Digestive protease activities, growth and feed utilisation in Atlantic salmon (*Salmo salar* L.)

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Dissertation for the degree doctor scientiarium (Dr. Scient.)

at the University of Bergen

03.03.2006

Scientific environment

It is not my intention to fool the readers into thinking that this work was the product of only one person. Without the opportunity kindly provided to me by the staff and directors of the Institute of Marine Research (IMR) and the (then) Department of Aquaculture, this would not have been possible. Most of the experiments were carried out at the Institute of Marine Research, Matre. Dr. Dave A. Higgs at the Directorate of Fisheries and Oceans (DFO) in Vancouver, Canada also kindly offered the use of their facilities for one of the experiments.

Financial support was in part provided by the Commission of the European Communities through their Agriculture and Fisheries specific RTD program FAIR-CT96-1329, “Effect of processing technology on the quality of aquaculture feeds”. Financing in the later writing stages was kindly provided by IMR.

Acknowledgements

IMR not only provided the facilities necessary to carry out this work, but my colleagues in Matre added the magic ingredient: a pleasant and inspiring work environment. Thank you!

One person in particular deserves special thanks, my supervisor in Matre, Dr. Krisna Rungruangsak-Torrissen, for her day-to-night discussions and dedication to her work. Without her previous work and ideas, this study would never have been started and thanks to her encouragement — it was finalised. I would also like to thank my supervisor Dr. Harald B. Jensen at the Institute of Molecular Biology for his support and inspiration, and the staff at the Institute of Molecular Biology and the Faculty of Mathematics and Natural Sciences for doing all the paperwork. Dr. Anders Kiessling and senior scientist Rolf Erik Olsen at Matre were also very helpful in their contribution.

Thank you to my family for having the patience to see me through the entire process.

And finally, thank you to my wife Pamela for just being the wonderful woman that you are.
Elsker deg!

Abstract

The specific activities of trypsin and chymotrypsin in the pyloric caeca were investigated in individually labeled Atlantic salmon (*Salmo salar* L.). Photoperiod (natural or 24 h) and feed protein quality (two levels of digestibility) were used as growth affecting factors in two grow-out experiments. Parameters indicative of protein growth and metabolism, i.e. plasma and white muscle free amino acid (FAA) concentrations, white muscle RNA concentrations, and white muscle protein synthesis capacity (RNA/protein ratio) were also measured. The feasibility of using free hydroxyproline (Hyp) concentration in the white muscle as an indicator of the rate of tissue protein breakdown (turnover) was assessed.

Further, an *in vitro* digestibility assay was developed to evaluate the effects of industrial processing conditions on feed protein digestibility. Digestion using crude pyloric caecal extracts was standardised by trypsin activity and compared with growth experiments to predict the effects of feed protein quality on specific growth rate (SGR) and feed conversion efficiency (FCE). Finally, dorsal aorta cannulation of fish was assessed as a tool for evaluating feed protein quality (containing no free or supplemented amino acids) through repeated measurements of plasma FAA concentrations after feeding.

Trypsin (T) and chymotrypsin (C) showed high covariation in all experiments, regardless of whether growth was affected indirectly (through photoperiod manipulation) or directly (through feed protein quality). Groups exhibiting different feed conversion efficiencies (FCE) had different activity ratios of trypsin to chymotrypsin (T/C ratio). The T/C ratio seemed to be more sensitive than growth measurements to slight differences in feed protein quality and might have an application as an indicator of growth performance.

In salmon reared under different photoperiods, SGR correlated with trypsin activity and T/C ratio on individual basis. These correlations could possibly be explained by a predominant influence of feed intake on growth under these conditions. In contrast to trypsin activity, chymotrypsin activity was uncorrelated to SGR variation. Plasma essential (EAA) and total (TFAA) free amino acid concentrations did not show consistent relationships with other biochemical parameters and growth rate. White muscle EAA, however, decreased with SGR, while white muscle TFAA and Hyp concentrations showed an increasing trend. Of all measured parameters, Hyp level in white muscle showed the highest correlation with growth rate. An observed inverse relation between SGR and white muscle RNA concentrations indicated a lower relative protein synthetic activity at higher growth rates and could indicate a shift to a higher importance of lipid deposition at high feed intakes. This was consistent with an increased protein turnover, indicated by elevated white muscle free Hyp levels, suggesting a lower efficiency of protein retention at high growth rates and that a higher fraction of ingested amino acids were used as energy substrates.

Fish meal raw material, drying temperature and duration of drying affected feed protein digestibility in vitro. This reduction in digestibility was concomitant with a higher incidence of disulphide bond formation in the feed proteins, demonstrating a negative effect of disulphide bond formation on feed protein quality. Digestibility of the experimental diets measured in vitro correlated with SGR and FCE after three months of feeding, but differences in SGR between feed quality groups did not reach statistical significance in either 150 g or 2 kg salmon. However, 2 kg salmon fed restricted rations showed significantly higher FCE in the ‘high’ protein quality feed groups. Differences in FCE at the end of the experiment seemed to be preceded by differences in trypsin and chymotrypsin specific activities one month earlier. Trypsin activity was unaffected by feed protein quality, possibly only reflecting the similar feed intake in the experimental groups. This resulted in a relationship between chymotrypsin activity and feed in vitro digestibility when standardised by trypsin activity.

Fish groups given feeds of ‘high’ protein quality had relatively higher RNA concentrations in the white muscle than groups given ‘low’ quality feeds, indicating a positive effect of feed protein digestibility on muscle ribosome concentration, and possibly protein synthetic activity. At the same time, white muscle Hyp concentrations were significantly lower in the ‘high’ quality dietary groups, indicating a lower protein turnover rate and potentially higher protein retention efficiency in these fish. However, this was not detectable as a difference in fillet protein content after three months of feeding. White muscle ratios of essential to non-essential free amino acids (EAA/NEAA ratio) were higher in groups with higher FCE, whereas plasma values showed no specific pattern, except after starvation and refeeding, where EAA/NEAA ratios were higher with higher FCE. No correlations were found between digestive protease activity and other parameters on an individual basis. A possible explanation for these findings could be that growth rate in this case was limited by feed protein digestibility and unrelated to feed intake.

High and low quality feeds were selected from the protein quality study in order to investigate amino acid uptake following feeding. Feed intake was positively correlated with the sum of EAA in plasma 6 h post-feeding. Variation in plasma FAA profiles was to a large extent explained by individual differences in feed intake, but individual differences in metabolism of specific amino acids were indicated. However, feeds of different protein qualities could still be distinguished through significant differences in plasma EAA profiles after statistical correction for these factors.

The results indicate that the relationship between trypsin and chymotrypsin activity (T/C ratio) may have an application as an indicator of differences in growth performance between groups of fish, both when growth is affected by external factors and diet quality. The different mechanisms through which growth differences were affected under the two specific experimental setups suggest that the T/C ratio could have a broader application also outside the limitations of the current studies. In particular, the method may be useful for determining the nutritional and growth status

of fish in the wild where food consumption cannot be measured. However, this remains to be validated. The relationship between white muscle Hyp concentrations and protein turnover deserves further investigation, as this parameter showed strong correlation with growth rates. Further studies of the hormonal and genetic mechanisms regulating trypsin and chymotrypsin activity, and how they are affected by dietary and exogenous factors need to supplement future studies in this field.

These studies demonstrated the suitability and efficiency of small-scale assays for the evaluation of feed protein quality. Further development of such methods is recommended as alternatives or supplements to regular time-consuming growth experiments.

List of publications

This dissertation consists of the following papers, referred to in the text by their Roman numerals:

Paper I:

Sunde, J., Taranger, G.L. & Rungruangsak-Torrissen, K. 2001. Digestive protease activities and free amino acids in white muscle as indicators for feed conversion efficiency and growth rate in Atlantic salmon (*Salmo salar* L.). *Fish Physiol. Biochem*., **25**, 335-345.

Paper II:

Sunde, J., Eiane, S.A., Rustad, A., Jensen, H.B., Opstvedt, J., Nygård, E., Venturini, G. & Rungruangsak-Torrissen, K. 2004. Effect of fish feed processing conditions on digestive protease activities, free amino acid pools, feed conversion efficiency and growth in Atlantic salmon (*Salmo salar* L.). *Aquacult. Nutr.*, **10**, 261-277.

Paper III:

Rungruangsak-Torrissen, K, Rustad, A., **Sunde, J.**, Eiane, S.A., Jensen, H.B., Opstvedt, J., Samuelsen, T.A., Mundheim, H., Luzzana, U. & Venturini, G. 2002. *In vitro* digestibility based on fish crude enzyme extract for prediction of feed quality in growth trials. *J. Sci. Food Agric.*, **82**, 644-654.

Paper IV:

Sunde, J., Kiessling, A., Higgs, D., Opstvedt, J., Venturini, G. & Rungruangsak-Torrissen, K. 2003. Evaluation of feed protein quality by measuring plasma free amino acids in Atlantic salmon (*Salmo salar* L.) after dorsal aorta cannulation. *Aquacult. Nutr.*, **9**, 351-360.

Contents

1. Introduction

1.1 Atlantic salmon in aquaculture

Atlantic salmon (*Salmo salar* L.) is one of the major fish species in aquaculture, with annual production exceeding 850 000 tons in 2000 (Storebakken 2002). Part of this success may be attributed to the carnivorous characteristics of this species, in particular efficient digestion of high dietary loads of protein (Anderson *et al.* 1995). Commercial pelleted diets are mainly based on fish meal of marine origin and may contain as much as 40–45 % crude protein; this high feed protein requirement currently represents the largest cost component. The International Fishmeal and Fish Oil Organisation (IFFO) predicts that, at the current industry development rate, demand from the aquaculture industry for fish meal as a protein source will reach 48% of global production by 2010 (Pike & Barlow 2002). The high protein content of aquaculture feeds represents a major source of nutrient pollution of the surrounding environment if not properly managed (Braaten *et al.* 1983), and it is therefore in both commercial and environmental interests that protein utilisation in farmed fish is optimised. To achieve this, a better understanding of the mechanisms underlying the digestion and utilisation of proteins is needed. This thesis focuses on how digestive protease activities can be utilised to aid understanding of these mechanisms.

1.2 The digestion process

1.2.1 Protein digestion

The control of digestive processes in vertebrates is complex, and involves a range of mechanical, sensory and hormonal stimuli. Digestion is characterised by the cleaving of longer chains of proteins, lipids and carbohydrates by designated substrate-specific digestive enzymes into smaller molecules that are readily absorbed into the blood

stream, either by active or passive transport mechanisms. The first step in the digestive process is the unspecific digestion of foodstuffs in the stomach after secretion of gastric hydrochloric acid (HCl) from gastric mucosa glands in the stomach lining. This secretion is activated by a combination of visual and olfactory cues, distension of the stomach walls caused by food present in the stomach, and the presence of proteins in the stomach (Wallace 1991). With few differences, digestive enzymes isolated and characterised from fish have been found to be functionally equivalent to those in other higher vertebrates.

1.2.2 The digestive proteases

The digestive proteases can be classified based on their substrate specificity (Figure 1.1); endopeptidases are able to break peptide bonds within the protein (polypeptide) chains while exopeptidases are restricted to breaking peptide bonds at the end of polypeptide chains. Following the secretion of HCl, the acidic endopeptidase pepsin is released from oxyntopeptic cells lining the stomach mucosa. Pepsin release is a rapid process that peaks within one hour of feeding in pre-starved Atlantic salmon (Einarsson *et al.* 1996).

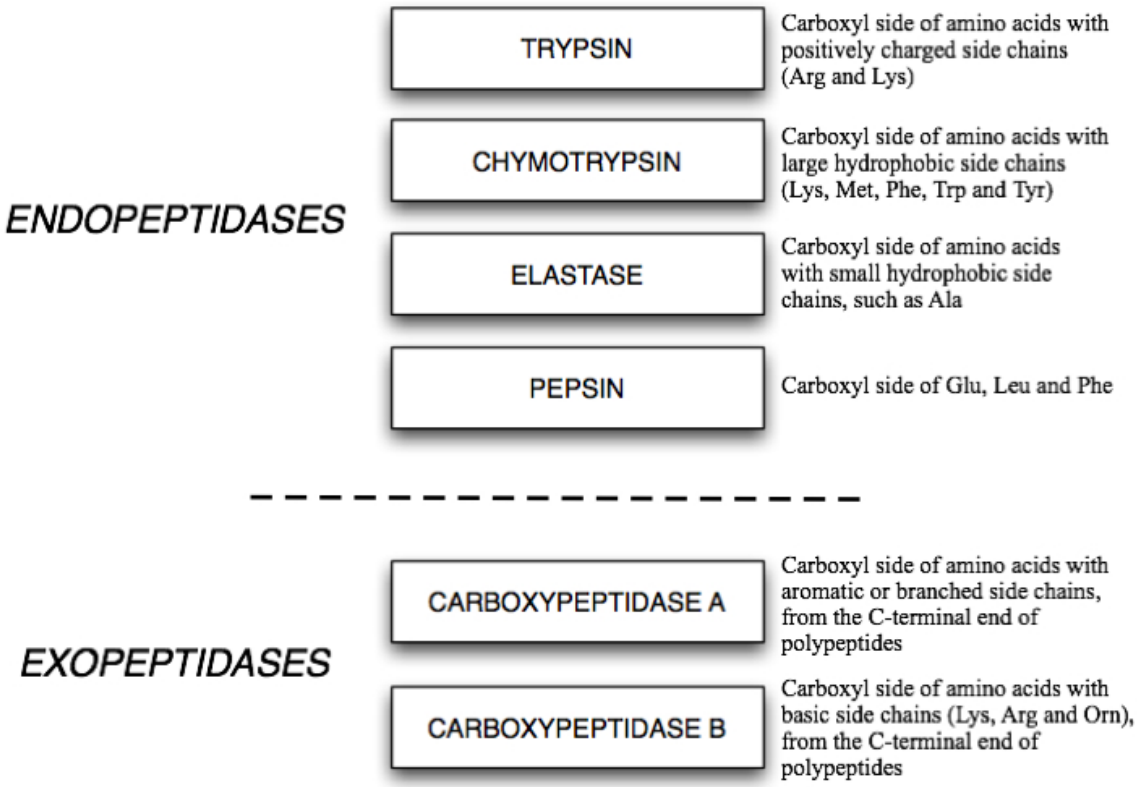

Figure 1.1: Digestive proteases and specific cleavage sites. Adapted from Wallace (1991).

Secretion and activation of digestive pancreatic proteases in Atlantic salmon follows the common pathway seen in other vertebrate species (Figure 1.2). As food enters the small intestine, proteases synthesised in the pancreas are secreted together with sodium bicarbonate ($NaHCO_3$), thereby neutralising the stomach acid and providing a slightly alkaline environment in the intestine.

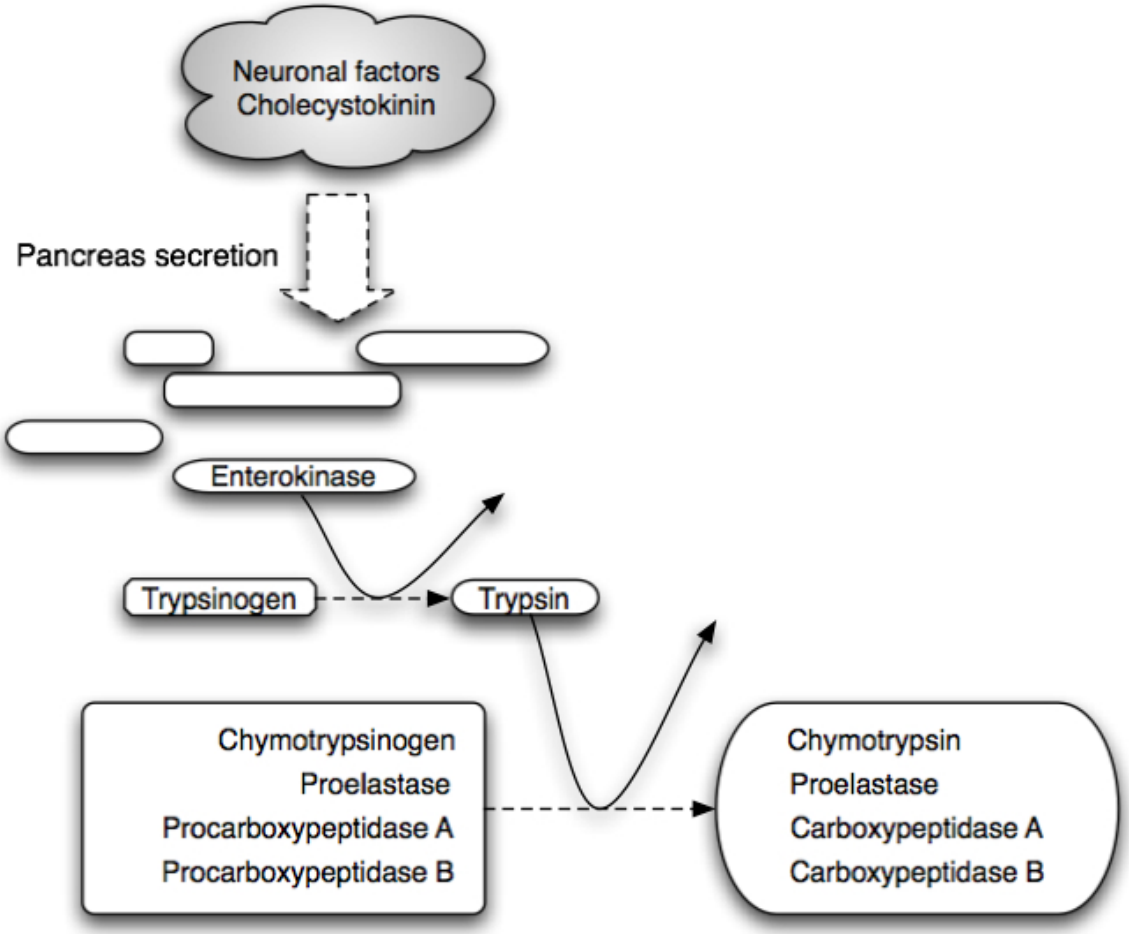

Figure 1.2: A schematic representation of pancreatic protease activation following ingestion of food.

1.2.3 The pyloric caeca in salmonids

A crucial step in the digestion process is the absorption of nutrients across the brush border of the epithelial cells in the intestine. Dietary nitrogen is absorbed as amino acids or peptides via three basic mechanisms: (1) passive transport or diffusion, a non-saturable component independent of carrier molecules, (2) carrier-mediated transport, dependent on carrier molecules, but independent of ion gradients and (3) energy-dependent carrier-mediated transport coupled to ion gradients (Collie & Ferraris 1995). In addition, endocytosis of intact low-molecular weight proteins occurs in the distal region of the intestine (Sire & Vernier 1992). The absorption capacity of the intestine is therefore dependent both on the available surface area and the surface density of carrier molecules. In Atlantic salmon, passive diffusion seems

to account for the majority of uptake of amino acids and peptides (Bakke-McKellep *et al.* 2000), and intestinal surface area thus could be a determining factor for amino acid uptake. A prominent feature of the salmonid digestive system is the pyloric caeca. These are blind diverticulae in the most proximal part of the intestine, and increase the absorption surface of an otherwise comparatively short intestinal tract (Buddington & Diamond 1987; Buddington *et al.* 1987; Bakke-McKellep *et al.* 2000). More than half of the dietary protein is digested and absorbed here (Krogdahl *et al.* 1999).

The pyloric caeca contributes most of the intestinal length in Atlantic salmon (Bakke-McKellep *et al.* 2000), and caecal surface increases with fish size, evidenced both as an increase in caecal mass (Pringle *et al.* 1992) and in the number of pyloric caeca (Bergot *et al.* 1981). However, there does not seem to be a direct relationship between the surface area of the pyloric caecal tissue and the efficiency of nutrient absorption (Carter *et al.* 1993b). In fact, *in vivo* studies on farmed Atlantic salmon indicated that only 59% of the amino acid N in commercial salmon feed is digested and absorbed by the end of the proximal intestine (Krogdahl *et al.* 1999). This is in contrast to the estimated N absorption capacity of the pyloric caeca of 84-92 %, calculated from *in vitro* studies of amino acid and peptide absorption (Bakke-McKellep *et al.* 2000). This discrepancy perhaps only illustrates the complex interaction between dietary components of composite feedstuffs during *in vivo* digestion, but could also suggest that the capacity of digestive proteolysis, rather than surface area available for absorption can influence or even limit amino acid and peptide absorption during high dietary loads of protein. For instance, positive correlations between trypsin activity, specific growth rate and feed conversion efficiency were found in cod, *Gadus morhua* L., when growth was manipulated through growth hormone injection and fish were given free access to feed (Lemieux *et al.* 1999).

1.3 The pancreatic digestive proteases

1.3.1 The serine proteases

Trypsin and chymotrypsin belong to the serine protease family of pancreatic proteases, a family characterised by a unique highly conserved catalytic amino acid triad (histidine, aspartic acid and serine) (Stryer 1988). The serine protease family is characterised by strong conservation both structurally and functionally in a range of organisms, from bacteria to mammals. Thus, although amino acid sequence identity between trypsin and chymotrypsin can be as low as 40%, they show almost identical tertiary structures (Bartunik *et al.* 1988). Substrate specificity however, differs significantly between the two. Other serine proteases include carboxypeptidase A and B and elastase (Figure 1.1).

1.3.2 Trypsin synthesis and secretion

Among the pancreatic proteases, trypsin is of particular importance as it serves the crucial function of activating the other proteases during the digestion process (Figure 1.2). The development of trypsin activity in early life stages of fish is therefore commonly used as an indicator of the development of the digestive system and exogenous feeding activity in larvae (*e.g.* Infante & Cahu 1993; Ueberschär 1993). Pancreatic protease synthesis in Atlantic salmon is located in pancreas-like tissues associated with the fat surrounding the pyloric caeca (Einarsson & Davies 1996), as salmonids lack a well-defined pancreatic gland. The exocrine acinar cells are evenly distributed throughout the pyloric caecal tissues and the surrounding fat (Pringle *et al.* 1992; Einarsson & Davies 1996), and synthesised proenzymes are transferred through the pyloric caecal tissues via a multiductal system (Einarsson & Davies 1997). The zymogens are stored in secretory tissue in the pyloric caecal walls before they are secreted to the lumen during digestion. In mammals, synthesis and secretion of pancreatic digestive zymogens is governed in a large part by cholecystokinin (CCK), a hormone-like peptide (Liddle 1995, 2000). In salmon, CCK is produced in

cells scattered among the epithelial cells lining the intestine (Einarsson *et al.* 1997a), and secretion is stimulated by the presence of digestion products of both fat and proteins (Liddle 2000). In addition, amino acids such as tryptophan (Trp) and phenylalanine (Phe) (Liddle 2000) as well as lysine (Lys) (Grendell & Rothman 1981) all stimulate trypsin secretion from mammalian acinar cells. Evidence suggests that CCK secretion is regulated by a negative feedback mechanism involving trypsin, and that CCK release is stimulated only when digestion of a CCK-releasing factor by trypsin is outcompeted by feed proteins (Liddle 1995, 2000).

The secreted inactive trypsinogen is converted to active trypsin by removal of a short amino acid sequence from its carboxylic end by cleavage between a Lys or Arg residue and an Ile residue (Light & Janska 1989). This step is facilitated by either enterokinase or already activated luminal trypsin. Once trypsin is activated, it subsequently triggers a cascade of activation of other proenzymes (Figure 1.2).

1.4 Trypsin, growth and feed utilisation

1.4.1 The principle of isozymal variation

During their lifetime, most organisms experience large variations in their physical as well as nutritional environment. The presence of an enzyme in multiple varieties, *i.e.* isozymes (enzymes that perform the same functions, but are structurally different), can provide the organism with the necessary metabolic flexibility to meet the challenges caused by an ever-changing environment. This flexibility may seem particularly important for anadromous fish species, faced with the 'ultimate' challenge of making the transition between fresh and seawater at two critical stages in their lives: after smoltification and during spawning. This radical change of environment not only poses metabolic challenges related to osmoregulation, but is accompanied by changes in available prey organisms. This may lead to changes in foraging behaviour as well as to the digestive system.

1.4.2 Isozymal variation in digestive enzymes

The importance of digestive proteases in providing metabolic flexibility is reflected in the variety of digestive protease expression seen in a wide range of animal species. In fish, the existence of multiple trypsin digestive enzymes have been described in Atlantic salmon (Torrissen 1984, 1987; Male *et al.* 1995), rainbow trout, *Oncorhynchus mykiss* (Torrissen 1984; Kristjánsson 1991), Arctic charr, *Salvelinus alpinus* (Torrissen & Barnung 1991), chum salmon, *Oncorhynchus keta* (Uchida *et al.* 1984), capelin, *Mallotus villosus* (Hjelmeland & Raa 1982), cod, *Gadus morhua* (Ásgeirsson *et al.* 1989; Raae & Walther 1989), Japanese flounder, *Paralichthys olivaceus* (Suzuki *et al.* 2002), and Japanese anchovy, *Engraulis japonicus* (Ahsan *et al.* 2001; Ahsan & Watabe 2001). A similar variation in chymotrypsin-like digestive proteases is found in species such as rainbow trout (Kristjánsson & Nielsen 1992), cod (Raae & Walther 1989) and Japanese flounder (Suzuki *et al.* 2002). The advantage of several coexistent trypsin enzymes can be to provide an arsenal of enzymes with different catalytic properties (Ásgeirsson *et al.* 1989; Dimes *et al.* 1994; Outzen *et al.* 1996) or different substrate specificity. In poikilotherm organisms like fish, and particularly those inhabiting cold marine environments, trypsin enzymes show considerably higher catalytic efficiencies than their mammalian counterparts (Ásgeirsson *et al.* 1989; Taran & Smovdyr 1992; Outzen *et al.* 1996; Ahsan & Watabe 2001); a possible evolutionary response to increase substrate binding at low temperatures. Adaptation to increased efficiency at lower temperatures may however, have serious repercussions for other enzyme properties, such as stability at higher temperatures (Ásgeirsson *et al.* 1989; Dimes *et al.* 1994; Outzen *et al.* 1996) or low pH (Outzen *et al.* 1996).

1.4.3 Trypsin isozyme expression and feed utilisation

Protein digestion, and in particular protein digestion by trypsin, has been proposed by several authors to be a potentially limiting factor of growth rate and feed utilisation in fish (Torrissen & Shearer 1992; Blier *et al.* 1997; Lemieux *et al.* 1999;

Rungruangsak-Torrissen *et al.* 1999; Rungruangsak Torrissen & Male 2000; Rungruangsak-Torrissen *et al.* 2005). This hypothesis is based in part on the substantial amount of work that has accumulated on the association of specific genetic variation in trypsin-like enzymes ("trypsin isozymes"[1], Torrissen 1987) with increased growth rate and feed utilisation in Atlantic salmon, *Salmo salar* L. in aquaculture (for a review, see Rungruangsak Torrissen & Male 2000). The possession of specific trypsin isozymes may improve feed utilisation at low water temperatures (Torrissen & Shearer 1992; Rungruangsak-Torrissen *et al.* 1998) or improve utilisation of feed proteins of low digestibility (Bassompierre *et al.* 1998). The specific growth-promoting trypsin isozymes have however yet to be isolated and identified, and their specific kinetic properties have not been characterised.

Attempts at selective breeding for specific trypsin expression patterns have revealed a complex non-Mendelian pattern of inheritance (Torrissen *et al.* 1993). This opens the possibility that the observed patterns may result from different post-translational modifications of the same trypsin enzymes. However, trypsin patterns seem to be inducible during specific 'windows of opportunity' during development by either environmental or dietary factors. The relative frequency of expressed trypsin isozymes was for instance affected by start feeding temperature in Atlantic salmon parr (Rungruangsak-Torrissen *et al.* 1998). These induced expression patterns are apparently retained throughout the life cycle, even though environmental conditions and diet change at a later stage (Torrissen 1987, 1991; Rungruangsak Torrissen & Male 2000; Rungruangsak-Torrissen & Sundby 2000).

1.4.4 Factors affecting digestive protease activity

Trypsin activity is influenced by a range of factors, *e.g.* fish size (Pringle *et al.* 1992; Torrissen *et al.* 1994; Sveier *et al.* 2000; **paper II**), time after feeding (Pringle *et al.*

[1] Note: The term 'trypsin isozyme' is used throughout this thesis for readability purposes and denotes enzymes with trypsin-like activity that are resolved based on differences in physiochemical properties. This expression does not mean that these enzymes can be detected as separate trypsin genes.

1992; Torrissen *et al.* 1994; Einarsson *et al.* 1996; **paper I**), seasonal variation (Torrissen & Torrissen 1985; Einarsson *et al.* 1997b), nutritional status (Torrissen & Torrissen 1985; Torrissen *et al.* 1994; Rungruangsak-Torrissen *et al.* 2005), water temperature (Einarsson *et al.* 1997b; Rungruangsak-Torrissen *et al.* 1998) and dietary trypsin inhibitor content (Krogdahl *et al.* 1994; Olli *et al.* 1994; Sveier *et al.* 2001; Krogdahl *et al.* 2003). Most studies of digestive enzymes in fish have however focused on the effects of feeding on pre-starved individuals or groups (*e.g.* Pringle *et al.* 1992; Carter *et al.* 1993b; Torrissen *et al.* 1994, 1995; Einarsson *et al.* 1996) or on the effects of feed protein replacement by alternative sources (Krogdahl *et al.* 1994; Olli *et al.* 1994; Krogdahl *et al.* 2003). In contrast, comparatively little research has been carried out on other digestive proteases such as chymotrypsin (Rungruangsak & Utne 1981; McLeese & Stevens 1982; Pringle *et al.* 1992; Rungruangsak-Torrissen & Sundby 2000). Chymotrypsin exhibits a high activity in the pyloric caeca of Atlantic salmon (Rungruangsak Torrissen & Male 2000; **paper I**; **paper II**), and may play an important role in the early stages of larval development, as observed in red drum, *Scieanops ocellatus* (Applebaum *et al.* 2001). The present study focuses on the relative contributions of trypsin and chymotrypsin activity to digestive protease activity in post-smolt Atlantic salmon and investigates the effect of environmental and dietary factors and growth rate on both trypsin and chymotrypsin specific activity.

1.4.5 Links between trypsin specific activity, growth and feed utilisation

In aquaculture, the expression of a specific trypsin isozyme ('TRP-2*92', Torrissen 1987) is associated with higher growth rates and feed utilisation in several salmonid species (Torrissen 1987, 1991; Torrissen & Barnung 1991). There is evidence suggesting that the improved growth characteristics of these phenotypes are linked to differences in post-prandial FAA absorption (Torrissen *et al.* 1994) and insulin response following feeding (Rungruangsak-Torrissen *et al.* 1999). However, whether this is coupled with an increased ability to absorb feed nitrogen is less clear. One

study was unable to detect differences in apparent digestibility between phenotypes, at least when highly digestible aquaculture feeds were used (Torrissen & Shearer 1992). Alternatively, the increased feed conversion efficiency can be brought about by an increase in protein utilisation efficiency, which has been observed in several studies (Torrissen & Shearer 1992; Rungruangsak-Torrissen *et al.* 1998). A lower maintenance protein synthesis rate may in addition reduce the metabolic cost of protein accretion (Rungruangsak-Torrissen *et al.* 1999).

Hormonal peri- and post-prandial responses could be affected by the rate and pattern of appearance of digestion products in the blood stream, thereby causing differences in protein metabolism and conceivably appetite regulation mechanisms. Rungruangsak-Torrissen & Sundby (2000) showed that the possession of TRP-2*92 led to a more rapid insulin response after feeding, apparently caused by a more rapid absorption of certain amino acids (Torrissen *et al.* 1994), some of which are known to have strong insulinotropic properties (Plisetskaya *et al.* 1991). *In vitro*, the rate and pattern of amino acid release during enzyme digestion was modulated by the ratio between trypsin and chymotrypsin activity (Kristinsson & Rasco 2000). Further, FAA absorption patterns *in vivo* were affected by trypsin expression patterns (Torrissen *et al.* 1994, 1995), trypsin inhibition (Sveier *et al.* 2001) and the activity ratio of trypsin to chymotrypsin (Rungruangsak-Torrissen & Sundby 2000). Modulation of FAA absorption patterns can in turn have significant effects on growth processes and protein utilisation by influencing protein metabolism, as demonstrated by Sveier *et al.* (2001).

These observations open up the possibility that a more general relationship can exist between digestive protease activities, growth rate and feed utilisation, and that digestive protease activities may have a use as an estimate of differences in growth rate and feed utilisation. However, the causal relationship between these parameters is difficult to determine. The relationship with growth rate and feed utilisation may be the *result* of specific aspects of digestive protease action that directly affect growth, or changes in digestive protease specific activity could be *caused by* changes in

growth rate and/or changes in protein metabolism and appetite mechanisms.

1.4.6 The digestion process and the concept of the anabolic drive

Following digestion of feed proteins, amino acids and short peptides are released in the gut lumen before they are absorbed across the intestinal wall and enter the blood stream through the hepatic portal vein (Ash 1985). The liver is central in amino acid metabolism and regulates the flow of FAA to other tissues. Although FAA pools of tissues are believed to be under strong regulation, mainly by protein synthesis, these pools increase after feeding (Espe *et al.* 1993a; Torrissen *et al.* 1994; Carter *et al.* 1995). This increase not only acts as substrate for protein synthetic activity; the presence of FAA in tissues is also thought to have an additional function as a stimulator of growth and anabolic processes. This effect has been termed 'the anabolic drive' (Millward & Rivers 1988). In higher vertebrates, anabolic activity and nutrient partitioning are regulated by complex interactions between feeding, the appearance of digestion products in the blood stream and endocrine secretions, the most central of the latter being thyroid hormones (Leatherland 1994), insulin (Rungruangsak-Torrissen *et al.* 1999) and growth hormone (Reddy & Leatherland 1995). For more detailed reviews of endocrine regulation in fish, see *e.g.* Leatherland (1994) and Björnsson (1997). Several protein digestion products can conceivably act as 'trigger' molecules to facilitate the anabolic drive; aromatic amino acids are for instance precursors of important neurotransmitters (Millward & Rivers 1988; Millward 1989) and tissue concentrations may influence the rate of synthesis of these molecules (Pogson *et al.* 1986). Further, a direct endocrine response to the injection of specific amino acids has been demonstrated in fish. Arginine administration in salmonids stimulated both insulin secretion (Gutiérrez & Plisetskaya 1991; Plisetskaya *et al.* 1991) and the insulin binding capacity of the liver (Gutiérrez & Plisetskaya 1991). Arginine injection in brown trout (*Salmo trutta*) also decreased the extraction of glucagon and insulin by the liver (Carneiro *et al.* 1993), whereas glutamine injection reduced protein catabolism in mice (MacLennan *et al.* 1987).

1.5 Growth and protein metabolism

1.5.1 Free amino acid metabolism

Measuring plasma EAA profiles is a common technique in studies of feed protein utilisation that has been applied in several fish species (Kaushik & Luquet 1979; Ogata 1986; Walton & Wilson 1986; Espe *et al.* 1993a; Torrissen *et al.* 1994, 1995; Shuhmacher *et al.* 1995, 1997; Gunasekara *et al.* 1997; Yamamoto *et al.* 1998). There is often a high correlation between feed EAA composition and plasma EAA profiles, and there seems to be a preferential absorption of EAA during the first 24 hours following feeding (Espe *et al.* 1993a; Torrissen *et al.* 1994; Carter *et al.* 2001). However, values show large variation between experiments and between species, probably due to a complex interaction of external and internal variables (Carter *et al.* 2001). Tissue EAA profiles have similarly been used as a tool for determining nutritional protein status in animals (Pion 1976) and tissue FAA pools may be employed to identify amino acids limiting to protein utilisation (Carter *et al.* 2000; Yamamoto *et al.* 2000). Tissue free EAA pools are regulated to low levels, suggesting that the accumulation of essential amino acids in tissues may have harmful effects (Millward & Rivers 1988). Protein synthesis is likely the major mechanism maintaining tissue FAA homeostasis (Houlihan *et al.* 1993; Carter *et al.* 1995).

Although changes in white muscle FAA pools are small following feeding, differences are often observed in conjunction with large physiological and metabolic changes associated with life stage transitions or changes in nutritional status. For instance, white muscle histidine and anserine concentrations differed between parr and smolts of masu salmon, *Oncorhynchus masou masou* (Ogata & Murai 1994), whereas histidine was preferentially metabolised in the white muscle of milkfish, *Chanos chanos*, during starvation (Shiau *et al.* 2001). One might therefore conceivably find measurable differences in white muscle FAA pools between fish exhibiting different protein growth efficiencies or different growth rates. For instance, free glutamine was higher in white muscle of salmon with high growth

efficiency (Torrissen *et al.* 1994), whereas intracellular glutamine concentrations correlated with protein synthesis rate in the hind limb of rat (MacLennan *et al.* 1987).

1.5.2 Growth and protein synthesis correlates in fish

In order to investigate the relationship between growth, digestive protease activities and protein metabolism, it was important to select appropriate parameters for estimating protein synthesis and breakdown. Protein synthesis and deposition represents a substantial part of total energy expenditure in fish (Houlihan *et al.* 1993; Lyndon *et al.* 1992), higher than that of the other main body constituents (lipids and carbohydrates). The rate of protein synthesis is significantly different between different body tissues, and among the lowest rates are those measured in the white muscle (McMillan & Houlihan 1989). However, white muscle (fillet) constitutes the majority of somatic tissue in fish, and protein accretion in this tissue is the primary contributor to growth of the whole organism (Carter & Houlihan 2001). There is therefore a close relationship between whole-body growth rate and protein synthesis rate in white muscle tissue (Smith 1981; Houlihan 1991; Houlihan *et al.* 1993).

Protein synthesis in fish is influenced by a multitude of factors such as growth rate (Houlihan *et al.* 1986, 1988), age (Peragón *et al.* 2001), water temperature (de la Higuera *et al.* 1997), and diet composition (Peragón *et al.* 1999). Tissue protein synthesis rate is a function of ribosomal concentration and the specific activity of these ribosomes. Ribosomal RNA constitutes approximately 85% of total tissue RNA (Pain & Clemens 1980). This has led to the widespread use of total tissue RNA as an estimation of tissue protein synthetic activity. A relationship between white muscle RNA concentration and whole-body growth rate has been established for feeding larvae and juveniles of several fish species (Houlihan *et al.* 1993). The RNA concentration can alternatively be expressed as relative to the protein content of the tissue (the ratio of RNA/protein), and is often referred to as the tissue capacity for protein synthesis (Sugden & Fuller 1991), *i.e.* the theoretical maximum tissue protein synthesis rate. White muscle protein synthesis capacity has been found to correlate

positively with growth rate in young trout (Houlihan *et al.* 1993), and protein consumption and synthesis rates in salmon (Carter *et al.* 1993b). Alternatively, RNA concentrations can be expressed as relative to tissue DNA content, a factor that is considered constant in fish no longer undergoing hyperplasia (Pelletier *et al.* 1995).

1.5.3 Protein turnover rate and implications for growth and feed utilisation

Factors that have an influence on whole-body growth rate often are detected in white muscle tissue as changes in protein synthesis rate, protein breakdown rate or both. These two processes are closely linked, and the term protein turnover is used to describe the relationship between the two; in growing organisms the rate of protein turnover is defined as being equivalent to the rate of protein breakdown (Weisner & Zak 1991). Protein breakdown rate can however be difficult to quantify and is usually estimated as the difference between protein accretion and synthesis rates (Houlihan 1991). The retention efficiency of synthesised proteins is variable; in salmonids, estimates range from 23-62% (Houlihan *et al.* 1995; Owen *et al.* 1999). The large variability in this parameter could be the reason behind the large variation seen in individual protein growth efficiency (Carter *et al.* 1993a, 1993b, 1998). In addition to protein synthesis, breakdown of body protein represents an energetic cost to the animal (Hawkins 1991), and reducing this cost by reducing protein turnover may be advantageous to protein growth efficiency (Carter *et al.*1993b). A total reduction in the cost of protein accretion may be achieved by reducing both protein synthesis and turnover rates (Carter *et al.* 1993a, 1993b, 1998, 2000; McCarthy *et al.* 1994), although a minimum (maintenance) rate of protein synthesis is considered necessary for the organism in order to replace the continuous loss of proteins through *e.g.* shedding of epithelial cells of the skin and intestine, and nitrogen loss through gill excretion (Houlihan 1991). Individual variation in protein turnover rates can have long-term impacts on growth rate and feed conversion efficiency (Carter *et al.* 1998) and has been linked to variation in trypsin expression in salmon (Rungruangsak-Torrissen *et al.* 1999).

Millward (1989) suggested that the limiting step in the growth of white muscle tissue might be the remodelling of the structural framework. Collagen is a major component of the intramuscular connective tissue in fish muscle, in salmon comprising about 0.66 % of wet weight (Eckhoff *et al.* 1998), and consists of the amino acids proline and its hydroxylated form hydroxyproline. Whereas the essential amino acid proline can be of dietary origin, it can also originate from breakdown of body proteins. The appearance of hydroxyproline in free form in plasma or muscle tissue, however, can indicate mobilisation of collagen (Torrissen *et al.* 1994; Toyohara *et al.* 1997). Free hydroxyproline concentrations may therefore be proportional to the relative rate of protein breakdown in the tissue. Mobilisation of white muscle collagen was for instance different between maturing and non-maturing male and female ayu, *Plecoglossus altivelis* (Toyohara *et al.* 1997) and indicated that Hyp can be utilised as an energy source under conditions of high metabolic demand, as described by van Waarde (1988).

2. Aims of the study

The aims of this study were:

- to examine the variation of proteolytic activity in the pyloric caeca, quantified as the specific activities of trypsin and chymotrypsin, as well as the activity ratio between these enzymes, with growth rate and feed utilisation in Atlantic salmon reared under different conditions (**paper I**; **paper II**)
- to determine to what extent digestive proteolytic activity, as specified above, was affected by dietary protein quality (**paper II**)
- to analyse covariation of digestive proteolytic activity and FAA concentrations in plasma and muscle tissue and the protein synthetic capacity of white muscle (**paper I**; **paper II**)
- to test the initial hypothesis that digestive protease activities might affect growth through controlling the supply of amino acids available for protein synthesis (**paper I**; **paper II**) or by modifying protein turnover
- to assess the effects of feed processing conditions on protein *in vitro* digestibility and correlate it with results from growth studies (**paper III**)
- to evaluate feed protein quality by measuring plasma FAA concentrations in cannulated fish, including the effects of variation in feed intake (**paper IV**)

3. Results and discussion

3.1 Methodological considerations

3.1.1 Experimental design and statistical treatment

By collecting data from a range of fish sizes (**paper II**), fish with different growth rates (**paper I**), fish fed different protein qualities (**paper II**) and fish held under different environmental conditions (**paper I**), multivariate analysis was made possible of the measured parameters and their co-variation with digestive protease activities and growth rate. This analysis aimed at identifying factors of importance for growth rate and feed utilisation, in particular whether changes in digestive protease activity related to changes in other parameters (**paper I**; **paper II**), how digestive protease activity related to long-term growth and feed utilisation (**paper I**; **paper II**), how protein quality (**paper II**) and photoperiod (**paper I**) affected digestive protease activities, how protein quality affected feed *in vitro* digestibility **(paper III**), and how differences in feed intake and feed quality affected amino acid uptake (**paper IV**). Analysis of hormonal and genetic factors was thus outside the scope of this study.

Paper I used a screening-type experimental design without replicate groups. Photoperiod and fish ploidy were factors in a 2×2 matrix design, aiming for a sample population with a wide range of growth rates, feed intake and feed utilisation. A sub-sample of individuals were selected and tagged within each un-replicated experimental group. The use of ANOVA or similar statistical tests that assume sample independence (Sokhal & Rolf 1995) to test for treatment effects between such un-replicated groups is statistically invalid, and commonly referred to as pseudo-replication (Hurlbert 1984). One way of circumventing this problem could be to treat triploid groups as diploid replicates, a reasonable assumption considering the similar growth performance of diploids and triploids in seawater (Galbreath & Thorgaard 1995; McCarthy *et al.* 1996). The analysis thus can be reduced to a one-way ANOVA

with photoperiod as the only treatment. Comments on the effect of both treatments in **paper I** can only be considered speculation and is treated as such in this thesis. However, the experimental group nomenclature from **paper I**, based on the treatments received by each experimental group, is carried on in this text. The reader is advised not to draw his/her own conclusions on treatment effects based on this nomenclature. The principal component analysis (PCA) and correlation analysis treated all groups as similar, assuming that appropriate indicators of growth rate would show covariation with growth rate, regardless of treatment (**paper I**).

In **paper II,** classical feeding trials were set up with either three (Experiment 1) or two (Experiment 2) replicates for each dietary treatment. This allowed a statistical evaluation of the effect of each of the two main feed processing parameters, as well as their interaction on the measured parameters using a two-way ANOVA (Sokhal & Rolf 1995). In order to elucidate the effects of inter-individual and day-to-day variation in feed intake, the experiments in **paper IV** were specifically designed to allow for statistical correction for these factors by employing individual measurements of feed intake and repeated treatments on the same individual, and a general linear model analysis (*proc glm*, SAS Institute 1993) was employed to construct least squares means plasma FAA profiles. A similar approach was applied to further analyse individual plasma and muscle FAA data from **paper I** and **paper II** (see sections 3.2.4. and 3.3.4.).

3.1.2 Feed intake measurements

Due to the large scale of most trials in this study, individual feed intake measurements were not performed in **paper I**, **paper II** & **paper III**. This unfortunately lessened the quality of the data acquired, as we were unable to quantify the effect of feed intake on the measured parameters. Ration size affects a range of physiological responses to feeding, such as *e.g.* protein synthesis rate (Lyndon *et al.* 1992; Carter *et al.* 1993b) and plasma FAA concentrations (Carter *et al.* 2000; **paper IV**). In contrast, the effects of ration size on digestive enzyme activities are less clear.

Lemieux *et al.* (1999) found a correlation between chymotrypsin specific activity and feed intake in GH-treated cod, whereas trypsin specific activity was unrelated to feed intake. McLeese & Stevens (1982) on the other hand, reported no effect of ration size on either trypsin or chymotrypsin specific activity in rainbow trout given two ration levels. Individual feed intake measurements ought to be performed where feasible to properly quantify the contribution of this factor to the measured parameters. Non-invasive methods utilising radiography (Talbot & Higgins 1983) can be performed with minimal disturbance, but add to the experimental cost by requiring specially prepared feeds containing radio-opaque particles, such as lead glass 'Ballotini' beads (*e.g.* McCarthy *et al.* 1993) or iron powder (*e.g.* Talbot & Higgins 1983).

A complicating factor in estimating feed intake was the use of sea cages in Experiment 2 in **paper II**. Whereas experiments usually are performed in indoor tanks in order to have control over several environmental factors, rearing fish in sea cages is the industry norm and was therefore chosen for comparison. However, in an open system affected by currents and other variables, accurate feeding data can be difficult to obtain. Even though methods are developed that utilise sensory feedback mechanisms to regulate the distribution of feed (*e.g.* Juell 1991; Juell *et al.* 1993), they are in limited use in research, due to their high investment and maintenance cost. Feed waste was therefore kept minimal by feeding *ad libitum*. However, as there was no available means of registering feed waste in this setup, we cannot exclude that it occurred. The data obtained therefore probably represent an overestimation of the actual feed intake in these cages. However, growth and feed conversion in this experiment compared well with reference values from similarly sized fish reared at the same temperatures (Austreng *et al.* 1987).

3.1.3 Protease extraction

Pyloric caeca with contents were removed by incisions anterior and posterior to the pyloric caeca, separating this tissue from the oesophagus and stomach. However, pancreatic tissue in salmon is not limited to the sampled section. Munro *et al.* (1984)

detected additional pancreatic tissue around the stomach, bile duct, anterior spleen and upper intestine, and it is not yet clear whether these cells represent a significant contribution to total pancreatic digestive enzyme activity. The stomach was inspected to exclude individuals with empty stomachs and a high incidence of intestinal parasites from the sample material, as this could affect measurements.

Enzymes were extracted by homogenising the sample in 5:1 (w/v) 1 mM HCl according to Rungruangsak & Utne (1981). The enzyme extracts used in the *in vitro* digestion study (**paper III**) deviated from this procedure, instead using pH 8.0 Tris-buffer (adopted from Bassompierre *et al.* 1998). However, in both cases specific activity was measured under basic assay conditions (pH 8.2), in the presence of Ca^{2+}. Of a total of five types of trypsin identified in Atlantic salmon, four are negatively charged (anionic) and one is positively charged (cationic) (Male *et al.* 1995). Outzen *et al.* (1996) characterised three anionic and one cationic variant from salmon in detail and found the anionic variants to be unstable at low pH. Thus, extracting trypsin from salmon pyloric caeca at low pH may render some or all of the anionic variants inactive and result in an underestimation of enzyme specific activity. However, trypsin-specific staining of caecal extracts from Atlantic salmon after isoelectric focusing (IEF) indicated residual trypsin specific activity for both cationic and anionic variants even after acid extraction (Rungruangsak Torrissen & Male 2000), and a comparison of acidic and alkaline caecal extracts suggests that inactivation, if it takes place, may be negligible or restored under basic assay conditions (Rungruangsak-Torrissen & Male 2000). This assumption is supported by the consistency between the results of the *in vitro* (**paper III**) and growth studies (**paper II**), where enzymes were extracted under both basic and acidic conditions, and differences measured *in vitro* correlated with observed differences *in vivo*.

3.1.4 Trypsin and chymotrypsin assay conditions

Trypsin and chymotrypsin specific activities were determined as their amidase activity at 50 °C and 40 °C, respectively, using enzyme-specific substrates, a

principle first described by Erlanger *et al.* (1961). These assay temperatures are significantly higher than what are commonly used in measurements of trypsin and chymotrypsin specific activity in fish (McLeese & Stevens 1982; Stevens & McLeese 1988; Pringle *et al.* 1992; Dimes *et al.* 1994; Einarsson *et al.* 1996; 1997a; 1997b; Outzen *et al.* 1996; Ahsan & Watabe 2001), but corresponds well with the peaks of proteolytic activity (52.5 ºC and 45 ^{o}C; Torrissen 1984) and trypsin- and chymotrypsin-like activity (Rungruangsak Torrissen & Male 2000) in the gastrointestinal tract of Atlantic salmon. Purified extracts of trypsin and chymotrypsin from rainbow trout show peak activities at even higher temperatures, 60 ºC and 45 °C, respectively (Kristjánsson 1991; Kristjánsson & Nielsen 1992).

An increase in assay temperature increases the specific activities of both trypsin and chymotrypsin, in both rainbow trout (McLeese & Stevens 1982; Stevens & McLeese 1988; Dimes *et al.* 1994) and Atlantic salmon (Torrissen 1984; Outzen *et al.* 1996; Kristinsson & Rasco 2000; Rungruangsak Torrissen & Male 2000) but at the same time can lead to an increase in denaturation and autolysis (Dimes *et al.* 1994; Outzen *et al.* 1996; Ahsan & Watabe 2001). However, the experimental protocols used in most denaturation studies involve pre-incubation of enzyme extracts for extended periods before activity is measured, and may be irrelevant for our discussion seen in relation to the short reaction times used in our assay (**paper I**; **paper II**; **paper III**). For instance, the loss of activity after incubation of the enzyme extract for 30 minutes at temperatures above 40 °C was dramatic both for trypsin and chymotrypsin (Figure 3.1A). In contrast, when a shorter assay time of 10 minutes was used, measured activity was higher and activity peaked at 50 °C and 40 °C for trypsin and chymotrypsin, respectively (Figure 3.1B). It is therefore reasonable to assume that the effect of temperature on enzyme denaturation was negligible in the current studies, where the incubation at elevated temperatures was even shorter (90 seconds).

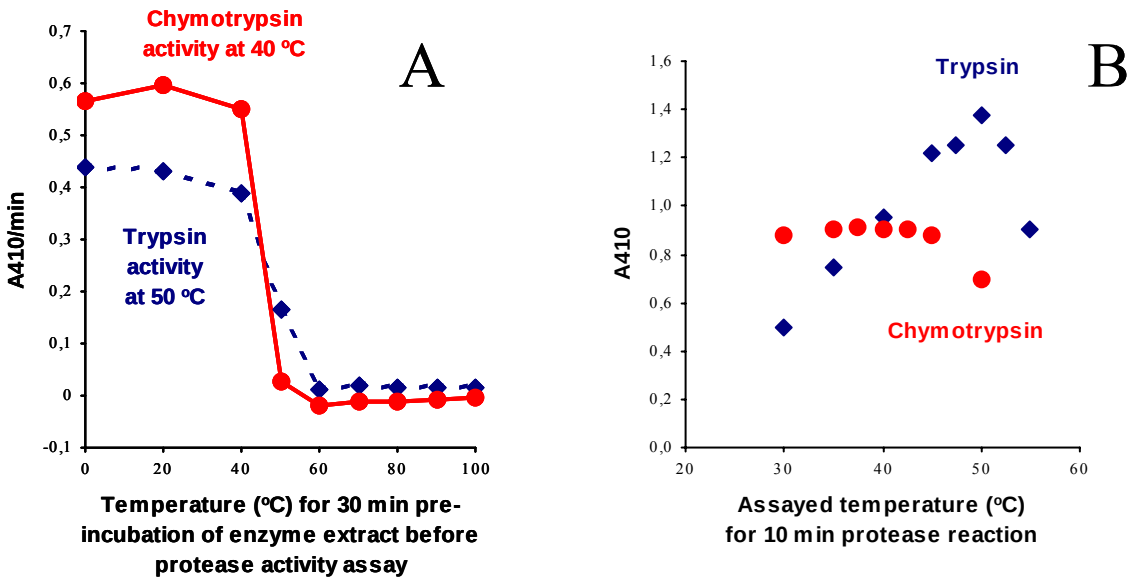

Figure 3.1: (A) Initial specific activity of trypsin and chymotrypsin (A_{410}/min) in Atlantic salmon pyloric caecal extract (1mM HCl) after 30 minutes pre-incubation at different temperatures before assaying (Rungruangsak-Torrissen K., unpublished data), and (B) specific activity of trypsin and chymotrypsin (A_{410}) as a function of assay temperature measured after stopping the enzyme reaction after 10 minutes (adapted from Rungruangsak Torrissen & Male 2000). In both cases, trypsin and chymotrypsin specific activities were measured as the rate of appearance of *p*-nitroaniline (λ_{max}= 410 nm) using enzyme-specific synthetic substrates as described in **paper I**.

3.1.5 Interpreting measured digestive protease activity

The assays used for measuring enzyme activities in this study (**paper I**; **paper II; paper III**) do not differentiate between the active enzymes and their inactive proenzyme forms (trypsinogen and chymotrypsinogen). Activation of proenzymes is likely to occur during either the extraction procedure or assaying due to the presence of active trypsin in the extract. All measurements therefore might be interpreted as the 'potential proteolytic activity' of the fish at the time of sampling. However, regarding measured differences in enzyme activity strictly as differences in enzyme secretion/synthesis may not be the only possible interpretation. An alternative explanation may be that measured differences reflect a shift to production of other isozymal variants with different kinetic or structural properties that are detectable at the high assay temperatures used in this study. Outzen *et al.* (1996) found significant

differences in thermal and pH stability between trypsin variants isolated from rainbow trout, and several enzymes isolated from fish exhibit properties typical of cold adaptation, *i.e.* reduced temperature and pH stability. This renders fish enzymes particularly sensitive to thermal breakdown. Small differences in thermal stability or associated differences in kinetic properties may be accentuated under high-temperature conditions, and could make the enzyme assay used in the present study (**paper I**; **paper II**) an indicator of changes in expressed isozyme properties.

Analytical data in this study were expressed either as units (molecular or weight) per wet weight tissue or as substrate-specific proteolytic activity on a protein basis. The latter denotation required an additional step for determination of protein concentration in the extract. When enzymes were extracted for this study, the caecal tissue was excised and homogenised together with the luminal contents. This process makes the expression of enzyme activity on protein basis problematic. When expressing substrate-specific enzyme activity as activity in this manner, changes in extract protein content that are unrelated to changes in enzyme concentration, *e.g.* undigested and partly digested feed proteins, could lead to a lower measured enzyme activity in the sample. However, this will not affect enzyme activity expressed as the ratio between trypsin- and chymotrypsin-specific activities (the T/C ratio). This parameter has the advantage of being independent of an external reference.

Immunological techniques such as ELISA (Enzyme-Linked Immuno Specific Assay), or measuring mRNA expression specific to trypsin and chymotrypsin would be more appropriate methods of measuring changes in enzyme activity related to enzyme secretion and activation. Alternatively, digestive enzyme activity may be expressed as total enzyme activity in the sample (activity units in total extraction volume), as activity relative to intestinal weight or as enzyme activity relative to body weight. In fish larvae during development, the maturation of the digestive system was only detectable when pancreatic enzyme activity was expressed as an intestinal segment specific basis, due to changes in larval protein content (Zambonini Infante *et al.* 1996; Ma *et al.* 2005).

The methodology for determining digestive enzyme activities varies between the works referred to in this thesis. Enzyme activity was for instance determined in pyloric caecal tissue (Pringle *et al.* 1992; Carter *et al.* 1993b; Torrissen *et al.* 1994; Rungruangsak Torrissen & Male 2000; Rungruangsak-Torrissen *et al.* 2005), in caecal contents (*e.g.* Torrissen *et al.* 1994), caecal tissue with contents (Rungruangsak Torrissen & Male 2000; **paper I**; **paper II**; Rungruangsak-Torrissen *et al.* 2005) or in faeces (Krogdahl *et al.* 1994; Olli *et al.* 1994; Rungruangsak-Torrissen *et al.* 2004). This must be taken into account when results are discussed.

3.1.6 Free amino acid measurements

A large variability exists in reported data on peak plasma concentrations of single AA, probably due to a complex interaction of several internal and external factors (Walton & Wilson 1986; Espe *et al.* 1993a; Torrissen *et al.* 1994, 1995; Carter *et al.* 2000). In the current studies, plasma samples were either taken at a specified time (6 h) after feeding (**paper IV**), 6 h after feeding with pre-starvation (**paper II**) or on days following regular feeding, *i.e.* from 18 to 26 hrs after the last meal (**paper I**, **paper II**). The variability in sampling time in the last case was a result of practical problems related to the scale of the experimental setup. The effect of feeding time (or nutritional status) on FAA concentrations is reflected in our results; samples taken from regularly feeding fish were different from plasma samples taken 6 h after feeding following three days of starvation (**paper II**, see section 3.3.7. for further discussion).

3.1.7 The Lowry method

The most accurate method available for determining the protein content of a sample is to acid hydrolyse the tissue or solution and run amino acid analysis of the hydrolysate. However, the costs of amino acid analyser equipment and analysis are high and the analysis is time consuming and complex. This has led to the development of less accurate, but inexpensive and rapid colorimetric methods. Most

colorimetric methods for protein determination are based on the principle of a reaction between one or more amino acid residues in the protein and a chromophoric compound. Protein concentration can subsequently be determined by spectrophotometry, *i.e*. by measuring the absorption of light at specific wavelengths. Light absorption by the sample will be linearly proportional to the protein content of the samples within the limitations of Beer's law (Walker 2000). A frequently cited method for protein determination is the Lowry method (Lowry *et al.* 1951). In an alkaline medium, peptide bonds in the protein reduce divalent copper ions to Cu^{+}, which reacts with the Folin reagent (the biuret reaction). The sensitivity of the reaction is increased by the addition of the Folin-Ciocalteau reagent. This poorly understood reaction reduces phosphomolybdotungstate to heteropolymolybdenum blue by a copper-catalyzed oxidation of aromatic amino acid residues (Lowry et al. 1951; Layne 1957). The Lowry method was used to measure protein content in the pyloric caecal extracts (**paper I**; **paper II**; **paper III**) and also for calibration against protein UV absorption at 280 nm (**papers I**; **paper II**). Certain limitations apply when this method is used in the analysis of complex protein mixtures such as tissue homogenates. The formation of the coloured complex is dependent on the number of peptide bonds in the protein as well as the content of aromatic amino acid residues, and thus will be dependent on protein amino acid composition. The choice of appropriate standard proteins is therefore important to avoid over- or underestimation. Due to the higher cost of production, or perhaps due to less demand, no single standard protein of marine origin has been proposed. Instead standard curves are constructed using mammalian proteins of known composition, *e.g.* bovine serum albumin. We consider this estimation as being of sufficient accuracy for our purposes, but are aware that few studies have actually been carried out on the subject.

3.1.8 Protein determination by UV absorption

The absorption of ultraviolet (UV) light by proteins is dependent on their content of the aromatic amino acids tryptophan (Trp) and tyrosine (Tyr), and to a lesser degree the number of phenylalanine (Phe) and cystine (Cys-Cys) residues (Gill & von

Hippel 1989). Protein concentration in the muscle tissue was determined after ultrasonic homogenisation of the muscle sample in the TRIzol® reagent (Life Sciences, NY, USA) and later isolation of the protein fraction (**paper I**; **paper II**). Other compounds with similar absorption maxima may interfer with measurements. In particular, contamination with nucleic acids can be critical, since extinction coefficients are ten times higher than for amino acids. Protein was isolated from the lower organic phase, while RNA was dissolved in the upper aqueous phase after TRIzol® extraction. DNA was distributed in the intermediate layer between the two phases. This made contamination with either nucleic acid avoidable. Drying and re-dissolving the protein pellet after isolation were steps that may be problematic and could have contributed to the low recovery of bovine serum albumin (BSA) after extraction (69.0 ± 1.0 %; **paper I**). Recovery was however stable and showed a linear response across a wide range of protein concentrations. A comparison between the two methods of protein determination (Lowry and UV) showed similar values with our sample material, and a linear response within the range of the sample protein concentrations. Muscle protein values in the current study were however lower (9-14%) than previously reported values from salmon white muscle (between 15-20% by weight, Jobling 2001). These low values might be caused by a low recovery of protein during the analysis. Later measurements of rainbow trout and salmon white muscle protein concentrations in our laboratory using the same TRIzol® reagent, were around 20 % (Rungruangsak-Torrissen *et al.* 2004), and could suggest that the low values in the present study were due to incomplete precipitation or solution of protein isolated from the 200 µl fraction used, compared to the later modification, where only 50 µl was removed for precipitation. Incomplete dissolution of protein could lead to light diffraction in the sample.

3.1.9 RNA determination in white muscle

RNA was isolated from muscle homogenates using the TRIzol® reagent, at the same time as protein, but in the upper aqueous phase (**paper I**; **paper II**). RNA concentration was later determined by UV absorption at 260 nm (Ashford & Pain

1986). Recovery of yeast rRNA after extraction was 68.0 ± 0.7 % (**paper I**), and showed a linear correlation with start RNA concentration. Purity of the RNA isolate (amount of DNA in the sample) was tested for each sample by measuring at the dual wavelengths of 260 and 280 nm, and was within the limits considered adequate. White muscle RNA concentrations corresponded well with previous studies in rainbow trout (McMillan & Houlihan 1989) and salmon (Carter *et al.* 1993b; Rungruangsak-Torrissen *et al.* 1999). Our study did not include estimations of RNA activity, *i.e.* g synthesised protein per g RNA (Sugden & Fuller 1991), or measurements of actual protein synthesis rate using the radioisotope "flooding" method of Garlick *et al.* (1980). Carter *et al.* (1998) found short-term measurements of protein syntesis rate to correspond well with long-term growth rate and feed utilisation in *Pleuronectes flesus.* In contrast, Sveier *et al.* (2000) found short-term protein synthesis rate measurements to correspond poorly with long-term growth rates obtained in salmon feeding experiments.

3.1.10 *In vitro* digestion assay

The *in vitro* method for measuring digestibility developed in **paper III** was based on Bassompierre *et al.* (1998), but was standardised by trypsin rather than chymotrypsin activity. Comparison of caecal extracts before, during, and at the end of the experiments in **paper II** showed that increases in measured specific chymotrypsin activity *in vivo* reflected that of feed digestibility *in vitro*, whereas specific trypsin activity on the other hand remained largely constant during the experiments. The extent of protein digestion was estimated by measuring free amino groups at peptide ends (released DL-alanine equivalents) after overnight enzyme digestion at 15 °C. This approximation shows good correlation with feed protein digestibility measured in other ways (Bassompierre *et al.* 1997, 1998; Opstvedt *et al.* 2003; **paper III**). Our experimental feeds, although based on different fish meal qualities, were similar in amino acid composition, and composed according to nutritional needs of salmon (National Research Council 1993).

3.1.11 Dorsal aorta cannulation

Several studies have evaluated the long-term effects of dorsal aorta cannulation in fish, and concluded that the procedure induces minimal stress (Kiessling *et al.* 1995; Sohlberg *et al.* 1996; **paper IV**) and a minimal immunological response (Lo *et al.* 2003). Vianen *et al.* (2003) concluded that cannulation of the dorsal aorta was insufficient to quantify digestion due to the quick metabolisation of amino acids in the liver. Our results in contrast showed that post-prandial amino acid profiles could be separated in the same individual when given different feeds (**paper IV**), leading us to suggest that the single cannulation method may be suitable also for studies of digestion processes. However, a possible drawback of this technique in digestion studies lies in the difficulty of interpreting the results. For instance, diets with high levels of free or supplemented amino acids can result in high plasma FAA levels although growth and protein synthesis is low due to amino acid imbalance (Espe *et al.* 1993; Torrissen *et al*. 1995; Rungruangsak Torrissen & Male 2000). The fish may have to be pre-starved or be given low rations for plasma FAA levels to better correlate with the digestibility of the feeds. Other, perhaps more promising indicators of diet quality could be growth rate correlates that are hormonal in nature (*e.g.* Dyer *et al.* 2004). The use of dorsal aorta cannulation in metabolic studies is however still interesting due to its minimal detrimental effects on the animal and the long-term durability of the cannula (Kiessling *et al.* 1995; **paper IV**).

3.1.12 Stress during handling and sampling

Salmon are sensitive to handling, and netting alone leads to a rapid increase in blood cortisol levels (Olsen *et al.* 1995). The use of anaesthetics immediately before sampling, but after the critical procedures of netting and handling, may therefore be insufficient to avoid a stress response in the animal (**paper I**; **paper II**). An improved handling protocol was used in **paper IV** (from Kiessling *et al.* 1995), in which the fish received mild anaesthetic in the tank before netting and subsequent transfer to a stronger anaesthetic solution. This protocol is difficult to implement in open systems

like the sea cages in Experiment 2 in **paper II,** but may significantly reduce the effects of stress in experiments. The choice of benzocaine as anaesthetic (**paper I**; **paper II**) may also be questioned, when metomidate (used in **paper IV**) is proven to reduce holding stress (Kreiberg & Powell 1991) and inhibit cortisol synthesis in salmonids (Olsen *et al.* 1995), and will therefore be more suited to experimental work by reducing the impact of handling stress. The physiological effects of the stress response include mobilisation of glucogen stores and changes in gill permeability, affecting plasma osmolyte concentrations (*e.g.* Diouf *et al.* 2000). It is unknown whether any of the parameters measured in this study are significantly affected by a short-term stress response to handling.

3.2 Effects of photoperiod (paper I)

3.2.1 Photoperiod manipulation in aquaculture

The strongest environmental cue entraining endogenous rhythms in biological organisms is photoperiod (Gwinner 1986). Photoperiod is classified as a *directive* factor controlling growth (Fry 1971), meaning that it exerts its influence through a modification of the organism's response to the environment. Salmon is sensitive to changes in photoperiod both in the freshwater and seawater stage, and manipulation of day length through application of artificial light has been successfully utilised in aquaculture to increase feed intake and growth rate during winter months (Kråkenes *et al.* 1991; Hansen *et al.* 1992; Endal *et al.* 2000), and to delay the onset of sexual maturation, which is otherwise triggered by the autumnal shortening of day length (Hansen *et al.* 1992; Taranger *et al.* 1998). The physiological changes associated with the use of artificially extended day lengths are not yet fully understood (Boeuf & Le Bail 1999), and much work remains to fully elucidate the mechanisms underlying the observed changes.

3.2.2 Effects on growth and feed conversion

Fish experiencing the continuous light (LL) regime showed significantly higher growth rates than those experiencing the simulated natural photoperiod (SNP) regime during the experimental period (**paper I**). This is similar to other reports of the application of artificially extended daylengths to salmon in seawater during winter/spring (see discussion in Oppedal 2002). A seasonal trend of an increasing feed intake during the experimental period in both LL groups (results not shown here) is in line with results from Oppedal *et al.* (2003) using an identical experimental setup in the same period (late winter/early spring). In contrast, Nordgarden *et al.* (2003) observed a higher feed intake in smaller SNP salmon smolts compared to LL groups during the same spring months, also after application of LL from January. A higher FCE in LL groups was indicated in **paper I,** and differences between SNP and LL groups in plasma (**paper I**; **paper IV**) and white muscle (**paper I**) FAA pools, as well as white muscle RNA concentrations (**paper I**) suggest that differences in nutrient utilisation and possibly FCE may exist, although differences in feed intake and nutrient partitioning is probably confounding factors (see section 3.2.5). Nordgarden *et al.* (2003) reported a shift in seasonal patterns of nutrient utilisation between salmon smolts reared under SNP and LL regimes.

3.2.3 Effects on digestive enzyme activity

Few studies have investigated the effects of photoperiod on digestive protease activities, and to our knowledge no evidence supports an endogenous rhythm of secretion and/or synthesis of these enzymes. Instead studies from both fish larvae, juvenile and adult fish seem to agree that once exogenous feed intake commences, this is the predominant factor determining the secretion of both trypsin and chymotrypsin, for instance in start feeding red drum larvae (Lazo *et al.* 2000; Applebaum *et al.* 2001; Applebaum & Holt 2003). In adult salmon, feed intake has been found to affect both trypsin (Pringle *et al.* 1992; Torrissen *et al.* 1994; Einarsson *et al.* 1996) and chymotrypsin activity (Einarsson *et al.* 1996; Rungruangsak-

Torrissen *et al.* 2005), whereas only chymotrypsin activity was correlated with feed intake in Atlantic cod, *Gadus morhua* (Lemieux *et al.* 1999).

In general, the difficulties in interpreting the data from **paper I** lie in the dissociation of the specific effects of photoperiod from the effects of that of the other co-variables. For instance, the discovery of a seasonal variation in digestive trypsin activity in salmon in sea cages was attributed to seasonal patterns in growth rate, *i.e.* the influence of water temperature and feed intake, rather than changes in day length (Torrissen & Torrissen 1985; Pringle *et al.* 1992; Einarsson *et al.* 1997b). Likewise, diel variations in chymotrypsin activity in red drum larvae were attributed to photoperiod-induced feeding behaviour rather than photoperiod alone (Lazo *et al.* 2000). Growth rate is strongly correlated with feed intake (Brett 1979) and this leads us to suggest that the correlation between trypsin activity and growth rate in **paper I** rather was a correlation with feed intake, although lack of replicate groups and individual measurements of feed intake makes us unable to confirm that this was the case.

Feeding hierarchies affect feed intake of individuals and promotes size heterogeneity within a group (*e.g.* McCarthy *et al.* 1993; Alanärä & Brännäs 1996). Although large day-to-day variation in feed intake is commonly seen in fish (Carter *et al.* 2001), fish having higher growth rates within a group likely have a higher mean feed intake during the same period. Caeca weights (with content) of individual fish were strongly correlated ($r = 0.85$, $p < 0.001$) with whole body weights (Sunde *et al.*, unpublished data from **paper I**), a relationship similar to what Pringle *et al.* (1992) reported for weights of the caecal tissue (without content). Likewise, trypsin specific activities (per mg protein) were correlated with caecal weights ($r = 0.38$, $p < 0.001$), suggesting a relation between trypsin activity and stomach filling. One may suggest that individual rank within the feeding hierarchy could explain how trypsin activity measured at a fixed sample point can correspond with long-term growth rates. No similar relationship was found with caeca weights and chymotrypsin specific activity. On the other hand, Lemieux *et al.* (1999) found trypsin specific activity to correlate

with FCE, but to be independent of feed intake. Instead chymotrypsin specific activity was correlated with feed intake, but not with FCE. These observations may however have been affected by the hormonal treatments utilised in that study, as GH treatment affects nutrient partioning (Fauconneau *et al.* 1996), and affect body composition towards a leaner body mass with a higher protein to lipid ratio (Cook *et al.* 2000). The results may also indicate that this response could be species specific, as speculated by Blier *et al.* (2002).

Starvation does not affect digestive trypsin mRNA transcription in black shrimp (Lehnert & Johnson 2002). Digestive trypsin synthesis in salmon is also continued during periods of starvation (Pringle *et al.* 1992; Einarsson *et al.* 1997a; Rungruangsak-Torrissen *et al.* 2005), although secretion is reduced (Einarsson *et al.* 1996; Rungruangsak-Torrissen *et al.* 2005). There is evidence that trypsin and chymotrypsin secretion is affected differently by starvation, since chymotrypsin activity seems to increase in the pyloric caeca during feed deprivation (Rungruangsak-Torrissen et al. 2005), in contrast to trypsin specific activity, which remains constant. This can suggest that the synthesis of chymotrypsin is controlled by other factors than trypsin synthesis. In the 3n-SNP group, a significantly depressed feed intake and growth rate was coupled with a markedly lower trypsin specific activity, suggesting that the low enzyme activity in this case reflected the generally poor nutritional condition of the fish. The coefficient of variation (100 × standard deviation/mean; Jobling 1994) may be interpreted as an index of inter-individual variation, and was lower in the LL groups (pooled) for both enzyme activities and could be due to either less variation in feed intake between individuals under these conditions, or could indicate a convergence towards limit values under conditions of rapid growth.

3.2.4 Effects on FAA pools in plasma and muscle

Several studies have measured amino acid flux following feeding, the majority focusing on blood or plasma concentrations (Walton & Wilson 1986; Espe *et al.*

1993a; Torrissen *et al.* 1994, 1995; Shuhmacher *et al.* 1995; Carter *et al.* 2000). FAA pools in plasma and tissues are a result of both digestion of exogenous protein and breakdown of endogenous protein, and post-prandial changes in amino acid concentrations after feeding may therefore be the result of increases in both protein synthesis and breakdown (Lyndon *et al.* 1992, 1993). However, several studies present evidence for a preferential absorption of EAA during the first 24 h following feeding (Murai *et al.* 1987; Espe *et al.* 1993a; Torrissen *et al.* 1994, 1995; Carter *et al.* 1995), and that there is a correlation between indispensible amino acid (IAA) profiles of the feed and those of the blood or plasma (Nose 1972; Espe *et al.* 1993a; Carter *et al.* 1995; Shuhmacher *et al.* 1995).

Analysis of our data showed that changes in EAA concentrations were responsible for most of the observed changes in FAA concentrations, both in plasma and white muscle (**paper I**). This was also characteristic of other results from salmon (Espe *et al.* 1993a, Torrissen *et al.* 1994; Carter *et al.* 2001; **paper IV**). Considering that plasma free EAA sums are directly proportional to the ingested amount of protein, at least 6 h after feeding (**paper IV**), the correlations between growth rate, EAA and TFAA in **paper I** are not unexpected, if we assume feed intake was the main growth-affecting factor. Much of the reason for the correlation of these values with growth rate was however due to the significantly lower plasma EAA and TFAA concentrations and the corresponding low growth in the 3n-SNP group, suggesting that this correlation may be weaker, if existent at all, in fish in a good nutritional condition. The results from **paper II** can be read in light of this explanation.

Plasma EAA/NEAA ratios were stable across all groups (**paper I**), suggesting that neither the lack of a standardised sampling time after feeding, nor differences in feed intake had any significant effect on this parameter. Most plasma amino acid concentrations return to pre-feeding levels after 72 hours (Torrissen *et al.* 1994) and a stable feeding regime with feeding twice daily (**paper I**; **paper II**) may obscure differences related to the time of feeding. Rather the level of FAA in the plasma may reflect the amount of feed commonly consumed by each individual, as suggested by

the correlation between growth rate and plasma EAA and TFAA pools (**paper I**). A predominant effect of feed intake may also be behind the correlation between trypsin activity and T/C ratio and plasma FAA sums.

Plasma FAA pools in **paper I** (individual FAA data not shown) were dominated by alanine (Ala) and glycine (Gly), but the highest concentrations were measured for taurine (Tau), a stabiliser of cell membranes that is not found in proteins (Huxtable 1992). This is in line with reports from salmon (Espe *et al.* 1993a; Torrissen *et al.* 1994, 1995) and rainbow trout (Walton & Wilson 1986; Carter *et al.* 1995). Supplementation of fishmeal-based diets with methionine and cysteine has been shown to increase taurine concentrations in plasma in Atlantic salmon (Nordrum *et al.* 2000), suggesting that it is synthesised from sulfur-containing amino acids. Some amino acids, such as asparagine (Asn) and tryptophan (Trp) were only registered intermittently in **paper I**. The lack of Trp peaks were in contrast to Torrissen *et al.* (1994), who detected low post-prandial concentrations during a 72 h experiment using the same method of amino acid determination as in the present study. On the other hand, Walton & Wilson (1986) reported Asn, but not Trp, figures, whereas Shuhmacher *et al.* (1995) and Yamamoto *et al.* (1998) reported both amino acids in rainbow trout plasma. In salmon fed diets with varying degrees of hydrolysed protein, Espe *et al.* (1993a) and Torrissen *et al.* (1994) were unable to detect Asn in plasma after feeding. The reason for these discrepancies in reported values cannot be readily explained, but large variations are probably due to a range of methodological, biological and environmental factors.

Post-prandial changes in tissue FAA pools (Espe *et al.* 1993a, Torrissen *et al.* 1994, 1995; Carter *et al.* 2000) are only slightly affected by feeding, and white muscle concentrations in particular are less likely to be affected by feeding than those of plasma (Carter *et al.* 2000). Protein synthesis is probably the major homeostatic mechanism regulating tissue FAA pools (Carter *et al.* 1995; Carter *et al.* 2001), and muscle EAA show decreases after feeding (Carter *et al.* 1995; Espe *et al.* 1993a; Torrissen *et al.* 1994), as protein synthesis is stimulated (Lyndon *et al.* 1992, 1993).

The change in FAA pools after feeding is however small when compared to the relative increases seen in protein synthesis rates (McMillan & Houlihan 1992). In fact, a high degree of conservation of white muscle FAA pool size and composition is seen in the hours following feeding (Torrissen *et al.* 1994, 1995; Carter *et al.* 2000). This observation suggests that tissue FAA pools are under strong regulation, and protein synthesis rate may play a central part in this regulation (Carter *et al.* 2001). However, few data are available on the diel variation in tissue FAA concentrations in fish experiencing stable feeding regimes.

A negative correlation between muscle EAA/NEAA ratios and growth rates was similar to that reported by Torrissen *et al.* (1994) between groups of fish with different feed conversion efficiency, and can be interpreted as a higher transport of dietary EAA from the blood and incorporation into muscle protein with increasing growth rates. The EAA contribution to the TFAA concentration in the muscle was significantly higher than in the plasma. Muscle pools were dominated by the dipeptide anserine (Ans) and methionine (Met), but showed low concentrations of Tau. This is in contrast to other studies in salmon that found high concentrations of both Ans (Espe *et al.* 1993a, Torrissen *et al.* 1994) and Tau (Espe *et al.* 1993a, Torrissen *et al.* 1994; Carter *et al.* 2000). Low white muscle concentrations of Met, involved in initiation of protein synthesis (Stryer 1988), were found in all studies. Ans and other histidine-related compounds are thought to be responsible for muscle buffering capacity (Abe *et al.* 1985; Van Waarde 1988), whereas Tau may have an osmoregulatory function (Boyd *et al.* 1977), so the low incidence of Tau in the muscle in **paper I** is somewhat surprising, but similar to data from **paper II**. However, high muscle concentrations of Ans and its constituent β-alanine (β-Ala) can suggest that these molecules to some degree substitute for osmoregulatory function. High concentrations of Tau in the plasma may indicate mobilisation of this molecule from muscle and may also be related to its function as an osmoregulatory molecule.

In order to further elaborate the results from **paper I**, variance analyses of the

individual amino acids were run for plasma (Table 3.1) and muscle (Table 3.2) data using the statistical software SAS System 8.02 for Windows (SAS Inc., Cary, USA). A general linear model (*proc glm*) was used to evaluate variation in the individual amino acids, with light and ploidy as class variables and SGR, trypsin specific activity, chymotrypsin specific activity, white muscle RNA concentration and white muscle RNA/protein ratio as continuous variables.

In general, photoperiod treatment, growth rate and digestive protease activities all correlated with several FAA concentrations in plasma, but the variation in most cases seemed random. Not all significant correlations can be interpreted as describing a causal relationship between the variables. Further, other correlations may go undetected due to large variation between individuals or quick metabolisation of amino acids after absorption. For the sake of argument, the following discussion will therefore focus only on compounds for which plasma concentrations correlated either with SGR or both enzyme specific activities.

One could conceivably expect to find an influence of trypsin specific activity on the plasma concentrations of Lys and Arg, as these residues are the sites of proteolysis by trypsin and therefore are susceptible to further breakdown by exopeptidases. This seemed to be the case for Lys ($r = 0.31$, $p < 0.004$, Table 3.1), also observed by Torrissen *et al.* (1994), but not for Arg ($r = 0.11$, $p = 0.33$, Table 3.1). This could be due both to its intermittent appearance in plasma (it was not detected in all individuals) as well as the fact that the Arg peak in many cases could not be separated from that of γ-aminobutyric acid (γ-ABA) in the HPLC analyses. Chymotrypsin activity, however, did not correlate with plasma concentrations of any of the amino acids associated with its preferred cleavage sites. The levels of glutamine (Gln) and threonine (Thr) showed weak, but significant correlations with both trypsin and chymotrypsin specific activities. Gln concentrations in plasma correlated significantly with trypsin ($r = 0.40$, $p < 0.001$, Table 3.1), but not chymotrypsin ($r = 0.10$, $p = 0.35$, Table 3.1), specific activities. Leucine is reported to increase upon degradation of mammalian muscle (Millward *et al.* 1976), but did not show any correlations with

any of the parameters in this study. Collagen contains high amounts of Gly and Hyp residues, and appearance of these amino acids can be expected to be the result of proteolysis (Van Waarde 1988).

In white muscle (Table 3.2), asparagine (Asn) and anserine (Ans) elevations were associated with trypsin and chymotrypsin specific activities, while Ala and Lys elevations were associated only with trypsin, and histidine (His) elevation was associated only with chymotrypsin. A higher anserine concentration may indicate increased metabolism in the muscle, as this molecule is important for the tissue buffering capacity (Abe *et al.* 1985).

Table 3.1. The effects of measured and external factors on the concentrations of FAA and other non-protein nitrogenous compounds in plasma (**paper I**). F-values and associated probabilities of each factor from a general linear model (*glm*) are shown, using photoperiod (SNP or LL) and ploidy (diploid or triploid) as class factors, and specific growth rate (SGR, [% day^{-1}]), trypsin activity [μmol hr^{-1} mg $protein^{-1}$] , chymotrypsin activity [μmol hr^{-1} mg $protein^{-1}$], white muscle RNA concentration [μg mg^{-1}] and white muscle protein synthesis capacity (RNA/protein, [μg mg^{-1}]) as continuous factors. Significant effects are marked in bold.

Amino acid	Photoperiod		Ploidy		SGR		Trypsin		Chymotrypsin		RNA		RNA/protein	
	F	***p***	**F**	***p***	**F**	***p***	**F**	***p***	**F**	***p***	**F**	***p***	**F**	***p***
Asp	6,07	**0,0162**	0	0,9707	0,19	0,6618	1,75	0,1907	3,45	0,0676	0,08	0,7793	0,43	0,5135
Glu	0,05	0,8185	1,88	0,1747	2,56	0,1144	0,67	0,4152	0,7	0,4053	0,33	0,565	0	0,9812
Hyp	6,43	**0,0135**	8,5	**0,0048**	6,84	**0,0109**	1,24	0,2687	0,14	0,7073	0,29	0,5917	1,17	0,2826
Ser	1,36	0,2479	2,39	0,1267	2,41	0,1251	0,16	0,6948	0,22	0,6421	0,77	0,3842	1,09	0,2995
Asn	0,27	0,6044	0,2	0,6527	2,81	0,0984	0,59	0,4449	1,75	0,1899	0,02	0,885	1,97	0,165
Pea	0,71	0,4013	0,05	0,8233	0,18	0,6731	1,41	0,2384	0,01	0,9356	0,01	0,9086	0,2	0,6562
Gly	0,03	0,8575	7,56	**0,0076**	3,56	0,0633	3,75	0,057	2,94	0,0908	0,15	0,6992	0,01	0,9245
Gln	4,06	**0,0479**	5,52	**0,0217**	1,1	0,2981	23,52	**<0.0001**	8,44	**0,0049**	0,1	0,7567	0,18	0,6698
β-Ala	9,84	**0,0025**	18,49	**<0.0001**	0,19	0,6647	0,83	0,3657	0,01	0,9304	0,81	0,3714	0,64	0,4276
His	0	0,9746	0	0,9843	0,09	0,7696	3,96	0,0507	1,8	0,1841	0,01	0,9108	0,36	0,5531
Tau	0,17	0,6847	15,11	**0,0002**	5,84	**0,0184**	1,24	0,2702	0,64	0,4249	0,13	0,7244	0,84	0,3629
Thr	10,54	**0,0018**	4,98	**0,0288**	8,25	**0,0054**	8,44	**0,0049**	6,43	**0,0135**	0	0,9871	0,05	0,8156
Cit	3,43	0,0683	9,89	**0,0024**	6,89	**0,0107**	1,22	0,2737	0,15	0,6988	0,04	0,8486	0,23	0,6319
Ala	0,31	0,5824	3,25	0,0759	0,11	0,7395	6,13	**0,0158**	4,34	**0,0409**	0,95	0,3324	1,09	0,3001
γ–ABA +Arg	3,01	0,0873	4,16	**0,0453**	0,04	0,8412	6,65	**0,0121**	4,6	**0,0355**	0	0,955	0,57	0,4521
Carn	3,35	0,0714	4,41	**0,0393**	1,02	0,3159	3,62	0,0612	3,03	0,0863	0,02	0,8815	0,72	0,4004
β–AIBA	32,4	**<0.0001**	0	0,9784	4,4	**0,0396**	2,35	0,1298	0,4	0,5295	2,57	0,1132	5,86	**0,0181**
Crea	0,37	0,5425	9,91	**0,0024**	1,21	0,2743	4,59	**0,0357**	1,19	0,279	0,02	0,8947	0,1	0,7522
Pro	5,61	**0,0206**	28,73	**<0.0001**	2,56	0,114	4,47	**0,0381**	2,69	0,1054	0,08	0,7724	1,69	0,1985
Cys	3,5	0,0656	0,54	0,4645	0,08	0,7821	4,99	**0,0287**	1,26	0,2653	2,75	0,1021	0,2	0,6577
Cyst1	4,22	**0,0437**	0,01	0,9114	2,02	0,1602	1,79	0,1852	0,51	0,4762	0,41	0,5263	2,32	0,1321
Cyst2	0,77	0,3818	0,08	0,781	0,17	0,6851	0,26	0,6131	0,1	0,758	4,13	**0,0461**	0,22	0,6426
Tyr	1,04	0,3108	1,36	0,2467	7,11	**0,0095**	0,09	0,7669	0,07	0,7914	0,04	0,8389	0,93	0,3381
Val	0,28	0,5989	1,08	0,3016	2,07	0,1545	3,43	0,0685	2,84	0,0964	0,8	0,3742	0,95	0,3336
Met	1,83	0,1803	0,81	0,3703	3,23	0,0766	0,02	0,8769	0,36	0,5478	0,01	0,9035	0	0,973
Hyl1	25,22	**<0.0001**	1,69	0,1981	0,94	0,3368	0,82	0,3682	0,12	0,7346	0,02	0,8958	0	0,9578
Hyl2	1,2	0,2775	1,28	0,2624	0,13	0,7235	0	0,9707	0,07	0,7885	1,64	0,2042	0,01	0,9259
Ile	1,97	0,1648	0,85	0,3594	0,8	0,3729	5,94	**0,0174**	4,46	**0,0384**	0,69	0,4075	0,23	0,6336
Leu	0,07	0,7952	3,63	0,0608	0	0,9903	1,74	0,1917	2,58	0,113	0,9	0,3464	3,24	0,0761
Lys	5,38	**0,0234**	0,79	0,3784	0,93	0,3385	11,46	**0,0012**	3,3	0,0737	0,66	0,4205	0,46	0,502
Phe	0,06	0,8009	6,77	**0,0113**	0,8	0,3742	2,31	0,1332	2,32	0,1323	0,11	0,7405	1,8	0,1843
Trp	3,07	0,0842	4,21	**0,0441**	0	0,9817	0,5	0,4832	0,01	0,9053	1,38	0,2441	0,28	0,6007

(Asp=aspartic acid, Glu=glutamic acid, Hyp=hydroxyproline, Ser=serine, Asn=Asparagine, Pea=Phosphoethanolamine, Gly=glycine, Gln=glutamine, β-Ala=β-alanine, His=histidine, Tau=taurine, Thr=threonine, Cit=citrulline, Ala=alanine, γ-ABA=γ-amino butyric acid, Arg=Arginine, Carn=carnosine, β-AIBA=β-aminoisobutyric acid, Crea=creatinine, Pro=proline, Cys=cystein, Cyst1=cystathionine peak 1, Cyst2=cystathionine peak 2, Tyr=tyrosine, Val=valine, Met=methionine, Hyl1=hydroxylysine peak 1, Hyl2=hydroxylsyine peak 2, Ile=isoleucine, Leu=leucine, Lys=lysine, Phe=phenylalanine, Trp=tryptophan)

Table 3.2. The effects of measured and external factors on the concentrations of FAA and other non-protein nitrogenous compounds in white muscle (**paper I**). F-values and associated probabilities of each factor from a general linear model (*glm*), using photoperiod (SNP or LL) and ploidy (diploid or triploid) as class factors, and specific growth rate (SGR, [% day^{-1}]), trypsin activity [μmol hr^{-1} mg $protein^{-1}$] , chymotrypsin activity [μmol hr^{-1} mg $protein^{-1}$], white muscle RNA concentration [μg mg^{-1}] and white muscle protein synthesis capacity (RNA/protein, [μg mg^{-1}]) as continuous factors. Significant effects are marked in bold.

Amino acid	Photoperiod		Ploidy		SGR		Trypsin		Chymotrypsin		RNA		RNA/PROTEIN	
	F	**p**	**F**	**p**	**F**	**P**	**F**	**p**	**F**	**p**	**F**	**p**	**F**	**p**
Asp	1,11	0,2954	3,39	0,0699	0,25	0,6213	0,03	0,5861	0,1	0,7575	3,13	0,0814	0,01	0,9404
Glu	10,48	**0,0019**	0	0,9955	1,79	0,1854	0,25	0,6166	0,01	0,9186	0,51	0,4755	1,52	0,2225
Hyp	13,84	**0,0004**	2,34	0,1306	13,13	**0,0006**	0,03	0,8673	0	0,9725	0,94	0,3359	0,01	0,9101
Ser	6,43	**0,0135**	1,23	0,272	0,71	0,4027	0,39	0,5333	0,1	0,7559	0,01	0,9173	0,43	0,5165
Asn	3,41	0,069	0,05	0,8245	1,28	0,2615	11,42	**0,0012**	5,47	**0,0223**	0,02	0,8826	1,46	0,2313
Pea	11,34	**0,0013**	3,03	0,0863	1,24	0,2701	1,12	0,2932	0,4	0,5318	0,17	0,6856	0,9	0,3469
Gly	12,86	**0,0006**	9,74	**0,0026**	0,51	0,4756	0,14	0,7105	0,29	0,5926	1,24	0,2702	0,03	0,8665
Gln	1,23	0,2704	1,84	0,179	3,23	0,0766	1,13	0,2911	2,82	0,0977	0,11	0,7376	4,75	**0,0328**
β-Ala	0,45	0,5058	8,96	**0,0038**	1,71	0,1956	0,79	0,3778	0,21	0,6472	3,82	0,0547	0,28	0,5982
His	8,78	**0,0042**	14,57	**0,0003**	1,41	0,2392	2,8	0,0987	5,26	**0,0249**	1	0,3211	0,76	0,3876
Tau	0,12	0,728	3,05	0,0853	0,09	0,7609	0,05	0,8308	0,024	0,6236	0,01	0,9203	1,89	0,1739
Thr	1,57	0,214	1,59	0,2118	10,87	**0,0016**	1,55	0,2175	2,7	0,1048	0,1	0,7543	0,11	0,7389
Cit	9,42	**0,0031**	9,56	**0,0029**	5,85	**0,0183**	0,99	0,3224	0,56	0,4551	0,39	0,5322	1,54	0,2187
Ala	6,81	**0,0111**	3,93	0,0516	20,12	**<0,0001**	6,09	**0,0161**	0,75	0,3896	0,18	0,6716	0,21	0,649
γ-ABA+ Arg	27,13	**<0.0001**	2,45	0,1224	3,4	0,0697	0,55	0,4589	0,01	0,9397	0,6	0,4421	7,1	**0,0096**
Carn	4,32	**0,0415**	5,8	**0,0187**	0,29	0,5892	2,2	0,1427	0,53	0,4691	4,47	**0,0382**	4,72	**0,0333**
Ans	13,74	**0,0004**	11,83	**0,001**	0,51	0,479	5,8	**0,0188**	4,24	**0,0433**	5,05	0,0279	2,44	0,1233
Pro	2,2	0,1427	0	0,9705	8,44	**0,0049**	0,02	0,8983	2,08	0,1536	1,23	0,2712	1,94	0,1683
Cyst1	0,22	0,6431	17,94	**<0.0001**	0,03	0,8656	0,05	0,8306	0,85	0,3588	3,77	0,0564	8,38	**0,0051**
Cyst2	0,35	0,5549	9,79	**0,0026**	11,04	**0,0014**	0	0,9648	0,01	0,9249	0,16	0,6906	0,39	0,5338
Tyr	4,63	**0,035**	24,97	**<0.0001**	20,35	**<0.0001**	2,73	0,1033	0,87	0,3537	2	0,1622	0,02	0,882
Val	3,69	0,0589	3,52	0,0648	0,02	0,8817	0,81	0,3725	3,87	0,0534	0,74	0,3927	3,29	0,0742
Met	7,14	**0,0094**	9,41	**0,0031**	2,27	0,1362	0,22	0,6379	0,91	0,3446	4,32	**0,0414**	0,1	0,7575
Ile	7,72	**0,0071**	3,03	0,0862	0,82	0,3677	2,07	0,1547	4,76	**0,0327**	0,9	**0,3466**	4,24	**0,0434**
Orn	2,03	0,1586	7,34	**0,0085**	0,03	0,8529	2,03	0,159	5,81	**0,0186**	3,18	0,0789	10,93	**0,0015**
Lys	4,35	**0,0407**	0	0,9574	9,74	**0,0026**	0,36	0,5498	3,74	0,0574	1,78	0,1861	20,07	**<0.0001**
Phe	8,6	**0,0046**	6,71	**0,0117**	0,95	0,3326	11,91	**0,001**	3,89	0,0525	0	0,9616	8,64	**0,0045**

(Orn=ornithine, otherwise see Table 3.1. for abbreviations

Excluding compounds that did not show correlation with SGR, only Thr, Hyp and β-AIBA correlated with both SGR and photoperiod (Table 3.1, Table 3.2). Further, of these three compounds, only Hyp concentrations showed a significant variation with photoperiod and growth rate in both plasma and muscle, both concentrations increasing with growth rate and under continuous light, indicating mobilisation of muscle collagen under these conditions. Glycine (Gly) level in white muscle was also significantly affected by photoperiod, and was higher under continuous light (Table 3.2). Proteolysis may be stimulated both under anabolic and catabolic conditions (Millward 1989), and may or may not increase with growth rate and feed utilisation (**paper I**; **paper II**). Taken together, higher concentrations of both free Hyp (in plasma and muscle) and free Gly (in muscle) seem to suggest a higher mobilisation of collagen tissue, and may indicate higher rates of protein turnover. Fish adopting a low protein turnover life strategy, show higher protein growth efficiency and long-term growth rates than others (Carter *et al.* 1998; Rungruangsak-Torrissen *et al.* 1999). Conversely, a high turnover rate is associated with lower protein retention (Carter *et al.* 1993b). A correlation between turnover rate and SGR, as seen in **paper I**, therefore can indicate a relative decrease in protein growth efficiency at high feed intakes. This is in accordance with the lower feed conversion efficiency observed for these fish (**paper I**). Induction of triploidy could have an influence on protein metabolism, as indicated by feed conversion values and FAA levels. Glutamine (Gln) is a central N donor in synthesis of a variety of compounds and proteins (Stryer 1988) and its intracellular concentration has been found to correlate positively with protein synthesis rate in mice (MacLennan *et al.* 1987). We found that Gln in free (non-protein bound) form in white muscle correlated with tissue protein synthesis capacity (RNA/protein ratio), but that it was unrelated to SGR (Table 3.2). The FAA concentrations that were found to relate to the protein synthesis rate (RNA content) and the capacity for protein synthesis (RNA/protein ratio) in the white muscle were Gln, carnosine (Carn), cystathione peak 1 (Cyst1), methionine (Met), Ile, Ornithine (Orn), Lys, phenylalanine (Phe) and possibly Arg, regardless of photoperiod and ploidy. On the other hand, elevations of hydroxyproline (Hyp), Thr, citrulline (Cit),

Ala, Pro, cystathione peak 2 (Cyst2), tyrosine (Tyr) and Lys were related to SGR of the fish.

3.2.5 Effects on RNA concentrations and protein turnover

In cod, RNA/protein ratio was positively correlated with feed intake (Houlihan *et al.* 1988), and data from salmon (Rungruangsak-Torrissen *et al.* 1999) found an inverse relationship between white muscle RNA concentration and protein turnover, similar to our findings (**paper I**). In larvae and juveniles of cod, the concentration of RNA in white muscle is significantly correlated with whole-body growth rates (Houlihan *et al.* 1988). This relationship has been established also in other fish species (Houlihan *et al.* 1993). However, this relationship may depend on age, species and growth stage of the fish (Carter *et al.* 1993a, 1993b; Pelletier *et al.* 1995).

A high protein synthesis rate may have its advantages under conditions of stress or large environmental changes (Houlihan 1991). However, an inverse correlation was found between growth rates and RNA concentrations in the white muscle in salmon exposed to different photoperiods (**paper I**). In agreement with our findings (**paper I**; **paper II**), Pelletier *et al.* (1995) did not find white muscle RNA concentrations to be positively correlated with growth rates in adult cod. The authors postulated that this could be due to the fact that growth in adult cod is dominated by hypertrophy. Muscle growth is dominated by hypertrophy also in large Atlantic salmon (Johnston *et al.* 2003), and could explain the lack of a positive correlation between white muscle RNA concentrations and growth rate in our study. On the other hand, Carter *et al.* (1993b) did find protein synthesis capacity to correlate with rates of growth and protein consumption in Atlantic salmon with mean start weights of about 100 g, comparable to the smaller fish in one of our studies (**paper II**). Protein synthesis rate is however a product of both tissue ribosome content (RNA concentration) as well as the translation efficiency of the ribosomes, the RNA activity (Sugden & Fuller 1991). Both these parameters have been reported to increase with increasing growth rates and food consumption (Carter *et al.* 1993b), and a complex picture emerges when it

becomes clear that RNA activity in addition is affected by other factors such as age (Peragón *et al.* 2001), feeding (Lyndon 1990) and temperature (Foster *et al.* 1992). However, RNA concentrations rather than RNA activity is thought to be the dominant factor determining tissue protein synthetic activity (Houlihan 1991; Peragón *et al.* 2001), and we assume that this was also the case in our studies. A possible interpretation of the results may therefore be that protein synthesis rate did not increase with increasing growth rates. Rather our observations may be explained by a shift to a relative increase in lipid deposition at high feed intakes, utilising amino acids as energy substrates, and a higher breakdown of body proteins. The higher Hyp concentrations in white muscle may indicate increased proteolytic activity in this tissue. There is evidence that endogenous rhythms entrained by photoperiod can affect nutrient utilisation, as protein utilisation was different between fish fed in the morning and in the afternoon (Bolliet *et al.* 2000), and also between salmon smolts subjected to SNP and LL photoperiod (Nordgarden *et al.* 2003). These rhythms may be affected by the use of extended photoperiods.

3.2.6 The influence of triploidy

Triploidisation can have effects on physiological properties of the fish, such as oxygen-carrying capacity (Benfey 1991), but the majority of studies have concluded that triploid growth performance is comparable to that of diploids (*e.g.* Galbreath & Thorgaard 1995; McCarthy *et al.* 1996). We found, however, indications that triploid groups showed lower feed conversion efficiency than diploid groups, at least during the experimental period (**paper I**). Oppedal *et al.* (2003) did not find feed conversion efficiency to be different between triploid and diploid groups when held for a longer period, but found differences in seasonal growth patterns between the two genotypes similar to our results. However, triploids differ from diploids in lipid content in the digestive tract tissue (Fauconneau *et al.* 1990) and average cell size is larger (Hyndman *et al.* 2003). This might presumably affect membrane permeability and nutrient transport across intestinal and cellular membranes. However, comparison of protein metabolism between diploid and triploid salmonids did not find any

differences in protein utilisation (Oliva-Teles & Kaushik 1987). Our results do indicate differences in amino acid flux and that there could be possible differences in nutrient partitioning between diploids and triploids (**paper I**), but the lack of replicate groups and the confounding effect of higher feed intakes in the LL groups prevent us from making further comments.

3.3 The effects of feed protein digestibility (paper II)

3.3.1 Factors determining feed digestibility after processing

During processing of fish raw material for feed production, proteins in their native state are denatured by drying and grinding. This can increase digestibility of the protein by exposing a larger surface area to digestive enzymes, but on the other hand high temperature and pressure introduce several chemical reactions that are detrimental to protein digestibility. Heat induced disulphide bond cross-linking (Opstvedt *et al.* 1984), amino acid racemisation and formation of lysoalanine residues (Friedman *et al*. 1981) all reduce the digestibility of the feed proteins. Interactions with other feed constituents, in particular lipids and carbohydrates, may further accelerate these reactions (Opstvedt *et al.* 1984). Sulphydryl groups and disulphide bonds are important in maintaining the structure and function of native proteins (Saxena & Wetlaufer 1970), and oxidation of sulphydryl groups to form disulphide cross-links between protein chains is thought to decrease the digestibility of the protein by hindering access to peptide bonds by proteolytic enzymes. In our studies, *in vitro* digestion of the experimental feeds with salmon extracts (**paper III**) confirmed a negative correlation between the degree of disulphide bond formation and the extent of digestion by crude enzyme extracts (measured as free amino groups produced at peptide ends). This is in line with results reporting a correlation between disulphide bond content and mink digestibility of the fish meals used in production of our experimental feeds (Opstvedt *et al.* 2003).

3.3.2 Effects on growth and feed utilisation

Fish meal origin and treatment did not significantly affect growth rate in either experiment, although SGR correlated positively with feed protein digestibility measured both in mink (**paper II**) and *in vitro* (**paper III**). Trends of higher growth rates in the high quality diet groups were seen in both experiments, and an effect of diet quality on FCE was seen in Experiment 2. When a comparison was made between the tagged subset of fish from each group, fish meal quality had a significant effect on SGR (Experiment 2, **paper II**). This indicated that the fish selected for tagging was of a better condition than the average of the group. This was confirmed by the significantly higher mean weights of these fish (**paper II**). Feeding a limited ration, as in Experiment 2 in **paper II**, may have introduced a larger variability in feed intake between individuals, due to stronger competition for food (McCarthy *et al.* 1993), and may have obscured the differences found in the tagged subset in the group as a whole.

3.3.3 Effects on digestive protease activities

The digestive system of fishes is capable of adapting nutrient transport mechanisms in response to changes in the diet, albeit this capability seems more limited in carnivorous than herbivorous species (Buddington *et al.* 1997). Protease, lipase and amylase secretions are proportional to the amount of their respective substrates present in the diet, *i.e.* protein, lipids and carbohydrates. Feed protein characteristics were found to affect trypsin activity in mammals (*e.g.* Lhoste *et al.* 1994) as well as in fish (*e.g.* Haard *et al.* 1996), and high feed amino acid content affected an increased protease production in rats (Hara *et al.* 2001). In juvenile channel catfish (*Ictalurus punctatus* R.) given starter diets with low quality protein replacement, trypsin activity was suppressed compared to controls (El-Saidy *et al.* 2000).

We found no significant effect of diet digestibility on trypsin specific activity by the end of the experiment (**paper II**). On the other hand, FCE differences at the end of the experiment on day 90 seemed to be preceded by differences in trypsin and

chymotrypsin specific activities on day 60. Although samples were limited to three fish from each dietary group and showed large variation, these results are similar to previous studies (Rungruangsak-Torrissen *et al*. 1999; Rungruangsak Torrissen & Male 2000), in which changes in trypsin specific activity was followed by growth differences one month later. Most studies on trypsin in fish deal with protein sources of vegetable origin, often containing significant amounts of trypsin inhibitors that affect trypsin activity and feed digestibility (Krogdahl *et al.* 1994; Olli *et al.* 1994; Sveier *et al.* 2001). However, feed intake data are often not given and we can therefore not exclude that some of the reported changes were accompagnied by changes in feed intake.

Regulation of digestive enzyme activity is complex and most likely works simultaneously at several levels, by regulation of transcription, translation as well as post-translational modification. Protease activities are affected by the nature of the dietary proteins: diets containing high-quality proteins increased chymotrypsin activity in the gut of rats compared to low-quality diets (Johnson *et al.* 1977; Lhoste *et al.* 1994). This is in accordance with our results after introduction of new diets (**paper II**). Studies of insect larvae show that dietary factors can exert a strong influence on transcription of alternative digestive proteases (Broadway 1997; Paulillo *et al.* 2000; Mazumdar-Leighton & Broadway 2001). This response may however be more prevalent in herbivorous species feeding on diets rich in natural protease inhibitors. Comparatively few studies have demonstrated similar effects in fish, although Haard *et al.* (1996) found trypsin from coho salmon to have a reduced sensitivity to inhibition by a soybean trypsin inhibitor after feeding on diets containing soybean meal. The apparent increase in chymotrypsin activity in this study (**paper II**) could however be seen as being related to methodology (see discussion in section 3.1.4). Unrelated to dietary factors, Rungruangsak-Torrissen *et al.* (2005) measured an increase in chymotrypsin activity during food deprivation as well as during periods with a reduction in growth rate using the same methods, and chymotrypsin digestive activity increased with maturation of the digestive system in larval red drum (Applebaum *et al.* 2001), independent of live feed composition.

The reason for the higher chymotrypsin values in **paper II** compared to **paper I** is not immediately clear. However, the two experiments were conducted at different times of the year and chymotrypsin activity may show significant seasonal variation similar to that observed for trypsin activity (Einarsson *et al.*1997b; Torrissen & Torrissen 1985). A size effect (see section 3.3.6) or a diet effect could also be involved. It is interesting to note that differences in T/C ratios are related to fish growth efficiency, independent of the levels of trypsin and chymotrypsin specific activities (**paper I**; **paper II**).

3.3.4 Effects on FAA pools in plasma and muscle

In **paper II**, fish meal quality had a positive effect on EAA, NEAA and TFAA concentrations in the plasma of the fish showing differences in FCE (Experiment 2), whereas no differences were seen in Experiment 1 where FCE differences were not detected. However, measurements of post-prandial plasma profiles after starvation and re-feeding, showed lower plasma EAA/NEAA ratios in fish feeding on high quality protein diets in both experiments, suggesting a higher assimilation of EAA for protein synthesis as described in Torrissen *et al*. (1994) and Rungruangsak Torrissen & Male (2000). The variation in individual amino acid concentrations in **paper II** are shown in Tables 3.3 and 3.4, treated statistically as described in the previous section (section 3.2.4), but with fish meal quality and extrusion conditions as external fixed effects (class factors).

Table 3.3. The effects of measured and external factors on the concentrations of FAA and other non-protein nitrogenous compounds in the plasma (experiment 2 in **paper II**). F-values and associated probabilities of each factor from a general linear model (*glm*), using fish meal quality (FM3 or FM4) and extrusion conditions (ET1, ET2 or ET3) as class factors, and specific growth rate (SGR, [% day^{-1}]), trypsin activity [µmol hr^{-1} mg $protein^{-1}$] , chymotrypsin activity [µmol hr^{-1} mg $protein^{-1}$], white muscle RNA concentration [µg mg^{-1}] and white muscle protein synthesis capacity (RNA/protein, [µg mg^{-1}]) as continuous factors. Significant effects are marked in bold.

Amino acid	FM		ET		SGR		TRP		CTRP		RNA		RNA/PROTEIN	
	F	***P***	**F**	***p***	**F**	***p***	**F**	***p***	**F**	***p***	**F**	***p***	**F**	***p***
Asp	0,56	0,4544	0,78	0,4604	12,17	**0,0007**	0,06	0,8101	0	0,9795	0,54	0,4646	0,11	0,7389
Glu	3,91	**0,0499**	6,27	**0,0024**	26,22	**<0.0001**	0,03	0,8706	0,05	0,8251	3,37	0,0685	0,11	0,7365
Hyp	2,29	0,1322	18,83	**<0.0001**	57,97	**<0.0001**	0,19	0,6604	0,19	0,6597	0,93	0,3375	0,1	0,7564
Ser	1,32	0,252	11,81	**<0.0001**	7,24	**0,0079**	1,03	0,3128	0,53	0,4697	4,68	**0,032**	0,03	0,8618
Asn	1,19	0,2768	1,81	0,1675	0,13	0,7201	0,5	0,4796	0,14	0,7099	0,07	0,7945	1,41	0,2373
Pea	0,56	0,4559	0,81	0,4487	0,04	0,8377	0,11	0,7398	1,1	0,2955	0	0,9708	0,15	0,7015
Gly	1,25	0,2651	8,04	**0,0005**	3,03	0,0837	0,01	0,9277	0,26	0,6106	0,28	0,5955	0,99	0,321
Gln	11,73	**0,0008**	7,82	**0,0006**	1,72	0,1914	0,28	0,5994	0,07	0,787	5,15	0,0247	0,01	0,9099
β-Ala	3,22	0,0747	1,69	0,1871	15,89	**0,0001**	0,1	0,7513	0,35	0,5522	0,19	0,6622	0,73	0,3947
His	1,04	0,3099	2,17	0,1176	0,07	0,7902	0,22	0,6401	0,31	0,579	7,39	0,0073	4,06	**0,0456**
Tau	2,66	0,1049	2,75	0,0669	5,12	0,0251	0,49	0,4841	0,11	0,7415	0,52	0,4719	1,31	0,2533
Thr	1,29	0,2579	6,87	**0,0014**	2,89	0,091	0,42	0,518	1,17	0,2805	5,62	**0,019**	0,41	0,5225
Cit	10,08	**0,0018**	5,88	**0,0035**	2,03	0,1567	0,93	0,3371	0,42	0,5162	1,23	0,2698	0,01	0,9349
Ala	1,26	0,2633	10,23	**<0.0001**	2,07	0,1527	0,67	0,4146	0,44	0,5103	4,69	**0,0318**	0,39	0,5351
γ-ABA+Arg	0	0,9979	5,62	**0,0044**	1,94	0,1662	0,9	0,3441	1,12	0,2918	6,98	**0,0091**	1,48	0,225
Carn	3,28	0,0722	4,44	**0,0133**	1,19	0,2778	0,42	0,5167	0,58	0,446	0,12	0,7305	0,9	0,3433
His1m	0	0,9675	2,31	0,1028	0,8	0,3735	1,42	0,2347	0,44	0,5062	0,49	0,4857	0,52	0,4724
β-AIBA	1,5	0,2226	5,02	**0,0078**	4,83	**0,0295**	0,19	0,6597	0,02	0,8949	0,03	0,8737	0,76	0,3841
His3m	3,81	0,0529	0,65	0,5221	1,95	0,1647	0,07	0,7876	0,43	0,5121	0,01	0,9177	3,04	0,0835
Ans	0,97	0,3265	1,35	0,2616	1,74	0,1897	0,69	0,4067	1,06	0,3048	0,64	0,4257	0	0,9932
Crea	2,87	0,0923	8,26	**0,0004**	1,81	0,18	0,65	0,4202	2,7	0,1024	12,04	**0,0007**	1,14	0,288
Pro	0,01	0,9148	5,41	**0,0054**	6,74	**0,0103**	1,37	0,2443	0,59	0,4432	2,11	0,1487	0,35	0,5576
Tyr	0,24	0,6256	8,08	**0,0005**	16,89	**<0.0001**	0,23	0,6333	1,77	0,1855	2,56	0,1119	1,45	0,2297
Val	2,2	0,1398	2,46	0,0884	8,76	**0,0036**	0,07	0,7986	0	0,9884	2,27	0,1343	0,14	0,7083
Cystine	3,85	0,0516	1,27	0,2847	0	0,956	1,22	0,2719	0,15	0,6995	0,33	0,5656	0,1	0,7548
Met	5,51	**0,0202**	10,67	**<0.0001**	32,82	**<0.0001**	0,35	0,5554	0,15	0,6973	8,72	**0,0036**	0,86	0,3561
Hyl1	7,27	**0,0078**	2,51	0,0847	0,15	0,7036	0,22	0,6424	0,18	0,6691	1,51	0,221	9,5	**0,0025**
Hyl2	1,55	0,2151	0,52	0,5931	0,31	0,581	0,01	0,9163	0,48	0,4911	1,08	0,3004	0,73	0,3949
Ile	0,64	0,4257	3,19	**0,0438**	5,78	**0,0174**	0,03	0,8608	0	0,983	4,4	**0,0376**	0,15	0,6995
Leu	18,22	**<0.0001**	7,05	**0,0012**	2,53	0,114	0,28	0,6003	1,25	0,2649	18,04	**<0.0001**	0,45	0,5042
Orn	11,56	**0,0009**	2,73	0,0681	0,55	0,4605	0,94	0,3334	2,74	0,0998	6,46	**0,0121**	2,32	0,1297
Lys	16,98	**<0.0001**	10,85	**<0.0001**	0,53	0,4676	0	0,9672	0,7	0,4024	7,36	**0,0074**	0,06	0,802
Phe	1,27	0,2606	6,03	**0,03**	13,33	**0,0004**	0,01	0,932	0,12	0,7331	6,05	**0,015**	0,81	0,3706

(His1m=1-methyl-histidine, His3m=3-methyl-histidine, Orn=ornithine, otherwise see Table 3.1 for abbreviations)

Table 3.4. The effects of measured and external factors on the concentrations of FAA and other non-protein nitrogenous compounds in the white muscle (experiment 2 in **paper II**). F-values and associated probabilities of each factor from a general linear model (*glm*), using fish meal quality (FM3 or FM4) and extrusion conditions (ET1, ET2 or ET3) as class factors, and specific growth rate (SGR, [% day^{-1}]), trypsin activity [μmol hr^{-1} mg $protein^{-1}$] , chymotrypsin activity [μmol hr^{-1} mg $protein^{-1}$], white muscle RNA concentration [μg mg^{-1}] and white muscle protein synthesis capacity (RNA/protein, [μg mg^{-1}]) as continuous factors. Significant effects are marked in bold.

Amino acid	FM		ET		SGR		TRP		CTRP		RNA		RNA/PROTEIN	
	F	***p***	**F**	***p***	**F**	***p***	**F**	***p***	**F**	***p***	**F**	***p***	**F**	***p***
Asp	6,46	**0,0122**	7,48	**0,0008**	0,08	0,7816	0,06	0,8029	0,02	0,88	0,5	0,4807	0,33	0,5657
Glu	16,47	**<0.0001**	13,99	**<0.0001**	0,27	0,6047	1,06	0,3055	0,2	0,656	2,39	0,1247	3,18	0,0767
Hyp	8,66	**0,0038**	36,59	**<0.0001**	42,93	**<0.0001**	0	0,9731	3,03	0,0842	0,34	0,5607	6,82	**0,0101**
AAA	1,94	0,1656	11,05	**<0.0001**	0,51	0,4783	0,06	0,8032	0,26	0,6076	0,32	0,5752	0,02	0,8919
Ser	2,39	0,1248	0,91	0,4056	0	0,9483	0,21	0,6492	0,99	0,3214	0,09	0,7604	1,53	0,2179
Asn	1,13	0,2897	0,92	0,4009	0,78	0,3801	0,97	0,327	0,42	0,517	0,46	0,4981	0,42	0,5192
Pea	0,01	0,9268	0,24	0,7868	1,57	0,2121	0,08	0,7825	0,03	0,8572	4,59	**0,034**	0,32	0,5741
Gly	6,57	**0,0115**	10,65	**<0.0001**	1,13	0,2888	0,03	0,8548	2,38	0,1255	0,42	0,518	3,55	0,0617
Gln	2,18	0,1418	0,12	0,8841	0,61	0,4374	0	0,973	0,02	0,8897	1,18	0,2797	3,12	0,0798
Bala	2,11	0,1486	5,2	**0,0067**	1,38	0,2417	3,19	0,0762	1,16	0,2835	3,12	0,0799	4,97	**0,0275**
His	1,04	0,3101	0,49	0,6144	0,4	0,5287	2,2	0,1403	0,97	0,3274	0,32	0,5738	0,17	0,6779
Tau	2,06	0,1537	0,14	0,866	0,09	0,7691	0,04	0,8351	0,05	0,8314	0,56	0,4557	0,81	0,3693
Thr	5,4	**0,0216**	1,19	0,3066	0,73	0,396	2,92	0,0898	1,27	0,2623	0,6	0,4401	0,58	0,4495
Ala	3,01	0,0853	2,75	0,0674	1,85	0,1759	0,33	0,5653	0,15	0,6974	2,02	0,1579	0,61	0,4367
γ-ABA	14,29	**0,0002**	3,13	**0,0468**	3,15	0,0782	0,03	0,8635	0,11	0,7384	14,17	**0,0003**	0,07	0,7915
Carn	17,68	**<0.0001**	2,21	0,1139	8,17	**0,005**	0,38	0,537	0,34	0,5585	0,03	0,8563	0,02	0,8846
Ans	15,86	**0,0001**	4,74	0,0102	16,82	**<0.0001**	0,02	0,894	0,12	0,7337	0,03	0,8721	0,19	0,6659
Pro	1,6	0,2081	0,6	0,5506	6,63	**0,0111**	1,11	0,2949	0,22	0,639	0,02	0,877	0,12	0,7328
Cystha	1,3	0,2562	0,66	0,5192	2,56	0,1118	0,01	0,9176	0,66	0,4178	11,37	**0,001**	0,04	0,841
Tyr	0,7	0,4059	2	0,139	0,03	0,869	0,38	0,5371	0,43	0,5151	3,26	0,0733	0,24	0,6222
Val	0,07	0,7983	0,98	0,3791	0	0,9981	0,29	0,5922	0,68	0,4125	0,16	0,6897	0,33	0,5692
Met	0,61	0,4357	5,6	**0,0046**	3,33	0,0703	2,15	0,1446	2,24	0,1369	1,97	0,1626	0,04	0,85
Hyl1	0,08	0,7785	0,24	0,7863	0,44	0,5089	0,41	0,5242	0,82	0,3666	1,41	0,238	3,43	0,0663
Hyl2	1,11	0,2935	2,73	0,0689	0,72	0,3989	0,34	0,5593	0,83	0,3639	0,28	0,5971	0,26	0,6097
Ile	6,55	**0,0116**	2,11	0,1249	3,19	0,0765	0,19	0,6624	1,31	0,2552	4,21	**0,0422**	0,35	0,5566
Leu	5,54	**0,0201**	3,75	**0,0261**	6,07	**0,0151**	1,49	0,2247	2,21	0,1392	0,01	0,9111	2,85	0,0939
Orn	1,13	0,2893	2,53	0,0834	0,2	0,6532	0,05	0,8234	0,04	0,8399	2,85	0,0938	0,04	0,8348
Lys	17,77	**<0.0001**	26,18	**<0.0001**	9,43	**0,0026**	1,47	0,2273	2,42	0,1222	7,19	**0,0083**	6,22	**0,0139**
Phe	0,27	0,6019	5,78	**0,0039**	8,24	**0,0048**	0,06	0,8008	3,1	0,0808	0,02	0,8773	1,33	0,2506

(AAA=α-aminoadipic acid, Orn=ornithine, otherwise see Table 3.1 for abbreviations)

Unfortunately, due to computer hardware failure after analysis, plasma individual amino acid data were available for the 2 kg salmon only. In general, however, no effect was found of trypsin or chymotrypsin specific activities on any of the FAA concentrations in plasma and muscle. This was in contrast to the observations in **paper I**, and could be due to lack of differences in growth rate in **paper II** or suggest that the correlations seen in **paper I** did not describe causal relationships of metabolic significance.

Interestingly, the same correlations between SGR and Hyp concentrations were seen in both plasma ($r = 0.52$, $p < 0.0001$, Table 3.3.), and white muscle ($r = 0.22$, $p < 0.008$, Table 3.4.). Gln concentrations in the plasma were also significantly correlated with RNA concentrations in the white muscle ($r = 0.20$, $p < 0.01$), suggesting a possible link between the two. It is possible that when fish are fed a low ration as in Experiment 1 in **paper IV**, plasma FAA levels are more responsive to changes in feed quality. There may be a similar difference during periods of higher growth, although difficult to detect due to rapid metabolisation. In contrast to **paper I**, no correlations were seen between digestive enzyme specific activities and any of the amino compounds found in the plasma and the muscle. Neither was there a correlation between individual growth rates and digestive protease specific activities in any of the two sizes of fish. This may suggest that the fish were in a different growth phase than in **paper I**, where growth rate increased during the experimental period. Of the two factors that determined feed protein quality, the less influential extrusion conditions, rather than fish meal quality, seemingly affected several of the FAA concentrations in plasma (**paper II**). This could indicate different amino acid availability in pellets extruded under different conditions. However, this effect was not large enough to be detected as differences in growth in our experiments.

Rainbow trout that were given diet containing high and low levels of soybean proteins and showed different protein retention efficiency, protein synthesis rates and protein consumption rates did not show significant differences in TFAA pools or individual amino acid concentrations in either white muscle or liver (Martin *et al.*

2003). This is in contrast to our results (**paper II**). However, the cause of the reduced protein retention efficiency was associated with soybean meal in the diet, and a strong effect of dietary protein on expressed liver enzymes involved in a range of metabolic pathways were demonstrated.

3.3.5 Effects on RNA concentrations and protein turnover

Dietary protein quality influences protein metabolism in both liver and white muscle (de la Higuera *et al.* 1999; Martin *et al.* 2003), and may affect complex changes in metabolism (Martin *et al.* 2003). Further, diets deficient in EAA may depress protein synthesis in the white muscle (de la Higuera *et al.* 1999). In the present study, dietary protein quality had a significant effect on white muscle RNA concentrations (**paper II**). This could be interpreted as differences in protein synthesis rates between the two main dietary groups. There was no correlation between RNA concentrations in the white muscle and SGR on the individual level. However, this relationship presumes that an increased growth rate is concomitant with an increase in tissue ribosomes and consequently protein synthesis rate. The relationship between protein synthesis rate and SGR may rather be driven by the correlation with feed intake (Carter *et al.* 1993b; Houlihan *et al.* 1993), which was similar for all groups in our study, explaining the lack of correlation. The tendency of increased feed conversion efficiency in the 'high quality' diet groups in both Experiment 1 and Experiment 2 was likely due to increased protein utilisation efficiency. This can be supported by the significantly lower white muscle free Hyp concentrations in these groups. Rungruangsak-Torrissen *et al.* (1999) found an inverse relationship between white muscle RNA concentration and RNA activity in salmon fed similar rations, whereas Sveier *et al.* (2001) did not find the same relationship in salmon fed excess rations.

3.3.6 Influence of fish size

Trypsin and chymotrypsin specific activities were higher in small fish (Experiment 1) than in larger fish (Experiment 2), indicating a higher digestive activity in the smaller

fish. Torrissen *et al.* (1994) reported a similar size effect on trypsin specific activity in salmon. This is in accordance with the observation that metabolic rate in fish decreases with size and age (*e.g.* Jobling 1994). Similarly, a fall in protein turnover rate with age has been reported in fish (Houlihan *et al*. 1986). Muscle free Hyp concentrations were approximately twice as high in 150 g fish as in 2 kg fish when expressed as a fraction of the muscle TFAA pool (**paper II**). This is in line with the differences Torrissen *et al.* (1994) found between plasma values of 100 g and 400 g salmon. Taken together, these observations support the interpretation of muscle free Hyp concentrations as an indicator of protein turnover rate in this tissue. However, one must bear in mind that this relationship probably is valid only for tissues where restructuring of the collagen framework could be limiting growth of the tissue (Millward 1989), and may therefore not be appropriate for other tissues. For instance, intestine and liver show high protein synthesis and turnover rates (McMillan & Houlihan 1989), but comparatively low Hyp concentrations (Carter *et al.* 1993b).

3.3.7 Regular feeding versus pre-starvation values

Most feeding studies assess the effects of a single meal given after a short period of starvation. It is unclear to what extent pre-starved fish differ from fish that receives regular feeding, as few comparison studies have been published (*e.g.* Torrissen *et al.*, cited in Torrissen *et al.* 1994). The experimental design in **paper II** allowed for comparison between regular feeding and pre-starved fish from the same groups and showed that while pre-starved fish showed different EAA/NEAA ratios in the plasma between groups fed on low and high protein quality feeds, this was not the case when samples were taken during regular feeding. Instead, regular feeding fish showed differences in total and essential FAA concentrations depending on diet protein quality, in accordance with observations in Experiment 1 in **paper IV**, *i.e.* higher concentrations in the fish feeding on high quality feeds. One can postulate from these observations that fish that are either pre-starved (day 93 in Experiment 2, **paper II**) or have a low feed intake (Experiment 1, **paper IV**) show plasma FAA relationships according to feed protein digestibility or growth rate. Rungruangsak-Torrissen *et al*.

(2005) reported differences in the trypsin-chymotrypsin relationship between 5-7 h post-prandial and regularly feeding fish.

3.4 In vitro digestibility using fish enzyme extracts (paper III)

The extent of disulphide bond formation (measured as a decrease in free sulphydryl groups, % SH/S-S) and aspartic acid racemisation (measured as an increase in % D-Asp/DL-Asp), due to feed processing conditions, corresponded with a decreased *in vitro* digestibility.

When extracts from fish fed a common holding feed (day 0) was used for the *in vitro* assay, *in vitro* digestibility of the experimental diets (liberated free amino groups in the digest) correlated with SGR of each corresponding diet group (Experiment 2; **paper III**). The method was thus able to rank the feeds correctly according to growth results achieved *in vivo*, and may represent a significant cost saving over regular feeding experiments for evaluating feed protein quality. In contrast, when enzyme extracts were prepared from diet-adapted fish and used for *in vitro* digestion of the respective feeds, free amino ends after digestion at 15 °C was similar for all feeds (Experiment 2, **paper III**). This leads us to put forward a few presumptions: Firstly, the results indicate that the nature of the adaptation to diets probably did not include changes in absolute total digestive proteolytic activity, at least not detectable at the temperature where *in vitro* digestion was performed. The assay is however meant to approximate digestion at the optimal growth temperature for salmon. Instead it is tempting to suggest that the higher chymotrypsin activity that was measured as substrate-specific activity at 40 °C may be a consequence of expression of different chymotrypsin isozymes, which our methods cannot discriminate between. Secondly, it supports the hypothesis that digestive enzyme profiles, rather than absolute proteolytic activity may be important in determining feed utilisation efficiency. This is in accordance with the results of Torrissen & Shearer (1992), who could not confirm that total N uptake (measured as the apparent digestibility coefficient, ADC)

was higher in fish with trypsin phenotypes associated with higher feed conversion efficiency.

Interestingly, the feeds used in Experiment 1 (**paper III**), did not differ in *in vitro* digestibility when using enzyme extracts prepared from fish given the 'neutral' holding feed (day 0). On the other hand, when extracts from fish adapted to the respective feeds were prepared, the feeds could be separated according to their *in vitro* digestibility. This could suggest that adaptation may take longer in these fish (or with these feeds), and that FCE differences between the diet groups might have been detected if the experimental period was extended.

Gut microflora was found to have an effect on the inhibition of digestive protease activity in rat given diets of different protein origin (Boisen *et al.* 1985). Part of the adaptation of the gut to the feed might include a change in the gut microflora and this may have an effect on the observed differences.

3.5 Assessing feed quality by dorsal aorta cannulation (paper IV)

We were able to show, by repeated sampling of the same individuals, that the variation in plasma post-prandial profiles could be decomposed in components related both to feed intake as well as to variation in amino acid metabolism between individuals. Specific amino acids, such as Hyp and Gln were identified as possible indicators of differences in growth and protein metabolism (**paper IV**). However, this remains to be verified. The difference in plasma FAA profiles between Experiments 1 and 2 could be attributed to the use of different photoperiods, and probably was an effect of other associated variables such as appetite. The fish that had a higher mean feed intake (Experiment 2), showed lower FAA concentrations when given the feed with higher digestibility (**paper IV**), contrary to that of Experiment 1, where the fish showed a lower appetite. In order to be able to assess feed quality correctly the fish may have to be given a low ration as in Experiment 1 (**paper IV**) or we could suggest

starvation of the fish prior to the experiment. Incorrect evaluation of feed quality could also occur if the feeds contain free or supplemented amino acids, as a high elevation of plasma FAA not necessarily may indicate a high quality feed (Torrissen *et al*. 1995; Rungruangsak Torrissen & Male 2000; see section 3.1.11). The lack of individual feed intake data from the experiments in **paper I** and **paper II** makes it difficult to compare plasma profiles between experiments. Besides, plasma profiles between fish with different feed intake or in different growth phases, may not be directly comparable, as seen in **paper I**, **paper II** and **paper IV**. However, some observations were made that deserve comment. Plasma profiles were dominated by Tau also in **paper IV**, and Met and Gln concentrations were correlated with size of the meal immediately after sampling and 18 h later, respectively, hinting at an involvement of these amino acids in either growth processes and/or appetite regulation.

3.6 General discussion

3.6.1 Feed composition

The diet of fish in the wild have a high protein content (Bowen 1987), however due to the relatively high ability of salmonids to utilise lipids, most of the dietary energy in artificial salmon feeds can be supplied in this form. Adding lipids to the feed increases the utilisation of the dietary protein, a phenomenon often referred to as the 'protein sparing' effect. In contrast, carbohydrate utilisation in salmon is poor (Hemre *et al.* 1995), and carbohydrates are only included in artificial diets as a substrate matrix. However, a minimum amount seems necessary to avoid an increase in breakdown of muscle proteins and a depression of fish growth, as demonstrated by Peragón *et al.* (1999) in rainbow trout. The feeds used in this study had lipid and carbohydrate contents that were well within recommended values for salmon (National Research Council 1993).

3.6.2 Other factors

Although experiments can be designed with the purpose to reduce unwanted variation in both physical and physiological variables, a multitude of factors have the ability to affect growth and feed utilisation in fish. These range from properties of the feed (*e.g.* raw material, processing, macronutrient composition, particle size), feeding regime used (*e.g.* time, duration, ration size), and tank physical environment (*e.g.* light, temperature, salinity, season), to biological properties of the experimental animals themselves (*e.g.* size, age, genetic constitution, previous nutritional history, life stage, hierarchy rank, stress level) (for a review, see Brett 1979).

3.6.3 The relation between Hyp and protein turnover

In the current studies, individual concentrations of white muscle free hydroxyproline, used as an indicator of protein breakdown rate, showed the highest correlation of all parameters with growth rate. RNA concentrations on the other hand showed poor correlation with long-term growth rates. This is in line with Sveier *et al.* (2000), who found poor correlation between long-term growth and measurements of amino acid incorporation and specific white muscle RNA activity, suggesting instead that protein breakdown is more important than synthesis in controlling growth rate. Increased food intake and growth rates are associated with higher rates of both protein synthesis and breakdown in cod (Houlihan *et al.* 1987). LL groups of salmon showed higher muscle Hyp concentrations than SNP groups, in association with a higher feed intake in these groups (**paper I**). Other factors influencing protein turnover rate include age and size of the fish (Houlihan *et al.* 1986), temperature and cold acclimation (Watt *et al.* 1988). Size effects on muscle Hyp concentrations were indicated in **paper II** (see section 3.3.6).

Relative differences in white muscle concentrations of free Hyp seemed to indicate a higher protein turnover under LL regimes (**paper I**), but at the same time growth rates and FCE were improved. Feed conversion efficiency increased and the ratio of EAA/NEAA decreased at higher growth rates. These findings could be explained not

only by an increased assimilation of EAA for protein synthesis, but also a shift to increased fat deposition and a reduction of protein retention efficiency at higher growth rates. Growth rates increase with increasing feed intakes, but above a certain feed intake, growth rates reach a plateau (Brett 1979). This suggests that there are limitations in the ability of the fish to efficiently utilise high-energy diets used in aquaculture, either in the digestive system or in the mechanisms of protein synthesis and deposition.

Both rates of protein synthesis and amino acid oxidation increase following feed intake (Houlihan 1991), and the amount of protein synthesised exceeds the amount of protein retained for growth (Houlihan 1991). More specifically, improved growth efficiency seems to relate to lower rates of protein turnover in the tissues (Carter *et al.* 1993b). Rungruangsak-Torrissen *et al.* (1999) found high protein growth efficiency to be associated with low protein turnover rate in the white muscle. In **paper II**, RNA concentrations in the white muscle were different between the high and low quality feed groups. However, there were no correlations between individual growth rates and RNA concentrations, not even within groups given the same diet. On group basis, fillet ratios of protein to lipid correlated with RNA concentration, suggesting a higher relative protein deposition rate in the fish feeding on feeds with higher protein digestibility, possibly as a result of less oxidation of dietary amino acids, and a higher retention of synthesised protein. This is in accordance with the findings of Espe *et al.* (1993b), where ribosomal RNA in the white muscle increased with whole body ratio of protein to lipid (Figure 3.2.) in fish fed feed with different degrees of pre-hydrolysed protein.

Growth hormone (GH) transgenic salmon showed a leaner body composition than controls (Cook *et al.* 2000). It would therefore be interesting to measure GH profiles in relation to RNA concentrations and body/muscle composition ratio of protein to lipid. Rungruangsak Torrissen & Male (2000) found higher RNA concentration in the white muscle inversely correlated with the RNA activity (g protein synthesised g RNA^{-1} day^{-1}), and RNA concentrations did not correlate with growth rates, as in

paper I. Other studies have also reported deviations from the relationship between growth rate and white muscle RNA concentrations (Pelletier *et al.* 1995; Foster *et al.* 1993).

Few studies have measured both body composition and ribosomal RNA concentration in salmon white muscle. However data from Espe *et al.* (1993b), shows a weak, but significant, relationship between the body ratio of protein to lipid and ribosomal RNA concentration in salmon white muscle (Figure 3.2.). This supports the speculations in **paper I** that lower white muscle RNA concentrations in LL groups may be due to a relative increase in lipid deposition, resulting in a changed body composition. **Paper II**, in line with these observations, showed higher white muscle RNA and slightly higher ratio of protein to lipid (P/L ratio) in the FM3 high quality diet groups.

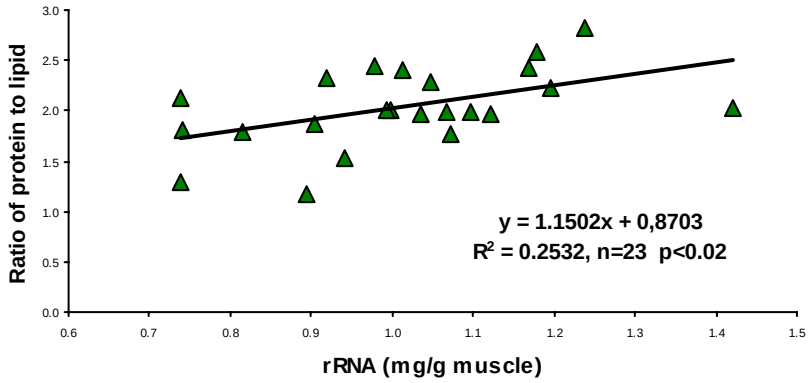

Figure 3.2. Relationship between ribosomal RNA (rRNA) concentration in the white muscle (µg) and whole body ratio of protein to lipid (P/L ratio) in individual Atlantic salmon (150 g) fed diets with different degrees of pre-hydrolysed protein (pooled for all diets). The fish were kept at 11ºC, salinity 17 and fed for 7 weeks at a ration of 0.5% bw day^{-1}. Data were adapted from Espe *et al.* (1993b).

3.6.4 The T/C ratio

Based on a comprehensive theoretical study of the capacity of oxidative and digestive processes in fish, Blier *et al.* (1997) suggested that digestive processes, rather than tissue aerobic capacity, had the ability to limit growth of the whole organism.

Lemieux *et al.* (1999) later found trypsin activity to be correlated with feed utilisation in cod when fish were injected with growth hormone (GH). Other studies have found the T/C ratio to be different between fish with different growth capacities (Blier *et al.* 2002) or growth rates (Rungruangsak-Torrissen & Fosseidengen 2002; Rungruangsak-Torrissen *et al*. 2005; Rungruangsak-Torrissen *et al*. submitted). Changes in this parameter seem to be detectable before differences in growth can be measured (**paper II**; **paper III**). This is consistent with the time lag seen between changes in feed intake and measurable differences in growth. The current working hypothesis therefore proposes that by measuring digestive protease specific activities, and more specifically the T/C ratio, one can estimate or even predict differences in growth rate and/or feed utilisation (**paper III**), possibly even in growth conditions where feed consumption data are unavailable (Rungruangsak-Torrissen *et al*. 2005).

Linear correlations between trypsin and chymotrypsin activity were significant in both SNP and LL groups, and the elevation of the regression (the T/C ratio) was significantly different between the two (Figure 3.3A). This is consistent with the activation of chymotrypsin by trypsin, and similar to calculations of individual T/C ratios using the same data (**paper I**). The trypsin-chymotrypsin correlation showed the same trends as that of trypsin specific activity and growth rate, and was poorer in the SNP groups and poorest in the 3n-SNP group (**paper I**). However, chymotrypsin specific activity was not correlated to growth rate. Taken together, the results suggest that chymotrypsin activity in salmon may be regulated by additional mechanisms that can lead to a response to changes in internal and external variables that are different from that of trypsin.

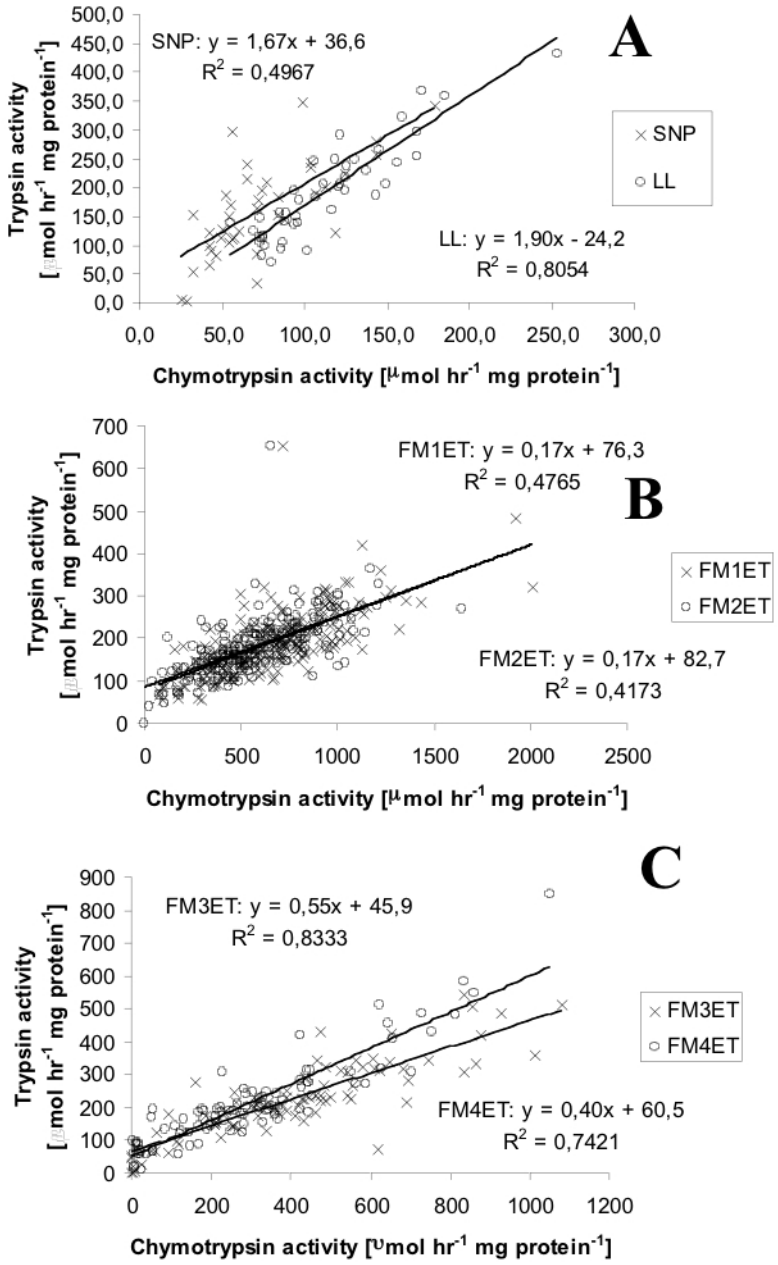

Figure 3.3: The relationship between trypsin and chymotrypsin specific activities [µmol hr^{-1} mg $protein^{-1}$], (A), grouped by photoperiod (data from **paper I**) (SNP=simulated natural photoperiod; LL: continuous light), and grouped by feed quality (data from **paper II**), for both 150 g (B) and 2 kg (C) salmon. (FM1ET and FM3ET = high quality protein diets; FM2ET and FM4ET = low quality protein diets).

Similar to **paper I**, trypsin and chymotrypsin specific activities were also significantly correlated in **paper II** (Figure 3.3B, Figure 3.3C). This correlation may disappear during periods of growth rate increase (Rungruangsak Torrissen & Male 2000; Rungruangsak-Torrissen *et al*. 2005). The T/C ratios calculated from

regression lines in general showed the same pattern as those calculated from individual activities; significant differences were present between groups of fish showing significant differences in feed utilisation (**paper II**; Figure 3.3C), However, the T/C ratios of the FM1ET and FM2ET diet groups in **paper II** were not significantly different when calculated from regression lines (Figure 3.3B). Interestingly, unlike the FM3ET and the FM4ET groups, the FM1ET and FM2ET groups did not show different feed conversion efficiencies.

The results seem to indicate that two different mechanisms were limiting growth in the two main studies (**paper I**; **paper II**). Still, significant differences in the activity ratio of trypsin to chymotrypsin (the T/C ratio) indicated significant differences in feed utilisation in both cases. When one feed quality was given and the feeding was adjusted *ad libitum* (**paper I**), feed intake differed between experimental groups and trypsin activity showed a strong correlation with growth rate. This could suggest that growth under those conditions was limited by appetite regulating factors and the subsequent ability to digest nutrients (a result of both feed intake and digestive capacity). This setup was similar to that of Lemieux *et al.* (1999), where individual cod with different feed intakes showed a similar correlation between trypsin activity, growth rate and feed utilisation. Feed intake was similar in all groups in **paper II**, and growth differences could therefore only result from differences in the abilities to utilise the feed given or differences in feed quality.

Such growth increases are more likely to be affected through differences in protein utilisation efficiency (Rungruangsak-Torrissen *et al.* 1999), rather than through differences in absolute digestive proteolytic activity or a higher absorption efficiency of N *per se*. Instead, a balanced simultaneous presence of amino acids in the plasma may be important (Murai *et al.* 1982), possibly by minimising the extent of postabsorptive loss of FAA through oxidation and/or protein turnover leading to a larger proportion of the ingested protein being retained as tissue growth. Rungruangsak-Torrissen & Sundby (2000) suggested that the ratio of the two enzymes (the T/C ratio) affected the rate and pattern of amino acid absorption, based

on the observation that the T/C ratio and plasma EAA/NEAA ratio correlated following feeding of Atlantic salmon. Kristinsson & Rasco (2000) demonstrated a similar enzyme ratio effect when digesting a standard protein *in vitro*. Altering the ratio of digestive proteases gave rise to different patterns of released amino acids and peptides. Temperature also influenced the time course and result of feed proteolysis. Sveier *et al.* (2001) showed that different degrees of dietary trypsin inhibitor inclusion resulted in different amino acid absorption patterns in salmon, and further showed that inhibition of trypsin activity had a positive effect on feed utilisation under conditions of high dietary protein loads. These results also seem to suggest that the type rather than the total proteolytic activity is important for feed utilisation.

The pattern and rate of amino acid absorption is influenced by addition of trypsin inhibitors to the feed, although this does not necessarily resulting in a negative effect on growth (Sveier *et al.* 2001).

3.7 Suggestions for future research

3.7.1 Full characterisation of digestive protease expression and properties

Although not included in the present work, variation in digestive protease expression, and in particular trypsin isozyme expression, has been included in the discussion of the results to illustrate some of the principles that could explain the observed differences. At present only partial characterisation of the catalytic properties of expressed salmon trypsin isozymes has been performed (Outzen *et al.* 1996). Furthermore, although several trypsin genes have been identified in salmon (Male *et al.* 1995), more research remains on the control of trypsin gene expression. Likewise, the effects of external and internal variables on isozyme expression are only partly understood (Rungruangsak-Torrissen *et al.* 1998; Rungruangsak-Torrissen & Stensholt 2001). Trypsin phenotyping by caecal biopsy as described by Torrissen (1991) is a relatively simple procedure with minimal impact on animal health and

survival that has been shown to aid the interpretation of experimental data (for a review, see Rungruangsak Torrissen & Male 2000). The relationship between protein utilisation and patterns of trypsin and chymotrypsin expression should be investigated further for a deeper understanding of the effects of digestive protease expression on growth mechanisms. The methods suggested in section 3.1.5. could provide a starting point.

3.7.2 Digestive proteases and possible control of hormonal secretion

Although the present work cannot be conclusive regarding a general relationship between digestive protease activities and growth performance, certain relationships are present under the specific experimental conditions present in this study (**paper I**; **paper II**). The digestive proteases themselves and/or the products of protein digestion are likely to be involved in the regulation of the growth process (*e.g.* Millward 1989; Rungruangsak-Torrissen *et al*. 2005). A natural progression from the current work would therefore be to describe the underlying mechanisms linking digestive protease activities with the hormonal regulation of growth and protein utilisation. One study has already indicated that the insulin response after feeding may be affected (Rungruangsak-Torrissen *et al.* 1999), possibly mediated in part by different patterns of amino acid absorption. It would be of interest to see if other hormones involved in growth regulation are affected or associated with changes in digestive protease expression.

The major hormones involved in regulation of growth processes in mammals and fish are growth hormone (GH), insulin, thyroxin (T_3 and T_4) and insulin growth factor IGF-1. All of the above exert both anabolic and catabolic influences on the organism. For instance, GH increases both after feed intake (Reddy & Leatherland 1995) and during feed deprivation (Björnsson 1997). In concert with IGF-1, which is down-regulated during feed deprivation (Moriyama *et al.* 1994), GH affects nutrient partitioning (Fauconneau *et al.* 1996), and GH transgenic fish have a leaner body mass and a higher protein to lipid ratio, compared to controls (Cook *et al.* 2000).

Recent work has found relations between circulating plasma IGF-1 concentrations and growth rates in several species of fish (Dyer *et al.* 2004). The relation between digestion of feedstuffs and the stimulation of hormone secretion is a field of study that deserves further attention and still poses interesting physiological questions. Future experiments should seek to further investigate the relationship between digestive protease activity, protein turnover and protein retention efficiency, possibly by exploring these together with hormonal responses.

4. Conclusions

The specific activities of trypsin and chymotrypsin or the ratio between these activities (the T/C ratio) in the pyloric caeca of salmon show considerable variation with growth rate under different conditions. A response to dietary factors was mainly observed in the specific chymotrypsin activity.

Growth rate was higher under continous light than natural photoperiod. Individual growth rates were correlated with trypsin specific activity (and T/C ratio), regardless of photoperiod. This may have been related to feed intake being the major growth-affecting factor under these conditions. The T/C ratio was different between groups with different feed conversion efficiency (FCE).

When FCE was affected by dietary protein digestibility, and feed intake was similar in all groups, no correlations were found between digestive protease activities and individual growth rates. Diet *in vitro* digestibility showed correlation with chymotrypsin specific activity *in vivo*, and the fish with higher FCE showed higher chymotrypsin specific activity (resulting in lower T/C ratio) than fish with a lower FCE.

When the growth increase was concomitant with increases in protein turnover (increased muscle hydroxyproline concentration) and decreases in muscle RNA concentrations, individual growth rates were inversely correlated with white muscle ratios of essential to non-essential amino acids (EAA/NEAA), indicating increased protein synthetic activity. This was associated with decreased feed conversion efficiency, consistent with lower protein retention efficiency, and might indicate a simultaneous change in nutrient partioning, *i.e.* an increased lipid deposition rate.

When the growth increase was concomitant with a decreased protein turnover and increased white muscle RNA concentrations, individual growth rates were uncorrelated to white muscle EAA/NEAA ratios. This indicated an increased protein synthetic activity and a possible increase in the ratio of protein to lipid deposition.

This was associated with increased FCE, consistent with higher protein retention efficiency.

In vitro digestion of diets using caecal extracts from fish given an external standard feed, and standardised by their trypsin activity, correctly predicted fish growth rate within the next three months of feeding. Differences in feed *in vitro* digestibility resulted in different uptake patterns of amino acids to the blood following feeding. Feeds could be ranked according to their protein qualities by studying absorption of FAA in the plasma, and feed intake correlated with plasma sums of EAA and TFAA (total free amino acids). The method demonstrated the use of cannulation in studies of feed quality.

The T/C ratio as well as muscle free hydroxyproline could be of use in determining the growth status of the fish, and more precision in predicting protein growth efficiency can be obtained when these parameters are analysed together with other protein metabolism parameters. Further investigations ought to look into the mechanisms affecting digestive enzyme expression, and the relationship between activity, protein digestion and anabolic hormone secretion.

Source of data

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