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Chapter 7

ATLANTIC SALMON, *SALMO SALAR* L.: FOOD UTILIZATION, PROTEIN GROWTH EFFICIENCY AND MATURATION

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ABSTRACT

Atlantic salmon, *Salmo salar* L., is an anadromous carnivore living in fresh water during the first year(s) of life and migrating to the sea after smoltification. The early life period in fresh water and the first sea winter are the critical periods when genetic expressions, food utilization, and growth can be affected by the environmental conditions.

Studies using modified and newly developed biochemical techniques have provided significant insights into growth mechanisms and genetics of growth in connection with food utilization under different environmental conditions. The evidence indicate that the utilization of dietary protein is the primary key biological process that influences nutrients influx (absorption and transport of amino acids), hormone insulin secretion, protein growth efficiency (ratio of protein to lipid – P/L ratio), health, maturation, and behaviour. The process is affected by internal factors (genetics, age, growth stage) and external factors (temperature, light, vaccine, quality and availability of food). Fish with higher growth capacity have higher protein growth efficiency, and higher dietary protein level affects an increase in skeletal growth (increase in length).

Trypsin and chymotrypsin specific activities are important indicators for dietary protein levels and consumption rates. The protease activity ratio of trypsin to chymotrypsin (T/C ratio) is developed to indicate digestive efficiency and growth, independent of the specific activity levels of the two enzymes. Moreover, *in vitro* protein digestibility technique, for prediction of dietary quality on fish growth, is improved for comparisons of dietary quality effects within and between different fish species by standardizing the dialyzed crude enzyme extracts used for digestion of raw materials and diets with respect to trypsin activity.

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Maturing Atlantic salmon have higher food consumption rate than immature salmon at the early period of the spawning year. This is indicated by higher total protease specific activity (peptic and tryptic specific activities) around April, and females have greater response than males. The protease specific activities become lower later due to lower consumption from summer until maturity in winter. Fish with higher growth rate (higher pyloric caecal T/C ratio) have faster maturation rate, and will have a higher reduction in growth rate with lower pyloric caecal T/C ratio at maturation. Females with higher maturation rates have higher oocyte T/C ratio of trypsin-like to chymotrypsin-like activities in spite of lower trypsin-like and chymotrypsin-like specific activities in their oocytes.

The T/C ratio is the unique key indicator in pyloric caeca for somatic growth as well as in oocytes for oocyte development (maturation rate), independent of the specific activity levels of the two proteases.

Keywords: Trypsin/chymotrypsin ratio, free amino acids, insulin, protein/lipid ratio, RNA/protein ratio, pyloric caeca, muscle, oocytes

INTRODUCTION

Atlantic salmon, *Salmo salar* L., is the most important economic species for aquaculture industry in Norway.

According to the Directorate of Fisheries in Norway [2012], the production of Atlantic salmon has been highly increasing since 2006, and in 2011 the production of juveniles for growout in sea water was around 288 millions individuals with a value of around 2,540 millions NOK (Figure 1).

Growout production was more than one million metric tonnes with a value of around 26,924 millions NOK (Figure 1). The production and value of juveniles for production in fresh water (around 24% and 60%, respectively, of those for production in sea water) as well as of roe production in 2011 are also illustrated (Figure 1).

Atlantic salmon is an anadromous species living its early life stage in fresh water (one to several years) before it undergoes smoltification to start its oceanic life stage where it spends up to four years before returning to its natal river to spawn (Holm et al. 2004). Spending time in each period depends on its genetics, water temperature, and feeding condition. Genetic studies of trypsin phenotypes (Torrissen 1984, 1987; Torrissen et al. 1993; Rungruangsak-Torrissen et al. 1998, 2006; Rungruangsak Torrissen and Male 2000) have provided evidence to indicate that utilization of dietary protein is the primary key biological process for growth performance, health, maturation, and behaviour (see Chapter 6).

The research also indicates that trypsin is the key protease for growth while chymotrypsin plays a major role when growth is interrupted or limited, and the protease activity ratio of trypsin to chymotrypsin (T/C ratio) is the important biological parameter newly developed for digestive efficiency and growth. An increase in growth rate is affected by increased trypsin specific activity and/or decreased chymotrypsin specific activity which will result in an increase in T/C ratio.

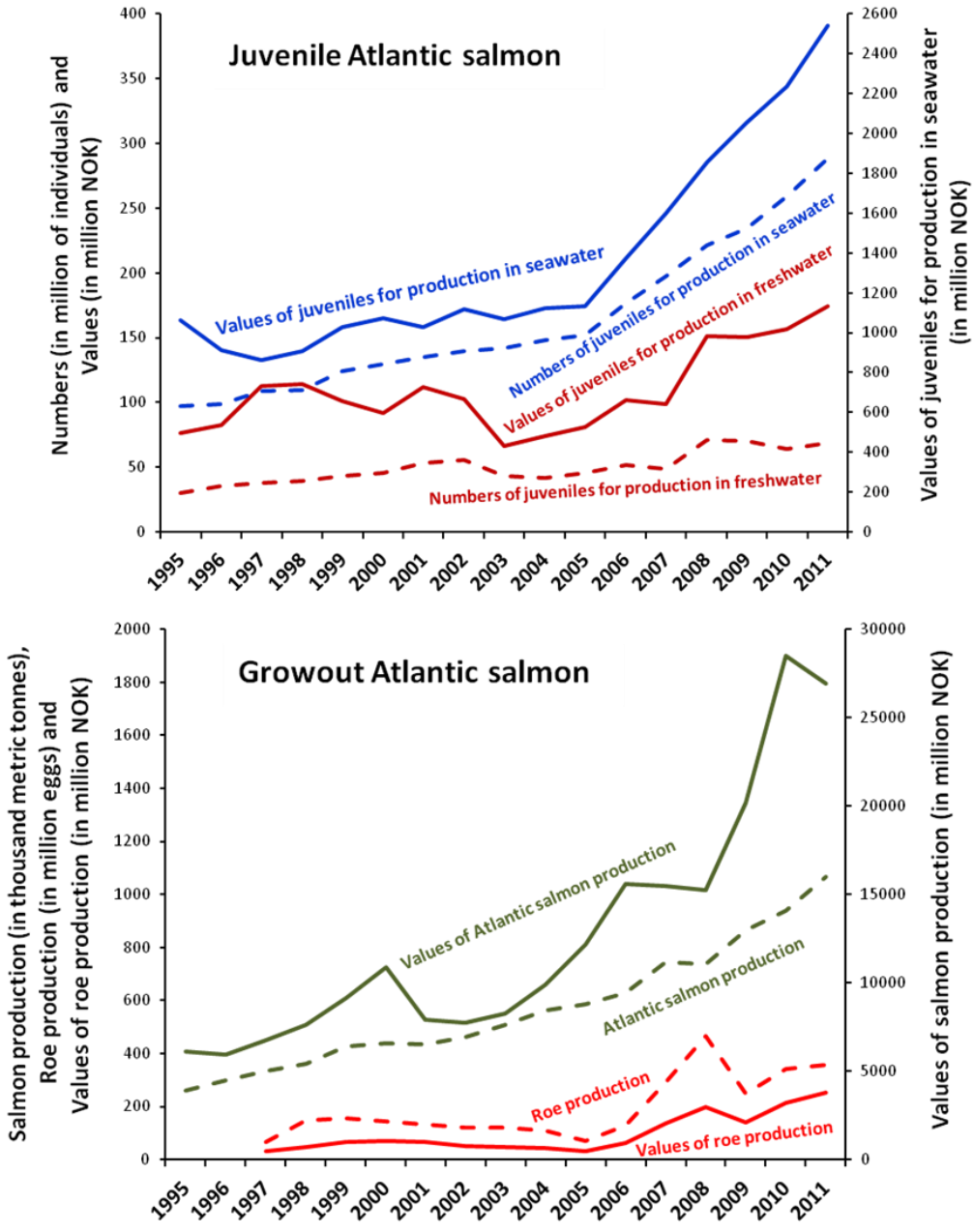

Figure 1. Statistical data during the years 1995–2011 of Norwegian aquaculture for production of Atlantic salmon from juveniles until growout stage for food production. (Adapted data from Directorate of Fisheries in Norway [2012]).

Different biochemical techniques were modified and developed for practical purposes to study food utilization, protein growth efficiency and maturation in aquaculture and natural ecosystems, especially under the conditions where food consumption rate and growth rate of the fish cannot be directly measured. The techniques are unique and can indicate digestive

efficiency and growth, growth status at sampling (reducing growth phase, steady growth phase, or growing phase) and maturation rate. The aim of this chapter is to review these studies in Atlantic salmon as well as in other fish species.

POPULATION STUDIES IN ATLANTIC SALMON

Development of Trypsin Activity and Growth

Different proteases in crude extracts from fish digestive systems were used earlier to study the effects of dietary protein quality on fish growth (Rungruangsak and Utne 1981). The same substrate was used to compare different protease reactions, and the main enzyme activity will be indicated by its optimum temperature characteristics; for example, in Atlantic salmon, the optimum temperatures for pepsin activity in the stomach (Torrissen 1984), and for trypsin and chymotrypsin activities in the pyloric caeca and intestine (Torrissen 1984; Rungruangsak Torrissen and Male 2000), are 37.5 ^{o}C, 50–52.5 ^{o}C, and 40–45 ^{o}C, respectively. The optimum temperature characteristics are species specific, and have to be determined for each species. It is important to use the optimum temperature and not habitat temperature to assay enzyme activities for practical comparisons.

Using casein as a substrate (Torrissen and Torrissen 1984, tryptic reaction in the pyloric caeca showed much higher specific activity (> 100 µmol tyrosine h^{-1} mg $protein^{-1}$), compared to peptic activity in the stomach (< 0.3 µmol tyrosine h^{-1} mg $protein^{-1}$) and tryptic activity in the intestine (< 5 µmol tyrosine h^{-1} mg $protein^{-1}$) (Figure 2A). Therefore, in Atlantic salmon, the tryptic activity of trypsin in the pyloric caeca (at around 50 ^{o}C) is the main protease activity in the digestive system, followed by chymotrypsin activity (at around 40 ^{o}C). Similar results on significantly more pronounced protease activities in the intestine than the stomach were also observed in Nile tilapia, *Oreochromis niloticus* L., with optimum temperatures of 50 ^{o}C for trypsin activity and 60 ^{o}C for chymotrypsin activity (Rungruangsak-Torrissen et al. 2010). The expressions of trypsin specific activity varied according to the genetic feature of each salmon strain and rearing temperatures (Figure 2A). Among the six different temperatures studied, the specific activities of trypsin were relatively high in the pyloric caeca at rearing temperature of 9.0 ^{o}C, especially the Ekso and Lærdal fingerlings. At rearing temperatures of ≥ 10 ^{o}C, the Alta strain showed relatively higher pyloric caecal trypsin specific activity than the other strains (Figure 2A). At 12.6 ^{o}C, trypsin specific activities in the intestine were activated to be in the same range as in the pyloric caeca, and those from the Imsa strain were most activitated (Figure 2A). Pepsin specific activity in the stomach tended to decrease when the rearing temperatures increased, and was not influenced by rearing temperatures.

Studies of growth rates, during 171 days after hatching at a rearing temperature of 12.7±1.5 ^{o}C, indicated that fingerlings from Lærdal and Alta strains had higher growth rates than those from Ekso, Figgjo and Imsa strains (Figure 2B), and their total protease (mainly trypsin) specific activities are illustrated in Figure 2C. During the first 70 days after hatching, the Lærdal and Alta strains had higher digestive enzyme levels than the other strains, and the Lærdal strain additionally showed a faster and higher response of total protease specific activity than the Alta strain (Figure 2C). The fish did not hatch at the same time and most of

the samples were collected at temperatures about 12–13 °C, except for day 171 of Ekso and Alta strains when the temperature decreased to about 10 °C. For the Lærdal strain, the temperature was around 10 °C on day 130 and decreased to 5 °C on day 171. The total protease specific activity should have remained higher in the Lærdal strain if the temperatures did not decrease during the last 41 days. However, alevins from the Ekso, Figgjo and Imsa strains, had very slow digestive enzyme development during the first 70 days after hatching. The Figgjo and Imsa strains seemed to response to the first feeding faster than the other strains but the total protease specific activities remained at lower levels, while the Ekso strain showed a slower response but the total protease specific activity remained at a higher level. The responses in protease expressions after the first feeding are different in the salmon strains (Figure 2C) which indicate the strain with faster digestive enzyme development and faster response to feeding with higher total protease (mainly trypsin) specific activity will grow faster (Figures 2B and 2C).

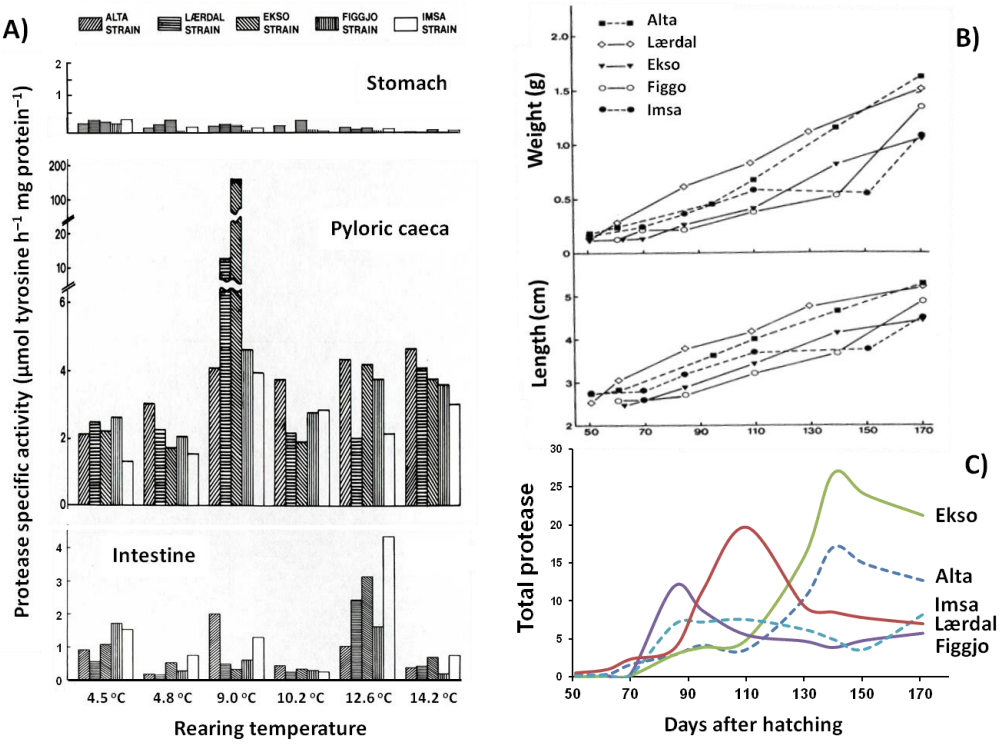

Figure 2. (A) Protease specific activities (using casein as substrate) of peptic activity (at 37.5 °C) in the stomach and tryptic activities (at 52.5 °C) in the pyloric caeca and intestine of Atlantic salmon fingerlings from five different river strains after 10 days of feeding at different rearing temperatures, and (B) their early growth rates (shown in weight and length) as well as (C) the development of total protease specific activity (sum of peptic and tryptic activities), which was mainly trypsin specific activity, during rearing at 12.7±1.5 °C. (Adapted from Torrissen and Torrissen [1984], with permission from Elsevier B.V.).

Three different salmon populations were studied (Torrissen et al. 1993) for the frequency of trypsin variant TRP-2*92 and growth at 2–3 ^{o}C (Table 1). These populations have differences in age of maturation and been used for sea-ranching experiments in Norway. Atlantic salmon with different trypsin genotypes have different food utilization efficiency, and the *TRP-2*92* salmon have better growth performance at low temperatures of ≤ 8 ^{o}C, especially below 6 ^{o}C (Rungruangsak-Torrissen et al. 1998; Rungruangsak Torrissen and Male 2000). The Dale strain comprised a mixture of grilse and two-sea-winter salmon, the Lonevåg strain consisted almost exclusive of grilse, while the Voss strain was mostly two-sea-winter salmon (Torrissen et al. 1993). Atlantic salmon parr from these three strains showed variations in the frequency of the variant TRP-2*92 at 0.29, 0.10, and 0.09, respectively. Within each strain, the salmon with the trypsin variant TRP-2*92 had apparently higher growth rates than the salmon without the variant, especially in the Dale and Lonevåg strains. Since the Voss strain grew much faster in the sea than in fresh water, a significant growth difference between the two genotypic groups in the Voss strain might be observed later if the experiment had been prolonged in the fast-growing sea phase. The *TRP-2*92* salmon from Dale strain were larger than the other strains, while the salmon lacking the variant were similar in size (Table 1). The low temperature of 2–3 ^{o}C had a positive effect on optimal feed utilization in the *TRP-2*92* salmon, especially in the homozygote *TRP-2*92/92* genotype, while it had a negative effect on the genotype lacking the variant. More details on the association between trypsin genotypes and optimal temperatures for efficiency of food utilization and growth have been previously described (Rungruangsak-Torrissen et al. 1998, 2006; Rungruangsak Torrissen and Male 2000; Rungruangsak-Torrissen and Stensholt 2001), and is also reviewed in Chapter 6.

Table 1. Average weights of Atlantic salmon parr from three different river strains with different frequencies of trypsin variant TRP-2*92 during 4 months at low water temperature of 2–3 ^{o}C. Within the same period, the values with different superscripts and with asterisk (*) are significantly different ($P < 0.04$). (From Torrissen et al. [1993])

Salmon river strains	*TRP-2*92* frequency	Initial weight (g) in October		Final weight (g) in February	
		With	Without	With	Without
Dale	0.29	52.7±2.2^{a}	42.6±1.2^{b}	64.3±2.9^{a}	50.2±1.5^{b}
Lonevåg	0.10	47.9±2.8^{a}	40.7±0.8^{b}	55.0±4.0*	47.5±1.0^{b}*
Voss	0.09	40.3±1.7^{b}	40.5±0.7^{b}	51.2±2.0^{b}	49.0±0.7^{b}

The protease activities in the digestive tract are induced by feeding, affected by rearing temperatures and genetic feature of individuals, and increased to a level of normal immature fish after two months of first-feeding (Torrissen and Torrissen 1984). Trypsin activity is the main protease activities in the digestive system, and genetic variations in trypsin genotype affect trypsin specific activity, food utilization, and growth in Atlantic salmon. Genetic variations in the frequency of trypsin variant TRP-2*92 affect food utilization and growth rate in salmon populations.

Development of Trypsin Activity and Maturation

Atlantic salmon populations have differences in the development of digestive proteases (especially trypsin) and growth. Those from four different river populations showed variations in growth rates after two years in the sea, with significantly higher weights in the salmon from Namsen and Årøy rivers than those from Ordo and Neiden rivers (Torrissen and Torrissen 19840. Fish were sampled in June, and maturing salmon had started the development of eggs and melt. Total protease (mainly trypsin) specific activities were significantly higher in maturing than immature salmon, regardless of weight and population, and maturing females had greater response than maturing males (Figure 3A). No significant differences in the total protease specific activities were observed among immature salmon, and between immature males and females, from the different river strains.

Changes in protease specific activities in the digestive tract during maturing process were studied (Torrissen and Torrissen 1985). The total protease specific activities (sum of peptic and tryptic specific activities) of immature salmon were similar (5.52±1.93 μmol tyrosine h^{-1} mg $protein^{-1}$) throughout the year (Figure 3B). However, the maturing salmon had significantly higher total protease specific activity at the early stage of maturation in the spring (around April) (Males: 5.56±1.91 μmol tyrosine h^{-1} mg $protein^{-1}$, Females: 6.75±4.36 μmol tyrosine h^{-1} mg $protein^{-1}$) than immature fish (4.08±1.54 μmol tyrosine h^{-1} mg $protein^{-1}$). The enzyme levels of maturing salmon decreased later to levels similar to immature fish in the summer, and were significantly lower than immature fish in the autumn (around August). Regardless of sex, the enzyme specific activities in maturing salmon remained about 10–20% in September, and were maintained at these low levels until the fish were sexually mature in November and December. The tryptic activity was more influenced than peptic activity by the sexual maturation process, especially at the early stage of maturation (Figure 3B). The reduction in tryptic activity at the late stage of maturation was also affected by the disappearance of some trypsin isozymes (see Figure 4 in Chapter 6).

Moreover, the increase in trypsin specific activity was also related to the increase in astaxanthin levels in the plasma, which indicated a higher feed digestion and absorption of astaxanthin from the feed (Torrissen and Torrissen 1985). This led to a higher astaxanthin concentration in the white muscle. An indication of protein mobilization from white muscle to oocytes during sexual maturation was observed, as the concentration of muscle astaxanthin (bound to protein) significantly decreased with a significantly higher total astaxanthin in the ovary, compared to the immature salmon (Torrissen and Torrissen 1985).

It is possible to distinguish maturing and immature Atlantic salmon by the levels of tryptic activity and the development of gonads in the spring of the spawning year. The higher protease specific activities in maturing Atlantic salmon indicate higher energy requirement for maturation through higher consumption rates, compared to immature salmon.

During sexual maturation, the fish reduce somatic growth and optimize oocyte maturation through minimum feeding (Yoneda and Wright 2005; Rungruangsak-Torrissen 2007; Rungruangsak-Torrissen and Fosseidengen 2007; Rungruangsak-Torrissen et al. 2009a0 as well as reducing body temperature (see Rungruangsak-Torrissen 2007) by diving to more than 600 m depth before homing to freshwater spawning areas (Star–Oddi 2006). Although higher water temperature would promote higher maturation rate in fish populations (Rungruangsak-Torrissen et al. 2012), it is possible that a natural behaviour of vertical movement (distribution) within the same population may be influenced by maturation. Less

mature fish may tend to stay at a deeper water level where the temperature is lower to reduce somatic growth and increase maturation rate.

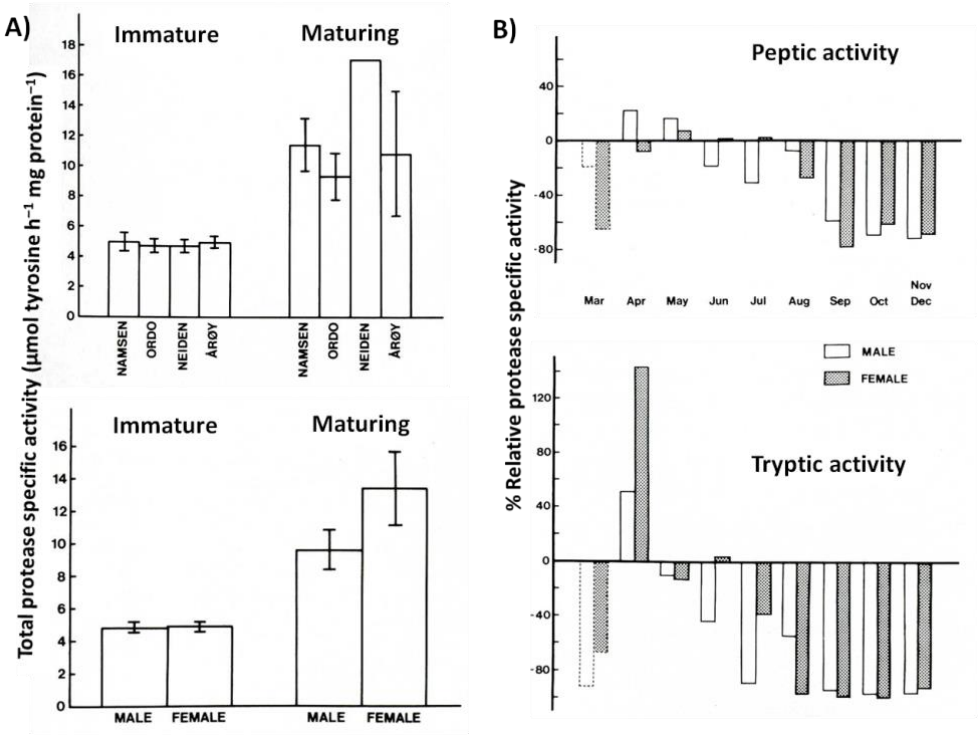

Figure 3. (A) Total protease specific activities of immature and maturing Atlantic salmon among four different river populations after two years in the sea and sampled in June with weight range of 0.7–4.8 kg, and (B) Percent relative protease specific activity of peptic activity in the stomach (at 37.5 ^{o}C) and tryptic activity in the pyloric caeca and intestine (at 52.5 ^{o}C) of maturing males (1.9–15 kg) and females (1.9–10.5 kg) Atlantic salmon compared to immature salmon (1.4–11.6 kg). (Adapted (A) from Torrissen and Torrissen [1984], and (B) from Torrissen and Torrissen [1985], with permission from Elsevier B.V.).

PERFORMANCE QUALITIES OF GROWTH AND MATURATION

Trypsin Activity, Insulin Secretion, Protein Growth and Maturation

Atlantic salmon with genetic differences in food utilization abilities (trypsin genotypes) have different temperature preferences for optimizing digestive efficiency, and show variations in trypsin specific activity as well as the protease activity ratio of trypsin to chymotrypsin (T/C ratio) in the pyloric caeca. These have resulted in the differences in maintenance ration, insulin secretion, protein synthesis capacity, growth rate, and temperature distribution in nature (see Chapter 6). Increased trypsin specific activity accompanied with increased plasma insulin level occurred at least one month before enhanced growth rate (Rungruangsak-Torrissen et al. 1999). At the time of the apparent mean growth differences between two fish

groups, the fish with higher growth rates had significantly higher concentrations of plasma insulin than the fish with slower growth rates, and trypsin specific activities were at low levels in both groups, but trypsin genotypes were not included in the study. The results indicated the digestion of dietary protein by trypsin as the primary mechanism, followed by plasma insulin secretion, for protein synthesis and growth in Atlantic salmon (Rungruangsak-Torrissen et al. 1999; Rungruangsak-Torrissen 2012). This is similar to the elevation of free amino acids in the plasma after feeding was observed to stimulate plasma insulin secretion (Rungruangsak-Torrissen and Sundby 2000; Rungruangsak-Torrissen 2012).

Protein synthesis capacity was studied in white muscle and oocytes during immature and maturing stages in Atlantic salmon (Rungruangsak-Torrissen 2007) as well as in Atlantic mackerel, *Scomber scombrus* L. (Rungruangsak-Torrissen and Fosseidengen 2007). A highly reverse power correlation was observed between the concentrations of protein and RNA/protein ratio in the white muscle of both Atlantic salmon (Figure 4A) and Atlantic mackerel (Figure 4B), regardless of feeding groups. The concentration of protein in white muscle influenced the rates of synthesis and turnover of protein in the white muscle. Interestingly, the relationship profiles were similar between the two species and indicated higher capacity for protein synthesis and turnover when the concentration of white muscle protein is below 150 mg g^{-1} (Figures 4A and 4B). A positive relationship was observed between the concentrations of protein and RNA in the white muscle (Rungruangsak-Torrissen and Fosseidengen 2007) (see Figure 7D in Chapter 6). However, they were not always highly correlated such as in white muscle during slow growth rates in Atlantic salmon with limited feeding and during maturing process, and in oocytes during oocyte development (Figure 4C). The relationships between the concentrations of RNA and RNA/protein ratio were affected by decreasing protein concentrations (Figure 4D) due to limited feeding, mobilization of muscle protein during maturing process, and hydrolysis of oocyte protein by its trypsin-like activity during oocyte maturation (Rungruangsak-Torrissen 2007, 2012). This indicates high protein turnover rates in both white muscle and oocytes during maturation.

Muscle protein concentration increases during growth (Rungruangsak-Torrissen 2007; Rungruangsak-Torrissen et al. 2009a). Increases in white muscle RNA concentration and capacity for protein synthesis result in synthesis and growth (Mathers et al. 1992; Carter et al. 1993; Houlihan et al. 1993). Protein growth occurs when protein synthesized exceeds the amount of protein retained (Houlihan 1991), and the efficiency of retention of synthesized protein is important for protein growth efficiency irrespective of the amount of protein synthesized (Carter et al. 1993). In addition, higher protein growth efficiency associates with lower protein turnover rate (Houlihan 1991; Hawkins 1991; Rungruangsak-Torrissen et al. 1999). Small differences in protein turnover that were not statistically significance could still result in differences in protein growth efficiency between individual salmon feeding at similar rates (Carter et al. 1993).

Studies in combinations with different biochemical parameters in different fish species further indicated that an increase in growth rate is not always associated with higher RNA concentration in the white muscle. It depends on the changes in body protein/lipid (P/L) ratio, whereas higher growth rate with increased lipid deposition would reduce muscle RNA concentration (Sunde et al. 2001). Increase in RNA/protein ratio could indicate higher protein synthesis capacity if the RNA level is increasing (Rungruangsak-Torrissen et al. 1999), and it could also indicate higher protein turnover rate if the protein level is decreasing (Rungruangsak-Torrissen and Male 2000; Rungruangsak-Torrissen 2007, 2012;

Rungruangsak-Torrissen and Fosseidengen 2007; Rungruangsak-Torrissen et al. 2012), especially during maturing process in mobilization of muscle protein and oocyte development (see Figure 4). Moreover, differences in growth rate are not always due to variations in food consumption, but it could also be due to differences in food utilization, as observed in Atlantic salmon with different trypsin genotypes (see Tables 3–5 in Chapter 6), and in diploid Atlantic salmon having better food utilization efficiency than the triploid counterparts (Sunde et al. 2001; Figure 11 in Chapter 60.

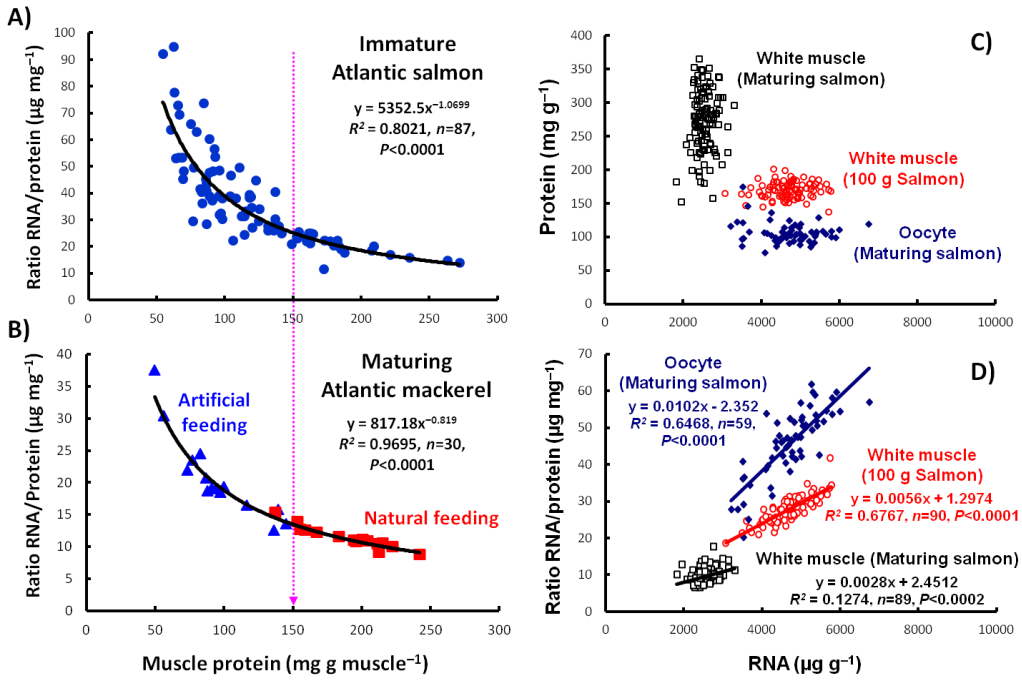

Figure 4. The relationships (A and B) between concentrations of protein and ratio of RNA to protein in the white muscle, (C) between concentrations of RNA and protein in white muscle and oocytes, and (D) between concentrations of RNA and ratio of RNA to protein in white muscle and oocytes. (From (A and B) Rungruangsak-Torrissen and Fosseidengen [2007], and (C and D) Rungruangsak-Torrissen [2007], with permission from John Wiley and Sons, Inc.).

Development of Oocytes during Maturation in Different Fish Species

Studies on maturation have been mainly on oocyte development, not only in Atlantic salmon (Rungruangsak-Torrissen 2007) but also in other species, such as Atlantic mackerel (Rungruangsak-Torrissen and Fosseidengen 2007) and Northeast Arctic cod, *Gadus morhua* L. (Rungruangsak-Torrissen et al. 2012). Females with higher maturation rates have lower specific activity levels of trypsin-like (T) as well as chymotrypsin-like (C) in the oocytes, with higher oocyte T/C ratio (in spite of the lower specific activity levels of the two proteases), regardless of species, and the trends are similar in aquaculture and in natural ecosystems (Table 2 and Figure 5).

The concentrations of RNA, protein, and RNA/protein ratio in the oocytes were dependent on the quality and amount of the food consumed (Table 2), whereas under similar feeding, their levels were lower in the oocytes of females with higher maturation rates (Figure 5). After fertilization, these values were still lower in the fertilized eggs than the oocytes, except for the protein synthesis capacity (RNA/protein ratio) that became higher in the fertilized eggs (Figure 5). The maturing fishes had different feedings (Table 2).

In Atlantic salmon (Rungruangsak-Torrissen 2007), the negative effects of 100% fishmeal replacement by krill meal were shown on feed conversion efficiency, pyloric caecal trypsin specific activity, and with a possibility of delayed maturation rate indicated by a higher oocyte trypsin-like specific activity (Table 2).

In Atlantic mackerel (Rungruangsak-Torrissen and Fosseidengen 2007), artificial feeding promoted fish growth, but affected on decreased muscle protein concentration and delayed maturation rate. A higher protein turnover rate (RNA/protein ratio) for mobilization from white muscle to oocytes was observed with higher levels of free amino acids (FAA) as well as the ratio of essential to non-essential FAA, but lower levels of RNA and RNA/protein ratio in the oocytes (Table 2).

In Northeast Arctic cod (Rungruangsak-Torrissen et al. 2012), the cod in area B living closer to coastal area with higher maturation rate (higher oocyte T/C ratio) showed lower feeding (lower trypsin as well as chymotrypsin specific activity in the pyloric caeca). They had lower somatic growth (lower pyloric caecal T/C ratio) with lower oocyte protein turnover rate (oocyte RNA/protein ratio). A higher oocyte protein of cod in this area might be due to a higher muscle protein mobilization (Table 2).

Correlation studies among different biochemical parameters in relation to oocyte parameters were summarized by Rungruangsak-Torrissen [2012] who indicated the importance of food utilization efficiency during sexual maturation for protein deposition in the white muscle where the protein would be mobilized to oocytes and hydrolyzed by trypsin-like activity in the oocytes into amino acids reserved as food for the offspring. Also, fish with different feedings and growth statuses have differences in growth performance quality including mobilization of white muscle protein. The different biochemical relationships provided more understanding on performance qualities of growth and maturation that would be varied under different environmental conditions and growth statuses of the fish (Rungruangsak-Torrissen 2012).

During the growing phase, the levels of pyloric caecal T/C ratio could affect the levels of white muscle free amino acids, but this was not observed during maturation when growth was reduced. Instead, the T/C ratio levels in the pyloric caeca were correlated with feed efficiency, and feed efficiency correlated with the level ratio of protein to lipid (P/L ratio) in the oocytes, which suggested an important of protein digestive efficiency for oocyte quality (Rungruangsak-Torrissen 2012).

The levels of white muscle free amino acids from nutrient transport could influence the levels of protein and protein turnover in the white muscle, but this was not observed in the oocytes due to free amino acids in the oocytes were from the hydrolysis of the oocyte self-protein. A reduced protein synthesis in the white muscle suggested a higher lipid deposition during growing phase as RNA levels related with RNA/protein ratio but not with protein levels in the white muscle, or suggested more protein mobilization during maturing process as RNA levels related positively with protein levels but negatively with RNA/protein ratio.

Table 2. Biological parameters of maturing females in Atlantic salmon without and with 100% fishmeal replacement by krill meal, in Atlantic mackerel without and with artificial feeding, and in Northeast Arctic cod in three studied areas in the Barents Sea. The specific activities of trypsin, chymotrypsin, trypsin-like, and chymotrypsin-like are expressed as µmol *p*-nitroaniline h^{-1} mg $protein^{-1}$. Within the same species, the values with different superscripts or with asterisk (*) are significantly different ($P < 0.05$). FAA, free amino acids. (Data from Rungruangsak-Torrissen [2007] and Rungruangsak-Torrissen and Fosseidengen [2007], with permission from John Wiley and Sons, Inc., and Rungruangsak-Torrissen et al. [2012], with permission from Bentham Open)

Parameters	Atlantic salmon		Atlantic mackerel		Northeast Arctic cod		
	Without	With	Without	With	Area A	Area B	Area C
Weight (kg)	1.827±0.098	1.661±0.075	0.296±0.01*	0.502±0.02*	1.36±0.36	0.85±0.35	0.90±0.17
Feed conversion efficiency (FCE)	1.10±0.02*	0.83±0.03*	–	–	–	–	–
Pyloric caeca							
Trypsin (T)	11.8±1.2*	8.7±0.7*	23.52±3.70	22.51±3.35	86.84±9.94[a]	52.17±2.96[b]	69.45±5.63[a]
Chymotrypsin (C)	23.9±3.3	16.6±1.5	196.0±42.9	212.8±24.7	189.9±14.8	162.94±0.20	175.70±9.62
T/C ratio	0.53±0.03	0.55±0.04	0.124±0.006	0.108±0.006	0.45±0.02[a]	0.32±0.02[b]	0.39±0.02[b]
White muscle							
RNA (µg g^{-1})	2,684±64	2,590±67	2,044±43	1,867±71	3,393±125[a]	2,319	2,993±101[b]
Protein (mg g^{-1})	245.3±11.2	260.1±11.9	187.8±24.1*	99.3±12.8*	364±38	109	349±27
RNA/Protein ratio (µg mg^{-1})	11.2±0.4	10.4±0.7	11.46±1.56*	20.87±2.96*	11.04±1.38	21.25	11.02±1.10
Oocytes							
Trypsin-like (T)	0.44±0.04	0.63±0.10	6.33±1.59*	15.38±2.48*	16.27±1.29[a]	13.15±0.44[b]	22.80±2.24[a]
Chymotrypsin-like (C)	–	–	–	–	16.99±1.12[a]	13.38±0.41[b]	25.15±2.27[c]
T/C ratio	–	–	–	–	0.96±0.04[ab]	0.98±0.00[a]	0.91±0.03[b]
Total FAA (µmol g^{-1})	–	–	42.73±0.48*	69.86±9.06*	–	–	–
Essential FAA (EAA) (µmol g^{-1})	–	–	4.36±0.29*	14.09±2.91*	–	–	–
Non-essential FAA (NEAA) (µmol g^{-1})	–	–	38.37±0.25	55.77±6.21	–	–	–
EAA/NEAA ratio	–	–	0.11±0.01*	0.24±0.03*	–	–	–
RNA (µg g^{-1})	4,895±322	4,753±266	8,309±570*	5,355±404*	17,501±957	18,944±116	18,766±1,098
Protein (mg g^{-1})	107.1±3.8	101.7±2.7	167.2±13.3	157.5±8.0	393±46[a]	526±27[b]	424±43[a]
RNA/Protein ratio (µg mg^{-1})	46.4±4.0	46.7±2.3	50.64±5.34*	34.49±3.02*	56.54±8.94[a]	36.11±2.05[b]	53.60±8.32[a]

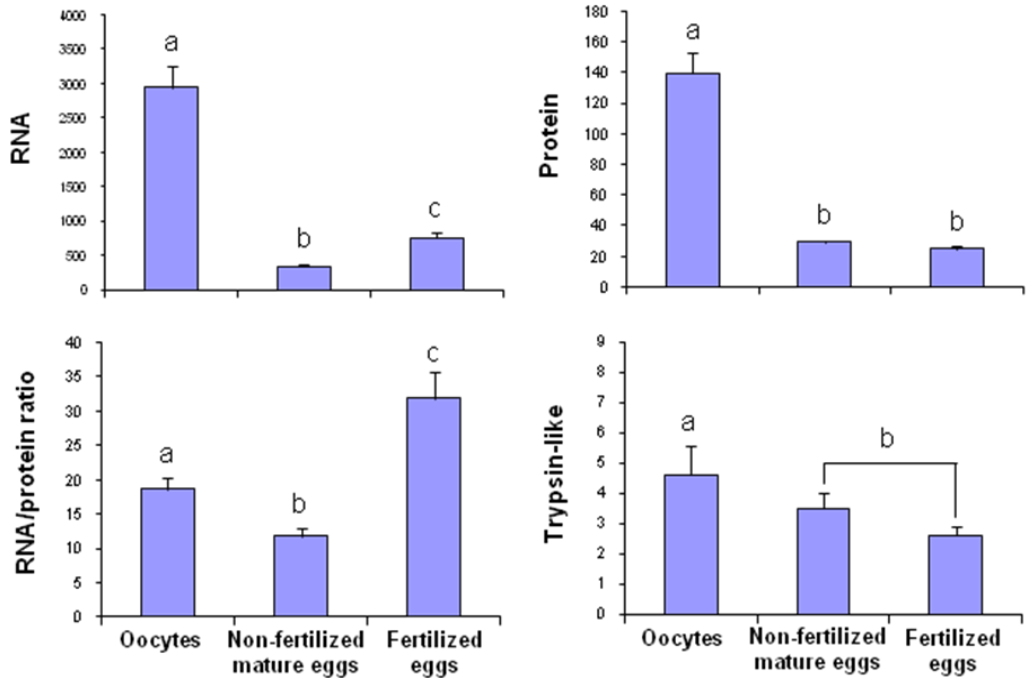

Figure 5. Concentrations of RNA (µg g^{-1}), protein (mg g^{-1}), RNA/Protein ratio (µg mg^{-1}), and specific activity of trypsin-like (µmol *p*-nitroaniline h^{-1} mg $protein^{-1}$) in oocytes, non-fertilized eggs, and fertilized eggs with more than 8 cells, from maturing Northeast Arctic cod. The bars with different superscripts are significantly different ($P < 0.05$). (From Rungruangsak-Torrissen et al. [2012], with permission from Bentham Open).

However, an increased protein synthesis in the white muscle suggested a higher protein deposition or less protein mobilization during maturing process (Rungruangsak-Torrissen 2012). Moreover, the increase in the ratio of protease activities of trypsin-like to chymotrypsin-like (T/C ratio) in the oocytes indicated a higher oocyte maturation rate, in spite of the lower specific activities of these oocyte proteases at higher maturing stage (Rungruangsak-Torrissen et al. 2012). A negative correlation between trypsin-like specific activity and glycine levels was observed in the oocytes, and suggested that specific activity of trypsin-like is reduced when most oocyte protein is hydrolyzed at higher maturing stage, as glycine is the essential amino acid in collagen and could be a precursor for synthesis as well as a catabolic product of connective tissues. This is in accordance with the indications of lower protein levels with lower trypsin-like specific activity in eggs at higher maturing stage (Figure 5). More details of these relationships were described in Rungruangsak-Torrissen [2012].

In order to reserve energy for the maturing process, maturing Atlantic salmon will have high consumption rate early in spring of their spawning year. During the maturing process in females, white muscle protein is mobilized to oocytes where higher oocyte trypsin-like specific activity will result in higher protein hydrolysis in the oocytes, and the enzyme specific activity will become lower when oocyte protein is lower in fish with higher maturation rate (Figure 5).

The T/C ratio level in the pyloric caeca is associated with fish growth, while the T/C ratio level in the oocytes is associated with oocyte growth (oocyte development), regardless of the specific activity levels of the two proteases (Rungruangsak-Torrissen et al. 2012; Rungruangsak-Torrissen 2012). This indicates the extraordinary advantage of studying the T/C ratio levels in both digestive system and oocytes. Such studies have not yet been performed elsewhere.

It is very important to utilize different biochemical techniques simultaneously, so that the statuses of fish growth (reducing growth phase, steady growth phase, or growing phase) are known and the direction of biological changes of the studied fishes could be precisely interpreted.

ENVIRONMENTAL EFFECT STUDIES IN ATLANTIC SALMON

Effects of Starvation, Re-feeding, and Temperature on Digestive Proteases

Development of specific activities of pepsin (in stomach) and trypsin and chymotrypsin (in pyloric caeca) were investigated in Atlantic salmon during starvation and re-feeding (Rungruangsak-Torrissen et al. 20060. During starvation (limited food), specific activities of pepsin and trypsin (T) decreased and were maintained at low levels while those of chymotrypsin (C) increased and were maintained at high levels which resulted in low levels of the T/C ratio. After diet deprivation, a decrease in trypsin activity is common in fish, while a decrease as well as an increase in chymotrypsin activity is observed (reviewed in Rungruangsak-Torrissen 2012). The increase in activity of chymotrypsin after diet deprivation is due to fish consuming natural food in the water (Rungruangsak-Torrissen 2012). Therefore, the activity of chymotrypsin could increase when there was a reduction in growth rate (Rungruangsak-Torrissen et al. 2006, 2009a, 2009b), which was unexpected. The T/C ratio value would be higher in the fish with higher growth potential if they were at a growing phase (Sunde et al. 2001; Rungruangsak-Torrissen et al. 2006), while during a reduced or steady growth phase, the fish with higher growth would show lower T/C ratio value due to relatively higher increased chymotrypsin specific activity (Rungruangsak-Torrissen et al. 2009a; Rungruangsak-Torrissen and Fosseidengen 2007).

Trypsin and chymotrypsin expressions could be explained in a similar way as a car driven that higher acceleration (trypsin specific activity) is needed to increase car speed (fish growth), and more braking capacity (chymotrypsin specific activity) is necessarily for stopping the car (fish) at higher speed (higher growth) (Rungruangsak-Torrissen and Fosseidengen 2007; Rungruangsak-Torrissen 2012). The increase in the T/C ratio value is independent of the specific activity levels of trypsin and chymotrypsin.

The other indication showing increasing chymotrypsin specific activity during slower growth is illustrated in Figure 6. During ordinary routine feeding and growth, trypsin activated higher chymotrypsin at 6 ^{o}C than at 10 ^{o}C (Figure 6A) while the T/C ratio values were higher at 10 ^{o}C than at 6 ^{o}C (Figure 6B). The relationship between specific activities of trypsin and chymotrypsin could be observed when growth was limited (Rungruangsak-Torrissen 2007), during food deprivation (Rungruangsak Torrissen and Male 2000; Rungruangsak-Torrissen et al. 2006) and during maturation (Rungruangsak-Torrissen et al. 2009a; Rungruangsak-

Torrissen and Fosseidengen 2007). This relationship disappeared at post-feeding after starvation (Rungruangsak Torrissen and Male 2000) and during a growing phase (Figure 6A). According to different studies (Rungruangsak-Torrissen et al. 2006, 2009a, 2009b, 2012; Rungruangsak-Torrissen 2007; Rungruangsak-Torrissen and Fosseidengen 2007), the relationship between trypsin specific activity and the T/C ratio can indicate growth status of the fish whether it is growing phase (positive relationship; Figure 6B), steady growth phase (no relationship), or reducing growth phase (negative relationship; Figure 7A).

These protease parameters (specific activities of trypsin and chymotrypsin, T/C ratio) were also studied in the faeces, and they could also indicate fish digestive efficiency, but could not predict fish growth status (Rungruangsak-Torrissen 2007), as shown in Figure 7B with the opposite direction of the relationship compared to the actual growth reduction shown in Figure 7A.

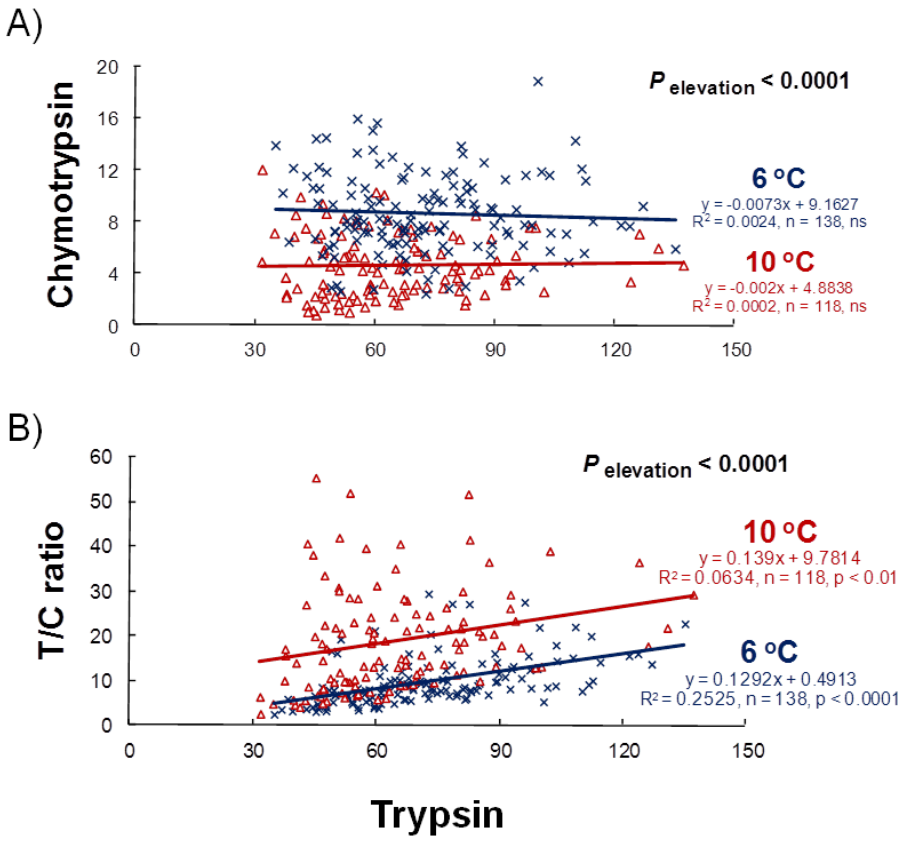

Figure 6. Effect of water temperatures on the relationships of trypsin (A) with chymotrypsin and (B) with the activity ratio of trypsin to chymotrypsin (T/C ratio), in Atlantic salmon parr reared at 6 ^{o}C (×) and 10 ^{o}C (Δ). The enzyme specific activities of trypsin and chymotrypsin in the pyloric caeca are expressed as µmol *p*-nitroaniline h^{-1} mg $protein^{-1}$. ns, not significant. (Adapted from Rungruangsak-Torrissen et al. [2006], with permission from Springer Corp.).

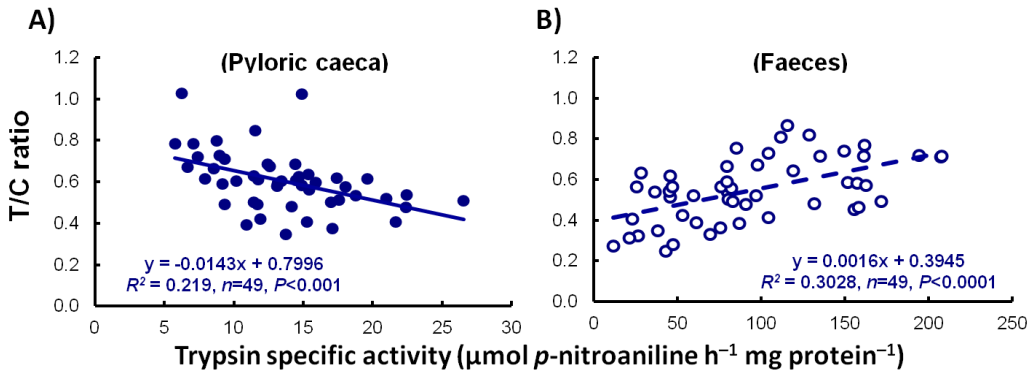

Figure 7. The relationship between trypsin specific activity and T/C ratio (A) in the pyloric caeca, compared to (B) in the faeces, in Atlantic salmon of the same individuals. (Adapted data from Rungruangsak-Torrissen [2007], with permission from John Wiley and Sons, Inc.).

Effects of Light Regime and Vaccine Type on Food Utilization and Growth

The effects of different light regimes in combination with vaccination were studied in Atlantic salmon (Rungruangsak-Torrissen et al. 2009b). The additional continuous light rigime (NL-LL group) for manipulating fish growth in sea cages affected food utilization through trypsin and chymotrypsin specific activities in the pyloric caeca (Figure 8). It took 70 days for the fish to adjust to the new environment as indicated by the return to low trypsin and chymotrypsin specific activities. At the time when the weights were different between the control (NL group) and the NL-LL group, the T/C ratio peaked in spite of low specific activity levels of trypsin and chymotrypsin, with the crossing of the regressions of slope T/C ratios, and the changes in directions of specific growth rate (SGR) between the two groups (Figure 8). The slope T/C ratio was calculated from the slope of the regression between the specific activities of trypsin and chymotrypsin (see Figure 10).

The relatively higher T/C ratio in the NL-LL group on day 70 affected the SGR during days 70–106, compared to the NL group. This indicated the effect of T/C ratio on SGR over a period of 1–2 months, and it is independent of the specific activity levels of trypsin and chymotrypsin. The decrease in T/C ratio value due to increased chymotrypsin specific activity at the end of the experiment would predict a later reduction in SGR of the NL-LL group if the experiment had been prolonged for 1–2 months. However, the slope T/C ratio indicated fish growth at sampling, as its relationship with fish weight was observed (Figure 9A), while such relationship was not observed with T/C ratio when the T/C ratio predicted future direction of SGR.

The study indicates that the additional continuous light stimulates fish growth rate during winter to spring when the natural day length is short, but reduces fish growth rate later during summer when the natural day length is long.

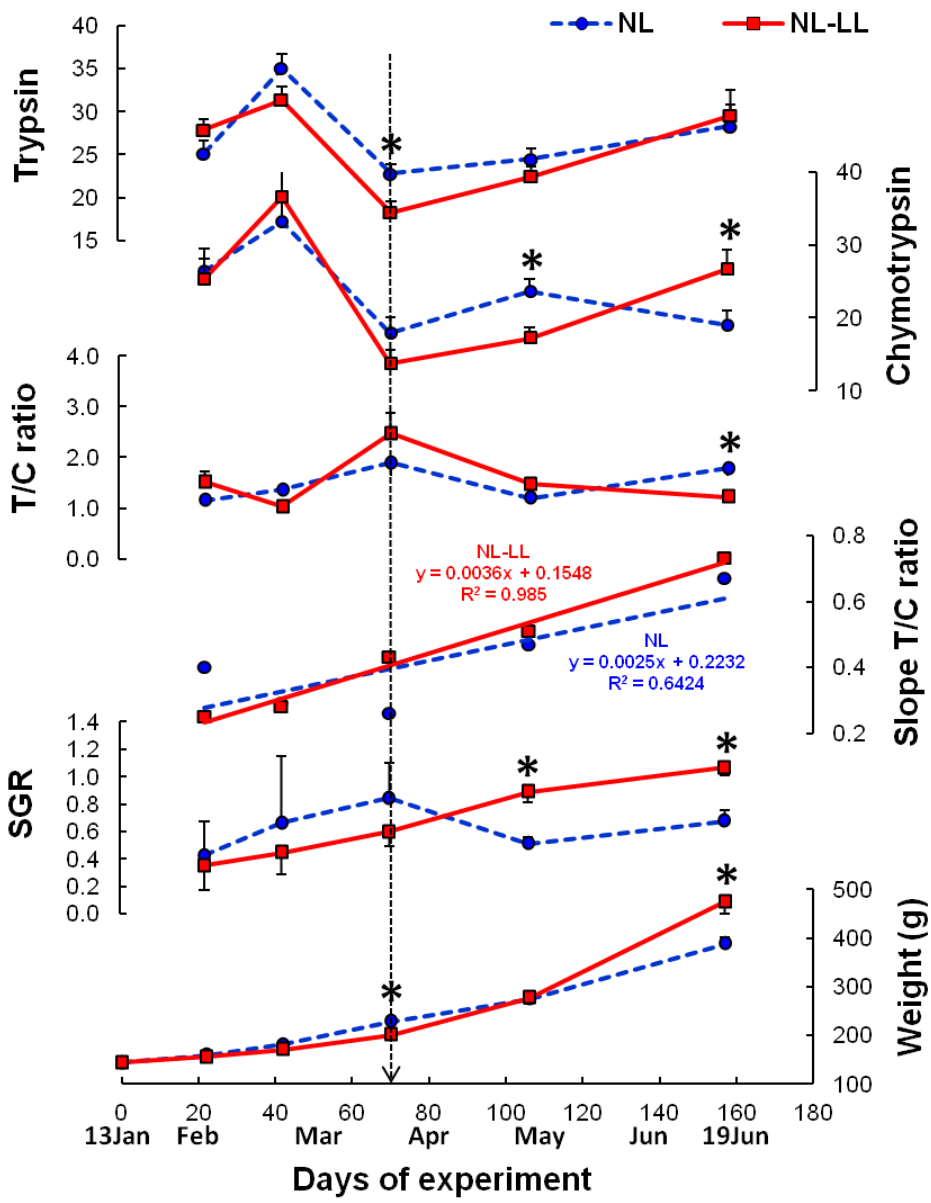

Figure 8. Weight (g), specific growth rate (SGR: % day^{-1}), and trypsin (T) and chymotrypsin (C) specific activities (expressed as µmol *p*-nitroaniline h^{-1} mg protein^{-1}) as well as T/C ratio during the entire experimental period, between Atlantic salmon reared under natural light regime (NL) and natural light with additional continuous light (NL-LL). The slope T/C ratios were calculated from the slopes of the regressions between specific activities of trypsin (*Y*-axis) and chymotrypsin (*X*-axis) at each sampling period. The asterisk (*) indicates significant differences between the two fish groups at that time ($P < 0.05$). The arrow indicates the change in digestive efficiency accompanied with the change in growth rate. (Data from Rungruangsak-Torrissen et al. [2009b], with permission from Springer Corp.).

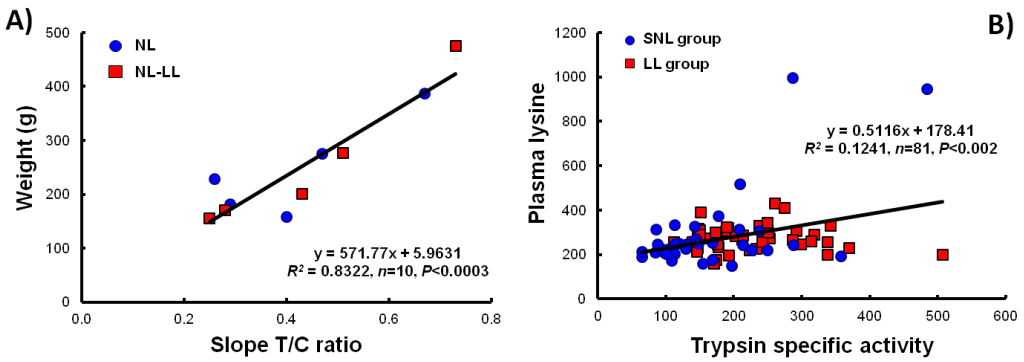

Figure 9. The relationships (A) between slope T/C ratio and mean fish body weight, regardless of light regimes, and (B) between trypsin specific activity (µmol *p*-nitroaniline h^{-1} mg $protein^{-1}$) and free lysine levels in the plasma (nmol ml^{-1}), regardless of light regimes. Atlantic salmon were reared under different light regimes; natural light (NL), natural light with additional continuous light (NL-LL), simulated natural light (SNL), and continuous light (LL). (Data from Rungruangsak-Torrissen et al. [2009b], with permission from Springer Corp.).

For muscle quality, only RNA concentration was higher in the NL-LL group (Rungruangsak-Torrissen et al. 2009b). For oocytes, the continuous light had no significant effect on quality at this stage, except for a tendency of higher oocyte trypsin-like specific activity that might influence later on a delay in oocyte maturation rate, compared to the NL group (Rungruangsak-Torrissen et al. 2009b).

The effects of mineral oil adjuvanted multiple vaccines (vaccine 1 was against furunculosis, vibriosis and cold water vibriosis and vaccine 2 also protected against winter ulcer) were studied (Rungruangsak-Torrissen et al. 2009b). Under the natural light regime, there were no significant effects by the vaccines on fish growth and T/C ratio. The similar T/C ratio predicted a similar later growth rate between the two fish groups. However, the additional continuous light, that enhanced the effects of both vaccines and fish growth, had a negative impact on vaccine 1 on the slope T/C ratio (0.459), compared to vaccine 2 (0.648; Figure 10A). A higher impact on vertebral growth (incidence of short tail) was concomitantly observed in salmon with vaccine 1 (25%) than those with vaccine 2 (12%), independent of light regimes (Rungruangsak-Torrissen et al. 2009b).

A similar experiment was studied indoor on the effects of continuous light (LL) compared to simulated natural light (SNL) (Rungruangsak-Torrissen et al. 2009b). Continuous light did not only stimulate fish growth rate, trypsin specific activity, and T/C ratio, but also increased the levels of non-essential free amino acids (NEAA) in plasma and white muscle. However, the levels of the ratio of essential to non-essential free amino acids (EAA/NEAA ratio), RNA and RNA/Protein ratio in white muscle were lower, which indicated lower protein growth efficiency in NL-LL group, as also observed a double fillet lipid content in these salmon (see Rungruangsak-Torrissen et al. 2009b). Plasma free lysine levels were affected by light regime and trypsin specific activity (Figure 9B). The levels of free hydroxyproline in both plasma and white muscle (affecting SGR) and of other free amino acids (involving in protein synthesis) were influenced by light regime (Rungruangsak-Torrissen et al. 2009b). The slope T/C ratio values were similar between the two light regimes with significantly higher elevation of trypsin specific activity in the LL group (Figure 10B).

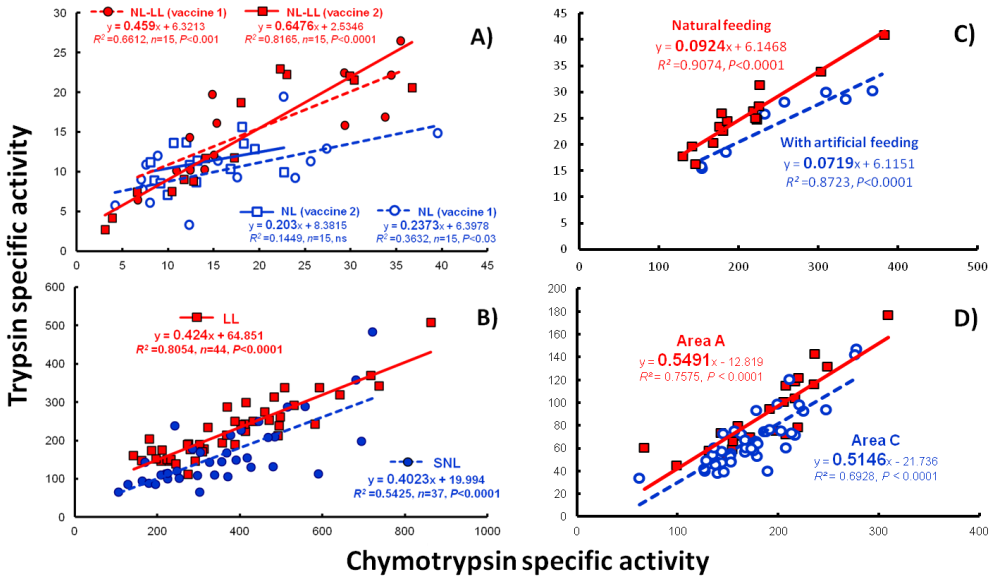

Figure 10. The relationships between trypsin and chymotrypsin specific activities (μmol *p*-nitroaniline h^{-1} mg $protein^{-1}$) in the pyloric caeca of Atlantic salmon, (A) effects of natural light (NL), natural light with additional continuous light (NL-LL) and vaccine types, and (B) effects of simulated natural light (SNL) and continuous light (LL); (C) of maturing Atlantic mackerel with different feedings; and (D) of maturing Northeast Arctic cod in different Barents Sea areas. The slopes of the regressions indicate the activity ratio of trypsin to chymotrypsin (slope T/C ratio). ns, not significant. (From (A and B) Rungruangsak-Torrissen et al. [2009b] with permission from Springer Corp., (C) adapted data from Rungruangsak-Torrissen and Fosseidengen [2007], and (D) Rungruangsak-Torrissen et al. [2012] with permission from Bentham Open).

Interestingly, slope T/C ratio could provide the advantageous illustrations of whether the two treatments had been under overlapping (Figure 10A) or distinct (Figure 10B) living conditions. The regressions of slope T/C ratio between Atlantic mackerel without and with artificial feedings (Figure 10C) and between Northeast Arctic cod living in different Barents Sea areas (Figure 10D) indicated the two fish groups in each species living in separate environments. The lower elevation of trypsin specific activity observed in artificially fed mackerel (Figure 10C) was due to a higher growth reduction during maturation in higher growth fish (Rungruangsak-Torrissen and Fosseidengen 2007; Rungruangsak-Torrissen et al. 2009a), similar to the observation in maturing rainbow trout, *Oncorhynchus mykiss* Walbaum (see Figure 16A). The Northeast Arctic cod population living in Area A with higher maturation rate (Table 2) also showed higher consumption rate and growth within 1–2 months period during the time of being caught, compared to the population in Area C (Figure 10D). It was expected to have a higher growth reduction at later stage of maturation as shown by a non-significant regression between T/C ratio (*Y*-axis) and trypsin specific activity (*X*-axis) which indicated stopping somatic growth, while the population in Area C still had somatic growth shown by a significant positive regression (Rungruangsak-Torrissen et al. 2012). More biological informations can be found in Table 2.

A higher elevation of trypsin specific activity indicates a higher consumption rate of diet and/or dietary protein, and a higher T/C ratio (independent of specific activity levels of trypsin and chymotrypsin) or slope T/C ratio indicates higher digestive efficiency and growth rate.

DIETARY QUALITY STUDIES IN ATLANTIC SALMON

Effects of Feed Processing Conditions on Protein Digestibility Quality *In Vitro*

Dietary quality is dependent on both chemical and nutritional properties. For carnivorous species like Atlantic salmon, the quality of dietary protein is very important for growth performance that will result in the quality of the food for human consumption.

Rungruangsak-Torrissen et al. (2002) studied the association between chemical and nutritional properties of fish materials processed under different conditions (Figure 11), and showed that the biochemical structure of the dietary protein (contents of reactive sulphydryl (SH) group, disulphide (S–S) bond, ratio of SH/S–S, and *D*-aspartic acid) indicates digestibility of the dietary protein, and is changed under different processing conditions.

The *in vitro* protein digestibility (using a dialyzed crude enzyme extract from the pyloric caeca of Atlantic salmon) correlated positively with the reactive SH content (Figure 11A) as well as the content ratio of SH/S–S that negatively correlated with the concentration of *D*-Asp (Figure 11B). The *D*-Asp level is caused by the racemization of *L*-Asp during processing (Luzzana et al. 1996, 1999).

Increases in S–S bond formation and Asp racemization reduced the nutritional quality of the dietary protein (Rungruangsak-Torrissen et al. 2002) (see Figure 12).

The relationship between *in vitro* protein digestibility and these dietary parameters has made the *in vitro* protein digestibility technique useful for detection an extent of difference among the raw materials and formulated diets as usually detected by the animal itself (Rungruangsak-Torrissen et al. 2002).

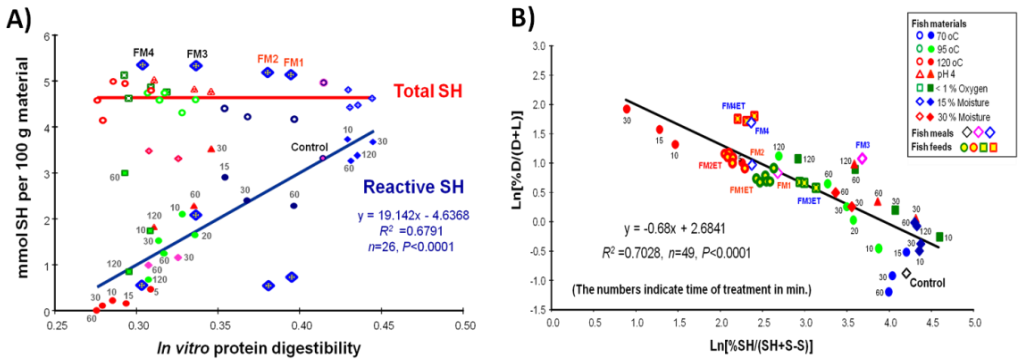

Figure 11. (A) Effect of the reactive SH content on *in vitro* protein digestibility (µmol *DL*-alanine equivalent liberated reactive amino group of peptides produced per 100 µg fish material) by Atlantic salmon pyloric caecal crude enzyme extract, and (B) relationship between SH and *D*-aspartic acid contents, in 27 fish materials produced under different processing conditions. The values of different fish meals (FM1–FM4) and fish feeds (produced from fish meals FM1 and FM2) are also illustrated, but not included in the regressions. The numbers indicate time of treatment in min. (From Rungruangsak-Torrissen et al. [2002], with permission from John Wiley and Sons, Inc.).

In vitro Digestibility of Dietary Protein in Different Fish Species

Dialyzed crude enzyme extracts from different ages of Atlantic salmon, rainbow trout, and European seabass *Dicentrarchus labrax* L., were compared using an *in vitro* protein digestibility technique (Rungruangsak-Torrissen et al. 2002). By standardizing trypsin activity (the key protease) in the crude enzyme extracts, the results could be comparable and indicated that different fish species and fish ages have different digestion ability to the same feed types (Figure 12), and the effective time for feed utilization and growth is dependent on fish sensitivity and the extent of difference in digestibility between the feeds consumed (Rungruangsak-Torrissen et al. 2002).

Significant differences in feed quality were due to fish meal types (Figure 12A), and not due to extrusion conditions (Figure 12B). Therefore, the quality of the protein meal used for feed formulation is very important for dietary quality. The rank of sensitivity of the crude enzymes to feed quality was Atlantic salmon > rainbow trout > European seabass, with rainbow trout enzymes showing a higher *in vitro* protein digestion ability (Figure 12). This suggests higher feed utilization in rainbow trout than the other species under the same conditions. Younger salmon digest the feeds better than older salmon (Figure 12A). Rainbow trout enzymes differentiated the experimental feeds similarly to Atlantic salmon enzymes, while the enzymes from European seabass did not. Therefore, it is important to use the enzyme extract from a specific species and at the age of interest to test the nutritional quality of the experimental diets.

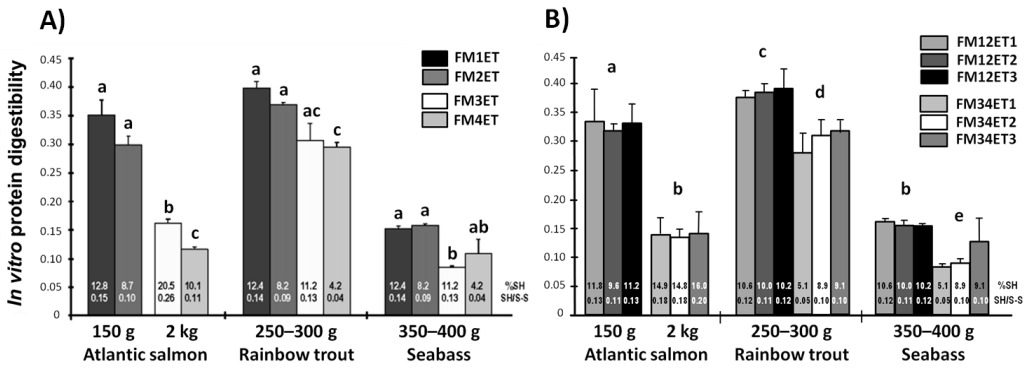

Figure 12. Association of SH content and SH/S–S content ratio of different experimental feeds with *in vitro* protein digestibility (µmol *DL*-alanine equivalent liberated reactive amino group of peptides produced per 100 µg feed) by crude enzyme extracts from different fish species that had never been fed the experimental feeds before. The dialyzed crude enzyme extracts were standardized by trypsin activity for all species. *In vitro* protein digestibilities are (A) grouped by fish meal types (FM1–FM4), regardless of extrusion conditions, and (B) grouped by fish meals (FM1,2 and FM3,4) and extrusion conditions (ET1–ET3). The bars or group bars with different superscripts are significantly different ($P < 0.05$). (From Rungruangsak-Torrissen et al. [2002], with permission from John Wiley and Sons, Inc.).

Rungruangsak-Torrissen [2007] also used the *in vitro* protein digestibility technique for quality assessment of salmon feeds containing different levels of fish meal replacement by krill meal (Figures 13A and 13B). At the same trypsin activity, the crude enzyme extracts from younger fish utilized the diets better than older fish (Figures 13A and 13C) similar to the observation shown in Figure 12A. Increasing krill meal levels showed an adverse effect on the

digestibility (Figures 13A and 13B). Moreover, studies in rainbow trout families with different growth capacities (Rungruangsak-Torrissen et al. 2009a) indicated that the digestive enzyme extracts from high growth families resulted in higher *in vitro* protein digestibility for all studied diets, compared to the enzymes from slower growth families (Figure 13D).

If there are significant differences in the chemical parameters of dietary protein (contents of SH group, SH/S–S ratio, and *D*-Asp), significant differences in feed utilization and growth are expected.

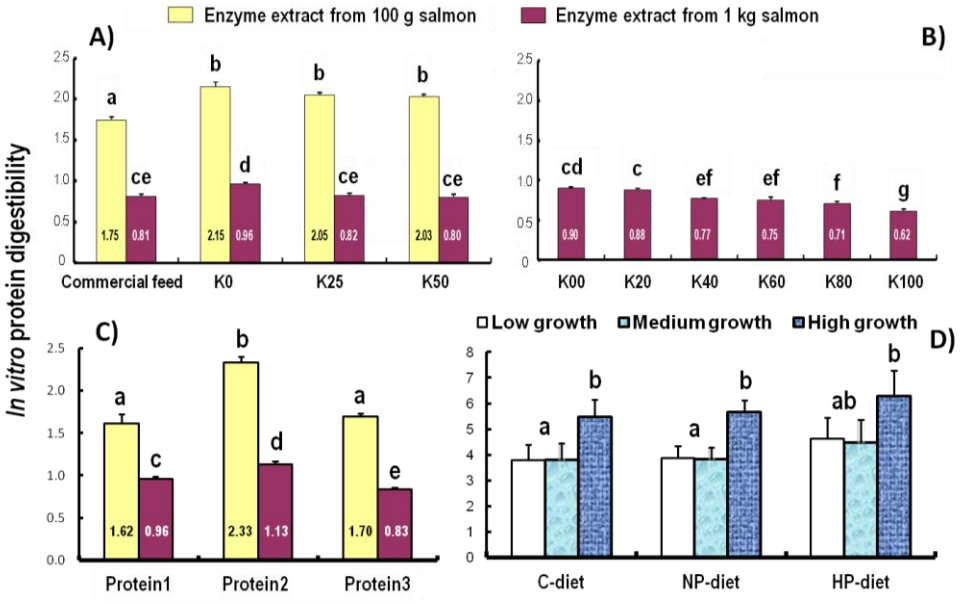

Figure 13. *In vitro* protein digestibility of different experimental diets (expressed as µmol *DL*-alanine equivalent per mg feed) and standardized by trypsin activity. (A and B) Different experimental diets (with numbers indicating different levels of fish meal replacement by krill meal) in comparison with commercial feed, and (C) three feed materials with different protein qualities. The crude enzyme extracts were from 100 g and 1 kg Atlantic salmon. (D) Two experimental diets with normal (NP-diet) and high (HP-diet) protein levels, compared to control diet (C-diet), where crude enzyme extracts used were from rainbow trout families with low, medium and high growth performance. The bars with different superscripts are significantly different ($P < 0.05$). (From (A and B) Rungruangsak-Torrissen [2007] with permission from John Wiley and Sons, Inc., (C) unpublished data, and (D) Rungruangsak-Torrissen et al. [2009a] with permission from Hindawi).

However, if significant differences between diets are observed in the *in vitro* protein digestibility by using an animal crude enzyme extract, it can be used to predict an actual time effect on potential growth differences within the next three months, as the *in vitro* protein digestibility associated with feed conversion efficiency (FCE) within 3 months of feeding, which is a common experimental period for growth study (Rungruangsak-Torrissen et al. 2002; Rungruangsak-Torrissen 2007). If a different *in vitro* protein digestibility is observed without statistical differences (Figure 13A of enzyme extract from 100 g salmon), it will take more than three months to affect FCE and SGR of the fish if there will be any effect of dietary quality (Rungruangsak-Torrissen 2007).

The *in vitro* protein digestibility technique is the most practical, quick and reliable method for testing nutritional quality of dietary protein, and it also reduces the number of

experimental animals used. Comparisons can be performed both within and between species if the crude enzyme extracts are standardized by trypsin activity, and shall be performed before starting up growth experiments that may not be necessary (Rungruangsak-Torrissen et al. 2002, 2009a; Rungruangsak-Torrissen 2007).

Effects of Digestibility Quality of Dietary Protein on Growth

The usefulness of *in vitro* protein digestibility technique has been tested on fish growth (Rungruangsak-Torrissen et al. 2002, 2009a; Sunde et al. 2004; Rungruangsak-Torrissen 2007), and showed its relationships with final fish weight, FCE and T/C ratio (Figure 14). The values declined when the level of krill meal was increased in the diets, which indicated an adverse effect of krill meal at a certain level in the diets on the digestive efficiency and growth of fish (Figure 14). Antarctic krill meal had less impact on fish growth than Atlantic krill meal (Rungruangsak-Torrissen 2007).

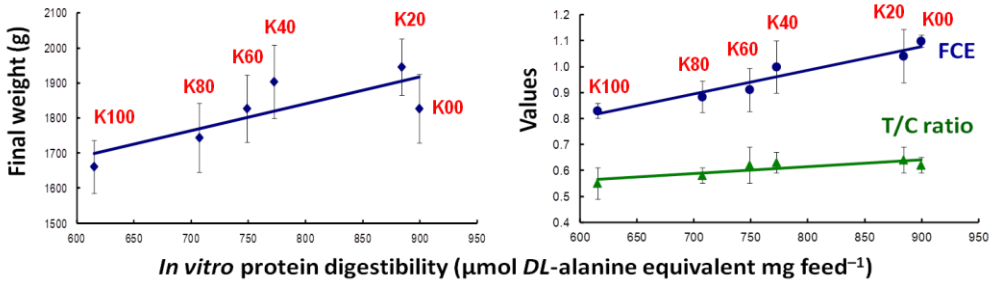

Figure 14. The relationships between *in vitro* protein digestibility (standardized by trypsin activity) of different experimental diets (with numbers indicating different levels of fish meal replacement by krill meal), with final salmon weight (R^2 = 0.6369, P = 0.057), feed conversion efficiency (FCE) (R^2 = 0.9304, P < 0.002), and protease activity ratio of trypsin to chymotrypsin (T/C ratio) (R^2 = 0.6443, P = 0.05). (Adapted from Rungruangsak-Torrissen [2007], with permission from John Wiley and Sons, Inc.).

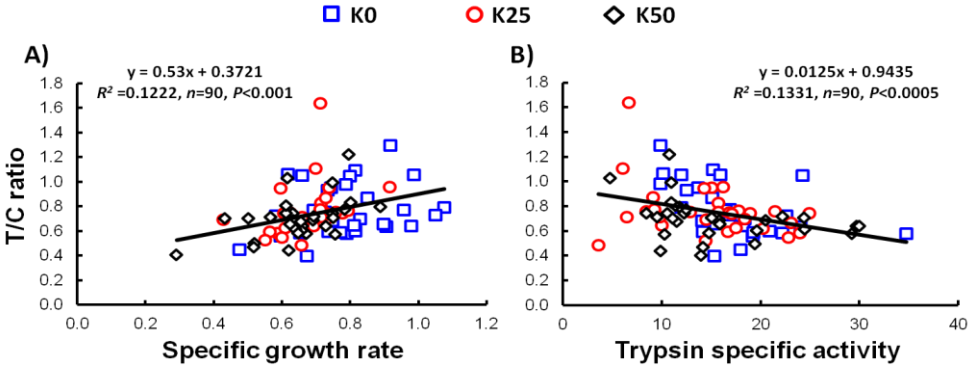

Figure 15. The relationships between T/C ratio (A) with specific growth rate, and (B) with trypsin specific activity showing growth status (reducing growth phase) of the Atlantic salmon. The experimental diets show numbers indicating different levels of fish meal replacement by krill meal. (Adapted data from Rungruangsak-Torrissen [2007], with permission from John Wiley and Sons, Inc.).

With *ad lib* feeding, the SGR was not correlated with either FCE or *in vitro* protein digestibility (Rungruangsak-Torrissen 2007). However, T/C ratio values correlated positively with SGR (Figure 15A), but negatively with trypsin specific activity (Figure 15B), which indicated a reduction in growth rate of the fish in the experiment.

The T/C ratio is more sensitive and more advantageous parameter than trypsin specific activity as it is independent on external factors, such as changes in the protein content and enzyme concentration in the crude enzyme extracts (Rungruangsak-Torrissen 2007).

A highly significant correlation between T/C ratio and SGR, although at a low correlation coefficient value (Figure 15A and also in Chapter 6 in Figure 13B), indicated that an increase in trypsin expression as well as a decrease in chymotrypsin expression in the digestion process is the mediator that stimulates growth (Rungruangsak Torrissen and Male 2000; Rungruangsak-Torrissen et al. 2006; Rungruangsak-Torrissen 2007).

Studies of muscle growth and muscle quality indicated that differences in the composition ratio of protein to lipid (P/L ratio) in the diets affected muscle composition of the fish. Muscle protein increases during growth (Rungruangsak-Torrissen 2007; Rungruangsak-Torrissen et al. 2009a), and the muscle P/L ratio is doubled at late stage with respect to the dietary P/L ratio (Rungruangsak-Torrissen et al. 2009a).

Krill meal diets seem to increase muscle protein through increasing protein retention as capacity for protein synthesis is reduced (Rungruangsak-Torrissen 2007). Increasing levels of krill meal also delay oocyte maturation in salmon females (Rungruangsak-Torrissen 2007).

Dietary quality tests using *in vitro* protein digestibility corresponded with growth and indicated a possibility of inclusion of krill meal at 50–60% replacements. Larger fish were more sensitive to dietary quality than smaller ones. At 80–100% replacements, *in vitro* protein digestibility and FCE were reduced, and oocyte quality changed through increased trypsin-like specific activity probably because of less or abnormal oocyte development (Rungruangsak-Torrissen 2007).

During oocyte development, trypsin-like specific activity in the oocytes is affected by different feedings, and this has also been observed in Atlantic mackerel (Rungruangsak-Torrissen and Fosseidengen 2007) and Northeast Arctic cod (Rungruangsak-Torrissen et al. 2012). More details are shown in Table 2.

Effects of Dietary Protein to Lipid (P/L) Ratio on Fish Growth Performance

Two diets containing normal protein (NP) level of P/L ratio 1.2–1.5 and high protein (HP) level of P/L ratio 2.1–2.7 were provided to rainbow trout from juvenile to maturity, and the HP-diet fish showed higher deposition of protein in body and white muscle than the NP-diet fish (Rungruangsak-Torrissen et al. 2009a).

Protein deposition associated more with body length than with body weight, as the condition factor was lower in the HP-diet fish especially during autumn (Figure 16A) and inversely correlated with the body P/L ratio (Figure 16B).

Pair comparisons indicated higher *in vitro* protein digestibility of the HP-diet than the NP-diet, regardless of the enzyme extracts used (Rungruangsak-Torrissen et al. 2009a). Trypsin and chymotrypsin specific activities were related to dietary protein levels, and the T/C ratio was related to intestinal weight and growth rate independent of the specific activity levels of trypsin and chymotrypsin (Rungruangsak-Torrissen et al. 2009a).

At maturation, fish growth rates were decreased in both dietary groups and the HP-diet fish with relatively higher growth showed lower growth rate (Figure 16A) with lower T/C ratio and feed efficiency (Rungruangsak-Torrissen et al. 2009a).

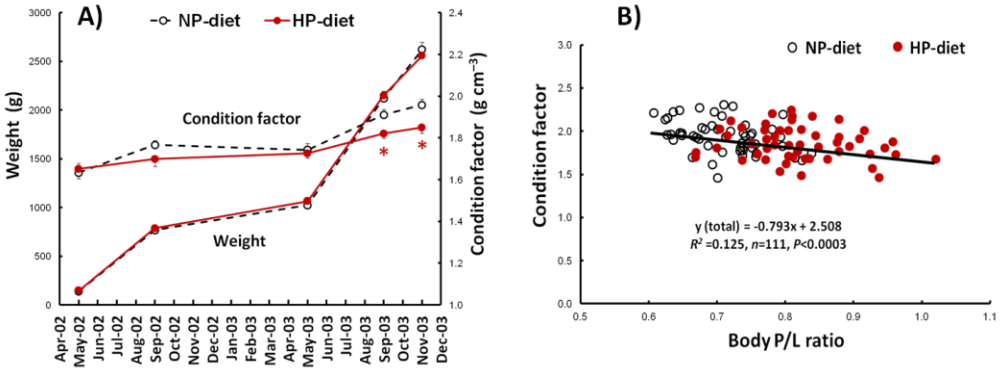

Figure 16. (A) Growth in weight and condition factor of rainbow trout fed the diet with normal protein (NP) or high protein (HP) level, and (B) the relationship between body composition ratio of protein to lipid (P/L ratio) and condition factor (100×Weight Length^{-3}), regardless of dietary groups. The values with asterisk (*) show significantly different between the two dietary groups ($P < 0.05$). (Adapted data from Rungruangsak-Torrissen et al. [2009a] with permission from Hindawi).

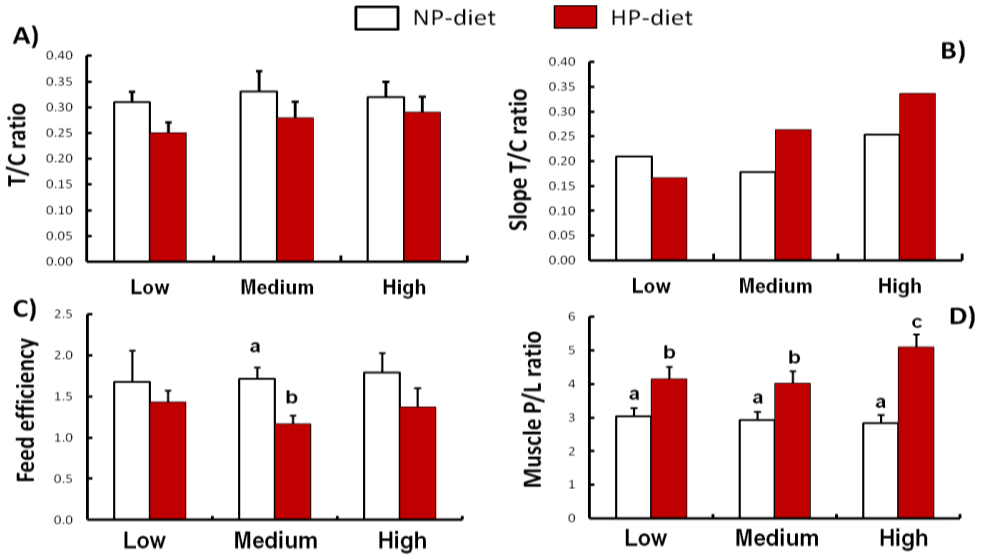

Figure 17. (A) Protease activity ratio of trypsin to chymotrypsin (T/C ratio), (B) slope T/C ratio, (C) feed efficiency, and (D) white muscle composition ratio of protein to lipid (P/L ratio), in groups of rainbow trout families with low, medium, and high growth capacity, subdivided according to feeding the diet with normal protein (NP) or high protein (HP) level. The values with different superscripts are significantly different ($P < 0.05$). (Adapted data from Rungruangsak-Torrissen et al. [2009a] with permission from Hindawi).

Rainbow trout families were divided into 3 groups according to low, medium, and high growth capacity. The T/C ratio values were similar, showing similar growth rates over a period of 1–2 months at maturation regardless of diet types (Figure 17A). However, the slope T/C ratios indicated a relatively higher growth of the HP-diet fish at sampling in the groups of medium and high growth capacity while the low growth group seemed to have less capacity to utilize the HP-diet for growth (Figure 17B). The HP-diet fish showed lower feed efficiency than the NP-diet fish, but the difference was significant only in the medium growth group (Figure 17C). The white muscle P/L ratio was higher in the HP-diet fish in all groups, and the high growth group had a higher capacity to deposit protein than the low and medium growth groups (Figure 17D). Atlantic salmon and rainbow trout usually have similar protein utilization and growth performance (Figure 12).

The associations have been observed among dietary P/L ratio, fish digestive ability (trypsin and chymotrypsin specific activities, T/C ratio, *in vitro* protein digestibility), white muscle P/L ratio, growth parameters (fish weight, condition factor, SGR), and variations in growth capacity of different family membership. The research also provides more insight on dietary protein affecting skeletal growth (length) as well as the interaction between genetics and nutrition that affects digestive ability and growth performance quality of the animal (Rungruangsak-Torrissen et al. 2009a).

Effects of Anti-Oxidant on Atlantic Salmon Fillet Quality

A non-toxic substance with anti-oxidant property, *N*-acetylcysteine, has been used to test its effects on improving fillet quality of Atlantic salmon during storage (unpublished data). Atlantic salmon fillet was mixed with *N*-acetylcysteine at 14 mmol or 35 mmol per 100 g flesh. The mixtures were heated at 95 ^{o}C for 45 min, and dried at 60 ^{o}C overnight before storage in a refrigerator for one week. The red colour of the mixtures before heating was visually reduced in the presence of *N*-acetylcysteine, compared to the control, but the differences in colour disappeared after the mixtures were heated and stirred.

In vitro protein digestibility and protein structure study by 2D-electrophoresis were applied to test the flesh quality in the presence and absence (control) of *N*-acetylcysteine. The results indicated that protein digestibility of salmon flesh was higher in the presence of *N*-acetylcysteine (Figure 18A), and *N*-acetylcysteine protected the loss of intact protein during heating and storage, as shown using the 2D-electrophoresis (Figure 18A). Moreover, *N*-acetylcysteine also showed anti-protease activities even on ice at around zero ^{o}C (Figures 18B and 18C), and it could preserve protein by preventing autolysis of krill for months in refrigerator (Figure 18D). *N*-acetylcysteine showed an anti-microbial property, which could be used for preservation of protein on ice as well as at ambient temperature.

The effects of *N*-acetylcysteine were further studied in association with rigor mortis on the quality of smoked salmon fillets. The fillets were obtained from Atlantic salmon without and with 4 days rigor mortis, and treated by spraying with 5% or 12.5% of *N*-acetylcysteine solution. The treated fillets were smoked and stored in vacuum packages at 5 ^{o}C for 2 weeks. The smoked fillets treated with *N*-acetylcysteine showed more firmness of texture than the control, regardless of rigor mortis (K. Rungruangsak-Torrissen, K. Storsæter and A. Kiessling (unpublished data)). In addition, *N*-acetylcysteine and its concentration (not rigor mortis) affected the colour of smoked fillets (Figure 19A), but it was rigor mortis (not *N*-acetylcysteine)

that caused weight loss and fat drip in smoked fillets (Figure 19B). Test panel of these smoked salmon fillets indicated a well acceptable quality for consumption of *N*-acetylcysteine treated smoked fillets (K. Rungruangsak-Torrissen and K. Storsæter (unpublished data)).

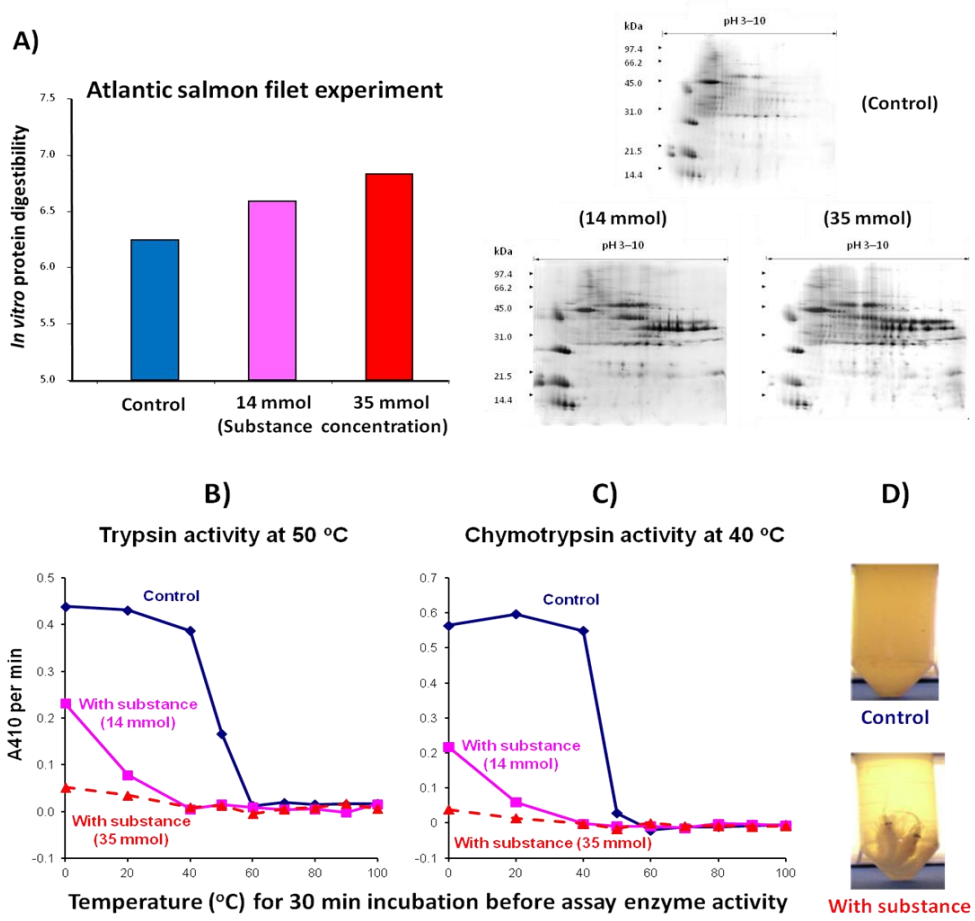

Figure 18. (A) An *in vitro* protein digestibility test of Atlantic salmon flesh mixed with *N*-acetylcysteine (NAC) at two different concentrations of 14 mmol and 35 mmol per 100 g flesh with 2D-electrophoresis showing an anti-autolysis of protein structure of the flesh in the presence of NAC, and the effects of NAC on the activities of (B) trypsin and (C) chymotrypsin after 30 min incubation at different temperatures, and (D) preservation of krill showing an anti-autolysis of krill in the presence of NAC dissolved in sea water. The *in vitro* protein digestibility was expressed as µmol *DL*-alanine equivalent per g fillet. The molecular weights (kDa) of the separated proteins are shown on the side of the gel pictures. The activities of trypsin and chymotrypsin were determined at 50 °C and 40 °C, using initial reaction rate with benzoyl-*L*-arginine-*p*-nitroanilide (BAPNA) and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPNA) as specific substrates, respectively. (Adapted from Rungruangsak-Torrissen [2012], with permission from Nova Science Publishers).

Since *N*-acetylcysteine can inactivate the protease activities during cold storage as well as at ambient temperature and protect firmness of protein, it can be used as a non-toxic anti-autolysis and anti-microbial agent for preserving food protein raw materials both before and during processing. It can also protect protein (in terms of digestibility and amino acid availability) during heating, and its use can be an advantage for food and feed industries.

Moreover, *N*-acetylcysteine can replace formalin for preservation of tissue proteins as it has "antiseptic property" but without toxicity, and it is easier to use and work with than formalin.

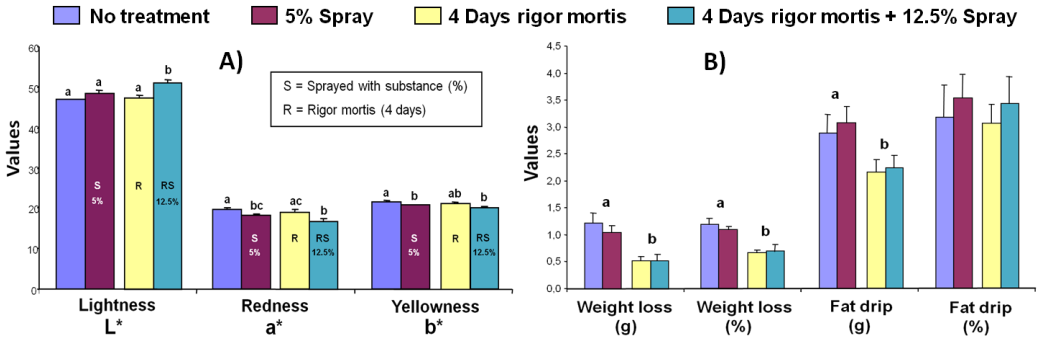

Figure 19. Effects of *N*-acetylcysteine on fillet quality of smoked salmon. The solution at 5% or 12.5% of *N*-acetylcysteine was used for spraying onto salmon fillet with or without 4 days of rigor mortis before smoking process. (A) The colour, and (B) the weight loss and fat drip, were tested after vacuum packing and stored at 5 ^{o}C for 2 weeks. The values in each test with different superscripts are significantly different ($P < 0.05$). (K. Rungruangsak-Torrissen, K. Storsæter and M. Bjørnevik (unpublished data)).

CONCLUSION

The studies on biochemical changes in fish, especially Atlantic salmon, in associations with genetics, environmental conditions, and dietary quality have provided a unique and significant knowledge on growth mechanisms in organisms (Figure 20).

Growth of organisms is dependent on the levels of consumption and how well the diets are utilized. Its performance also depends on the dietary quality that leads to protein deposition, and results in health and maturation processes. These studies indicate close associations between the expressions of digestive proteases (trypsin and chymotrypsin) including the ratio of trypsin activity to chymotrypsin activity (T/C ratio), free amino acids influx, and hormone secretion (especially insulin), for protein synthesis and growth. Thus, trypsin expression and T/C ratio are the primary biological key factors, which affect different levels of nutrient influx for stimulating growth including hormone insulin secretion and immune responses for promoting fish growth as well as promoting oocyte maturation (Figure 20).

The higher T/C ratio relates to higher growth rates, which can be from an increase in trypsin specific activity and/or a decrease in chymotrypsin specific activity. The T/C ratio is a very important factor, as higher levels of T/C ratio in the pyloric caeca (Sunde et al. 2004; Rungruangsak-Torrissen 2007; Rungruangsak-Torrissen et al. 2009b, 2012) and higher T/C ratio (activity ratio of trypsin-like to chymotrypsin-like) in the oocytes (Rungruangsak-Torrissen et al. 2012) indicate higher development in somatic growth and oocyte maturation, respectively, independent of the specific activity levels of the two proteases.

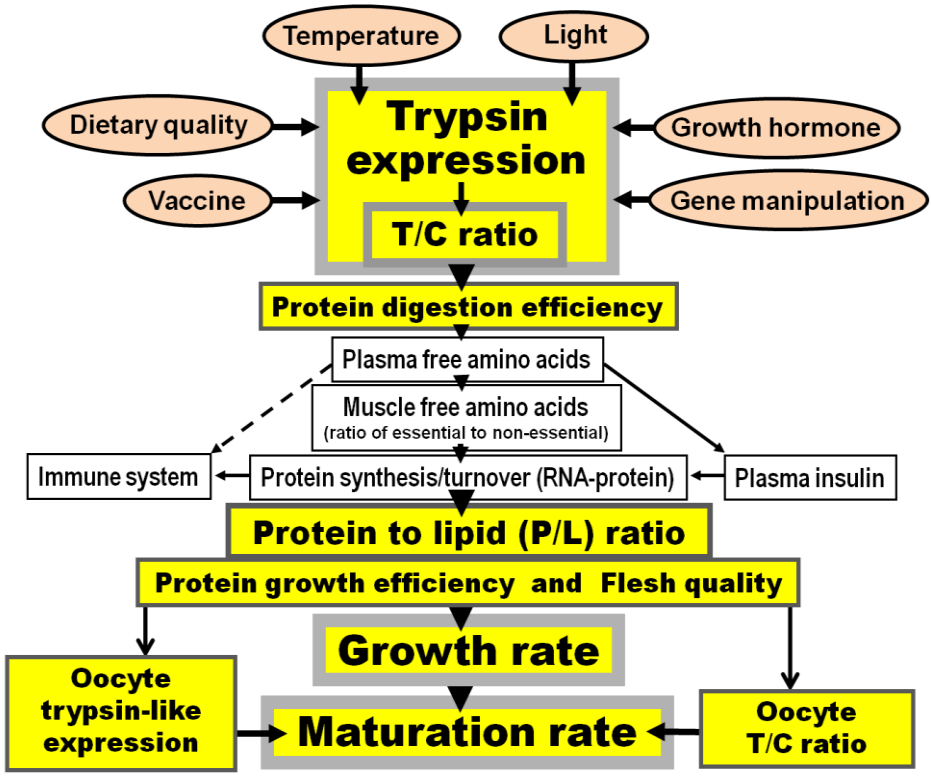

Figure 20. Diagram showing the importance of trypsin for a series of growth mechanisms through its role as the key enzyme activity in protein digestion process. Trypsin expression and the protease activity ratio of trypsin to chymotrypsin (T/C ratio) in the pyloric caeca (affecting nutrient influx that subsequently influences capacity for protein synthesis and immune system and growth) is influenced by temperature and dietary quality (Rungruangsak Torrissen and Male 2000; Rungruangsak-Torrissen et al. 2006; Sunde et al. 2004) as well as by light regime (Sunde et al. 2001; Rungruangsak-Torrissen et al. 2009b), growth hormone (Rungruangsak-Torrissen et al. 2009b together with Nordgarden et al. 2006; Blier et al. 2002), gene manipulation (Sunde et al. 2001; Blier et al. 2002), and vaccine type (Rungruangsak-Torrissen et al. 2009b). Transport rate and level of free amino acids to target tissues (affecting plasma insulin secretion and protein synthesis in the white muscle) indicate protein utilization efficiency and flesh quality of the fish resulted by the ratio of protein to lipid (P/L ratio). Besides from this chapter and Chapter 6, more details of the effects of trypsin expression and the T/C ratio on protein utilization and muscle capacity for protein synthesis and growth have been described in Rungruangsak Torrissen and Male [2000] and Sunde et al. [2001, 2004], and on maturation in Rungruangsak-Torrissen [2007], Rungruangsak-Torrissen and Fosseidengen [2007] and Rungruangsak-Torrissen et al. [2009a, 2012]. (Modified from Rungruangsak-Torrissen [2012] with permission from Nova Science Publishers, based on Rungruangsak-Torrissen et al. [2006]).

Growth hormone has a connection with trypsin and chymotrypsin activities and growth (Lemieux et al. 1999; Blier et al. 2002; Nordgarden et al. 2006 and Rungruangsak-Torrissen et al. 2009b). Although abdominal injection of growth hormone did not induce differences in trypsin and chymotrypsin activities and growth rates in Atlantic cod (Lemieux et al. 1999), it did affect transgenic coho salmon, *Oncorhynchus kisutch*, by higher growth rate and higher T/C ratio in the pyloric caeca due to lower chymotrypsin activity (Blier et al. 2002). Moreover, the profiles of plasma growth hormone levels (Nordgarden et al. 2006) were

similar to the profiles of trypsin and chymotrypsin expressions during adaptation period, and later similar to the T/C ratio profiles during on-growing (Rungruangsak-Torrissen et al. 2009b) in Atlantic salmon.

Fish with higher food utilization efficiency and growth have higher elevation of trypsin specific activity and/or higher T/C ratio in the pyloric caeca (and intestine). Higher protein growth efficiency is indicated by higher *in vitro* protein digestion ability for food materials and higher protein to lipid (P/L) ratio in body and white muscle. Higher dietary protein level affects an increase in skeletal growth (increase in length), and fish with higher growth capacity can deposit higher protein that results in higher white muscle P/L ratio. The T/C ratio can be used to predict growth rate over a period of 1–2 months, while the slope T/C ratio (from the regression of trypsin and chymotrypsin specific activities) indicates growth rate at sampling. The interaction between genetics and nutrition affects digestive ability and growth performance.

During maturation, fish growth is reduced and fish with higher growth have higher growth reduction as indicated by lower pyloric caecal T/C ratio. Females with higher maturation rate have higher activity ratio of trypsin-like to chymotrypsin-like (T/C ratio) in oocytes, in spite of lower specific activity levels of the two proteases in their oocytes.

Besides Atlantic salmon (Sunde et al. 2001, 2004; Rungruangsak-Torrissen 2007; Rungruangsak-Torrissen et al. 2009b), different combinations of the various biochemical techniques have been used in aquaculture as well as in natural ecosystems in other fish species such as rainbow trout (Rungruangsak-Torrissen et al. 2009a), Atlantic mackerel (Rungruangsak-Torrissen and Fosseidengen 20070, Northeast Arctic cod (Rungruangsak-Torrissen et al. 2012), Nile tilapia (Rungruangsak-Torrissen et al. 20100, and Siamese fighting fish *Betta splendens* (Thongprajukaew et al. 2011, 2013). These studies can help to elucidate the performance qualities of growth and maturation.

Moreover, studies of trypsin specific activity and the T/C ratio can provide information of whether the low level of food availability observed in nature is due to really low food availability or due to high grazing, and whether empty stomachs are due to starvation or complete food evacuation. These are questions that cannot be explained without studying the protease parameters. This approach can be important new strategy in future ecological studies for a better understanding of living resources in natural ecosystems. Variated data of food digestion and utilization due to climate change will also serve as significant future tools for environmental impact assessment on fish production in nature.

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