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Chapter 2

FREE-HYDROXYPROLINE AS GROWTH AND MATURATION INDICATORS

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ABSTRACT

Hydroxyproline is the hydroxylated form(s) of non-essential amino acid proline. It is found in collagen, a connective tissue which has a directive role in developing tissues and is hydrolyzed by collagenases during growth and remodeling. Proline can be hydroxylated only in a protein molecule, not in the free form. Therefore, the free-hydroxyproline observed in a physiological system must be resulted from a degradation of protein molecules. Variations in free-hydroxyproline concentrations in the plasma and white muscle of fishes, with genetic differences in growth rates and with different feedings, could indicate differences in protein degradation rates for tissue remodeling and growth. Since the synthesis of the connective tissue framework of muscle is a rate-limiting step in muscle growth, the concentrations of white muscle free-hydroxyproline

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could be reliable for growth studies, as its levels are correlated with fish specific growth rates (SGR) and feed efficiency. The concentration ratios of essential to non-essential free amino acids (EAA/NEAA) in the white muscle are also correlated with SGRs but with reverse effects, because of higher assimilation of free amino acids, especially EAA, for protein synthesis in higher growth fish. Moreover, females with lower free-hydroxyproline concentrations in their oocytes have higher oocyte maturation rates, due to low or no oocyte protein degradation at maturity.

The protease activity ratio of trypsin to chymotrypsin (T/C ratio) has been successfully implemented as the digestive efficiency for studies of somatic growth (through the pyloric caecal and small intestinal samples) and oocyte growth (for maturation rate in females). Free-hydroxyproline and EAA/NEAA ratio in the white muscle should be studied along with the T/C ratio, and the growth status of the animals which is important for correctly interpreting the results. However, a growth status study could be performed only by using the T/C ratio through its regression with trypsin specific activity in the pyloric caeca or small intestine. The T/C ratio study is more practical because of more simple instrumentation, however, muscle biopsy is more practical than intestinal biopsy. Free-hydroxyproline concentrations in the white muscle and oocytes could be as useful indicators as the T/C ratio for studies of growth rate and maturation rate, especially for animals in the wild where their food availabilities, consumption rates and growth rates are unknown.

Keywords: free amino acids, T/C ratio, RNA, protein, RNA/protein ratio, pyloric caeca, white muscle, oocytes, Atlantic salmon, Atlantic mackerel, Arctic charr

INTRODUCTION

Utilization of dietary protein is the primary key process for growth performance quality of living organisms. The knowledge is based on the uniquely intensive studies on a series of growth mechanisms by Rungruangsak-Torrissen and her research team through genetic variations of trypsin phenotypes affecting the expression levels of protease enzymes trypsin and chymotrypsin in the pyloric caeca and small intestine, especially the activity ratio of trypsin to chymotrypsin (T/C ratio), resulting in variations in digestive efficiency and growth of individuals under differences in food availabilities and environmental conditions

(Rungruangsak-Torrissen, 2012, 2014a, 2014b, 2018). Moreover, it is evident that protein digestibility is the primary important key factor determining food quality and growth of living organisms, followed by carbohydrate digestibility as the secondary factor, regardless of feeding habits (carnivores, omnivores, or herbivores).

Protein metabolism has been studied and emphasized through facilitation of free amino acids (FAA) in plasma and white muscle of both Atlantic salmon, *Salmo salar* L. (Torrissen et al., 1994, 1995a) and Arctic charr, *Salvelinus alpinus* L. (Rungruangsak Torrissen and Male, 2000) possessing different trypsin phenotypes and capabilities for growth under different food availabilities and environmental conditions. In addition, FAA have been studied in white muscle and oocytes of maturing Atlantic mackerel, *Scomber scombrus* L. showing differences in FAA levels in these tissues between fish groups with different feedings (Rungruangsak-Torrissen and Fosseidengen, 2007). One of FAA observed to have significantly different levels in plasma and white muscle (Torrissen et al., 1994, 1995a) and oocytes (Rungruangsak-Torrissen and Fosseidengen, 2007) in different fish groups, with surprisingly its levels in white muscle showing significant relationships with specific growth rate (SGR) and digestive efficiency (the activity ratio of trypsin to chymotrypsin as T/C ratio) in Atlantic salmon (Sunde et al., 2001), is free-hydroxyproline.

Hydroxyproline is the hydroxylated form(s) of non-essential amino acid proline that can be hydroxylated only in a protein molecule, not in the free form, especially in newly synthesized collagen (Stryer, 1988). Therefore, free-hydroxyproline will only be found in the products from protein breakdown.

DETERMINATION OF PHYSIOLOGICAL FREE AMINO ACIDS

The physiological free amino acids could be determined in different biological tissues, such as plasma, white muscle and oocytes, using the developed technique based on the AccQ•Tag Amino acid Analysis Method (Millipore, 1993) with modified buffers in the gradient system according to

Rungruangsak-Torrissen and Sundby (2000). The instrument for amino acids analysis was Alliance High Pressure Liquid Chromatography (HPLC) System consisting of Waters 2690 Separations module, Waters 474 Scanning Fluorescence Detector, Millennium 996 Photodiode Array Detector and Millennium Chromatography Manager System (Waters Corporation, Milford, MA, USA). The free amino acids and nitrogen-containing compounds react with the AccQ•Fluor Reagent and become fluorescent derivatives that can be detected at an excitation wavelength of 250 nm and emission wavelength of 395 nm. As shown in Figure 1, the separation profiles of more than 40 fluorescent derivatives of standard amino acids and nitrogen-containing compounds were achieved, with the internal standard α-aminobutyric acid including the modified gradient system illustrated. However, tryptophan derivative was not reliably detected. Free-hydroxyproline derivative is early detected within 30 min of running time, with clear separation from the other derivatives (see Figure 1), and can be easily and precisely calculated.

The developed technique for the analysis of free amino acids and nitrogen-containing compounds has been described by Rungruangsak-Torrissen and Sundby (2000) with more detail description in Rungruangsak-Torrissen (2018). The samples of blood, epaxial white muscle, and oocytes, must be orderly collected to prevent contaminations after the fish was immediately killed by a blow to the head. Plasma was separated and immediately kept frozen at –80°C together with white muscle and oocytes.

It is important that all glassware must be cleaned properly by being pyrolyzed at 500°C for at least 4 h, and double distilled water must be used for chemical preparations. In addition, the samples of white muscle and oocytes must be frozen immediately at –80°C, and weighed when they are still frozen to avoid the leakage and loss of free amino acids and nitrogen-containing compounds when performing the analysis. The internal standard α-aminobutyric acid must be added to the sample (plasma, white muscle, or oocytes) before deproteinization, and the filtrate or supernatant obtained was derivatized by using the Waters AccQ•Tag Chemistry Package. The concentrations of fluorescent derivatives of free amino acids

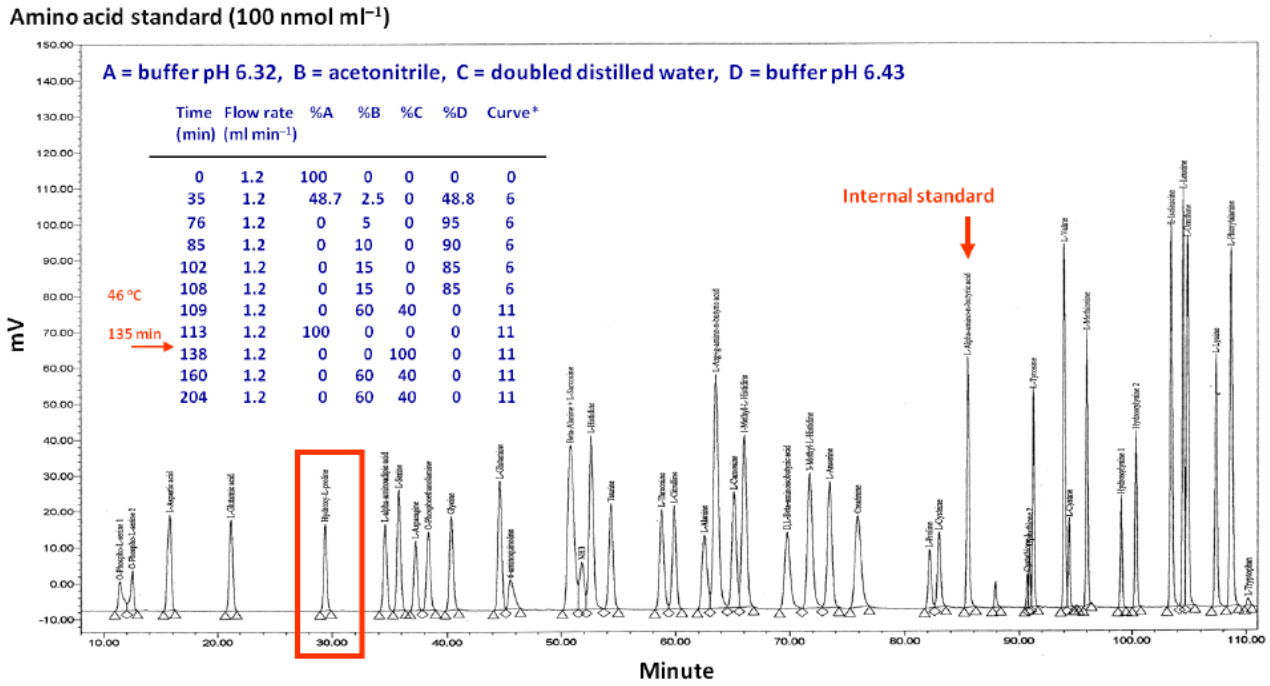

Figure 1. Chromatogram of the fluorescent derivatives of standard amino acids and nitrogen-containing compounds at a concentration of 100 nmol ml^{-1}, derivatized by using the Water AccQ•Tag Chemistry Package and using the gradient system shown in the picture for separation. The asterisk (*) shows the rates at which the solvent is to change to the new proportions (6: linear gradient and 11: no gradient). The sample injection volume is 10 µl with Nova-Pak C18 column (60 Å, 4 µm, 3.9×300 mm) at 46ºC and 135 min running time. (From Rungruangsak-Torrissen and Sundby, 2000, with permission from Springer Corp.).

and nitrogen-containing compounds were determined by using the Waters Alliance HPLC System with Nova-Pak C18 column (60 Å, 4 µm, 3.9×300 mm) at 46°C and 135 min running time with the buffer gradient system shown in Figure 1. The concentrations of FAA and nitrogen-containing compounds in the samples could be calculated by comparing with the calibration standards (Figure 1) by using the response level of the internal standard (α-aminobutyric acid) for standardization.

ABSORPTION AND TRANSPORT OF FREE-HYDROXYPROLINE

The physiological free amino acids (FAA) could be determined in different biological tissues. To study the absorption and transport of FAA in relation to protein utilization and growth, plasma and epaxial white muscle were the target tissues.

The technique was emphasized for studying the effects of different dietary qualities and genetic variations in protein digestive efficiency (through genetic expression of trypsin phenotypes) on absorption and transport of free amino acids in the plasma and white muscle of Atlantic salmon (Torrissen et al., 1994, 1995a) and Arctic charr (Rungruangsak Torrissen and Male, 2000) with and without the trypsin variant TRP-2*92 (Figure 2). More details on trypsin phenotypes in fish pyloric caeca have been summarized in Rungruangsak-Torrissen (2012, 2014a, 2014b, 2018).

The Atlantic salmon possessing the trypsin variant TRP-2*92 (patterns 2 and 2’ shown in Figure 2) grew faster than those without the variant (pattern 1 shown in Figure 2) at the temperature less than 8°C, especially below 6°C. Interestingly, the fast-growing anadromous Arctic charr (Hammerfest strain) from Storvannet in Northern Norway has possessed the trypsin variant TRP-2*92, while the slow-growing non-anadromous Skogseid strain from Skogseidvannet with somewhat higher temperature in Southern Norway does not possess the trypsin variant (see Figure 2).

The presence and the absence of the trypsin variant TRP-2*92 in trypsin phenotypes of the fishes were focused for studies.

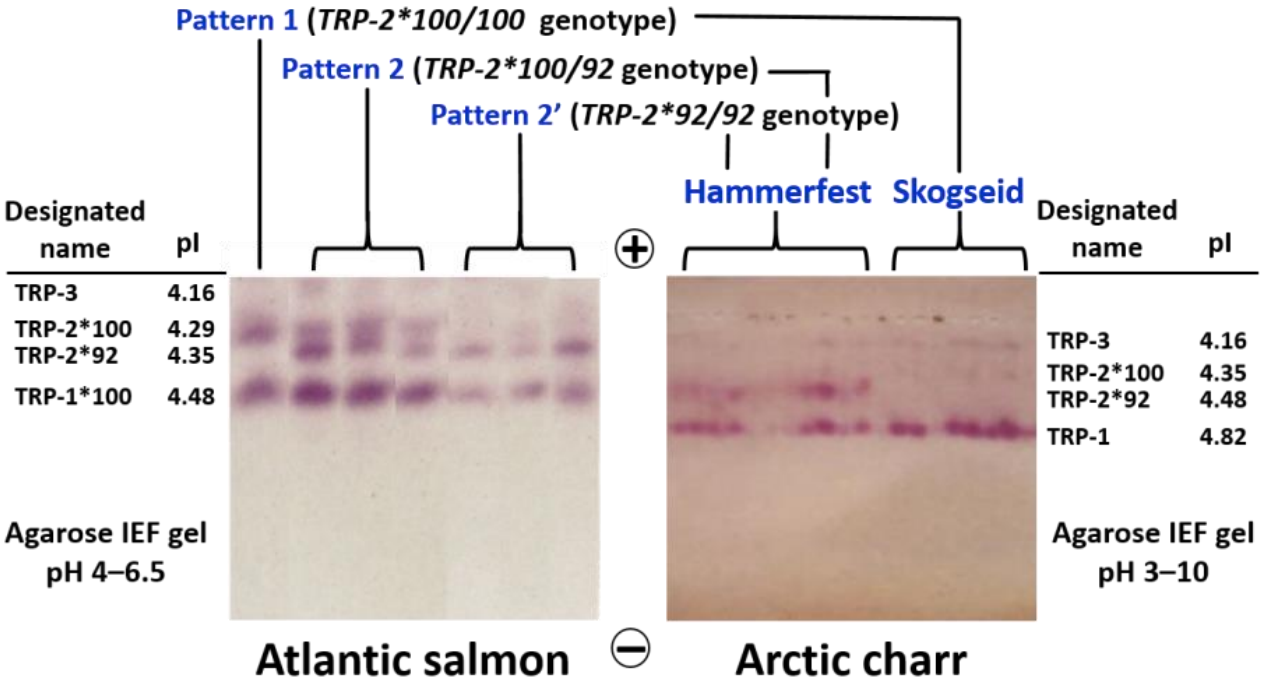

Figure 2. Electrophoretic zymograms on Agarose IEF gels of trypsin phenotypes, with (patterns 2 and 2') and without (pattern 1) the trypsin variant TRP-2*92, in the pyloric caeca of Atlantic salmon and two strains of Arctic charr (Hammerfest: the fast-growing northern anadromous strain, and Skogseid: the slow-growing southern landlocked strain). The isoelectric points (pI) were adapted from Rungruangsak-Torrissen et al. (1998), according to the designated names of trypsin isozymes based on Torrissen et al. (1993) for Atlantic salmon, and Torrissen and Barnung (1991) for Arctic charr.

Genetic Effects of Trypsin Phenotypes

The first study was to see the effect of genetic differences in trypsin phenotypes on protein utilization through absorption in plasma and transport to white muscle of free amino acids in Atlantic salmon (Torrissen et al., 1994). Trypsin phenotypes of individuals were identified through biospsy of the pyloric caeca at smolt period and analysis by the isoelectric focusing technique, as shown in Figure 2. The two trypsin phenotypic groups (with and without the trypsin variant TRP-2*92) were reared together at the salinity 18.9 ± 0.1 with the temperature 10.7 ± 0.2°C, and individually labelled with Floy anchor tags (Floy Tag & Manufacturing, Inc., Seattle). Postprandial levels of FAA in plasma and white muscle, after a single feeding subsequent to 2 days starvation, are shown in Figure 3. During two-week experiment, the 100-g salmon had an average specific growth rate of 0.38 ± 0.04% per day, about one-third of the normal range due to the continuous light regime during a period when natural photoperiod should affect the highest growth rate. The 400-g salmon did not grow and lost weight (–0.28 ± 0.04% per day), therefore, the FAA levels in the white muscle were not studied in these groups. The comparisons were made between the trypsin phenotypes, with and without the trypsin variant TRP-2*92.

The amino acid absorption was apparently better in the Atlantic salmon possessing the trypsin variant TRP-2*92, as their concentrations of total FAA (TFAA) in the plasma during the whole time course were significantly higher due to higher essential FAA (EAA), in both 100-g and 400-g salmon, without significant differences in non-essential FAA (NEAA) levels (Torrissen et al., 1994). It was suggested that the *TRP-2*92* salmon had a higher food digestion rate, since the fish of both phenotypic groups were fed the same ration. This could also be seen in the levels of plasma lysine (the EAA involving in trypsin digestion) that showed significantly higher in the *TRP-2*92* salmon of both sizes (Figure 3) showing a higher metabolism of lysine in these fish groups. Since lysine cannot be synthesized by the fish, the initial increases in the plasma were

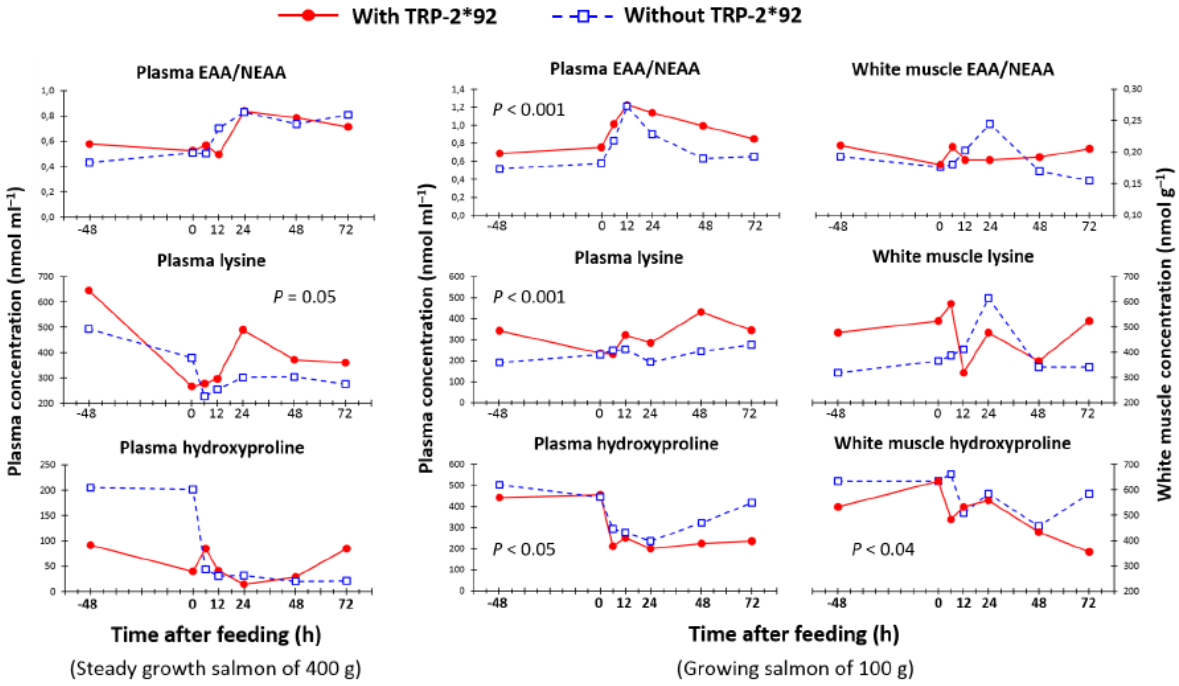

Figure 3. Postprandial concentrations of the ratio of essential to non-essential free amino acids (EAA/NEAA), free-lysine and free-hydroxyproline, in the plasma and epaxial white muscle after a single feeding subsequent to 2 days starvation, in 400 g Atlantic salmon with steady growth period and 100 g Atlantic salmon with growing phase, with and without the trypsin variant TRP-2*92. Probability values indicate significant differences between the two phenotypic groups during the whole-time course. Trypsin phenotypes are illustrated in Figure 2. (Adapted data from Torrissen et al. 1994, with permission from Academic Press).

the result of absorption from the gut, while the prolonged elevations could be the result from either absorption or protein degradation or both. However, the plasma ratio of EAA/NEAA was significantly higher only in the growing 100-g *TRP-2*92* salmon, but not in the non-growing or steady growth 400-g salmon (Figure 3). Plasma levels during the whole time course of free-hydroxyproline were significantly lower in the 100-g *TRP-2*92* salmon, but not different between the two phenotypic groups in the 400-g salmon (Figure 3).

The amino acid transport to white muscle for protein synthesis and growth was faster (6 h of EAA/NEAA and free-lysine levels) in the 100-g *TRP-2*92* salmon, compared to 24 h after feeding in the other phenotype, albeit insignificant during the whole time course (Figure 3). In fact, the white muscle levels of EAA and NEAA were significantly higher in the 100-g *TRP-2*92* salmon, and almost all FAA increased with a peak at 6 h post-feeding (Torrissen et al., 1994). Therefore, faster and higher transport of FAA could increase the assimilation of FAA at the time of protein synthesis. However, the levels of free-hydroxyproline in the white muscle (due to remodeling and growth) were significantly lower in the 100-g *TRP-2*92* salmon during the whole time course (Figure 3). This might be due to the negative effect of continuous light regime during a period when natural photoperiod should affect the highest growth rate, and/or the high rearing temperature of 10.7 ± 0.2°C that was known later by Rungruangsak-Torrissen et al. (1998) to provide a disadvantage condition for growth of the *TRP-2*92* salmon.

Effects of Trypsin Phenotypes and Dietary Qualities

The interaction between trypsin phenotype and dietary quality was studied in order to see its effect on absorption and transport of free amino acids for protein metabolism and growth in Atlantic salmon (Torrissen et al., 1995a). The two trypsin phenotypic groups (with and without the trypsin variant TRP-2*92) were reared together at the salinity 16.9 ± 0.2 with the temperature 10.8 ± 0.1°C, and individually labelled with Floy

anchor tags (Floy Tag & Manufacturing, Inc., Seattle) near their dorsal fins. The fish were fed with a diet containing non-hydrolyzed protein (Feed A), or containing the proteins with partially pre-hydrolysis by pepsin for 6 h (Feed B) or 48 h (Feed C), at a ration of 1.5% body weight per day for studies of absorption and transport of FAA, and at a restricted ration of 0.5% body weight per day for digestibility and growth studies. Postprandial levels of FAA in plasma and white muscle, after a single feeding subsequent to 2 days starvation, are shown in Figure 4. The biological parameters for digestibility and growth studies are shown in Table 1.

Variations in dietary protein qualities due to differences in the degree of pre-hydrolysis by pepsin affected the digestion of protein and absorption and transport of free amino acids in Atlantic salmon with different trypsin phenotypes (Figure 4). The effect was positively significant for Feed B in the presence of trypsin variant TRP-2*92, as the *TRP-2*92* salmon fed with Feed B had higher specific growth rate (0.72% per day), compared to the fish of both phenotypic groups fed either Feed A or Feed C (0.51 – 0.69% per day) (Table 1). The analysis of variance with two dependent variables, feed and trypsin TRP-2*92, indicated the interaction between the variables was very close to significance (P = 0.054, n = 5 for each phenotypic group of each feed) for specific growth rate (SGR). Significant differences in the SGR between the two phenotypic groups were only observed in Feed B (Table 1). The productive protein values (PPV) increased with the degree of pre-hydrolysis of proteins in the feeds (Table 1). The productive fat value (PFV) was very high in the fish fed with Feed A, showing that the apparently high SGRs in these fish groups were due to higher fat deposition which is common in fish raised with commercial feeds of non-hydrolyzed proteins.

The usefulness of dietary protein is limited to the relative concentration of its EAA. A higher protein quality in Feed B resulted in faster and higher absorption of EAA (resulting in a higher ratio of EAA to NEAA) and free-lysine levels in the plasma, with lower levels of plasma free-hydroxyproline (Figure 4). The transport of FAA to white muscle was

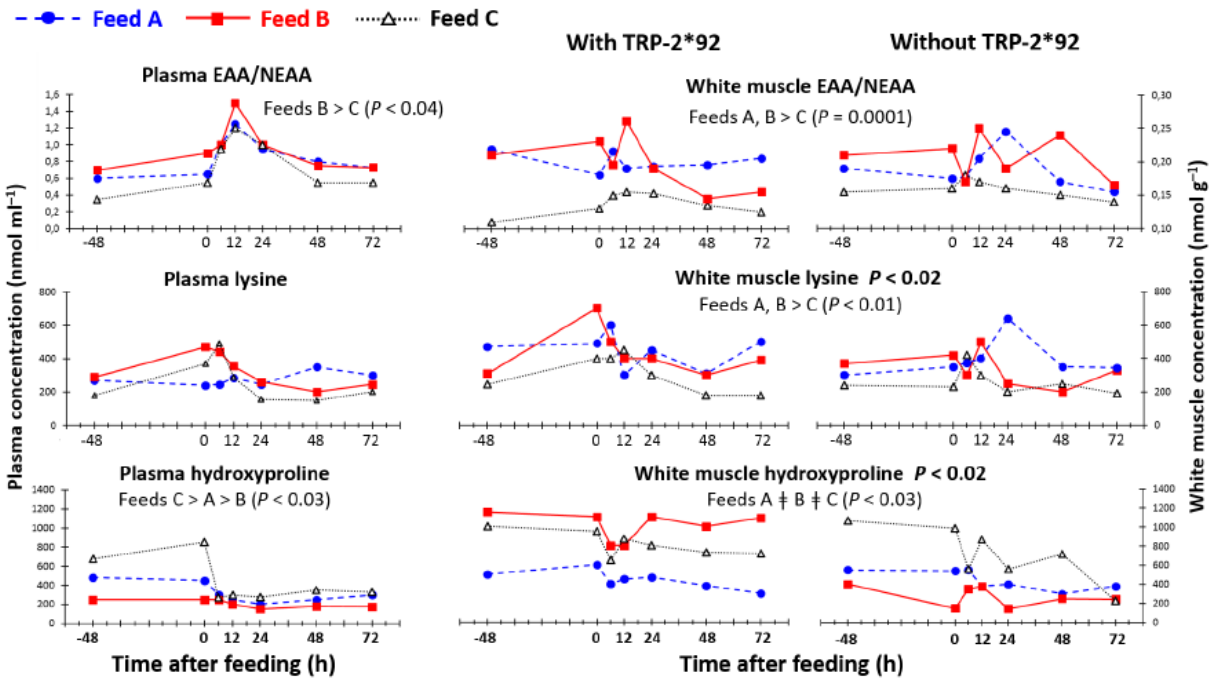

Figure 4. Postprandial concentrations of the ratio of essential to non-essential free amino acids (EAA/NEAA), free-lysine and free-hydroxyproline, in the plasma and epaxial white muscle after a single feeding subsequent to 2 days starvation, in growing Atlantic salmon with and without the trypsin variant TRP-2*92 and fed on a diet containing protein meal with non-hydrolyzed protein (Feed A), or pre-hydrolysis by pepsin for 6 h (Feed B), or for 48 h (Feed C). Probability values indicate significant differences between the feeds in the plasma (regardless of trypsin phenotypes), and both between the two phenotypic groups and the feeds (in brackets) in the white muscle, during the whole-time course. Trypsin phenotypes are illustrated in Figure 2. (Adapted data from Torrissen et al. 1995a).

Table 1. Apparent digestibility coefficient (ADC) of amino acids and protein, specific growth rate (SGR), productive protein value (PPV) and productive fat value (PFV) of Atlantic salmon, with and without the trypsin variant TRP-2*92, fed on a diet containing protein meal with non-hydrolyzed protein (Feed A), or pre-hydrolysis by pepsin for 6 h (Feed B) or 48 h (Feed C) at a restricted ration of 0.5% body weight per day for 7 weeks. The values with the asterisk (*) are significantly different between the two phenotypic groups ($P < 0.03$). Trypsin phenotypes are illustrated in Figure 2. (From Torrissen et al., 1995a)

% ADC of individual amino acids	Non-hydrolyzed protein (Feed A)		6 h-Pre-hydrolyzed protein (Feed B)		48 h-Pre-hydrolyzed protein (Feed C)	
	With	Without	With	Without	With	Without
Aspartic acid	92.2	93.8	93.6	91.7	92.1	90.8
Glutamic acid	96.7	97.4	97.7	96.8	96.4	95.3
Hydroxyproline	95.0	92.3	96.7	93.2	88.6	90.9
Serine	92.3	93.3	93.4	92.0	92.1	92.4
Glycine	95.0	95.7	96.2	95.1	93.2	93.7
Histidine	95.6	96.2	95.5	94.8	100.0	94.2
Threonine	94.4	93.8	95.1	92.7	91.5	93.4
Alanine	95.4	95.9	96.6	94.9	94.0	94.8
Arginine	96.2	96.3	97.0	95.6	95.3	96.0
Proline	94.8	94.2	95.5	93.6	89.4	93.2
Cysteine	90.8	85.0	84.6	74.2	46.9	86.9
Tyrosine	94.7	95.7	95.8	93.5	93.9	93.9
Valine	94.9	95.6	96.3	94.6	94.0	94.5
Methionine	95.9	96.5	97.2	95.6	94.8	95.8
Isoleucine	95.0	95.1	96.0	93.9	92.1	93.9
Leucine	95.7	96.0	96.7	95.1	95.1	94.9
Lysine	96.4	96.6	98.5	95.7	94.9	96.5
Phenylalanine	94.9	96.0	96.2	94.4	94.6	94.6
ADC of protein (%)	94.3	94.8	95.4	93.0	89.9	93.2
SGR (% per day)	0.62 ± 0.04	0.69 ± 0.04	0.72 ± 0.07*	0.51 ± 0.06*	0.52 ± 0.06	0.52 ± 0.06
PPV (%)	31.82		35.75		47.13	
PFV (%)	51.91		–6.69		5.52	

also faster with higher levels of EAA/NEAA ratio, free-lysine, and free-hydroxyproline in the white muscle of the *TRP-2*92* salmon fed with Feed B (Figure 4). The apparent digestibility coefficient (ADC) of protein was also apparently higher in this fish group, with higher digestion of every single amino acid (including hydroxyproline) in Feed B than the fish lacking the variant TRP-2*92 (Table 1). Regardless of feed types, the elevations of total free amino acids in the white muscle during the whole time course of the *TRP-2*92* salmon were significantly higher than those lacking the variant (P = 0.0001). This indicated that more amino acids were available after digestion in the presence of trypsin variant TRP-2*92.

Genetic variations in protein utilization (trypsin phenotypes) and differences in dietary protein qualties (degree of pre-hydrolysis) have caused differences in the rates of protein digestion and absorption of FAA. However, the ratio of EAA/NEAA in the plasma was always under physiological control, as changes in the profiles after feeding were similar and peaked at 12 h for all feeds with highest levels for the best quality feed (Feed B) (Figure 4). Although the absorption of FAA was high in the plasma of the slower growth fish fed with Feed C, the elevations of them were not high in the white muscle of these fish, compared to those fed with Feeds A and B, especially in the levels of EAA/NEAA ratio and free-lysine (Figure 4). This was because of the imbalance of amino acids in Feed C caused by too long pre-hydrolysis time. Therefore, a high absorption of FAA in the plasma is not always a good indicator for a high quality feed. The most reliable indicator for feed quality and growth study is the levels of transported constituent FAA, especially EAA, to the white muscle (the target tissues for growth) at the precise time for protein synthesis of around 6 – 9 h after the meal (Fauconneau et al., 1989; McMillan and Houlihan, 1989).

A higher assimilation of FAA for protein synthesis in the white muscle was observed at 6 h post-feeding in the fish group with higher SGR (*TRP-2*92* salmon fed with Feed B) (Figure 4). The elevations of FAA in their white muscle were faster, immediately after feeding (0 h), with apparently higher levels of EAA and the EAA/NEAA ratio within 12 h after feeding Feed B, compared to the other feeds. Nutritional status of the

*TRP-2*92* fish (Figures 3 and 4) and fed with Feed B (Figure 4) seemed to be better than that of the other phenotypic fish and fed with Feeds A and C, as their plasma free-hydroxyproline was significantly lower due to less catabolism of collagen as an energy source. There were no differences in the white muscle free-hydroxyproline levels between the two phenotypic groups, with no differences in SGR, fed with either Feed A or Feed C (Figure 4 and Table 1). However, the differences were observed between the two phenotypic groups fed with Feed B (Figure 4) which had different SGR (Table 1), indicating significantly higher levels of white muscle free-hydroxyproline in higher growth fish possessing trypsin variant TRP-2*92. This is because of higher growth fish having higher collagen metabolism for remodeling of growing tissues in the white muscle, in contrast to their lower levels of plasma free-hydroxyproline due to lower mobilization of collagen as an energy source (Torrissen et al., 1994, 1995a). The synthesis of the connective tissue framework of white muscle was suggested to be a rate-limiting step in muscle growth (Millward, 1989). Therefore, the level of free-hydroxyproline in the white muscle could become a reliable factor for nutritional status and growth of the fish.

Comparisons of white muscle FAA levels were also performed in Atlantic salmon and Arctic charr (Table 2), with and without the trypsin variant TRP-2*92 (see Figure 2), and fed with a diet containing non-hydrolyzed protein or pre-hydrolyzed protein for 6 h by pepsin. The results indicated that most FAA involving in nitrogen metabolism were differences between the two trypsin phenotypic groups in their levels in the white muscle. The total FAA (TFAA) levels were different due to EAA or NEAA levels or both. However, the levels of EAA/NEAA ratio were under physiological control. The fish with higher growth potentials had higher nitrogen metabolism with mainly higher levels of FAA involving in nitrogen metabolism and growth (Table 2).

It is important to note that the levels of free-hydroxyproline in the white muscle were significantly higher in the *TRP-2*92* salmon fed with the diet containing 6 h pre-hydrolyzed protein, and also in the *TRP-2*92* Hammerfest charr fed with the diet containing non-hydrolyzed protein,

Table 2. Free amino acids in the white muscle (µmol per g wet weight) of Atlantic salmon and Arctic charr, with and without trypsin variant TRP-2*92, fed on a diet containing protein meal with non-hydrolyzed protein or with pre-hydrolysis by pepsin for 6 h. Within the same species and the same feed, the values with the asterisk (*) are significantly different between the two phenotypic groups ($P < 0.05$) by paired analysis during the whole time course (–48 h to 72 h post-feeding). Trypsin phenotypes are illustrated in Figure 2. (Original data from Torrissen et al., 1995a, 1995b)

White muscle free amino acids (µmol g^{-1})	Non-hydrolyzed protein		6 h-Pre-hydrolyzed protein		Non-hydrolyzed protein	
	Salmon with	Salmon without	Salmon with	Salmon without	Hammerfest with	Skogseid without
TFAA	21.75 ± 0.43*	19.84 ± 0.51*	24.47 ± 0.63*	20.70 ± 0.81*	33.89 ± 0.81*	25.48 ± 0.07*
EAA	3.56 ± 0.18*	3.16 ± 0.18*	3.99 ± 0.27	3.52 ± 0.22	3.40 ± 0.31*	3.96 ± 0.33*
NEAA	18.19 ± 0.35*	16.68 ± 0.31*	20.48 ± 0.54*	17.18 ± 0.64*	30.50 ± 2.12*	21.53 ± 0.87*
EAA/NEAA	0.20 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.11 ± 0.01	0.18 ± 0.01
Alanine	2.98 ± 0.11*	2.34 ± 0.09*	3.45 ± 0.13*	2.91 ± 0.11*	3.12 ± 0.08	2.97 ± 0.11
β-Alanine	0.50 ± 0.03	0.42 ± 0.02	0.53 ± 0.05*	0.34 ± 0.03*	0.70 ± 0.06*	1.34 ± 0.11*
Glutamic acid	3.57 ± 0.09*	3.15 ± 0.13*	2.82 ± 0.14*	2.44 ± 0.42*	3.06 ± 0.11*	4.16 ± 0.11*
Glutamine	0.22 ± 0.03*	0.19 ± 0.02*	0.38 ± 0.04*	0.26 ± 0.04*	0.20 ± 0.02	0.17 ± 0.01
Glycine	5.18 ± 0.17*	4.94 ± 0.23*	6.07 ± 0.38	5.56 ± 0.50	8.74 ± 0.41*	6.21 ± 0.38*
Hydroxyproline	0.51 ± 0.04	0.58 ± 0.04	1.00 ± 0.05*	0.38 ± 0.06*	0.75 ± 0.08*	0.39 ± 0.01*
Proline	0.25 ± 0.03*	0.20 ± 0.03*	0.31 ± 0.03*	0.25 ± 0.04*	0.24 ± 0.01	0.25 ± 0.02
Taurine	3.53 ± 0.16	3.45 ± 0.09	4.27 ± 0.19*	3.66 ± 0.09*	4.16 ± 0.20*	3.62 ± 0.10*
Anserine	11.86 ± 0.02*	11.62 ± 0.03*	16.45 ± 0.13*	12.86 ± 0.43*	10.51 ± 0.24	not detected

TFAA (total free amino acids), EAA (essential free amino acids), NEAA (non-essential free amino acids)

compared to the other phenotype with slower growth and fed with the same feed (Table 2). The white muscle free-hydroxyproline levels were not different between the two phenotypic groups with no growth differences and fed with the diet containing non-hydrolyzed protein (Table 2).

Sunde et al. (2004) have reported about free-hydroxyproline in Atlantic salmon fed on diets with different protein qualities due to differences in concentrations of free (reactive) sulphydryl (SH) group. During a steady growth phase, the fish fed with a higher quality feed (containing protein with higher level of SH group), and having a higher feed efficiency, had higher white muscle RNA levels, and their white muscle TFAA levels inversely correlated with pyloric caecal trypsin specific activity. However, they had lower levels of white muscle free-hydroxyproline because of lower protein turnover rate during steady growth. It is important to know the growth status of the fish in order to correctly interpret the results.

White Muscle Free-Hydroxyproline as a Growth Indicator

The aim of this experiment was to screen several biochemical indices in fish including their interrelations in order to select variables for future studies of growth rate and feed efficiency (Sunde et al., 2001). Diploid (2n) and triploid (3n) Atlantic salmon were reared indoors under simulated natural light period (SNP) or continuous light regime (LL) during February to April for 75 days with the water temperature of 8.7 ± 0.1°C. The biological factors which correlated with specific growth rate (SGR) are shown in Figure 5. Regardless of ploidy and light regime, the SGR values were significantly correlated positively with the protease specific activity of trypsin (Figure 5A) and the activity ratio of trypsin to chymotrypsin (T/C ratio) (Figure 5B) in the pyloric caeca, including the levels of free-hydroxyproline in the white muscle (Figure 5C). However, the SGR values were significantly correlated negatively with the concentrations of EAA/NEAA (Figure 5D), RNA (Figure 5E), and RNA/protein ratio (Figure 5F) in the white muscle, because higher growth caused higher FAA

assimilation, and increased growth by continuous light regime was not caused by an increased protein deposition rate.

A principal component analysis (PCA) explained 80.6% of the variance in the data, and white muscle free-hydroxyproline levels showed the highest correlation, alone explaining 55% of SGR variability.

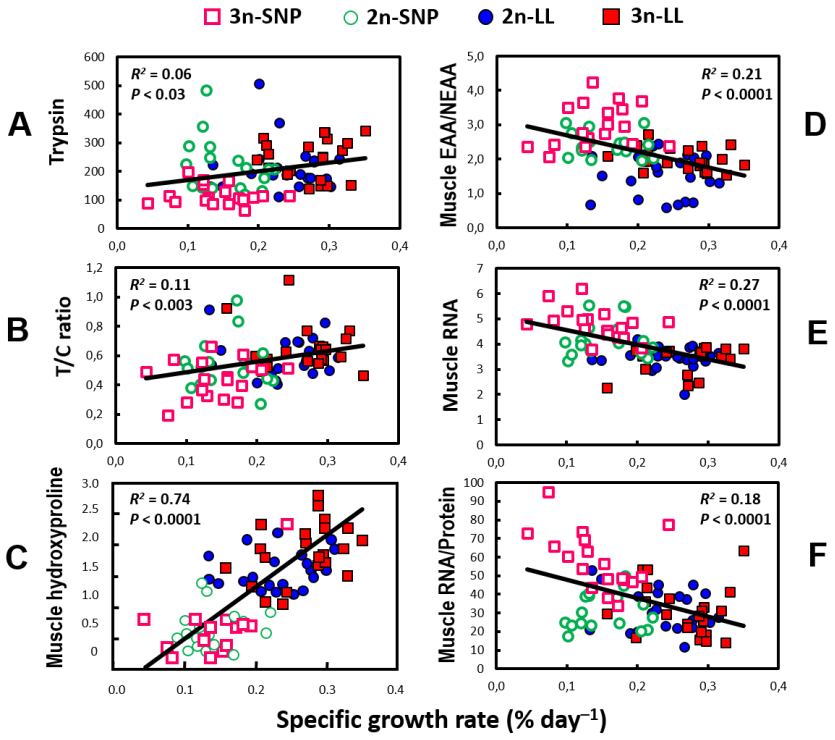

Figure 5. The relationships between specific growth rate with (A) trypsin specific activity (µmol *p*-nitroaniline h^{-1} mg $protein^{-1}$), (B) the activity ratio of trypsin to chymotrypsin (T/C ratio), and with epaxial white muscle concentrations of (C) free-hydroxyproline (nmol mg^{-1}), (D) the ratio of essential to non-essential free amino acids (EAA/NEAA), (E) RNA (µg mg^{-1}), and (F) the ratio of RNA to protein (µg mg^{-1}), in diploid (2n) and triploid (3n) Atlantic salmon (about 1 kg) reared for 2.5 months at 9 °C in seawater tanks under simulated natural light period (SNP) or 24 h light regime (LL). (Adapted from Rungruangsak-Torrissen 2014a, with permission from Nova Science Publishers, Inc., original data from Sunde et al. 2001).

Most importantly, feed efficiency (on tank basis) correlated positively (r = 0.97) with SGR, T/C ratio and white muscle free-hydroxyproline levels, and negatively (r = –0.90) with white muscle EAA/NEAA ratio (Sunde et al., 2001). According to feed efficiency and correlation coefficient, the most convenient and reliable biological factors for studying in relation to SGR were the T/C ratio in the pyloric caeca (independent of the specific activity levels of trypsin and chymotrypsin) and the free-hydroxyproline concentration in the white muscle. These two growth factors, the pyloric caecal T/C ratio (for digestive efficiency) and the white muscle free-hydroxyproline (for muscle remodeling and growth) were also positively correlated.

Therefore, the levels of both pyloric caecal T/C ratio and white muscle free-hydroxyproline could be used as the biological growth indicators for studies and comparisons of SGR of living resources, especially in the wild where their food availabilities, consumption rates and growth rates are unknown. So far, the pyloric caecal T/C raio has been successfully implemented for studying growth and growth status (a regression of trypsin specific activity and T/C ratio) of aquatic animals in different species in aquaculture and in the wild (see Rungruangsak-Torrissen, 2012, 2014a, 2014b, 2018). A positive regression indicates a growing phase, a negative regression indicates a reducing growth period, and a regression with no correlation indicates a steady growth phase. The levels of white muscle free-hydroxyproline could be used for growth study, but not for growth status study. However, it has not yet been utilized for growth study. It could be a new perspective for future growth comparison study of animals, because muscle biopsy is easier and more practical than intestinal biopsy, if the animals are needed to be alive after samplings.

FREE-HYDROXYPROLINE IN OOCYTES

Free amino acids (FAA) have been studied in the white muscle and oocytes to observe muscle growth and the development of oocytes during maturation of maturing Atlantic mackerel feeding on only natural food in

the sea or provided with an artificial diet at a ration of 1% of body weight per day (Rungruangsak-Torrissen and Fosseidengen, 2007). The experiment was performed with the fish of about 250 g for 273 days from September to May at the average temperature of 8.4 ± 0.5°C with the salinity 32. All of sampled mackerel were at a late maturing stage, and the results are shown in Table 3. The artificially fed mackerel grew faster with the body weight about 1.7 times heavier than the naturally fed mackerel, but with less white muscle protein concentrations (0.5 time) (Table 3). During maturation, the development of oocytes and muscle for growth occurred concurrently in higher growth mackerel fed with artificial diet, while the development of oocytes dominated in slower growth fish fed on only natural food. The pyloric caecal T/C ratio values were significantly lower in the artificially fed mackerel (0.100 ± 0.005), due to higher reduction in their growth rates at maturation than the fish with natural feeding (0.124 ± 0.003). There were no correlations between trysin specific activity and the T/C ratio in any fish groups, indicating that the fish were at a limited or steady growth phase at maturation. The information on growth status study is described in Rungruangsak-Torrissen (2012, 2014a, 2014b, 2018).

Specific activities of trypsin-like (T) and chymotrypsin-like (C) enzymes including T/C ratio were also studied in the oocytes (Rungruangsak-Torrissen et al., 2012; Rungruangsak-Torrissen, 2012, 2018). Females with higher maturation rates have higher T/C ratio values in their oocytes, despite the lower oocyte specific activities of trypsin-like and chymotrypsin-like enzymes. Higher levels of trypsin-like specific activity (indicating slower maturation rates) with higher FAA levels were observed in the oocytes from the artificially fed mackerel, compared to the naturally fed females (Table 3). This suggested differences in the development and quality between the gametes of the fish with different feedings.

Most FAA levels were relatively higher in the white muscle and oocytes of the artificially fed mackerel. However, the significantly higher levels were observed only on the EAA in the oocytes, resulting in

Table 3. Concentrations of free amino acids (FAA) and nitrogen-containing compounds (nmol g^{-1}), RNA, and protein in the white muscle and oocytes, including trypsin-like specific activity (μmol *p*-nitroaniline h^{-1} mg $protein^{-1}$) in the oocytes, from maturing Atlantic mackerel with natural feeding in comparison with those with artificial feeding. Significant differences are indicated by bold values ($P \leq 0.05$). (Adapted from Rungruangsak-Torrissen and Fosseidengen 2007, with permission from John Wiley and Sons, Inc.)

Parameters (n = 4 – 7)	White muscle			Oocytes		
	Natural	Artificial	*P*	Natural	Artificial	*P*
Essential FAA (EAA)						
Histidine	9330 ± 942	11787 ± 2362	ns	537 ± 27	1504 ± 180	**< 0.004**
Isoleucine	121 ± 5	163 ± 15	**< 0.03**	571 ± 122	2378 ± 676	< 0.082
Leucine	176 ± 32	184 ± 45	ns	99 ± 33	183 ± 13	**< 0.02**
Lysine	133 ± 23	270 ± 76	ns	111 ± 16	84 ± 18	ns
Methionine	120 ± 6	187 ± 19	**< 0.01**	324 ± 53	1576 ± 419	< 0.056
Phenylalanine	1376 ± 192	1643 ± 375	ns	1145 ± 133	4023 ± 563	**< 0.005**
Threonine	167 ± 63	478 ± 49	**< 0.001**	767 ± 91	1555 ± 491	ns
Valine	249 ± 8	284 ± 35	ns	810 ± 154	2790 ± 701	< 0.069
Non-essential FAA (NEAA)						
Alanine	2121 ± 104	1686 ± 201	< 0.097	2842 ± 521	5724 ± 172	ns
β-Alanine + Sarcosine	2982 ± 125	2793 ± 452	ns	1638 ± 140	2484 ± 290	< 0.067
γ-Aminobutyric acid	nd	205 ± 146		nd	nd	
Anserine	223 ± 126	4330 ± 642	**< 0.0001**	nd	244 ± 138	
Arginine	106 ± 35	281 ± 51	**< 0.02**	1029 ± 294	3326 ± 435	**< 0.006**
Asparagine	nd	130 ± 100		3865 ± 453	1584 ± 450	**< 0.01**
Aspartic acid	nd	5 ± 3		479 ± 72	1009 ± 281	ns
Carnosine	nd	nd		nd	nd	
Citrulline	nd	nd		nd	862 ± 565	
Creatinine	79 ± 60	1859 ± 576	**< 0.02**	908 ± 133	1160 ± 93	ns
Cystathione	99 ± 36	594 ± 189	**< 0.04**	2015 ± 594	852 ± 271	< 0.07

Table 3. (Continued)

Parameters (n = 4 – 7)	White muscle			Oocytes		
	Natural	**Artificial**	***P***	**Natural**	**Artificial**	***P***
Non-essential FAA (NEAA)						
Cysteine	7 ± 2	9 ± 1	ns	24 ± 4	16 ± 3	ns
Cystine	nd	522 ± 246		443 ± 226	161 ± 30	ns
Glutamic acid	769 ± 91	1030 ± 103	< 0.083	1795 ± 342	2533 ± 474	ns
Glutamine	4 ± 2	28 ± 7	ns	78 ± 19	253 ± 73	ns
Glycine	1273 ± 92	856 ± 130	**< 0.03**	3179 ± 897	2046 ± 423	ns
Hydroxy-lysine	nd	nd		62 ± 13	312 ± 118	< 0.091
Hydroxy-proline	nd	205 ± 55		nd	24 ± 15	
1-Methyl-histidine	nd	nd		nd	39 ± 25	
3-Methyl-histidine	29 ± 14	nd		251 ± 26	167 ± 39	**< 0.04**
Ornithine	146 ± 45	303 ± 39	**< 0.02**	1018 ± 158	3644 ± 912	< 0.064
Phosphoethanolamine	37 ± 16	76 ± 37	ns	3239 ± 1910	1992 ± 480	ns
Phosphoserine	nd	nd		nd	nd	
Proline	253 ± 72	1212 ± 289	**< 0.02**	959 ± 117	1866 ± 423	ns
Serine	235 ± 55	220 ± 40	ns	1350 ± 324	4171 ± 1349	ns
Taurine	3326 ± 306	1750 ± 211	**< 0.0002**	11846 ± 659	17280 ± 820	**< 0.002**
Tyrosine	102 ± 5	108 ± 12	ns	370 ± 157	1113 ± 298	ns
Total FAA (µmol g^{-1})	23.71 ± 1.31	34.81 ± 11.21	ns	42.73 ± 0.48	69.86 ± 9.06	**= 0.05**
EAA (µmol g^{-1})	11.68 ± 2.05	14.78 ± 5.10	ns	4.36 ± 0.29	14.09 ± 2.91	**< 0.04**
NEAA (µmol g^{-1})	12.04 ± 0.76	20.03 ± 6.30	ns	38.37 ± 0.25	55.77 ± 6.21	ns
EAA/NEAA ratio	1.02 ± 0.26	0.71 ± 0.12	ns	0.11 ± 0.01	0.24 ± 0.03	**< 0.02**
RNA (µg g^{-1})	2044 ± 43	1876 ± 71	ns	8309 ± 570	5355 ± 404	**< 0.0001**
Protein (mg g^{-1})	187.8 ± 24.1	99.3 ± 12.8	**< 0.0001**	167.2 ± 13.3	157.5 ± 8.0	ns
Trypsin-like specific activity	–	–	–	6.33 ± 1.59	15.38 ± 2.48	**< 0.0001**

Tryptophan was not reliably detected by the method. nd, not detected. ns, not significant.

significantly higher levels of total FAA and EAA/NEAA ratio in the oocytes (Table 3). For free-hydroxyproline, it was only observed in the white muscle and oocytes of the artificially fed mackerel (Table 3). Free-hydroxyproline level in the white muscle is higher in the fish with higher growth rate because of higher protein turnover rate for remodeling of connective tissues for muscle growth (Torrissen et al., 1994; Rungruangsak-Torrissen and Male, 2000; Sunde et al., 2001). The non-detectable levels observed in both white muscle and oocytes of the naturally fed mackerel indicated no or very low breakdown of muscle protein for remodeling muscle for growth in these fish including no or very low oocyte protein breakdown at a high maturity stage. The artificially fed mackerel still had somatic growth, showing slower oocyte development and lower protein synthesis capacity (lower oocyte RNA levels, shown in Table 3), with higher oocyte trypsin-like specific activity for higher protein degradation resulting in higher free-hydroxyproline levels in the oocytes, compared to the naturally fed mackerel. The females with higher oocyte development (higher maturation rate) have lower trypsin-like specific activity and free-hydroxyproline levels in the oocytes, due to lower oocyte protein levels for protein degradation at the late maturing stage (Rungruangsak-Torrissen et al., 2012; Rungruangsak-Torrissen, 2018).

CONCLUSION

Free-hydroxyproline is a product from protein degradation. Its levels in the white muscle and oocytes could be used as indications of muscle growth and oocyte maturation rate, respectively. The levels of T/C ratio in the pyloric caeca (regardless of specific activity levels of trypsin and chymotrypsin) and in the oocytes (despite the lower specific activity levels of trypsin-like and chymotrypsin-like enzymes at higher maturity) have been successfully implemented for respective studies of growth and maturation rates of living resources in aquaculture and in the wild (see Rungruangsak-Torrissen, 2012, 2014a, 2014b; Rungruangsak-Torrissen et al., 2012; Rungruangsak-Torrissen, 2018). However, the levels of free-

hydroxyproline in the white muscle and oocytes have not yet been utilized for these purposes, and the growth status of the animals is important for correctly interpreting the results. This chapter provides the ideas for a new possibility for future studies in using the higher white muscle concentration of free-hydroxyproline for a higher somatic growth and the lower level in the oocytes for a higher oocyte growth (maturation rate), along with the T/C ratio values that always positively correlate with somatic growth and oocyte maturation rate.

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