Development of an *In vitro* Assay for the Assessment of Alternative Protein Sources for Use in Artificial Diets for Farmed Southern Bluefin Tuna (*Thunnus maccoyii*)

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Table of Contents

Non-Technical Summary

95/068	Development of an *in vitro* assay for the assessment of alternative protein sources for use in artificial diets for farmed Southern Bluefin Tuna (*Thunnus maccoyii*)

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OBJECTIVES:

1. To enhance the development of artificial diets for caged SBT by using *in vitro* analysis of feed ingredients to determine protein, amino acid and energy digestibility. This will allow formulation of manufactured diets of maximum nutritive value using the most cost-effective ingredients. It will also allow accurate matching of the diet specifications to the nutrient requirements of the SBT.
2. To determine the ultrastructure of the SBT small intestine to a) identify the presence of macromolecule transport apparatus and b) to establish the existence of bacteria associated with the intestinal epithelium.
3. To evaluate the enzyme profile of the small intestine and the epithelial associated bacteria and identify bacteria associated with biofilms.
4. To estimate the intestinal capacity to transport the major groups of amino acids and peptides.

NON-TECHNICAL SUMMARY

Apart from the physical characteristics of manufactured feeds for Southern Bluefin Tuna (SBT) we need to develop an understanding of the nutritional quality of various feed ingredients if we are to improve feed efficiency and maintain product quality. If a suitable bait fish replacement is to be found, a number of other protein sources (animal proteins, grain legumes, cereals) must be evaluated. This will ensure the most efficient use of manufactured diets by the fish and will maintain or improve the current levels of production achieved with the bait fish. Feed evaluation *in vivo* (ie. in the fish) is very difficult to achieve and development of an *in vitro* (ie. laboratory-based) system is very desirable.

The research aims were to develop and validate an *in vitro* method for predicting the digestibility (ie the proportion of nutrients removed from the gut) of feed ingredients so that the information could be used to more accurately formulate feeds for Southern Bluefin Tuna (*Thunnus maccoyii*). *In vitro* multi-enzyme digestion of feeds and feed ingredients using different enzyme systems to measure crude protein and dry material digestibility and the multi-enzyme pH change assay for protein digestibility were used. *In vitro* digestibility values were compared to *in vivo* digestibility values measured in salmon and SBT. The *in vitro* digestibility values for the 1997 tuna feeds showed a number of trends. The feeds had different digestibility values; the highest values were obtained using the commercial enzymes, then the crude enzyme extracts from salmon and the extracts from the tuna were generally lowest. For each feed and enzyme system the digestibility for crude protein was higher than for dry material. There were statistically significant ($P<0.05$) correlations between the three enzyme systems, the salmon and tuna systems showed the strongest correlation ($r = +0.85$; $P<0.01$). The assays were sensitive enough to distinguish between complete feeds and because the tuna enzyme

system had the widest range of values it could be considered the most sensitive of the three enzyme systems. The ingredients reflected the trends in digestibility observed with the complete feeds by showing a range of values, lower values for dry material than for crude protein, differences between the different enzyme systems and greater sensitivity (range of values) of the fish enzyme systems. The multi-enzyme pH change assay provided a range of crude protein digestibility values but these did not correlate with the digestibility values from the multi-enzyme digestion assays. *In vivo* digestibility was measured for eight 1997 tuna feeds and nine feed ingredients and compared with *in vitro* values. There were no significant correlations between the *in vivo* and *in vitro* digestibility values for the 1997 feeds. There were significant correlations between *in vivo* and *in vitro* digestibility values for the ingredients. The commercial (porcine) enzyme system produced the strongest correlations with *in vivo* protein ($r=+0.83$; $P<0.01$) and dry material ($r=+0.86$; $P<0.01$) digestibility. Furthermore, limited evidence suggested the use of a tuna enzyme system predicted *in vivo* digestibility values in tuna.

1. Multi-enzyme digestion systems can be used to indicate the *in vitro* dry material and crude protein digestibility of feeds and feed ingredients.
2. The sensitivity of the enzyme system is expressed by the range in digestibility values for a given set of feeds and ingredients and relates to the source of the enzymes.
3. Enzyme systems using fish enzymes, from salmon and tuna, were more sensitive than the system using porcine enzymes.
4. Digestibility of feeds determined from both ingredient and complete feeds were shown to be similar and demonstrated that ingredient digestibility values were additive using salmon and tuna enzyme systems.
5. Correlations between *in vivo* and *in vitro* digestibility values, the ability to indicate poor quality protein sources and the additivity of ingredient digestibility values demonstrated that salmon and tuna enzyme systems are suitable for predicting the digestibility of feeds from ingredient digestibility and therefore the values can be used in formulating tuna feeds.

In addition to the above, the following outcomes were achieved as part of this research project:

- The isolation of brush border membranes is possible from SBT intestinal tissue. The characterization of transport demonstrates the presence of at least two transporters for the marker amino acids, alanine and proline. However, additional work is required to evaluate the true effect of diet on transport capacity of the Pyloric Caeca for amino acids.
- In terms of nutrient uptake, the Pyloric caeca does posses the transport capacity for amino acids however this occurs in conjunction with endocytosis. Given this coupled with the apparent regional difference in function and microbial population of the pyloric caeca it is recommended that freeze dried extracts are used in digestibility assays *in vitro*. Doing this the environment of pyloric caeca can best mimicked.
- The Pyloric Caeca of the SBT is responsive to diet and such changes will impact on the digestive and absorptive physiology of this organ. Whether such changes are solely due to level of nutrient intake or may be induced by specific dietary components binding to and influencing the cellular function in the Pyloric caeca remains to be determined.
- Amylase activity is significant in the pyloric caeca but not in the small intestine. This means that feeds containing starch should be formulated such that the starch was on the outside where it can be digested fully within the pyloric caeca. Endoglucanase activity was not detected, suggesting that cellulose should be kept to a minimum in diet formulations. Lipase activity was greatest in the small intestine where most digestion of fats appears to occur. Lipids should therefore be located on the inside of feed pellets. Protease activity is high in both the pyloric caeca and the small intestine, although specific activity per mg of protein was greatest in the pyloric caeca. DPP-IV activity was present in the pyloric caeca but not in the small intestine. This correlates with the protease locations and supports the concept that protein degradation occurs mainly in the pyloric caeca.

KEYWORDS: Southern Bluefin tuna, aquaculture, nutrition, digestion

Background

Southern Bluefin Tuna (*Thunnus maccoyii*; SBT) farming continues to develop with production in 1997/98 valued at $AUD110 million. A major constraint to the development of this industry is feed costs, feeding methods and a suitable replacement for bait fish which currently represent the only sourced of added nutrients for these farmed fish. The fact that the tuna farming industry relies entirely on a single food source makes it particularly vulnerable in the event that bait fish supply (ie pilchards, Jack Mackerel) is impeded or restricted.

Developing an efficient feeding system

The ultimate goal of feeding SBT must be to match diet specifications as closely as possible to fish nutrient requirements with the aim of reducing feed costs, minimising environmental pollution, ensuring even growth rates, maximising product quality, exploiting desirable carcase traits, and allowing selection of the most cost effective ingredients. To accomplish this, a better understanding of the interactions between the physical and chemical properties of a food matrix and the subsequent effects on nutrient utilisation is required. While a considerable amount of research is currently being invested establishing a suitable diet form for SBT, little research has been directed towards establishing the relative value of feed ingredients and the capacity of SBT to digest these ingredients.

Proximate analysis and total amino acid profiles have been used to characterise most feed ingredients used in manufactured diets for SBT in the past. To clearly define the nutritional quality of a feed ingredient, and thus facilitate subsequent assessments of nutritional value, information pertaining to nutrient availability to the fish (ie digestion, uptake and use) is required.

Research to date has demonstrated the numerous difficulties associated with feeding SBT manufactured diets (acceptance, utilisation, resulting product quality). Even in cases when desirable feed intakes are achieved with manufactured feeds, performance levels of the fish are still below expectations. Many reasons could be ascribed to this phenomenon, however, binding properties of the manufactured feed and digestibility of ingredients used in the feed are likely to be major causes. For this reason, improved knowledge of the digestibility of feed ingredients will not only allow the selection of the most cost-effective ingredients, but will improve our ability to predict SBT performance and reduce feed conversion ratios.

The use of in vitro analysis to characterise feed ingredients

Evaluation of the digestibility of nutrients in a variety of feed ingredients will provide feed manufacturers with the capacity to alter ingredients within manufactured diets without influencing subsequent nutrient supply to the fish. For obvious reasons it is difficult to determine the nutritional quality of feed ingredients for use in SBT manufactured feeds using *in vivo* techniques. As a consequence, development of an *in vitro* assay is a logical alternative. For example, protein digestibility can be measured from the quantity of nitrogen or amino acids released by the enzymatic hydrolysis of protein sources.

An *in vitro* assay for nutrient digestibility based on enzyme digestion systems is the most desirable approach. Other systems such as those involving isotope dilutions are undesirable on the basis of cost and complexity. Enzyme based systems can also be applied routinely in any laboratory for minimal cost and an equivalent level of accuracy.

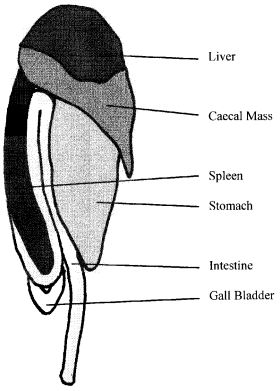
Boison and Eggum (1991) suggested a multi-enzyme *in vitro* system for the evaluation of feeds. This system included chemical analysis of dry matter, ash, N, energy and amino acids combined with two parallel multi-enzyme systems for predicting digestibility of amino acids and energy. A technique like this has been used successfully with other fish species (eg Anderson *et al.* 1993). A similar

system for evaluating feedstuffs for SBT can be developed and would prove invaluable, however, if this system is to be useful it must simulate *in vivo* digestion as closely as possible and account for microbial contributions to the digestive processes.

The need to understand intestinal physiology

Animal growth and development are dependent not only on the digestibility of feeds, but also their ability to capture and utilise nutrients. A major obstacle to improving the feeding management of SBT is the paucity of information regarding protein digestion and uptake in this fish. Given the exceptionally short digestive tract of the fish (R. van Barneveld, personal communication (see below)) important questions regarding intestinal digestive function in SBT need to be resolved. These include:

1. What is the activity profile of luminal and epithelial associated proteases and petidases ?
2. Is there a significant amount of macromolecular uptake and intracellular digestion of intact or partially digested proteins in this fish ?
3. What is the effect of raised temperature/fluctuations in temperature with food intake on the activity profiles of digestive enzymes ?
4. What is the function of the "blind" gut sac ? (eg. pH, absorptive function)
5. What is the function/action of the stomach in the digestive process ?



In addition, there is little or no information about the potential contribution of the epithelial associated bacteria to the welfare and nutrition of SBT. If bacteria are proven to play a significant role, they could provide tools for manipulating the nutritional status of the fish. In addition to providing the basis for a longer term research program, this specific information is extremely important to the development of a suitable *in vitro* assay for the assessment of ingredients for use in manufactured diets for farmed SBT.

Protein digestion and uptake by the posterior intestine

It is now well established that protein digestion is initiated in the lumen by proteases derived from pancreas (Alpers, 1994) with the typical pancreatic enzyme profile being trypsin, chymotrypsin, elastase and the carboxypeptidases. Luminal proteolysis results in a final digesta composition of 40% free amino acids and 60% peptides of 2-6 amino acids in length. Peptide digestion is completed by peptidases expressed in the brush border membrane of epithelial cells that line the posterior intestine (Smith, 1990). Substrate specificity is determined by amino acid class and four major peptidase families exist, these being, the endopeptidases, aminopeptidases, carboxypeptidases and dipeptidases. The presence and level of expression of these enzymes is unknown in SBT.

Transport of amino acids across the intestinal cell wall is of fundamental importance to the growth and development of animals. To date, 13 amino acid transport systems have been characterised and the distribution of these systems is dependent upon tissue and cell type (MicGivan and Pasto-Anglanda,

1994). Characterisation of transporters identified to date has revealed a high stereospecificity, a low substrate specificity and either Na dependence or independence. Major systems present in the epithelial cells of the small intestine are , L and YO+ (James *et.al*., 1987). These can be monitored by alanine, leucine and lysine uptake respectively. In contrast, our knowledge of peptide transporters is limited, but they are H-dependent rather than Na dependent and have a similar affinity to a large number of di-and tri- peptides (Smith, 1990). The majority of peptides entering the intestinal epithelial cells are digested to amino acids intracellularly. The capacity of the small intestine to absorb amino acids and peptides needs to be assessed in the membrane vesicles prepared from SBT intestines using representative amino acids and peptides resistant to hydrolysis.

In both young and adult terrestrial animals, large and small amounts of protein can be endocytosised respectively (Smith, 1990). These may provide a significant component of the nitrogen intake. This non-selective macromolecular transport is predominantly associated with the distal small intestine and results in supranuclear vacuoles. The contents of these are subsequently hydrolysed by lysosomal enzymes. Given the length of the SBT intestinal tract and hence the probable short transit time of digesta, macromolecular uptake by the intestine may be an important scavenging system for partially digested protein in the diet of SBT. The presence of such a system can be determined by localisation of intracellular features associated with macromolecule transport using electron microscopy. The potential capacity of macromolecular transport can be quantified *in vitro* by radio-labelled proteins using small intestine explants.

Microbial biofilms: Importance to digestion and uptake

Direct electron microscopic examination of washed animal tissues has shown the presence of attached bacteria on many tissues in the digestive system. (Savage and Blumershine, 1974; Bauchop *et al.* 1975; McCowan *et al.* 1978). The stratified squamous epithelium of the rumen is colonised by a complex continuum as much as 5Omm thick covering the tissue surface. The crops and true stomachs of other animals have been shown to colonised by virtually monospecific populations of acid tolerant, adherent, surface associated bacteria. The bacteria are often covered by a mucus blanket.

Our understanding of the role of surface associated bacterial biofilms in digestive processes is fragmentary. Nevertheless, information available indicates their role may be other than passive (Cheng *et al.* 1981).

1. Bacterial populations lining the rumen epithelium express urease activity that mediates passage of urea nitrogen from the bloodstream and helps maintain an anaerobic ruminal environment by scavenging oxygen.
2. Tissue protein recycling by digestion of mammalian intestinal epithelial cells is mediated by bacterial biofilms.
3. Biofilms protect against colonisation of the gut by potentially pathogenic bacteria. Bacterial populations that are associated with biofilms in the digestive tract include Proteus, Vibrio and Fusobacterium.

Electron microscopic examinations show that biofilm populations are embedded in an extracellular polysaccharide glycocalyx. Nevertheless, there are microchannels that the biofilm, providing an access route for nutrients to reach the intestinal epithelium. It is also possible that the biofilm protects receptors on the epithelial surface from the abrasive action of particulate digesta material and from the degradative effect of digestive enzymes. Whether the bacterial populations play an active role in mediating nutrient uptake is not known.

In SBT, almost nothing is known about microbial populations that may inhabit the digestive tract, or what role they may play. Evidence from salmonids suggest the major bacterial species found in marine fish are species of Rennibacterium, Vibrio, Aeromonas and Citrobacter. However, whether these organisms occur as free living bacteria, or in a surface associated biofilm is not known.

Need

Due to the infancy of the SBT farming industry, there are many nutritional questions that must be addressed before a cost-effective efficient feeding system can be developed. Concurrent research is underway to develop a manufactured feed or bait fish substitute that is easy to store and handle and is readily accepted by the caged tuna. Apart from the physical characteristics of the manufactured feed, however, we NEED to develop an understanding of the nutritional quality of various feed ingredients for the caged tuna if we are to improve feed efficiency and maintain product quality.

If a suitable bait fish replacement is to be found, a number of other protein sources (animal proteins, grain legumes, cereals) must be evaluated. This will ensure the most efficient use of manufactured diets by the fish and will maintain or improve the current levels of production achieved with the bait fish. At present, feed evaluation *in vivo* is very difficult to achieve due to:

1. The high value of the fish limiting destructive experiments
2. Difficulties associated with handling the fish
3. The small number of research cages limiting replication
4. Difficulties associated with getting the fish to accept an experimental diet
5. Little potential for routine faeces or digesta collection

For these reasons, the development of an *in vitro* assay based on the digestive enzyme type, level and activity of SBT, knowledge of microbial contributions to protein digestion and validation using *in vivo* digestion studies is a logical option for the evaluation of the nutritional quality of alternative feedstuffs. In addition, when conducting feed evaluations, there are an infinite number of feed sources (species, variety and cultural differences). The limited *in vivo* tuna research facilities limit our ability to conduct these evaluations.

It is also very important that a specific *in vitro* assay is developed for the SBT. The SBT is characterised by a unique digestive morphology and a highly specific diet. As a consequence, it is unlikely that *in vitro* assays that have been developed for other fish species or terrestrial animals will have any relevance to the SBT.

Objectives

1. To enhance the development of artificial diets for caged SBT by using *in vitro* analysis of feed ingredients to determine protein, amino acid and energy digestibility. This will allow formulation of manufactured diets of maximum nutritive value using the most cost-effective ingredients. It will also allow accurate matching of the diet specifications to the nutrient requirements of the SBT.

2. To determine the ultrastructure of the SBT small intestine to a) identify the presence of macromolecule transport apparatus and b) to establish the existence of bacteria associated with the intestinal epithelium.

3. To evaluate the enzyme profile of the small intestine and the epithelial associated bacteria and identify bacteria associated with biofilms.

4. To estimate the intestinal capacity to transport the major groups of amino acids and peptides.

Methods

In vivo digestibility studies

The *in vivo* digestibility studies were completed by Dr Robert van Barneveld and Ms Bronwyn Davis from the South Australian Research and Development Institute.

Knowledge of the digestibility of nutrients in feed sources is important if insights are to be gained into the availability of nutrients for SBT and for the *in vivo* validation of *in vitro* digestibility assays, that are being developed. The estimation of the digesta transit time is also important for setting appropriate *in vitro* incubation times. The development of *in vitro* digestibility assays will facilitate the selection of the most cost effective feed ingredients, satisfying one of the above aims in feeding caged SBT.

The objectives of this experiment were to:

1. Establish techniques for *in vivo* digestibility and transit time experiments using caged SBT.
2. Estimate the transit time of Peruvian fish meal fed to caged SBT, using coloured plastic beads and serial sampling techniques.
3. Determine the digestibility of protein and amino acids in Peruvian fish meal fed to caged SBT, using destructive sampling techniques.
4. Determine the digestibility of energy in Peruvian fish meal fed to caged SBT, using destructive sampling techniques.
5. Compare acid-insoluble ash and n-hexatriacontane as digestibility markers in *in vivo* experiments using SBT.

Diets: The experiment was based on a Peruvian fish meal diet. Initially, this experiment was also designed to assess soybean meal as an alternative protein. This assessment was not pursued, because the caged SBT would not accept the diet. The diet must be of the correct sensory characteristics, or the tuna will reject the diet.

Peruvian fish meal was the only source of amino acids. Jack mackerel oil was the main source of non-protein energy. Squid oil was also included as an energy source and an attractant. The diet contained vitamins, minerals and carboxymethylcellulose as a binder. Celite was included as an acid-insoluble ash digestibility marker and n-hexatriacontane was included as a hydrocarbon digestibility marker. Coloured plastic beads were also included for the transit time study (Table 1).

The Peruvian Fish meal diet was contained in a "sausage skin" to prevent disassociation of the diet, on contact with the water.

Fish and cages: Sixty fish were randomly allocated between six 12 m diameter research sea-cages, (n=10) anchored in Boston Bay, Port Lincoln, South Australia. The cages and fish were provided by the Tuna Boat Owners Association of Australia (TBOAA).

Transit time and digestibility: The experimental diet was fed for a period of seven days prior to the harvest of the tuna. On the first day of collection the tuna were fed the Peruvian fish meal diet with the addition of white plastic beads. Three of the cages were sampled 2.5 hours after feeding the beaded diet. Four fish out of each cage were taken at this time. The other three cages were sampled 5 hours after feeding the Peruvian fish meal beaded diet. Four fish were again taken from each cage.

The fish were caught by divers with the aid of a net reducing the swimming area. Once on the boat, the tuna were immediately killed by pithing the brain and coring the spine, as per industry practice.

The digestive tract was then removed and divided into regions with coarse string. The tracts were then individually bagged and stored on ice until the return to the laboratory. The tracts were then emptied region by region into sample containers, frozen and flown back to Adelaide. The regions that were sampled were the stomach, pyloric caeca, proximal intestine and distal intestine. The proximal intestine was the section of the intestine leading from the pyloric caeca to the second bend of the intestine. The distal intestine was the section from the second bend to the anus.

The above procedure was repeated the following day. The diet fed on the second collection day contained yellow plastic beads rather than white, to see if any of the previous days feed was retained.

All samples were freeze dried, and ground in preparation for proximate, energy, marker and amino acid analysis. Two fish per cage, per sampling time were pooled so that sufficient digesta was obtained for analysis.

Table 1. *Composition of the experimental diet (g/kg, air-dry basis)*

Ingredient	Inclusion
Carboxymethylcellulose	2.0
Mineral pre-mix[a]	1.0
Vitamin pre-mix[b]	0.50
Jack mackerel oil	300.0
Squid oil	20.0
Celite (acid insoluble ash)	20.0
n-hexatriacontane	0.20
Starch	60.0
Water	300.0
Peruvian fish meal	296.3
Beads	200/kg

Chemical analyses: The dry matter, crude protein (N x 6.25), light petroleum extract (bp 40° - 60°), and ash were determined by the methods of the Association of Official Analytical Chemists (1984). Gross energy was determined by adiabatic bomb calorimetry. Acid-insoluble ash was determined by the method of Choct *et al*, 1996. *n*-hextriacontane was determined by gas chromatography using the method of Choct *et al*, 1996, after extraction of the hydrocarbon. Amino acids were separated by ion exchange chromotography and measured after reaction with ninhydrin. Norleucine was utilised as an internal standard for amino acid analysis. Analysis for all amino acids, except methionine and cystine, was completed following hydrolysis at 110°C for 24 hours with constant boiling point hydrochloric acid, under nitrogen. No pre-oxidation occurred and therefore there are no results for methionine and cystine.

Experimental design and statistics: The experiment was based on a randomised block design and was analysed using a general linear model in SAS. There were three replications per time period per day for the digestibility and transit time study.

Digestibility coefficients were obtained, for pairs of fish, using acid-insoluble ash and n-hexatriacontane as digestibility markers. The average difference between each measurement was tested to see if it was significantly different from zero (similar to a paired *t* test). Differences were considered for the Peruvian fish meal diet. Date and cage variation was eliminated using analysis of variance.

To further the knowledge of digestibility of manufactured diets in SBT and to provide more information for *in vivo* validation of *in vitro* digestibility techniques, a second digestibility and transit time study was performed.

Results from the previous digestibility and transit time study show that the digestibility coefficients of dry matter, nitrogen and energy in the stomach and pyloric caeca were very low and the digestibility coefficients in the proximal intestine were low.

Estimation of the mean digesta transit time is important to the development of an *in vitro* digestibility assay, so that an appropriate incubation time can be set. The previous transit time study, using coloured beads, failed due to the size of the beads. Two chemical markers were used to estimate the transit time in this experiment. Chromic oxide was used to estimate the transit time of the solid phase of the digesta and polyethylene glycol (PEG) was used to estimate the transit time of the liquid phase of the digesta.

The objectives of this experiment were to:

1. Further establish techniques for *in vivo* digestibility experiments with caged Southern Bluefin Tuna.
2. Obtain digesta transit time information using two chemical markers - chromic oxide and polyethylene glycol, using destructive sampling techniques.
3. Obtain amino acid digestibility data for a manufactured diet and pilchards fed to caged SBT, using destructive sampling techniques.
4. Obtain energy digestibility data for a manufactured diet and pilchards fed to caged SBT, using destructive sampling techniques.

Diets: Two diets were compared in this experiment (Table 2). The first diet was based on the Cooperative Research Centre (CRC) - Aquaculture sub-program 2B - Tuna nutrition diet (a manufactured diet) and the second diet was pilchards with the addition of guar gum. The diets used for the transit time experiment contained chromic oxide and polyethylene glycol markers (diets 1 and 3). The diets used for the digestibility study did not contain chromic oxide or polyethylene glycol, but contained coloured plastic beads (diets 2 and 4). Both diets were bound in a sausage skin to prevent disassociation of the moist pellet on contact with the water.

The protein sources for the manufactured diet were Chilean fish meal, Inual Antartic krill meal and Blood meal. The protein source for the pilchard diet was whole pilchards. See Table 5 for chemical composition of protein sources, and Table 6 for amino acid composition of protein sources.

Celite was included as an acid insoluble ash marker for the digestibility section of the experiment. Chromic oxide was included as solid phase transit time marker. Polyethylene glycol (PEG) was included as a liquid phase marker.

Blue coloured plastic beads were added to the diets that contained no chemical transit time markers. These beads were added, so that the pellet intake of the tuna could be estimated. The correlation between number of sausages and number of beads in the stomach was shown to be acceptable in the preliminary assessment, and was reassessed in this experiment.

Fish and cages: There were 55, 40, 25 and 32 fish in four 12 m diameter sea-cages, anchored in Boston Bay, Port Lincoln, South Australia. The cages and the fish were provided by the Tuna Boat Owners Association of Australia (TBOAA).

Transit time and digestibility: The two diets, containing markers, were fed to SBT, distributed between the four cages. The manufactured diet was fed to two randomly allocated cages and pilchards were fed to the other two cages. These diets were fed, once daily, for approximately three weeks

before the harvests occurred. During this time, three fish per diet were harvested, to obtain the equilibrium marker excretion rate.

The fish in the cages allocated to the manufactured diet were fed Diet 1 at 5.00 pm, the day before the harvest. The fish in the cages allocated to the pilchard diet were fed Diet 2 at 5.45 pm, the day before the harvest. The fish were fed again on the morning of the harvest, as part of the transit time experiment, to provide a diluent, so the dilution of the marker could be assessed (Robertson, 1992). At 7.00 am, on the day of harvest, Diet 3 was fed to the fish in the cages allocated to the manufactured diet and at 7.45 am, Diet 4 was fed to the fish in the cages allocated to the pilchard diet.

Three fish per cage were harvested approximately 1.5, 3, 5 and 6 hours after feeding diets 3 and 4 respectively. This allowed for three fish per diet, per time period, with a cage as a replicate. The whole procedure was also repeated one week later.

The fish were hooked and poled onto the boat. Once on the boat, the tuna were immediately killed by pithing the brain and coring the spine, as per industry practice. The fish were weighed, on capture. The digestive tract was removed and the section between the proximal and distal intestine were sutured with coarse string. The tracts were placed on ice, to prevent any further digestion occurring, and were flown to Adelaide. Stomach contents were examined and coloured plastic beads and sausages were counted. The contents of the distal intestine was stripped into a labelled jar which was subsequently frozen for freeze drying. After the samples were dry, they were ground for analysis. Marker analysis was performed on individual fish, but equal amounts from each fish (3) in a cage (same diet and harvest time) were pooled to provide sufficient digesta for analysis.

Chemical analyses: The dry matter, crude protein (N x 6.25), light petroleum extract (bp 40° - 60°), and ash were determined by the methods of the Association of Official Analytical Chemists (1984). Gross energy was determined by adiabatic bomb calorimetry. Acid insoluble ash was determined by the method of Choct *et al*, 1996. Chromic oxide in the digesta was determined by atomic absorption spectrophotometry (Kimura and Miller, 1957). Polyethylene glycol was determined by turbidimetric analysis, using gum arabic as an emusifier (Malawer and Powell, 1967). Amino acids were separated by ion exchange chromotography and measured after reaction with ninhydrin. Norleucine was utilised as an internal standard for amino acid analysis. Analysis for all amino acids, except methionine and cystine, was completed following hydrolysis at 110°C for 24 hours with constant boiling point hydrochloric acid, under nitrogen. No pre-oxidation occurred and therefore there are no results for methionine and cystine.

Experimental design and statistics: The experiment was based on a randomised block design and was analysed using a general linear model in SAS. The digestibility and transit time studies were based on two replications per diet (two diets) per time period (four times).

Table 2 *Composition of experimental diets 1 - 4 (g/kg, air-dry basis)*

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4
Blood meal (90% CP)	20.00	-	20.00	-
BO11C Pre-gel starch	141.74	-	153.34	-
Chilean fish meal (65% CP)	324.41	-	324.41	-
Choline chloride (50%)	2.00	-	2.00	-
DL methionine	2.49	-	2.49	-
Guar gum	-	20.00	-	20.00
Inual Antarctic krill meal	20.00	-	20.00	-
Jack mackerel oil	140.00	-	140.00	-
Lethicin for aquatic diets	0.28	-	0.28	-
Pilchards	-	944.80	-	956.40
Pre-mix minerals[a]	2.00	-	2.00	-
Pre-mix vitamins[b]	1.00	-	1.00	-
Squid flavouring	0.40	-	0.40	-
Squid oil for tuna diets	30.00	-	30.00	-
Stay-C coated vitamin C	0.48	-	0.48	-
Water	250.00	-	250.00	-
Wheat gluten	30.00	-	30.00	-
Celite	20.00	20.00	20.00	20.00
Chromic oxide	5.00	5.00	-	-
n-hexatriacontane	0.20	0.20	-	-
Polyethylene glycol	10.00	10.00	-	-
Coloured plastic beads[c]	-	-	3.60	3.60

[a] See Table 3
[b] See Table 4
[c] Beads included at 200 beads per kilogram (100 beads = 1.8 grams)

Table 3. *Composition of mineral pre-mix used in experimental diets.*

Mineral	Activity (g/tonne)	Inclusion (mg/kg diet)
Cobalt sulphate (21%)	0.50	2.35
Potassium Iodide	1.10	1.57
Copper sulphate pentahydrate	3.00	11.76
Magnesium oxide 58.5%	148.50	248.82
Manganous oxide 60%	13.00	21.18
Ferrous sulphate monohydrate	30.00	94.90
Zinc oxide 80%	7.87	9.60
Limestone	820.938	804.90
Prebase	820.939	804.90
Total		2000

Table 4. *Composition of vitamin pre-mix used in experimental diets.*

Vitamin	Potency (%)	Activity (g/tonne)	Inclusion (mg/kg diet)
Vitamin A	50.0	5.00	9.80
Thiamine HCL (B1)	100.0	2.00	1.96
Riboflavin (B2)	80.0	15.00	18.43
Pyridoxine (B6)	100.0	12.00	11.76
Cyanocobalamin (B12)	1.0	0.20	19.61
Microvit H Promix 2000	2.0	0.30	14.71
Vitamin D3	50.0	4.80	9.41
Menadione (K3)[a]	22.7	2.00	8.63
Calcium-D-pantothenate	100.0	40.00	39.22
Nicotinic acid	100.0	20.00	19.61
Inositol	100.0	450.00	441.18
Vitamin E	50.0	100.00	196.08
Folic acid	100.0	2.00	1.96
Ethoxyquin	100.0	150.00	147.06
Limestone	100.0	30.00	29.41
Prebase	100.0	31.839	31.18
Total			1000

[a] Hetrazeen - 50% Menadione di-methyl pyrimidinol bisulphite (54% menadione activity)

Table 5. *Chemical composition of protein sources used in experimental diets (g/kg, air dry basis).*

Component	Protein source			
	Chilean fish meal	Inual Antarctic krill meal	Blood meal	Whole pilchards
Crude protein (Nx6.25)	644.5	594.2	885.3	220.9
Dry matter	885.5	886.9	933.7	283.6
Light petroleum extract (bp 40-60°C)	72.4	152.6	14.6	46.9
Ash	145.1	94.6	85.2	43.9
Gross energy	192.3	224.5	230.1	73.1

Table 6. *Amino acid composition of protein sources used in experimental diets (g/kg, dry matter basis).*

Amino acid	Protein source			
	Chilean fish meal	Inual Antarctic krill meal	Blood meal	Whole pilchards
Aspartic acid	15.73	29.59	51.48	18.94
Threonine	8.72	13.54	27.24	9.57
Serine	9.12	13.25	27.04	9.59
Glutamic acid	30.80	38.28	48.53	28.99
Proline	15.34	13.73	23.21	13.05
Glycine	16.78	11.25	20.94	12.95
Alanine	9.06	8.05	21.77	7.71
Valine	13.04	12.60	39.47	10.14
Isoleucine	14.19	14.14	5.10	9.47
Leucine	26.64	26.80	59.83	17.28
Tyrosine	12.68	11.68	15.24	7.27
Phenylalanine	16.42	14.08	37.65	10.39
Lysine	30.76	21.52	44.70	20.54
Histidine	12.94	6.26	32.21	12.11
Arginine	26.41	18.30	23.11	17.39

Figure A1. Weight variation of the Southern Bluefin Tuna sampled for protein and energy digestibility and transit time studies.

In vitro digestibility studies

The *in vitro* studies were completed and reported by Dr Chris Carter and Mr Matthew Bransden from the University of Tasmania.

The traditional method for assessing the performance of ingredients and feeds is to conduct long-term growth experiments. These are likely to be the most dependable methods but tend to be slow and prevent the analysis of a wide number of potential ingredients and therefore preclude rapid screening. They are also expensive with extensive requirements for time and fish rearing facilities. The need to rapidly screen large numbers of feed ingredients and to test a variety of feeds is especially important for the development of tuna feeds, but growth trials with large expensive tuna have to be restricted to testing a few feeds. This situation excludes the testing of large numbers of complete feeds or the screening of potential ingredients. The difficulties in working with tuna also excludes the routine use of *in vivo* digestibility procedures. Several studies have demonstrated a strong correlation between *in vitro* and *in vivo* digestibility values and since ingredient digestibility has a major effect on nutrient utilisation and growth the use of *in vitro* digestibility methods have potential for use in the development of tuna feeds.

A considerable amount of research has been conducted on the use of *in vitro* systems for predicting the digestibility of feeds and feed ingredients. Much of this research has focused on farm animals (Boisen & Eggum, 1991; Fuller, 1991), particularly ruminants, and it is clear that the development of systems for studies on fish requires modifications although the general principles are the same. Several studies have used methods for fish (Grabner, 1985; Grabner & Hofer, 1985; Eid & Matty, 1989; Rotter *et al.*, 1990; Anderson *et al.*, 1991; Anderson *et al.*, 1993; Oshodi & Hall, 1993; Romero *et al.*, 1994; Ostrowski *et al.*, 1996) and the most complete are by Dimes and colleagues (Dimes *et al.*, 1994a; Dimes & Haard, 1994; Dimes *et al.*, 1994b). The principle issues relate to the sensitivity of the assays and whether they have the potential to separate different feed ingredients and how accurately they predict the measured *in vivo* digestibility values. A number of studies have demonstrated significant correlations between *in vitro* and *in vivo* digestibility (Eid & Matty, 1989; Dimes & Haard, 1994; Dimes *et al.*, 1994b) but this has not always been the case (Romero *et al.*, 1994). Thus, the aims of this research were to measure the *in vitro* digestibility of a range of feeds and ingredients, compare this with *in vivo* digestibility measurements and validate a method which could be used to select ingredients with potential and to use ingredient digestibility to formulate tuna feeds.

Ingredients and feeds: The ingredients were obtained from a variety of sources (Table 7). Selection was based on their use in current tuna feeds or because of their potential for inclusion in tuna feeds. The tuna feeds were fed to tuna during 1997 as part of the TBOAA / CRC for Aquaculture trials or in smaller trials organised by the TBOAA or private companies.

The effect of heat damage was investigated in fish meal, dehulled soybean meal, and extruded and non-extruded pea concentrate. The meals were autoclaved under standard conditions (121°C) in Schott bottles, before being placed in glass petri dishes in a fan-forced oven at 160°C until charred (approximately 4.5 hours).

Multi-enzyme digestion: Three enzyme preparations were used: commercial enzymes, trypsin (T8128), chymotrypsin (C4129) and protease (P4630) were all supplied by Sigma and all of porcine origin; extracts from the pyloric caeca of Atlantic salmon (Salmo salar); extracts from the pyloric caeca of southern bluefin tuna (Thunnus maccoyii). The pylorus was removed from the fish, washed clean of feed material and homogenised (10 g / 50 ml distilled water). Care was taken to remove several whole caeca rather than a smaller part of many caeca. This accounted for differences in enzymes and activities along the length of the caeca. The homogenate was centrifuged (10 000g, 4°C, 20 min) and the supernatant stored at -70°C. The trypsin activity of each batch of crude enzyme extract was measured using the BAPNA linked assay (Preiser, 1975; Pringle, 1992) and used to

standardise the amount of crude enzyme extract added to make up the buffered enzyme solution. A standard activity of 0.05 μmoles pNA/ min / ml buffered enzyme solution was used.

The multi-enzyme digestion method used was adapted from Hsu *et al.* (1977) and Eid and Matty (1989). Samples of feed and ingredients were dried and then ground in a domestic spice mill. The sample was added to 25 ml of buffered enzyme solution, at a rate of 1 mg nitrogen / ml of enzyme solution, in a 100 ml conical flask. The solution was then incubated in a shaking water bath at 25 ^{o}C for 12 hours to match the expected temperature and residence time of ingesta in tuna. The proteins and peptides are then precipitated by the addition of 14 % sulphosalicyclic acid and the solution shaken for a further 20 min. After centrifugation (20 000g, 4^{o}C, 5 min) the supernatant is discarded, 30 ml of distilled water added and the resulting mixture re-centrifuged: this procedure was repeated 4 more times. The mixture was then filtered through a 1.2 μm Millipore filter, air dried, weighed for dry material and analysed for nitrogen. The insoluble nitrogen fraction was assumed to equate to the non-digestible nitrogen so that nitrogen (crude protein) digestibility is calculated as 100% (N_{diet} - $N_{insoluble}$ / N_{diet}).

Multi-enzyme pH change assay: The decrease in pH of the buffered enzyme solution plus ingredient was measured to determine whether this technique provided a more rapid method of achieving similar results to the 12 hour digestion method described above (Hsu *et al.*, 1977; Parsons, 1991; Oshodi & Hall, 1993). It was assumed that the hydrolysis of peptide bonds releases carboxyl groups which then cause the release of hydrogen ions and a decrease in pH. Samples were prepared as described above and the buffer adjusted to pH 8 before the enzyme solution (buffered at pH 8) was added. Addition of the enzyme resulted in a decrease in pH that was measured over 10 min. Use was made of the general formula Y = 210.46 -18.10 X, presented by Hsu *et al.* (1977) and used in other studies (Oshodi & Hall, 1993), where Y is *in vitro* nitrogen (crude protein) digestibility (%) and X the pH after 10 min.

In vivo digestibility: The *in vivo* digestibility values obtained from tuna are discussed elsewhere in this report. In order to validate the technique *in vivo* digestibility was measured in 8 tuna feeds used in 1997 and in 9 feed ingredients: fish meal (jack mackerel); pilchards; squid meal; krill meal; extruded pea protein concentrate (at two inclusions levels of 15 and 30 %); wheat gluten; pre-gel starch; wheat flour and dehulled soybean meal. The experiment was conducted at the Department of Aquaculture, University of Tasmania. Atlantic salmon parr were obtained from Wayatinah Salmon Hatchery (SALTAS) and stocked into 200-L tanks. The tanks were held in a constant environment room in which temperature (15.9 ± 0.1 ^{o}C) and photoperiod (12: 12) were maintained. The fish were held in recirculating freshwater. Water was treated through physical and biofilters with 50 % replacement twice a week. Water quality parameters (DO, pH, ammonia, nitrate and nitrite) were monitored to ensure water quality remained well within limits recommended for Atlantic salmon. A settlement system (Cho *et al.*, 1982) was attached to each tank to collect the faeces in the effluent water.

Duplicate groups of salmon were fed the experimental feeds containing chromic oxide (10 g. kg^{-1}) and faecal samples taken on days 7 and 21 (1997 tuna feeds) or after two and four weeks (ingredients). Ingredient digestibility was measured by mixing a reference diet with the ingredient and chromic oxide at a ration of 69: 30: 1 (Cho *et al.*, 1982). Faecal samples were collected from the settlement trap between the evening and morning feeds (17:00 to 09:00), freeze dried and used in the analysis of chromic oxide, nitrogen and dry material. The apparent digestibility coefficients (ADC) were calculated using the standard formula ADC (%) = 100 - [100. (% I_{diet} / %I_{faeces}) x (%N_{faeces} / %N_{diet}) (Maynard & Loosli, 1969) where I is the inert marker and N the nutrient.

Validation of in vitro digestibility: Validation of the *in vitro* method was based on the correlation between *in vitro* digestibility and *in vivo* digestibility. The data base included the eight 1997 tuna feeds and nine feed ingredients detailed above. In addition, the *in vitro* digestibility values obtained using different enzyme systems and the pH change assay were compared with each other.

Table 7. *Feeds and Ingredients.* Apparent dry matter and nitrogen digestibility coefficients for *in vivo* (*Salmo salar* at day 7at 30 % ingredient inclusion) and *in vitro* analyses using commercial enzymes (Enz.Com) or *S. salar* crude enzymes (Enz.Sal) for selected tuna feeds. All data are % mean ± standard deviation.

Test material	Supply	Source	Comments
Feeds			
CRC 97	SARDI	SARDI	
NBT 97	SARDI	SARDI	
Extruded	SARDI	SARDI	
Gibson salmon feed	Gibsons	Gibsons	
Commercial A	SARDI	Kensway	
Commercial B	SARDI	Chubpack	
Gibson tuna mash	Gibsons	Gibsons	
Telba	SARDI	Tiggerman	
Extruded base	SARDI	SARDI	
Japanese mash[d]	SARDI	SARDI	
Ingredients			
Fish meal - Triabunna	Gibsons	Triabunna	Jack Mackerel
Fish meal - Chilean	Gibsons	South America	
Fish meal - SARDI	SARDI	Ridley	
Fish meal - white	NSW Fisheries	Denmark	
Pilchard - Californian	SARDI		
Pilchard - WA	NFL, L'ton	WA	
Krill meal	SARDI		
Squid - SARDi	SARDI		
Squid - Ridley	Ridley		
Casein	UTAS		

Nutrient transport studies and ultra structure analysis

The nutrient transport studies and ultrastructure analysis were completed by Dr David Tivey and Mr Robert Kemp from the University of Adelaide.

In the development of new feeds for SBT it is imperative that an effective *in vitro* assay is developed to screen potential novel ingredients. However, to achieve this a detailed knowledge of digestive tract physiology is required. In this regard, the importance of the pyloric caeca in fish has been recognised early.

"Most fishes have a large number of caecal appendages by the side of the stomach, in which to store up the food as if in additional cellars and there to putrefy it and concoct it" Aristotle 345BC.

Buddingtion and Diamond (1987) have since confirmed this opinion. They observed that the more carnivorous a species is, the greater the percentage of nutrient uptake is mediated by the pyloric caeca. In this respect, SBT have large and uniquely shaped pyloric caeca suggesting this organ can perform the major proportion of nutrient digestion and transport. However, in the SBT little or no information is available on whether this organ can, in fact, transport the end products of digestion which range from single amino acids to small peptides. Knowledge of transport system(s) and their capacity, eg amino acid transporters compared with endocytosis and intracellular digestion of intact peptides, will have significant implications to the design of an appropriate digestion assay *in vitro*. For example, given the latter situation there may be a need to include a cathepsin digestion step in order to mimic digestion of proteins intracellularly.

To meet these goals it was imperative, in the first instance, to develop a system is to evaluate the absorptive characteristics of the Pyloric Caeca. Secondly, such an assay should be used to determining the presence of amino acid transport in this species.

Despite best efforts, new feed ingredients may have profound effects *in vivo* that can not be predicted from their digestibility as determined *in vitro*. Such responses may be a direct response to new feed ingredients that the fish has not experienced before. Ingredients binding to the lining of the intestinal tract and evoking structural and function changes in the tract may mediate such events as;

1. increased mucus production, thus increasing the barrier to nutrient absorption
2. structural changes in the villi and microvilli of the pyloric caeca thereby affecting the surface area available for digestion and absorption of feed
3. potential changes in the micro - environment that would alter the microbiology of the pyloric caeca.

Such events may be independent of how digestible the fed proves to be *in vitro* but would contribute to poor utilisation of feed *in vivo*. Furthermore, the changes that may occur are most likely reflected in the barrier function of the gastrointestinal tract; as such, they may effect the risk of enteric disease. Therefore, the final objective of this section was to examine Pyloric Caeca morphology in SBT maintained on feeds varying in composition.

Specific aims of this section of the program were

1. to investigate the capacity of the pyloric caeca to transport amino acid and or peptides
2. determine the ultra - structure of this organ and
3. evaluate the effect of age and diet on these parameters.

Mr. Robert Kemp describes most methods in his Honours thesis "Amino Acid transport in the Pyloric Caeca of Southern Blue Fin Tuna", University of Adelaide, 1997 (Appendix III). Those not described in this work are detailed, where appropriate in the results and discussion section as method development.

Microbiology and enzymology

The microbiology and enzymology components of this project were completed by Dr John Brooker from the University of Adelaide and Mr David West from Flinders University.

Microorganisms play an important role in digestive processes in most terrestrial species, and there is good evidence that microorganisms also exist in the digestive tract of fish. However, it is not clear what role those bacteria play. They could play a passive role in restricting the establishment of significant numbers of pathogenic bacteria in the gut, or a more active role by promoting or enhancing digestion of the normal diet. When fish are fed an artificial diet containing components not normally found in the wild, the action of microbial populations may be essential in achieving optimal digestion and growth response. The objective of this component of the project was to determine whether a significant microbial population existed in the digestive tract of Southern Bluefin Tuna, to characterise that microbial population with regard to their regional distribution, and to determine what potential digestive functions the microorganisms may carry out.

Digestive enzymes, whether of microbial or endogenous origin are the key to understanding digestive processes in the intestinal tract. However, not only is the total digestive profile important, it is also essential to understand the regional localisation of specific digestive enzymes so that the structure of feed pellets can be matched with digestive capabilities. In SBT, the digestive tract is divided into 2 major functional regions - the pyloric caeca and the small intestine. In order to maximise the digestibility and absorption of nutrients, we need to know which digestive enzymes have the greatest activities, where they are located and whether they change in level with changes in feed. Integration of the microbial and enzyme components of the project should provide useful information about the capability of SBT to digest artificial feeds, and help in the structural and compositional design of a feed pellet.

Most methods are described in the Honours thesis submitted by Mr David West, "Analysis of microbial populations in the digestive tract of Southern Bluefin Tuna". Those not described in this thesis are outlined below.

Preparation of homogenised fish extract: Frozen pyloric caeca tissue samples were thawed on ice. Approximately 5g weight samples were placed in a 10ml polycarbonate tube and 5mls of 0.1M phosphate buffer pH7.2 was added. Samples were homogenised on ice/water using a Kinematica polytron 1/2" probe until no visible tissue existed.

The homogenate was centrifuged at 8000g for 20 minutes and the cell debris discarded. Small intestine samples were thawed on ice and cut to expose the villas. The mucosal layer and contents were collected by scraping the surface with a spatula. 3mls of 0.1M phosphate buffer pH 7.2 was added and the samples were homogenised on ice/water using the polytron 1/2" probe.

Samples were placed in a 200ml round bottom glass flask and snap frozen in Liquid N_2, swirling the flask to ensure even freezing over a large surface area. Samples were freeze dried in a Dynavac freeze drying apparatus. The freeze dried extracts were placed in a sterile tube and stored at -80°C.

DPPIV test tube assay

*Substrate***:** 3mM gly-pro p- nitroanilide hydrochloride (Sigma) dissolve in 20g/LTriton X-100 . {N.B. Substrate may be stored at 4°C for 7 days)

Reagents: 0.3M Glycine/NaOH pH 8.7, Acetate buffer (Walpole) pH 4.2, 0.05M Sodium Acetate, 0.15M Sodium Chloride, pH to 4.2 with Acetic acid

Standards: 3mM p-nitroaniline dissolve in 20ml/L Methanol first then add water containing 20ml/L Triton X-100. {N.B. Standard may be stored at 4˚C for 7 days). Sample protein concentration was determined using the Bradford method. A control sample extract was boiled for 10 minutes to denature protein and incubated separately.

Reaction: 262.5 µl reactions (***done in duplicate or triplicate***). 62.5 µl Glycine/NaOH buffer, 125µl 3mM gly-pro p-nitroaniline substrate, 62.5µl water, 12.5µl enzyme (extract containing 50µg protein) or boiled enzyme as control.

Incubate at 32°C for 30 mins. Add 750µl acetate buffer. Read OD at 380 nm. Use water as auto zero. Minus control from enzyme standard. Read absorbance from standard curve using p-nitroaniline 50-200nmol.

Bacteria assayed

Samples selected for assaying included:

- 45 Mixed bacteria from Pyloric caeca of CRC feed fish
- 51 Mixed bacteria from Small Intestine of CRC feed fish
- 30 Mixed bacteria from Pyloric caeca of pilchard feed fish
- 32 Mixed bacteria from Small Intestine of pilchard feed fish
- 14 Mixed bacteria from Pyloric caeca of wild fish
- 12 Mixed bacteria from Small Intestine of wild fish

Protein determination (Bradford method)

Reagents: Bradford solution, 0.15 M NaCl, 0.5 mg/ml BSA

*Standards **(in duplicate):*** Add 0,5,10,15,20,30,40 µl of BSA (0.5 mg/ml) to give protein concentration of 0, 2.5,5,7.5,10,15, and 20 µg.

Samples: Add 10 ul of cell extract, lysed or unlysed. Make to 100 µl with 0.15 M NaCl. Add 1 ml Bradford's solution and vortex. Allow to stand at RT. for 2 mins. Read OD at 595 nm. Plot standard curve and read off protein concentration of samples.

Sample extraction: Over night cultures grown in 5mls BHI where spun at 3000 rpm for 10 mins. Remove media. Resuspend cells in 0.1M potassium phosphate buffer 2.5 mls. Remove 500 ul for whole cell analysis. Lyse remainder of cells by sonication.

Sonication: Using Branson® Model 450 Sonifer. Set output control to #4. Don't let meter reading exceed 15 (= 25 Watts). 5 x 15 secs sonication on 50% duty cycle at 25 W with 1 min rests between treatments. After sonication centrifuge at 12, 000 rpm for 10 min in JA20.

Results and Discussion

In vivo digestibility studies

Preliminary assessment of methodology to determine protein and energy digestibility and digesta transit time in feed ingredients offered to caged southern bluefin tuna (SBT)

Establishment of in vivo techniques: The destructive sampling technique allowed the collection of sufficient quantities of digesta from each section of the tract. Samples from more than one fish were required to facilitate chemical analysis.

Sampling was expensive due to boat hire and employment of divers and crew to assist with the capture, killing and removal of the digestive tracts. There was a need to sample many fish, to provide replication and sufficient digesta. Factors such as these emphasise the need for an *in vitro* digestibility assay.

Estimation of digesta transit time: Transit time could not be determined as the coloured plastic beads were too large to pass through the pyloric sphincter, between the stomach and the pyloric caeca. Most beads remained in the stomach but some were ejected from the stomach, observed by divers when harvesting the tuna.

Beads and sausage skins remaining in the stomach were counted. A small number of white beads remained in the stomach, from the first feeding, suggesting that a near total regurgitation of feed and/or beads occurred overnight. The amount of yellow beads found in the stomach was correlated to the number of sausage skins found in the stomach 2.5 hours after feeding (Figure A2) and 5.0 hours after feeding (Figure A3). No beads were found beyond the stomach. A moderately low correlation ($r^2 = 0.48$) was found between number of beads and sausages 2.5 hours after feeding. A higher correlation ($r^2 = 0.65$) was found 5.0 hours after feeding.

Digestibility of protein, amino acids and energy: Univariate analysis revealed a normal distribution of the data. With the exception of pyloric caeca dry matter digestibility, there was no significant difference ($P>0.05$) between the digestibility results determined from each collection date. There were no date by time interactions, so the data was pooled to compare the effect of time of digesta collection on the digestibility coefficients.

Dry matter, nitrogen and energy digestibility increased as the digesta moved through the gastro-intestinal tract. There was a significant difference between the digestibility coefficients obtained in the four regions ($P<0.001$). With the exception of pyloric caeca dry matter digestibility ($P<0.01$) and stomach energy digestibility ($P<0.05$), there was no significant difference between the digestibility coefficients obtained 2.5 or 5.0 hours after feeding the Peruvian fish meal diet (Table 8).

The amino acid digestibility was only measured in the distal intestine, as the nitrogen digestibility coefficients showed that there was significant digestion of protein in this region.

Amino acid digestibility coefficients were obtained using acid-insoluble ash as an indigestible marker (Table 9). Digestibility of all amino acids was low, but positive with the exception of alanine, valine and isoleucine at 5 hours. There was no significant difference between digestibility coefficients determined 2.5 and 5.0 hour after feeding.

n-hexatriacontane was also used as an indigestible marker to obtain digestibility coefficients for amino acids (Table 10). Digestibility was again low but positive, except for isoleucine after 5.0 hours. There was a significant difference ($P<0.05$) between aspartic acid and isoleucine, for each sampling time.

There was no significant difference (P>0.05) in digestibility coefficients between the sampling times for the remaining amino acids.

Comparison of digestibility markers: The proximal and distal intestine were analysed for acid-insoluble ash content and *n*-hexatriacontane content. These results were correlated, with an r^2 of 0.79 (Figure A4).

Dry matter, nitrogen and energy digestibility difference was also compared using the acid-insoluble ash and n-hexatriacontane markers (Table 11). There was no significant difference from zero, between the two markers for both time periods, for dry matter, nitrogen or energy digestibility.

The difference between acid-insoluble ash and *n*-hexatriacontane was (Table 12). There was no significant difference from zero, between the two markers for both time periods, suggesting they behave similarly in the digestive tract.

All comparisons showed that the coefficients obtained using n-hexatriacontane were slightly higher than the coefficients obtained using acid-insoluble ash. All results were negative after the n-hexatriacontane coefficient was subtracted from the acid-insoluble ash coefficient.

Discussion

Establishment of in vivo techniques: Destructive sampling is an efficient way of obtaining digesta samples from any section of the digestive tract. Unfortunately, this method of sampling does not allow multiple sampling, from one fish, over time. A greater amount of fish have to be sampled to gain estimates of digestibility and transit time. This increases errors due to fish variation.

The acceptance of experimental diets is proving to be a problem associated with *in vivo* techniques. Often the SBT will eat a moist pellet, but regurgitate it soon after. The diets appear to be unpalatable either by taste, texture or smell, even though the fish are initially attracted to the diet. The development of an *in vitro* assay will remove the difficulties associated with getting the fish to accept diets. Separate research trials will be carried out to ascertain the factors required for SBT to accept diets containing protein sources, deemed to be suitable by the *in vitro* digestibility assay.

The digestibility and transit time experiment was costly and time consuming. The objectives of this experiment were not fully met as a result of the problems associated with *in vivo* digestibility and transit time determinations using SBT. These factors also indicate the need for an *in vitro* digestibility assay, as *in vivo* experiments are too costly when inaccurate results are the outcome.

Estimation of digesta transit time: Coloured plastic beads, of approximate dimension 2mm x 1mm, are not suitable for transit time studies as the beads are too large to pass through the pyloric sphincter. It is possible that smaller beads may pass through the sphincter, allowing physical determination of the transit time. The use of a chemical marker may be more appropriate for the measurement of the transit time in caged SBT.

As most of the beads remained in the stomach, they could have served as an estimate of intake. It is impossible to assess the intake in these fish because the cage is too large, and there are too many fish to observe individual intake. An estimate of intake would be useful for growth rates and feed conversion rates.

Beads were removed from the stomach contents. Sausage skins, that encapsulated the diet, were also remaining in the stomach. These were also counted. The number of beads present was correlated against the number of sausage skins present. It was assumed that the number of beads present would increase as the number of sausages eaten increased, because there was an average number of beads per sausage.

Separate correlations were performed for both time periods. The slope and the r^2 were slightly different for both time periods. The correlation for 5.0 hours shows that there were more beads found per sausage consumed. This is unusual because beads were observed to be floating in the water after 2.5 hours, leading to the assumption that there would be less beads per skin counted. The difference in slope may be due to digestion of sausage skins after 5.0 hours, or regurgitation of skins. The r^2 values were both reasonable, but not high enough to encourage the use of beads to measure approximate intake.

Digestibility of protein, amino acids and energy: It is not necessary to sample from all the regions of the tract for digestibility studies, because significant digestion occurs in the proximal and distal intestine. It is reasonable to base digestibility on the estimates from the distal intestine, as this is the last region of the gastro-intestinal tract, and hence the last region of digestion.

Although the digestibility coefficients, for all examined nutrients, increased as the digesta moved through the gastro-intestinal tract, the results in the distal intestine are lower than expected. In pigs, the apparent ileal amino acid digestibilities of fish meal are all approximately 0.90, (Table 13). Values for digestibility of lysine in Peruvian fish meal are 0.50 (acid-insoluble ash marker) and 0.56 (n-hexatriacontane marker). Most of the amino acid digestibility coefficients are less than 0.50 in SBT.

Reasons for low digestibility estimates may include:

1. Peruvian fish meal was relatively indigestible to SBT.
2. There was a large contribution of endogenous nitrogen, which suppressed the digestibility values.
3. Digestion is inefficient because digesta is moving too rapidly through the gastro-intestinal tract.

The first reason can only be clarified with future digestibility studies.

Endogenous nitrogen contribution could be assessed by feeding a protein free diet. In this case, it would be impractical as the SBT would not readily accept this type of diet. A disadvantage with using protein free diets to quantify endogenous nitrogen is that different amounts of endogenous nitrogen are produced due to the unphysiological nature of the protein-free state (Low, 1980). Other methods include chemically labelling dietary protein by converting lysine to homoarginine (Rutherfurd *et al*, 1990), feeding a semi-synthetic diet containing enzymically hydrolysed casein, which simulates the natural products of digestion (Darragh et al, 1990) or feeding protein sources that are naturally devoid of certain amino acids. Zein, derived from maize only contains small traces of lysine, so most lysine in the digesta would be of endogenous nature (Butts et al, 1993).

The inefficiency of digestion due to rapid transit times may be clarified, in the future, by comparing the digestibility of diets to the digesta transit time. Factoring in growth rates obtained on diets may also indicate whether the nutrients in the diet are being absorbed and utilised efficiently.

Comparison of digestibility markers: Digestibility markers such as acid-insoluble ash and n-hexatriacontane have been compared favourably in previous experiments with poultry. They also compared favourably in this experiment, as shown by the regression. There were close correlations between acid-insoluble ash and *n*-hexatriacontane levels in the proximal and distal intestine of SBT (r^2 = 0.79). This compares well with the experiment conducted with poultry, where r^2 = 0.63 in ileal digesta and r^2 = 0.91 in excreta (Choct *et al*, 1996).

The close relationship, between acid-insoluble ash and *n*-hexatriacontane, was further supported by comparing the difference (acid-insoluble ash minus *n*-hexatriacontane) from zero of the amino acid digestibility coefficients. The statistical analysis showed that there was no significant difference between the amino acid digestibility coefficients derived from acid-insoluble ash or *n*-hexatriacontane digestibility markers. n-hexatriacontane was the more accurate indigestible marker because the

standard error was lower than the standard errors for coefficients obtained with acid-insoluble ash. The chemical analysis for n-hexatriacontane contributed to the accuracy because the analysis was strictly controlled with an internal standard, which was a hydrocarbon of similar chain length. Analysis of acid-insoluble ash was susceptible to error due to multiple weighing of the sample through the analysis process, and no standard control was used.

It was hypothesised that the salt content of the water would affect the acid-insoluble ash contents of the diet and digesta by raising the concentration. The comparison of acid-insoluble ash to n-hexatriacontane, supported by an acid-soluble ash analysis of sea salt, which contains zero percent acid-insoluble ash, disproved the hypothesis.

Figure A2. Correlation between the number of sausage skins and coloured plastic beads found in outhern Bluefin Tuna stomachs 2.5 hours after feeding a diet containing beads.

Figure A3. Correlation between the number of sausage skins and coloured plastic beads found in outhern Bluefin Tuna stomachs 5.0 hours after feeding a diet containing beads.

Table 8. *Dry matter, nitrogen and energy digestibility in the stomach, pyloric caeca, proximal intestine and distal intestine, in a Peruvian fish meal based diet fed to caged Southern Bluefin Tuna, determined 2.5 and 5.0 hours after feeding.*

	Collection time (hours)		Statistics		
	2.5	5.0	SEM (2.5h)	SEM (5.0h)	Time
Dry matter digestibility					
Stomach	0.00	0.06	0.021	0.021	NS
Pyloric caeca	-0.08	-0.24	0.024	0.029	**
Proximal intestine	0.17	0.06	0.065	0.088	NS
Distal intestine	0.42	0.35	0.048	0.048	NS
Region*Time	NS				
Region	***				
SEM	0.044				
Nitrogen digestibility					
Stomach	-0.13	-0.07	0.020	0.019	NS
Pyloric caeca	0.02	-0.05	0.076	0.082	NS
Proximal intestine	0.11	-0.01	0.062	0.077	NS
Distal intestine	0.38	0.33	0.062	0.059	NS
Region*Time	NS				
Region	***				
SEM	0.047				
Energy digestibility					
Stomach	0.12	0.20	0.023	0.022	*
Pyloric caeca	0.21	0.19	0.068	0.073	NS
Proximal intestine	0.41	0.29	0.049	0.060	NS
Distal intestine	0.62	0.59	0.041	0.039	NS
Region*Time	NS				
Region	***				
SEM	0.039				

NS, not significant; * P<0.05; *** P<0.001; SEM, standard error of the mean

Table 9. *Digestibility of amino acids in a Peruvian fish meal based diet, fed to caged Southern Bluefin Tuna, determined 2.5 and 5.0 hours after feeding, using acid insoluble ash as an indigestible marker.*

	Collection time (hours)		Statistics		
Amino acid	2.5	5	SEM (2.5h)	SEM (5h)	Time
Aspartic acid	0.31	0.14	0.063	0.085	NS
Threonine	0.26	0.10	0.070	0.095	NS
Serine	0.31	0.16	0.066	0.090	NS
Glutamic acid	0.38	0.24	0.054	0.074	NS
Glycine	0.45	0.32	0.049	0.067	NS
Alanine	0.14	-0.04	0.078	0.105	NS
Valine	0.16	-0.04	0.076	0.103	NS
Isoleucine	0.09	-0.16	0.088	0.119	NS
Leucine	0.45	0.36	0.051	0.069	NS
Tyrosine	0.31	0.14	0.062	0.083	NS
Phenylalanine	0.39	0.30	0.055	0.075	NS
Lysine	0.50	0.39	0.044	0.059	NS
Histidine	0.51	0.38	0.047	0.063	NS
Arginine	0.53	0.46	0.044	0.059	NS

SEM, standard error of the mean; *, P<0.05; NS, not significant;

Table 10. *Digestibility of amino acids in a Peruvian fish meal based diet, fed to caged Southern Bluefin Tuna, determined 2.5 and 5.0 hours after feeding, using n-hexatriacontane as an indigestible marker.*

	Collection time (hours)		Statistics		
Amino acid	2.5	5	SEM (2.5h)	SEM (5h)	Time
Aspartic acid	0.40	0.25	0.040	0.054	*
Threonine	0.34	0.21	0.046	0.063	NS
Serine	0.39	0.26	0.043	0.059	NS
Glutamic acid	0.46	0.34	0.039	0.052	NS
Glycine	0.52	0.41	0.032	0.044	NS
Alanine	0.25	0.09	0.053	0.072	NS
Valine	0.27	0.10	0.051	0.069	NS
Isoleucine	0.20	-0.01	0.053	0.072	*
Leucine	0.54	0.46	0.033	0.044	NS
Tyrosine	0.39	0.25	0.048	0.065	NS
Phenylalanine	0.49	0.40	0.034	0.046	NS
Lysine	0.56	0.46	0.031	0.042	NS
Histidine	0.57	0.47	0.034	0.046	NS
Arginine	0.60	0.52	0.032	0.043	NS

SEM, standard error of the mean; * $P<0.05$; NS, not significant;

Figure A4. Comparison of the recovery of the indigestible markers, acid-insoluble ash and n-hexatriacontane, in the proximal and distal intestine of Southern Bluefin Tuna.

Table 11. *Comparison of the difference in dry matter, nitrogen and energy digestibility coefficients in a Peruvian fish meal based diet, determined in the proximal and distal intestine of Southern Bluefin Tuna, using acid insoluble ash and n-hexatriacontane as indigestible markers.*

	Collection time (hours)		Statistics		
	2.5	5.0	SEM (2.5h)	SEM (5.0h)	Significance
Proximal intestine					NS
Dry matter digestibility	-0.83	-0.93	0.065	0.087	NS
Nitrogen digestibility	-0.18	-0.18	0.033	0.044	NS
Energy digestibility	-0.13	-0.13	0.023	0.031	NS
Distal intestine					NS
Dry matter digestibility	-0.58	-0.64	0.048	0.048	NS
Nitrogen digestibility	-0.08	-0.13	0.071	0.071	NS
Energy digestibility	-0.05	-0.08	0.044	0.044	NS

SEM, standard error of the mean; NS, not significant;

Table 12. *Comparison of the differences (acid insoluble ash minus n-hexatriacontane) between amino acid digestibility coefficients, for a Peruvian fish meal based diet fed to caged Southern Bluefin Tuna, determined 2.5 and 5.0 hours after feeding, using acid insoluble ash or n-hexatriacontane as indigestible markers.*

	Collection time (hours)		Statistics		
Amino acid	2.5	5.0	SEM (2.5h)	SEM (5.0h)	Significance
Aspartic acid	-0.09	-0.12	0.083	0.112	NS
Threonine	-0.09	-0.11	0.096	0.130	NS
Serine	-0.08	-0.10	0.090	0.121	NS
Glutamic acid	-0.08	-0.09	0.077	0104	NS
Glycine	-0.07	-0.09	0.065	0.083	NS
Alanine	-0.11	-0.13	0.106	0.144	NS
Valine	-0.11	-0.13	0.102	0.138	NS
Isoleucine	-0.11	-0.15	0.114	0.155	NS
Leucine	-0.09	-0.09	0.056	0.075	NS
Tyrosine	-0.08	-0.11	0.088	0.119	NS
Phenylalanine	-0.10	-0.10	0.064	0.087	NS
Lysine	-0.06	-0.07	0.064	0.086	NS
Histidine	-0.06	-0.08	0.059	0.080	NS
Arginine	-0.05	-0.06	0.058	0.078	NS

SEM, standard error of the mean: NS, not significant;

Table 13. *Apparent ileal digestibility coefficients of fish meal in the pig.*

Nutrient	Digestibility coefficient
Crude protein	0.87
Lysine	0.94
Threonine	0.92
Methionine	0.93
Cystine	0.80
Tryptophan	0.85
Arginine	0.92
Histidine	0.92
Isoleucine	0.91
Leucine	0.92
Phenylalanine	0.90
Tyrosine	0.87
Valine	0.89

(From Jondreville *et al*, 1995).

The distal intestinal digestibility of amino acids and energy and the mean transit time of a manufactured diet and pilchards fed to caged Southern Bluefin Tuna

Establishment of in vivo techniques: The weight of the fish sampled in this experiment was greatly variable (Figure A5). This variation may affect the accuracy of transit time and digestibility estimates.

The recovery of blue plastic beads was zero in many instances, compared with the number of sausage skins. The correlation between number of beads and number of sausages in the stomach was not attempted due to the poor recovery of beads.

Estimation of digesta transit time: There was not enough digesta present for the analysis of polyethylene glycol. Polyethylene glycol is a liquid phase marker, so analysis is performed on a wet sample. Most of the wet digesta sample would need to be centrifuged, to produce enough juice for analysis. This would leave minimal samples for digestibility determinations.

The concentration of chromic oxide in the digesta was plotted against time after feeding the unmarked diet (the diluent). A line of best fit was taken from the average equilibrium excretion rate. An estimation of 80% marker excretion was calculated for the manufactured diet (Figure A6). The transit time was the point of intersection of the line of best fit with the line of 80% marker excretion. The transit time was estimated to be 5.30 hours. One observation was removed from the manufactured diet data, as only one fish was harvested at one of the time periods, whereas the rest of the data was an average of three fish per cage per time period.

A transit time estimation did not occur with the pilchard diet. The concentration of chromic oxide, in the digesta, did not fall during the time period of sampling (Figure A7). This indicated that the transit time of pilchards is longer than the transit time of the manufactured diet, due to the difference of the particle size within the diet.

Digestibility of protein, amino acids and energy: Univariate analysis revealed a normal distribution of the data. Dry matter and nitrogen digestibility, in the distal intestine, was negative for both the manufactured diet and the pilchards. Energy and amino acid digestibility was positive, but low, for the manufactured diet and negative for the pilchards (Table 14). There were no diet*time, time*day, diet*day or diet*day*time interactions ($P>0.05$).

Discussion

Establishment of in vivo techniques: Destructive sampling appears to be the most efficient way to obtain digesta samples from the SBT. The tuna would not respond well to other sampling methods involving confinement in small collection tanks or excessive handling that would result if digesta was being stripped through the anus.

A problem, that may occur with the stripping of digesta from the gastro-intestinal tract, is that of excessive inputs of nitrogen and energy of endogenous origin. Digesta was squeezed from the intestine using the fingers. The distal intestine was stripped vigorously to obtain sufficient digesta. It is possible that the squeezing is forcing cells and mucous that line the wall of the intestine into the collection jar, along with the digesta. A yellow-white substance was frequently observed on the last strip of a section of distal intestine. This was in contrast to the bright green of the digesta.

Results may be improved by less vigorous stripping of the digesta, or the removal of digesta by infusing distilled water into the open ends of the intestine. The use of water will increase the water content of the digesta, incurring a longer freeze drying period.

Very small numbers of plastic beads were recovered, compared to the number of sausage skins that were observed in the stomach of the Southern Bluefin Tuna. In this instance, beads should not be used

as an estimate of intake. The poor recovery of beads may be due to regurgitation of the beads or the non-consumption of the beaded diet. The experimental SBT are hand fed and observed to see if they were consuming most of the feed. The consumption of the beaded diets were reported to be "poor" to "bad", whereas consumption of the marked diet was reported to be "good" to "average". The acceptance of the beaded diets should not have been a factor, as the diets were in the same form and had the same chemical composition apart from the subtraction of chemical markers and the addition of plastic beads. The SBT had been eating the marked diet for three weeks prior to the harvest of the trial. The factor that caused the difference is the time of feeding. The SBT were accustomed to being fed around 5.00pm and were not adapted to feeding in the early morning. It is concluded that the SBT did not eat much of the beaded diet, and the sausage skins that were observed in the digestive tract were the remains from the previous day.

The variability of fish weight within the experiment was large and it would be desirable to avoid this in future experiments. There is, however, very limited opportunity to be highly selective when sampling SBT, as the cage environment and handling problems does not facilitate easy and accurate weighing and transferral to different cages. The individual fish weights could not be compared to the results of chemical analysis because the concentration of chromic oxide was measured over time and digesta, from three fish, was pooled for chemical analysis of digestibility. Both transit time and digestibility estimations should be done on a similar sized fish, and it is assumed that the differences in weight did affect the results.

Estimation of digesta transit time: The analysis for polyethylene glycol required a sample of gut juice (Malawer and Powell, 1967). The sample must be centrifuged to obtain gut juice with no solids. It is not possible to use the solid digesta after the sample is centrifuged, because the digesta is more concentrated after the removal of the juice. This would lead to erroneous digestibility coefficients and solid phase transit time determinations. On this basis, the analysis of polyethylene glycol was not done, and so the transit time of the liquid phase of the digesta was not estimated.

An estimate of 80% marker excretion was obtained, using chromic oxide as a solid phase marker, in the manufactured diet. This means that the marked diet was diluted to 20% of its original concentration, in 5.30 hours, by the diet that was fed on the morning of the harvest. The transit time, 5.30 hours, was obtained using the equation of the line of best fit, substituting 20% of equilibrium marker excretion as the y-value. This was shown as the intersection between the line of best fit and the line of 80% marker excretion.

The transit time of the manufactured diet was short for the following reasons:

1. The length of the gastro-intestinal tract is very short in the Southern Bluefin Tuna.
2. The particle size of the manufactured diet was small, and proceeded quickly through the gastro-intestinal tract, as it does not need to be broken down to smaller sized particles in the stomach.

The transit time estimation was not possible for the pilchard diet. The concentrations of chromic oxide in the digesta randomly spread around the average equilibrium excretion throughout the period of serial sampling. If the sampling time was extended beyond six hours, a decrease in the concentration of marker may have been observed.

The longer transit time of the pilchard diet may be related to particle size. Unlike the manufactured diet, the pilchard diet may have to undergo more mechanical and chemical digestion, once ingested, before movement from the stomach into the pyloric caeca.

The chromic oxide concentrations were variable between fish harvested from the same cage at the same time. This may be attributed to diet mixing difficulties. One of the problems with the use of chromic oxide as a marker is the inability to mix homogeneously (Choct *et al*, 1996). Chromic oxide was mixed with some success with the manufactured diet because all of the ingredients are powders apart from Jack mackerel oil, lethicin, squid oil and water. The mixer used to mix the diets for tuna

can mix powder homogenously. The problem occurs when trying to mix chromic oxide, a powder, with pilchards. The particle size of the pilchards was minimally decreased by blending in a food processor, but the mixer was still unable to mix the pilchards and chromic oxide homogenously. Feed mixing technology proved to be a limitation.

Digestibility of protein, amino acids and energy: The negative dry matter digestibility values for both the manufactured diet and pilchards suggest the digestibility estimates should be interpreted with caution.

A number of factors may be contributing to the low digestibility estimates determined during this experiment. The variability of the weights of the fish affected the accuracy of the determinations. Regurgitation and poor consumption of experimental diets would also decrease the accuracy and the reliability of the results.

Endogenous nitrogen contributions may be suppressing the digestibility values, and the endogenous nitrogen contribution needs to be quantified before the *in vitro* digestibility assays can be designed correctly.

Feed mixing difficulties may be contributing the poor digestibility results. The current feed mixing technology is not sufficient to combine the diets and markers homogenously, as discussed previously in estimation of digesta transit time.

Figure A5. Estimation of digesta transit time of a manufactured diet using a chromic oxide dilution technique. Fish were sampled over four time periods to gain an estimate of the excretion of marker to a point where 20% of the equilibrium marker concentration remained.

Figure A6. Estimation of digesta transit time of pilchards using a chromic oxide dilution technique. Fish were sampled over four time periods to gain an estimate of the excretion of marker to a point where 20% of the equilibrium marker concentration remained.

Table 14. *Digestibility of dry matter, nitrogen, energy and amino acids in a manufactured diet and pilchards determined in the distal intestine of caged Southern Bluefin Tuna*

	Diet		Statistics	
	2	4	Diet	SEM
Dry Matter	-0.06	-2.05	*	0.719
Nitrogen	-0.31	-2.09	*	0.736
Energy	0.22	-2.07	*	0.677
Aspartic acid	0.22	-0.85	NS	0.467
Threonine	0.13	-1.42	NS	0.587
Serine	0.18	-1.21	NS	0.547
Glutamic acid	0.44	-0.84	NS	0.436
Proline	0.22	-1.91	NS	0.656
Glycine	0.43	-0.58	NS	0.337
Alanine	0.31	-0.90	NS	0.453
Valine	0.18	-1.32	NS	0.539
Isoleucine	0.15	-1.41	NS	0.544
Leucine	0.29	-1.21	NS	0.538
Tyrosine	0.22	-1.33	NS	0.600
Phenylalanine	0.37	-1.01	NS	0.466
Lysine	0.13	-0.95	NS	0.507
Histidine	0.37	-0.24	NS	0.310
Arginine	0.27	-0.61	NS	0.459

NS, not significant; *, P<0.05; SEM, standard error of the mean;

In vitro digestibility studies

Digestibility values from multi-enzyme digestion

1997 Tuna Feeds: The *in vitro* digestibility values for the 1997 tuna feeds showed a number of trends. The feeds had different digestibility values; the highest values were obtained using the commercial enzymes, then the crude enzyme extracts from salmon and the extracts from the tuna were generally lowest. There were statistically significant correlations between the three enzyme systems, the salmon and tuna systems showed the strongest correlation. For each feed and enzyme system the digestibility for crude protein was higher than for dry material. This demonstrated that the assays were sensitive enough to distinguish between complete feeds. The tuna enzyme system had the widest range of values and could be considered the most sensitive of the three enzyme systems.

The *in vitro* digestibility using commercial enzymes varied between 66 and 77 % for dry material and between 83 and 91 % for crude protein (Table 15). Similarly, the ranges were 62-73% and 74-89% using salmon enzymes (Table 15) and 57-68% and 65-84% using the tuna enzymes (Table 16). The NBT 97 and Gibson's salmon feeds generally had higher dry material and crude protein digestibility values where as the Commercial A and B feeds had lower values. There were differences between the enzyme systems and the salmon and tuna enzyme systems showed the Extruded Feed to have a low digestibility but this was not shown by the commercial enzyme system. However, the three systems gave generally similar results over the range of feeds. The correlations between all enzymes systems were significant ($P < 0.05$) (Figure. 2) and the strongest was between the fish enzyme extracts ($r = +0.85$; $P < 0.01$).

The range in crude protein digestibility was 8% using the commercial enzymes and compared with ranges of 15 % and 19 % using the salmon and tuna enzyme systems, respectively. The wider range of values suggested that the crude fish enzyme systems provided more sensitive assays than the commercial (porcine) enzyme system.

Ingredients: The ingredients reflected the trends observed with the complete feeds by showing a wide range of values, lower values for dry material than for crude protein, differences between the different enzyme systems and greater sensitivity of the fish enzyme systems (Tables 17-20).

Animal meals: The dry material and crude protein values were higher using the commercial enzyme system and ranged between 54-87% and 65-94%, respectively, where as the ranges were 36-76% and 47-83% using salmon (Table 18) and 27-78% and 54-83% using tuna enzymes (Table 17). Squid meal (source - Ridley) was identified as having the lowest values by all systems, pilchards the highest dry material and fish meal (jack mackerel) the highest crude protein by the fish enzyme systems. There were relatively large differences between the three systems for some animal meals such as squid, krill and fish meal (source - SARDI) compared with other meals such as pilchards and fish meal (source - Triabunna) (Figure 3). Digestibility values from the fish crude enzyme extracts tended to be closer than from the commercial enzyme system.

Plant meals: The digestibility of plant meals was assessed using the commercial and salmon crude enzyme systems but not the tuna enzymes. Trends were similar to those reported for the feeds and animal meals although the differences between the two systems appeared more pronounced (Figure 4). Dry material digestibility was lower than crude protein digestibility (Table 19) and values were lower for lupin and pea meals than for the soybean meals. The salmon system demonstrated that processing affected digestibility. Dehulling the lupin meal increased digestibility. Extrusion significantly increased the dry material digestibility of the pea meal concentrate. The dehulled pea meal had a very low dry material digestibility of 22% compared with 32-30% for the protein concentrate and 66% for the extruded concentrate. Plant meals contain large amounts of starch and non-starch polysaccharides which contributes to low dry material digestibility. In contrast, the crude protein digestibility values were considerably higher. Values from the commercial enzyme system only varied by 7% (88-95%)

compared with a range of 58 % (37-95%) using the salmon system (Table 19). This suggested the salmon system was more sensitive. Consequently, the salmon system showed clear differences due to processing. Dehulling lupin meal increased digestibility as did extrusion of the pea meal concentrate. Excessive heating of the meals also had a considerable effect on crude protein digestibility and reduced values to very low levels (Table 21). For example, fish meal digestibility, using all three enzyme systems, was reduced to about 25% of the untreated value.

As has been reported above the commercial enzyme system gave higher digestibility values than the salmon enzyme system and this was apparent for animal, plant and heat damaged meals (Figure 5). Furthermore, the animal and damaged meals appeared to show the same relationship between the two system but the majority of the plant meals fell outside this (Figure 5). This suggested that the porcine enzymes were more suited to a wider range of feed ingredients than the fish enzymes and would be predicted since salmon, carnivores, occupy a different trophic level to that of pigs, omnivores. Digestibility values for highly processed meals, such as the extruded pea protein concentrate and the wheat gluten, were similar between the two enzyme systems. It appears likely that the high carbohydrate content in the less processed plant meals resulted in decreased digestibility of the crude protein as well as of the dry material by the salmon enzymes.

Digestibility values from pH change assay

The multi-enzyme pH change assay provides a value for crude protein digestibility and was used on a variety of feeds, animal and plant meals (Table 22). Ingredient values ranged between 70% (krill meal) to 86 % (dehulled lupin and wheat gluten), a range of 16%. Purified proteins BSA and Casein had a higher value of 95%. As reported previously the ranges obtained using the enzyme systems were considerably larger than this and partly explained the lack of correlation between digestibility predicted by the pH assay and the enzyme systems (Figure 7).

In vivo digestibility

Apparent dry material and crude protein digestibility for the eight 1997 tuna feeds, measured *in vivo* using Atlantic salmon, varied between feeds and was lower for dry material than for crude protein (Table 15). The crude protein digestibility of the Extruded Feed was 86% whereas the other feeds had values between 91 and 97 % (Figure 8). The dry material digestibility was low for the Commercial B (70%) and Extruded (74%) feeds but higher for the remaining feeds (82-86%) (Table 15). There were no significant correlations between *in vivo* and *in vitro* digestibility values. The largest differences between the *in vivo* and *in vitro* ranks of respective feeds were for the Extruded, Telba and Salmon feeds.

The *in vivo* digestibility values for selected ingredients were lower for dry material than for crude protein. Higher dry material values of over 80% were measured for fish meal, wheat gluten and pre-gel starch at a l5% inclusion level (Tables 18-20). Wheat flour had very low values of less than 20%. Crude protein digestibility values were higher, over 90%, for fish meal, pea protein concentrate, soybean meal and wheat gluten (Figure 9). The effect of time, measurements were made after 7 and 21 days feeding on the diets, differed between ingredients. Fish meal (dry material only) and squid meal showed a greater than 5% decrease whereas extruded pea protein concentrate and wheat flour showed a greater than 5% increase. The other ingredients showed less than a 5% change and the digestibility values were correlated between the two days for both crude protein (Figure 10) and dry material (Figure 13).

The main reason for measuring *in vivo* digestibility in salmon was to investigate the correlations with the *in vitro* measurements. There were a number of significant correlations and the strength of the correlation depended on the digestibility measure (crude protein or dry material) and the assay used. *In vivo* crude protein digestibility was positively and significantly correlated with the porcine (2 and 4 weeks) and salmon (4 weeks) enzymes (Figures 11 and 12) and with pH change (2 weeks). The correlation was very close to being significant for salmon enzymes (2 weeks) and tuna enzymes (2 and

4 weeks) (Table 23). *In vivo* dry material digestibility was positively and significantly correlated with only the porcine enzymes (Figure 14).

Discussion

The principle aim of the research was to develop a method for predicting the digestibility of ingredients to allow more accurate formulation of new tuna feeds. This was achieved and the values were validated using salmon as a surrogate for tuna. The positive and significant correlations between *in vivo* and *in vitro* digestibility values indicated that the commercial (porcine) enzyme system provided the best predictor of dry material and crude protein digestibility. The results for both *in vivo* and *in vitro* digestibility of fish meals using the porcine enzyme system were similar to other studies in which both *in vitro* and *in vivo* values were available (Eid & Matty, 1989; Dimes *et al.*, 1994b) (Figure 15). However, values from the fish enzyme systems may have been more sensitive to differences between ingredients since the values covered a wider range and the significant (and nearly significant) correlations with the *in vivo* values indicated that these should not be discounted at this stage. For example, the only piece of data on the digestibility of fish meal for southern bluefin tuna (Barneveld *et al.*, 1997) suggests the tuna enzyme system may be a more accurate predictor of crude protein digestibility than porcine or salmon enzymes (Figure 15). Dry material digestibility values are also similar being 35-42 % for *in vivo* (Barneveld *et al.*, 1997) and 54 % from the tuna enzyme system.

To further test the applicability of the multi-enzyme digestion systems the crude protein digestibility of two 1997 tuna feeds, CRC and NBT, was predicted from the digestibility values of the component ingredients. Values obtained from each enzyme system as well as the *in vivo* values were compared with the values determined by each system for the complete feed. In most cases the two values were very similar (Figure 16). This provides an internal assessment and suggests that ingredient digestibility is, generally, additive. This supports the use of ingredient digestibility values to predict the digestibility of complete feeds and therefore to predict *in vivo* digestibility. The difference between the two values was large using the commercial enzyme system. At this stage it is not clear what caused this although it appears to relate to the high digestibility values for pilchards and fish meal.

Digestibility is clearly important in determining the nutrients available to fish for growth. Consequently, information on digestibility will provide an indication of the potential of a feed ingredient for supporting growth. There are, however, many post-absorptive factors that influence nutrient utilisation and growth. Some studies have been able to assess feed ingredients in relation to growth performance. The effect of fish meal quality, source and processing, was tested on juvenile dolphin fish and growth performance was higher with *in vitro* pepsin digestibility values of over 90% (Ostrowski *et al.*, 1996). Pepsin digestibility was shown to correlate with feed efficiency in rainbow trout fed a range of fish meals (Miyazono, 1989) and the degree of protein hydrolysis correlated with the growth of chinook salmon when a wide variety of fish and plant meals and treatments were compared (Dimes *et al.*, 1994b). Thus, there is support form the literature that digestibility values provide a biologically meaningful assessment of the nutritional value of feeds and feed ingredients.

Figure 1. ***In vitro*** **crude protein digesibility for 1997 tuna feeds using the three enzyme systems**

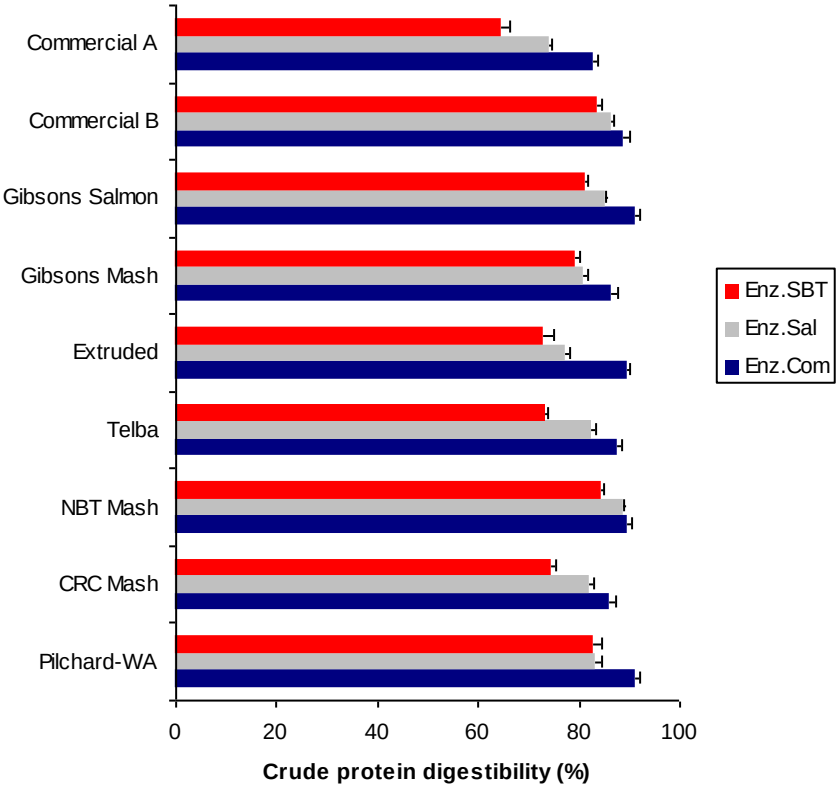


Figure 2. Correlations between crude protein digestibility (%) for the commercial enzyme and salmon (r=+0.69; P<0.05) or SBT (r=+0.75; P<0.75) enzyme systems.

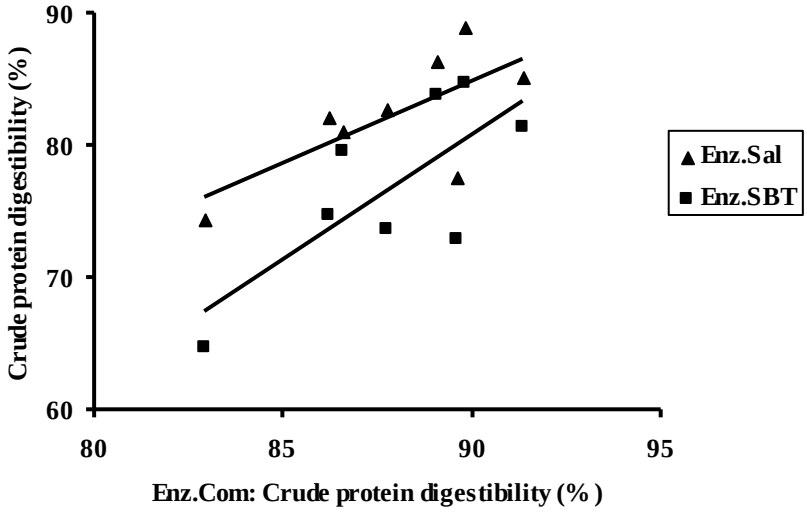


Figure 3*In vitro* crude protein digestibility for animal using three enzyme

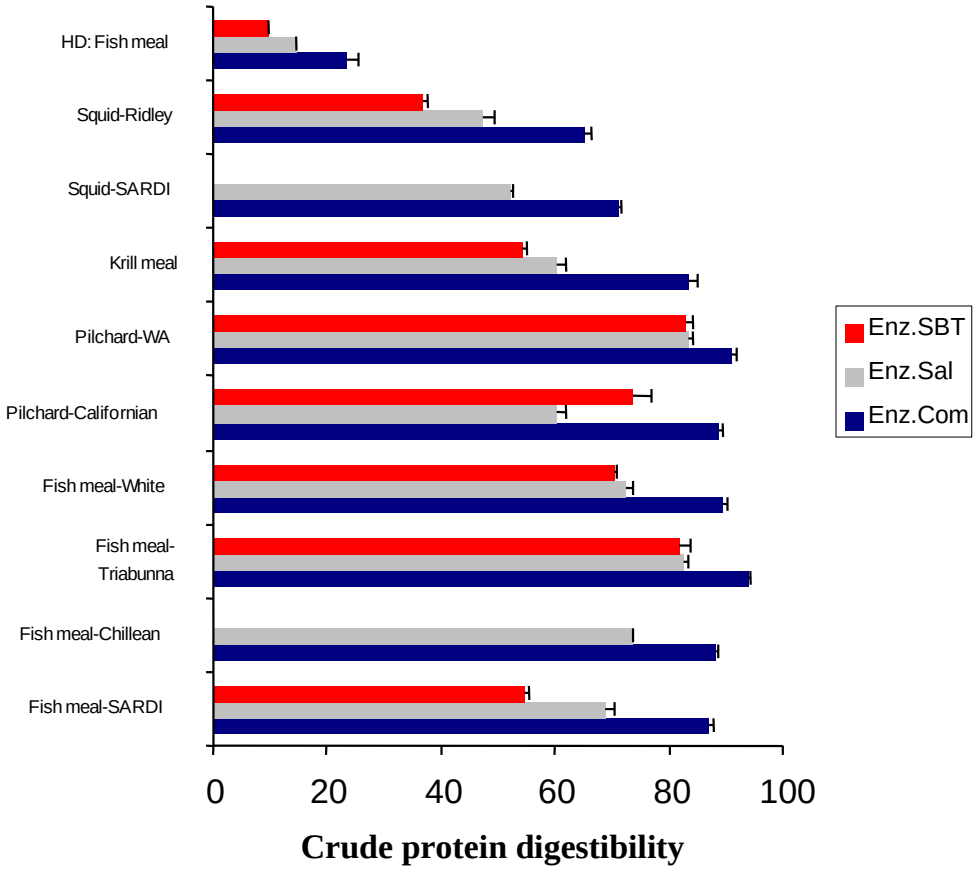


Figure 4. *In vitro* crude protein digestibility for plant meals using the three enzyme systems.

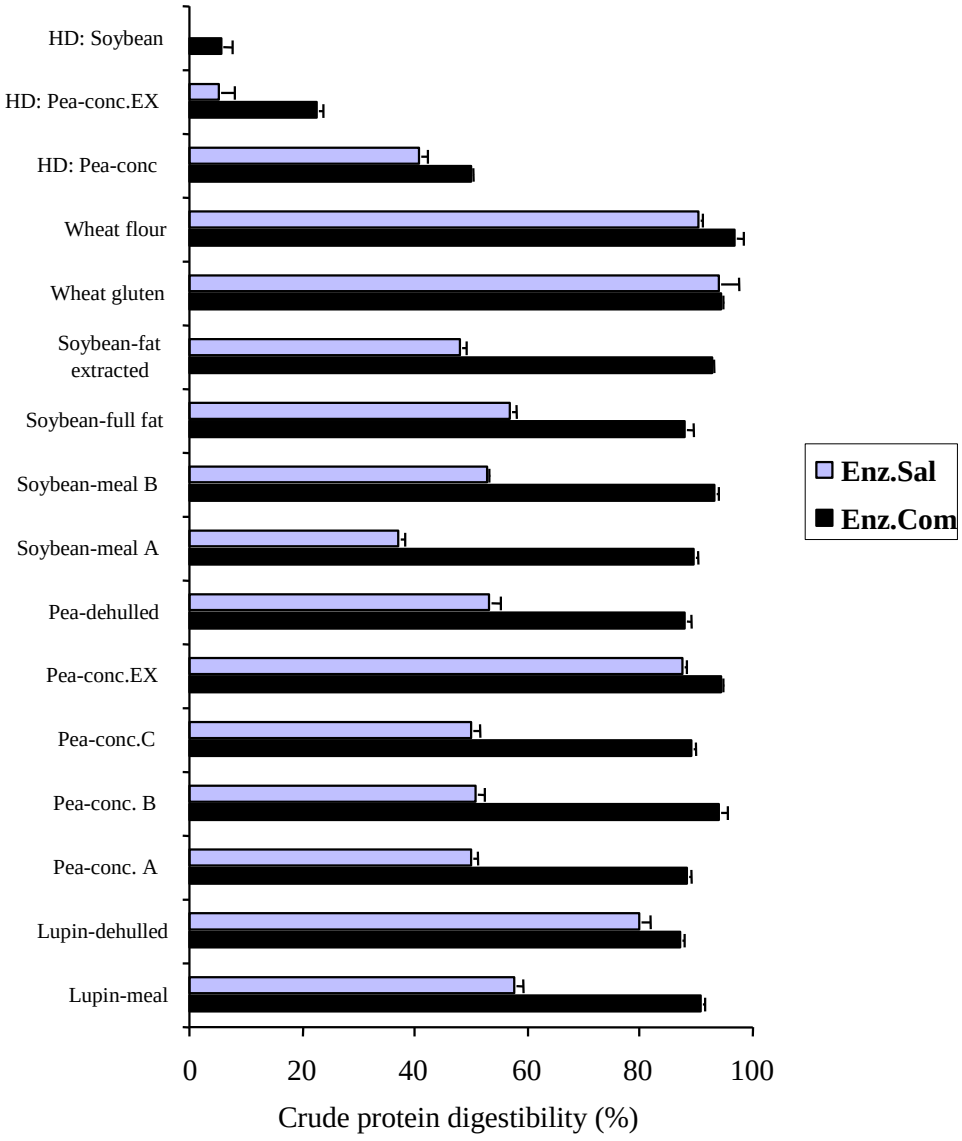


Figure 5. Comparison between *in vitro* crude protein digestibility of feed ingredients from two enzyme systems

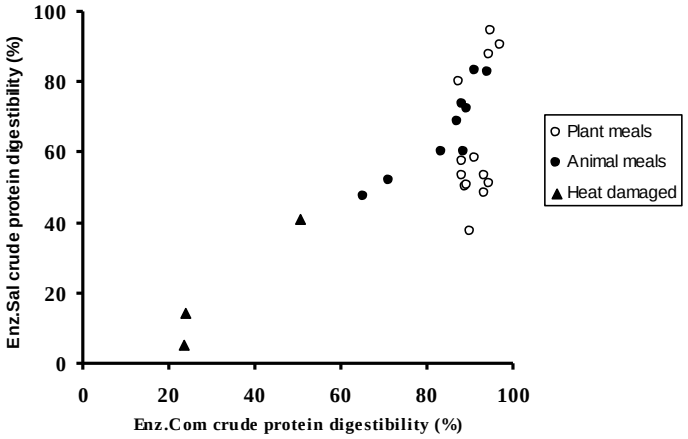


Figure 6. *In vitro* crude protein digestibility for ingredients using the pH change

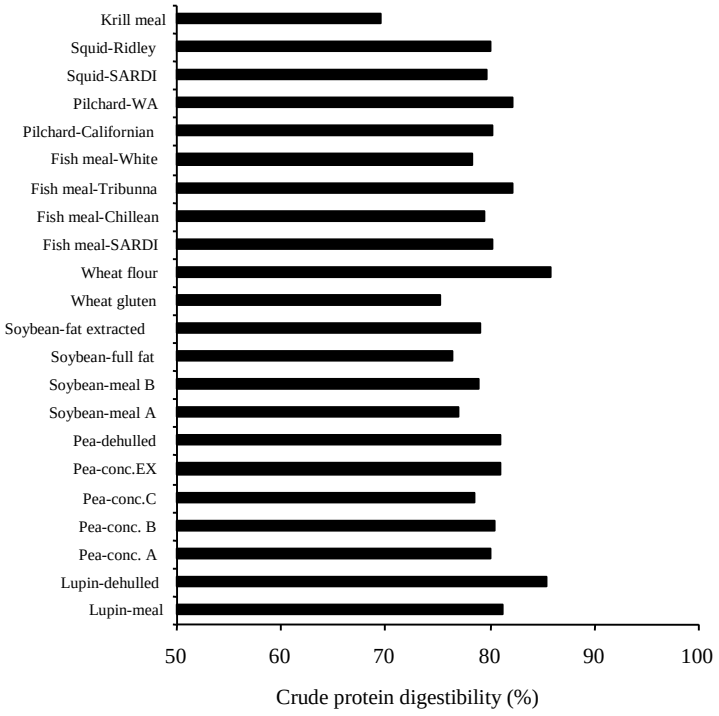


Figure 7. Comparison between crude protein digestibility using pH change assay and two enzyme systems

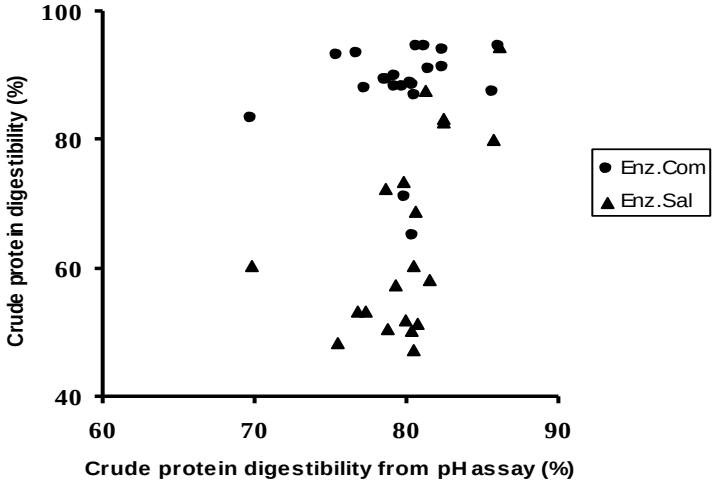


Figure 8. The *in vivo* apparent crude protein digestibility for 1997 tuna feeds

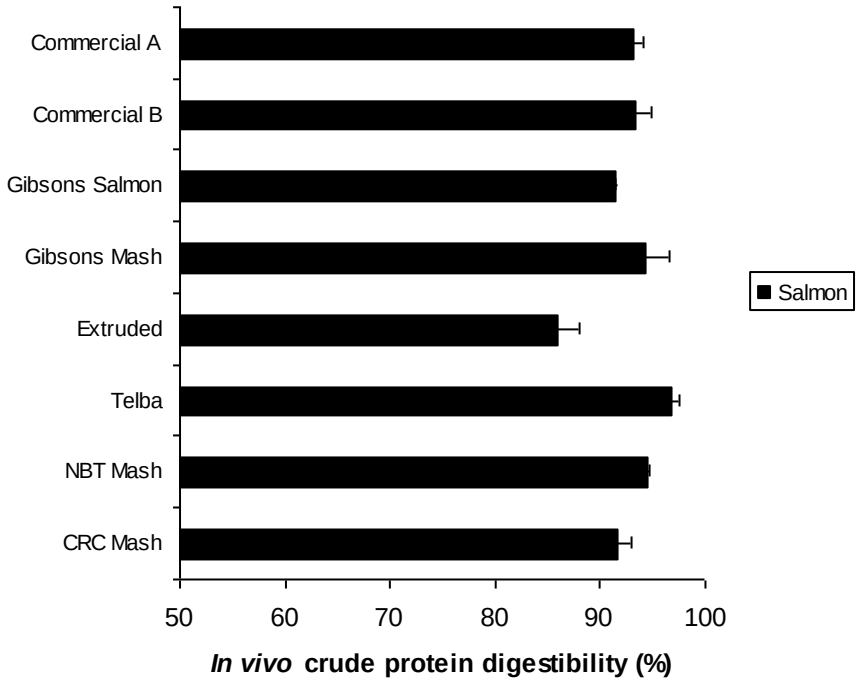


Figure 9. The *in vivo* apparent crude protein digestibility for feed ingredients

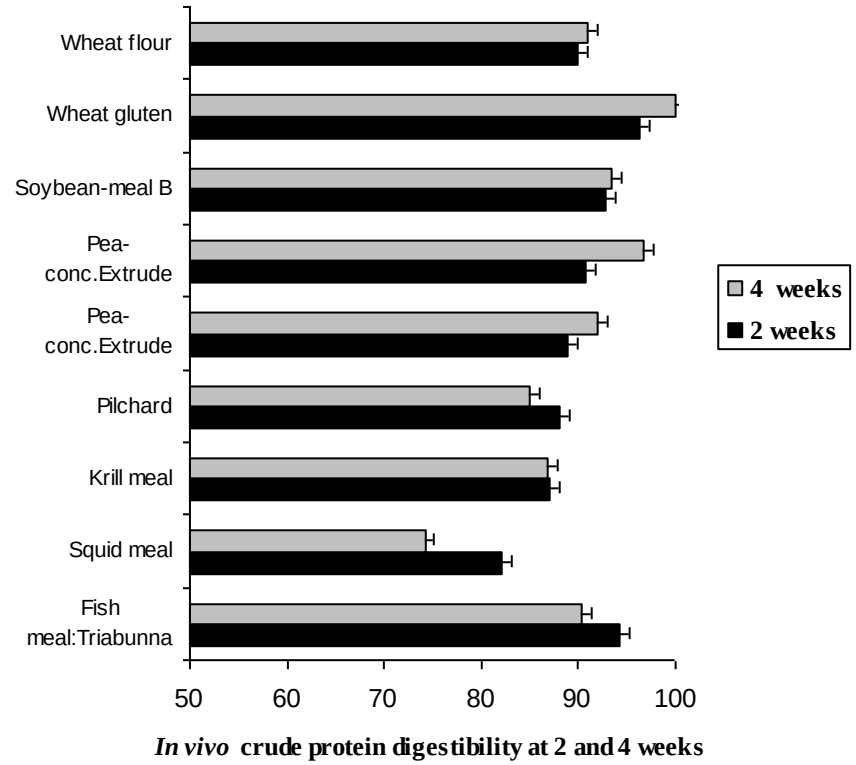


Figure 10. The correlation (r=+0.88; P<0.01) between *in vivo* crude protein digestibility at 2 and 4 weeks.

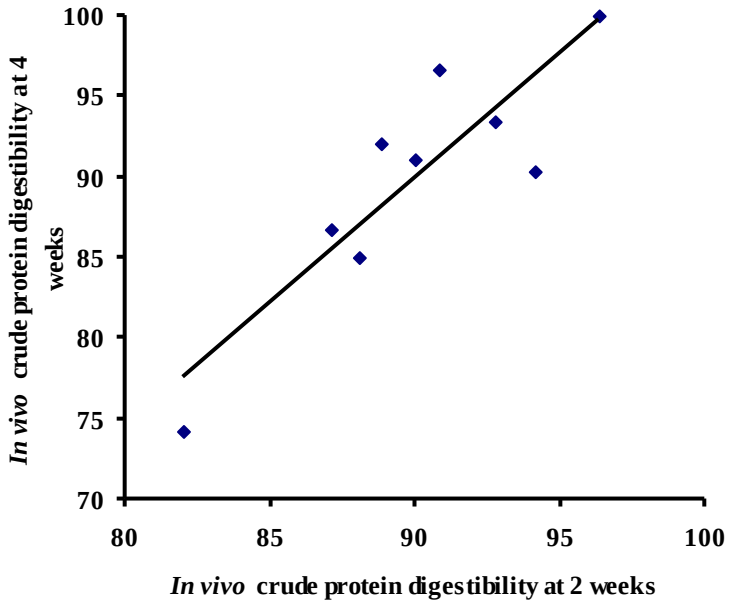


Figure 11. The correlation between crude protein digestibility using *in vivo* after 2 weeks and *in vitro* using Enz.Com (r=+0.83; P<0.01) and Enz.Sal.

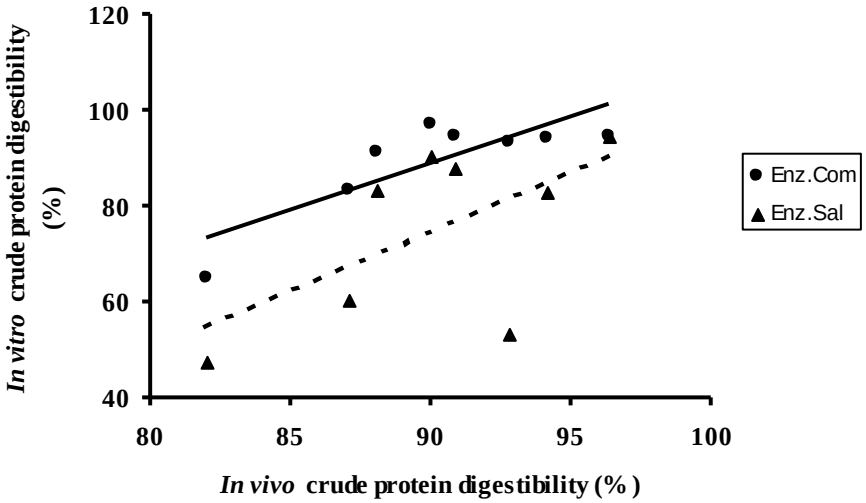


Figure 12. The correlation between crude protein digestibility *in vivo* after 4 weeks and *in vitro* using Enz.Com (r=+0.86; P<0.01) and Enz.Sal (r=+0.65; P<0.05).

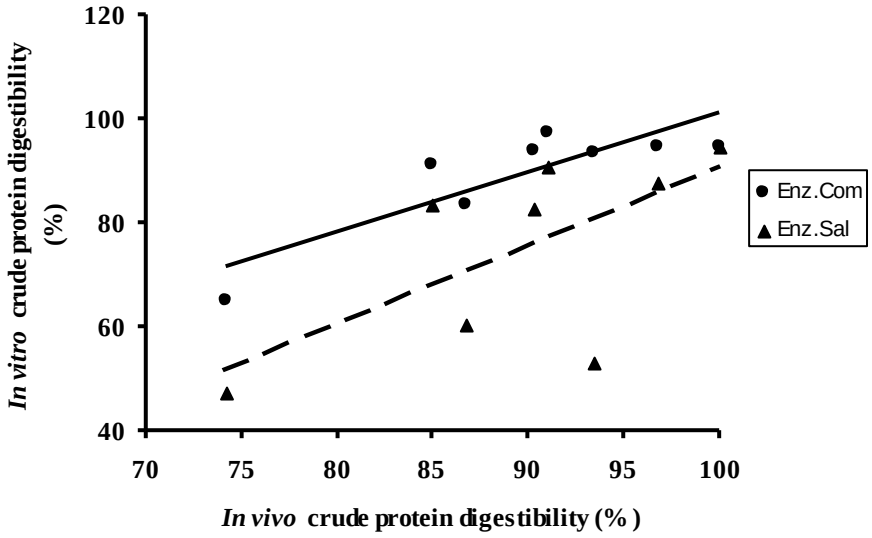


Figure 13. The correlation (r=+0.95; P<0.001) between *in vivo* dry material digestibility at 2 and 4 weeks

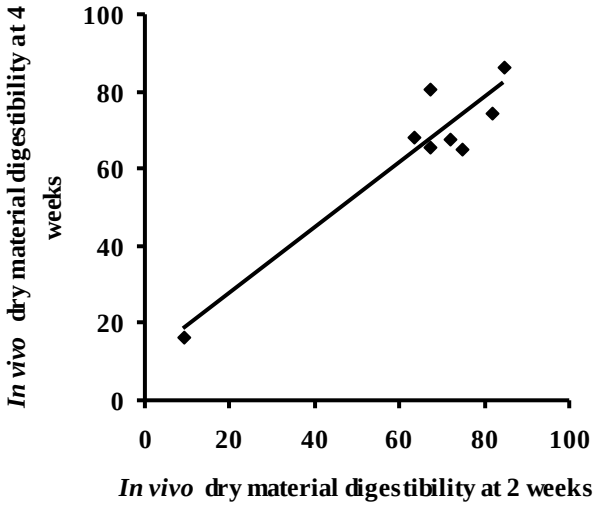


Figure 14. The correlation between *in vitro* dry material digesibility measured using porcine enzyme digestion and *in vivo* measurements after 7 (r=+76; P<0.05) and 21(r+0.80; P<0.05) days.

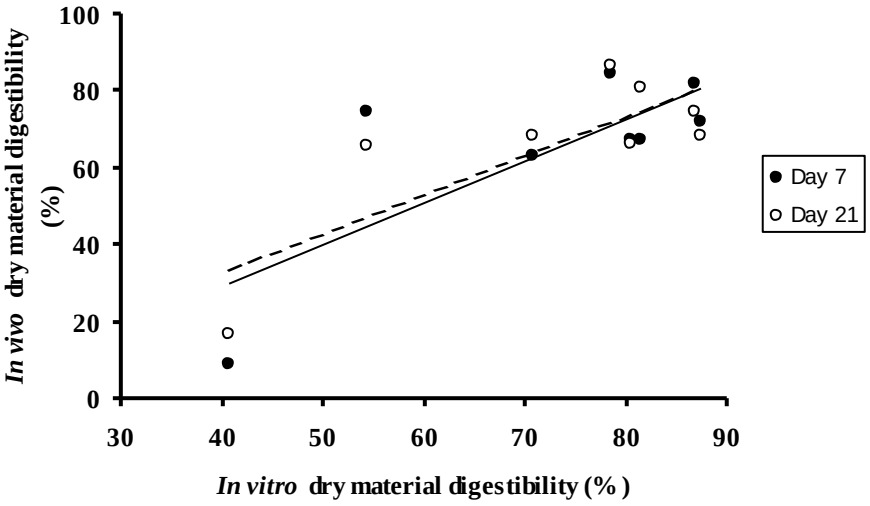


Figure 15. *In vivo* and *in vitro* crude protein digestiblty for a variety of fish meal and enzyme systems

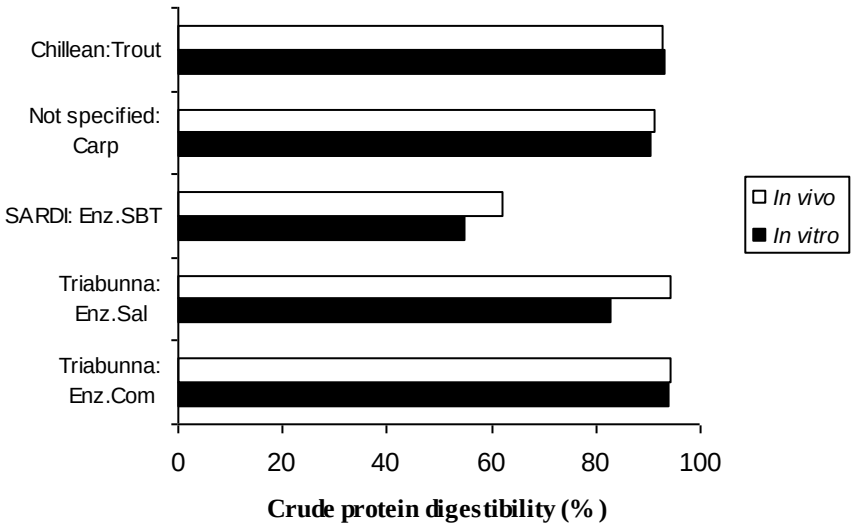


Figure16. Compariso betwee crudeprotei digestibilit calculate fromingredien digestibilit andmeasure in the complete feed using the different digestibility systems. CRC96 (plain shading); NBT (patterned)

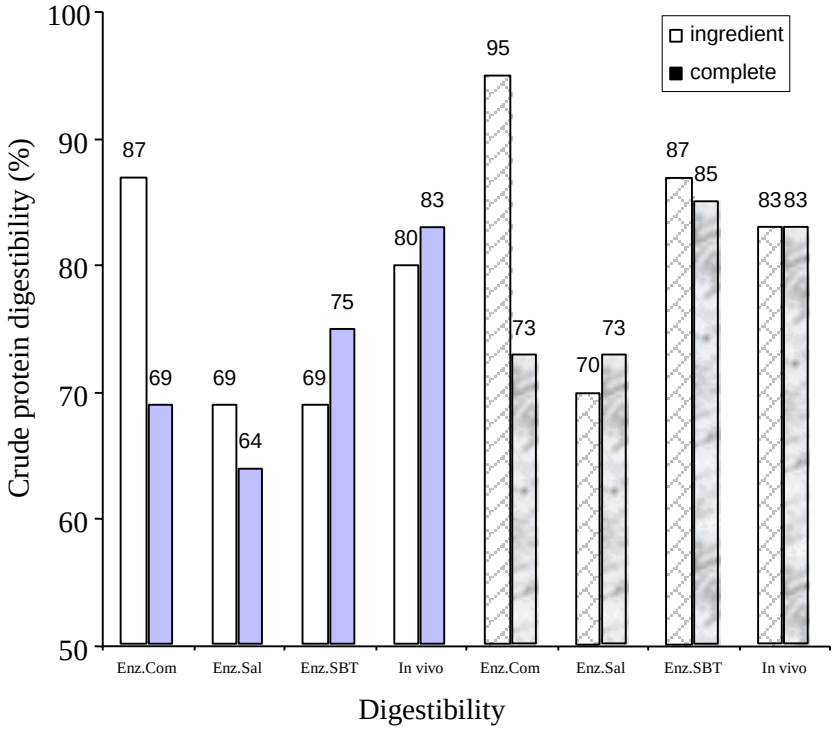


Table 15. *Complete feeds.* Apparent dry matter and nitrogen (crude protein) digestibility coefficients for *in vivo* (*Salmo salar* at day 7at 30 % ingredient inclusion) and *in vitro* analyses using commercial enzymes (Enzyme commercial) or *S. salar* crude enzymes (Enzyme salmon) for selected tuna feeds. All data are % mean ± standard deviation.

	in vivo	*in vitro*	
Diet	7d	Enzyme commercial	Enzyme salmon
Dry matter			
CRC 97	82.94 ± 1.93	69.02 ± 1.89	64.09 ± 0.62
NBT 97	82.55 ± 0.35	72.79 ± 3.02	72.74 ± 1.17
Extruded	74.40 ± 0.09	74.05 ± 2.35	63.12 ± 2.47
Gibson salmon feed	82.38 ± 1.17	77.67 ± 1.30	68.52 ± 0.11
Commercial B	70.67 ± 3.56	65.78 ± 1.43	61.74 ± 2.44
Commercial A	83.43 ± 0.89	74.34 ± 0.90	64.5 ± 2.06
Gibson tuna mash	85.87 ± 0.33	74.03 ± 0.90	67.39 ± 3.19
Telba	83.93 ± 2.17	67.66 ± 4.61	66.28 ± 7.04
Extruded concentrate[d]		74.34 ± 0.01	55.68 ± 0.43
Japanese mash[d]		82.03 ± 1.55	76.12 ± 1.01
Nitrogen			
CRC 97	91.73 ± 1.15	86.21 ± 1.15	82.06 ± 0.74
NBT 97	94.47 ± 0.14	89.80 ± 0.59	88.91 ± 0.03
Extruded	85.92 ± 2.03	88.08 ± 0.47	77.52 ± 0.74
Gibson salmon feed	91.44 ± 0.01	91.33 ± 0.80	85.15 ± 0.21
Commercial B	93.43 ± 1.37	89.04 ± 0.95	86.41 ± 0.45
Commercial A	93.20 ± 0.94	82.96 ± 0.82	74.32 ± 0.21
Gibson tuna mash	94.28 ± 2.36	86.57 ± 0.94	81.08 ± 0.52
Telba	96.73 ± 0.84	87.76 ± 0.92	82.67 ± 0.47
Extruded concentrate[d]		84.61 ± 0.15	62.38 ± 1.23
Japanese mash[d]		91.23 ± 0.16	85.39 ± 1.03

[d] Ingredient

Table 16. *1997 Tuna Feeds*: Apparent dry matter and nitrogen (crude protein) digestibility coefficients from *in vitro* analyses using SBT crude enzymes (Enzyme SBT) for 1997 tuna feeds. All data are % mean ± standard deviation.

	Enzyme SBT	
Diet	Dry Material	Nitrogen[b]
CRC mash	58.00 ± 1.70	74.63 ± 0.84
NBT mash	67.75 ± 1.11	84.66 ± 0.44
Extruded	56.84 ± 3.09	72.89 ± 1.98
Gibson salmon feed	62.31 ± 1.94	81.33 ± 0.49
Commercial B	58.15 ± 1.40	83.72 ± 0.61
Commercial A	58.07 ± 3.25	64.67 ± 1.49
Gibson tuna mash	66.76 ± 1.57	79.53 ± 0.64
Telba	61.49 ± 1.43	73.59 ± 0.22

Table 17. *Tuna Feed Ingredients.* Apparent dry matter and nitrogen (crude protein) digestibility coefficients from *in vitro* analyses using SBT crude enzymes (Enzyme SBT) for 1997 tuna feed ingredients. All data are % mean ± standard deviation.

	Enzyme SBT	
Diet	Dry Material	Nitrogen
Dry matter (%CP)		
Fish meal-SARDI(60.1%)	53.69 ± 0.21	54.70 ± 0.91
Fish meal- Triabunna (65.2%)	74.67 ± 0.45	81.67 ± 2.01
Fish meal - White (64.3%)	72.49 ± 1.19	70.35 ± 0.47
Krill meal-(54.2%)	42.93 ± 0.31	54.20 ± 0.66
Squid meal-Ridley (64.3%)	27.21 ± 1.74	36.69 ± 1.08
Pilchard-Californian(67.3%)	63.85 ± 4.18	73.57 ± 3.28
Pilchard-WA (71.5%)	78.34 ± 2.73	82.83 ± 1.52
Wheat gluten(67.1%)	71.39 ± 1.90	94.68 ± 2.39
Caesin (77.6%).	97.80 ± 2.03	98.31 ± 0.19

Table 18. *Animal meals.* Apparent dry matter and nitrogen (crude protein) digestibility coefficients for *in vivo* (*Salmo salar* at 2 and 4 weeks at 30 % ingredient inclusion) and *in vitro* analyses using commercial enzymes (Enzyme commercial) or *S. salar* crude enzymes (Enzyme Salmon) for animal meals. All data are % mean ± standard deviation.

	In vivo		*In vitro*	
Ingredient (%CP[d])	2 weeks	4 weeks	Enzyme commercial	Enzyme salmon[b]
Dry matter				
Fish meal-SARDI(60.1%)			82.07 ± 1.11	63.42 ± 1.43
Fish meal-Chillean(57.1%)			78.94 ± 3.35	67.81 ± 1.04
Fish meal-Triabunna (65.2%)	81.75 ± 0.33	74.63 ± 1.28	86.87 ± 0.15	74.17 ± 2.14
Fish meal-White(64.3%)			81.60 ± 2.33	72.75 ± 2.94
Pilchard-Californian(67.3%)			78.64 ± 1.27	73.17 ± 4.42
Pilchard-WA(71.5%)	71.75 ± 0.86	68.00 ± 3.38	87.34 ± 0.72	76.41 ± 1.16
Krill meal-(54.2%)	63.06 ± 0.30	68.39 ± 0.76	70.81 ± 2.57	51.36 ± 1.10
Squid-SARDI(62.2%)			63.22 ± 3.70	42.39 ± 0.08
Squid-Ridley(64.3%)	74.37 ± 1.92	65.37 ± 1.93	54.43 ± 1.86	36.33 ± 0.73
Nitrogen				
Fish meal-SARDI(60.1%)			86.88 ± 0.94	68.99 ± 1.60
Fish meal-Chillean(57.1%)			88.19 ± 0.55	73.61 ± 0.09
Fish meal-Triabunna (65.2%)	94.17 ± 0.56	90.30 ± 0.36	94.01 ± 0.17	82.75 ± 0.60
Fish meal-White(64.3%)			89.35 ± 0.84	72.59 ± 1.06
Pilchard-Californian(67.3%)			88.50 ± 0.84	60.40 ± 1.42
Pilchard-WA(71.5%)	88.05 ± 1.73	84.95 ± 0.61	91.25 ± 0.63	83.28 ± 1.07
Krill meal-(54.2%)	87.09 ± 0.63	86.76 ± 0.03	83.36 ± 1.73	60.40 ± 1.42
Squid-SARDI(62.2%)			71.18 ± 0.66	52.09 ± 0.53
Squid-Ridley(64.3%)	82.03 ± 1.70	74.20 ± 0.27	65.06 ± 1.27	47.38 ± 1.84

Table 19. *Plant meals:* Apparent dry matter and nitrogen (crude protein) digestibility coefficients for *in vivo* (*Salmo salar* at 2 and 4 weeks at 30 % ingredient inclusion) and *in vitro* analyses using commercial enzymes (Enzyme commercial) or *S. salar* crude enzymes (Enzyme salmon) for selected plant meals. All data are % mean ± standard deviation.

	In vivo		*In vitro*	
Ingredient (%CP)	2 weeks	4 weeks	Enzyme commercial	Enzyme salmon
Dry matter				
Lupin-meal(35.1%)			57.40 ± 0.43	42.55 ± 0.85
Lupin-dehulled(27.9%)			50.34 ± 1.60	49.12 ± 2.34
Pea-conc. A(37.5%)			56.71 ± 1.08	33.50 ± 2.50
Pea-conc. B(41.0%)			64.94 ± 1.10	39.49 ± 2.39
Pea-conc.C(40.6%)			63.86 ± 1.51	32.12 ± 2.14
Pea-Ex.conc.(41.0%)[d]	55.91 ± 1.79	60.39 ± 8.19	81.54 ± 0.05	66.02 ± 0.22
Pea-Ex.conc.(41.0%)	67.00 ± 9.90	80.76 ± 1.30	81.54 ± 0.05	66.02 ± 0.22
Pea-dehulled(21.8%)			45.02 ± 3.54	22.10 ± 1.76
Soybean-meal A(41.1%)			72.93 ± 0.89	43.57 ± 1.88
Soybean-meal B(40.4%)	67.19 ± 8.49	66.01 ± 0.98	80.43 ± 0.26	47.05 ± 0.32
Soybean-full fat(32.4%)			71.23 ± 0.07	50.95 ± 1.99
Soybean-fat extracted(43.8%)			80.64 ± 0.02	44.77 ± 0.09
Wheat gluten(67.1%)	84.38 ± 13.11	86.40 ± 0.96	78.52 ± 0.78	70.19 ± 0.92
Nitrogen				
Lupin-meal(35.1%)			91.05 ± 0.49	58.23 ± 1.00
Lupin-dehulled(27.9%)			87.34 ± 0.56	80.20 ± 1.80
Pea-conc. A(37.5%)			88.91 ± 0.35	50.27 ± 1.14
Pea-conc. B(41.0%)			94.48 ± 1.23	51.33 ± 1.14
Pea-conc.C(40.6%)			89.34 ± 0.63	50.51 ± 0.91
Pea-Ex.conc.(41.0%)[d]	88.85 ± 0.57	92.03 ± 1.20	94.61 ± 0.28	87.83 ± 0.64
Pea-Ex.conc.(41.0%)	90.84 ± 0.58	96.71 ± 0.25	94.61 ± 0.28	87.83 ± 0.64
Pea-dehulled(21.8%)			88.12 ± 0.96	53.45 ± 1.64
Soybean-meal A(41.1%)			89.97 ± 0.54	37.46 ± 0.98
Soybean-meal B(40.4%)	92.79 ± 1.24	93.38 ± 0.24	93.46 ± 0.48	53.25 ± 0.14
Soybean-full fat(32.4%)			88.33 ± 1.33	57.38 ± 0.75
Soybean-fat extracted(43.8%)			93.28 ± 0.04	48.50 ± 0.80
Wheat gluten(67.1%)	96.33 ± 9.43	101.04 ± 0.90	94.64 ± 0.13	94.55 ± 3.09

d 15% inclusion level of *in vivo* trial

Table 20. *Feedstuffs*: Apparent dry matter and nitrogen (crude protein) digestibility coefficients for *in vivo* (*Salmo salar* at 2 and 4 weeks at 30 % ingredient inclusion) and *in vitro* analyses using commercial enzymes (Enzyme commercial) or *S. salar* crude enzymes (Enzyme salmon) for selected ingredients. All data are % mean ± standard deviation.

	In vivo		*In vitro*	
Ingredient (%CP)	2 weeks	4 weeks	Enzyme commercial	Enzyme salmon
Dry matter				
BSA(93.6%)			82.56 ± 3.18	7.47 ± 3.90
Casein(77.6%)			94.19 ± 1.63	92.86 ± 0.43
Wheat flour(10.6%)	9.02 ± 8.26	16.73 ± 10.93	40.64 ± 1.88	66.12 ± 0.78
Pre-gel starch[d]	83.31 ± 5.40	84.25 ± 0.12		
Pre-gel starch	72.85 ± 1.28	68.64 ± 3.92		
Nitrogen				
BSA(93.6%)			88.39 ± 0.36	33.00 ± 2.44
Casein(77.6%)			97.07 ± 0.63	98.52 ± 0.08
Wheat flour(10.6%)	89.99 ± 1.42	91.03 ± 1.70	97.19 ± 1.14	90.62 ± 0.68
Pre-gel starch[d]	95.84 ± 2.61	93.72 ± 0.33		
Pre-gel starch	95.48 ± 1.40	93.09 ± 1.41		

d 15% inclusion level of *in vivo* trial

Table 21. *Heat Damage*. The effect of heat damage on apparent nitrogen (crude protein) digestibility coefficient for *in vitro* analyses using commercial enzymes (Enzyme commercial), *S. salar* crude enzymes (Enzyme salmon) or SBT crude enzymes (Enzyme SBT) for ingredients that had been damaged by excessive heat (see text for details). All data are % mean ± standard deviation.

	In vitro		
Diet	Enzyme commercial	Enzyme salmon	Enzyme SBT
Fish meal - Tribunna	23.53 ± 1.91	14.46 ± 0.06	9.6 ± 0.17
Pea-Ex Conc	23.17 ± 0.64	5.61 ± 2.63	
Pea-Conc	50.21 ± 0.09	41.23 ± 1.09	6.94 ± 1.49
Soybean	6.24 ± 1.22	0	

Table 22. Apparent nitrogen digestibility (ADC_N) calculated using the multi-enzyme pH change assay (crude protein content in parentheses).

Animal meals	ADC_N	Plant meals	ADC_N
Fish meal-SARDI(60.1%)	80.50 ± 0.77	Lupin-meal(35.1%)	81.41 ± 0.63
Fish meal-Chillean(57.1%)	79.73 ± 2.83	Lupin-dehulled(27.9%)	85.66 ± 2.18
Fish meal-Petuna(65.2%)	82.34 ± 0.87	Pea-conc. A(37.5%)	80.23 ± 1.69
Fish meal-White(64.3%)	78.51 ± 1.79	Pea-conc. B(41.0%)	80.62 ± 1.51
Pilchard-Californian(67.3%)	80.44 ± 3.82	Pea-conc.C(40.6%)	78.69 ± 0.31
Pilchard-WA(71.5%)	82.36 ± 0.52	Pea-Ex.conc.(41.0%)[d]	81.23 ± 1.01
Krill meal-(54.2%)	69.73 ± 1.15	Pea-dehulled(21.8%)	77.24 ± 1.05
Squid-SARDI(62.2%)	79.90 ± 2.36	Soybean-meal A(41.1%)	79.19 ± 0.27
Squid-Ridley(64.3%)	80.24 ± 0.59	Soybean-meal B(40.4%)	76.70 ± 0.26
BSA(93.6%)	95.33 ± 0.45	Soybean-full fat(32.4%)	79.23 ± 0.54
Casein(77.6%)	94.76 ± 0.32	Soybean-fat extracted(43.8%)	75.43 ± 0.82
		Wheat gluten(67.1%)	86.11 ± 2.30
1997 Tuna Feeds			
CRC 97	79.87 ± 0.38		
NBT 97	81.86 ± 1.92		
Extruded	78.33 ± 1.02		
Gibson salmon feed	82.22 ± 0.64		

[c] Method and equation converting pH to digestibility based on Hsu *et al.*, 1977.

Table 23. Correlation coefficients between *in vivo* and *in vitro* digestibility values.

Assay	*In vivo* crude protein		*In vivo* dry material		n	$P_{0.05}$
	Day 7	Day 21	Day 7	Day 21		
Enzyme commercial	0.83	0.86	0.76	0.80	8	0.64
Enzyme salmon	0.61	0.65	0	0.07	8	0.64
Enzyme SBT	0.90	0.86	0.32	0.53	5	0.90
pH	0.66	0.55			8	0.64

Table 24**.** Trypsin activity[a] (µmoles pNA/min/ml extract) of southern bluefin tuna crude enzymes[b] after feeding on experimental diets. All values are mean ± standard deviation.

Diet	Activity	
	Salmon	SBT
Fishmeal[*]	0.075 ± 0.009	
Fishmeal + soybean[*]	0.088 ± 0.008	
Fishmeal + soybean + enzyme[*]	0.095 ± 0.008	
Gibsons Salmon	0.03 - 0.06	
CRC mash[b]		0.140 ± 0.006
NBT mash[b]		0.174 ± 0.006
Pilchard-Californian[b]		0.076 ± 0.002
Telba[b]		0.069 ± 0.002

[a] Method based on modified Preiser *et al.* (1975) and Pringle *et al.*(1992).
* (Carter *et al.*, 1994)
[b] Assay conducted on distal section of March 1997 samples.

Nutrient transport studies and ultra structure analysis

Nutrient transport studies – Results and discussion

Amino acid transport: To establish whether the Pyloric Caeca of SBT can transport the terminal products of protein digestion, it was first necessary to develop appropriate methodology to study amino acid transport. Due to logistic difficulties of working with SBT and the anatomical peculiarities of their Pyloric Caeca (plate 1), the decision to use Brush Border Membrane Vesicles (BBMV) was taken. However, the isolation of such vesicles from Pyloric Caeca of this species has not been reported previously. Much of the initial work has been reported in Mr. Robert Kemp's thesis.

Apical Membrane vesicle preparation: The method relies on the differential precipitation of membranes in the presence of divalent ions and subsequent separation by centrifugation. Success of isolation is determined by evaluating the enrichment of enzymes that are associated with the membrane of interest, in this case the apical membrane of the intestinal lining (Brush Border). These enrichments are the specific enzyme activity in the isolated membrane fraction relative to that observed in the crude homogenate of gut tissue. A comparison of results with those of mammals, birds and other aquatic animals is given in the Table 25.

Enrichment of Alkaline Phosphatase activity is low compared with other species (Table 25). This discrepancy can be ascribed to a high activity of this enzyme associated with pancreatic tissue that intercalates with the tubules (Plate 1). Indeed, subsequent preparations using individual Pyloric Caeca tubules, cleaned of pancreatic tissue, yielded enrichments close to those observed in other species (data not shown). Demonstrating that, in this case, the low enrichments do not reflect low purity, thus the standard isolation protocols for membrane vesicles can be applied to Pyloric Caeca from SBT without prior removal of pancreatic tissue. Subsequent transmission electron microscopy demonstrated the presence of intact vesicles.

Characterization of membranes from varying regions of the Pyloric caeca: Only limited work was conducted on this comparison. However, the results indicated that that the proportion of protein associated with the brush border membrane is higher for enterocytes derived from proximal versus distal pyloric caeca; 9.33± 0.30 *vs.* 6.83 ± 0.30 % (mean ± SE), respectively. Furthermore, enzymes associated with the terminal digestion of nutrients had higher specific activities in the proximal compared with distal pyloric caeca, as reported by Kemp, 1997 (Table 2.2, page 32). Indicating a greater total digestive capacity per gram of tissue. The significant amino peptidase activity observed indicates that single amino acids are produced during terminal digestion of protein in this organ.

The effect of diet on the preparation of membranes: Samples used in this section were obtained from fish maintained on diets yielding maximum difference in growth, Pilchard *vs* Telba fed fish (chapter 4, Kemp 1997). In brief, specific activity of alkaline phosphatase in Brush Border Membranes was not effected by diet type. However, the activity of this enzyme in the crude homogenate was increased in Telba - fed fish. This activity was associated with non-absorptive tissue and may indicate an effect of diet on pancreatic tissue located between tubules.

Preliminary assessment of transport capacity for amino acid by the Pyloric caeca: Results reported by R. Kemp demonstrate that pyloric caeca of SBT has transport systems for alanine, lysine and proline, Figure 17. These amino acid where chosen to screen for the known neutral and cationic amino acid transporters. Assignment of transport to any given transporter is based on relative inhibition studies, pH sensitivity and cation dependency. Initial characterization of the relative sensitive of proline and alanine transport to media pH indicates the presence of at least two discrete systems. These being system ASC and imino for alanine and proline respectively. For data see chapters 3 and 4 of Kemp, 1997. However, this work requires refinement.

Ultra-structure studies – Results and discussion

Visual assessment: Light Microscopy: Plate 1 demonstrates the gross morphological structure of Pyloric Caeca tissue. Strikingly, pancreatic tissue is intercalated between the tubules. Within each tubule villus – like structures can be observed. These are in fact ridges that extend the length of the tubule rather than discrete finger like projections. The zone of proliferation is located at the base of troughs between ridges as shown by the cell proliferation marker PCNA (proliferation cell nuclear antigen, plate 2). Unlike many mammalian species these regions do not form true crypt structure. However, such zoning indicates that epithelial in the Pyloric Caeca of SBT is replaced in a similar manner to that seen in mammals. Application of this method will allow the assessment of feed on cell renewal in this highly proliferative tissue.

Effect of diet: Ridge height*:* A common, yet robust, measure of epithelial integrality is villus/ridge height. In many mammalian species ingestion of plant material can cause villus atrophy resulting in a reduced surface area for absorption of nutrients. To evaluate whether the dietary treatments tested in 1997 affected gross structure of the small intestine, quantitative image analysis was performed. Results indicate that no significant differences were observed between treatment groups, table 26. However, ridges are taller in the proximal than compared with distal regions of the Pyloric Caeca. Such difference along the axis of the Pyloric Caeca indicates that there may be region specialization of function in this organ.

Microvillus structure: Aside from ridge height, the major determinant of surface area for digestion and absorption is microvillus size. In the present study, microvilli were observed to be disrupted when fish were maintained on the pelleted diet, telba (plate 3). This assessment is on a qualitative basis from scanning electron micrographs. The difference between these diet and whole pilchards is the nutrient density and form in which the are presented to the animal. This overall effect may be due to under nutrition as reflected in growth rates, but the possibility of processing or the source of fish effects can not be ruled out. Intriguingly, microvilli structure on diets SBT and NBT where intermediate between Pilchard and telba diets.

Other features: Endocytosis: Significantly, proximal Pyloric caeca was observed to be mediating endocytosis of potential feed particles (plate 4). Such data as implications to degree of digestion necessary in an *in vitro* assay. This type of transport may be in response to poor nutrition in an attempt to obtain nutrients from the diet. The stimuli for switch from transport of single amino acid to endocytosis, if this occurs, is not known. However, such a change in nutrient supply will have several consequences. First, a simple pancreatic enzyme extract will not mimic the digestive processes that occur in this fish. Secondly, if endocytosis occurs only during nutritional stress, it provides a potential route of immunoreactive molecules to be presented to the lymphoid tissue or pathogens to enter the animal. To evaluate the true significance of this present observation, a range of samples taken from fish of known age and nutritional history need to evaluated at the transmission electron microscopy level. Such information is vital to determine the effectiveness of diets that provide both adequate nutrition and maintain digestive tract health.

Evidence of immune function for the Pyloric Caeca: Throughout the morphological studies, features of the pyloric caeca were observed that are most probably associated with immune function. First, and the most dramatic is the presence of lymphoid – like tissue (plate 1, lower panel). This structure bares similarity to lymphoid tissue present in mammalian intestinal tracts. In addition, transmission electron micrographs revealed the presence of putative intraepithelial lymphocytes and rodlet cells (plate 5). The former of these cell types is known to increase at weaning in mammals and are thought to play an integral role in the adaptations to solid food with minimum food intolerance. The latter are features associated with fish. Their number does vary with disease state but the true nature and purpose of these cells is still in debate.

Table 25. Comparison of enzyme markers of membrane enrichments for SBT pyloric caeca with other species. [a]Relative specific enzyme activity (product produced/mg protein/hour) in isolated membranes relative to total gut homogenate, values greater than 1 indicate enrichment.

		Enrichment[a]	
Marker enzyme	Membrane	SBT	Other species
Alkaline phosphatase	Brush border	2.93 - 3.54	Rat 7[1] - 15[2] Human 8 - 13[3] Trout 8.1[4] Eel 13[5] Pig 21[6] Chicken 25[7]
Acid phosphatase	Lysosomes	1.40 - 1.73	Trout 3.44[4] Chicken 0.6[3]
Na/K ATPase	Basolateral	0.41 - 0.62	Eel 0.4[8] Rat 0.4[1] - 1.5[9] Chicken 1[3] Sea bass 1.2[10]
Protein		Recovery 7.5 - 8.5%	Rat 2.3[2] Human 0.97[11] Chicken 1.8[3] Eel 1.8[8] African Tilapia 5.5 - 6.1[12]

1(Sanchis et al 1994). 2(Kessler et al 1978) 3(Shirazi - Beechey et al 1990) 4(Freire et al 1995) 5(Vilella et al 1988) 6(Maenz and Patience 1992) 7(Debiec et al 1990) 8(Storelli et al 1986) 9(Lynch and McGiven 1987) 10(Balacco et al 1993) 11(Malo and Berteloot 19991) 12(Reshkin and Ahearn 1987).

Table 26. Comparison of ridge height from proximal and distal regions of Pyloric caeca from fish maintained on either whole pilchards or processed diets.

	Pyloric Caeca region Ridge height (μm)		
	Proximal	Distal	SEM
Diet	199.3	203.5	20.79[ns]
Telba	225.6	178.1	20.79[ns]
NBT mash	214.6	180.9	20/79[ns]
SBT mash	177.5	163.8	20.79[ns]
Pooled	203.0	180.6	8.05[a]

[ns] No interaction of diet x sample region was observed. Data are the mean of 5 tubules with 5 individual ridges recorded per tubule. A minimum of three fish were used for these determinations.
[a] Significant difference between region was observed between pooled means at the $P<0.05$ level.

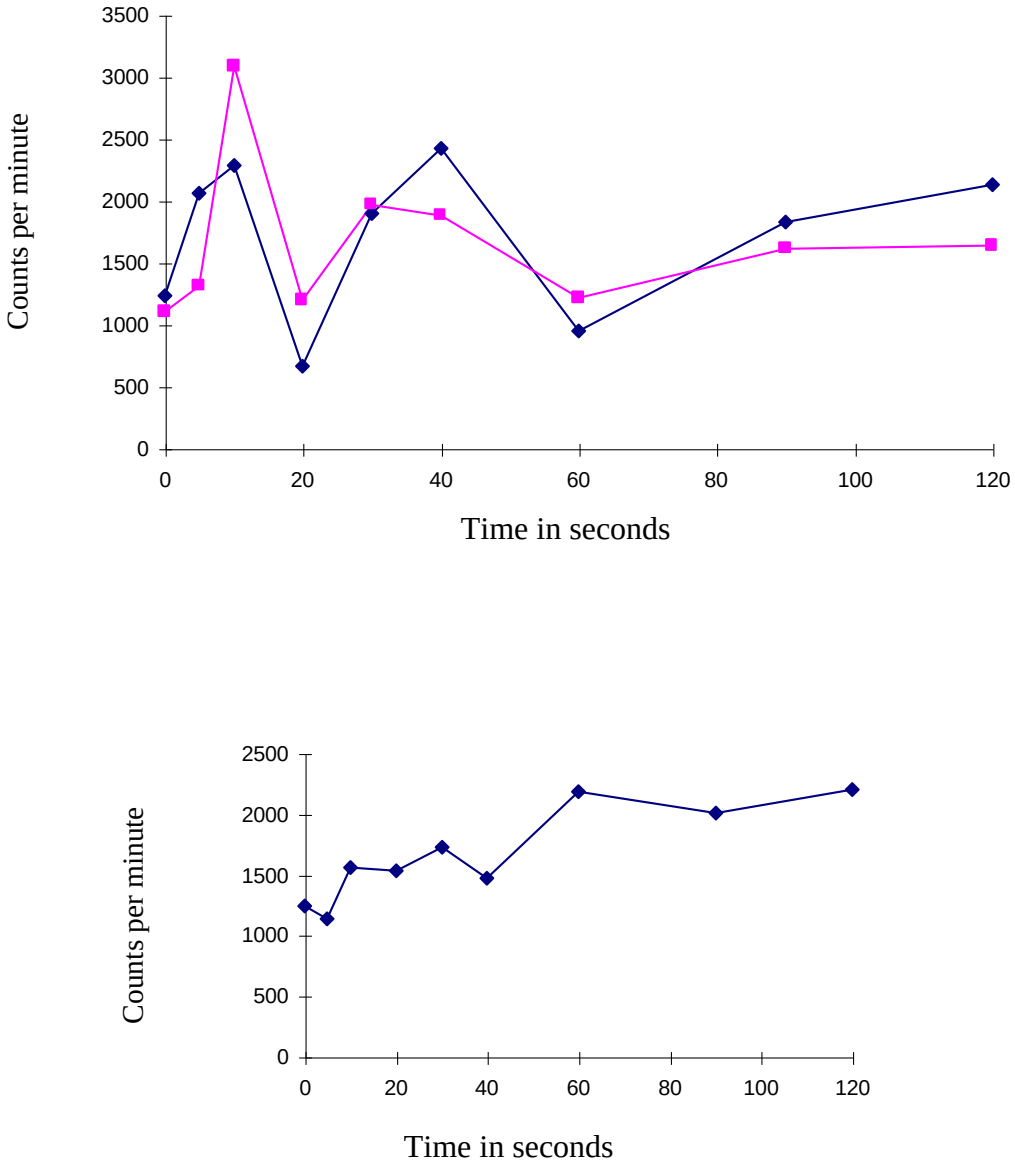


Figure 17. Typical time dependent uptake of (upper panel) L-Alanine, ♦ and L- Proline ■ and (lower panel) L-lysine by Brush Border Membrane Vesicles prepared from Pyloric Caeca taken from Southern Blue Fin Tuna. Uptakes were performed in the presence of sodium. Vesicles were stored in buffer (pH6.0) containing (in mM) Mannitol 300, Hepes 10, and Mg_2SO_4 0.1. Reactions were started by adding 25µl of vesclie membrane suspension (50µg protein) to 50µl of uptake buffer containing (in mM) Mannitol 100, Hepes 20, NaCl 100, unlabeled amino acid 0.1. [^{3}H] – amino acids were added to give 0.02 µCi/µl. Reactions were stopped using 1ml of ice-cold buffer containing 100mM amino acid followed by rapid filtration. Temperature was maintained at 15°C. Data are the mean ± SE of 3 separate membrane preparations with each being assayed in triplicate.

p

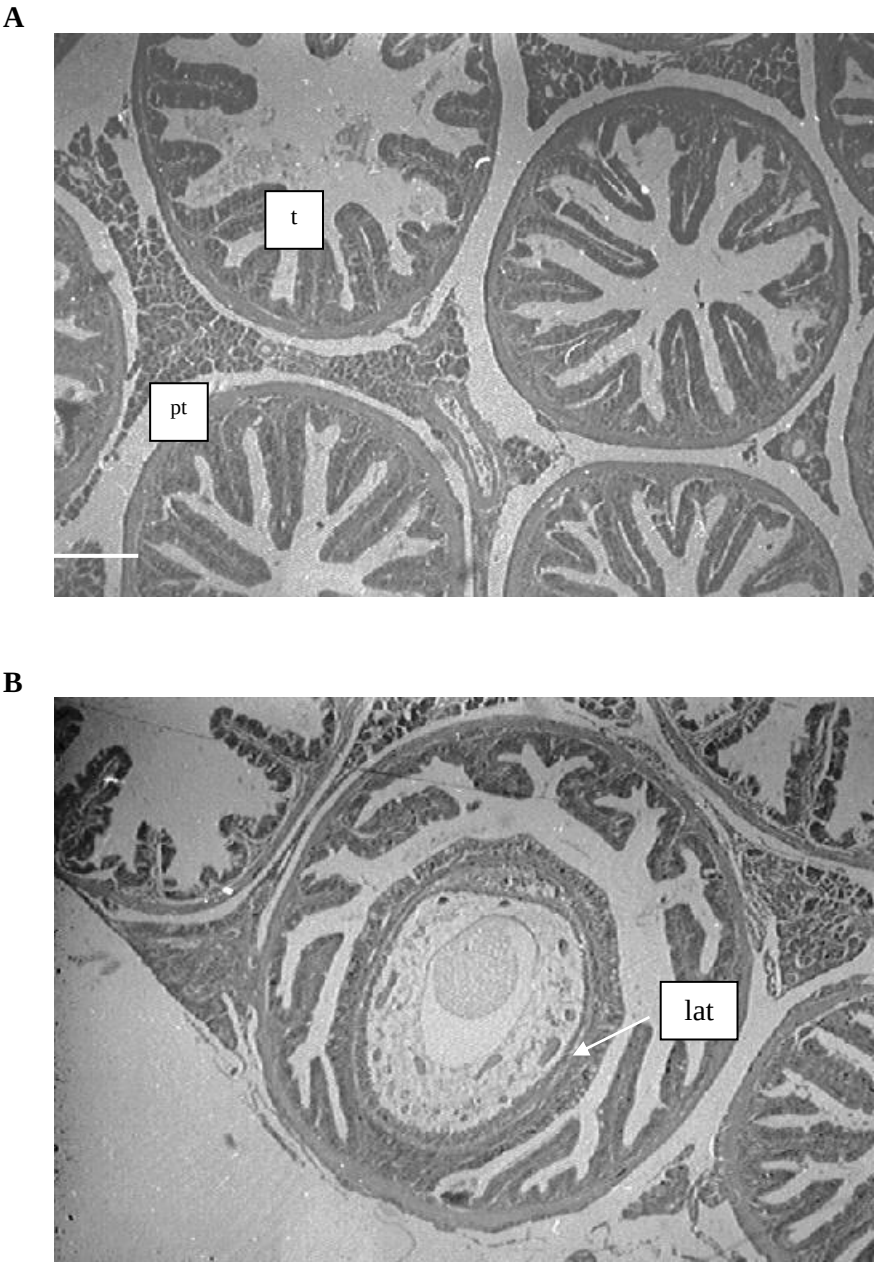


Plate 1. Light micrographs of proximal Pyloric Caeca of SBT (x80; bar = 100μm). Upper panel, shows normal tubules (t) with intercalated pancreatic tissue (pt). Lower panel, shows putative lymphoid - associated tissue (lat). Tissues were formalin fixed and paraffin embedded. Sections were cut at 5μm and stained with haematoxylin and eosin.

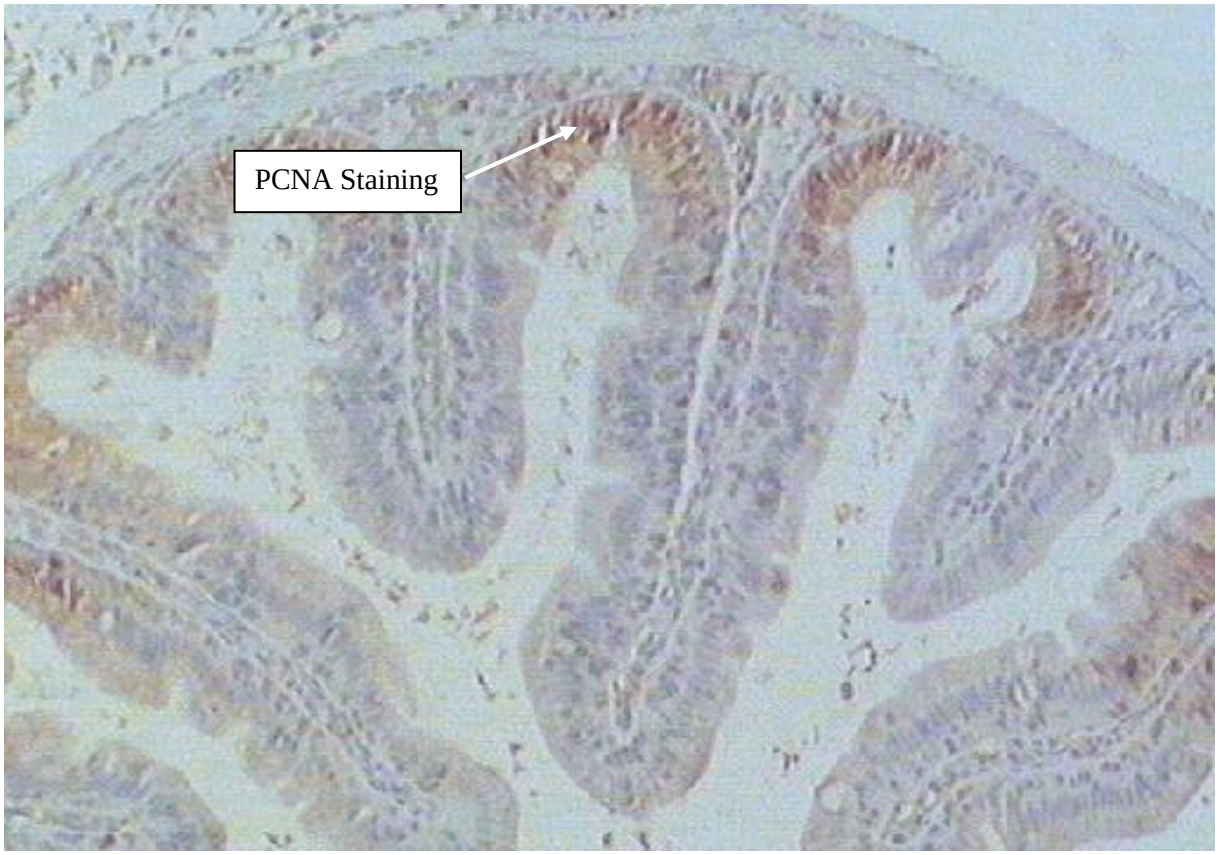
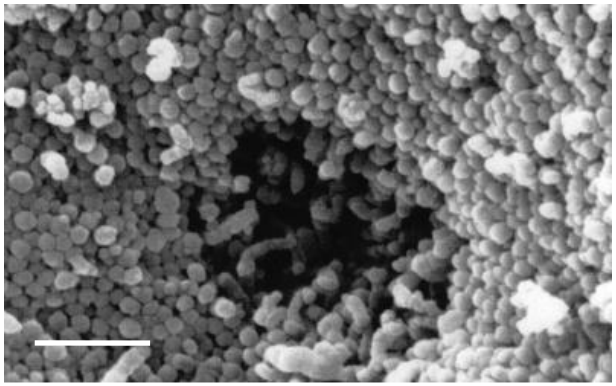
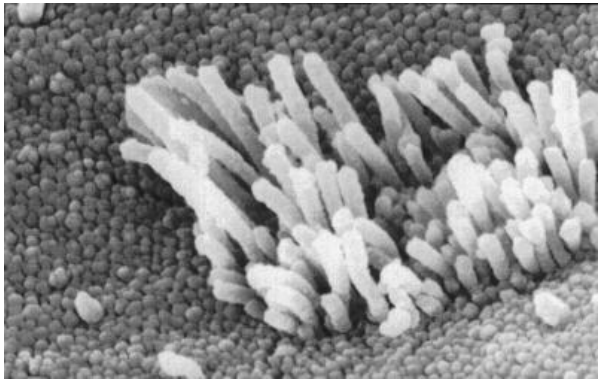

PCNA Staining

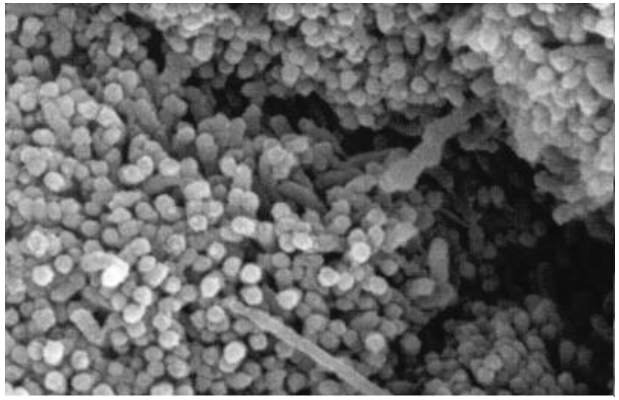
Plate 3. Scanning electron micrographs of microvilli associated with proximal pyloric caeca enterocytes. SBT were maintained on the specified diets for 3 months. Micrographs are typical views (Magnification x19800; bar = 1μm).



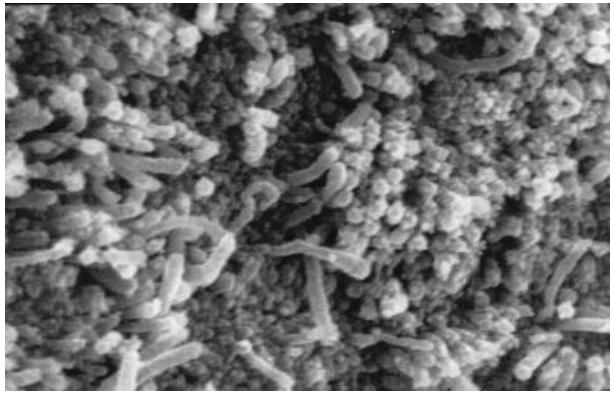
A: Pilchard fed fish



B: SBT mash fed fish



C: NBFT mash fed fish



D: Telba fed fish

Pilchards = complete fish
SBT = Southern Blue Fin Tuna mash;
NBFT = Northern Blue Fin Tuna mash
Telba = Commercial experimental diet

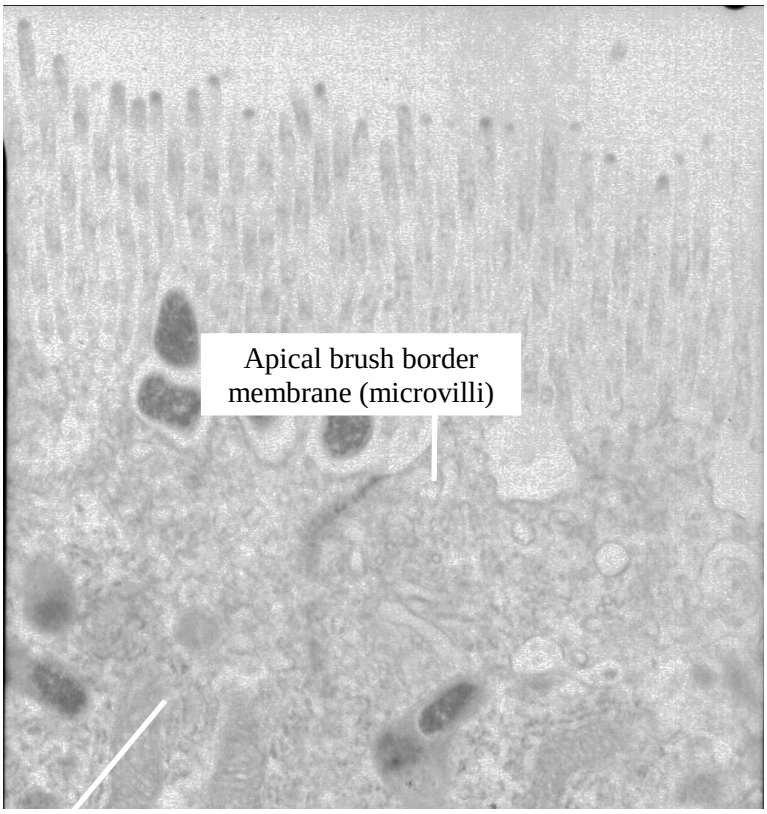


Plate 4. Endocytosis of feed particles. Typical electron micrograph of proximal Pyloric Caeca taken from fish that have been maintained on the TELBA diet for 3 months (bar = 1μm).

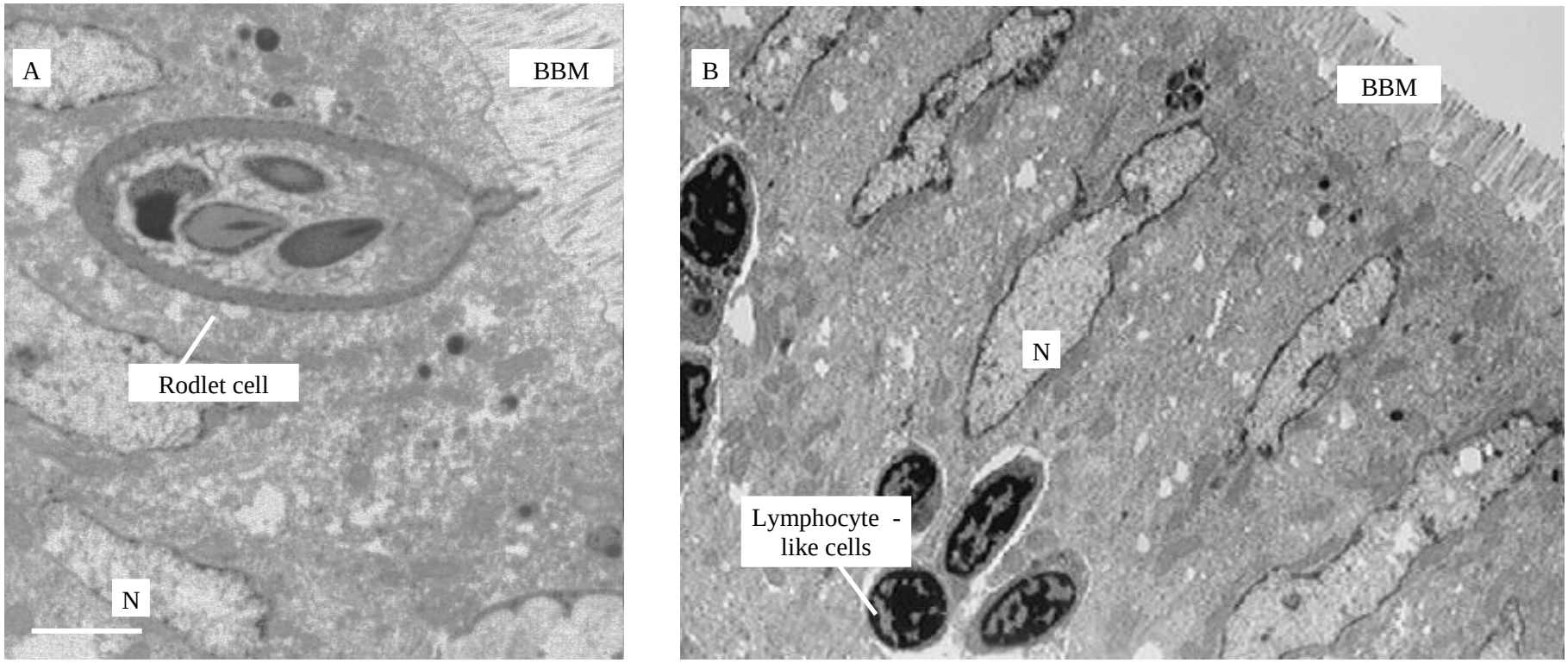


Plate 5: Structure that indicates a potential immune function for the Pyloric Caeca in the SBT.
Typical transmission electron micrograph (a) the presence of Rodlet cells (b) intraepithelial lymphocyte - like cells.
Bar indicate 1 and 4 μm for panels a and b respectively. N and BBM denote nucleus and brush border membrane respectively

Microbiology and enzymology

Mr. David West, an honours student from the Biotechnology course of Flinders University contributed a significant amount to the following results and his thesis has been submitted as a formal record of his achievements. Other work was carried out by Dr. N. Nili, who was responsible for most of the electron microscopy work and Mrs. J. McCarthy who established the crude enzyme extracts and carried out the enzyme activity measurements. The following is a summary of that work.

Electron microscopy.

Sectioning of regions of the pyloric caeca and small intestine of pilchard-fed SBT followed by scanning electron microscopy (SEM) showed regional differences. The pyloric caeca contained microbial populations mainly attached to small particles of feed in the lumen (see thesis for full description and figures). In contrast, the small intestine contained microbial populations that appeared to be attached to the intestinal wall. In some cases, the feed particles were covered with mucin-like material and bacteria were seen trapped within this layer. The predominant bacteria visible were cocci although some rods were also seen. There were differences in feed particle composition with different diets. Spherical particles were mainly seen when the artificial feed was used. This suggests that feed composition may have a significant effect on the rate of digestion. Bacterial attachment to feed particles may also be significant in the ability of SBT to digest artificial feeds, especially those that have fibrous, starchy or non-starch polysaccharide (NSP) components.

Bacterial isolation

Samples of material from the pyloric caeca and the small intestine were tested for the presence of bacteria. Up to 10^8 bacteria / ml of digesta were detected. Crude samples inoculated into rich broth grew vigorously as a mixed culture. Platings either directly or from broth culture showed a rich diversity of microorganisms. These organisms were not derived from the feed and appeared to be resident populations in the digestive tract. The populations differed depending on the diet but were highest in the pyloric caeca. Individual colonies were picked and purified for further analysis.

Bacterial characterisation

Individual cultures were grown and characterised morphologically, metabolically and by Gram stain. Counts of culturable bacteria compared with direct counts from the samples indicated that a significant population of bacteria may not be capable of growing under the culture conditions used, or require the interaction with other bacterial species for optimal growth *in vitro*. Therefore the number of isolates obtained would be only a conservative estimate of the microbial diversity in the SBT digestive tract. Isolated bacteria were tested for enzymatic activity. Bacteria isolated from the pyloric caeca were mainly proteolytic, with some being amylolytic as well. Only a few isolates expressed lipase activity or grew on lipid-containing plates. In contrast, many bacterial isolates from the small intestine were either lipolytic or proteolytic and lipolyic. This suggests that there is a separation of functions in the digestive tract and that maybe the bacteria were partly responsible for the digestion of lipid. However, until we quantify the bacterial populations and the total enzymatic activities, it is not possible to say how much of a contribution the bacteria make to overall digestion. Individual bacterial species were identified by staff at the IMVS using an API bacterial identification kit. Some isolates which demonstrated significant levels of enzymatic activity are shown in table 27.

Comparison of fish fed pilchards versus an artificial diet suggested that a greater amount of mucin was produced in response to the artificial diet and the relative proportions of some of the bacteria were changed. In particular, lipase expressing bacteria were more prominent when the SBT were fed a pilchard diet than an artificial feed. Because of the time and cost involved in achieving a full characterisation of all bacterial species, only a few examples have been characterised. However, at a

later date it would be possible to establish genetic phylogeny mapping using molecular techniques to obtain a more complete picture of microbial diversity in the SBT digestive tract. This technique is now being used in Adelaide to characterise microbial samples from animal digestive tracts.

Table 27. Isolates from the digestive tract of SBT which showed significant levels of enzymatic activity.

Isolate	Enzymatic profile	Feed	Identification
1DA	Amylase (small intestine)	Wild SBT	*Vibrio* sp.
31B	Protease (pyloric caeca)	Pilchard	*Proteus vulgaris*
32D	Lipase (small intestine)	Pilchard	*Staphyloccus* sp
32M	Protease (small intestine)	Pilchard	*Proteus mirabilis*
51H	Lipase, amylase (Small intestine)	CRC	*Vibrio* sp.

Bacterial ecology

In order to estimate the bacterial diversity in the SBT digestive tract, a direct analysis procedure was developed. This was based on the use of gene primers for conserved regions of ribosomal DNA and a Polymerase Chain Reaction (PCR) to amplify a variable region between the primers. When one primer contained a fluorescent label, PCR products (digested with a restriction enzyme) can be analysed on a DNA sequencing gel. A single fluorescent band is observed and the size of that band is characteristic of the bacterial species. Therefore the number of different organisms in a crude mixture can be estimated in a single reaction (see thesis for details).

We were able to establish that this method was valid and that different organisms could be detected in a crude mixture. Unfortunately, we were not able to complete this section of the work due to lack of fish samples and Mr. West has now completed his studies. To obtain this information, a separate student project would need to be established. Nevertheless, the results validated the technique, and has provided a valuable experimental tool for studying changes in microbial populations as a consequence of changed feeding regimes and will permit conclusions to be drawn about the relative importance of specific bacterial species to the digestion of artificial feeds by SBT.

Enzymatic activity

To determine the digestibility and likely nutritive value of artificial feeds, an *in vitro* system needed to be established. To mimic as closely as possible, the conditions in the SBT digestive tract, the experimental system must contain enzymes extracted from SBT. For repeatability of assays, these extracts must be able to be stored and retain enzymatic activity on thawing. Crude tissue extracts from the pylori caeca and the small intestine have been prepared and tested for enzyme activity over a prolonged period of time. Methods for these assays are attached. Results showed that extracts could be prepared and freeze dried without any significant loss of protease or lipase activity. This procedure will be used to prepare extracts for use in the *in vitro* experiments.

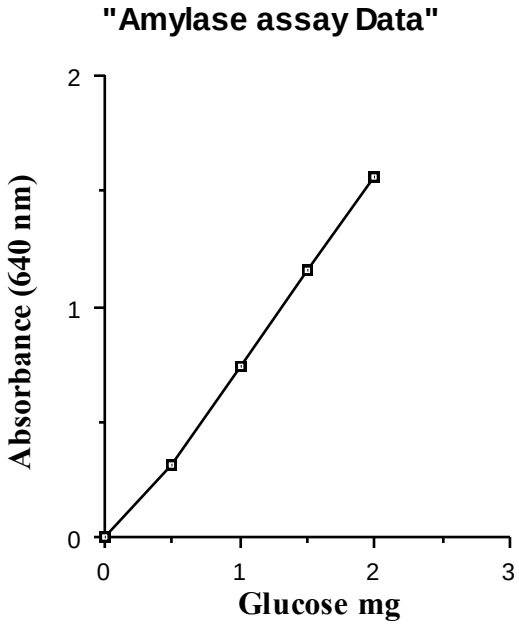
Enzyme activity in the pyloric caeca – Experiment 1.

(a) Amylase activity in Wild SBT

Results are from freeze dried homogenate sample stored at -80°C for 1 month = 0.287 ± .018 U/mg protein

One enzyme unit is defined as 1µmole of glucose equivalent formed per minute. The amount of product formed was linear with respect to the amount of protein added between 25-100µg protein.

The specific activity is equilivent to the specific activity of *S.bovis* intracellular amylase = 0.276 U/mg protein.



Reference: The method was as described by Cotta M.A. (1987). Amylolytic activity of selected species of ruminal bacteria. *Applied and Environmental Microbiology* **54,** 772-776. Modifications were that the buffer was adjusted to pH 7.2 and the assay mixture was incubated at 27°C for 10 minutes.

The activity of several digestive enzymes in tissue extracts of SBT fed different diets were also assayed.

Amylase activity in the pyloric caeca of farmed SBT: Original results are from freeze dried homogenate sample stored at -80°C for 1 month from pilchard fed fish = 287 U ± 18.3

Enzyme specific activity (Units/mg protein) is summarised below:

Fish no.	Pilchards	NBT mash	SBT mash	TELBA
1	167			
2	67			
3		11		
4		222		
10			156	
16				189

(b) Endoglucanase activity in Wild SBT

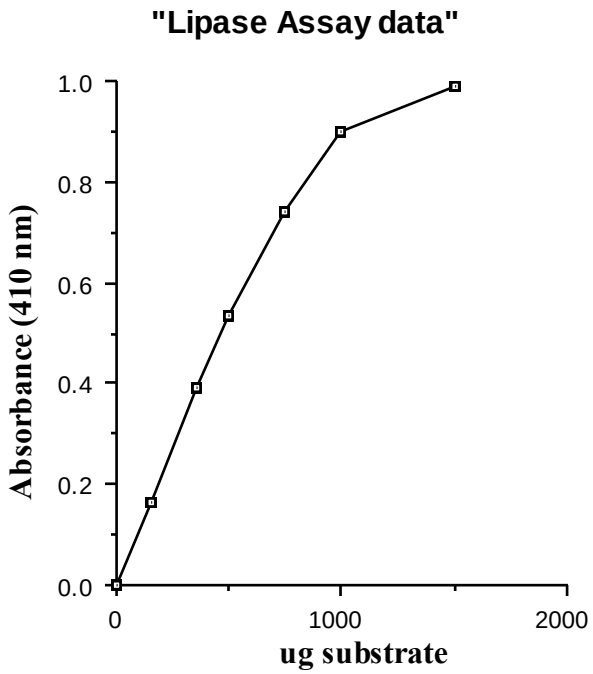
There was no endoglucanse activity dectected from freeze dried homogenate samples stored at -80°C for 1 month using a range between 25µg-1.5mg of protein

Reference: Miller , Glennon and Burton (1960). Measurement of Carboxymethylcellulase Activity. *Analytical Biochemistry* **2**, 127-132. Modifications were that the buffer was adjusted to pH 7.2 and the assay mixture was incubated at 27°C for 30 minutes.

(c) Lipase activity in Wild SBT

Results are from freeze dried homogenate stored at -80°C for 1 month = 40.65 ± 8.85 U/mg protein

One enzyme unit is defined as 1µmole of p-nitrophenylpalmitate released per minute. The specific activity is equilivent to the specific activity of *Pseudomonas aeruginosa*, 36 U/mg protein.



Reference: Winkler and Stuckmann (1978). Glycogen, Hyaluronate, and some other Polysaccharides Greatly Enhance the Formation of Exolipase by *Serratia marcescens.* *Journal of Bacteriology* **138,** 663-670.

Enzyme activity in the pyloric caeca – Experiment 2.

(a) Lipase activity in farmed SBT

One unit is expressed as 1nmol p-nitrophenol enzymatically released from the substrate p-nitrophenyl palmitate / mg protein/min.

Fish	Pilchard P2	Pilchard D2	NBT P2	NBT D2	SBT P2	SBT D2	TELBA P2	TELBA D2
1	2.5±0.57	1.7±0.5						
2	2.3±0.42	2.4 ±0.35						
3			3.0± 1.1	4.9 ±1.1				
4			5.1± 0.83	4.1 ±1.5				
5			4.7± 1.5	4.4 ±2.2				
6	2.9±0.75	4.7±0.77						
7	2.7±1.06	4.0 ±1.7						
8	2.7±0.18	4.1±0.6						
9	1.7±0.34	5.9 ±1.7						
10					4.3±1.79	3.9±0.53		
11					1.4±0.26	4.7±0.84		
12					1.4±1.38	4.0±0.97		
13					0.64±0.91	3.2 ±2.9		
14					5.1 ±2.0	5.6±0.4		
15					2.1±0.3	4.1 ±1.5		
16							2.9 ±0.39	2.3 ±.2.4
17							1.9 ±1.3	3.4 ±1.6
18							2.5 ±0.55	2.9 ±1.8
19							3.1 ±0.43	3.6±0.43
20							3.1 ±0.9	3.6 ±1.2
Mean	2.5±0.27	3.4 ± 0.55	4.3 ±0.91	4.5 ± 0.33	2.75 ± 1.4	4.4 ± 0.65	2.7 ± 0.7	3.16±0.49

P2 =Proximal pyloric caeca; D2 = Distal Pyloric caeca

(b) DPPIV activity in farmed SBT

One unit is expressed as 1nmol p-nitroaniline enzymatically released from the substrate gly-pro-p-nitroaniline / mg protein/min. (P2 = Proximal Pyloric caeca; D2 = Distal Pyloric caeca)

Fish	Pilchard P2	Pilchard D2	NBT P2	NBT D2	SBT P2	SBT D2	TELBA P2	TELBA D2
1	62 ±4.0	54 ±1.7						
2	50 ±3.3	27 ±2.0						
3			54 ± 6.5	37 ±2.8				
4			43 ± 4.3	47 ±7.7				
5			35 ± 5.5	23 ±4.2				
6	41 ±3.9	48 ±2.9						
7	46 ±5.7	40 ±2.4						
8	51 ±3.0	41 ±1.9						
9	58 ±2.2	54 ±3.2						
10					48 ±4.4	39 ±3.5		
11					51 ±2.8	35 ±1.5		
12					39 ±1.7	32 ±2.9		
13					37 ±2.8	40 ±2.8		
14					44±0.50	50 ±3.5		
15					47 ±1.5	38 ±2.6		
16							61 ±3.4	53±.001
17							51±0.	56 ±3.7
18							56±1.62	47 ±2.6
19							33 ±5.	33 ±3.0
20							41 ±6.0	24 ±2.7
Mean	52.5 ± 6.2	43.5 ± 9.6	44 ±7.8	36± 9.8	45 ± 3.8	40.5 ± 4.6	48 ± 10.2	43 ± 12.2

Reference: Hino M. *et al. (*1976). X-Prolyl Dipeptidyl-Aminopeptidase Activity, with X-Proline p-Nitroanilides as substrates, in Normal and Pathological Human Sera. *Clinical Chemistry*. **22/8**, 1256-1261.

(c) Protease activity in farmed SBT

One unit is expressed as 1ug substrate protein (casein) digested/ mg protein/ minute.

Fish	Pilchard P2	Pilchard D2	NBT P2	NBT D2	SBT P2	SBT D2	TELBA P2	TELBA D2
1	383 ±1.6	315 ±2.0						
2	310 ±4.4	330 ±1.8						
3			240 ± 6.6	393 ±1.9				
4			350 ± 4.4	497 ±0.3				
5			388 ± 3.6	320 ±2.4				
6	388 ±14.7	520 ±0.33						
7	411 ±2.3	347 ±4.9						
8	453 ±8.8	357 ±4.3						
9	372 ±1.7	423 ±2.2						
10					320 ±2.1	383 ±4.4		
11					414 ±6.0	450 ±2.7		
12					127 ±11.5	150 ±6.2		
13					311 ±0.9	300 ±1.4		
14					403 ±3.1	308 ±1.6		
15					354 ±2.5	417 ±1.2		
16							350 ±7.2	433 ±.2.3
17							238 ±4.0	375 ±1.2
18							214 ±14.7	283 ±4.0
19							370 ±3.9	433 ±1.5
20							418 ±2.8	288 ±0.7
Mean	377±33	222±38	326±63	403±73	336±63	342±83	318±78	363±66

P2 = Proximal Pyloric caeca; D2 = Distal Pyloric caeca

Fresh homogenate

13/9/96 418µg ±2.8

Freeze dried sample activity kept at following temperatures

19/9/96	+4°C	412µg ±4.5
8/10/96	+4°C	522µg± 25.5
25/10/96	+4C°	427µg ±11.9
19/9/96	-20°C	418µg±9.4
8/10/96	-20°C	484µg± 3.2
25/10/96	-20°C	460µg±2.3
19/9/96	-80°C	476µg ± 3
8/10/96	-80°C	508µg± 7
25/10/96	-80°C	413µg±4.4

Enzyme activity in the small intestine

(a) Lipase activity in farmed SBT

One unit is expressed as 1nmol p-nitrophenol enzymatically released from the substrate p-nitrophenyl palmitate / mg protein/min.

Fish No	Pilchard	NBT	SBT	TELBA
1	0			
2	0			
3		0.80 ±0.8		
4		0.27 ±0.7		
5		0.07 ±0.16		
6	0			
7	0			
8	0.13 ±0.8			
9	0.47 ±0.5			
10			0.8 ±1.5	
11			1.3 ±0.6	
12			0.93 ±0.5	
13			0.4 ±0.8	
14			0.67 ±0.4	
15			2.4 ±1.6	
16				0.53 ±0.6
17				0.47 ±0.1
18				0.60 ±0.3
19				0.67 ±0.4
20				0.27 ±0.6
Mean	0.30±0.75	0.38±0.55	1.2±0.55	0.51 ±0.13

Reference: Winkler and Stuckmann (1978). Glycogen, Hyaluronate and some other Polysaccharides greatly enhance the formation of Exolipase by *Serratia marcescens. Journal of Bacteriology* **138,** 663-670.

(b) Protease activity in farmed SBT

One unit is expressed as 1ug substrate protein (Casein) digested/ mg protein/ minute.

Fish No.	Pilchard	NBT	SBT	TELBA
1	150 ±1.7			
2	130 ±1.4			
3		167 ±9.2		
4		127 ±3.5		
5		245 ±1.1		
6	258 ±3.7			
7	283 ±0.3			
8	100 ±1.7			
9	384 ±0.21			
10			367±1.3	
11			283 ±1.4	
12			346 ±1.8	
13			233 ±1.0	
14			348 ±1.56	
15			178 ±1.2	
16				378 ±1.6
17				173 ±0.72
18				426 ±0.7
19				270 ±0.1
20				217 ±0.9
Mean	198±15	180 ± 49	285 ± 69	293 ± 96

Reference: Bickerstaff and Zhou (1993). Protease activity and auto digestion (autolysis) assays using Coomassie blue dye binding. *Analytical Biochemistry*. **210**, 155-158.

*Summary of enzyme work (farmed SBT***)**

Location (FD extracts)	Amylase	Endo-glucanase	Lipase	Protease	DPP-IV
Pyloric Caeca					
P	117	0	2.9	372	48
NBT	117	0	4.4	365	40
SBT	156	0	3.6	339	43
TELBA	189	0	2.9	341	46
Small Intestine					
P	0	0	30	140	ND
NBT	0	0	38	180	ND
SBT	0	0	85	285	ND
TELBA	0	0	51	293	ND

P = Pilchard; NBT = NBT mash; SBT = SBT mash; TELBA = TELBA diet

Benefits

The results of this project will benefit a variety of industry sectors including:

Southern Bluefin Tuna farmers
This sector can use these results to assist with the incorporation of cost-effective manufactured tuna diets with a reduced reliance on imported whole bait fish.

Manuafacturers of tuna feeds
Feed manufacturers can use these results to substitute ingredients in manufactured feeds with no compromise to nutrient supply. The best example from this research is variations in nutrient supply from fish meals used in tuna feeds.

Researchers focussing on feed evaluation for fish
Researchers focussing on feed evaluation can use results from this research to develop similar techniques for the assessment of feed ingredients for other species.

Other aquaculture enterprises
Other aquaculture enterprises can use results from this research to develop alternative feed formulation systems based on nutrient digestibilities.

Grain growers and ingredient suppliers
Ingredient suppliers can use techniques developed as part of this research to define the nutritive value of their produce to support its inclusion in manufactured feeds for SBT. Results from this research can also be used to identify those ingredients with limited potential for use in SBT diets.

Results from this research, along with developments in feed manufacturing technology and tuna husbandry, have combined to reduce the feed conversion efficiency of farmed SBT from more than 25:1 in 1994/95 to less than 8:1 in 1998. If 4000 tonnes of farmed tuna are sold in any one year at least doubling in size from the time of capture, this equates to a reduction in feed usage (pilchards or manufactured feeds) from 50 000 tonnes/annum to 16 000 tonnes/annum. If a mean value of feed of $1000 per tonne is used, then this research has contributed to savings or more than $34 million/annum.

The benefits and beneficiaries compare well with those listed in the original FRDC application.

Further Development

- Since the completion of this project, a new project aimed at developing manufactured feeds for Southern Bluefin Tuna has been commenced as part of the FRDC Tuna Aquaculture Subprogram. This project utilises results from the current research in all diet formulations.

- Further *in vitro* analysis of feed ingredients could be conducted to develop a more extensive database on the potential nutritive value of feed ingredients for SBT.

- Techniques developed as part of this project are being used to assess diets for large scale *in vivo* tuna experiments prior to commencement in an attempt to identify those formulations with limited potential, thus saving considerable research funds and effort.

Conclusions

***In vivo* digestibility studies**

The results indicate that destructive sampling is an efficient way of obtaining digesta for digestibility and transit time studies. However, the costs and time involved with such practices for minimal gain of information, reveal the need for *in vitro* digestibility assays. Transit time was not determined, but the presence of beads in the stomach were used for feed intake correlations, with moderate success.

In general, the digestibility of all nutrients were lower than expected. Changes in experimental methods are required to improve the accuracy of the digestibility and transit time studies. Endogenous nitrogen contributions and digesta transit times need to be quantified to explain the lower than expected results.

Acid-insoluble ash and n-hexatriacontane are comparable markers that are both suitable for use as digestibility markers in SBT diets. The use of n-hexatriacontane is advantageous, due to the higher degree of accuracy, compared with acid-insoluble ash.

The disadvantages of stripping the digesta from the intestine were apparent from this research, with indications that there is a high endogenous nitrogen contribution. The destructive sampling method is still the most efficient, if the removal of the digesta from the intestine is done by the infusion of distilled water, rather than manual squeezing.

Factors that affect the accuracy and the reliability of transit time and digestibility results were also revealed. The variability in fish weight is a difficult factor to overcome as handling the SBT for weighing is difficult. Poor consumption and regurgitation of diets can only be overcome with research into the palatability of diets. The digestibility results may be improved if the level of endogenous nitrogen was quantified, allowing the use of true digestibility results rather than apparent results. An improvement in feed mixing technology may also improve the uniformity of marker distribution in the diet.

In vitro digestibility studies

1. Multi-enzyme digestion systems can be used to indicate the *in vitro* dry material and crude protein digestibility of feeds and feed ingredients.
2. The sensitivity of the enzyme system is expressed by the range in digestibility values for a given set of feeds and ingredients and relates to the source of the enzymes.
3. Enzyme systems using fish enzymes, from salmon and tuna, were more sensitive than the system using porcine enzymes.
4. Digestibility of feeds determined from both ingredient and complete feeds were shown to be similar and demonstrated that ingredient digestibility values were additive using salmon and tuna enzyme systems.
5. Correlations between *in vivo* and *in vitro* digestibility values, the ability to indicate poor quality protein sources and the additivity of ingredient digestibility values demonstrated that salmon and tuna enzyme systems are suitable for predicting the digestibility of feeds from ingredient digestibility and therefore the values can be used in formulating tuna feeds.

Nutrient uptake studies

The isolation of brush border membranes is possible from SBT intestinal tissue. However, the refinement of the initial isolation protocols will yield greater enrichments. The characterization of transport demonstrates the presence of at least two transporters for the marker amino acids, alanine and proline. However, additional work is required to evaluate the true effect of diet on transport

capacity of the Pyloric Caeca for amino acids. Such diet interactions could be profound. A true understanding of how feed ingredients and how specific ingredients, e.g. lectins, interact with the brush border membrane will be invaluable in assessing the nutritive value of both established and novel ingredients. To this end, isolated brush border membranes and their use in transport studies provide an excellent species specific, yet rapid, methodology to determine the level of compounds in diets that can bind to and affect membrane function.

Ultrastructure studies

In terms of nutrient uptake, the Pyloric caeca does posses the transport capacity for amino acids however this occurs in conjunction with endocytosis. Given this coupled with the apparent regional difference in function and microbial population of the pyloric caeca it is recommended that freeze dried extracts are used in digestibility assays *in vitro*. Doing this the environment of pyloric caeca can best mimicked.

The Pyloric Caeca of the SBT is responsive to diet and such changes will impact on the digestive and absorptive physiology of this organ. Whether such changes are solely due to level of nutrient intake or may be induced by specific dietary components binding to and influencing the cellular function in the Pyloric caeca remains to be determined. What is clear is that intestinal tract of the SBT possesses structure similar to those seen in mammals that are know to react to food and alter function of the gut. Such changes could include appropriate adaptation to new feed ingredients. Further to this, such reactivity may depend on dietary history. Given the nature of SBT farming and the dependence on wild caught fish and hence only limited knowledge of their previous feeding may introduce significant variations in acceptance and tolerance of novel feeds. However, such speculation needs to be verified by establishing trials in a model species. In these trails dietary interactions on intestinal structure and function can be evaluated. Therefore, future work on dietary evaluation needs to include how dietary components interact with the digestive tract and hence determine its function. The presence of such compounds is not detectable by assays designed to assess digestibility of the diet.

Microbiology and enzymology

Amylase activity is significant in the pyloric caeca but not in the small intestine. This means that feeds containing starch should be formulated such that the starch was on the outside where it can be digested fully within the pyloric caeca. Endoglucanase activity was not detected, suggesting that cellulose should be kept to a minimum in diet formulations. Lipase activity was greatest in the small intestine where most digestion of fats appears to occur. Lipids should therefore be located on the inside of feed pellets. Protease activity is high in both the pyloric caeca and the small intestine, although specific activity per mg of protein was greatest in the pyloric caeca. DPP-IV activity was present in the pyloric caeca but not in the small intestine. This correlates with the protease locations and supports the concept that protein degradation occurs mainly in the pyloric caeca. These results may be useful in designing a feed structure that would permit maximum digestion of a feed whilst protecting other component from premature degradation. It will be important to understand where maximum absorption of degradation products occurs. It is likely that bacterial populations, which appear to express the same enzymatic activities as the tissue, will also play a role in the digestive processes. To what extent bacterial processes complement tissue processes requires further investigation.

Because of the difficulty in controlling the environment and diet of SBT, we were unable to carry out definitive experiments in which diet and intake could be correlated with bacterial activity. We were therefore only able to establish a likely role of bacteria in digestion of feeds and to identify some of the bacterial species present. Experiments that will complete these studies are:

1. Determine specific enzyme activity on extracts from pure bacterial cultures. This is to relate bacterial enzyme activities to tissue activities, especially protease and lipase.

2. Determine the microbial diversity at specific locations within the digestive tract. It is also important to understand tissue functions and absorptive processes in SBT.

It is clear from these conclusions that this research has:

- Enhanced the development of manufactured feeds for SBT by using *in vitro* analysis of feeds.
- Determined the ultrastructure of SBT intestine.
- Evaluated the enzyme profile of SBT intestine and the epithelial associated bacteria.
- Estimated the intestinal capacity to transport the major groups of amino acids and peptides.

All objectives have therefore been met.

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Appendix I: Intellectual Property

Intellectual property arising from this research could include:

- Techniques for the *in vitro* assessment of feed ingredients for SBT (and other aquaculture species). This information should be protected and may have some commercial value.

- Information pertaining to the digestibility of nutrients in feed ingredients for SBT diets. While this data is likely to have limited commercial value, it is valuable and will have a significant effect on the ability of nutritionists to optimise the nutrient supply from diets.

- Data on enzyme profiles would be of value to those seeking to utilise exogenous enzymes in tuna feeds.

- Techniques developed as part of the nutrient uptake studies could have further application for the assessment of the nutritive value of feed ingredients for fish. Mr Robert Kemp is pursuing these developments as part of his PhD studies.

Valuable information arising from the research includes:

- Techniques and data from *in vivo* digestibility and transit time studies.

- Ultrastructure analysis.

With the above in mind, three reports are recommended. Apart from the full report, an unrestricted version should be prepared without details of the *in vitro* assays. A third report including a summary and the results from the *in vitro* analysis only should be prepared for use by all sectors of the industry.

To the knowledge of the Principal Investigator, this report does not affect third party intellectual property. Certainly, no third party IP was declared prior to the commencement of the research.

Appendix II: Staff

The following staff have been engaged on this project:

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- Dr Chris Carter
- Mr Matthew Bransden

Appendix III

Honours Thesis

Mr R Kemp

Appendix IV

Honours Thesis

Mr D West

Appendix V

Honours Thesis

Ms B Davis

Appendix VI
Publications