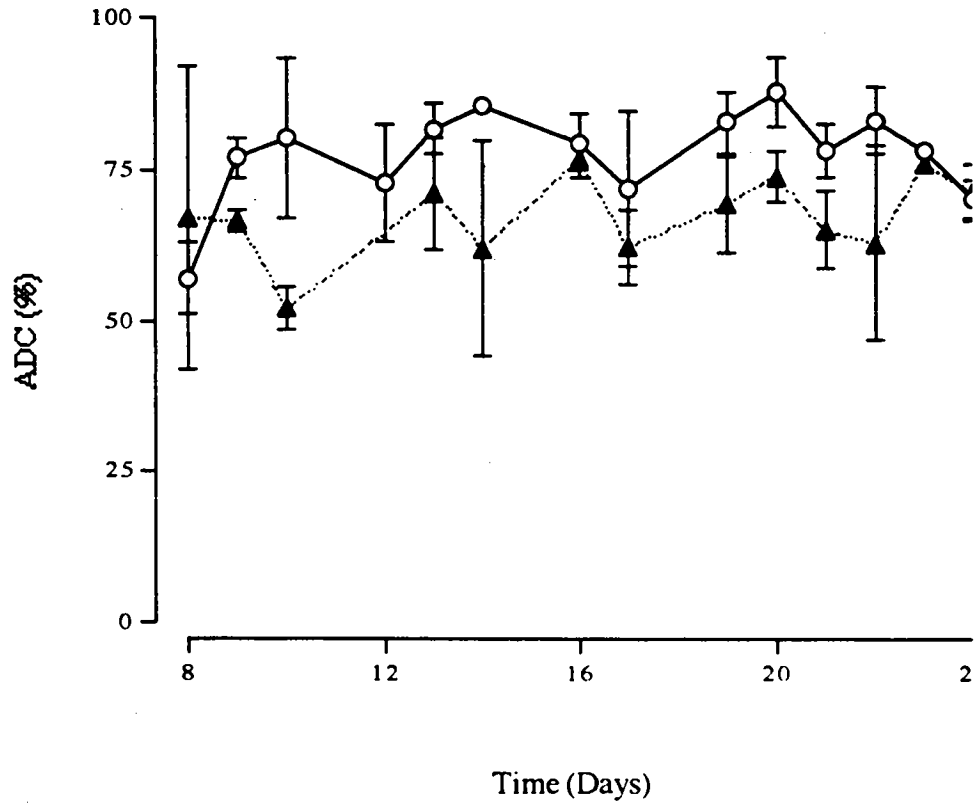
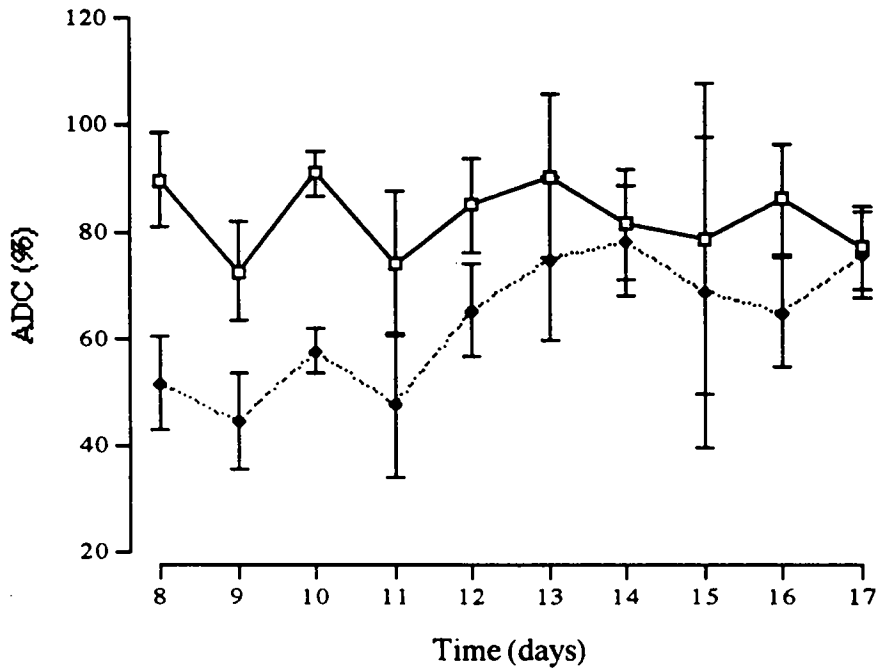

100
75
50
25
0
ADC (%)
8
12
16
20
2
Time (Days)

Figure 7.6
Daily apparent digestibility coefficients (%) of tuna oil included at 10% (□) and 15% (◆) into the reference diet versus time (days). Values are mean ± standard deviation (n = 5).


120
100
80
60
40
20
ADC (%)
8
9
10
11
12
13
14
15
16
17
Time (days)

as 17.0% (between days 8 and 9) at 10% inclusion and 13.2% (between days 9 and 10) at 15% inclusion. The degree of variability in tuna oil digestibility did not differ with the inclusion of the test ingredient, with the mean variation between consecutive days being 10.7 ± 5.4% and 9.3 ± 4.4% at 10% and 15% inclusion respectively.

Digestibility of tuna oil included at 15% appeared to increase over the experimental period (Figure 7.6). ADC's over days 8 to 11 ranged from 44.2% to 57.4%, while values from days 14 to 17 ranged from 64.4% to 78.0%. The digestibility of tuna oil at a 10% inclusion did not appear to increase over the duration of the trial. As a result the large difference between digestibility of tuna oil at 10% and 15% inclusion, present at the beginning of the trial, decreased and values were similar at the conclusion of the experiment.

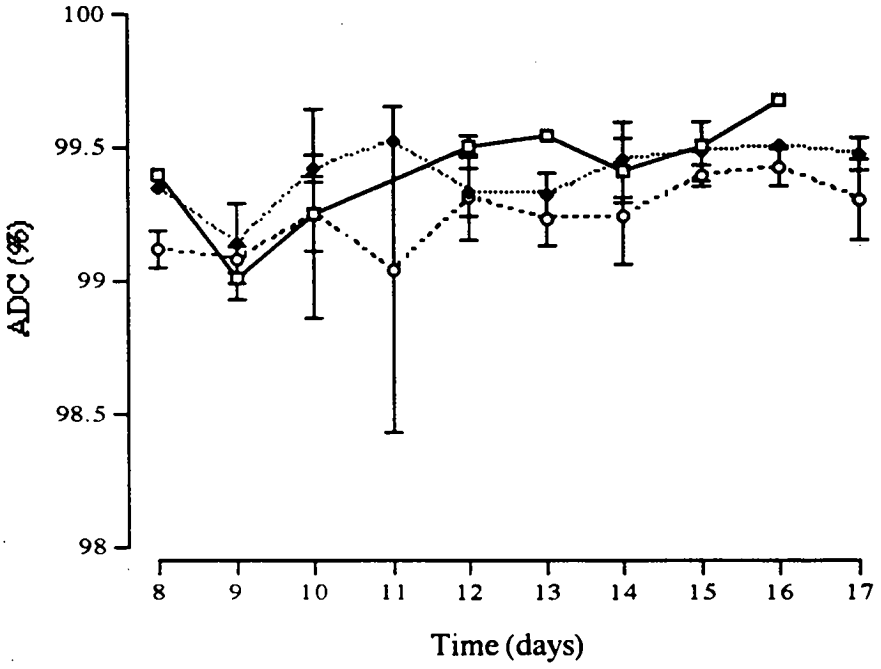
7.3.3 Digestibility of Protein in the Experimental Diets

Tuna oil digestibility trial:

The daily ADC's for protein from the experimental diets used in the graded tuna oil digestibility trial are shown in Figure 7.7. Protein digestibility was high, greater than 99.0% for the three experimental diets at all time points measured. The high and consistent protein digestibility did not allow for large daily variation in digestibility, however ADC's still ranged from 99.0 ± 0.1% (day 9) to 99.7 ± 0.0% (day 16) (mean 99.4 ± 0.2%) for the reference diet, 99.1 ± 0.2% (day 9) to 99.5 ± 0.0% (day 16) (mean 99.4 ± 0.1%) for the 10% inclusion test diet and 99.0 ± 0.6% (day 11) to 99.4 ± 0.1% (day 16) (mean 99.2 ± 0.1%) for the 15% inclusion test diet.

Daily variability of protein digestibility was fairly uniform for the experimental diets, with the largest changes in ADC's for dietary protein between consecutive days being 0.4% (days 8 and 9), 0.3% (days 9 and 10) and 0.3% (days 11 and 12) for the reference diet, 10% inclusion test diet and 15% inclusion test diet respectively. Mean daily variation in dietary protein digestibility also did not show a difference for the diets, being 0.2 ± 0.1% for the reference diet and 0.1 ± 0.1% and 0.1 ± 0.1% for the 10% inclusion test diet and 15% inclusion test diet respectively.

Figure 7.7
Daily apparent digestibility coefficients (%) of crude protein from the reference diet (□), 10% inclusion diet (◆) and 15% inclusion diet (○) used in the graded tuna oil digestibility trial versus time (days). Values are mean ± standard deviation (n = 5).


100
99.5
99
98.5
98
ADC (%)
8
9
10
11
12
13
14
15
16
17
Time (days)

7.3.4 Digestibility of Lipid in the Diets

Tuna oil digestibility trial:

The daily ADC's for dietary lipid from the experimental diets used in the graded tuna oil digestibility trial are shown in Figure 7.8. Dietary lipid digestibility showed a marked daily variation, with ADC's ranging from 58.1 ± 9.1% (day 9) to 78.7 ± 1.0% (day 14) (mean 69.9 ± 7.6%) for the reference diet, 58.8 ± 3.3% (day 9) to 81.0 ± 1.9% (day 15) (mean 75.5 ± 6.6%) for the 10% inclusion test diet and 53.8 ± 1.8% (day 9) to 82.1 ± 2.9% (day 17) (mean 69.5 ± 8.7%) for the 15 % inclusion test diet. Differences in dietary lipid digestibility between consecutive days did not vary greatly between the diets, with the maximum and mean differences between consecutive days being 10.0% (between days 9 and 10) and 4.2 ± 3.2% for the reference diet, 14.4% (between days 8 and 9) and 4.2 ± 5.8% for the 10% inclusion test diet, and 11.5% (between days 9 and 10) and 3.8 ± 3.6% for the 15% inclusion test diet.

Dietary lipid digestibility for all three experimental diets showed a tendency to increase over the duration of the digestibility trial (Figure 7.8).

7.3.5 Daily Faecal Collection and Digestibility Calculations

Daily faeces collection rates for the graded cellulose, ground SBM and tuna oil digestibility trials are detailed in Tables 7.1, 7.2 and 7.3 respectively. From these tables it can be seen marked variation existed in faecal collections both on individual days and over the duration of the digestibility trial for each experimental replicate. On several occasions replicates in all three digestibility trials produced no faeces during the daily collection period, while maximal faeces collection (dry weight) were 1.58g (day 7, 10% inclusion test diet with antibiotics, replicate 1) for the graded cellulose digestibility trial, 1.89g (day 14, reference diet replicate 1) for the ground SBM digestibility trial and 3.26g (day 8, 10% inclusion test diet, replicate 2) for the graded tuna oil digestibility trial. Total faecal collection (dry weight) for each experimental replicate ranged from 1.49g (reference diet, replicate 4) to 12.69g (10% inclusion diet with antibiotics, replicate 1) (mean 3.83 ± 2.01g) for the graded cellulose digestibility trial, 0.38g (40% test diet, replicate 3) to 12.81g (reference diet, replicate 1) (mean 5.49 ± 4.31g) for the ground SBM digestibility trial and 3.24g (10% inclusion test diet, replicate 1) to 19.14g (15% test diet, replicate 5) (mean 10.07 ± 4.83g) for the tuna oil digestibility trial. The proportion of the total faeces supplied by a single collection day was also highly variable, with the maximum percentage of total

Figure 7.8
Daily apparent digestibility coefficients (%) of crude lipid from the reference diet (□), 10% inclusion diet (◆) and 15% inclusion diet (○) used in the graded tuna oil digestibility trial versus time (days). Values are mean ± standard deviation (n = 5).

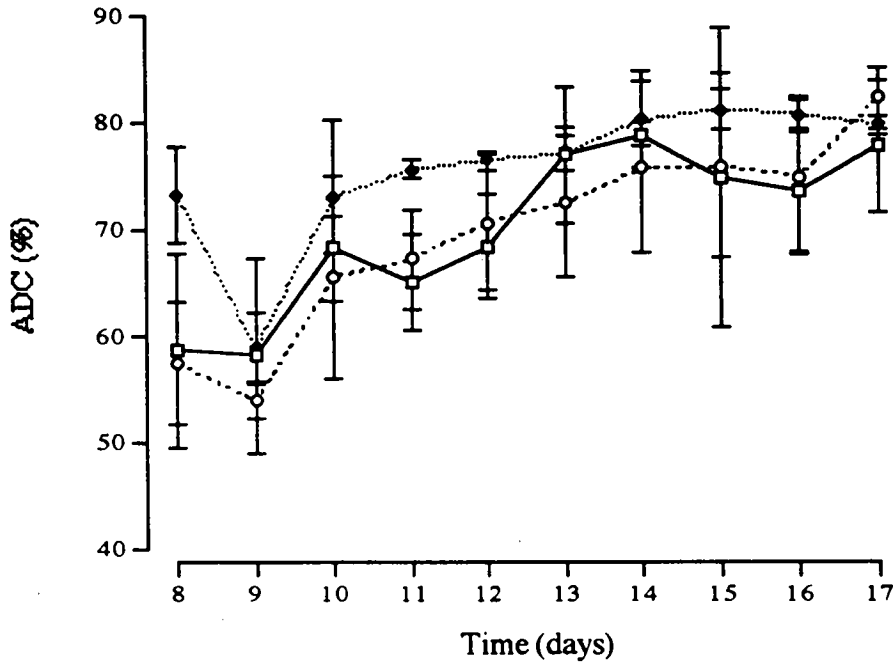

90
80
70
60
50
40
ADC (%)
8
9
10
11
12
13
14
15
16
17
Time (days)

Table 7.1 Dry weight faeces (g) collected for analysis during the graded cellulose digestibility trial. Values for the percentage of total faeces supplied by daily collections are in parentheses.

Day	Dry Weight Faeces Pooled for Analysis (g)															
	Reference				10% Cellulose				20% Cellulose				30% Cellulose			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
7	0.01	0.60	0.12	0.04	0.19	0.24	0.15	0.18	0.38	0.64	0.29	0.08	0.01	0.42	0.25	0.23
	(0.37)	(11.72)	(4.88)	(2.68)	(4.26)	(6.52)	(9.32)	(6.08)	(9.74)	(11.31)	(8.10)	(3.90)	(0.44)	(7.65)	(9.36)	(8.33)
8	0.04	0.44	0.14	0.01	0.37	0.07	0.08	0.32	0.10	0.18	0.17	0.07	0.06	0.14	0.18	0.16
	(1.50)	(8.59)	(5.69)	(0.67)	(8.30)	(1.90)	(4.97)	(10.81)	(2.56)	(3.18)	(4.75)	(3.41)	(2.62)	(2.55)	(6.74)	(5.80)
10	0.03	0.21	0.12	0.10	0.20	0.02	0.08	0.05	0.16	0.08	0.18	0.07	0.08	0.10	0.10	0.39
	(1.12)	(4.10)	(4.88)	(6.71)	(4.48)	(0.54)	(4.97)	(1.69)	(4.10)	(1.41)	(5.03)	(3.90)	(3.49)	(1.82)	(3.75)	(14.13)
12	0.22	0.34	0.20	0.05	0.19	0.27	0.04	0.30	0.33	0.49	0.29	0.11	0.28	0.60	0.32	0.28
	(8.24)	(6.64)	(8.13)	(3.36)	(4.26)	(7.34)	(2.48)	(10.14)	(8.46)	(8.66)	(8.10)	(5.37)	(12.23)	(10.93)	(11.99)	(10.14)
13	0.34	0.42	0.27	0.18	0.48	0.25	0.16	0.28	0.52	0.56	0.47	0.19	0.39	0.40	0.26	0.25
	(12.73)	(8.20)	(10.98)	(12.08)	(10.76)	(6.79)	(9.94)	(9.46)	(13.33)	(9.89)	(13.13)	(9.27)	(17.03)	(7.29)	(9.74)	(9.06)
14	0.33	0.43	0.33	0.09	0.65	0.22	0.14	0.38	0.38	0.46	0.43	0.31	0.16	0.49	0.27	0.37
	(12.36)	(8.40)	(13.41)	(6.04)	(14.57)	(5.98)	(8.70)	(12.84)	(9.74)	(8.13)	(12.01)	(15.12)	(6.99)	(8.93)	(10.11)	(13.41)
15	0.30	0.48	0.24	0.40	0.34	0.92	0.59	0.23	0.57	0.66	0.35	0.40	0.26	0.32	0.18	0.21
	(11.24)	(9.83)	(9.76)	(26.85)	(7.62)	(25.00)	(36.65)	(7.77)	(14.62)	(11.66)	(9.78)	(19.51)	(11.35)	(5.83)	(6.74)	(7.61)
16	0.29	0.44	0.28	0.23	0.48	0.57	0.18	0.42	0.44	1.31	0.37	0.28	0.22	0.77	0.17	0.22
	(10.86)	(8.59)	(11.38)	(15.44)	(10.76)	(15.49)	(11.18)	(14.19)	(11.28)	(23.14)	(10.34)	(13.66)	(9.61)	(14.03)	(14.61)	(7.97)
19	0.12	0.12	0.14	0.03	0.19	0.46	0.02	0.18	0.20	0.53	0.23	0.12	0.16	0.77	0.04	0.20
	(4.49)	(2.34)	(5.69)	(2.01)	(4.26)	(12.50)	(1.24)	(6.08)	(5.13)	(9.36)	(6.42)	(5.85)	(6.99)	(14.03)	(9.74)	(7.25)
20	0.11	0.25	0.12	0.03	0.19	0.12	0.01	0.18	0.22	0.16	0.26	0.16	0.13	0.25	0.13	0.08
	(4.12)	(4.88)	(4.88)	(2.01)	(4.26)	(3.26)	(0.62)	(6.08)	(5.64)	(2.83)	(7.26)	(7.80)	(5.68)	(4.55)	(8.99)	(2.90)
21	0.29	0.61	0.31	0.12	0.66	0.37	0.12	0.25	0.43	0.46	0.30	0.22	0.41	0.59	0.30	0.18
	(10.86)	(11.91)	(12.60)	(8.05)	(14.80)	(10.05)	(7.45)	(8.45)	(11.03)	(8.13)	(8.38)	(10.73)	(17.90)	(10.75)	(6.74)	(6.52)
22	0.40	0.38	0.16	0.12	0.45	0.12	0.01	0.09	0.17	0.08	0.16	0.00	0.09	0.16	0.34	0.13
	(14.98)	(6.45)	(6.50)	(8.05)	(10.09)	(3.26)	(0.62)	(3.04)	(4.36)	(1.41)	(4.47)	(0.00)	(3.93)	(2.91)	(0.75)	(4.71)
23	0.19	0.45	0.03	0.09	0.07	0.05	0.03	0.10	0.00	0.05	0.08	0.03	0.04	0.48	0.35	0.06
	(7.12)	(8.79)	(1.22)	(6.04)	(1.57)	(1.36)	(1.86)	(3.38)	(0.00)	(0.88)	(2.23)	(1.46)	(1.75)	(8.74)	(0.75)	(2.17)
Total	2.67	5.12	2.46	1.49	4.46	3.68	1.61	2.96	3.90	5.66	3.58	2.05	2.29	5.49	2.67	2.76
Mean	2.94 ± 1.54				3.18 ± 1.21				3.80 ± 1.31				3.30 ± 1.47			

Table 7.1 (cont.)

Day	Dry Weight Faeces Pooled for Analysis (g)															
	Reference*				10% Cellulose*				20% Cellulose*				30% Cellulose*			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
7	0.09	0.28	0.32	0.18	1.58	0.96	0.15	0.37	0.39	0.57	0.30	0.28	0.45	0.42	0.12	0.41
	(2.21)	(7.63)	(9.67)	(5.44)	(12.45)	(6.52)	(9.20)	(8.17)	(8.92)	(15.08)	(11.11)	(5.88)	(9.43)	(7.65)	(6.15)	(8.40)
8	0.47	0.35	0.44	0.32	2.03	0.83	0.23	0.37	0.45	0.34	0.33	0.28	0.43	0.14	0.27	0.34
	(11.52)	(9.54)	(13.29)	(9.67)	(16.00)	(1.90)	(14.11)	(8.17)	(10.30)	(8.99)	(12.22)	(5.88)	(9.01)	(2.55)	(13.85)	(6.97)
10	0.29	0.23	0.39	0.42	0.73	0.43	0.23	0.77	0.35	0.04	0.26	0.09	0.32	0.10	0.22	0.27
	(7.11)	(6.27)	(11.78)	(12.69)	(5.75)	(0.54)	(14.11)	(17.00)	(8.01)	(1.06)	(9.63)	(1.89)	(6.71)	(1.82)	(11.28)	(5.53)
12	0.01	0.21	0.06	0.01	0.57	0.31	0.00	0.44	0.15	0.21	0.05	0.46	0.22	0.60	0.12	0.29
	(0.25)	(5.72)	(1.81)	(0.30)	(4.49)	(7.34)	(0.00)	(9.71)	(3.43)	(5.56)	(1.85)	(9.66)	(4.61)	(10.93)	(6.15)	(5.94)
13	0.34	0.32	0.26	0.30	1.09	0.80	0.12	0.28	0.43	0.23	0.33	0.40	0.43	0.40	0.14	0.25
	(8.33)	(8.72)	(7.85)	(9.06)	(8.59)	(6.79)	(7.36)	(6.18)	(9.84)	(6.08)	(12.22)	(8.40)	(9.01)	(7.29)	(7.18)	(5.12)
14	0.25	0.32	0.33	0.34	1.11	1.05	0.13	0.43	0.41	0.44	0.15	0.59	0.26	0.49	0.14	0.34
	(6.13)	(8.72)	(9.97)	(10.27)	(8.75)	(5.98)	(7.98)	(9.49)	(9.38)	(11.64)	(5.56)	(12.39)	(5.45)	(8.93)	(7.18)	(6.97)
15	0.23	0.67	0.18	0.76	0.52	0.38	0.21	0.52	0.20	0.40	0.17		0.54	0.32	0.18	0.36
	(5.64)	(18.26)	(5.44)	(22.96)	(4.10)	(25.00)	(12.88)	(11.48)	(4.58)	(10.58)	(6.30)		(11.32)	(5.83)	(9.23)	(7.38)
16	0.47	0.31	0.17	0.17	0.99	0.76	0.16	0.61	0.46	0.49	0.20	0.66	0.51	0.77	0.22	0.87
	(11.52)	(8.45)	(5.14)	(5.14)	(7.80)	(15.49)	(9.82)	(13.47)	(10.53)	(12.96)	(7.41)	(13.87)	(10.69)	(14.03)	(11.28)	(17.83)
19	0.00	0.17	0.04	0.02	0.83	0.08	0.00	0.19	0.11	0.33	0.04	0.43	0.30	0.77	0.03	0.39
	(0.00)	(4.63)	(1.21)	(0.60)	(6.54)	(12.50)	(0.00)	(4.19)	(2.52)	(8.73)	(1.48)	(9.24)	(6.29)	(14.03)	(1.54)	(7.99)
20	0.12	0.18	0.13	0.03	0.47	0.19	0.04	0.10	0.21	0.23	0.16	0.45	0.23	0.25	0.15	0.42
	(2.94)	(4.90)	(3.93)	(0.91)	(3.70)	(3.26)	(2.45)	(2.21)	(4.81)	(6.08)	(5.93)	(9.45)	(4.82)	(4.55)	(7.69)	(8.61)
21	0.37	0.31	0.30	0.24	1.35	0.42	0.10	0.30	0.49	0.20	0.40	0.56	0.55	0.59	0.21	0.47
	(9.07)	(8.45)	(9.06)	(7.25)	(10.64)	(10.05)	(6.13)	(6.62)	(11.21)	(5.29)	(14.81)	(11.76)	(11.53)	(10.75)	(10.77)	(9.63)
22	0.51	0.14	0.34	0.17	1.38	0.28	0.15	0.02	0.42	0.18	0.24	0.35	0.40	0.16	0.07	0.29
	(12.50)	(3.81)	(10.27)	(5.14)	(10.87)	(3.26)	(9.20)	(0.44)	(9.61)	(4.76)	(8.89)	(7.35)	(8.39)	(2.91)	(3.59)	(5.94)
23	0.93	0.18	0.35	0.35	0.04	0.19	0.11	0.13	0.30	0.12	0.07	0.20	0.13	0.48	0.08	0.18
	(22.79)	(4.90)	(10.57)	(10.57)	(0.32)	(1.36)	(6.75)	(2.87)	(6.86)	(3.17)	(2.59)	(4.20)	(2.73)	(8.74)	(4.10)	(3.69)
Total	4.08	3.67	3.31	3.31	12.69	3.68	1.63	4.53	4.37	3.78	2.70	4.76	4.77	5.49	1.95	4.88
Mean	3.59 ± 0.37				3.63 ± 4.86				3.90 ± 0.90				4.27 ± 1.58			

* Antibiotics incorporated into the experimental diets.

Table 7.2 Dry weight faeces(g) collected for analysis during the graded ground SBM digestibility trial. Values for the percentage of total faeces supplied by daily collections are in parentheses.

Day	Dry Weight Faeces Pooled for Analysis (g)														
	Reference			10% Ground SBM			20% Ground SBM			30% Ground SBM			40% Ground SBM		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
9	0.05	0.03	0.01	0.11	0.01		0.04	0.02		0.06	0.04	0.10		0.05	0.04
	(0.40)	(0.28)	(0.12)	(0.97)	(0.48)		(0.61)	(0.59)		(1.51)	(2.04)	(14.29)		(2.23)	(10.53)
10	0.09	0.06	0.24	0.17	0.04	0.13	0.20	0.06	0.10	0.26	0.07	0.05	0.10	0.08	0.02
	(0.71)	(0.56)	(2.80)	(1.49)	(1.92)	(1.16)	(3.07)	(1.76)	(3.60)	(6.55)	(3.57)	(7.14)	(2.76)	(3.57)	(5.26)
12	0.18	0.09	0.35	1.20	0.03	0.46	0.19	0.09	0.05	0.08	0.02	0.01	0.22	0.10	0.00
	(1.37)	(0.84)	(4.08)	(10.54)	(1.44)	(4.11)	(2.91)	(2.64)	(1.80)	(2.02)	(1.02)	(1.43)	(6.08)	(0.45)	(2.63)
13	0.40	0.93	0.35	0.56	0.01	0.44	0.36	0.11	0.10	0.16	0.04	0.00	0.08	0.00	0.00
	(3.11)	(8.69)	(4.08)	(4.92)	(0.48)	(3.93)	(5.52)	(3.23)	(3.60)	(4.03)	(2.04)	(0.00)	(2.21)	(0.00)	(0.00)
14	1.89	1.27	1.68	1.22	0.32	1.31	0.98	0.25	0.43	0.62	0.20	0.14	0.38	0.26	0.05
	(14.76)	(11.87)	(19.58)	(10.71)	(15.38)	(11.70)	(15.03)	(7.33)	(15.47)	(15.62)	(10.20)	(20.00)	(10.50)	(11.61)	(13.16)
15	1.73	0.31	0.80	0.82	0.10	0.79	0.37	0.12	0.03	0.09	0.04	0.00	0.03	0.02	0.00
	(13.47)	(2.90)	(9.32)	(7.20)	(4.81)	(7.05)	(5.67)	(3.52)	(1.08)	(2.27)	(2.04)	(0.00)	(0.83)	(0.89)	(0.00)
16	1.62	1.23	1.12	1.46	0.18	1.24	1.25	0.98	0.26	0.56	0.56	0.13	1.06	0.28	0.10
	(12.68)	(11.50)	(13.05)	(12.82)	(8.65)	(11.07)	(19.17)	(28.74)	(9.35)	(14.11)	(28.57)	(18.57)	(29.28)	(12.50)	(26.32)
17	1.33	1.35	0.53	0.94	0.03	1.48	0.47	0.05	0.11	0.10	0.02	0.02	0.07	0.07	0.02
	(10.37)	(12.62)	(6.18)	(8.25)	(1.44)	(13.21)	(7.21)	(1.47)	(3.96)	(2.52)	(1.02)	(2.86)	(1.93)	(3.13)	(5.26)
19	1.29	1.05	0.43	1.08	0.15	1.09	0.78	0.35	0.18	0.43	0.45	0.02	0.82	0.48	0.03
	(10.10)	(9.81)	(5.01)	(9.48)	(7.21)	(9.73)	(11.96)	(10.26)	(6.47)	(10.83)	(22.96)	(2.86)	(22.65)	(21.43)	(7.89)
20	1.06	0.96	0.23	1.01	0.10	1.01	0.35	0.18	0.38	0.33	0.21	0.02	0.26	0.24	0.01
	(8.24)	(8.97)	(2.68)	(8.87)	(4.81)	(9.02)	(5.37)	(5.28)	(13.67)	(8.31)	(10.71)	(2.86)	(7.18)	(10.71)	(2.63)
21	0.87	0.88	0.70	0.71	0.20	0.83	0.66	0.20	0.76	0.44	0.04	0.13	0.19	0.62	0.05
	(6.78)	(8.22)	(8.16)	(6.23)	(9.62)	(7.41)	(10.12)	(5.87)	(27.34)	(11.08)	(2.04)	(18.57)	(5.25)	(27.68)	(13.16)
22	0.92	0.93	0.62	0.70	0.19	0.84	0.24	0.13	0.14	0.14	0.04	0.02	0.04	0.06	0.01
	(7.20)	(8.69)	(7.23)	(6.15)	(9.13)	(7.50)	(3.68)	(3.81)	(5.04)	(3.53)	(2.04)	(2.86)	(1.10)	(2.68)	(2.63)
23	0.88	0.93	0.99	0.79	0.57	0.65	0.38	0.72	0.02	0.53	0.18	0.00	0.23	0.02	0.01
	(6.88)	(8.69)	(11.54)	(6.94)	(27.40)	(5.80)	(5.83)	(21.11)	(0.72)	(13.35)	(9.18)	(0.00)	(6.35)	(0.89)	(2.63)
24	0.50	0.68	0.53	0.62	0.15	0.93	0.25	0.15	0.22	0.17	0.05	0.06	0.14	0.05	0.03
	(3.92)	(6.36)	(6.18)	(5.44)	(7.21)	(8.30)	(3.83)	(4.40)	(7.91)	(4.28)	(2.55)	(8.57)	(3.87)	(2.23)	(7.89)
Total	12.81	10.70	8.58	11.39	2.08	11.20	6.52	3.41	2.78	3.97	1.96	0.70	3.62	2.24	0.38
Mean	10.70 ± 2.12			8.22 ± 5.32			4.24 ± 2.00			2.21 ± 1.65			2.08 ± 1.63		

Table 7.3 Dry weight faeces (g) collected for analysis during the graded tuna oil digestibility trial. Values for the percentage of total faeces supplied by daily collections are in parentheses.

Day	Dry Weight Faeces Pooled for Analysis (g)														
	Reference					10% Tuna Oil					15% Tuna Oil				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
8	0.82	0.34	0.04	0.19	0.52	0.04	3.26	3.04	?	2.10	0.80	1.13	0.07	1.80	1.24
	(12.44)	(4.76)	(0.52)	(2.63)	(8.95)	(1.23)	(58.74)	(29.04)	(?)	(12.57)	(8.26)	(6.02)	(0.68)	(14.94)	(6.48)
9	1.95	1.90	1.70	1.24	1.38	0.42	1.59	1.56	2.02	2.90	2.10	2.95	2.39	2.42	4.12
	(29.59)	(26.57)	(22.28)	(17.17)	(23.75)	(12.96)	(28.65)	(14.90)	(18.84)	(17.35)	(21.67)	(15.71)	(23.25)	(20.08)	(21.53)
10	0.86	0.53	0.41	0.60	0.33	0.10	0.02	1.20	1.02	2.14	1.35	2.50	1.26	1.70	1.64
	(13.05)	(7.41)	(5.37)	(8.31)	(5.68)	(3.09)	(0.36)	(11.46)	(9.51)	(12.81)	(13.93)	(13.31)	(12.26)	(14.11)	(8.57)
11	0.30	0.48	0.17	0.02	0.04	0.02	0.00	0.13	0.36	1.71	0.46	2.01	0.79	1.09	2.02
	(4.55)	(6.85)	(2.23)	(0.28)	(0.69)	(0.62)	(0.00)	(1.24)	(3.36)	(10.23)	(4.75)	(10.70)	(7.68)	(9.05)	(10.55)
12	0.00	1.19	1.46	0.53	0.73	0.42	0.17	0.89	1.30	1.61	1.18	1.59	1.30	0.94	1.93
	(0.00)	(16.64)	(19.13)	(7.34)	(12.56)	(12.96)	(3.06)	(8.50)	(12.13)	(9.63)	(12.18)	(8.47)	(12.65)	(7.80)	(10.08)
13	0.69	0.54	1.10	0.25	0.15	0.40	0.02	1.28	1.31	1.39	1.12	2.22	1.40	0.59	1.92
	(10.47)	(7.55)	(14.42)	(3.46)	(2.58)	(12.35)	(0.36)	(12.23)	(12.22)	(8.32)	(11.56)	(11.82)	(13.62)	(4.90)	(10.03)
14	0.99	0.92	1.08	1.10	1.11	0.42	0.14	0.79	1.42	1.22	0.83	1.96	1.36	0.86	2.02
	(15.02)	(12.87)	(14.15)	(15.24)	(19.10)	(12.96)	(2.52)	(7.55)	(13.25)	(7.30)	(8.57)	(10.44)	(13.23)	(7.14)	(10.55)
15	0.33	0.51	0.73	0.34	0.42	0.38	0.04	0.62	0.92	1.34	0.5	1.76	0.74	0.90	1.88
	(5.01)	(7.13)	(9.57)	(12.74)	(7.23)	(11.73)	(0.72)	(5.92)	(8.58)	(8.02)	(5.26)	(9.37)	(7.20)	(7.47)	(9.82)
16	0.39	0.51	0.75	0.62	0.73	0.79	0.31	0.63	1.22	0.88	0.82	1.40	0.64	1.29	1.80
	(5.92)	(7.13)	(9.83)	(16.90)	(12.56)	(24.38)	(5.59)	(6.02)	(11.38)	(5.27)	(8.46)	(7.45)	(6.23)	(10.71)	(9.40)
17	0.26	0.22	0.19	0.12	0.40	0.25	0.00	0.32	1.15	1.42	0.52	1.26	0.33	0.46	0.57
	(3.95)	(3.08)	(2.49)	(15.93)	(6.88)	(7.72)	(0.00)	(3.15)	(10.73)	(8.50)	(5.37)	(6.71)	(3.21)	(3.82)	(2.98)
Total	6.59	7.15	7.63	7.22	5.81	3.24	5.55	10.47	10.72	16.71	9.69	18.78	10.28	12.05	19.14
Mean	6.88 ± 0.70					9.34 ± 5.22					13.99 ± 4.62				

faecal collection supplied by a single time point being 36.65% (day 15, 10% inclusion test diet, replicate 3) for the graded cellulose digestibility trial, 29.28% (day 16, 40% inclusion test diet, replicate 1) for the graded ground SBM digestibility trial and 58.47% (day 8, 10% inclusion test diet, replicate 2) for the graded tuna oil digestibility trial.

The mean faeces collection for the experimental treatments also showed marked variation (Tables 7.1, 7.2 and 7.3). In the graded cellulose and tuna oil digestibility trials, faeces collection tended to increase with the level of test ingredient incorporation. During the graded cellulose digestibility trial mean faeces collection increased from 2.94 ± 1.54g (reference diet) to 3.80g ± 1.48 (20% inclusion test diet) for diets supplemented with antibiotics and 3.59 ± 0.37 (reference diet) to 4.27 ± 1.58g (30% inclusion test diet) for diets not supplemented with antibiotics. Mean faecal collection in the tuna oil digestibility trial increased from 6.88 ± 0.70g for the reference diet to 13.99 ± 4.62g for the 15% inclusion test diet. Mean faecal collection for the ground SBM digestibility trial tended to decrease with the inclusion of ground SBM from 10.70 ± 2.12g for the reference diet to 2.08 ± 1.63g for the 40% inclusion test diet.

7.3.6 Mean Daily ADC's Versus Predicted ADC's

The mean of the daily whole diet ADC's for each experimental replicate, and the mean values for each treatment, of the graded cellulose, ground SBM and tuna oil digestibility trials are detailed in Tables 7.4, 7.5 and 7.6 respectively. Also detailed in these tables are the "expected" ADC's approximating digestibility determined from pooled faeces (refer Equation 7.1, Section 7.2.4). From Table 7.4 it can be seen that good agreement existed between the mean of the daily ADC's and the "expected" ADC's for all treatments of the graded cellulose digestibility trial, the largest difference being 0.7% for the 20% inclusion test diet. The comparison of the mean daily ADC's and "expected" ADC's for the experimental replicates used in the graded cellulose digestibility trial also showed good agreement with the greatest difference being 1.3% for replicate 3 of the 30% inclusion test diet with antibiotic supplementation.

The mean daily ADC's and expected ADC's for the graded ground SBM digestibility trial also showed good agreement with the exception of 20%, 30% and 40% inclusion test diet for days 8 to 17 (Table 7.5). Differences between the two ADC values for these treatments were 1.2%, 1.4% and 2.6% respectively with the "expected" ADC's being the higher in all three cases. The difference in values for the remaining treatments varied from 0.0% (20% inclusion test diet, days 19 to 24) to 0.7% (30%

Table 7.4 Mean daily ADC's (%), calculated "expected" ADC's (%) and ADC's measured from pooled faeces for the experimental diets used in the graded cellulose digestibility trial.

Diet Type	Replicate	Mean Daily ADC's	Calculated "Expected" ADC's[a]	Measured Digestibility Pooled Faeces
Reference	1	83.0	83.5	82.4
	2	84.8	84.8	84.5
	3	84.6	85.0	84.3
	4	84.1	84.4	82.8
	Mean	84.1 ± 0.8	84.4 ± 0.7	83.5 ± 1.1
10% Cellulose	1	76.9	76.6	76.8
	2	76.4	76.6	76.6
	3	76.8	77.6	76.9
	4	75.7	76.7	77.7
	Mean	76.4 ± 0.5	76.9 ± 0.5	77.0 ± 0.5
20% Cellulose	1	67.6	68.2	69.6
	2	69.2	69.9	69.6
	3	68.1	68.5	69.5
	4	67.7	68.9	69.0
	Mean	68.2 ± 0.8	68.9 ± 0.7	69.4 ± 0.3
30% Cellulose	1	57.8	57.0	64.9
	2	61.8	62.5	63.8
	3	61.8	61.8	65.8
	4	60.9	61.0	64.6
	Mean	60.6 ± 1.9	60.6 ± 2.5	64.8 ± 0.8
Reference/AB	1	84.0	84.1	83.5
	2	83.8	83.8	84.2
	3	83.3	83.9	83.9
	4	83.0	83.7	84.3
	Mean	83.5 ± 0.4	83.9 ± 0.2	84.0 ± 0.4

Table 7.4 (cont.)

Diet Type	Replicate	Mean Daily ADC's	Calculated "Expected" ADC's[a]	Measured Digestibility Pooled Faeces
10%	1	73.0	73.1	74.5
Cellulose/AB	2	72.6	72.7	74.6
	3	72.5	72.2	74.6
	4	71.4	70.9	73.6
	Mean	72.4 ± 0.7	72.2 ± 0.9	74.3 ± 0.5
20%	1	68.6	68.8	71.1
Cellulose/AB	2	67.4	67.2	70.8
	3	68.3	69.0	71.0
	4	69.0	69.0	70.6
	Mean	68.3 ± 0.7	68.5 ± 0.8	70.9 ± 0.2
30%	1	60.5	60.5	64.6
Cellulose/AB	2	59.2	58.4	65.4
	3	63.0	61.7	64.3
	4	62.3	61.8	64.6
	Mean	61.3 ± 1.7	60.6 ± 1.5	64.6 ± 0.7

[a] Expected digestibility values were calculated by multiplying the proportion of total faeces collected daily by the daily ADC's and summing the resultant values per replicate (refer Equation 7.1, Section 7.2.4).

Table 7.5 Mean daily ADC's (%), calculated "expected" ADC's (%) and ADC's measured from pooled faeces for the experimental diets used in the graded ground SBM digestibility trial.

Diet Type	Replicate	Mean Daily ADC's	Calculated "Expected" ADC's[a]	Measured Digestibility Pooled Faeces
Days 8 to 17				
Reference	1	81.1	80.9	80.2
	2	82.2	83.1	83.0
	3	82.4	83.1	82.7
	Mean	81.9 ± 0.7	82.4 ± 1.3	82.0 ± 1.5
10% Ground	1	82.2	82.1	82.1
SBM	2	82.7	81.7	82.4
	3	82.5	81.7	82.5
	Mean	82.4 ± 0.2	81.8 ± 0.2	82.4 ± 0.2
20% Ground	1	80.4	81.8	82.4
SBM	2	80.5	82.7	82.0
	3	81.3	81.4	82.5
	Mean	80.8 ± 0.5	82.0 ± 0.6	82.3 ± 0.3
30% Ground	1	77.9	79.9	80.2
SBM	2	78.0	79.5	78.4
	3	76.9	77.7	81.3
	Mean	77.6 ± 0.6	79.0 ± 1.2	80.0 ± 1.5
40% Ground	1	72.7	78.9	78.6
SBM	2	75.7	77.4	79.0
	3	79.6	79.6	
	Mean	76.0 ± 3.4	78.6 ± 1.1	78.8 ± 0.3
Days 19 to 24				
Reference	1	82.9	82.7	84.1
	2	83.2	83.1	82.7
	3	83.0	83.4	83.1
	Mean	83.0 ± 0.1	83.1 ± 0.3	83.3 ± 0.7

Table 7.5 (cont.)

Diet Type	Replicate	Mean Daily ADC's	Calculated "Expected" ADC's[a]	Measured Digestibility Pooled Faeces
10% Ground	1	83.4	83.5	84.8
SBM	2	83.7	84.0	82.8
	3	83.0	82.9	83.3
	Mean	83.7 ± 0.4	83.5 ± 0.6	83.6 ± 1.0
20% Ground	1	82.6	82.7	79.9
SBM	2	82.5	82.6	79.7
	3	82.0	81.8	82.3
	Mean	82.4 ± 0.3	82.4 ± 0.5	80.6 ± 1.4
30% Ground	1	80.8	81.0	79.8
SBM	2	79.1	80.9	79.4
	3	80.9	80.9	
	Mean	80.3 ± 1.0	81.0 ± 0.0	79.6 ± 0.2
40% Ground	1	77.9	76.2	79.0
SBM	2	77.2	79.1	78.7
	3			
	Mean	77.5 ± 0.5	77.7 ± 2.0	78.8± 0.2

[a] Expected digestibility values were calculated by multiplying the proportion of total faeces collected daily by the daily ADC's and summing the resultant values per replicate (refer Equation 7.1, Section 7.2.4).

Table 7.6 Mean daily ADC's (%), calculated "expected" ADC's (%) and ADC's measured from pooled faeces for the experimental diets used in the graded tuna oil digestibility trial.

Diet Type	Replicate	Mean Daily ADC's	Calculated "Expected" ADC's[a]	Measured Digestibility Pooled Faeces
Reference	1	73.5	73.0	74.8
	2	73.4	72.1	76.5
	3	75.3	75.0	77.7
	4	74.8	74.5	77.1
	5	76.0	74.7	78.3
	Mean	74.6 ± 1.1	73.9 ± 1.2	76.9 ± 1.3
10% Tuna Oil	1	74.4	74.8	77.6
	2	75.0	71.2	78.0
	3	75.4	75.3	76.8
	4	75.6	74.1	77.1
	5	76.2	74.9	78.0
	Mean	75.3 ± 0.7	74.1 ± 1.6	77.5 ± 0.5
15% Tuna Oil	1	72.8	71.7	73.9
	2	71.0	69.6	71.8
	3	73.8	71.9	74.6
	4	73.2	72.0	74.7
	5	73.1	72.0	74.1
	Mean	72.8 ± 1.1	71.4 ± 1.1	73.8 ± 1.2

[a] Expected digestibility values were calculated by multiplying the proportion of total faeces collected daily by the daily ADC's and summing the resultant values per replicate (refer Equation 7.1, Section 7.2.4).

inclusion test diet, days 19 to 24), with no real trend for one value to be higher than the other. Comparison of the mean daily ADC's and "expected" ADC's for each experimental replicate followed a similar trend, with values differing by greater than 1.00% in the 20% inclusion test diet (replicate 1 and 2), 30% inclusion test diet (replicate 1 and 2) and 40% inclusion test diet (replicate 1 and 2) on days 8 to 17 and the 30% inclusion test diet (replicate 2) and 40% inclusion test diet (replicate 1 and 2) for days 19 to 24. The greatest variation between mean daily ADC and expected ADC was 6.18% for replicate 1 of the 40% inclusion test diet on days 8 to 17. For the replicates in which the difference was greater than 1.00% the "expected" ADC was higher on all occasions with the exception of replicate 1 for the 40% inclusion test diet on days 19 to 24.

Comparison of the mean daily diet ADC's and the calculated "expected" ADC's for the dietary treatments showed a greater difference between values for the graded tuna oil digestibility study (Table 7.6). The expected ADC's were consistently less than the mean daily ADC's, with the difference increasing from 0.7% (reference diet) to 1.4% (15% inclusion test diet) with the level of inclusion of the test ingredient. Comparison of the mean daily ADC's and the "expected" ADC's for each replicate showed all mean daily ADC's to be higher, with the exception of the replicate 1 of 10% inclusion diet. The greatest differences between the daily mean ADC and "expected" ADC were 1.3% (replicate 2), 3.8% (replicate 2) and 1.9% (replicate 3) for the reference diet, 10% inclusion test diet and 15% inclusion test diet respectively.

7.4 Discussion

The present study has determined the daily digestibility of cellulose, SBM and tuna oil, demonstrating the effects of time on digestibility of diets, dietary ingredients and nutrients.

The apparent digestibility coefficients for all the experimental diets used showed daily variation in digestibility. The daily variation in digestibility was generally greater at higher inclusion of the test ingredient for the graded cellulose and graded SBM digestibility trials, while for the graded tuna oil digestibility trial, daily variation in digestibility tended to be uniform at all levels of inclusion. The digestibility of the test ingredients also exhibited a marked daily variation. The variation between consecutive days was generally higher at the lower levels of inclusion of the test ingredient for the graded cellulose and graded SBM digestibility trials, whereas there was no difference between the two levels of inclusion used in the tuna oil digestibility trial. The observed daily variations in digestibility for common carp in the present study are also of a similar magnitude as the variations found in studies on milkfish (Ferraris *et al.*, 1980), green chromid (De Silva and Perera, 1983), Nile tilapia (De Silva and Perera, 1984) and rainbow trout (de la Noüe *et al.*, 1986).

The daily variation in digestibility did not appear to follow any clear pattern, however there was seldom more than two consecutive days of change in any one direction. That is, a day or two of higher digestibility was generally followed by a day or two of lower digestibility. This rhythm is similar to that described by De Silva and Perera (1983; 1984), although the rhythm found by de la Noüe *et al.* (1986) showed a cycle lasting approximately 7 days. The presence of a rhythmicity in the variation in digestibility further suggests that the daily variation in digestibility reflects the presence of a natural rhythm of the digestive processes of the animal, as previously suggested by De Silva and Perera (1983; 1984).

The digestibility of dietary protein and crude lipid from the experimental diets used in the graded tuna oil digestibility trial were also shown to exhibit daily variation. The highly digestible nature of the protein from the casein-based reference diet resulted in only slight variation in the digestibility of dietary protein. The variability in protein digestibility found in the present study was similar to that described by de la Noüe *et al.* (1986), where the digestibility of protein from fishmeal for rainbow trout was also high, greater than 90%. It would be expected that a highly digestible nutrient would show a reduced variability in digestibility. De Silva and Perera (1983, 1984) found the daily digestibility of dietary protein to vary markedly, although the digestibility of

dietary protein in those studies was considerably less, 61% to 86%, than that observed in the present digestibility trial.

The digestibility of crude lipid in the graded tuna oil study was found to exhibited marked daily variability, in particular at 15% inclusion. Previous studies have not determined daily variability in the digestibility of dietary lipid, however they have described a tendency for the degree of variability in dietary nutrient digestibility to increase as the digestibility of the nutrient decreased (de la Noüe *et al.*, 1986). The digestibility of dietary lipid in the present study showed a variability similar to that described by de la Noüe *et al.* (1986) for starch by rainbow trout, although the digestibility of starch in that study was considerably lower, ranging between 15% to 35%. Variation in dietary lipid digestibility in the present study also approximated that described by De Silva And Perera (1983; 1984) for dietary protein which was of similar digestibility.

Apart from the variation observed in daily ADC's, the digestibility of the experimental diets appeared to increase over the duration of the trial, suggesting a continued adaptation of the experimental animals to the diets used. This effect was most marked for the 15% inclusion diet for the tuna oil digestibility trial. The marked increase in tuna oil digestibility at 15% inclusion over the duration of the trial resulted in the digestibility of tuna oil at 10% and 15% inclusion becoming similar at the end of the trial. Such an increase in digestibility with time may explain the observed effects of the level of inclusion of tuna oil on digestibility found in Trial 2 of Chapter 5 when pooled faeces were used to determine tuna oil digestibility. It would appear from the current experiment, that the effect of the level of inclusion on tuna oil digestibility may have been an artefact resulting from the extended time required for adaptation of the fish to the experimental diet containing a larger proportion of dietary lipid. The adaptation to the dietary lipid is also exemplified by the ADC's of crude lipid from the experimental diets used in the graded tuna oil digestibility trial. Digestibility of lipid from the 10% inclusion diet appeared fairly uniform, allowing for the daily variation, throughout the trial. However, the increase in lipid digestibility for the reference diet is difficult to explain, although it may be due to the need to adaptat to high levels of complex carbohydrate present in the diet (Steffens 1989; Médale *et al.*, 1991; Fagberno, 1992).

An increase in the digestibility of the test ingredient over the duration of the trial was also observed in the graded cellulose digestibility trial. The digestibility of cellulose was initially negative at all inclusion levels, however over the duration of the cellulose digestibility trial increased to be positive and to approximate the value observed in

Chapter 3, with the exception of the 10% inclusion in the presence of antibiotics. The initial low cellulose digestibility in this trial meant the mean of cellulose digestibility throughout the trial was also low, which may account for the differences between the ADC's measured for cellulose in Trials 3 and 4 of Chapter 3. The extremely low nature of the digestibility measured for cellulose at 10% inclusion has been discussed in Chapter 3.

Increases in nutrient digestibility observed over the duration of digestibility trials have been previously reported by De Silva and Perera (1983). Such increases in diet digestibility with time probably reflecting an adaptation of the digestive physiology of the animal to the experimental diets. The fact that in the present study the increase in digestibility with time was more evident for the "physiologically extreme" diets, that is diets containing high levels of lipid or cellulose, would appear to support this hypothesis.

When discussing daily variation in digestibility it is important to consider the effects that such variation may have on the measurement of digestibility coefficients. It is common practice to pool faeces over a number of consecutive days in order to acquire adequate sample for proximate analysis. For sampling methods where total faecal collection is achieved, the effect of pooling faeces exhibiting varying digestibility would be of no consequence. However, where sampling is not complete and the input of faeces from each particular day to the calculation of total digestibility is found to vary, there is the potential for daily variation in digestibility to markedly bias digestibility determinations. Collection of faeces by siphoning is one such situation. In the present study the ADC's of the test diets were calculated as means of the daily digestibility coefficients and as "expected" ADC's adjusted for the amount of faeces collected each day, thereby reflecting digestibility calculated from pooled faeces. From these calculations it can be seen that differences existed between the mean daily digestibility and the expected digestibility of the experimental diets. The differences in the digestibility coefficients was generally low for dietary treatments, although larger variations were found in the values for experimental replicates. Where larger differences existed between the mean daily ADC and the expected ADC it was generally found that faeces collected was dominated by several days which corresponded to either high or low digestibility. Therefore it can be seen that daily variation in digestibility may potentially bias digestibility coefficients measured from pooled faeces if faeces is not collected in consistent amounts over the sampling period.

In the present study it was found that a daily variability existed in whole diet and dietary ingredient digestibility. This digestibility was also found to be present in the

digestibility of dietary nutrients. The potential for the daily variation in digestibility to effect the determination of digestibility coefficients from pooled faeces has also been shown.

Chapter 8

General Discussion

8.1 Introduction

Digestibility coefficients provide a relative measure of the extent to which ingested diets, dietary ingredients and their constituent nutrients have been digested and absorbed by the animal. Assuming all biotic and abiotic effects to be minimal, digestibility coefficients may be used as determinants of biological availability. Digestibility coefficients are therefore among the most important tools in determining feed evaluation, a knowledge of the digestibility of dietary ingredients and nutrients being essential to the formulation of nutritionally adequate, cost-effective diets. Unfortunately, however, digestibility coefficients are most difficult to measure. Therefore the development of methods for accurately and dependably measuring digestibility is vital to the study of fish nutrition.

There are several major areas of concern regarding the accurate measurement of ADC's for fish. The two areas receiving most attention are faeces collection and digestibility markers. As fish live in an aquatic environment, nutrient loss from food and faeces occurs through leaching, the degree to which leaching occurs being related to the time which feed and faeces remain in the water. When determining the effects of various parameters on digestive physiology of fish it is most important that methods of faeces collection are consistent and performed to a rigid regime. Although the rapid collection of faeces using modern automatic faecal collection methods minimises the amount of leaching, it does not prevent leaching from occurring. The apparatus required for automated faecal collection is also prohibitively expensive. The collection of faeces by siphoning is the simplest and least expensive method of faecal collection. Although siphoned faeces is more susceptible to result in the overestimation of digestibility coefficients as a result of the effects of leaching, it provides information on ADC's and indicates changes in digestibility. Therefore siphoning was chosen as the method of faecal collection in the present study.

Digestibility markers must be indigestible and move along the gut at the same rate as the rest of the food material. The greatest debate over the use of markers thus relates to marker digestibility and differential movement. Chromium oxide is the most commonly used external marker in the study of digestibility in both terrestrial and aquatic animals. Although concerns have been raised over its digestibility (Saha and Gilbreath, 1993) and differential movement (Bowen, 1978; Lorico-Querijero and Chiu, 1989), other studies have shown quantitative collection of chromium oxide (de la Noüe and Choubert, 1982) and digestibility measurement comparable with direct determination (de la Noüe and Choubert, 1982; Hajen *et al.*, 1993a). Chromium oxide was therefore used as the external marker in the present study.

Apart from the choice of a faecal collection method and digestibility marker the actual method of determining ingredient and nutrient digestibility needs further investigation. Digestibility coefficients are currently reported as single values, implying that the digestibility is constant (Henken *et al.*, 1985). However, digestibility is influenced by both biological and environmental factors, although a paucity of information is available on these aspects of digestibility (De Silva and Anderson, 1995).

The generally accepted method for determining the digestibility of dietary ingredients and their nutrients for fish is by incorporating the test ingredient into a reference diet at a known inclusion level and comparing test diet and reference diet digestibility. Assuming the test ingredients do not alter the digestibility of the reference diet. To date digestibility coefficients are most commonly determined using a fixed inclusion level. In the majority of studies a 30% inclusion of test ingredient is used, however it has been suggested that 15% or 20% inclusion may be preferable for some ingredients (De Silva *et al.*, 1990). The justification for choosing a level of inclusion is based upon the changes in ingredient digestibility and statistical variance with inclusion. The change in variance is simply a function of distributing experimental error across a greater value, resulting in reduced variation with inclusion. Changes in test ingredient digestibility with inclusion suggests either ingredient digestibility is altered with inclusion or that elevated levels of test ingredient alters the digestibility of the reference diet, either of which has marked ramifications in digestibility determination. Thus a compromise between biological constraint and statistical reliability is necessary. However, it is also necessary to identify any changes in digestibility occurring with the level of inclusion.

Digestibility coefficients are also generally only measured at a single time point, usually represented by pooled faeces. Debate exists over the effects of faeces pooling on digestibility estimations and the time of commencement of faeces collection.

The aims of the present study were to provide information on the effects of inclusion level, time and the pooling of faeces on the estimation of the digestibility of a range of dietary ingredients and nutrients for fish. The effects of ingredient inclusion on the digestibility of other dietary nutrients was also of interest. To gain a broad understanding of these effects the digestibility of an inert filler - cellulose, a purified lipid source - tuna oil, a carbohydrate source - corn starch, and a plantaceous protein source - solvent extracted soybean meal, were determined.

8.2 Effect of Inclusion Level on Digestibility

The purified wood cellulose BW 40 Solka floc is generally considered to be an inert filler in artificial diets for fish. Although cellulase activity has been identified in fish (refer Section 1.2.4.3), few studies have determined the digestibility of cellulose for fish (Shcherbina and Kazlauskene, 1971; Smith, 1971; Bergot, 1981). Incorporation of BW 40 Solka floc into the diet of the carnivorous Murray cod, *Maccullochella peelii peelii*, appeared to have a positive effect on digestibility at 10% inclusion, while at higher levels it tended to inhibit the processes of digestion. The mechanisms of these processes were not investigated. Conversely cellulose appeared to be digestible for the omnivorous common carp, *Cyprinus carpio*, at a level of nutritional significance although the level of digestibility was not consistent. A decrease in digestibility at higher inclusion levels suggested the mechanism of cellulose digestion was saturable. Previous studies have suggested that the resident intestinal micro-flora and/or micro-flora introduced with food items are responsible for the production of intestinal cellulase in fish (Stickney and Shumway, 1974; Prejs and Blaszcyk, 1977, Lindsay and Harris, 1980). Although the failure of antibiotics to affect cellulose digestibility in the present study suggests intestinal micro-flora were not responsible for the production of intestinal cellulase and therefore cellulose digestion, microbiological analyses indicated a failure of the antibiotic treatment employed to effectively eliminate gut bacteria.

The inclusion of cellulose at levels up to 40% into diets did not consistently alter the digestibility of dietary protein or ash for the common carp, however lipid digestibility decreased linearly with the level of cellulose incorporation. Although in previous studies the digestibility of dietary nutrients has been reported to be affected by the inclusion of antibiotics (Soivio and Koskela, 1992), in the present study inclusion of antibiotics into the experimental diets did not affect the digestibility of dietary ash, lipid, protein or energy.

The present study has shown two effects of cellulose incorporation into experimental diets for fishes. It has shown a negative effect produced by cellulose on the digestive physiology of the carnivorous Murray cod, and an ability of the omnivorous carp to utilise dietary cellulose as a nutrient source. Such a discrepancy in physiological reactions to dietary cellulose could be expected due to the natural diets of the species. The Murray cod feeds exclusively on prey items while the large proportion of the diet of carp consists of cellulose laden detritus and aquatic flora. Cellulose inclusion was also shown to affect the digestibility of other dietary nutrients. These results indicate that cellulose is not an inert filler and that the effects of cellulose on the digestive

physiology of the species of interest must be determined prior to its incorporation into experimental diets. Thus ensuring that changes observed are due in fact to the intended dietary manipulation and are not due to a confounding physiological effect introduced by the cellulose filler.

The effects of the method of processing and inclusion level on the digestibility of solvent-extracted soybean (SBM) were determined. An initial trial suggested that the digestibility of ground SBM was significantly lower than that of sifted SBM or ground SBM middlings. However, a later trial showed the digestibility of ground SBM to be higher than originally thought and similar to the values obtained for sifted SBM and ground SBM middlings (70% to 75%). The initial low value for ground SBM digestibility probably reflected the short time allowed for the wild animals to adapt to the experimental conditions and artificial diets. The method of processing did not significantly effect the digestibility of protein or energy. Mean values for protein digestibility for ground SBM, sifted SBM and ground SBM middlings being 89.5%, 91.0% and 93.1% respectively. The digestibility coefficients of crude energy were similar to those of dry matter digestibility being 80.1%, 75.8% and 77.3% for ground SBM, sifted SBM and ground SBM middlings respectively. A negative effect of inclusion level on the digestibility of ground SBM was found, digestibility deceasing significantly at greater than 20% inclusion. The decrease in ground SBM digestibility may have been related to either the presence of anti-nutritional factors or the elevated levels of indigestible complex carbohydrate. Digestibility of protein from ground SBM included at graded levels was high, greater than 95%, and showed a trend to decrease with the level of inclusion, however there was no significant difference found. The inclusion of ground SBM did not affect the digestibility of dietary lipid. Soybean oil digestibility was found to be extremely variable, 47% to 75%, showing no relationship to the level of incorporation. The high variability of SBM oil digestibility reflected the low levels of SBM oil present in the diets.

Tuna oil was used to investigate the digestibility of a purified lipid source. A preliminary trial indicated that 10% and 15% inclusion of tuna oil into a reference diet be used as an experimental protocol for measuring tuna oil digestibility, thus overcoming the problem of high variances associated with low inclusion and the reduced pelletability associated with elevated levels of dietary lipid. The apparent digestibility coefficients for tuna oil were 83% and 60% at 10% and 15% inclusion respectively, indicating a negative effect of inclusion level on tuna oil digestibility. However, analysis of daily faeces showed this effect was due to a prolonged period of adaptation to the experimental diets by the animals fed the higher level of tuna oil, as by the end of the feeding trial (17 days) the daily digestibility of tuna oil was similar at

both inclusion levels. The inclusion of tuna oil into the reference diets did not affect the digestibility of dietary protein or ash.

The digestibility of raw corn starch by common carp was high, greater than 79% at all inclusion levels. At a restricted feeding rate, 2% body weight.weight diet.day^{-1}, starch digestibility was found to increase with increased inclusion. A reduced palatability of the experimental diets with high inclusion of corn starch is thought to have led to a reduced daily intake of these diets and therefore the increase in digestibility with inclusion level may be due to the higher ratio of intestinal α-amylase to dietary starch. At a 20% inclusion, the digestibility of energy from starch was found to reflect the dry matter digestibility of the ingredient. Incorporation of starch into the reference diet at 20% inclusion also decreased protein digestibility, however this was not of a nutritionally significant level. Such a reduction in protein digestibility may reflect the increased level of endogenous α-amylase secretion in response to the elevated levels of dietary carbohydrate and the inability to resorb α-amylase due to the formation of enzyme-carbohydrate complexes.

From this data, it is clear that the level of inclusion of test ingredient into the reference diet can affect ingredient digestibility. The digestibility coefficients of cellulose, ground SBM and tuna oil for common carp were found to decrease with increased inclusion, although the decrease in tuna oil digestibility appears related to a prolonged adaptation period. While the digestibility of raw corn starch was found to increase with increased inclusion. De Silva *et al.* (1990) found the digestibility of leafmeal decreased with increased inclusion, however these authors proposed amending the level of inclusion of leafmeal into the reference diet used to determine digestibility rather than noting the potential adverse effects of inclusion on ingredient or reference diet digestibility. The change in the digestibility of ingredients with the level of inclusion indicates that it is necessary to determine the digestibility of the test ingredient over the range of inclusion levels intended for its use. Any change in digestibility with inclusion may then be allowed for in diet formulation, thus ensuring that the final diet is both nutritionally and economically optimal.

8.3 Effect of Ingredients on Other Dietary Nutrients

Apart from determining the digestibility of the test ingredient, it is important to determine, where possible, any effects on dietary nutrient digestibility caused by the inclusion of the test ingredient. When an ingredient is in itself a food, for example SBM, the effects of the test ingredient on dietary nutrient digestibility will be incorporated into the digestibility of the nutrients from the test ingredient. Therefore, unless the resultant digestibility coefficients are not physiological, that is negative or

greater than 100%, it is not possible to ascertain whether the ingredient is adversely affecting the digestibility of dietary nutrients. Even if unphysiological digestibility coefficients are calculated, it is not possible to apportion digestibility to nutrients from either the test ingredient or reference diet. However, when measuring the digestibility of purified ingredients, that is ingredients containing singly or predominantly one nutrient type, the effect of the test ingredient on the other dietary nutrients may be determined. In the present study the inclusion of cellulose, BW 40 Solka floc, into the reference diet adversely affected the digestibility of dietary lipid for common carp. Such a finding indicates that cellulose, apart from its own digestibility, may not be an inert filler in that it affects the digestibility of other dietary nutrients. Thus if cellulose were incorporated into a commercial diet as a filler then the level of available energy and fatty acids, and therefore the capacity of the diet to meet the nutritional requirements of the animal, would be reduced through a concomitant decrease in lipid digestibility. Inclusion of cellulose in diets for Murray cod would appear to have a similar effect at greater than 10% inclusion, although the exact manifestation of decreased diet digestibility was not identified.

8.4 Effect of Feeding Rate

Digestibility of dietary ingredients and nutrients have been shown to be affected by feeding rate (Windell *et al.*, 1978b; Henken *et al.*, 1985). Higher feeding rates are thought to increase the rate of passage of material through the digestive tract resulting in less time for the processes of digestion and absorption and thereby decreasing digestibility. The increased bulk of food through the gut may also overload the intestinal enzyme systems. Although not specifically tested in the present study, the enhanced digestibility of starch at higher inclusion levels most likely reflects the reduced palatability of the diet and therefore a decreased daily intake. Therefore it would appear that not only is it essential to make allowances for the level of incorporation of the test ingredient into the diet but it is also necessary to take into account the daily feeding rates to be employed in the culture system.

8.5 Effect of Time on Digestibility

The results from the present study have shown that there is an effect of time on digestibility for common carp. This effect has two components: an adaptation of the animals to the experimental diets and a daily variability in digestibility. The analysis of daily faecal samples allowed the degree to which these factors affect digestibility measurement to be exemplified.

The period required for adaptation to experimental diets has previously been investigated in rainbow trout (de la Noüe *et al.*, 1980) and milkfish (Ferraris *et al.*, 1986). As early as three days after changing the diet, digestibility for rainbow trout achieved characteristic values (de la Noüe *et al.*, 1980), while milkfish appeared to immediately adapt to a new diet (Ferraris *et al.*, 1986), thereby suggesting that there is no adaptation phenomenon for fish (de la Noüe *et al.*, 1980). Although diet digestibility earlier than day 7 was only determined for the preliminary trials, there appeared to be an adaptation period for common carp to diets containing elevated levels of lipid or cellulose. The duration of this period was highly variable.

Experimental diets with 20% inclusion of starch, cellulose or SBM all appeared to have acquired a characteristic value by day 3 and therefore exhibited no adaptation period. However, all experimental diets in the preliminary tuna oil trial showed a reduced digestibility at day 5, digestibility adopting a consistent value by day 8.

In the later study of variability in digestibility, the digestibility of experimental diets including SBM showed no tendency for adaptation after the initial time point, day 8. Similarly, the digestibility of the experimental diets incorporating cellulose at 10% and 20% and tuna oil at 10% showed no adaptation period after day 7 and day 8 respectively. However, the 30% cellulose inclusion test diet and 15% tuna oil inclusion test diet both showed longer periods of adaptation lasting 12 and 17 days respectively. The prolonged period of adaptation to diets containing high lipid appeared to be related to the ability to absorb lipid as dietary lipid digestibility followed whole diet digestibility. Although de la Noüe *et al.* (1980) found no continued adaptation to experimental diets, De Silva and Perera (1983) found an overall increase in dry matter and protein digestibility from a poorly digested aquatic macrophyte, *Hydrilla verticellata*, for the green chromid throughout a 20 day digestibility trial.

Daily variability in digestibility has previously been described for rainbow trout (de la Noüe *et al.*, 1980), milkfish (Ferraris *et al.*, 1986), green chromid (De Silva and Perera, 1983) and the Nile tilapia (De Silva and Perera, 1984). In the present study the digestibility of whole diets, ingredients and dietary nutrients for common carp were also shown to exhibit natural variance. The rhythm of the variability in digestibility in the present study was similar to that described by De Silva and Perera (1983; 1984), that is a day or two of high digestibility followed by a day or two of low digestibility. The cycle of this rhythm is also evident from the effects of faecal pooling, in that pooling faeces over 3 days or less did not mask the effect of natural variability in digestibility, whereas pooling over 5 days or greater generally eliminated the observed variability. However, masking the effects of variability in the digestive processes of

fish merely hides a phenomenon and does not allow explanations to be attained. A knowledge of the rhythm of digestibility in cultured species allows for the development of mixed feeding methodologies suited to the digestive cycle of the animal, thus reducing feed costs. Feeding alternate diets containing high and low protein levels has previously been shown to be effective in reducing feed costs in both laboratory and semi-intensive pond culture systems (De Silva and Anderson, 1995).

The occurrence of a natural physiological variance in the digestion of the experimental diets, test ingredient and dietary nutrients indicates that in order to accurately determine digestibility coefficients, faeces should either be pooled over a larger number of days, or multiple sampling and analysis should be performed on single day samples and mean values calculated. The latter is the preferred option as it also provides data on the rhythm of digestibility and the state of adaptation of the animals to the experimental diets, thus preventing the calculation of erroneous digestibility coefficients due to the effects of the adaptation phenomenon. Although the ability to analyse all the dietary nutrients on daily faecal samples has not been possible in many studies in the past, an increase in the number of experimental animals used in digestibility trials would overcome this difficulty. Where such an increase is prohibitive due to the availability of animals, facilities and/or cost, it is recommended that faeces be pooled over as few days as possible and multiple time points analysed.

If digestibility coefficients are to be determined using pooled faeces, it is suggested that pooling be performed over a period not less than five days, and preferably over a much greater number of days, in order to overcome error due to the daily variability in digestibility. It is also recommended that an adaptation period of greater than 7 days be allowed for the digestive physiology of the animals to adapt to the experimental diets. The contribution of faeces from each collection day to the final sample should also be consistent, thus eliminating the possibility of days were large amounts of faeces are collected biasing the calculation of digestibility coefficients.

8.6 Ash as a Digestibility Marker

The selection of markers for digestibility studies is presently a point of some conjecture, with debate existing as to the appropriateness of exogenous markers. Some authors have suggested that, where possible, it is best to select an endogenous marker (De Silva and Anderson, 1995). Ash is one such endogenous marker (Hickling, 1966; De Silva *et al.*, 1984). However, several authors have reported ash to be digestible for fish (Buddington, 1980; Bjorndahl, 1985). In the present study ash was consistently found to be digestible for common carp in the range of 20% to 40%, a level similar to that previously reported (Buddington, 1980; Bjorndahl, 1985).

Therefore it would appear that ash is not suitable as an endogenous marker for studying the digestibility of fish.

8.7 Conclusion

The findings from this study clearly indicate that diet and nutrient digestibility is not a constant. Ingredient digestibility appears to vary with the level of inclusion of the test ingredient and time. The inclusion of some dietary ingredients also adversely affect the digestibility of other dietary nutrients. These results indicate that the digestibility of dietary ingredients should be measured over the range of inclusion to be used and over a number of time points. It is also necessary to account for any adverse effects of the dietary ingredient on the digestibility of other dietary components. Thus the need to perform detailed digestibility experiments incorporating the range of conditions to be used in the culture system, both biological and environmental, is becoming increasingly apparent.

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