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**Nutritional requirements of fish with  
emphasis on Atlantic salmon and rainbow  
trout: A literature study by AKVAFORSK  
and NIFES**

Edited by B. Grisdale-Helland

# REPORT

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## Objective of the project

Review the state of knowledge about the nutrient requirements of fish, with a special emphasis on Atlantic salmon and rainbow trout.

## Summary

The growth rates of Atlantic salmon and rainbow trout in aquaculture are continuously increasing because of advances in husbandry, management, nutrition and genetic selection. Detailed evaluations of the nutrient requirements for these important species have not kept pace with these improvements in growth, however. In addition, many of the requirements determined may not be valid because the specialized diets used in these trials did not give growth rates in line with those obtained with a control diet or as expected under commercial conditions.

In this literature study, the nutrient requirements that have been determined for rainbow trout and Atlantic salmon are presented, with emphasis on the nutrients that most affect the growth, health and well-being of the fish and the quality of the final product. The contents of these nutrients in marine and different plant ingredients are discussed along with comments about any negative aspects these ingredients may have for fish performance or quality of the final product.

## keywords

Atlantic salmon; rainbow trout; nutritional requirements

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## Contributors

### **Grete Baeverfjord**

Senior Scientist  
Nofima  
6600 Sunndalsøra

### **Bjørn Bjerkeng**

Senior Scientist  
Nofima  
6600 Sunndalsøra

### **Barbara Grisdale-Helland**

Senior Scientist  
Nofima and Aquaculture Protein Centre  
6600 Sunndalsøra

### **Ståle J. Helland**

Senior Scientist  
Nofima and Leader, Amino Acid & Protein Metabolism Section, Aquaculture Protein Centre  
6600 Sunndalsøra

### **Bente Ruyter**

Senior Scientist  
Nofima  
1432 Ås

### **Bente E. Torstensen**

Senior Scientist  
NIFES  
5817 Bergen

### **Rune Waagbø**

Professor  
NIFES  
5817 Bergen

### **Trine Ytrestøyl**

Scientist  
Nofima  
6600 Sunndalsøra

## **1. Introduction**

*Barbara Grisdale-Helland and Ståle J. Helland*

The growth rates of Atlantic salmon and rainbow trout in aquaculture are continuously increasing because of advances in husbandry, management, nutrition and genetic selection. Detailed evaluations of the nutrient requirements for these important species have not kept pace with these improvements in growth, however. In addition, many of the requirements determined may not be valid because the specialized diets used in these trials did not give growth rates in line with those obtained with a control diet or as expected under commercial conditions. Because of the shortage of marine resources, fish meal and fish oil are currently being replaced by other protein and lipid sources. Many of these ingredients are not optimally balanced. In order to utilize these ingredients appropriately, either by blending them in the correct ratios or with the use of specific supplementations, the requirements of the fish must be known.

The requirements can be defined as: the combination of nutrients and energy that yields optimal performance. This will be reflected by growth rate, lack of nutrient deficiency symptoms, and an end product of optimal quality. This group of conditions is, naturally enough, difficult to fulfil at the same time, and a key phrase in this definition is: “the combination of nutrients”. This implies not only that knowledge about the minimum amounts of nutrients is needed. There can equally well be a toxic effect of a nutrient and it is obvious that the balance between the various nutrients is very important. The consequence of this is that a nutrient requirement can be defined in several ways, all dependent on its intentional use. How then, should the requirements be used and in what framework? Nutrient modelling is a mathematical setup used to predict performance. The value of the output from any model is dependent on the quality of the input, and it would ease the situation if the requirements were established in a framework that is coordinated with the inner workings of the models used. A model also requires a set of correction factors that quantify the influence of the different biotic and abiotic factors on the model. Therefore, it is important to generate these correction factors in a setting that is as little influenced by other factors as possible, or, in other words, to generate a mathematical relationship that is as simple as possible. In classical animal energetic modelling, for example, the requirements are separated into two components. The first is the amount of energy that is needed to maintain zero energetic balance, or, as it is also called, the maintenance requirement for energy. The other component is the energy required

for growth above maintenance. In an ideal modelling situation, each of these would be a constant. The biological situation, however, is somewhat more complicated and influencing factors must be taken into account.

In this literature study, the nutrient requirements that have been determined for rainbow trout and Atlantic salmon will be presented, with emphasis on the nutrients that most affect the growth, health and well-being of the fish and the quality of the final product. The contents of these nutrients in marine and different plant ingredients will be discussed along with comments about any negative aspects these ingredients may have for fish performance or quality of the final product.

## 2. Energy

*Ståle J. Helland*

Energy is not defined as a nutrient. It is a fuel that is released by the break-up of chemical bonds when the three macronutrients (fat, protein and carbohydrates) undergo biological oxidation. A description of the central energy terms used is given below:

*Gross energy* (GE) - the energy released by total combustion of a biological sample in a bomb calorimeter.

*Digestible energy* (DE) - the dietary energy that remains when the energetic losses in the faecal material are deducted.

*Metabolizable energy* (ME) - the energy that remains after the energy in the branchial and urinary losses is subtracted from DE. For fish, this consists mostly of the energetic losses associated with the nitrogenous end products from the oxidation of dietary protein, that is, mainly urea and ammonia. The latter is the largest component (75-80%). The energetic value of the ammonia in fish, however, is low, and therefore, in fish ME is not much lower than DE.

*Heat increment* (HI) or *specific dynamic action* (SDA) - the energy required for the digestion and metabolism of the dietary nutrients.

*Activity* - the energy loss associated with locomotion and maintaining position in the water column.

*Net energy* (NE) - the dietary energy that is available for the fish. This is defined as:  $NE = ME - (HI + Activity)$ . Traditionally, NE is split into that needed for maintenance ( $NE_m$ ), for growth ( $NE_g$ ) and for reproduction.

The majority of publications use  $DE_m$ , since this requires less complicated experimental setups than what is required for the determination of  $ME_m$ . In addition, as mentioned above, the numerical difference between DE and ME is small. The magnitude of experimental complications increases when going from  $ME_m$  to  $NE_m$ . HI, Activity and  $NE_m$  are all expressed in the form of chemically liberated energy (heat), and are often determined indirectly in respiration experiments through the measurement of oxygen consumption.

The size of the animals has often been a complicating factor in animal energetics. This problem has been circumvented by expressing the results on a Metabolic Body Weight basis (MBW), where the weight is raised to a certain power. The power used for energy is frequently 0.8 in fish. This factor may be different depending on the fish species.

The most frequently tabulated value for requirement is the minimum dietary inclusion level that is needed for optimal animal growth or the gain of a specific nutrient. The

experimental design used for establishing this type of requirement may be called Requirement by Dose Response (RDR). In this design, several diets are fed with only one nutrient is changed at a time. The result is a dilution of the other dietary components and thus, the nutrient in question is generally exchanged with another. It is important in such cases to maintain the required balance by exchanging the nutrient in question with another that has minimal impact on the desired response. The RDR design is not suitable for establishing the minimum energy required for optimal growth. This is because the diets needed for such a study have to be designed with confounding effects between energy and the macronutrients (protein, fat and carbohydrate).

Another design for establishing requirements can be termed Requirement by Ration Levels (RRL). This has also been termed the Factorial method. In this design, one diet is fed at different ration levels and the nutrient intake and growth response are expressed on a MBW basis. Both RDR and RRL can be used to determine the requirements for maintenance ( $DE_m$ ) by extrapolating to zero gain. The RDR relationship is often curvilinear. The strength of the RRL-design is that it is frequently a linear relationship, possibly allowing a more correct extrapolation to zero gain. Additionally, the nature of the RRL design is such that data points can be generated that are closer to the  $DE_m$  than what is the case with the RDR design, thus requiring a smaller extrapolation. The slope of the linear relationship between energy intake and gain ( $k_{DEg}$ ) describes the efficiency of utilization of digestible energy for growth above maintenance.

The major bulk of scientific information related to the needs of dietary energy is related to finding the optimal balance between the macronutrients and energy that gives highest growth. A problem with this pool of information is that it is comprised of data from different experimental conditions and the extraction of general conclusions from such data might be hampered by several non-quantified interactions from the different experiments. Rodehutsord and Pfeffer (1999) demonstrated this problem when looking at the maintenance requirement for energy in rainbow trout. They pooled data from 292 groups of fish from experiments conducted over 10 years, and re-evaluated the results by regression analysis. They found that the dietary fat content influenced both the efficiency of utilization of energy for growth and also  $DE_m$ . The authors concluded that “Maintenance can therefore, hardly be calculated by extrapolation of the regression line to zero energy retention. This can only be a rough estimate as it is not clear why maintenance requirement for digestible energy of a fish should depend on the fat level in the diet.” The concept of RRL assumes that the maintenance requirement is fixed for that experiment. The traditional way to establish the maintenance



requirement in an RRL-design is to use only one diet fed at different ration levels, as used by Lofgreen (1965) for beef cattle. One explanation for the influence of dietary fat level on the estimate of the maintenance requirement reported by Rodehutsord and Pfeffer (1999) could be related to differences in substrates available or preferred for energy metabolism. This substrate can either come from the turnover of body tissues or from the diet. McKenzie et al. (1998) demonstrated with Atlantic salmon that the ratio of fish oil : rapeseed oil in the diet influenced the fish's swimming performance. With the same maximum oxygen consumption, increasing the fraction of rapeseed oil in the diet resulted in increased maximum swimming speed. It is suggested that increased levels of oleic and linoleic acid in the muscle, from the rapeseed oil, might influence the efficiency of aerobic energy production by mitochondria during exercise. This experiment indicates that the choice/availability of substrates for energy metabolism influences the energy balance, and could therefore, also influence the energy required for maintenance.

Several investigators that have used RRL to determine the  $DE_m$  and  $k_{DEg}$  have questioned whether it is valid to extrapolate the relationship between energy intake and gain to the maintenance level, or if special fasting processes set in at this low level of intake. Helland et al. (2006) used 100 g Atlantic salmon smolt in a single diet RRL-design and fed the fish at four levels (100%, 73%, 53% and 20%) or fasted. The full-fed groups had growth rates 140% of that given in Austreng et al. (1987) and similar to that found in the higher producing commercial salmon farms. In the relationship between digestible energy intake and energy gain, 100% of the variation was accounted for ( $R^2=1$ ). The groups of fish that were fed at 20% of full-fed were at energy equilibrium (maintenance), giving evidence that the influence of any special energetic processes during low intake was negligible.

Extrapolation of regression lines outside the area covered by data should be approached with caution. Such exercises, however, give credit to a data set with little uncontrolled interactions if the maintenance estimates obtained by extrapolation are comparative to literature values. A maintenance requirement for rainbow trout of  $137 \text{ kJ kg}^{-0.8} \text{ d}^{-1}$  (Table 2.1) was reported by Rodehutsord and Pfeffer (1999). This is twice the value obtained by recalculating the trout data of Huisman (1976). Kaushik and Gomes (1988) fed 150 g rainbow trout a maintenance ration for 28 days and reported that  $DE_m$  was only 31 to 41  $\text{kJ kg}^{-0.8} \text{ d}^{-1}$ , or close to a quarter of the  $DE_m$  reported by Rodehutsord and Pfeffer (1999). The  $DE_m$  values of Kaushik and Gomes (1998) are similar to those reported for the other species listed in Table 2.1 (32-50  $\text{kJ kg}^{-0.8} \text{ d}^{-1}$ ). These values are approximately 10% of the energy released by fasting

Table 2.1. Digestible energy for maintenance ( $DE_m$ ) and efficiency of utilization of digestible energy for growth above maintenance ( $k_{DEg}$ ) in various fish species<sup>1</sup>

	Start weight, g	$DE_m$ , kJ MBW <sup>-1</sup> d <sup>-1</sup>	$k_{DEg}$	Temperature, °C	$R^2$	Remarks	Highest growth rate, % <sup>2</sup>	Reference
Atlantic salmon, <i>Salmo salar</i>	100	31.5	0.80	10	1.00		~140	Helland et al., 2006
Rainbow trout, <i>Oncorhynchus mykiss</i>	14-113	137		15 (12-17.5)				Rodehutscord and Pfeffer, 1999
	13	-0.001	0.42	6		Recalculated <sup>3</sup>	~80	Azevedo et al., 1998
	13	-0.002	0.56	9		Recalculated	~80	Azevedo et al., 1998
	13	-0.0002	0.39	12		Recalculated	~70	Azevedo et al., 1998
	13	0.0006	0.60	15		Recalculated	~70	Azevedo et al., 1998
	13	0.0014	0.57	6-15		Recalculated		Azevedo et al., 1998
	70	57	0.57	15		Recalculated	~40	Huisman, 1976 <sup>4</sup>
	140-150	31-41		18		Recalculated		Kaushik and Gomes, 1988
Atlantic cod, <i>Gadus morhua</i>	250	42.3	0.78	10	0.99			Hatlen et al., 2007
Gilthead seabream, <i>Sparus aurata</i>	17 - 32	43.8	0.50	21 - 24	0.94			Lupatsch et al., 2001a <sup>5</sup>
	30	46.0	0.65	19 - 27	0.97			Lupatsch et al., 2003
European sea bass, <i>Dicentrarchus labrax</i>	14 - 97	45.8	0.66	20-26	0.97			Lupatsch et al., 2001b <sup>6</sup>
White grouper, <i>Epinephelus aeneus</i>	10	33.2	0.69	19 - 27	0.99			Lupatsch et al., 2003
	13 - 90	32	0.64	22	0.98			Lupatsch and Kissil, 2005
		40	0.65	24	0.98			Lupatsch and Kissil, 2005
		50.3	0.68	27	0.98			Lupatsch and Kissil, 2005

<sup>1</sup>Exponent for metabolic body weight (MBW) is 0.8, unless otherwise indicated.

<sup>2</sup>Percentage of growth rates for salmon and trout given in Austreng et al. (1987).

<sup>3</sup>Recalculated: Used the authors' data and expressed the results on a DE basis using a MBW coefficient of 0.8.

<sup>4</sup>Have assumed an energy digestibility of 80% in this recalculation due to the high carbohydrate level in the diet.

<sup>5</sup>Used MBW coefficient of 0.83.

<sup>6</sup>Used MBW coefficient of 0.79.

homoeothermic animals ( $70 \text{ kcal kg}^{-0.75} \text{ d}^{-1}$ ; Kleiber, 1975). The maintenance values reported by Azevedo et al. (1998) were obtained by long extrapolations to zero, and seem to be unrealistic. The relatively high  $\text{DE}_m$  of Rodehutscord and Pfeffer (1999) could also be unrealistic and reflects an untraditional use of the RRL design. They did not use a single diet fed at several levels, but by pooled the uncontrolled variations obtained from a variety of experimental conditions and diets. The authors indicated that a larger database than the 292 experimental units was needed in order to separate out other effects than that of dietary fat level.

The requirements for DE above maintenance ( $k_{\text{DEg}}$ , Table 2.1) range from 0.39 to 0.80 kJ DE deposited per kJ consumed, with Atlantic salmon and Atlantic cod as the most efficient species. Apart from one observation on sea bream, rainbow trout seem to be the least efficient species depositing energy above maintenance. The recalculated values from Azevedo et al. (1998) change a fair amount. These authors state that temperature does not influence the  $k_{\text{MEg}}$ . This is in contrast to the results presented by Lupatsch and Kissil (2005), using white grouper. This discrepancy in conclusions between these papers, the large numerical spread in the  $k_{\text{DEg}}$  from the rainbow trout experiment, plus the unrealistic  $\text{DE}_m$  extrapolated from the data of Azevedo et al. (1998) warrants a re-evaluation of the effect of temperature on the growth and maintenance requirements of rainbow trout.

We have stated earlier that energy results obtained from nutrient and energy optimization experiments are of limited value when discussing the energy requirements in a general sense. The comparative slaughter technique, where the amount of consumed nutrients deposited or retained in the body is calculated, is a frequently used method in such studies. These values can also be defined as the amount of nutrients required for that particular level of production. A major reason why these values are of limited use in a general requirement discussion is that they are confounded with growth rate. With poorer growth, a higher proportion of the required energy is used to maintain energy balance ( $\text{DE}_m$ ). Rodehutscord and Pfeffer (1999) reported that close to 30% of the DE intake was used for maintenance in poor-growing groups of fish, whereas this was reduced to 10% among the fast-growing groups. In agreement, Helland et al. (2006) found that the energy required for maintenance changed from 100% of the DE consumed for fish fed at the 20% level to 20% of the DE consumed for the full-fed groups.

In conclusion, for rainbow trout and Atlantic salmon, little data is available concerning energy utilization for maintenance and growth above maintenance. The ultimate comparison

of the energetic utilization between these two species in an RRL design has not yet been conducted.

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### **3. Protein and amino acids**

*Barbara Grisdale-Helland*

#### **3.1. Protein**

Protein is a major component in fish diets, but there is no actual requirement for it. Rather, protein is consumed as a source of amino acids (AA). The amino acids are required for protein growth, as precursors of bioactive molecules such as hormones and neurotransmitters (D'Mello, 2003), or to replace these components in the body. Even if not in excess, protein will be used not only for growth, but also as a supply of energy. When protein is used for energy, ammonia is released from the amino acids and excreted, contributing to pollution of the environment. Thus, dietary formulations are generally designed to supply the minimum protein level for maximum growth along with an appropriate balance of the other nutrients to supply the required energy. When insufficient dietary protein is available to the fish, a reduction or lack of growth will generally be the result. Besides being species dependent, the demand for dietary protein, as summarized by Hardy (2002), may be affected by fish size, water temperature, activity level, reproductive status and dietary energy level.

Many trials with Atlantic salmon and rainbow trout have shown protein-sparing effects of lipids and/or carbohydrates (for example, Austreng, 1976a,b; Bergot, 1979; Pieper and Pfeffer, 1979; Watanabe et al., 1979; Kim and Kaushik, 1992; Johnsen et al., 1993; Hemre et al., 1995; Grisdale-Helland and Helland, 1997), and also possibly, nucleotides (Aas et al., 2006).

#### **3.2. Amino acids**

Ten specific AA have been found to be indispensable in the diet for all fish species studied so far, including rainbow trout (reviewed by Wilson, 1985, 2002). While specific studies have not been done to determine the essentiality in the diet of all of these 10 AA in Atlantic salmon, it is assumed, based on reports from the other fish species, that this species also requires these AA. Besides supplying the indispensable amino acids (IAA), dietary protein is also needed as a source of dispensable amino acids (DAA) or their precursors. The proportion of IAA and DAA in the diet affects growth and nitrogen (N) retention, and has been studied in several species (see section 3.5).

The IAA are: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. As described in Chapter 2, the minimum

dietary requirements for IAA have been determined using dose-response experiments. With this design, increasing increments of the AA under investigation are added to a basal diet deficient in that AA (D'Mello, 1982) and the AA concentration giving maximum response is assumed to be the requirement. The minimum IAA requirements may be affected by the age, life stage and genetics of the animal, dietary composition, feeding regime, growth rate and environmental variables. The response variable is usually either weight gain or protein gain, although other variables such as AA gain or feed efficiency may also be studied, depending on the production objectives. As seen in Table 3.1, the results of Rodehutschord et al. (1995b, 1997) show that the response variable greatly affects the estimated minimal AA requirements. In addition, the choice of mathematical model used to interpret the requirement may have a substantial effect on the result (Rodehutschord and Pack, 1999; Shearer, 2000; Encarnação et al., 2004). For rainbow trout, the minimal requirements for maximal weight gain have been determined using this method for all of the IAA. For Atlantic salmon, requirements by dose-response have been determined for arginine, histidine, lysine, methionine and threonine. Particularly for salmon, several of the requirements have only been published in abstracts or theses, but are considered valuable for the current report.

In dose-response trials, semi-synthetic diets have often been used in order to restrict the level of the AA under investigation. This has resulted in sub-optimal growth in comparison with current commercial conditions, and even the twenty-year old growth estimates of Austreng et al. (1987) (Tables 3.1 and 3.2). Recently, however, diets for use in AA requirement studies have been improved and growth rates have been achieved in rainbow trout (pers. comm., N. Bodin) and Atlantic salmon (Espe et al., 2007) at levels 90% or higher than those given by Austreng et al. (1987). The dietary lysine requirement for maximal protein growth determined by Espe et al. (2007) was 27-40% higher than that reported by Anderson et al. (1993) and Berge et al. (1998) (that had growth rates that were 35% and 60% respectively, of those given in Austreng et al. (1987)), but only 5% higher than that estimated by Rollin et al. (1994) [growth rate 60% of Austreng et al. (1987)], who used the diet-dilution method (see below). Han and Baker (1991) studied fast- and slow-growing broiler chicks and found that the amount of lysine required (consumed) per day was greater in the fast-growing poultry, but that the required dietary lysine concentration was not different for the two types. The authors noted that there was no difference in the protein concentration in the two chicken types. Interestingly, with rainbow trout, Chiu et al. (1988) showed that the dietary arginine requirement was lower when the fish were fed to satiation than when intake was restricted and that the daily intake of arginine per fish was not affected by the dietary arginine level. Thus,

Table 3.1. Dietary protein (N x 6.25) and indispensable amino acid (AA) requirements of rainbow trout for weight gain, protein gain or feed efficiency

Amino acid								Protein Source	Dietary protein (g kg <sup>-1</sup> DM)	Highest growth rate (%) <sup>§</sup>	Evaluation method	Reference
Response	Weight gain	Protein gain	Weight gain	Protein gain	Weight gain	Protein gain	FER					
	g AA kg <sup>-1</sup> dry diet*	g AA kg <sup>-1</sup> dry diet*	g AA kg <sup>-1</sup> dietary protein*	g AA kg <sup>-1</sup> dietary protein*	g AA kg <sup>-1</sup> gain*	g protein or AA kg <sup>-1</sup> protein gain*	g AA kg <sup>-1</sup> dry diet					
Protein						2280		CS	14-506	~75	Regression	Fournier et al., 2002
Arginine		14		35				FM, CS or egg	400		Protein gain	Ogino, 1980
			54-59					CG	470	~60	ANOVA	Ketola, 1983
	16		47					Milk, CG	330			Cho & Woodward, 1985 <sup>a</sup>
	16-18		36-40					FM, Zein	450	~55	Broken line	Walton et al., 1986
	14 (full-fed); 17 (restricted feeding)		35 (full-fed); 42 (restricted feeding)					CS, GEL	400	TLS	Polynomial	Chiu et al., 1988
	14		42	42				Milk, CG	300	~75	ANOVA	Cho et al., 1992
			30					Milk, CG	300	~75	4-PL	Cho et al., 1992
	14.1		40					CS	350	~80	Broken line	Kim et al., 1992a
			38									Forster, 1993
	11.1	11.6	33	35			8.45	WG	335	~70	Exp.	Rodehutsord et al., 1995b
	14.1-28.1		32-63					CS	468-477	~35	ANOVA	Riley et al., 1996
						138		CS	14 - 508	~75	Regression	Fournier et al., 2002
		16.8		48				FM	350	~55	Ideal protein	Green and Hardy, 2002
Histidine		6.4		16				FM, CS or egg	400		Protein gain	Ogino, 1980
	5.2	5.8	15	17			4.0	WG	338	>50	Exp.	Rodehutsord et al., 1997
		6.4		18				FM	350	~55	Ideal protein	Green and Hardy, 2002
Isoleucine		9.6		24				FM, CS or egg			Protein gain	Ogino, 1980
	14.1	13.7	43	41			10.4	WG	331	>55	Exp.	Rodehutsord et al., 1997
		9.9		28				FM	350	~55	Ideal protein	Green and Hardy, 2002
Leucine		17.6		44					400		Protein gain	Ogino, 1980
	13.4	13.6	39	40			10.9	WG	344	>50	Exp.	Rodehutsord et al., 1997
		17.5		50				FM	350	~55	Ideal protein	Green and Hardy, 2002

<sup>a</sup> Cited by Wilson (1989).



Table 3.1. cont'd

Amino acid								Protein Source	Dietary protein (g kg <sup>-1</sup> DM)	Highest growth rate (%) <sup>§</sup>	Evaluation method	Reference
Response	Weight gain	Protein gain	Weight gain	Protein gain	Weight gain	Protein gain	FER					
	g AA kg <sup>-1</sup> dry diet*	g AA kg <sup>-1</sup> dry diet*	g AA kg <sup>-1</sup> dietary protein*	g AA kg <sup>-1</sup> dietary protein*	g AA kg <sup>-1</sup> gain*	g protein or AA kg <sup>-1</sup> protein gain*	g AA kg <sup>-1</sup> dry diet					
Lysine		21.2		53				FM, CS or egg	400		Protein gain	Ogino, 1980
			61					CG	470	~60	ANOVA	Ketola, 1983
	19.5		43		18.9]			FM, gluten	450	~70	ANOVA	Walton et al., 1984a
	21.6		54		27.6]				399		Broken line	Lanari et al., 1991
		17.5		37	17.7]			WG	470	~75	Polynomial	Pfeffer et al., 1992
	13		37		15.7]			CS	350	~85	Broken line	Kim et al., 1992a
	23.2	27.7	69	82	21.1]		14.3	WG	338	>65	Exp.	Rodehutsord et al., 1997
		19.3		55				FM	350	~55	Ideal protein	Green and Hardy, 2002
	17.4-23.3		38-51		10.6 (16 MJ/kg diet), 6.0 (20 MJ/kg diet)	152 (16 MJ/kg diet), 129 (20 MJ/kg diet)		WG, FM, CG	460	>75	Broken line, 4-PL, Exp.	Encarnaçao et al., 2004
		15.2		56.9!		78		WG	DP, 286	~90!	Broken line	Bodin et al., 2006a
		20.5		53.8!		78		WG	DP, 430	~100!	Broken line	Bodin et al., 2006a
Methionine (Cysteine)		7.2 (3.6)		18 (9)				FM, CS or egg	400		Protein gain	Ogino, 1980
	10.1 (0)		20.2 (0)					CS, GEL	500	†	ANOVA	Walton et al., 1982
	5.1 (20.5)		10.2 (41)									
	5.5-7.5 (3)		16-21(8.6)					CS, GEL	350	<40	ANOVA	Rumsey et al., 1983
	11.3 (3.4)		29.7 (8.9)					SM	380	~60	Polynomial	Poston, 1986
	5.2 (3)		15 (8.6)					CS	350	~65	Broken line	Kim et al., 1992b
	6.0 (1.6)¶		15 (4)					FM, SM, WM	400	TLS	ANOVA	Cowey et al., 1992
	6.7 (3.0)	8.0 (3.0)						WG	340	~60	Exp.	Rodehutsord et al., 1995a
		8.1 (2.2)		23 (6)				FM	350	~55	Ideal protein	Green and Hardy, 2002
Phenylalanine (Tyrosine)		12.4 (8.4)		31 (21)				FM, CS or egg	400		Protein gain	Ogino, 1980
Phe + Tyr	15		43					CS, GEL	350	~65	Broken line	Kim, 1993
		13.7 (10.5)		39 (30)				FM	350	~55	Ideal protein	Green and Hardy, 2002
Threonine		13.6		34				FM, CS or egg	400		Protein gain	Ogino, 1980
	9.5	10.4	28	31			8.1	WG	335	~65	Exp.	Rodehutsord et al., 1995b
		12.3		35				FM	350	~55	Ideal protein	Green and Hardy, 2002
		10.5		27				WG	393	~100	Broken line	Bodin et al., 2006b

Table 3.1. cont'd

Amino acid								Protein Source	Dietary protein (g kg <sup>-1</sup> DM)	Highest growth rate (%) <sup>§</sup>	Evaluation method	Reference
Response	Weight gain	Protein gain	Weight gain	Protein gain	Weight gain	Protein gain	FER					
	g AA kg <sup>-1</sup> dry diet*	g AA kg <sup>-1</sup> dry diet*	g AA kg <sup>-1</sup> dietary protein*	g AA kg <sup>-1</sup> dietary protein*	g AA kg <sup>-1</sup> gain*	g protein or AA kg <sup>-1</sup> protein gain*	g AA kg <sup>-1</sup> dry diet					
Tryptophan		2		5				FM, CS or egg	400		Protein gain	Ogino, 1980
	2.3		5.8 <sup>‡</sup>					GEL	420	~45	Regression	Poston & Rumsey, 1983
	2.5		4.5					FM, GEL	550	~45	Broken line	Walton et al., 1984b
	2-2.5		5.7-7.1									Kim et al., 1987
	2.1-2.6		3.8-4.6					GEL, CS	560	~65	ANOVA	Johnston et al., 1990
	1.9	2.0	5.7	6.0			1.8	WG	331	>55	Exp.	Rodehutsord et al., 1997
		2.1		6				FM	350	~55	Ideal protein	Green and Hardy, 2002
Valine		12.4		31				FM, CS or egg	400		Protein gain	Ogino, 1980
	15.5	15.7	45	46			7.8	WG	344	>70	Exp.	Rodehutsord et al., 1997
		11.2		32				FM	350	~55	Ideal protein	Green and Hardy, 2002

Abbreviations: CG, corn gluten; CS, casein; DP, digestible protein; Exp., exponential model; FER, feed efficiency ratio; FM, fish meal; GEL, gelatine; TLS, too large change in weights for comparison with table in Austreng et al. (1987); SM, soybean meal; WG, wheat gluten; WM, wheat middlings; 4-PL, 4-parameter logistic equation.

<sup>§</sup>Percentage of growth rates for salmon and trout given in Austreng et al. (1987).

\*If not presented, then calculated from data given in the article.

†Water temperature not given.

‡From Hauler and Carter (2001b).

!Pers. comm., N. Bodin.

¶6 g Met/kg diet and 3.6 g Cys/kg diet required for prevention of lens abnormality.

‡6.3 g Trp/kg dietary protein required for prevention of pathology.

Table 3.2. Dietary protein (N x 6.25) and indispensable amino acid (AA) requirements of Atlantic salmon for weight gain or protein gain

Amino acid							Protein source	Dietary protein (g kg <sup>-1</sup> DM)	Highest growth rate (%) <sup>§</sup>	Evaluation method	Reference
Response	Weight gain	Protein gain	Weight gain	Protein gain	Weight gain	Protein gain					
	g AA kg <sup>-1</sup> dry diet*	g AA kg <sup>-1</sup> dry diet*	g AA kg <sup>-1</sup> dietary protein*	g AA kg <sup>-1</sup> dietary protein*	g AA kg <sup>-1</sup> gain*	g protein or AA kg <sup>-1</sup> protein gain*					
Protein						3050	FM	14-541	~55	Regression	Abboudi et al., 2006b
						1560	FM	540	~140	Regression	Helland et al., 2006
Arginine	16		41				CS, CG	400	~65	Broken line	Lall et al., 1994
	21.2	21.6	50	51			Zein	420	~35	Asymptotic	Berge et al., 1997
	18.2		40				FM	450	~60	Ideal protein	Rollin et al., 2003
						95	FM	540	~140	Regression	Helland et al., 2006
Histidine	8.1		20				CS, CG	460	~70	Broken line	Scott, 1998
	6.7		15				FM	450	~60	Ideal protein	Rollin et al., 2003
						31	FM	540	~140	Regression	Helland et al., 2006
Isoleucine						52	FM	540	~140	Regression	Helland et al., 2006
Leucine						98	FM	540	~140	Regression	Helland et al., 2006
Lysine	19.9		40		21.3]		FM, WG	500	~35	Broken line	Anderson et al., 1993
	18		42		15.4]		Zein	427	~60	Asymptotic	Berge et al., 1998
					19.3		Zein, FM	430	~40	Regression	Hauler and Carter, 2001a
	23.9		53				FM	450	~60	Ideal protein	Rollin et al., 2003
					22.5	152	FM	14-382	~55	Regression	Abboudi et al., 2006b
						105	FM	540	~140	Regression	Helland et al., 2006
		25.2		50.4			FM, WG, CG	500	~90	Exponential	Espe et al., 2007
Methionine (Cysteine)	11		24				CS	450		Broken line	Rollin et al., 1994
	11.4 (3.6)		24.2 (8)				FM, SM, WM	470	~60	Broken line	Scott, 1998
	8.7 (≥2.6)		20.3 (6)				FM, SM	410	100	Regression	Sveier et al., 2001
						40	FM	540	~140	Regression	Helland et al., 2006
Met & Cys	15.4		34				FM	450	~55	Ideal protein	Rollin et al., 2003
Phenylalanine & Tyrosine	25.1		56				FM	450	~55	Ideal protein	Rollin et al., 2003
Phenylalanine						51	FM	540	~140	Regression	Helland et al., 2006
Threonine	12.1		27				FM	450	~65	Ideal protein	Rollin et al., 2003
	11.4		28		9.3	66	WG	397	~80	Regression	Rollin et al., 2006
		10.6		27			WG	393	~85	Broken line	Bodin et al., 2006b
						55	FM	540	~140	Regression	Helland et al., 2006
Tryptophan	3.3		7				FM	450	~60	Ideal protein	Rollin et al., 2003
						19	FM	540	~140	Regression	Helland et al., 2006
Valine	14.1		31				FM	450	~60	Ideal protein	Rollin et al., 2003
						64	FM	540	~140	Regression	Helland et al., 2006

Abbreviations: CAS, casein; CG, corn gluten; CS, casein; FER, feed efficiency ratio; FM, fish meal; SM, soybean meal; WG, wheat gluten; WM, wheat middlings.

<sup>§</sup>Percentage of growth rates for salmon and trout given in Austreng et al. (1987).

\*If not presented, then calculated from data given.

]From Hauler and Carter (2001b).

the effect of growth rate on minimal AA requirements of fish has still not been resolved.

Several other methods have been used to determine AA requirements. Ogino (1980) used the daily increase in IAA in rainbow trout fed a 40% crude protein diet for estimating the requirement of all of the IAA. In calculating the requirements, Ogino (1980) reported a feed intake level of 3% body weight per day, but Kim et al. (1992b) suggests that this level is too low according to the data presented, resulting in an overestimation of the requirements (28% overestimation for methionine). Walton et al. (1986) also noted that Ogino (1980) did not take into account maintenance losses of AA and assumed that there were no oxidation or transformation losses of the dietary IAA.

Green and Hardy (2002) and Rollin et al. (2003) used the diet-dilution method to determine the optimum dietary IAA pattern in rainbow trout and Atlantic salmon. The diet-dilution method assumes that the reduction of a non-limiting IAA has no effect on N gain. When a single AA is limiting, the rate of body protein accretion is directly related to the level of that AA. The change in N gain measured on removal of a proportion of the dietary content of an IAA is used to calculate the appropriate AA pattern. The pattern was determined for all of the IAA in the study of Green and Hardy (2002) and all of the IAA except leucine and isoleucine in the study of Rollin et al. (2003). For the latter two IAA, no decrease in N gain was registered when either of these was diluted in the diet. Both groups used the IAA pattern to establish the dietary requirement for the IAA.

During the last decade, the factorial approach, or the requirement by ration level method (RRL; described in Chapter 2) has been used to determine the requirements for growth and maintenance for crude protein and energy in fish species such as African catfish, carp, European sea bass, gilthead seabream, rainbow trout, silver perch, white grouper, Atlantic cod (cited by Hatlen et al., 2007), brown trout (Mambrini et al., 2004) and Atlantic salmon (Helland et al., 2006). The method is dependent on accurate measurements of feed intake, digestibility and nutrient accretion. Usually, only one diet is used and it is offered at different levels of feeding or ration levels (Fig. 3.1). In the relationship between the intake of a nutrient and its deposition, or the deposition of a related nutrient (per unit of weight), the maintenance requirement is the amount of nutrient consumed (or absorbed) when gain is zero. This value may be estimated from a measured point of zero nutrient gain, or through an extrapolation to zero gain from higher intake levels. As defined by Beck and Gropp (1995), an approximate maintenance requirement reflects the loss of the nutrient during starvation, and is a value correlated with, but lower than the maintenance requirement. For crude protein and AA, the maintenance requirement includes AA losses from the skin and gastrointestinal

tract, ammonia loss from oxidation of amino acids in body protein, AA used for synthesis of other bioactive molecules, irreversible alteration of an AA (proline to hydroxyproline and lysine to hydroxylysine in protein), and the loss of AA in the urine (Moughan and Fuller, 2003). According to Boisen et al. (2000), the maintenance requirement for amino acids is difficult to determine because at N-equilibrium redistribution of tissue protein will occur. Muscle protein is degraded at low feed intake to compensate for the endogenous loss of amino acids. The methionine, cystine and threonine contents in muscle protein are lower than those in the endogenous losses, thus making the other AA in excess when used for replacing the endogenous losses. The excess of the other AA in this redistribution may result in an underestimation of the required amounts of these AA for maintenance (Boisen et al., 2000). Nevertheless, the dose-response method (Rodehutscord et al., 1997) has been used to estimate the maintenance requirements for the AA for trout. We are currently evaluating the suitability of the RRL method for estimating AA maintenance and growth requirements (Helland et al., 2006). The requirement for growth reflects the efficiency of deposition of the nutrient in the body per unit intake above that required for maintenance.

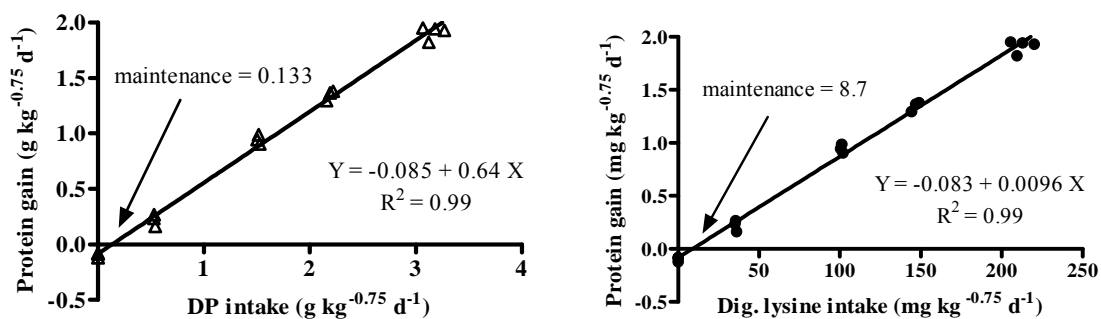


Figure 3.1. The relationships between digestible protein (DP) and digestible lysine intake on protein gain in Atlantic salmon post-smolt fed different rations (Helland et al., 2006).

### 3.3. Amino acid deficiency

Feed intake is generally reduced when there is a severe amino acid deficiency. Fish may compensate though, when the deficiency is not too severe and maintain feed intake. As reviewed by D'Mello (2003), acute amino acid deficiency, in animals fed to *ad libitum*, may result in decreased protein content of the liver, muscle and pancreas, increased free amino acid levels in the plasma, increased protein synthesis in the liver and blood, and decreased

protein synthesis in the muscle. In dose-response trials with trout, Rodehutsord et al. (1995a; 1995b; 1997) found that deficiencies of arginine, isoleucine, lysine, leucine, methionine, threonine or tryptophan resulted in increased lipid deposition, valine deficiency resulted in reduced lipid deposition, whereas no change in lipid deposition was found with histidine deficiency.

### **3.4. Amino acid requirements**

In the following summaries of AA requirements, priority has been given to the use of  $g\ AA\ kg^{-1}\ dietary\ protein$  as the independent variable and *protein gain* as the dependent or response variable.

#### **3.4.1. Lysine**

Most commonly, the first-limiting AA in protein sources used in fish diets is lysine. As a result, the lysine requirement has been evaluated most often. Hauler and Carter (2001b) reviewed the lysine requirements that have been determined in dose-response trials in different fish species. They concluded that when the requirement is expressed as a percentage of the diet, variation between the results, within species, could not be explained by experimental factors such as dietary formulation, growth rate, statistical model or response variable. In contrast, Hauler and Carter (2001b) found that if the requirement was expressed relative to weight gain (as in the factorial method), less variation between experiments was evident. Their calculations showed that for salmon (Table 3.2), the lowest requirement for lysine was  $15.4\ g\ kg^{-1}$  weight gain from the study of Berge et al. (1998), where 0.5 kg fish were studied. For 2 g salmon, Abboudi et al. (2006b) reported the highest value measured ( $22.5\ g\ lysine\ kg^{-1}$  weight gain) and showed a strong negative correlation ( $r=0.99$ ) between the lysine requirement ( $g\ kg^{-1}$  weight gain) and log body weight in the four available studies with Atlantic salmon. Hauler and Carter (2001b) also summarized data for rainbow trout and found that 15.7 to 21.1 g lysine was required per kg gain (the data of Lanari et al. (1991) was ignored because of overestimated feed intake). Later data with trout shows though, that only 6 to 11 g lysine were required per kg weight gain (Encarnaç o et al., 2004). Hauler and Carter (2001b) noted that this expression would overestimate AA requirements in fish depositing adipose tissue (such as in the study of Berge et al., 1998) and that the most appropriate responses would be protein or AA gain, instead of live weight gain. Using this response, Abboudi et al. (2006b) reported a lysine requirement of  $152\ g\ kg^{-1}$  protein gain in

salmon fry, whereas the data of Helland et al. (2006), with on-growing salmon, shows a lysine requirement of 105 g kg<sup>-1</sup> protein gain.

Encarnaç o et al. (2004) reported that there was no effect of the dietary energy level on the minimal dietary lysine requirement for trout and concluded that there was no reason to express the AA requirements in relation to the dietary digestible energy content. In contrast, the dietary energy level has a negative effect on the lysine requirement for protein gain in trout. A lysine requirement of 152 g kg<sup>-1</sup> protein gain was found when trout were fed a diet containing 16 MJ kg<sup>-1</sup> and 129 g kg<sup>-1</sup> protein gain was required when trout were fed a diet containing 20 MJ kg<sup>-1</sup> (Encarnaç o et al., 2004). The data of Bodin et al. (2006a) shows that in rainbow trout fry fed a diet containing an even higher level of energy (22-23 MJ kg<sup>-1</sup>), the lysine requirement was about 78 g kg<sup>-1</sup> protein gain. This suggests increasing AA-sparing through the catabolism of greater amounts of dietary non-protein energy sources. The effect of dietary protein level on the utilization efficiency of lysine in trout is not clear. Rodehutschord et al. (2000) reported a positive effect, whereas Bodin et al. (2006a) found no effect. Bodin et al. (2006a) showed though, that an increase in the digestible protein concentration from 286 to 430 g kg<sup>-1</sup> diet increased the dietary lysine requirement of rainbow trout from 15 to 20 g kg<sup>-1</sup> diet, and decreased the required lysine concentration in the dietary protein (pers. comm., N. Bodin).

Rodehutschord et al. (2000) showed significant, but slight, effects of dietary lysine concentration on organic matter and energy digestibility in trout. Encarnaç o et al. (2004) also found an effect of dietary lysine level on the digestibility of carbohydrates in trout, but noted that this may have resulted from experimental error because the carbohydrates were calculated by difference from the other organic diet materials. This also seems to be the case in the trial of Rodehutschord et al. (2000).

The minimum lysine requirements determined for rainbow trout vary from 37 to 82 g kg<sup>-1</sup> dietary protein, although most values are in the range of 40-60 g kg<sup>-1</sup> dietary protein (Table 3.1). The lysine requirement has not been determined as often for Atlantic salmon as for rainbow trout, but the four trials from which the salmon requirement can be expressed in relation to the dietary protein level, gave results in the range of 40-53 g kg<sup>-1</sup> dietary protein (Table 3.2), or quite similar to the values for rainbow trout. The lysine requirement values for Pacific salmon are also in this range (38-50 g kg<sup>-1</sup> dietary protein) (reviewed by Wilson, 2003).

The lysine maintenance requirement reported for rainbow trout (11.2 mg kg<sup>-0.75</sup> d<sup>-1</sup>) (Rodehutschord et al., 1997) is between the levels that have been estimated for Atlantic salmon



by Helland et al. (2006) ( $8.7 \text{ mg kg}^{-0.75} \text{ d}^{-1}$ ) and Abboudi et al. (2006b) ( $20 \text{ mg kg}^{-0.75} \text{ d}^{-1}$ ) (Tables 3.3 and 3.4).

### 3.4.2. Arginine

In rainbow trout, arginine deficiency causes reduced growth, high mortality and fin erosion (Ketola, 1983). Based on the increase in free arginine levels in the blood and muscle when the dietary arginine intake was met, Kaushik (1979) concluded that the arginine requirement of freshwater trout was  $12 \text{ g kg}^{-1}$  diet, but this was not confirmed in an accompanying growth trial. The requirement level estimated by Kaushik (1979) is, however, only slightly lower than the requirement levels subsequently determined, when weight gain was used as the response variable ( $14\text{-}28 \text{ g kg}^{-1}$  diet; Table 3.1), except that of Rodehutsord et al. (1995b) ( $11 \text{ g kg}^{-1}$  diet). Furthermore, when expressed in terms of the dietary protein level, the arginine requirement given by Kaushik (1979) ( $33 \text{ g kg}^{-1}$  dietary protein<sup>b</sup>) was in the range of that determined in most of the later trials ( $30\text{-}40 \text{ g kg}^{-1}$  dietary protein; Table 3.1). On a dietary basis, the arginine requirements estimated for Atlantic salmon ( $16\text{-}21 \text{ g kg}^{-1}$  diet) are similar to those found for trout, whereas on a protein basis ( $40\text{-}50 \text{ g kg}^{-1}$  dietary protein) these are a little higher. The arginine requirements reported for salmon species from the Pacific Ocean (chinook, chum and coho salmon) range from 32 to  $65 \text{ g kg}^{-1}$  dietary protein (reviewed by Wilson, 2003).

The maintenance requirement for arginine has been studied in two trials with rainbow trout (Table 3.3). Rodehutsord et al. (1997) reported an arginine requirement of  $15.5 \text{ mg kg}^{-0.75} \text{ d}^{-1}$ , whereas Fournier et al. (2002) could find little or no maintenance needs for arginine in rainbow trout, turbot or gilthead seabream. The arginine requirement for maintenance for Atlantic salmon (Table 3.4) determined by Helland et al. (2006) was  $8 \text{ mg kg}^{-0.75} \text{ d}^{-1}$ , approximately half of that determined by Rodehutsord et al. (1997) for trout. In the neonatal and immediate post-weaning phases of the pig, endogenous synthesis of arginine from glutamate/glutamine and proline occurs, covering about 50% of the daily requirement (Wu et al., 1997). It has been suggested that *de novo* synthesis of arginine may also occur in fish (Buentello and Gatlin, 2000, 2001), but this has not been unequivocally shown based on the presence of the necessary enzymes.

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<sup>b</sup> The dietary protein content was not given. This value is cited by Wilson (2003).

Table 3.3. Protein and indispensable amino acid (AA) requirements of rainbow trout for maintenance and growth

Amino acid	Maintenance requirement	Growth requirement	Protein source	Dietary protein (g kg <sup>-1</sup> )	Evaluation method	Reference
Response	Zero protein gain (mg protein or AA kg <sup>-0.75</sup> d <sup>-1</sup> )	For max. protein or weight gain (mg AA kg <sup>-1</sup> d <sup>-1</sup> )				
Protein	774					Kaushik et al., 1981
	325					Mambrini and Kaushik, 1995 (cited by Abboudi et al, 2006b)
	236		CS	14-506	Regression	Fournier et al., 2002
Arginine		210†	Zein, FM		Graphic	Kaushik, 1979
	15.5		WG	344	Exponential	Rodehutscord et al., 1997
	0		CS	14-506	Regression	Fournier et al., 2002
Histidine	6.0		WG	344	Exponential	Rodehutscord et al., 1997
Isoleucine	5.1		WG	344	Exponential	Rodehutscord et al., 1997
Leucine	47.8		WG	344	Exponential	Rodehutscord et al., 1997
Lysine	11.2		WG	344	Exponential	Rodehutscord et al., 1997
		640	WG	DP, 286 or 430	Regression	Bodin et al., 2006a
Methionine	9.0		WG	344	Exponential	Rodehutscord et al., 1997
Threonine	18.8		WG	344	Exponential	Rodehutscord et al., 1997
Tryptophan	6.0		WG	344	Exponential	Rodehutscord et al., 1997
		50	FM, GEL	550	Broken line	Walton et al., 1984
Valine	16.7		WG	344	Exponential	Rodehutscord et al., 1997

Abbreviations: CS, casein; DP, digestible protein; FM, fish meal; GEL, gelatine; WG, wheat gluten.

† Based on increase in free arginine in blood and muscle.

Table 3.4. Protein and indispensable amino acid (AA) requirements of Atlantic salmon for maintenance

Amino acid	Maintenance requirement	Protein source	Dietary protein (g kg <sup>-1</sup> )	Evaluation method	Reference
Response	Zero protein gain (mg protein or AA kg <sup>-0.75</sup> d <sup>-1</sup> )				
Protein	337	FM	14-541	Regression	Abboudi et al., 2006b
	133	FM	540	Regression	Helland et al., 2006
Arginine	7.9	FM	540	Regression	Helland et al., 2006
Histidine	2.6	FM	540	Regression	Helland et al., 2006
Isoleucine	4.5	FM	540	Regression	Helland et al., 2006
Leucine	8.3	FM	540	Regression	Helland et al., 2006
Lysine	20	FM	14-382	Regression	Abboudi et al., 2006b
	8.7	FM	540	Regression	Helland et al., 2006
Methionine	3.3	FM	540	Regression	Helland et al., 2006
Phenylalanine	4.4	FM	540	Regression	Helland et al., 2006
Threonine	6	FM, WG	398	Regression	Rollin et al., 2006
	4.7	FM	540	Regression	Helland et al., 2006
	9.9	FM		Regression	Abboudi et al., 2006a
	41.2	Acc. CAA†	398	Regression	Abboudi et al., 2006a
	5.4	WG	398	Regression	Abboudi et al., 2007
Tryptophan	1.7	FM	540	Regression	Helland et al., 2006
Valine	5.4	FM	540	Regression	Helland et al., 2006

Abbreviations: FM, fish meal; WG, wheat gluten.

†Pre-accustomed for 14 days to a crystalline AA diet.

### 3.4.3. Histidine

There is good agreement between the histidine requirements for Atlantic salmon and rainbow trout (15-20 g kg<sup>-1</sup> dietary protein) and these are also in line with most of the histidine requirements determined for other salmonid species (16-18 g kg<sup>-1</sup> dietary protein), except the 9 g kg<sup>-1</sup> dietary protein for coho salmon estimated by Arai and Ogata (1993) (reviewed by Wilson, 2003).

Breck et al. (2003) reported that elevated levels of dietary histidine (from 8 to 15 g kg<sup>-1</sup> diet) and/or Fe in the diet had an alleviating effect on cataract development in Atlantic salmon, rather than correcting a nutritional deficiency. Compared with the minimal requirement determined for maximum growth (7-8 g kg<sup>-1</sup> diet; Scott, 1998; Rollin et al., 2003), these results may indicate a higher requirement for normal eye development. In a later study, Breck et al. (2005a) found that the supplementation of a fishmeal-based diet with crystalline histidine (increasing the dietary histidine level from 12.6 to 19.0 g kg<sup>-1</sup> diet) resulted in a reduction in the severity of cataract scores, but no effect on the frequency of cataract development. The greatest effect of supplementary histidine was found when it was fed in the saltwater period, although some effect was also seen in the freshwater period. A reduction in cataract scores in salmon fed a diet containing 15.3 g histidine kg<sup>-1</sup> compared with those fed a control diet (9.6 g histidine kg<sup>-1</sup>) was also seen in the study of Breck et al. (2005b). Breck et al. (2005a) observed that despite 10% lower growth in the salmon fed the histidine-supplemented diet for six weeks in fresh water, growth was better in the fish fed the supplemented diet after a total of 26 weeks (including 17 weeks in saltwater), compared with those fed the control diet. The fish fed the histidine-supplemented diet also had better feed conversion than those fed the control diet.

As with arginine, the maintenance requirement for histidine in Atlantic salmon (2.6 mg kg<sup>-0.75</sup> d<sup>-1</sup>; Helland et al., 2006) was about half of that found for rainbow trout (6.0 mg kg<sup>-0.75</sup> d<sup>-1</sup>; Rodehutscord et al., 1997) (Table 3.3 and 3.4).

### 3.4.4. Isoleucine

The isoleucine requirements for rainbow trout determined by Ogino (1980) and Green and Hardy (2002) were 24 and 28 g kg<sup>-1</sup> dietary protein, respectively, whereas that estimated by Rodehutscord et al. (1997) was 41 g kg<sup>-1</sup> dietary protein. No dose-response study has been done to determine the isoleucine requirement for Atlantic salmon. As noted above, Rollin et al. (2003) used the diet-dilution method to estimate the IAA requirements in Atlantic salmon, but this was not possible for isoleucine and leucine, since no decrease in N gain was

registered when either of these AA was diluted in the diet. As reviewed by Wilson (2003), the isoleucine requirements for Pacific salmon and lake trout (12-26 g kg<sup>-1</sup> dietary protein) are similar to the lowest value determined for rainbow trout.

The isoleucine maintenance requirements are similar in Atlantic salmon (4.5 mg kg<sup>-0.75</sup> d<sup>-1</sup>; Helland et al., 2006) and rainbow trout (5.1 mg kg<sup>-0.75</sup> d<sup>-1</sup>; Rodehutscord et al., 1997) (Tables 3.3 and 3.4).

#### **3.4.5. Leucine**

The leucine requirements determined for rainbow trout vary from 40 to 50 g kg<sup>-1</sup> dietary protein, and are in the range of those determined for Pacific salmon and lake trout (35-46 g kg<sup>-1</sup> dietary protein) (reviewed by Wilson, 2003). As noted above, the leucine requirement for Atlantic salmon has not been determined.

The maintenance requirement for Atlantic salmon is 8.3 mg kg<sup>-0.75</sup> d<sup>-1</sup> (Helland et al., 2006) (Table 3.4). For rainbow trout, Rodehutscord et al. (1997) reported a value of 47.8 mg kg<sup>-0.75</sup> d<sup>-1</sup>, which, the authors noted, appeared surprisingly high compared to the level for the other AA (Table 3.3).

#### **3.4.6. Methionine**

The requirements for the sulfur-containing AA, methionine and cysteine, for rainbow trout are in the range 19-39 g kg<sup>-1</sup> dietary protein, with most values varying from 19 to 30 g kg<sup>-1</sup> dietary protein (Table 3.1). Trials with Atlantic salmon juveniles and smolt have shown that the methionine requirement for maximal growth is 24 g kg<sup>-1</sup> dietary protein (Rollin et al., 1994; Scott, 1998), whereas Sveier et al. (2001) reported that for 1 kg salmon, the methionine requirement was 20 g kg<sup>-1</sup> dietary protein. The total amount of the sulfuric AA in these diets was about 26-34 g kg<sup>-1</sup> dietary protein. For Pacific salmon, the total requirement for these AA (27 to 50 g kg<sup>-1</sup> dietary protein; reviewed by Wilson, 2002) is similar or higher than the results for Atlantic salmon and rainbow trout.

Methionine deficiency causes bilateral cataracts in trout (Poston et al., 1977), probably as a result of oxidation of the methionine in the lens (reviewed by Cowey et al., 1992). Cowey et al. (1992) found that trout fed a methionine-deficient diet exhibited cataracts and increased focal length variability in the lenses compared with normal fish. Although reductions in the number of cataracts occurred when the methionine level was increased in steps up to 19 g kg<sup>-1</sup> diet, an increase in the dietary cystine level to 3.6 g kg<sup>-1</sup>, at a methionine level of 6 g kg<sup>-1</sup>, was necessary before a well-defined focal point with little evidence of

spherical aberration was found in the trout (Cowey et al., 1992). Rodehutsord et al. (1995a) found no effect of increasing the dietary cystine level from 3.0 to 5.8 g kg<sup>-1</sup> (dry matter) on the growth and protein deposition of rainbow trout, both when the methionine supply was deficient or adequate. As shown by Kim et al. (1992b), cystine may be able to replace some of the methionine when the cystine level is much lower. Rodehutsord et al. (1995a) concluded that since the cystine level in trout feeds based on usual ingredients should not normally be lower than 3 g kg<sup>-1</sup> diet, recommendations for sulfur-containing AA should be specified in terms of methionine.

The methionine maintenance requirement has been estimated to be 9.0 mg kg<sup>-0.75</sup> d<sup>-1</sup> for rainbow trout (Rodehutsord et al., 1997) and 3.3 mg kg<sup>-0.75</sup> d<sup>-1</sup> for Atlantic salmon (Helland et al., 2006) (Table 3.3 and 3.4).

Increasing the dietary methionine content in trout diets from about 8 g kg<sup>-1</sup> up to 12 or 16 g kg<sup>-1</sup> (plus ~4 g kg<sup>-1</sup> cysteine) resulted in a tendency for decreased feed intake and significantly poorer gain (Gaylord et al., 2007).

#### **3.4.7. Phenylalanine**

Phenylalanine and tyrosine are aromatic amino acids. Tyrosine is a DAA because it can be synthesized from phenylalanine. Growth studies indicate that tyrosine can replace or spare about 48% of the phenylalanine requirement in rainbow trout (Kim, 1993).

The requirement for phenylalanine and tyrosine for rainbow trout is in the range of 43 (Kim, 1993) to 69 g kg<sup>-1</sup> dietary protein (Green and Hardy, 2002). The value for Atlantic salmon, determined by Rollin et al. (2003), is between these values (56 g kg<sup>-1</sup> dietary protein). The requirement values for phenylalanine and tyrosine for Pacific salmon are in a similar range (45-63 g kg<sup>-1</sup> dietary protein) (reviewed by Wilson, 2002).

The phenylalanine requirement for maintenance for Atlantic salmon is 4.4 mg kg<sup>-0.75</sup> d<sup>-1</sup> (Helland et al., 2006) (Table 3.4). This requirement has not been determined for rainbow trout.

#### **3.4.8. Threonine**

Only small differences have been reported in the threonine requirement for rainbow trout determined in four trials (27-35 g kg<sup>-1</sup> dietary protein; Table 3.1). The requirement for threonine for Atlantic salmon, determined in three trials, has showed little variation (27-28 g kg<sup>-1</sup> dietary protein; Table 3.2). These values are similar to those determined for Pacific salmon (20-30 g kg<sup>-1</sup> dietary protein) (reviewed by Wilson, 2002).

The maintenance requirement for threonine in rainbow trout (Table 3.3) has been reported to be  $18.8 \text{ mg kg}^{-0.75} \text{ d}^{-1}$  for rainbow trout (Rodehutsord et al., 1997). The maintenance requirement for threonine in Atlantic salmon (Table 3.4) has been determined using three of the methods described above. The dose-response (Rollin et al., 2006; Abboudi et al., 2006a) and diet-dilution methods (Abboudi et al., 2007) have been used for estimating the requirement in salmon fry, whereas Helland et al. (2006) used the factorial method with on-growing salmon. Together, these studies show that when protein-bound AA are used in the diet the threonine maintenance values range from 4.7 to  $9.9 \text{ mg kg}^{-0.75} \text{ d}^{-1}$  (Rollin et al., 2006; Helland et al., 2006; Abboudi et al., 2006a). When salmon are first accustomed to a crystalline AA before the start of the trial, however, the threonine maintenance was increased to  $41 \text{ mg kg}^{-0.75} \text{ d}^{-1}$  (Abboudi et al., 2006a). The reason for this difference has not been determined.

#### **3.4.9. Tryptophan**

Tryptophan deficiency may result in low feed intake and growth, scoliosis, lordosis, fin erosion, cataracts and mortality in rainbow trout (reviewed by Poston and Rumsey, 1983). Poston and Rumsey (1983) found that  $5.8 \text{ g tryptophan kg}^{-1}$  dietary protein resulted in maximum weight gain (95% confidence intervals), whereas  $6.3 \text{ g kg}^{-1}$  dietary protein was required for maximum predicted health score. The tryptophan requirement for Atlantic salmon ( $7 \text{ g kg}^{-1}$  dietary protein; Table 3.2) is at the upper end of the requirement values determined in various trials for rainbow trout ( $3.8\text{-}7.1 \text{ g kg}^{-1}$  dietary protein; Table 3.1). The tryptophan requirement values for Pacific salmon are similar to those for the other salmonids ( $5\text{-}7 \text{ g kg}^{-1}$  dietary protein; reviewed by Wilson, 2002).

The maintenance requirement for tryptophan is considerably higher in rainbow trout than in Atlantic salmon ( $6.0$  and  $1.7 \text{ mg kg}^{-0.75} \text{ d}^{-1}$ , respectively) (Rodehutsord et al., 1997; Helland et al., 2006) (Tables 3.3 and 3.4).

#### **3.4.10. Valine**

The valine requirement for Atlantic salmon ( $31 \text{ g kg}^{-1}$  dietary protein) (Rollin et al., 2003) is very similar to that determined in two trials with rainbow trout ( $31\text{-}32 \text{ g kg}^{-1}$  dietary protein; Ogino, 1980; Green and Hardy, 2002). Somewhat higher was valine requirement value for rainbow trout determined by Rodehutsord et al. (1997),  $46 \text{ g kg}^{-1}$  dietary protein.

Rainbow trout seem to require a higher level of valine for maintenance than Atlantic

salmon (16.7 and 5.4 mg kg<sup>-0.75</sup> d<sup>-1</sup>, respectively) (Rodehutsord et al., 1997; Helland et al., 2006) (Tables 3.3 and 3.4).

#### **3.4.11. Effect of salinity on protein and arginine needs**

Zeitoun et al. (1973) found that the minimum protein levels for optimum growth in rainbow trout fingerlings were 400 and 450 g kg<sup>-1</sup> when the salinity was 10 and 20 ppt, respectively. In contrast, based on free arginine levels in the blood and muscle, Kaushik (1979) concluded that the arginine requirement was decreased from 12 g kg<sup>-1</sup> diet for trout held in fresh water to 8 to 12 g kg<sup>-1</sup> at 20 ppt salinity and 8 g kg<sup>-1</sup> in sea water. Krogdahl et al. (2004) compared feed utilization in fresh water and sea water by rainbow trout and Atlantic salmon. The authors found that the protein concentration in the body of the trout was 0.5% higher in fish maintained in sea water compared with those maintained in fresh water, although this difference may have been related to the body lipid level. No differences in protein or lipid concentrations were found in the salmon. For the two species together, feed efficiency increased from 1.23 to 1.30 and the retention of digestible protein in the body increased by almost 5% when the fish were held in sea water compared with fresh water. Except for a slight decrease in protein digestibility in sea water compared with fresh water, these results do not seem to support a higher dietary protein need in sea water.

#### **3.4.12. Validation of IAA minimum requirements**

In an interesting study, Green and Hardy (2002) compared the growth and N retention of rainbow trout fed diets containing IAA patterns generated using different techniques. The IAA patterns used included 1) one that they themselves determined with the diet-dilution method (Green and Hardy, 2002), 2) the pattern reflecting the IAA composition of rainbow trout whole-body protein from Wilson and Cowey (1985), 3) the requirements published by the National Research Council (NRC, 1993), or 4) the requirements determined in the studies of Rodehutsord et al. (1995a; 1995b; 1997). The fish were fed to the same level (near-satiation for the fish with the least intake) in quadruplicate groups. The results showed that both the growth rate and N retention were significantly greater in the fish fed the NRC requirement pattern compared with those fed the Rodehutsord pattern. The other two diets yielded differences that were intermediate to the others. Green and Hardy (2002) concluded that the NRC pattern, derived from a large number of studies, was best matched to the optimum IAA pattern for rainbow trout, even though it was not statistically better than that derived from whole-body protein or determined with the diet-dilution method. Unfortunately,



the best growth rate in this comparison trial was under 50% of that estimated by Austreng et al. (1987) for the fish size and water temperature used. The pattern of IAA needed for maintenance is not the same as that for growth, and thus, it is not certain that the same conclusion would be obtained with higher growth rates.

### 3.4.13. Comparison of maintenance requirements for Atlantic salmon and rainbow trout

Seven of the nine IAA maintenance requirement values determined for trout by Rodehutsord et al. (1997), are two to six times higher than those found for salmon by Helland et al. (2006). The exceptions are for isoleucine and lysine that were only 10 and 30%, respectively, higher for trout than for salmon. Differences between these trials include the feed efficiency ratio achieved by each species and the digestible energy level of the diets used. The feed efficiency ratios in the dose-response trials for each AA for the trout were all about 1, whereas in the salmon trial, this ratio varied from 1.2 to 1.8, depending on the ration level. As seen in Fig. 3.2, greater gain with the same intake will result in a greater slope and a lower maintenance level. In addition, in the salmon trial, the digestible energy level of the diet was almost 24 MJ kg<sup>-1</sup>, whereas in the trout trials the DE levels were about 20 MJ kg<sup>-1</sup>. An increase in the dietary digestible energy content may spare protein (as, for example, shown in Grisdale-Helland and Helland, 1997), again suggesting a greater efficiency slope and lower maintenance level for the IAA. Direct comparisons between Atlantic salmon and rainbow trout show that salmon retain greater amounts of consumed or digested protein in the body than trout do (Refstie et al., 2000; Azevedo et al., 2004; Grisdale-Helland et al., 2007).

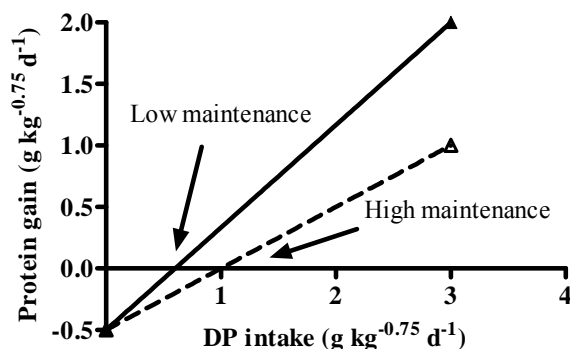


Figure 3.2. Higher feed efficiency (gain/intake) results in lower maintenance.

#### **3.4.14. Amino acid requirements for growth determined using the requirement by ration level (RRL) method**

The RRL method can be used to determine the AA requirements for maintenance and for AA or protein gain in fish (Helland et al., 2006; Table 3.2). This method does not give the minimum dietary requirement for an AA, but rather the efficiency of utilization of the AA for growth. Together with the maintenance requirement for the AA, the reciprocal of the efficiency coefficient can be used to determine the total requirement of the AA per kg growth. From Table 3.2, it can be seen that much less lysine and total protein were required per kg protein gain with post-smolt salmon (Helland et al., 2006) than with fry (Abboudi et al., 2006b). The data for the latter trial were determined using diets with different N and lysine levels and the best growth rate was about 55% of that estimated by Austreng et al. (1987). The AA requirement for growth above maintenance determined with the RRL method is, of course, independent of the growth rate of the fish. At the same time, the diet used in the study of Helland et al. (2006) seemed to be well balanced and led to excellent growth in the full-fed groups of fish. Our results with an RRL study with Atlantic cod (Hatlen et al., 2007) suggest that the utilization efficiency of the AAs will be negatively affected by an increase in the dietary protein level. This does not, however, seem to be the reason for the difference between the results in the two salmon studies described above. The protein levels in the diets used in the study of Abboudi et al. (2006b) ranged from 14 to 54%, whereas the dietary protein level used in the trial of Helland et al. (2006) was 54%.

To accurately determine the AA requirements for maintenance and growth of fish, the effects of both biotic and abiotic factors need to be evaluated. Fish size, stage of growth and genetics, diet ingredients and composition, and the environment are all assumed to be important in influencing these requirements. It is clear from this review of AA requirements that much research is necessary to generate data that can be used in modelling to predict the AA levels required in diets for fast-growing fish. In this context, the RRL-method is very interesting since it predicts both the requirements for maintenance and growth above maintenance for all the essential amino acids in one trial and, at the same time, permits an evaluation of the interaction between energy and amino acid metabolism.

#### **3.5. Dietary indispensable : nondispensable AA ratio**

NAA can be obtained from the diet or synthesized in the body. Diets lacking NAA, however, result in low growth of trout (Schuhmacher et al., 1995). These authors found that the growth rate of trout was equal when they were fed diets containing IAA:NAA ratios of

40:60 or 60:40. Nile tilapia (*Oreochromis niloticus*) had greater body and N growth when fed a diet with an IAA:NAA ratio of 40:60 compared with a diet with an IAA:NAA ratio of 25:75 (Mambrini and Kaushik, 1994). In a more detailed trial, Green et al. (2002) found that N retention in trout responded in a quadratic manner to an increasing dietary IAA:NAA ratio (from 23:77 to 66:34) and that 95% of the maximum N retention was found with a ratio of 46:54.

### **3.6. Amino acid antagonisms and toxicities**

In many animals, excess lysine impairs the utilization of arginine, whereas excess leucine reduces the utilization of the other two branched-chain AA, isoleucine and valine, even when they are not limiting in the diet. Reversal of these effects may be obtained by supplementation of the AAs that were negatively affected. The antagonism between lysine and arginine has not been demonstrated with growth studies in channel catfish, blue tilapia, rainbow trout, hybrid striped bass or yellow perch (reviewed by Wilson, 2002). In chicks fed excess lysine, an enhanced activity of arginase may occur, resulting in increased catabolism of arginine (Austic, 1986). In salmonids, some evidence indicates metabolic changes when excess levels of lysine or arginine are fed.

Excess branched-chain amino acids may reduce the brain pool of other amino acids, particularly phenylalanine and tryptophan, which are the precursors of the neurotransmitters (D'Mello, 1994). This may be the reason for the adverse effect of excess leucine on food intake in fish, as often seen when corn gluten is used in the diet. As reviewed by Wilson (2002), the interactions among the branched-chain AA seem to be different in the various species of fish.

### **3.7. Alternative protein sources**

Due to the increased demand in aquaculture for the restricted supplies of fish meal, plant feedstuffs are being increasingly used in diet production. An excellent overview of the utilization of plant products in diets for fish has recently been published (Gatlin et al., 2007). Important aspects from that review in relation to the present topic are included here.

Fish meal is an excellent dietary ingredient for aquafeeds because of its high protein content, balanced AA profile, high digestibility, lack of anti-nutrients and availability. Plant protein sources are all at some disadvantage to fish meal for their use in aquafeeds. As stated by Gatlin et al. (2007), the nutritional characteristics of candidate ingredients must include “low levels of fibre, starch, especially non-soluble carbohydrates and anti-nutrients, plus have

a relatively high protein content, favourable amino acid profile, high nutrient digestibility and reasonable palatability.”

The AA profile of diets can be adjusted by combining different plant sources, such as soybean meal and corn gluten meal for balancing the lysine content (Table 3.5), and/or supplementing with crystalline AA. Some plant products, however, have anti-nutritional factors that may or may not be possible to remove through processing. Much research has been done on the use of soybean meal in fish diets, but it is still not clear why salmonids have limited tolerance to this ingredient. As reviewed by Gatlin et al. (2007), impaired utilization of soybean meal in fish diets may be related to protein toxicity, as well as an immunological intolerance, exhibited as skin lesions, gastrointestinal tract alterations and excess mucus excretion in the faeces. Lectins, anti-nutritional factors present in soybean meal or other legumes, may bind with the intestinal wall, interfere with nutrient absorption and precipitate immune reactions causing cell death.

Although peas and lupins have high potential for use in aquafeed, as their protein digestibility is high, the low energy digestibility as a result of the high carbohydrate content is a negative factor.

Concerning the supplementation of crystalline AA to diets containing imbalanced AA profiles, a preliminary report indicates that the source of plant protein may affect the retention efficiency of supplemented lysine in diets for rainbow trout (Tran et al., 2006). The lysine retention efficiency in a diet containing wheat gluten was significantly higher than that in a diet containing both wheat gluten and sesame oil cake, or a diet containing maize gluten.

Table 3.5. Crude protein concentration (%) and amino acid profile [% of crude protein (CP)] in various protein sources<sup>1,2</sup>

	Fish meal, Norway	Casein	Gelatine	Wheat gluten meal	Corn gluten meal, 60% CP	Soybean meal, 48% CP	Lupin seed, Australia	Field peas
Crude protein	70.5	87.2	74.2	75.9	61.1	47.6	31.0	21.7
Lysine	7.4	8.0	4.3	1.5	1.6	6.1	4.6	7.1
Methionine	2.7	2.9	1.0	1.5	2.4	1.3	0.6	0.9
Cystine	0.9	0.5	0.3	<b>2.1</b>	1.7	1.5	1.3	1.4
Met+Cys	3.6	3.4	1.3	3.5	4.1	2.8	1.9	2.3
Threonine	4.1	4.3	2.2	2.4	3.3	3.9	3.4	3.7
Tryptophan	1.0	1.3	0.2	0.9	0.5	1.4	0.8	0.9
Arginine	6.1	3.5	7.9	3.3	3.2	7.3	<b>10.6</b>	8.4
Isoleucine	4.0	5.2	1.7	3.5	3.9	4.5	4.0	4.1
Leucine	7.1	9.5	3.8	6.7	<b>16.1</b>	7.6	6.6	7.1
Valine	4.8	6.6	2.8	3.8	4.5	4.7	3.9	4.6
Histidine	2.2	3.0	1.9	2.1	2.1	2.7	2.7	2.5
Phenylalanine	3.9	5.1	2.5	5.0	6.2	5.0	3.8	4.8
Tyrosine						3.2	5.8	3.7

<sup>1</sup>From AminoDat, v. 3.0, Degussa AG, Hanau-Wolfgang, Germany.

<sup>2</sup>Compared with fish meal, amino acids with low concentrations are shown in italics and high concentrations in bold.

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## **4. Fatty acids and lipids**

*Bente Ruyter and Bente E. Torstensen*

### **4.1. Introduction**

The fatty acid composition of most fish species in the wild is characterised by high concentrations of the n-3 fatty acids 20:5 and 22:6, and low concentrations of the fatty acids of the n-6 series (Ackman 1982, Robinson, 1992, Henderson and Tocher 1987). The proportion of n-3 polyunsaturated fatty acids (PUFA) relative to the proportion of n-6 fatty acids in fish is naturally much higher than in land mammals, where n-6 fatty acids, particularly 18:2 n-6 dominate (Bourre et al., 1990). This difference may be due to different fatty acid composition in the respective diets. Humans consume large quantities of saturated fatty acids from ruminants and 18:2 n-6 from plant seed oils, whereas fish consume marine food sources rich in the very long chain n-3 PUFAs, 20:5 n-3 and 22:6 n-3 (reviewed by Sargent 1997).

The fatty acid composition of body lipids, however, is not the same as that of the diet. Regardless of whether they are assimilated from the diet, or formed endogenously, fatty acids can be subjected to the various processes outlined in Figure 1 (Henderson 1996). They can, for example, be esterified into phospholipids, the structural lipid of biomembranes, or they may be incorporated into triacylglycerols, the neutral reserve lipid. The degradation of fatty acids via  $\beta$ -oxidation can also be used to provide energy. In addition, fatty acids can also be subjected to various modification processes such as elongation or desaturation. Polyunsaturated fatty acids can also be converted to eicosanoids.

### **4.2. Essential fatty acids**

Lipid requirements of fish came under investigation in the 1960s, following the growth in the aquaculture industry. Although n-3 polyunsaturated fatty acids are abundant in natural fish oils, early fish nutritionists assumed that the essential fatty acid requirement of fish was the same as that of land animals. They used plant oils rich in 18:2 n-6 as dietary lipids for farmed trout (Owen et al., 1972). They found that the use of plant oils rich in n-6 polyunsaturated fatty acids, however, produced a number of adverse effects on the growth and

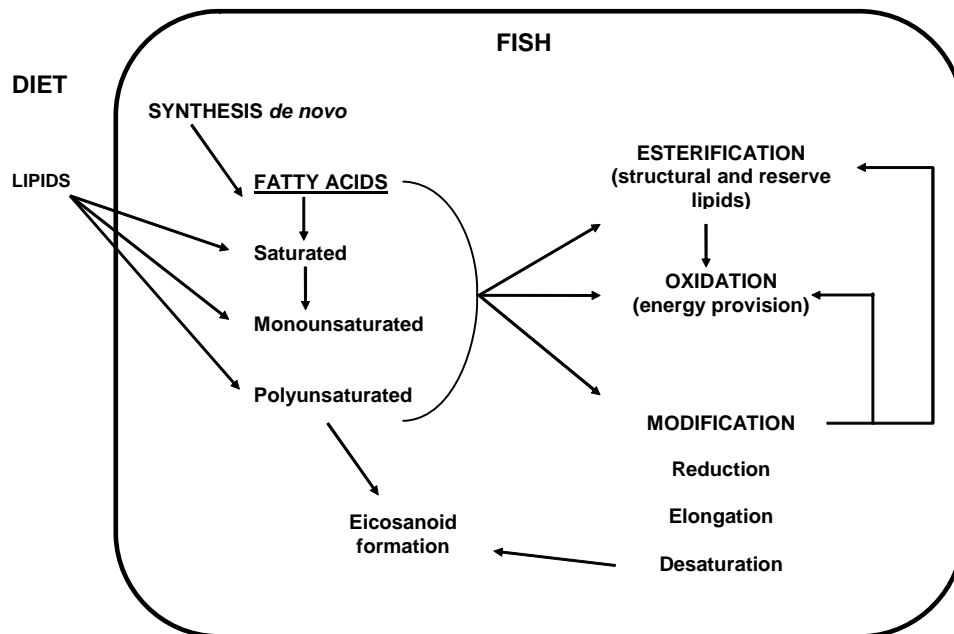


Figure 4.1. Schematic representation of fatty acid metabolism in freshwater fish (Henderson 1996).

behaviour of fish. Fingerling rainbow trout fed corn oil or soybean oil for 12 weeks grew poorly and had only 75% survival. They showed signs of essential fatty acid deficiency, such as fin erosion, swollen pale livers, heart myopathy and shock syndrome. Trout fed a diet which included n-3 fatty acids grew faster, with better feed efficiency and lower mortality (Lee et al., 1967). Similar studies were later performed on Atlantic salmon (Ruyter et al., 2000a,b), where fry were fed for 4 months on semi-synthetic diets containing fatty acid methyl esters of either 18:2 n-6, 18:3 n-3 or a mixture of equal amounts of 20:5 n-3 and 22:6 n-3. Increasing levels of dietary n-3 fatty acids up to 1% gave faster growth rates, and fish fed the mixture of 20:5 n-3 and 22:6 n-3 grew faster than fish fed only 18:3 n-3. Fish fed only 18:2 n-6 had the lowest growth rate and highest mortality. All studies so far have shown that fish require the n-3 PUFAs in their diet, but it is still not known whether the n-6 PUFAs are essential in some salmonids like rainbow trout (Castell et al., 1972a, b), cherry salmon (Thongrod et al., 1990) and Atlantic salmon (Ruyter et al. 2000 a,b). If so, they are probably needed only in small amounts, since pure 18:2 n-6 deficiency (with n-3 PUFA available in the diet) has little effect on growth in these fish species (Sargent et al., 1995; Ruyter et al., 2000a). It is shown though, that chum salmon (Takeuchi et al., 1979) and coho salmon (Yu

and Sinnhuber 1979) require dietary supplementation of n-6 fatty acids in order to achieve good growth.

The early investigations about the relative importance of n-3 and n-6 fatty acids were conducted without much consideration of the chain length of the fatty acids in the dietary lipid. Once it was clearly established that n-3 fatty acids were required, research emphasis shifted to longer chain polyunsaturated fatty acids and the processes of desaturation and elongation of fatty acids. Arachidonate (20:4 n-6) and docosahexaenoate (22:6 n-3) were classified as essential fatty acids (EFAs). This classification was made even though these fatty acids could normally be synthesised from 18:2 n-6 and 18:3 n-3. These longer chain polyunsaturated fatty acids became interesting particularly after it was shown that they are important membrane constituents of fish, and that metabolites from 20:4 n-6 and 20:5 n-3 are prostaglandins. These prostaglandins have more or less the same important biological functions in fish as in mammals (Bell et al., 1991b). Generally, prostaglandins and leukotrienes constitute a group of extracellular mediator molecules that are part of an organism's defence system. Prostaglandins and leukotrienes are formed during the inflammatory process, and if the inflammation is caused by invading bacteria, the formation of prostaglandins and leukotrienes will stimulate macrophages and other leukocytes to begin the process of destroying the bacteria. Prostaglandins can also act as hormones (reviewed by Hansen 1994).

#### **4.3. Desaturation, elongation and $\beta$ -oxidation of fatty acids**

The ability of tissues or organs to synthesise carbon-20 and carbon-22 PUFAs from carbon-18 fatty acids depends on the presence of active desaturase and elongase enzymes. The relative activities of these enzymes determine the relative amounts of products formed.

Three types of desaturases are distinguished in animals based on their desaturation site in the fatty acids:  $\Delta 9$ ,  $\Delta 6$  and  $\Delta 5$ , respectively (Wakil et al., 1961, Nugteren et al., 1962, Jeffcoat et al., 1979, Sprecher 1981) (Figure 4.2).  $\Delta 9$ -desaturase acts only on saturated fatty acids which it desaturates specifically between carbon-9 and carbon-10. 18:0 is the main substrate for this enzyme (Blomfield and Block 1960, Wakil 1961).  $\Delta 6$ -desaturase introduces an additional ethylene bond between carbon-6 and carbon-7 of poly- or mono-unsaturated fatty acids. Three fatty acids, 18:1 n-9, 18:2 n-6 and 18:3 n-3, compete for the same  $\Delta 6$ -desaturase (Brenner 1977). Which fatty acid becomes desaturated depends on affinities of the fatty acids for  $\Delta 6$ -desaturase, and on their relative proportions. Nevertheless, the affinity for the enzyme varies in the order 18:3 > 18:2 > 18:1, which illustrates the importance of a

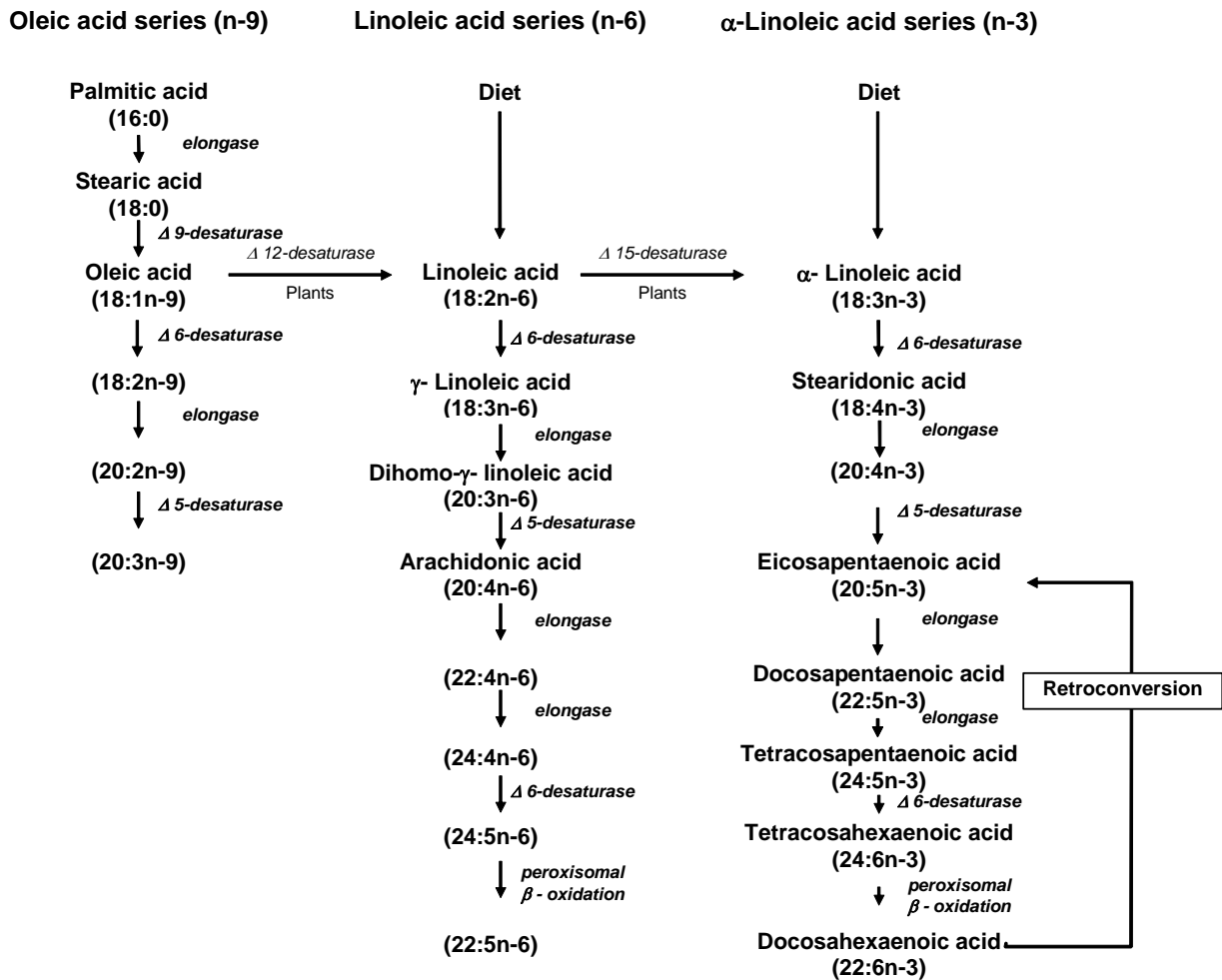


Figure 4.2. A simplified scheme showing the biosynthesis of mono- and polyunsaturated fatty acids, indicating pathways of elongation, desaturation and retroconversion (Modified from Pan et al., 1994).

balanced proportion of these three fatty acids in the feed. Only then can the essential carbon-20 and carbon-22 PUFAs of the n-6 and n-3 families be synthesised (Cook and Emken 1990).

The factors which influence desaturase activity can be classified into nutritional factors, environmental factors (temperature), hormonal factors and physiological factors (such as age). These factors are not independent and, in some cases, the effects of nutritional factors may be explained by changes in the hormonal status.

Atlantic salmon is an anadromous fish which begins life in fresh water and migrates to the sea after 1-2 years. It returns to fresh water to spawn after spending 1-3 years at sea. Atlantic salmon may not be able to desaturate and elongate 18-carbon EFAs during smoltification (Ackman 1986), but this inability is not apparent during the parr stage in fresh

water and the post-smolt seawater phase of the salmon life cycle (Bell et al., 1989). Atlantic salmon post-smolts in sea water are able to convert 18:2 n-6 and 18:3 n-3 to their higher homologs (Bell *et. al.*, 1993, 1997). High levels of DHA in the diets and in the different tissue lipids may, however, partly inhibit the conversion of 18:3 n-3 to EPA and DHA, which means that a fish that has a relatively low tissue concentration of DHA (like a parr in fresh water may show higher capacity to convert 18:3 n-3 to DHA than a post-smolt in sea water with higher tissue concentrations of DHA (Bell et al., 1993). Furthermore, 22:6 n-3 is shown to inhibit the  $\Delta$ 6-desaturase in rainbow trout, and thereby preventing the desaturation of both 18:2 n-6 and 18:3 n-3 (Leger et al., 1981).

The efficiency of the conversion of carbon-18 PUFAs to carbon-20 and carbon-22 PUFAs may differ between freshwater species as shown for different mammalian species. Rats, for instance, have a very efficient conversion, whereas man has a very slow rate (Blond et al., 1981). Species that have a relative low efficiency of the conversion of 18:3 n-3 to EPA and DHA, must be supplemented with carbon-20 and carbon-22 PUFAs in their diets. In Atlantic salmon for instance (Ruyter et al., 2000), very low dietary doses of 18:3 n-3 resulted in a moderate increase in the percentage of 22:6 n-3 in the liver. Higher dietary doses of 18:3 n-3 resulted mainly in accumulation of 20:5 n-3, and did not result in a further increase in the percentage of 22:6 n-3. Similarly, 22:5 n-6 was not detected in fish groups fed increasing dietary levels of 18:2 n-6, even though an accumulation of 20:4 n-6 was observed. The low conversion to carbon-22 fatty acids described above may be due to competition between 18:3 n-3 and 24:5 n-3 for the  $\Delta$ 6-desaturase. High dietary levels of carbon-18 PUFAs, as for instance from linseed oil, may inhibit the desaturation of carbon-24 fatty acids to 22:6 n-3. This means that Atlantic salmon cannot be fed a diet with 18:3 n-3 as the only EFA source (as from instance from linseed oil), but need also a dietary supplementation of the longer chain EPA and DHA.

Most marine fish lack or have a very low activity of  $\Delta$ 5-desaturase, and so they need a supply of carbon-20 and carbon-22 PUFAs in the diet. In contrast, the n-3 EFA requirement of pure freshwater fish can generally be met by 18:3 n-3 (Sargent 1997). Since 20:5 n-3 and 22:6 n-3 are the biologically active forms of n-3 EFA in vertebrates, we can conclude that 18:3 n-3 can be converted to 20:5 n-3 and 22:6 n-3 in freshwater fish, but not in marine fish. This is not, however, a general rule because at least one freshwater species, mature pike (*Essox lucius*), is known to be incapable of converting 18:3 n-3 to 22:6 n-3 (Henderson et al., 1995). It was recently reported that Atlantic cod express the  $\Delta$ 6-desaturase gene (Tocher et

al., 2007). The enzyme was barely functional, however, and the authors concluded that further studies are needed in order to understand this problem.

$\beta$ -oxidation of EFAs has not been as extensively investigated as their desaturation and chain-elongation pathway. Even though salmonids can oxidise EFAs, however, Sargent et al., (1989) postulated that they are only used as energy sources in fish when they are abundant and when their role as EFAs has been satisfied [for further details about how different dietary oils affect  $\beta$ -oxidation of EFAs, see sections about oils sources (4.7) and body composition (4.9)].

#### **4.4. Dietary requirement for n-3 and n-6 PUFAs**

The precise nutritional requirements of any animal species for n-6 and n-3 polyunsaturated fatty acids are not known with certainty (reviewed by Bezard et al., 1994). Moreover, the situation with fish is particularly complex since there is a wide variety of species and a wide range of natural habitats and diets. Not only do we need to determine the absolute requirements of the total amount of each series of PUFAs for a given species, and as an extension of this, to determine the optimal dietary n-6 to n-3 ratio. We also need to know the optimal balance between individual carbon-18, carbon-20 and carbon-22 fatty acids within a given PUFA series. In addition, the methods used for determination of the EFA requirement of a species are seldom optimal. The use of semi-synthetic diets with unbalanced lipid composition and the state of the fatty acid reserves in the body of the fish may influence the results.

Rainbow trout (Castell et al., 1972c, Yu and Sinnhuber, 1972, Rinchard et al., 2007) and Arctic charr (Yang and Dick 1993) require from 0.5 to 1% of n-3 PUFA in the feed to attain good growth, whereas dietary inclusion of 18:2 n-6 does not improve growth. This is different from coho salmon (Yu and Sinnhuber 1979) and chum salmon (Takeuchi et al., 1979) where 18:2 n-6 enhances growth significantly.

Atlantic salmon seemed to grow faster and have lower mortality with substantially lower dietary doses of 20:5 n-3 and 22:6 n-3 than with 18:3 n-3 (Ruyter et al., 2000a). The same effects are seen in rainbow trout, where 20:5 n-3 and 22:6 n-3 had higher EFA efficiency than did 18:3 n-3 (Takeuchi and Watanabe, 1979). Atlantic salmon fed diets supplemented with only 18:2 n-6 had lower growth rates than fish fed n-3 supplemented diets (Ruyter et al., 2000a). Thus, Atlantic salmon fry seem to have an EFA requirement similar to that of rainbow trout (Castell et al., 1972c, Yu and Sinnhuber, 1972) and Arctic charr (Yang and Dick 1993).

The requirement of Atlantic salmon for n-3 EFAs cannot, however, be directly transferred to fish in production. Firstly, the diet used in the EFA-deficient trials were semi-synthetic (Ruyter et al., 2000a,b), which may result in slower growth rates than commercial diets (Lochman and Gatlin 1993). Secondly, the EFA requirement probably changes during the life cycle of Atlantic salmon, and may also be influenced by other dietary fatty acids. Thirdly, the requirement may increase with increasing dietary lipid levels. Takeuchi and Watanabe (1977) reported that the 18:3 n-3 requirements of trout increased with increasing dietary lipid levels. Thus, the EFA requirement of farmed salmon, fed diets containing 30-40% fat, is probably different from that for salmon fed an 8% fat diet, as used in the trials to establish the EFA requirement (Ruyter et al., 2000 a,b). Fourthly, the ratio between the different n-6 and n-3 PUFAs is probably important in Atlantic salmon, as it is in rainbow trout (Bell et al., 1991b). In this study, rainbow trout were fed a sunflower oil diet which contained a considerable excess of n-6 polyunsaturated fatty acids compared to n-3 fatty acids. So although the diet was not deficient in n-3 PUFAs in an absolute sense, the ratio of n-3 to n-6 was low. Fish fed the sunflower oil diet had levels of 20:3 n-6 and 20:4 n-6 which were higher, and a 20:5 n-3 level which was lower, than the corresponding levels in fish fed a fish oil diet. Fish fed the sunflower oil diet developed severe heart lesions, and were also susceptible to transportation shock resulting in 30% mortality. This phenomenon is very similar to the fainting or shock syndrome described by Castell et al. (1972b), in trout fed a diet deficient in n-3 PUFA. A number of later studies, however, have shown no negative effects with 75-100% replacement of fish oil with a plant oil mix, through the whole production cycle (reviewed in section 4.7).

The activities of desaturases and elongases in salmonids are also affected by water temperature (Ruyter et al., 2003; Tocher et al., 2004). Fatty acids in phospholipids (PLs) are less saturated in fish exposed to low temperatures than in fish exposed to high temperatures, such that the membranes keep their fluidity (Sellner and Hazel, 1982; Cossins, 1983, 1987). This phenomenon is known as homeoviscous adaptation, and it ensures that the function of the membranes is unaltered during changes in water temperature (Sinesky, 1974). The increase in the degree of FA unsaturation at low temperature may be due to changes in both the desaturase and elongase capacities (Schuenke and Wodtke, 1983; Hagar and Hazel, 1985; Trueman et al., 2000; Ruyter et al., 2003).



#### **4.5. Concluding remarks to EFA requirement**

Atlantic salmon and rainbow trout fry require at least of 0.5 to 1% n-3 PUFA in the feed to attain good growth. These requirement results cannot, however, be directly transferred to fish in general. One must consider that the EFA requirement may change during the life cycle, as well as with the fatty acid composition and fat percentage of the diet. Atlantic salmon and rainbow trout are able to convert dietary 18:3 n-3 and 18:2 n-6 to C-20 and C-22 PUFAs, thus indicating that only the 18-carbon forms of these fatty acids are essential in the diet. High dietary levels of 18:3 n-3 inhibited, however, its conversion to 22:6 n-3, and salmonids may therefore, become deficient in this essential, long chain PUFA if 18:3 n-3 is the only n-3 PUFA supplied in the diet. Atlantic salmon seem to need a dietary supplementation of the longer chain n-3 PUFAs, EPA and DHA, in addition to 18:3 n-3. The ratio between different fatty acids may be more important than the actual level of each fatty acid, in securing the production of longer chain fatty acids. Future work on the aspects of Atlantic salmon dietary requirement for certain fatty acids must focus on finding the most optimal ratio between the different EFAs of different chain lengths in today's high fat diets.

It is important to define some early symptoms of EFA deficiency, since sub-deficiency states can result in increased health risks, even when not manifested by overt symptoms. This is particularly important under suboptimal conditions, like low water temperatures, stress, infections etc.

#### **4.6. Fatty acid peroxidation**

Fish are generally regarded as PUFA-rich organisms providing a virtually unique source of the very long chain n-3 fatty acids EPA and DHA. These EFAs, which are vital constituents of cell membrane structure and function, are very susceptible to attack by oxygen and other organic radicals. Resulting damage to PUFA in membrane phospholipids can have damaging consequences for cell membrane structure and fluidity (muscle quality), with potential pathological effects on cells and tissues (Sies et al., 1991). An efficient antioxidant protection system is essential for the physiological well-being of the animals. When parts of the antioxidant protective mechanism are placed under stress, however, perhaps due to dietary deficiencies of essential antioxidant nutrients or intake of oxidized (rancid) foodstuffs, then pathological consequences can result. Reduced growth, loss of appetite, decreased feed efficiency, reduced pigmentation and increased mortality have all been reported in Atlantic salmon (Ketola et al., 1989) and rainbow trout (Cowey et al., 1984) when fed oxidised lipids.

A number of histological lesions due to lipid peroxidation have been identified, including myopathy of skeletal muscle (Cowey et al., 1984). We have recently shown that very high levels of EPA and DHA in salmon diets may result in intracellular peroxidation inducing cell death both in liver, muscle and adipose tissue (unpublished results).

#### **4.7. Oil sources – Effects on body fatty acid composition**

Atlantic salmon and rainbow trout in aquaculture are increasingly being fed diets based on plant oils (PO) as a replacement for fish oil. In contrast to fish oil, PO do not contain fatty acids longer than 18 carbons and three double bonds and thus, lack the long chain n-3 PUFAs such as EPA (20:5 n-3) and DHA (22:6 n-3). PO for fish should contain a low level of 18:2 n-6. Furthermore, a high level of 18:1 n-9 is desirable, whereas the level of 18:3 n-3 should possibly be moderate. These criteria are currently best met by rapeseed oil, although even this oil contains 16-25% 18:2 n-6.

A number of studies have shown that complete or partial replacement of fish oil with a single PO, such as rapeseed oil, palm oil, linseed oil, or soybean oil, in parts of the salmon production cycle, and 75-100% replacement of fish oil with a PO mix, through the whole production cycle, does not affect growth negatively, but does affect the fatty acid composition of the edible portion (Bell et al., 2002; Bell et al., 2001; Bell et al., 2003a; Bell et al., 2003b; Regost et al., 2004; Rosenlund et al., 2001; Torstensen, 2000; Torstensen et al., 2005; Torstensen et al., 2004b; Waagbø et al., 1991; Waagbø et al., 1993). Furthermore, dietary fatty acid composition is also mirrored by the fish's organs and lipid stores (Bell et al., 2001; Bell et al., 2003b; Caballero et al., 2002; Nanton et al., 2007; Olsen et al., 1999; Stubhaug et al., 2007; Thomassen & Røsjø, 1989; Tocher et al., 2003; Torstensen et al., 2005; Torstensen et al., 2004a; Torstensen et al., 2004b; Torstensen et al., 2000; Waagbø et al., 1991). Thus, replacement of fish oil with PO and thereby increasing dietary 18:2 n-6, 18:1 n-9 and 18:3 n-3 and the marine n-3 fatty acids EPA and DHA, will be reflected in the organs and flesh of the fish. The magnitude, however, of fatty acid changes depends on the type of tissue (Bell et al., 2001; Bell et al., 2003a; Torstensen et al., 2004a; Grisdale Helland et al., 2002; Moya-Falcon et al., 2005) and the amount of phospholipids relative to neutral lipids in the tissue, since neutral lipids are reported to be more influenced by dietary fatty acid composition than polar lipids (Brodtkorb et al., 1997; Olsen & Henderson, 1997; Moya Falcon et al., 2005; Ruyter et al., 2006).

Fish fatty acid composition also depends on factors other than dietary fatty acid composition. Digestibility (Torstensen et al., 2000; Sigurgisladottir et al., 1992), transport and

uptake, elongation and desaturation processes (Bell et al., 2001; Bell et al., 2002) and  $\beta$ -oxidation of fatty acids (Frøyland et al., 2000; Torstensen et al., 2000) will affect the fatty acid composition in the membranes (polar lipids) and storage depots (neutral lipids).

Lipid digestibility in rainbow trout is fairly constant ( $\approx 90\%$ ) for lipid sources with melting points  $<10^\circ\text{C}$ , but is dramatically reduced for sources with higher melting points (Austreng et al. 1979). A practical consequence is that fats with low melting points (oils) are preferred in diets for salmonids and probably other coldwater fishes as well. This favours PO from oilseeds, which usually have melting points well below  $0^\circ\text{C}$ .

Studies of  $\beta$ -oxidation in fish suggest that there exist substrate preferences for saturated and monounsaturated fatty acids over polyunsaturated fatty acids (Kiessling & Kiessling, 1993; Henderson, 1996). A recent report from Stubhaug et al. (2007) showed that Atlantic salmon fed low levels of marine n-3 fatty acids selectively stored these in the tissue. Fish-oil fed fish stored about 20% EPA and 30% DHA, whereas PO-fed fish stored 70% EPA and 80% DHA during the fast growth period in sea water (Stubhaug et al., 2007). Thus, these results suggest a switch in fatty acid substrate used for  $\beta$ -oxidation when dietary levels of EPA and DHA are low; these PUFA are preferentially stored in membranes instead of being used as an energy substrate in PO-fed fish.

By introducing a finishing-diet period when fish are being fed 100% FO diet, the levels of marine n-3 PUFA increase whereas the typical PO fatty acids decrease to levels comparable with fish oil fed fish. Studies have shown that a period long enough for the fish to double its weight is necessary for obtaining fatty acid compositions comparable to fish oil fed fish (Bell et al., 2003a; Bell et al., 2003b; Torstensen et al., 2004b; Torstensen et al., 2005).

#### **4.8. Oil sources and effects on colour**

Flesh pigmentation is an important factor in perception of flesh quality in salmonids (Bell et al., 1998; Refsgaard et al., 1998). The flesh levels of lipid soluble nutrients, such as astaxanthin are dependent on the dietary composition (Lie, 2001), and it has been shown that both total lipid level and type of oil can affect carotenoid absorption in rodents (Clark & Furr, 2001; Clark et al., 2000) and colour characteristics in raw and smoked Atlantic salmon fillets (Regost et al., 2004). The majority of reports show no effects on flesh astaxanthin levels of using up to 100% PO (Bell et al., 2001; Bell et al., 2002; Torstensen et al., 2004b; Torstensen et al., 2005), however in none of these studies was fish oil replaced with soybean oil. As

reported by Regost et al (2004), dietary soybean oil resulted in reduced flesh astaxanthin, cantaxanthin and visual colour, especially after four months frozen storage.

#### 4.9. Effects of high fat diets on body lipid composition



Depending on the species, fish are able to accumulate fat in different tissues such as liver for Atlantic cod and abdomen and flesh for Atlantic salmon (see figure to the left) and rainbow trout. Fat stores may supply energy in periods of low feed availability and more specifically for use in periods of particularly high energy demand such as reproduction and

smoltification. Since most fish species have a limited capacity to utilize carbohydrates, a common way of reducing feed cost is to replace as much protein as possible with fat. Increased energy levels improve growth and feed utilization in most fish species. Increased dietary energy levels though, also increase fat deposition in the fish's fat storage organs and particularly increased visceral fat deposition reduces harvest yields (Hillestad et al., 1998). By preventing excessive fat deposition in cultivated fish, sustainability is strengthened, more marine fat is available for food production, feed efficiency is increased, and cost of fish production is reduced.

The use of high-lipid feed for cultured fish may also affect fish flesh quality by increasing the percentage of lipids stored in the edible muscle (Watanabe, 1982; Hemre & Sandnes, 1999; Arzel et al., 1993; Arzel et al., 1994; Bendiksen et al., 2003). Generally, increased dietary lipid also results in increased muscle lipid levels, however this is reported to level off so that increasing dietary lipid from 38% to 47% did not increase muscle total lipid levels beyond 16% (Hemre & Sandnes, 1999). Contradicting results are reported on the impact of dietary fatty acid composition on body composition. In Atlantic salmon, changing dietary fatty acid composition by replacing fish oil with a PO blend during both fresh- and seawater stages did not change the body lipid stores in any major way (Nanton et al., 2007). There was a trend towards decreased triacylglycerol (TAG)/PL ratio, however, in both visceral lipid stores and myosepta in 100% PO-fed Atlantic salmon. Muscle lipid and protein levels have been reported not to be affected by dietary fatty acid composition (Torstensen et al., 2005; Bendiksen et al., 2003).

Reports on the effects of changes in the dietary fatty acid composition through replacement of fish oil with PO show contradictory results regarding hepatic lipid storage in fish. By replacing fish oil with oleic acid enriched-sunflower oil and rapeseed oil, a slight increase in hepatic total lipid storage was reported (Torstensen et al., 2000; Bell et al., 2001), whereas no effects on hepatic lipid were found when replacing with palm oil (Bell et al., 2002; Torstensen et al., 2000) or a 1:1 rapeseed oil: linseed oil mix (Tocher et al., 2001). Further, Ruyter and co-workers (2006) have reported increased liver lipid when replacing fish oil with 100% soybean oil at 5°C, but not at 12°C. A long term feeding experiment reported increased hepatic lipid stores, especially at low water temperatures, in Atlantic salmon fed a PO blend (Jordal et al., 2007). Increased liver TAG stores are reported as one of the indicators of essential fatty acid deficiency in salmonids (Castell et al., 1972b; Takeuchi et al., 1979). Increased n-3 PUFA requirement during very high growth and/or low water temperatures in Atlantic salmon may be indicated by the increased liver TAG results when decreasing n-3/n-6 ratio and decreasing dietary marine n-3 fatty acids, however this needs further evaluation.

The digestibility of fatty acids has been reported to be affected by dietary fatty acid composition; specifically the amount of dietary saturated fatty acids influences the digestibility of lipids, especially at low water temperatures (Caballero et al., 2002; Ng et al., 2004; Olsen et al., 1999; Torstensen et al., 2000). As a consequence, at low water temperatures, the replacement of capelin oil with rapeseed oil leads to less saturated fatty acids in the diet, and increased monounsaturated fatty acids increase lipid digestibility, in both rainbow trout and Atlantic salmon (Caballero et al., 2002; Karalazos et al., 2007). Thus, changes observed in total liver lipids, especially at low water temperatures, may be related to increased lipid uptake from the diet. Also, increased protein retention has been reported at low temperatures when replacing dietary fish oil with PO (Bendiksen et al., 2003; Torstensen et al., 2005). If the increased protein retention was due to increased fatty acid digestibility or increased fatty acid  $\beta$ -oxidation capacity at low water temperatures remains to be elucidated. In conclusion, the impacts of dietary fatty acid composition on total body composition seem to be highly influenced by water temperature, both for Atlantic salmon and rainbow trout.

#### **4.10. Fish health and welfare**

In the growing fish farming industry there are always new challenges concerning fish health and fish diseases. Still, diseases and health problems lead to substantial losses every year. Fish farming has changed quickly, and fish growth has increased significantly throughout the last decade. This always brings new aspects of health and welfare for the fish,

where several of the diseases and production related disorders are proved to be preventable by correct nutrition.

Dietary fat and polyunsaturated fatty acids are important regulators of numerous cellular functions, including those related to inflammation and immunity. Although little is known for fish, there is convincing evidence from studies with mammals that both the type and level of fat in the diet have major impacts on several aspects of immune function and heart health. A large number of experiments have shown that a relatively high intake of high fat plant-oil diets under normal production conditions does not affect the growth and feed utilisation of the salmon to any notable degree. It still remains though, to find out how much and how long the fish can be fed different types of plant oils and high fat diets without it affecting fish health negatively, especially under suboptimal conditions such as fluctuating farming conditions, stress and lowered health condition. Combined with a low level of exercise, fish in aquaculture consume high energy diets, resulting in large lipid stores especially around the viscera. Importantly, high inclusion levels of plant ingredients in fish diets lead to reduced levels of the health-promoting, marine n-3 fatty acids. Together, these factors may push the system towards a state similar to metabolic syndrome in humans and the lifestyle diseases that follow. Epidemiologic human studies have shown that higher aerobic fitness is associated with lower incidence of inflammatory illnesses (Milne et al., 2008). In aquaculture, fish are kept in net pens during the grow-out phase where the degree of exercise depends on the local currents at the fish farm and, in most cases, these are low. This phenomenon may lead to low cardio-respiratory fitness and eventually lead to development of obesity related metabolic disorders. High mortality in slaughter size Atlantic salmon, due to handling stress, has in fact been reported as frequently occurring in aquaculture farms (Pope et al, 2004).

Further, obesity has been associated with reduced antioxidant defense mechanisms in humans, including decreased antioxidant enzyme activity, giving rise to increased oxidative stress in the body (reviewed by Milne et al., 2008). Although not known, a reduced antioxidant defense system in obese fish may have major implications also for the ability to recover and survive acute stresses such as handling or crowding. Stress and obesity are linked in several ways, chronic stress with hyper-activation of the hypothalamic-pituitary-adrenal (HPA) axis favors accumulation of visceral fat and vice versa (Balkan et al., 1993). Obese rats demonstrated an elevated response to mild stress compared to lean rats measured as plasma epinephrine and cortisol (Kyrou and Tsigos, 2007). Similarly in fish, the energy status significantly affected the cod's ability to recover from handling stress (Hemre et al., 1991).

A high fat level in the blood has proved to be a serious risk factor for the development of life style diseases, as for example heart diseases and diabetes II in humans. Several studies in humans have shown that dietary EPA and DHA decrease plasma TAG (Harris et al., 1983; Nestel, 1990) and protect against coronary heart diseases (Bang et al., 1971; Seierstad et al., 2005b). If this also is a risk factor in fish, is still uncertain, but it has been shown by using *in vitro* experiments that the lipoprotein secretion from liver cells is significantly higher in salmon fed with PO than salmon fed with fish oil (Vegusdal et al., 2005). Some of the most frequently used plant oils in salmon diets contain high levels of 18:1n-9 which has been shown in different cell systems (Ranheim et al., 1994; Vegusdal et al., 2005) and rats (Halvorsen et al., 2001) to affect liver lipid and lipoprotein metabolism. Further, both EPA and DHA are reported to affect hepatic triacylglycerol (TAG) metabolism and  $\beta$ -oxidation capacity (Berge et al., 1999; Madsen et al., 1999; Nossen et al., 1986; Willumsen et al., 1996) with especially EPA having a plasma lipid lowering effect in rats (Frøyland et al., 1997; Frøyland et al., 1996).

It is important to be conscious about possible heart health implications when increasing amounts of the fat in salmon feed are replaced with plant oils which have less of the healthy n-3 fatty acids. Studies on effects of reduced n-3 HUFA and increased n-6 PUFA on salmonid heart histology are, however, so far contradictory (Bell et al., 1993; Grisdale-Helland et al., 2002; Seierstad et al., 2005a) and predominantly show no negative effects of significantly reduced n-3 polyunsaturated fatty acids (PUFA) (EPA and DHA) in the diet (Seierstad et al., 2007). The dietary levels of phytosterols, however, provided from the plant oils used, were not considered in any of these studies. Phytosterols are reported to reduce intestinal cholesterol uptake, reduce plasma LDL-cholesterol and protect against cardiovascular disease in mammals (reviewed by Orzechowski et al., 2002). The amount and type of phytosterol varies depending on the type of plant oil and the degree of refinement (Weihrauch & Gardner, 1978), and to ensure that cardiovascular disease is not developed when different oils are used, the impact of dietary phytosterols needs to be evaluated in salmonids.

Examination of the literature shows that changing the concentration of dietary n-3 PUFA in fish feeds can have both beneficial and, in some instances, detrimental effects on disease resistance. When challenged with *Aeromonas salmonicida* and *Vibrio anguillarum* Atlantic salmon fed plant oils were less resistant to infection compared to fish oil fed fish (Thompson et al., 1996). In contrast, Erdal et al., (1991) found that increasing the amount of

dietary n-3 PUFA (by increasing 20:5 n-3 and 22:6 n-3) from 13 to 24% of total fatty acids actually had an immuno-suppressive effect on Atlantic salmon, and resulted in higher rates of mortality against *Yersinia ruckeri*. In line with results from Erdal et al., (1991), high n-3 PUFA has been reported to result in decreased response by the unspecific immune system in Atlantic salmon (Waagboe et al., 1993). In a study by Bransden et al. (2003) resistance to *V. anguillarum* was significantly impaired in salmon supplied feeds where fish oil was replaced by sunflower oil at different inclusion levels, although it remains unclear as to why some diets improved disease resistance in salmon, whereas others did not. This suggests an optimal dietary n-3/n-6 ratio probably exists for disease resistance, although not yet identified.

Head kidney tissue of fish has a high prevalence of macrophages and is hence, essential in the immune system of salmon. One mechanism by which macrophages remove invading microorganisms is through phagocytosis and subsequent presentation of antigens on major histocompatibility complex (MHC) molecules for other cells of the immune system. Macrophages also secrete interferons (IFNs) upon stimulation. Experiments with Atlantic salmon have shown that changes in the composition of fatty acids can affect the immune system through changes in the phagocytotic capacity of macrophages. These effects were especially clear at low water temperatures (5°C) (Gjøen et al., 2007). Another important macrophage function is to modulate the immune system by producing eicosanoids. Eicosanoids are a group of lipid mediators of inflammation that are derived mainly from arachidonic acid (20:4 n-6). After its release from membrane phospholipids by the action of enzymes like phospholipase A<sub>2</sub> (PLA<sub>2</sub>), it has been shown that dietary lipids may influence the eicosanoid production in both mammals (Brouard and Pascaud 1990) and fish (Bell et al., 1996; Gjøen et al., 2004). Furthermore, by feeding plant oils, the tissue n-6 levels increase and thus, possibly the amount of eicosanoids produced from arachidonic acid (ARA) compared to EPA increase (Gjøen et al., 2004; Gjøen et al., 2007). This is thought to have an impact on a number of responses such as stress response and smoltification (Bell et al., 1993; Bell et al., 1996; Bell et al., 1991a; Bell & Raynard, 1990; Bell et al., 1991b; Bell et al., 1992; Bell et al., 1997).

It has also been shown that fast growing salmon that had 75 to 100% plant oils (fish meal as protein source) in the feed, from start feeding and onwards, developed the eye disorder cataract two-fold greater compared to fish fed a diet containing 100% fish oil (Waagboe et al., in prep).



#### 4.11. Future Challenges within lipid nutrition of salmonids

- **Decrease obesity of cultured fish:**
  - It is necessary with increased knowledge about the mechanisms underlying obesity in fish in order to pave the way for new methods for obesity control and thus contribute to increase fish flesh quality and decrease the production losses due to fat offals.
  - It is necessary with increased knowledge about the impact of high lipid storage depots on fish health and stress tolerance.
  
- **Safe usage of plant ingredients in fish diets:**
  - When not only fish oil but also fish meal is replaced by plant oils and plant protein sources in combination, it is necessary with more knowledge on how these components in combination will affect the fish growth, health, metabolism and final quality.
  - The following questions need to be answered: What is the n-3 PUFA requirement for salmonids at different life stages and at different water temperatures? What is the impact of n-3/n-6 ratio on fish health, particularly under sub-optimal conditions?
  - The impact and function of bioactive components present in plant raw materials and the impact on fish metabolism and health need to be evaluated.
  - Studies on how to secure the nutritional quality of the fish, particularly related to the level of the healthy omega-3 fatty acids.

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## **5. Vitamins**

*Rune Waagbø*

### **5.1. Introduction**

Most vitamins defined in the literature have also been demonstrated to be essential for fish. A status of vitamin minimum requirements was published by National Research Council, USA (NRC 1993), and revisions were published by (Waagbø et al. 2001a; Halver 2002; Hardy 2002; Storebakken 2002). Changes in genetics, husbandry and management routines, and overall dietary composition, as well as increased feed utilization and use of novel feed ingredients in intensive fish farming has urged the need for revised recommendations on vitamin supplementation. We may assume that the minimum requirements, as well as situation dependent requirements have changed along with the other advances made in the aquaculture industry, including the development of modern diets. Micronutrient deficiencies are among the common category of diseases observed in aquaculture, and also the easiest to correct if they are identified (Hardy 2001).

In the present chapter, vitamin minimum requirements and recommendations are covered. The former expresses the vitamin level requirement needed to prevent overt signs of deficiency (NRC 1993), while the latter is the suggested vitamin level required for “optimum health and productivity” under practical conditions. Dietary vitamin recommendations are often several fold the minimum requirement, for safety. One has to remember that the minimum requirements often are established in juveniles and performed with suboptimal semi-synthetic diets (“pure ingredients”) that seldom supported optimal growth.

It is still natural to divide the vitamins into lipid soluble and water soluble vitamins, especially since these are inherent in the lipid rich and protein rich feed ingredients, respectively. It is important to consider both the vitamin contents and possible anti-nutrients and anti-metabolites in novel feed ingredients, as compared to the traditional marine feedstuffs, since these may interfere with the natural and supplemented vitamins in the feed (Table 5.1).



Table 5.1. Aspects to consider for vitamin requirements and recommendations for fish

Aspect	Cause
Processing and storage stability	Inherent chemical vitamin forms Additives with vitamins Vitamin derivatives Heat destruction Oxidation
Bioavailability	Inherent chemical vitamin forms Additives with vitamins Vitamin derivatives
Alternative feed resources	Inherent chemical vitamin forms Anti-nutrients Anti-metabolites Thiaminase; avidin
Nutrient interactions	Energy content Macronutrient content Oxidized lipids Minerals Antioxidants
Environmental interactions	Water temperature O <sub>2</sub> Salinity
Beyond minimum requirement during periods of production cycle	Health implications (prevention/repair) Smoltification Reproduction Larvae nutrition

The content of micronutrients in the feed varies with the type, treatment and quantity of feed ingredients (Hertrampf and Piedad-Pascual 2000), as well as the vitamin supplementation levels. The introduction of novel and sustainable feed resources calls for more attention to both inherent or natural vitamin contents and potential anti-nutrients. The latter has to be overcome by proper pre-treatment to destroy the adverse component or by vitamin over-fortification of the feed. In addition, strong biochemical interactions both within micronutrients (vitamin-vitamin; vitamin-mineral) and between micro- and macronutrients may influence the requirement of the vitamins. As examples, lipid utilization for energy purposes seems to be affected in Atlantic salmon parr fed high energy diets (30 % lipid) with riboflavin content close to the requirement ( $6 \text{ mg kg}^{-1}$ ), as compared to the same diet supplemented with  $20 \text{ mg riboflavin/kg}$  (Brønstad et al. 2002). Similarly, a variety of micronutrient interactions affect the requirement of vitamin C in fish (Waagbø et al. 2001b).

Recently, there has been an increasing focus on how changes in environment and husbandry management may affect vitamin requirements in farmed fish, for example related to changes in antioxidant protection [oxygenation; (Lygren 1999)], detoxification [pollutants; (Norrgren et al. 2001)] and disease resistance (Waagbø 2006). This focus also includes promising preventive measures by dietary vitamin regimes, of which vitamin C has been by far the most studied (Dabrowski 2001; Li and Robinson 2001), while vitamin E have been examined as a protective measure against outbreak of bacterial disease (Salte et al. 1988; Waagbø et al. 1993b).

The requirements for vitamins are normally given as  $\text{mg vitamin kg}^{-1}$  dry feed. Since many of the vitamins participate in the intermediary metabolism, questions have been raised if the requirement should be given on an energy basis, relative to growth or as a daily requirement intake ( $\text{mg vitamin kg}^{-1} \text{ fish day}^{-1}$ ) like in human recommendations (Woodward 1994; Kaushik et al. 1998; Masumoto 2002). Due to missing updated information on requirement studies for many vitamins, the requirements are given here as  $\text{mg kg}^{-1}$  dry feed.

## **5.2. Lipid soluble vitamins**

The term lipid soluble vitamins covers vitamins A, D, E and K, and suggested minimum requirements are available for salmonid species (Table 5.2), including symptoms of vitamin deficiencies (Hardy 2001; Halver 2002). In general, this group of vitamins follows the lipids in the digestion, intestinal absorption, transport, storage and excretion in the fish body, although specialized extracellular and intracellular proteins may be involved in transport and storage of the single vitamin. Consequently, any changes in diet or husbandry that impact

Table 5.2. Main functions, minimum requirements and recommendations of lipid soluble vitamins ( $\text{mg kg}^{-1}$  dry feed), including suggested or defined legal (vitamin D only) safe upper limits. Care should be taken to correct for vitamin purity of the supplemented products.

<b>Vitamin (common name) Main vitamin function</b>	<b>Minimum requirement (<math>\text{mg kg}^{-1}</math>)<sup>1</sup></b>	<b>Updates for salmonids (<math>\text{mg kg}^{-1}</math>)</b>	<b>Recommended target level (<math>\text{mg kg}^{-1}</math>)</b>	<b>Suggested safe upper limit (<math>\text{mg kg}^{-1}</math>)</b>
<b>Vitamin A</b> (Retinol) Growth, cell differentiation, vision	0.75	1.5 <sup>12</sup>	1.5 $\text{mg kg}^{-1}$	$> 37$ <sup>2</sup> $< 122$ <sup>3</sup>
<b>Vitamin D<sub>3</sub></b> (Cholecalciferol) Calcium regulation	0.06	0.013 <sup>13</sup> 0.06 <sup>12</sup> 0.05 <sup>14</sup>	0.1	0.075 (feed legislation) $> 57$ <sup>4</sup> (no effect) $< 29$ <sup>5</sup> (no effect) $< 6.25$ <sup>6</sup> (growth reduction) 1.25 <sup>4</sup> (consumer safety)
<b>Vitamin E</b> ( $\alpha$ -Tocopherol) Antioxidant	30-50	30 <sup>12</sup> - 60 <sup>8</sup>	100	700
<b>Vitamin K</b> (Menadione) Coagulation, bone composition	10	0.2 <sup>9</sup> 40 <sup>11</sup> 0.45 <sup>12</sup>	2.5 <sup>9</sup>	10 <sup>9,2</sup>

<sup>1</sup>(Halver 2002); <sup>2</sup>(Grisdale-Helland et al. 1991); <sup>3</sup>(Ørnsrud et al. 2002); <sup>4</sup>(Graff et al. 2002b);

<sup>6</sup>(Vielma et al. 1998); <sup>7</sup> <sup>8</sup>(Hamre and Lie 1995); <sup>9</sup>(Grahl-Madsen and Lie 1997) in Atlantic cod (*Gadus morhua*); <sup>11</sup>(Storebakken 2002) – practical addition; <sup>12</sup>(Woodward 1994);

<sup>13</sup>(Horvli and Lie 1994b); <sup>14</sup>(Barnett et al. 1982);

(Vitamin A IU=0.3  $\mu\text{g}$  all-trans retinol or 1 mg retinol  $\text{kg}^{-1}$  = 3333 IU  $\text{kg}^{-1}$ );

(Vitamin D IU= 0.025  $\mu\text{g}$  cholecalciferol or 1 mg cholecalciferol =40 000 IU)

lipid metabolism in a broad sense, may also influence the supply and need for these vitamins. Deficient or suboptimal dietary vitamin E is among the most commonly observed deficiencies in aquaculture, related to rancid diets and a general lack of antioxidants.

The safety levels of dietary vitamins are generally high, while dietary vitamins A and D are among the few vitamins that have been suspected to cause hypervitaminosis and toxicological symptoms in farmed salmonids (Graff 2002; Ørnstrud 2003). Bioaccumulation and magnification of these vitamins in the food web may result in extremely high levels of these vitamins in fish species used for fish meal and fish feed (Grisdale-Helland et al. 1991; Julshamn et al. 2003), which subsequently may result in exceeding the upper tolerable limits for these vitamins in feed for rapidly growing salmonids.

### **5.2.1. Vitamin A**

The standing minimum requirement of vitamin A for salmonids (NRC 1993) is 0.75 mg retinol kg<sup>-1</sup> (2500 IU kg<sup>-1</sup>), based on a study on rainbow trout recording growth and mortality (Kitamura et al. 1967) (Table 5.2). A feeding study on Atlantic salmon fry using a mixture of retinyl-acetate and retinyl-palmitate (1:1) in a semi-synthetic diet suggests that this recommendation may represent a low estimate (Christiansen et al. 1994). The latter may however, reflect reduced bioavailability of retinyl palmitate in salmon fry intestine, as observed for other vitamin palmitate derivatives (Albrektsen et al. 1988).

The inherent natural content of vitamin A in marine resources depends on fish species and catch season. Thus, the vitamin A content in fish meal may vary from approx. 1 to 70 mg vitamin A kg<sup>-1</sup>, while fish oils based on different fish species range from 6 to 690 mg vitamin A kg<sup>-1</sup> (Table 5.3). Based on the natural content in the marine resources there is no need for further vitamin A supplementation in fish feeds. Indeed, the concentration of retinol in commercial feed samples produced in Norway during the years 2002-2006 varied from 4 to 121 mg retinol kg<sup>-1</sup>; NIFES, Feed Surveillance Program, 2006). These surveys indicate that vitamin A deficiency in salmonids mediated through commercial feeds based on fish meal and oil is unlikely, while vitaminosis A is more likely. A change in dietary vitamin A levels relative to changes in major feed ingredients is not known (Table 5.3), however, there is less inherent vitamin A in the applied plant ingredients. Further, contributions of vitamin A that may arise from astaxanthin, as a provitamin A compound, is not known, but indications of approx. 10% vitamin activity have been suggested (Al-Khalifa and Simpson 1988). Astaxanthin, however, may only be important as a vitamin A source under vitamin A deprivation (Christiansen et al. 1994). The typical inclusion level of astaxanthin ranges

Table 5.3. Concentration range of fat soluble vitamins (mg kg<sup>-1</sup> dry feed) in fish meal, soybean meal, fish oil and linseed oil, illustrating need for attention

Vitamin	Fish meal (mg kg <sup>-1</sup> )	Soybean meal, extracted (mg kg <sup>-1</sup> )	Fish oil (mg kg <sup>-1</sup> )	Soybean oil (mg kg <sup>-1</sup> )	Linseed oil (mg kg <sup>-1</sup> )
Vitamin A (Retinol)	1-70 <sup>7</sup>	0.12-0.24 <sup>2</sup>	6-690 <sup>5</sup> Pro vitamin A pigments <sup>6</sup>	-	-
Vitamin D (Cholecalciferol)	0.006-0.179 <sup>1</sup>	-	2.5-5.0 (liver oils) <sup>3</sup>	-	-
Vitamin E ( $\alpha$ -Tocopherol)	3-10 <sup>2</sup> 5-22 <sup>4</sup>	3-9 <sup>2</sup>	-	27-95	75
Vitamin K (Menakinone)	2.4 <sup>2</sup>	-	-	5.4	-

<sup>1</sup>(Horvli and Lie 1994a); <sup>2</sup>(Hertrampf and Piedad-Pascual 2000); <sup>3</sup>(DTU 2007); <sup>4</sup>(NRC 1993); <sup>5</sup> (Opstvedt et al. 1997); <sup>6</sup> assumed approx. 10 % retinol equiv. (Al-Khalifa and Simpson 1988); <sup>7</sup>(Ørnsrud 2003)

between 30 and 60 mg kg<sup>-1</sup>, with an legally given upper limit of 100 mg kg<sup>-1</sup> in feed for salmonids beyond 6 months of age (LD/FD 2002).

Toxic levels of vitamin A result in mortality, reduced growth, and disturbances in development of fish fry, including backbone deformities (Primbs et al. 1971; Hilton 1983; Ørnsrud et al. 2002). The sensitivity for vitamin A intoxication may depend on stage of development, with juveniles as more sensitive (Cahu et al. 2003). In general, salmonids seem to tolerate high dietary levels of vitamin A. In rainbow trout diets, (Hilton 1983) found 270 mg kg<sup>-1</sup> (900 000 IU kg<sup>-1</sup>) to be the upper limit, using the retinyl palmitate form. In Atlantic salmon fry, Ørnsrud et al. (Ørnsrud et al. 2002) found that a dietary level of 122 mg kg<sup>-1</sup> (407 000 IU kg<sup>-1</sup>) retinyl acetate fed for 14 weeks reduced the growth and the fish showed

symptoms of metabolic stress. Thus, the upper limit for vitamin A in Atlantic salmon feed seems to be between 37 (Grisdale-Helland et al. 1991) and 122 mg kg<sup>-1</sup>.

### **5.2.2. Vitamin D**

Vitamin D deficiency was described in amago salmon fed a vitamin D free diet, as compared to a diet containing 0.5 mg kg<sup>-1</sup> (Taveekijakarn et al. 1996a). The minimum dietary requirement for vitamin D in salmonid feeds is set at 0.06 mg kg<sup>-1</sup> (2400 IU kg<sup>-1</sup>) (NRC 1993) (Table 5.2). Based on the natural content in marine resources there is no need for further vitamin D supplementation in marine based fish feeds (Table 5.3), while use of plant oils and meals do not support any vitamin D to the diet. The concentration of vitamin D in commercial feed samples produced in Norway during the years 2002-2006 varied from 0.1 to 1.04 mg vitamin D kg<sup>-1</sup> (NIFES feed reports, Feed Surveillance Program, 2006). The variation in vitamin D recorded in fish meals and fish oils varied 50 and 32 fold, respectively (Horvli and Lie 1994a; Opstvedt et al. 1997), indicating that fish diets based on traditional marine ingredients may be exposed to extremely high levels of vitamin D, including the active vitamin D metabolites.

To present knowledge, megadoses of vitamin D<sub>3</sub> supplementation are not harmful for fish. Feeding experiments with vitamin D doses of 57.5 mg kg<sup>-1</sup> did not affect growth and health in Atlantic salmon (Graff et al. 2002b). According to the Norwegian feed legislation (LD/FD 2002) vitamin D<sub>3</sub> supplementation should be below 0.075 mg kg<sup>-1</sup> (3000 IU kg<sup>-1</sup>), while inherent vitamin D is not included in the upper limit unless a supplementation is performed. An upper limit of vitamin D of 1.25 mg kg<sup>-1</sup> is suggested in Table 5.2, despite no harmful impacts on fish health observed far above this level. This limit reflects, however, more an aspect of seafood safety for humans. Salmon retain between 10-20 % of feed vitamin D in fillet. By feeding Atlantic salmon 1.25 mg vitamin D kg<sup>-1</sup> feed, the fillet will contain between 0.13-0.25 mg kg<sup>-1</sup>, and consumption of 200 g fillet will contain 50 µg vitamin D. While clinical vitamin D toxicity has been observed at daily intakes of 10-20 times this dose, selected parts of the population like children and elderly are more sensitive to toxicity.

### **5.2.3. Vitamin E**

A major essential role of vitamin E (tocopherols/tocotrienols) is to protect body lipids and biomembranes towards oxidation or rancidity (Hamre 1995). Thus, a state of vitamin E deficiency is characterized by anemia and accumulation of lipid oxidation products in the liver with liver degeneration, exudative diathesis and exophthalmia (Hamre et al. 1994).

Muscle degeneration is also observed, however mostly in dual vitamin E and selenium deficiency (Hamre 1995; Halver 2002). Vitamin E deficiency was commonly observed in early aquaculture history with low quality rancid diets, not protected with synthetic antioxidants nor added vitamin E. Estimated minimum requirement in coldwater species is 50-60 mg kg<sup>-1</sup> dry feed (NRC 1993; Hamre and Lie 1995). The requirement may, however, vary between 5 and 120 mg kg<sup>-1</sup>, depending on fish species and farming conditions (Cowey et al. 1981; Hamre and Lie 1995). Increasing levels of dietary polyunsaturated fatty acids (PUFA) increases the need for antioxidants and vitamin E (Watanabe et al. 1981). This includes increased requirement at lower water temperatures, since the fish adapts its biomembranes to lower temperatures by replacing saturated fatty acids with higher proportions of PUFA (Cowey et al. 1984). Most studies of vitamin E requirement are performed with low lipid levels (<10%) in feed to juvenile fish, while modern salmonid feeds contain 25-40 % lipid. Due to its protective role as antioxidant, recommended levels of vitamin E should be adjusted according to dietary lipid level. Further, micronutrient interactions may increase the requirement of vitamin E, including low dietary vitamin C levels (Hamre et al. 1997), low selenium levels (Poston et al. 1976) and oxidized lipid (Cowey et al. 1984). Environmental conditions, like water oxygenation, as well as infectious diseases, expose the fish to increased oxidative stress, which may be alleviated with elevated supplementation of vitamin E or tissue vitamin E status (Lygren et al. 2000). Based on established minimum requirements, and nutritional and environmental interactions, it is recommended to target 100 mg kg<sup>-1</sup> of vitamin E ( $\alpha$ -tocopherol) in practical diets for salmonids.

The muscle vitamin E level reflects the dietary vitamin E supplementation in a linear way, and this can be used to tailor the vitamin E content in the final product by use of finishing diets. This allows the producer to both increase the nutritional value of the produce, as well as improve the protection against oxidation during storage and processing (Frigg et al. 1990; Waagbo et al. 1993; Sigurgisladottir et al. 1994a; Sigurgisladottir et al. 1994b; Hamre et al. 1998). The finishing diet should be used for at least three months prior to slaughter (Hamre et al. 1998).

Processed marine feed ingredients are protected towards oxidation by synthetic antioxidants and normally contain low inherent levels of vitamin E. Plant oils and meals contain considerable concentrations of inherent vitamin E (Table 5.3). However, changes towards will also increase the relative contribution of plant specific tocopherols ( $\beta$ -,  $\gamma$ - and  $\delta$ -tocopherols) with adequate antioxidant properties, but with considerably lower biological

vitamin E activity (Hamre et al. 1993; Sigurgisladottir et al. 1994a; Hamre et al. 1998). Vitamin E toxicity is not observed in farmed fish, however elevated levels ( $> 700 \text{ mg kg}^{-1}$ ) should be avoided for extended feeding periods, since high doses of vitamin E may be pro-oxidative in antioxidant imbalances (Hamre et al. 1997).

#### **5.2.4. Vitamin K**

Vitamin K plays vital roles in blood coagulation and bone mineralization (Udagawa 2000). The minimum requirement is suggested to be  $2.5 \text{ mg kg}^{-1}$  using menadione (vitamin K<sub>3</sub>) as vitamin K source. A study on amago salmon, *Oncorhynchus rhodurus*, fed a vitamin K deficient, purified diet developed deficiency symptoms like mortality, anemia, increased blood clotting time and histopathological findings in liver and gills, which was recovered after feeding a diet containing vitamin K (Taveekijakarn et al. 1996b). Feeding  $10 \text{ mg kg}^{-1}$  of menadione sodium bisulphite (MSB; vitamin K<sub>3</sub>) increased the active form menakinone (MK-4; vitamin K<sub>2</sub>) in the Atlantic salmon liver compared to no supplementation ( $0.05 \text{ mg phyllokinones kg}^{-1}$ ; vitamin K<sub>1</sub>), while the latter diet did not result in vitamin K deficiency (Graff et al. 2002a). The MSB vitamin K form used in fish feed is unstable during feed processing and storage (Marchetti et al. 1999), and the dietary content may under unfavorable production and storage conditions reach critical low levels. Menadione nicotinamide bisulphite (MNB) has been suggested as a more stable and less toxic alternative for vitamin K supplementation (Marchetti et al. 1995). Recent unpublished data however, questions the availability of this form of vitamin K in feed for salmonids (Krossoy 2007). While a minimum requirement is probably  $<1$  and recommended target level are  $2.5 \text{ mg vitamin K kg}^{-1}$  (Table 5.2), there are analytical difficulties to correctly assessing inherent (phyllokinones) and supplemented forms (MSB or MNB) of vitamin K for exact determinations. Relevant feed ingredients of plant origin may cover the requirement for vitamin K (Table 5.3).

#### **5.3. Water soluble vitamins**

The expression water soluble vitamins covers eight B vitamins (Table 5.4) and vitamin C. Additionally, inositol and choline are often included as vitamin-like compounds since salmonids seem to require supplementations during juvenile stages with high growth rates. The minimum requirements have been estimated for most of the vitamins in salmonid species, although not always including Atlantic salmon and rainbow trout [(Halver 2002; Hardy 2002; Storebakken 2002); Table 5.5]. Although (Halver 2002) represents a recent publication on



Table 5.4. Names, importance in metabolism, sensitive organ at deficiency, and main biochemical function of the single B-vitamins

<b>Vitamin</b>	<b>Familiar name</b>	<b>Essential for</b>	<b>Sensitive organs</b>	<b>Biochemical function</b>
Vitamin B <sub>1</sub>	Thiamin	Carbohydrates	Nerve system, muscle tissue	Carboxylation, decarboxylation
Vitamin B <sub>2</sub>	Riboflavin	Lipid, protein, carbohydrate, energy	Skin, epithelium, nerves, eye tissues	Redox-reactions
Niacin	Nicotinic acid, Vitamin B <sub>3</sub>	Lipid, protein, carbohydrate, energy	Skin, epithelium, nerves, muscle cell	Redox-reactions
Vitamin B <sub>6</sub>	Pyridoxine	Protein, amino acids, lipid	Muscle cell, nerves, blood cell	Transaminations/ deaminations
Biotin	Vitamin H	Lipid, carbohydrates	Skin, gill, liver tissues	Carboxylation, decarboxylations
Pantothenic acid	Vitamin B <sub>5</sub>	Carbohydrates, proteins, fatty acids	Gills, skin, nerve system	Acetylations, acylations
Folate	Folic acid	Amino acids, nucleic acids	Blood cells, nerves, skin, cell division	1C- transfers
Vitamin B <sub>12</sub>	Cyano-cobalamin	Amino acids, nucleic acids, fatty acids	Blood cells, nerves, cell division	Methylations

Table 5.5. Minimum requirements, updates and recommended supplementation to target levels in commercial salmonid diets (mg kg<sup>-1</sup> dry feed). Water soluble vitamins are regarded safe at high dietary doses (minimum requirement x 100) and no current legal upper limits exist.

<b>Vitamin</b>	<b>Minimum requirement (mg kg<sup>-1</sup>)<sup>1</sup></b>	<b>Updates for fish species (mg kg<sup>-1</sup>)<sup>2</sup></b>	<b>Recommended target level (mg kg<sup>-1</sup>)</b>
Vitamin C	20	10	100
Thiamin	10-15	1	10
Riboflavin	20-30	4-6 <sup>5,6</sup>	15
Niacin	88-200 <sup>7</sup>	10 <sup>8</sup>	100
Pantothenic acid	40-50	19	50
Pyridoxine	10-20	2-8 <sup>9</sup>	10 <sup>2</sup>
Biotin	1.0-1.5	0.1-0.3 <sup>3,4</sup>	0.5
Folic acid	6-10	0.6 3.6 <sup>3</sup>	10
Vitamin B <sub>12</sub>	0.015-0.02	0.007 0.014	0.03
<i>Vitamin- like compounds</i>			
Inositol	200-400	250	500
Choline	2000-4000	430-4000	2000

<sup>1</sup> (Halver 2002); <sup>2</sup>(Woodward 1994); <sup>3</sup>(Koppe 1993) ; <sup>4</sup>(Mæland et al. 1998); <sup>5</sup>(Serrini et al. 1996); <sup>6</sup>(Brønstad et al. 2002); <sup>7</sup> (Chuang 1991); <sup>8</sup>(Poston and Wolfe 1985); <sup>9</sup> (Albrektsen et al. 1993)

water soluble vitamins, virtually no new information on this area is included since the previous 1989 book edition (Halver 1989).

Lack of B-vitamins is not normally considered as an obvious reason for deficiency in salmonid farming, and potential single vitamin deficiencies are relatively easily discovered (Hardy 2001). There seems to be even less chance for toxic conditions, with safety factors for humans over 100 times the RDA (recommended daily allowances) for most water soluble vitamins, and 50 times the RDA for folate (Marks 1989).

Basic roles and deficiency symptoms of the single vitamins are reported in detail by (NRC 1993; Halver 2002). All the B-vitamins participate in biochemical reactions in the intermediate energy metabolism, and high concentrations of the vitamins are found in metabolically active fish tissues [(Brækkan 1959); (Table 5.4)]. The roles of the single B-vitamins in the fish body are not fully known, and there is a large gap between historical requirement experiments and modern intensive farming conditions. Uncertainty is also illustrated by wide ranges in the suggested minimum requirements (Table 5.5). In human nutrition, beyond deficiency concepts are considered, and often include other roles and recently discovered novel essential functions of single vitamins. For B-vitamins, such functions include, for example, the role of folate derivatives in regulation of tissue differentiation and growth. Likewise, novel roles of pyridoxine in steroid hormone regulation in stress, smoltification and reproduction has been examined in Atlantic salmon (Albrektsen 1994). Symptoms of suboptimal vitamin supplies may be expressed under intensive farming conditions, like elevated water temperatures, manipulation of light regimes and extreme water gas regimes. Own unpublished data show up to three-fold fluctuations in liver and muscle status of selected B-vitamins in Atlantic salmon smolts reared at three different temperatures while given the same diet. This is probably related to differences in feed intake, metabolism and requirements for the vitamins. Research is needed to examine if B-vitamins introduce biological limitations in intensive farming of adult salmonids.

Changes in feed ingredients from marine towards plant ingredients may introduce changes both in dietary vitamin levels, their relative bio-availabilities and their content of anti-vitamins and anti-metabolites (Table 5.6). Vitamins are supplemented in different chemical forms, often protected towards harsh production conditions. The stability of crystalline and coated forms of water soluble vitamins during feed production (pelleting or extrusion) and storage identified greater loss of unstable vitamins during modern extrusion

Table 5.6. Concentrations (range) of water-soluble vitamins in fish meal, soybean meal and corn gluten meal ( $\text{mg kg}^{-1}$  dry ingredient). In cases of major fish meal replacement by plant ingredients, care should be taken to fulfill vitamin recommendations.

<b>Vitamin</b>	<b>Fish meal types (<math>\text{mg kg}^{-1}</math>)<sup>1,2</sup></b>	<b>Defatted soybean meal (<math>\text{mg kg}^{-1}</math>)<sup>1,2</sup></b>	<b>Corn gluten meal (60% protein) (<math>\text{mg kg}^{-1}</math>)<sup>1,2</sup></b>
Vitamin C	-	-	-
Thiamin	0.1-2	3-7	0.3
Riboflavin	5-10	3	2
Niacin	18-144 <sup>6</sup>	22-30	60
Pantothenic acid	8-30	15	4
Pyridoxin	3-6	5-7	7
Biotin	0.1-0.8 <sup>4</sup>	0.3 <sup>5</sup>	0.2
Folic acid	0.1-0.8 <sup>7</sup>	0.7-2.8	0.3
Vitamin B <sub>12</sub>	0.07-0.43 <sup>7</sup>	-	-
<i>Vitamin- like compounds</i>			
Inositol	700-800 <sup>3</sup>	-	-
Choline	3000-5300	2 700-2 800	350

<sup>1</sup>(Hertrampf and Piedad-Pascual 2000); <sup>2</sup>(NRC 1993); <sup>3</sup>(Waagbø et al. 1998); <sup>4</sup>(Mæland and Sandnes 1999); <sup>5</sup>(Lovell and Buston 1984); <sup>6</sup>(Blum 1991); <sup>7</sup>(Sandnes and Mæland 1994)

production than yesterday's pelleting production procedure, and subsequent storage (Gadient and Fenster 1994; Gabaudan and Hardy 2000). This holds especially for crystalline vitamin C, thiamine, pyridoxine, vitamin B<sub>12</sub> and folate.

### **5.3.1. Vitamin C**

Vitamin C or ascorbic acid deficiency has probably been the most widespread nutritional disease in aquaculture history due to the extreme instability of the vitamin during processing and storage (Sandnes 1991; Gadient and Fenster 1994; Gabaudan and Hardy 2000). Several protected vitamin compounds and derivatives have been examined for their stability, bioavailability and efficacy in salmonids, of which the phosphate derivatives seem to be superior (Waagbø et al. 1991; Sandnes and Waagbø 1991b; Sandnes et al. 1992). There is an extensive body of literature on vitamin C (ascorbic acid) qualitative (Mæland and Waagbø 1998) and quantitative requirements in fish species (Sandnes 1991; Dabrowski 2001), including the use of excess dietary vitamin C under stressful and unfavourable farming conditions. Severe vitamin C deficiency is characterized by bone deformities, anemia and massive mortalities. Detailed histopathological and descriptive deficiency symptoms are reviewed by (Meier and Wahli 1990; Dabrowski 2001). No vitamin C is normally found in processed feed materials due to its instability during feed manufacture (Table 5.6). The minimum requirement in Atlantic salmon is covered by 20 mg ascorbic acid kg<sup>-1</sup>, using stable phosphate derivatives or target levels of crystalline ascorbic acid [(Sandnes et al. 1992); Table 5.5]. The vitamin C status of fish easily reflects the dietary vitamin C concentration. At the same dietary level, however, fluctuations in vitamin C status have been observed over time, probably related to factors such as feed intake, rate of excretion or metabolism of ascorbic acid (Waagbø et al. 1993a). On this basis, 100 mg ascorbic acid equivalents kg<sup>-1</sup> is recommended (Table 5.5). Feed levels used to improve stress and disease resistance are most often in the order of magnitude > 1000 mg kg<sup>-1</sup> for limited feeding periods (Lim et al. 2001).

### **5.3.2. Thiamin**

Thiamin (vitamin B<sub>1</sub>) is a coenzyme vitamin (active form is thiamin pyrophosphate) participating in several biochemical pathways in the metabolism of carbohydrates and lipids. Thiamin deficiency is described in several salmonid and carp species, with heavy mortalities following initial behavioral and homeostatic disturbances (Morito et al. 1986; NRC 1993; Woodward 1994; Halver 2002). Juveniles are more susceptible to deficiency due to rapid metabolism and lack of storage capacity, while larger fish show more general and obscure

symptoms. Development of thiamin deficiency is accelerated at elevated temperatures relative to increased metabolism, and by increased dietary carbohydrates. Thiamin deficiency may be explored by studying activity of key thiamin-dependent enzymes, like  $\alpha$ -ketoglutarate dehydrogenase and transketolase, in selected tissues. The M74 syndrome, a reproduction disorder of Baltic salmon manifested as death of developing yolk-sac fry, seems to be related to thiamin deficiency (Amcoff et al. 2002). A similar disorder, “Cayuga syndrome”, has been described in the US (Fisher et al. 1995). Low salmon egg thiamin concentration is probably mediated through imbalanced broodstock nutrition or increased degradation of thiamin by thiaminase from the wild prey in the brood fish intestine. Consequently, brood fish have been injected with thiamin to improve egg thiamin status (Fitzsimons et al. 2005), while M74 affected fry have successfully recovered after a thiamin bath treatment (Bylund and Lerche 1995). Smolt mortalities shortly after seawater transfer has been associated with “energy deficiency” and a disturbed thiamin metabolism (Salte 1993). The thiamin contribution from marine ingredients is variable, probably depending on thiaminase activity in the minced fish raw material during meal production prior to heating. The dietary thiamin requirement is uncertain, but is suggested to be considerably lower than  $10 \text{ mg kg}^{-1}$ , earlier suggested by NRC (NRC 1993). Woodward suggested  $1 \text{ mg kg}^{-1}$ , while Morris & Davis estimated a thiamin requirement in excess of  $5 \text{ mg kg}^{-1}$  for gilthead seabream (*Sparus aurata* L.) fed a diet with moderately elevated lipid content (Woodward 1994; Morris and Davies 1995b). Besides variable inherent thiamin contents (Table 5.6), plant ingredients may contain heat stable antithiamin factors, like tannins and polyphenols (Tanphaichitr 1999). Thus, the suggested dietary thiamin concentration for modern salmonid diets should be targeted to be  $10 \text{ mg kg}^{-1}$  (Table 5.4).

### 5.3.3. Riboflavin

For rainbow trout (Takeuchi et al. 1980; Hughes et al. 1981; Woodward 1985; Amezaga and Knox 1990), blue tilapia, *Oreochromis aureus* (Soliman and Wilson 1992), red hybrid tilapia (Lim et al. 1993) and channel catfish, *Ictalurus punctatus* (Serrini et al. 1996), levels of 3-6 mg riboflavin  $\text{kg}^{-1}$  diet seemed to be sufficient for maximal growth and feed efficiency. Besides unspecific deficiency symptoms like growth depression, reduced feed conversion and dark pigmentation, cataract is a classical symptom of riboflavin deficiency (Hughes et al. 1981; Lim et al. 1993; Bjerkås et al. 2006). This led to increased focus on riboflavin during outbreaks of severe cataracts in Atlantic salmon farms during the nineties (Bjerkås et al. 1996; Waagbø et al. 1996; Midtlyng et al. 1999). Despite adequate inherent

riboflavin content in practical feeds with 50% fish meal (6-8 mg kg<sup>-1</sup>), Brønstad *et al.* found that riboflavin supplementation (20 mg kg<sup>-1</sup>) affected lipid metabolism in smolting Atlantic salmon fed a practical high energy diet (Brønstad *et al.* 2002). Similar conditions may also be valid for the feed and energy utilisation in adult salmon, where even higher lipid concentrations are used.

Variable amounts of bioavailable forms of riboflavin are found in most plant and animal feed ingredients (Table 5.6). For optimal growth, lipid metabolism and health in salmonid farming, a target level of 15 mg riboflavin kg<sup>-1</sup> should be achieved.

#### **5.3.4. Biotin**

Biotin functions as a coenzyme for carboxylases in metabolism of carbohydrates, lipids and some amino acids (Dakshinamurti and Cauhan 1989). Biotin deficiency symptoms in fish are reduced growth rate and increased mortality, as well as more specific symptoms such as abnormalities in skin, intestine and gill tissue (including “blue slime patch disease”) and reduced activity of biotin dependent enzymes in metabolically active tissues (Phillips *et al.* 1950; Lovell and Buston 1984; Woodward and Frigg 1989; Koppe 1993). One of the biotin dependent enzymes, pyruvate carboxylase (PC), converts pyruvic acid to oxaloacetic acid in glycolysis and this enzyme has been showed to be a sensitive indicator of biotin status in fish (Woodward and Frigg 1989). Among the proteins in raw egg white, avidin binds strongly to biotin and makes the vitamin inaccessible for absorption in the intestine (Mock 1999). Biotin deficiency due to intake of raw egg white is a part of the history on the discovery of biotin as a vitamin [reviewed by (Mock 1999)]. Supplementation of raw egg white or avidin to diets induces biotin deficiency symptoms in several fish species (Poston 1976; Casteldine *et al.* 1978; Lovell and Buston 1984; Mæland *et al.* 1998). The biotin requirement in Atlantic salmon has not been established. The minimum dietary requirement for biotin for optimal growth in other salmonids, such as rainbow trout and lake trout, *Salvelinus namaycush*, have been estimated to be 0.14 mg kg<sup>-1</sup> and 0.10 mg kg<sup>-1</sup>, respectively (Poston 1976; Woodward and Frigg 1989). Koppe (Koppe 1993) demonstrated that the biotin requirement was met in young rainbow trout fed a practical diet without any supplementation of biotin (0.11 mg biotin kg<sup>-1</sup> diet), while somewhat higher levels were needed for optimal lysozyme levels in serum and mucus (0.15 mg biotin kg<sup>-1</sup> diet). The biotin concentration in Norwegian fish meal ranges between 0.4 and 0.8 mg kg<sup>-1</sup> (Mæland and Sandnes 1999), depending on fish species and season. Practical feeds based on marine raw materials covered

the requirement for biotin for Atlantic salmon juveniles (Mæland et al. 1998). Similarly, practical feeds for channel catfish, based on dehulled soybean meal (75%) seem to contain enough biotin ( $0.35 \text{ mg biotin kg}^{-1}$ ) to meet the requirement (Lovell and Buston 1984). Elevated biotin levels, up to  $1 \text{ mg kg}^{-1}$ , may be favourable during salmon smoltification and seawater transfer (Waagbø et al. 1994). The bioavailability of biotin in feedstuffs varies greatly, between 0-100% in animal and plant ingredients (Frigg 1976; 1984). Considering variable bioavailability of biotin also in fish meals, a target level of  $0.5 \text{ mg biotin kg}^{-1}$  is suggested for salmonids (Table 5.5).

### **5.3.5. Pantothenic acid**

Pantothenic acid is a part of coenzyme A, essential in transfer of acetyl- and acyl-groups as substrates in energy production, the synthesis of the neurotransmitter acetylcholine, acetylation of aromatic amines and synthesis of cholesterol. Mitochondria rich tissues like the kidney and gills, are specially sensitive to pantothenic acid deficiency (NRC 1993; Halver and Hardy 2002). The NRC requirement is estimated to be in the range of  $10 - 50 \text{ mg kg}^{-1}$  (NRC 1993), while Smith et al. suggested  $10-15 \text{ mg kg}^{-1}$  in their review on comparative requirement of pantothenic acid (Smith and Song 1996). In blue tilapia (*Tilapia aurea*), requirement was estimated to be  $10 \text{ mg kg}^{-1}$  based on growth and lack of pathology (Roem et al. 1991). Fish meal contains between  $8 - 30 \text{ mg kg}^{-1}$ , while plant materials contain somewhat less (Table 5.6). Pantothenic acid is added in the form of a stable calcium salt. Due to its role in lipid and energy metabolism, a conservative target feed level of  $50 \text{ mg kg}^{-1}$  is suggested.

### **5.3.6. Niacin**

Niacin deficiency symptoms in fish include reduced growth, appetite, and feed utilization, dark pigmentation, skin lesions, muscular weakness, behavioral changes, edemas and mortalities (Chuang 1991; Halver 2002). Since niacin participates in plentiful enzymatic reactions (Table 5.4), deficiency may develop relative rapidly. Requirements in salmonids vary between  $10 - 175 \text{ mg kg}^{-1}$ , reflecting the degree of uncertainty in the estimates. A requirement level of  $63-83 \text{ mg kg}^{-1}$  was calculated for gilthead seabream using a semi-purified diet (Morris and Davies 1995a), while channel catfish required  $7.4 \text{ mg kg}^{-1}$  (Ng et al. 1997) and rainbow trout  $10 \text{ mg kg}^{-1}$  (Poston and Wolfe 1985). The given wide range in requirements among species suggests that the niacin requirement may vary according to dietary composition, like the lipid content and lipid metabolism. In contrast to land-living animals,



tryptophan have been shown to be inefficient as a niacin precursor in channel catfish (Ng et al. 1997). A major part of the niacin in cereals and oil seed (Table 5.6) is poorly utilized, probably due to bindings to indigestible components in the plant or seed (Blum 1991). According to these considerations, the requirement for energy rich salmonid diets should target a conservative level of 100 mg niacin kg<sup>-1</sup> feed (Table 5.5).

### 5.3.7. Folate and vitamin B<sub>12</sub>

Vitamin B<sub>12</sub> and folate are involved as coenzymes in a series of biochemical reactions in the intermediary metabolism, mainly as donors of one carbon units, and are essential for growth and health [(NRC 1993; Halver 2002); Table 5.4]. Folate deficiency symptoms such as impaired haematopoiesis, reduced growth, survival and resistance to infectious diseases have been observed in several fish species (NRC 1993; Waagbø et al. 2001a; Halver 2002; Hardy 2002), including coho salmon, *Oncorhynchus kisutch* (Smith 1968), rainbow trout (Cowey & Woodward 1993), channel catfish (Duncan *et al.* 1993) and Atlantic salmon (Waagbø et al. 2001a). Haematological effects of folate deficiency in teleosts include macrocytic anaemia with abnormal cell nucleus segmentation of the blood cells (Smith 1968; Waagbø et al. 2001a). The quantitative requirement of folate has been established in channel catfish (Duncan et al. 1993; Robinson and Li 2002) and rainbow trout (Cowey and Woodward 1993). In the latter study, the authors suggested that the dietary requirement of folate in rainbow trout was between 0.6-1.1 mg kg<sup>-1</sup>, depending on response criteria used like reduced concentration of folate in liver, kidney and whole blood (Cowey and Woodward 1993).

For vitamin B<sub>12</sub>, qualitative requirement studies exist for Japanese eel, *Anguilla japonica* and red sea bream, *Pagrus major* (Koshio 2002), yellowtail, *Seriola quinqueradiata* (Hosokawa 1999), cited by (Masumoto 2002), and European sea bass, *Dicentrarchus labrax* (Kaushik et al. 1998). Difficulties in determining quantitative requirements have been related to intestinal microbial vitamin synthesis (Sugita et al. 1990). Despite differences in chemical structure and biochemical actions of vitamin B<sub>12</sub> and folate, a close functional relationship exists between the two vitamins, especially in cell division. Requirements and interactions between vitamin B<sub>12</sub> and folate, and bioavailability of these vitamins from practical diets were studied in two experiments with Atlantic salmon (Sandnes and Mæland 1994). Feeding a purified diet without supplementation of folic acid showed growth reduction, reduced levels of folate in liver and muscle tissue, and anemia characterized by reduced blood hemoglobin concentration, enlarged immature erythrocytes with fragmented nuclei and reduced erythrocyte hemoglobin content (MCH). Vitamin B<sub>12</sub> deficient fish also showed anemia with

immature erythrocytes. Most severely, anemia was observed in fish fed a diet without both vitamin B<sub>12</sub> and folate supplementation. Dietary supplementation of 0.014 mg vitamin B<sub>12</sub> kg<sup>-1</sup> and 3.6 mg folate kg<sup>-1</sup> in purified diets prevented vitamin deficiency signs. By feeding a practical fish meal based diet without supplementation of both vitamins, reduced organ levels of folic acid, but not vitamin B<sub>12</sub> were demonstrated. All organs responded rapidly to a folate supplementation according to NRC recommendation (10 mg folate kg<sup>-1</sup>), while only plasma vitamin B<sub>12</sub> reflected extra supplementation of vitamin B<sub>12</sub> (0.2 mg kg<sup>-1</sup> vitamin B<sub>12</sub>). Fish meals contain marginal levels of folate, 0.3-1.0 mg kg<sup>-1</sup>, whereas the concentration of vitamin B<sub>12</sub> in fish meal from Norway has been found to be 0.2-0.4 mg kg<sup>-1</sup> [(Waagbø et al. 2001a) reported from (Sandnes and Mæland 1994)] which is ten times higher than the recommendation of NRC (0.02 mg kg<sup>-1</sup> diet). These data suggest that it is necessary to supplement folic acid in practical salmon diets based on marine ingredients, whereas the natural content of vitamin B<sub>12</sub> is high enough to cover its dietary requirement. Vitamin B<sub>12</sub> occurs in animal tissue, while not in plant ingredients (Table 5.6). Foliates are ubiquitous in nature and are present in nearly all feed items in varying amounts. However, both folate and vitamin B<sub>12</sub> are among the most sensitive vitamins in fish feed and considerable losses occur during ingredient processing, fish feed production (50-65 %) and storage (Gabaudan and Hardy 2000). Again conservative target levels are suggested; 0.03 mg kg<sup>-1</sup> for vitamin B<sub>12</sub> and 10 mg kg<sup>-1</sup> for folate (Table 5.5).

## **5.4. Vitamin-like compounds**

### **5.4.1. Inositol (myo-inositol)**

Myo-inositol is a structural component in living tissues, as well as an important participant in transmembrane signal transfer in the phospholipid (PL) form, phosphatidyl inositol (Aukema and Holub 1994). Inositol is classified as a vitamin-like nutrient and is often supplemented to fish diets. Fish and other vertebrates, or their intestinal microbial flora, may synthesize inositol (Burtle and Lovell 1989; Aukema and Holub 1994). Pathological conditions including fatty liver and muscle degeneration have been reported in some fish species due to inositol deficiency, as well as more general signs of poor appetite, anemia, growth reduction, fin erosion and skin lesions (NRC 1993). In carp (*Cyprinus carpio* L.), deficiency symptoms occurred after three to five weeks on an inositol deficient diet, including hemorrhages, skin and fin lesions and reduced growth and protein and energy retention (Meyer-Burgdorff et al. 1986). According to NRC (1993) the general requirements of inositol for fish seem to be in the range of 250 to 500 mg kg<sup>-1</sup>. In fatty fish species, lipid metabolism

may be affected by inositol deficient diets, as compared to lean species (Burtle and Lovell 1989; Waagbø et al. 1998). The latter authors showed that inositol concentrations in whole fish and liver were affected by dietary inositol inclusion level. The natural content in marine feed ingredients may fully meet the requirement reported by NRC (1993) for several fish species. A study on Atlantic salmon fry indicated that the requirement for inositol is covered through the natural content of inositol in practical feed ingredients at around 300 mg inositol kg<sup>-1</sup> (Waagbø et al. 1998). The authors advised to supplement starter diets with moderate amounts of inositol, around 200 mg kg<sup>-1</sup>, to compensate for fluctuations in inositol concentrations in natural ingredients, leaching loss of inositol from the diet and for any potential increased inositol requirement in salmon fry. By using plant ingredients, inositol may be supplied in the phytate form (phosphorylated inositol), considered an anti-nutrient (Francis et al. 2001). Enzymatic pre-treatment of plant feed ingredients or inclusion of phytase in plant based diets may reduce the negative impact of phytate and supply inositol.

#### **5.4.2. Choline**

Like inositol, choline is involved in the synthesis of phospholipids (PL), in form of the major membrane and transport PL, phosphatidyl choline. Besides roles in structural lipids, choline is also a component in synthesis of the neurotransmitter acetylcholine. It participates in one carbon transfer reactions, among others the methylation of homocysteine to methionine and the synthesis of betaine in the mitochondria. Marine ingredients normally cover the “requirement” for choline (Table 5.6), suggested to be between 2 and 4 g kg<sup>-1</sup>. Soy products also contain considerable amount of choline in form of lecithin (Hertrampf and Piedad-Pascual 2000). Since choline and PL also participate in digestion and metabolism of lipids, symptoms of deficiency have been related to malfunctions in lipid metabolism and organ hemorrhages (Halver 2002). Similarly, the required amount of choline depends on the dietary lipid content, age of the fish, and the rearing water temperature (Hertrampf and Piedad-Pascual 2000). In marine larvae feeding, focus has been placed on PL more than choline since problems seem to be related to the role of PL in intestinal micelle formation, lipid absorption and subsequently lipoprotein transport of lipids (Hamre et al. 2007). From its nature as a strong basic compound, choline may chemically react with other vitamins in feed mixtures (vitamins E and K). Betaine is also used as an additive in fish feed to improve osmoregulatory capacity, and betaine may replace choline since it is more chemically stable.

## 5.5. Summary

Vitamin requirements and recommendations must be seen in relation to fish species, stages of life cycle, overall feed composition, and ambient farming conditions. The single vitamins fulfill known and unknown roles in the integrated metabolism, like the oil in the body machinery. Clearly, care should be taken to be sure that the micronutrients are not limiting productivity and hazarding welfare in farmed fish. The present report suggests target levels of vitamins rather than supplementations, to minimize the risk for deficiencies from qualitative and quantitative changes in feed composition and from vitamin product purities. Still, there are uncertainties relative to vitamin bioavailability arising from inherent and supplemented vitamins and possible contents of antinutrients in novel ingredients. This means an increased focus and awareness on these aspects rather than passively adding a “safe” vitamin recommendation mixture.

## 5.6. References

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## **6. Dietary mineral requirements and skeletal deformities**

*Grete Baeverfjord*

### **6.1. Introduction**

The role of minerals in skeletal pathology was recently reviewed by Lall and Lewis-McCrea (2007), and where the impact of dietary calcium (Ca) and phosphorus (P) was addressed. In the present summary, current knowledge on mineral requirements in compound diets will be discussed in view of the relation between mineral deficiencies and skeletal development. In addition to Ca and P, magnesium (Mg) and zinc (Zn) are included. These two elements have shown variable and low values in whole-body analyses done on farmed Atlantic salmon within the last years, and have previously been associated with skeletal malformations.

### **6.2. Minerals of known relevance to skeletal development**

Calcium and phosphorus are closely related to the development and maintenance of the skeletal system, and the stability of the vertebrae and other skeletal structures is maintained by solid phases of calcium phosphates, hydroxyapatites (Lall and Lewis-McCrea, 2007). Fish and other aquatic organisms absorb Ca and P from water, and their Ca requirement is met partly by their ability to absorb Ca directly from water. The concentration of P is low in both freshwater and seawater and diet is the main source of P (Lall, 2002). Also, according to Lall (2002), the Ca:P ratio in the diet is less important in fish than in terrestrial animals. Of particular relevance is the fact that without available P, Ca cannot be deposited. P deficiency will lead to an accompanying decrease in whole-body Ca levels (Baeverfjord et al., 1998; Baeverfjord, 2005). In fish with proper mineralization of bone, whole-body levels of Ca and P are  $\geq 4000 \text{ mg kg}^{-1}$ , and the Ca:P ratio is  $\geq 1$  (Shearer et al., 1994; Baeverfjord et al., 1998). When P supply is insufficient, Ca and P levels are  $< 4000 \text{ mg kg}^{-1}$  and Ca:P  $< 1$ .

Most of the magnesium in fish is located in bone. Magnesium is also an essential cofactor in many enzymatic reactions in intermediary metabolism (Lall, 2002). Freshwater salmonids derive Mg ions by active uptake from water and from dietary sources. The uptake from water can partially meet the requirement for Mg in freshwater, depending on the water Mg concentration (El-Mowafi and Maage, 1998; Shearer and Åsgård, 1992). In seawater, Mg is abundant and fish obtain Mg by drinking; surplus Mg is excreted over the kidneys (Lall, 2002). Reference values (whole-body analyses) indicate that the normal level for both

rainbow trout and salmon is  $> 300 \text{ mg kg}^{-1}$  (Shearer, 1984; Shearer et al., 1994; Shearer and Åsgård, 1992).

Zinc is important for metabolic processes as part of metalloenzymes (Lall, 2002), and contributes to a high number of metabolic processes. Some of the deficiency signs observed in fish may relate to disturbances of nucleic acid and protein metabolism. Zinc deficiency is associated with shortening of the spine, “short body dwarfism”, in some flatfish species and, as reported in Baeverfjord (2005), an aggravation of phosphorus-induced deformities was observed when dietary zinc levels were restricted. Reference values for whole-body content of Zn given by Shearer for rainbow trout (Shearer, 1984) were  $20\text{-}30 \text{ mg kg}^{-1}$ . Similar values for Atlantic salmon were  $40\text{-}60 \text{ mg kg}^{-1}$  (Shearer et al., 1994).

### **6.3. Dietary mineral supply and skeletal deformities**

The relation between reduced mineralization and skeletal deformities was established for Atlantic salmon both in freshwater and seawater (Baeverfjord et al., 1998; Baeverfjord, 2005; Fjellidal et al., 2007). Corresponding studies in rainbow trout are currently in progress. In Baeverfjord (2005), it was demonstrated that suboptimal dietary phosphorus supply resulted in poor mineralization of bone as an acute response, whereas the long term effects were deformities of jaw and tail and a prominent shortening of the spinal column in a significant number of fish. The condition was aggravated by restricted dietary zinc levels. Growth was little affected. The gravity of skeletal defects increased with the duration of suboptimal mineral supply, but deformities in harvest-size fish were also observed in response to a transient period of undermineralization in the freshwater stage, in particular in the period from first feeding to 20 g size when the growth rates are extremely high (Baeverfjord, 2005). In a study from 1983, Satoh et al. reported shortened spine, or “short body dwarfism”, in response to zinc deficiency in rainbow trout. Likewise, magnesium deficiency in rainbow trout was associated with vertebrae deformity in a study with purified diets from 1978 (Ogino et al., 1978).

### **6.4. Reference values for whole-body contents**

Reference values for whole-body content of elements in various life stages of Atlantic salmon and rainbow trout were given by Shearer et al. (1994) and Shearer (1984) (Table 6.1). The Atlantic salmon values cited are for fish sizes from 1 g to 4 kg. For Ca, the levels seem to be somewhat lower and more variable in seawater than in freshwater. For rainbow trout, the values cited are for analyses of fish from 1 g to 1 kg reared in freshwater. The whole-body Zn

levels for rainbow trout seem inexplicably low. In Hardy and Shearer (1985), all corresponding values were  $> 40 \text{ mg kg}^{-1}$ .

Table 6.1. Reference values for whole-body content of calcium, phosphorus, magnesium and zinc in Atlantic salmon and rainbow trout. Data for Atlantic salmon were extracted from Figures 2 and 3 in Shearer et al. (1994), and for rainbow trout from Figure 3 in Shearer (1984).

	Ca ( $\text{mg kg}^{-1}$ )	P ( $\text{mg kg}^{-1}$ )	Mg ( $\text{mg kg}^{-1}$ )	Zn ( $\text{mg kg}^{-1}$ )
Atlantic salmon	4000-5200 (FW <sup>a</sup> ) 3000-4500 (SW <sup>b</sup> )	4000-5200	380-480	40-60
Rainbow trout	4000-6000	4000-5000	300-400	20-30

<sup>a</sup>Freshwater.

<sup>b</sup>Saltwater.

### 6.5. Current requirement estimates

The following dietary mineral requirements were suggested by Lall (2002) (Table 6.2).

Table 6.2. Requirement estimates for some elements (extracted from Lall, 2002). The estimates are given as amount per kg feed. Requirements were determined using purified or semipurified diets.

	Ca	P ( $\text{mg kg}^{-1}$ )	Mg ( $\text{mg kg}^{-1}$ ) <sup>a</sup>	Zn ( $\text{mg kg}^{-1}$ )
Atlantic salmon	not applicable	6000	500	15-30
Rainbow trout	not applicable	6000	400	37-67

<sup>a</sup>Freshwater.

The suggested dietary P requirement estimates were  $6000 \text{ mg kg}^{-1}$ , or 0.6% of the diet for both Atlantic salmon and rainbow trout. The estimates were based on experiments with purified or semipurified diets with highly available P sources, and Lall comments that factors affecting bioavailability must be considered when formulating fish feeds. The P requirement of juvenile Atlantic salmon was estimated to approximately 1.0-1.1 % of the diet, or  $10\text{-}11\ 000 \text{ mg kg}^{-1}$ , by Åsgård and Shearer (1997) in a study using semipurified diets, when whole-body levels of P was used as a criterion. In the study, P availability was calculated to be 86% and feed conversion ratio (FCR, feed given/weight gained) was 0.7. This study was

done with 1-5 g fish. This would correspond to a requirement of available P of 6000-6600 mg kg<sup>-1</sup> weight gain. A different approach was chosen by Vielma and Lall (1998), who estimated a P requirement of 0.28 mg available P per kJ digestible energy. Expressed as dietary concentration, the requirement estimated by Vielma and Lall (1998) was 0.83-0.86 % (8300-8600 mg kg<sup>-1</sup>) of the diet, when using a highly available P source.

The Zn requirement of Atlantic salmon seems to be severely underestimated by Lall (2002). A Zn requirement of 57-97 mg Zn kg<sup>-1</sup> dry feed was estimated by Maage et al. (1991) in order to sustain normal whole-body zinc levels in the period following first feeding. In Baeverfjord (2005), a dietary level of 50-80 mg Zn kg<sup>-1</sup> feed resulted in a severe depletion of whole-body zinc levels.

## **6.6. Monitoring of mineral status**

The difficulties associated with establishing mineral requirements in fish were reviewed by Lall (2002). Test methods that monitor specific biological functions are sparse in fish, and future development of methods that allow for more refined evaluation of mineral homeostasis are anticipated. Some studies related to expression of genes involved in P uptake have been presented (Sugiura et al., 2007), and this field is expected to expand rapidly.

In previous studies on mineral requirements, the recommendations were based on a range of different criteria (Rodehutscord, 1996; Vielma and Lall, 1998; Maage et al., 2001). Also, in view of the restrictions on P outlet from freshwater fish farms in some countries, it is reasonable to suggest that interpretation of some of the data may be considered restrictive, i.e. recommendations are in the lower range of what is indicated by the data. In view of recent results that identify the link between suboptimal mineralization of bone and deformities, the response variables used to monitor mineral status should include some measure of mineral contents of bone.

The most relevant and reliable measure for mineral content of fish appears to be whole-body mineral content (El-Mowafi et al., 1997). The methodology related to sampling and analysis contains few sources of error, and gives data that can be compared across sampling points and fish sizes. For Atlantic salmon and rainbow trout, relatively complete sets of reference values for different stages of the life cycle exist (Shearer, 1984; Shearer et al., 1994).

A range of other response variables have been used to describe mineral status in experiments. Plasma concentrations of elements describe the immediate effects related to absorption (Vielma and Lall, 1998), but are of little use for evaluation of long-term effects.

Bone mineral content is a highly relevant and much used variable, and is mentioned by Lall (2002) as the most sensitive criterion for evaluating P utilization. It is a problem that analytical values seem to vary inexplicably between experiments. Also, the data are reported in different ways, such as on a fat-free or a dry-weight basis. It is likely that sampling and preparation methods can be better standardized, but the method is still labour consuming and with a significant risk of unintended variation or misinterpretation. Fjelldal et al. (2007) and Albrektsen (2005) use the ash content of the bone, relative to the average content within the experiment, as a measure of mineralization, and link it with mechanical properties and morphology (Fjelldal et al., 2007). Without any reference to absolute values however, these data are of limited value.

In summary, based on the literature, whole-body elemental concentration is suggested as the standard response parameter. There is a strong correlation between mineral content in bone and whole-body levels (Baeverfjord et al., 1998), there are absolute reference values to compare with, and the methods for sampling and analysis are relatively foolproof. The value given should be mineral content in wet weight, as it was demonstrated by Shearer (1984) that values given on a dry-weight basis are too much influenced by variation in lipid content. The main weakness is that the values will be influenced by the fat and muscle content of the fish, but in commercially farmed salmonids, this variation is not expected to be very high.

### **6.7. Fish welfare versus environmental concerns**

Analyses done in 2006 and 2007 of whole-body mineral levels in farmed, freshwater salmon from commercial operations demonstrate that mineral levels vary widely (data from Nofima Marine). Some groups of fish have whole-body calcium and phosphorus concentrations that are comparable to reference values (Shearer et al., 1994), but more than half of the samples have concentrations that are lower than these values (Fig 6.1).

Similarly, many of the values for zinc and magnesium were low. The mean whole-body concentration of magnesium in the 25 samples shown in Figure 1 was 263 mg kg<sup>-1</sup> (SD 25.8, min 225, max 320), compared to reference values for Atlantic salmon of 380-480 mg kg<sup>-1</sup>. The mean whole-body zinc concentration in these samples was 32.7 mg kg<sup>-1</sup> (SD 4.1, min 23.2, max 41.1), compared to reference values of 40-60 mg kg<sup>-1</sup>. These data indicate that the mineralization status in Atlantic salmon juveniles in commercial production is far from satisfactory.

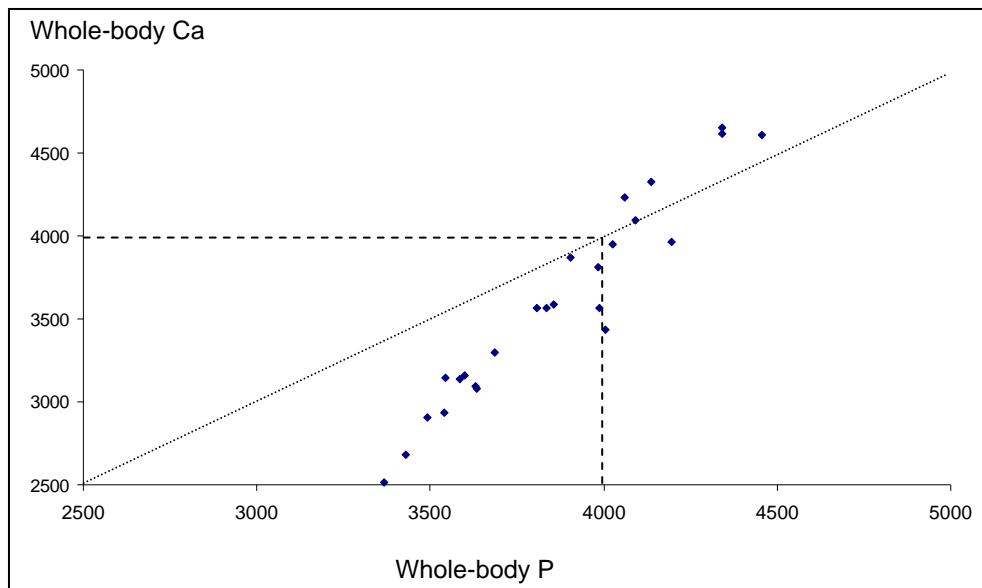


Figure 6.1. Whole-body concentrations of Ca and P in 25 samples of fish fed commercial diets in 2006 and 2007. Values are given as mg kg<sup>-1</sup> wet weight, in pooled samples of 10 fish per fish group. Fish weights were 20-60 g. The diagonal line represents Ca:P 1:1. Reference values for fish with normal mineralization of bone are Ca and P > 4000 (dotted lines) and Ca ≥ P. Data from Nofima Marine.

Phosphorus output from freshwater production sites is a major concern, and restrictions on P discharge from freshwater sites are in existence in several of the countries that produce rainbow trout in freshwater. Strategies to minimize P output were reviewed by Lall (2002), and recommendations include reductions in dietary P levels (without compromising the fish), as well as increasing P availability through different approaches. In the current situation, there is no reason to activate self-imposed restrictions on P discharge in freshwater effluents in Norway, and considering the observations of unsatisfactory mineralization presented above, such restrictions would represent a potential fish welfare risk.

### 6.8. Factorial model for determination of dietary requirements of elements

Shearer (1995) suggested a factorial model for determination of dietary requirements of essential elements in fishes. A simplified version of the model was suggested by Åsgård and Shearer (1997) for minerals where feed is the major source and endogenous loss is low:

$$C=(G/A)/F \quad \text{or} \quad G=C \cdot F \cdot A,$$

where G: Elemental concentration in fish, cfr reference values

C: Elemental concentration in feed

F: Expected feed conversion ratio, kg feed/kg weight gain

A: Predicted bioavailability of element in feed.

The model demonstrated that a requirement estimate given as concentration in the diet (C, e.g. mg P kg<sup>-1</sup> feed) is of little value, unless feed conversion ratio (F) and bioavailability (A) are specified. A range of other methods have been used to establish requirements, leading up to the authoritative recommendations given in NRC (1993) and Lall (2002). Some studies include comments or footnotes that specify that the requirement estimates refer to available amounts, others do not. Also, few, if any, of the studies acknowledge the importance of growth rate and feed efficiency for the relevance of the estimates. None of the estimates discuss safety margins.

### **6.9. Growth rates and feed efficiency – impact on requirement estimates**

In intensive rearing of salmonids, utilization of nutrients is extremely efficient. The two most common measures are feed efficiency (FE, weight gained/feed given) or the inverse value feed conversion ratio (FCR, feed given/weight gained).

FCR may be extremely low, especially in small fish in freshwater. Mean FCR values of 0.6 and lower are not uncommon in juvenile fish, at the stages where growth rate is highest. Expected growth rates for freshwater juveniles of Atlantic salmon and parr at 12 °C are between 3 and 5% per day, and at 16°C between 4 and 6% per day (Tvenning, 1998). In seawater, a close correlation between FE and thermal growth coefficient (TGC) ( $r=0.79$ ,  $P<0.001$ ) was observed by Thodesen et al. (2001b), and in consequence, a corresponding negative correlation exists between growth rate and FCR: High growth rates → low FCR.

A FCR value of 0.6 implies that no more than 600 g of feed is transformed to 1 kg of fish. Thus, the element concentration in the feed formulated to support such efficient growth must be relatively higher than when FCR is low. Requirement estimates should include this aspect. A different approach to this was chosen by Rodehutsord (1996) and Vielma and Lall (1998), who calculated P requirement in relation to kJ available energy. This approach may be equally correct, and relating mineral requirements to dietary energy content may give more precise estimates than using FCR. FCR, on the other hand, is a commonly recorded parameter, and represents a more practical choice.

In the context of dietary mineral supply versus growth rate, it is worth noting that growth rates of farmed salmonids are continuously high throughout the life cycle. As a result



of selective breeding, growth potential of farmed stocks continues to increase. This represents a desired development, and is part of the foundation for profitability in the production. Considering the data from Thodesen et al. (2001b) cited above, further increase in TGC is likely to be associated with a corresponding decrease in FCR. No data exist so far to determine whether there is an upper limit for growth rate at which normal development of skeletal tissues can be obtained.

### 6.10. Mineral bioavailability

The common fish meals and vegetable meals used in fish diets contain minerals in significant amounts (NRC, 1993). There is, however, no corresponding authoritative database available over P digestibility.

Two studies refer to variation in uptake of elements due to biological factors, both done in seawater. Thodesen et al. (2001a) demonstrates a genetic variation in mineral absorption among family groups of large seawater-adapted salmon (Table 6.3). Rydland (1998) presents individual digestibility of minerals, examined by repeated sampling of faeces in 15 large, seawater-adapted Atlantic salmon (Table 6.4).

Table 6.3. Apparent digestibility coefficients (ADC, %) for some elements in 82 full-sib family groups of Atlantic salmon (average weight 4.6 kg). Data are given as mean values (n=82), standard deviation (SD), coefficient of variation (CV) and minimum and maximum values. (Modified after Thodesen et al., 2001a)

Element	Mean ADC	SD	CV	Min	Max
Ca	-11.7	14.5	-123	-91.8	11.1
P	40.4	6.4	15.8	23.9	54.8
Mg	-260	91	-35.0	-768	-119
Zn	37.9	6.7	17.7	18.3	51.3

Table 6.4. Apparent digestibility coefficients (ADC, %) for some elements in 15 individual Atlantic salmon (average weight 1.7 kg). Analyses were done in 3 to 6 repeated samples per individual fish. Data are given as mean values (n=15), standard deviation (SD), coefficient of variation (CV) and minimum and maximum values. (Modified from Rydland, 1998)

Element	Mean ADC	SD	CV	Min	Max
Ca	-0.5	9.9	-1980	-20.9	20.7
P	38.9	5.8	14.9	30.4	49.2
Mg	-325	79	-24.0	-483	-251
Zn	44.7	7.8	17.4	32	57.7

The P digestibility coefficients of approximately 40% were low compared to some of the earlier studies. It should also be noted that Zn digestibility coefficients were at the same level, around 40%. The coefficients of variation for P and Zn digestibility are similar in these two studies, 15.8 and 14.9 for P, and 17.7 and 17.4 for Zn. In Thodesen et al. (2001a), the best family has a P digestibility coefficient of 54.8%, the worst 23.9%. The range from the best to the worst individual in Rydland (1998) was almost of the same magnitude. These values indicate that there should be some consideration of safety margins when applying mineral requirement estimates in feed formulation. The corresponding values for Ca and Mg are of little interest, since these were seawater-adapted fish.

Albrektsen (2005) presented data on the low availability of phosphorus in fish meals produced from blue whiting, thus confirming previous findings on the unpredictable availability of P from different fish meals. Also, availability of minerals may be severely impaired in diets containing vegetable ingredients, e.g. soybean meal (Storebakken et al., 1998). Thus, prediction of actual digestibility of P in formulated diets requires knowledge about feed ingredients and expected availability of P for each batch produced. The data presented above on zinc availability indicate that it may be necessary to do the same considerations also for zinc.

## 6.11. Recommendations

The following recommendations are suggested:

- New studies should be done to determine dietary mineral requirements using current fish stocks with growth potential, feed intake and feed efficiencies which are relevant for today's aquaculture
- Requirement estimates should include safety margins based on knowledge about within-population variation in digestibility and utilization
- In addition to P, Zn should have high priority
- Studies on development of bone quality in response to deficiency conditions are recommended, in order to characterize bone formed during deficient periods. This is of importance for monitoring of mineral status and evaluation of dietary history of fish
- Interrelation with other metabolic functions and requirements should be clarified
- Development of non-invasive indicators, including molecular markers, for mineral metabolism, should be given a high priority
- Updated requirement estimates should be given as available amount per unit of weight gain, in order to include consideration related to growth rate, feed conversion ratio and digestibility of elements
- Further development of methods for estimating digestibility of minerals in commercial feeds should be done
- Procedures for improving mineral availability from major feed ingredients should be further developed.

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## 7. Carbohydrates

*Barbara Grisdale-Helland*

Carbohydrates are not specifically required in the diet of fish, but a growth-promoting effect is found when, with an adequate dietary protein level, gelatinized starches are included in the diet at low levels (reviewed by Hemre et al., 2002). Starches are added to the diet mainly for binding and expansion during extrusion. But, increasing dietary starch levels reduce both starch and fat digestibility in salmon (reviewed by Storebakken, 2002). Similarly, feed utilization is reduced with increasing dietary starch levels in diets for Atlantic cod and Atlantic halibut (Hemre et al., 1993; Helland and Grisdale-Helland, 1998). The protein-sparing effect of carbohydrates varies with species, temperature, light regime, season, and the type and amount of carbohydrate included in the diet (reviewed by Hemre et al., 2002). Simple sugars in gelatinized forms are most effective for protein sparing (Hemre et al., 2002). Oligosaccharides in soybean meal may be indigestible and reduce the uptake of fat and minerals in salmon (Refstie et al., 1998; Refstie et al., 2005). In addition, dietary soybean non-starch polysaccharides may be responsible for reducing the digestibility of fat and protein in salmon (Refstie et al., 1999). According to Bæverfjord and Krogdahl (1996), enteritis in salmon caused by exposure to soybean meal is reversible.

As reviewed by Hemre et al. (2002), high glycogen deposition as a result of feeding diets with high starch levels may reduce liver function in rainbow trout. No effect of high carbohydrate diets on non-specific immunity has been found in trout, whereas serum haemolytic activity was negatively associated with dietary carbohydrate level in salmon. The effect of dietary starch level on mortality when fish are challenged with pathogens seems to vary with type of pathogen, environment and fish size.

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## 8. Carotenoids

*Trine Ytrestøyl and Bjørn Bjerken*

### 8.1. Introduction

The pink flesh colour unique to salmonid fishes is caused by deposition of carotenoids such as astaxanthin (3,3'-dihydroxy- $\beta,\beta$ -carotene-4,4'-dione) and canthaxanthin ( $\beta,\beta$ -carotene-4,4'-dione) in the muscle where the carotenoids are associated with the muscle protein fraction (Henmi et al., 1987, 1989). Salmonid fishes are able to deposit appreciable amounts of carotenoids in their muscle tissues. Carotenoids are synthesised *de novo* by plants, algae, certain types of bacteria and fungi. In plants, the main function of carotenoids is as light-harvesting accessory pigments and as protectants against photooxidation and quenchers of singlet oxygen. No animals, including fishes, are able to biosynthesise carotenoids and thus depend on a dietary supply. In animals, carotenoids are important in species-specific colouration of integuments and plumage and thus contribute to camouflage and communication (Brush 1981; Shahidi et al., 1998; Schiedt 1998). Colourful ornaments are often displayed by males and play an important role in reproduction through sexual selection. One of their most important physiological functions in animals is their action as vitamin A precursors. Almost all animals are able to enzymatically convert certain carotenoids into vitamin A. Canthaxanthin and astaxanthin serve as vitamin A precursors in salmonid fishes (Schiedt et al., 1985; Al-Khalifa and Simpson, 1988; Guillou et al., 1989; White et al., 2003). Carotenoids have also been shown to have beneficial effects on health and fecundity probably related to their effects as antioxidants, quenchers of free radicals such as singlet oxygen and as immunostimulants (Palace et al., 1999; Stahl and Sies, 2003; Amar et al., 2004; Fraser and Bramley, 2004; Ahmadi et al., 2006).

### 8.2. Reproduction and growth

Carotenoids accumulate in the reproductive organs of a wide range of organisms and their role in reproduction has been debated for more than 50 years (Goodwin, 1950; Craik, 1985; Palace and Werner, 2006). The carotenoids have been suggested to act as fertilisation hormones, to enhance growth, maturation and fecundity, as well as to reduce mortality during embryonic development and increase the ability to tolerate harsh environmental conditions such as high temperature, light and low oxygen (reviewed by Tacon, 1981; Craik, 1985; Choubert, 1986). The experimental evidence for this is conflicting and knowledge on this topic has been hampered by the lack of controlled fish experiments in which carotenoid

supply was the only variable. Some studies have found carotenoids to positively affect egg production and offspring growth or survival, whereas several others have failed to detect such effects. This could be due to the choice of parameters. Fertilization rate has often been used as an indicator of egg or embryo quality, but this may not always be the case. In the two-spotted goby (*Gobiusculus flavescens*), females fed diets supplemented with 135 mg astaxanthin per kg developed a stronger nuptial coloration, were more likely to spawn and also produced larvae that had a higher phototactic response compared to females fed diets without astaxanthin (Svensson et al., unpublished results). Phototaxis is crucial for survival of the larvae. However, commonly reported parameters for reproductive success, such as fertilization and hatching rates, were unaffected. In aquaculture, optimal broodstock diet formulation is essential for producing offspring with good quality. During sexual maturation, the carotenoids in the muscle of salmonid fishes are redistributed to the ovaries and accumulate in the eggs. The carotenoid concentration in the eggs usually reflects the concentration in the broodstock diet (Christiansen and Torrissen, 1997; Ahmadi et al., 2006). In female rainbow trout, dietary supplementation of astaxanthin to broodstock diets increased the fertilization rate, percentage of eyed and hatched eggs, and reduced mortality of eyed eggs. In males, astaxanthin supplementation had a positive effect on fertilization rate (Ahmadi et al., 2006). There seemed to be a critical dietary level between 0.07 and 12.5 mg astaxanthin per kg. Above 12.5 mg/kg there were no significant differences between the dietary astaxanthin concentrations. Thus, the astaxanthin concentration in broodstock diets for rainbow trout should be kept above this level. The critical concentrations in the rainbow trout eggs were somewhere between 2 and 10 mg/kg (Ahmadi et al., 2006). A critical level of 1-3 mg/kg in the eggs was suggested by Craik (1985) after reviewing many of the early studies on rainbow trout. In contrast, Tveranger (1986) fed diets containing astaxanthin concentrations between 1.4 and 4.2 mg/kg to rainbow trout broodstock and could not find any differences between the treatments on fertilisation rate, survival of eggs and alevins, and growth of fry during the start-feeding period. This is in accordance with Choubert et al. (1998) who fed diets supplemented with canthaxanthin to rainbow trout broodstock before spawning without observing any effect on the frequency of maturing females and the subsequent growth of fry. However, the carotenoid concentration of the control diet was not given in the study of Choubert and coworkers, and it could be above the critical level due to the contribution from the dietary ingredients. Deufel (1965) found an increase in both fertilization rate and the percentage fertile females after canthaxanthin supplementation (40 mg/kg) in the broodstock diet.



Astaxanthin supplementation to a broodstock diet (98.7 mg/kg vs. control level of 0.4 mg/kg) did not have a significant effect on the percentage of fertilised eggs, egg viability and embryo quality in Atlantic salmon (Christiansen and Torrissen, 1997). Torrissen (1984) did not find any effect of the carotenoid content of the eggs on survival of eggs and alevins in Atlantic salmon, but fry fed diets supplemented with 30 mg astaxanthin or canthaxanthin per kg grew faster than fry fed unsupplemented diets in the early start feeding period. Dietary supplementation of astaxanthin to semi-purified casein/gelatine based diets has later been shown to improve growth performance in first feeding fry, juveniles and parr of Atlantic salmon (Christiansen et al., 1994, 1995a,b; Christiansen and Torrissen, 1996). However, these studies were done without replication of some of the dietary treatments. Christensen et al. (1994, 1995a) found that astaxanthin had a positive effect on growth and survival in Atlantic salmon fry apart from its function as a source of vitamin A, but the authors questioned the bioavailability of the vitamin A source used in the study (a mixture of retinol palmitate and retinol acetate). A concentration of 5 mg/kg was recommended as a minimum in start feeding diets to secure maximum growth and survival (Christensen et al., 1995a). Torrissen and Christiansen (1995) suggested that astaxanthin and canthaxanthin should be considered as a vitamin for fish and supplemented in the diet at a level of minimum of 10 mg/kg dry diet. However, Atlantic salmon smolts fed diets supplemented with high levels of astaxanthin (48 mg/kg) had a reduced cataract frequency compared to smolts fed lower dietary astaxanthin levels (11 mg/kg) (Waagbø et al., 2003). Thus, dietary astaxanthin levels above 10 mg/kg might be necessary to secure optimal health in Atlantic salmon.

In his pioneering study, Steven (1949) believed that all the egg carotenoids of brown trout (*Salmo trutta*) were transferred to the embryo and fry where it was found predominantly as esters in the integument, but later studies with wild Atlantic salmon showed that about 30% of the egg astaxanthin disappeared during development to fry (Craik and Harvey, 1986). Irreversible transformation into vitamin A and retinoic acids and subsequent secretion via catabolic pathways may be responsible for at least part of the observed loss. In rainbow trout, the loss of egg carotenoids is about 60% when the fish reaches the start feeding stage (Bazyar Lakeh et al., unpublished results). Since these fishes are not eating at this stage the carotenoids must have been transformed to colourless metabolites. Astaxanthin is probably not an important vitamin A source at the egg stage, whereas it may serve as a precursor at the fry stage when a functional liver has been developed (Ørnsrud et al., 2004). The contribution of astaxanthin to the retinoid pool in eggs and fry of salmonid fishes remains to be determined. Effects of intact carotenoids or metabolites other than vitamin A should also be

considered. At present there is no knowledge on the biochemical basis of the effects of carotenoids during embryogenesis and later stages in salmonid fishes. However, a gene from zebrafish (*Danio rerio*) encoding the enzyme responsible for vitamin A formation,  $\beta,\beta$ -carotene-15,15'-oxygenase (*bcox*), has been cloned (Lampert et al., 2003). Targeted gene knockdown caused severe malformations of eyes, craniofacial skeleton and pectoral fins during embryonic development. Furthermore, retinoic acid formation depending on local *de novo* formation of retinal from provitamin A via *bcox* appeared to be essential. This suggests a crucial role of carotenoids in the early development of fish. The substrate requirements and putative roles of such enzymes in formation of retinoids in developing embryos of salmonid fishes and other farmed species require investigation.

### **8.3. Flesh quality**

In the seawater grow-out phase, the need for carotenoids is not defined by health and growth parameters but by the need to obtain a sufficient colouring of the flesh. Flesh colour is an important quality criterion for Atlantic salmon, and a mean astaxanthin level of 6-7 mg/kg in the muscle is considered sufficient to obtain an acceptable flesh colour. To achieve this target it is common practice to fortify diets with 20-75 mg carotenoids per kg during the entire seawater phase. The dietary dose may be changed during the grow-out phase and in general, small fish are fed higher doses of pigment than larger fish. In Norway, the main carotenoid source is synthetically manufactured astaxanthin. Canthaxanthin is also used by some feed producers, but the restrictions on the use of canthaxanthin posed by the EU (maximum 25 mg per kg feed) have reduced the importance of this carotenoid for the salmon industry. The muscle retention of carotenoids decreases with increasing dietary dose in a curvilinear fashion. For both Atlantic salmon and rainbow trout, the gain in flesh pigmentation is only minor when applying diet concentrations higher than 50-60 mg/kg (Bjerkeng et al., 1990; Torrissen et al., 1995; Forsberg and Guttormsen, 2006a). Apart from dietary concentration, the amount of carotenoid that is deposited in the flesh is also affected by several other factors such as diet composition, fish age, size, physiological status and genetics (Torrissen et al., 1989; Storebakken and No, 1992). In pre-smolts, the carotenoids are deposited mainly in the skin and the deposition in the muscle is limited (Storebakken et al., 1987; Bjerkeng et al., 1992). In post-smolts the carotenoid concentration in the muscle increases with size in a curvilinear fashion when the dietary concentration is kept constant throughout the seawater phase (Storebakken et al., 1987; Bjerkeng et al., 1992; Torrissen et al., 1995; Forsberg and Guttormsen, 2006a). The deposition of carotenoids ceases when the fish become sexually

mature, and instead there is a mobilisation of carotenoids from the muscle to the skin and reproductive organs (Bjerkeng et al., 1992; Schiedt, 1998). A pigmentation model for farmed Atlantic salmon based on published experimental data was recently published (Forsberg and Guttormsen, 2006a). This model may be used to predict the concentration of astaxanthin in the fillet as a function of fish weight and dietary astaxanthin concentration in the seawater phase. The model has been included in a mathematical program designed to predict the dietary astaxanthin concentration that gives an adequately pigmented fish at the minimum cost (Forsberg and Guttormsen, 2006b). The model is built on data from 6 different Norwegian pigmentation trials performed in the period 1993-2002. The model simulations show three general trends: 1) Smaller fish should be fed higher pigment concentrations than larger fish (fish of 0-2 kg are fed 20-40 mg more per kg diet than fish of 4-6 kg). 2) The optimal diet concentration is dependent on the flesh target concentration and increases by 10-30 mg/kg if the Salmofan score is increased from 27 to 28. This would increase the pigment cost by around 50%. 3) The optimal pigmentation strategy depends on the desired harvest size and thus how fast the targeted Salmofan score of 27 should be reached. The model of Forsberg and Guttormsen (2006a) assumes that fish weight and dietary astaxanthin concentration are the only variables that determine the astaxanthin concentration in the flesh. Specific dose-response relationships are in principle only valid under the experimental conditions under which they were obtained with respect to pigment dose and dietary composition, growth rate, temperature, genetic origin and physiological state of the fish material. Fish in small experimental units do not always grow as fast as fish under commercial production regimes and as the authors point out, the model should be used with caution and there is a need to validate the model on fast growing and well-performing Atlantic salmon.

How effective the dietary carotenoids are used for muscle pigmentation is defined as the bioavailability of the carotenoid (Jackson, 1997). A major determinant of nutrient bioavailability is the proportion absorbed from the gastrointestinal tract, often referred to as digestibility. The proximal intestine is the major site of absorption (Choubert et al., 1987; Al-Khalifa and Simpson, 1988; Torrissen et al., 1990; White et al., 2002). Digestion involves several steps from breakdown of the food matrix, solubilization of carotenoids into mixed bile salt micelles, movement across the unstirred water layer adjacent to the microvilli, uptake by the enterocyte and incorporation into chylomicrons (Furr and Clark, 1997; Tyssandier et al., 2001, 2002). Biotic or abiotic factors interfering with any of these steps may potentially have an effect on carotenoid bioavailability. The apparent digestibility of carotenoids in salmonid

fishes is quite low compared to that of essential nutrients, typically between 40 and 60% (Bjerkeng et al., 1997a; Bjerkeng and Berge, 2000; Ytrestøyl et al., 2005, 2006). The digestibility of carotenoids is negatively correlated with the dietary pigment concentration (Choubert and Storebakken, 1989; Bjerkeng et al., 1990; Torrissen et al., 1990, 1995) and recently, water temperature and feed intake were found to affect carotenoid digestibility. Reducing the ration level by 60%, from 0.44 to 0.17% of biomass, led to a 1.5 fold increase in astaxanthin digestibility (Ytrestøyl et al., 2006) whereas increasing the temperature from 8 to 12 °C improved the digestibility by 10% (Ytrestøyl et al., 2005). Thus, carotenoid digestibility and retention may vary with season, explaining the spring drop often observed in muscle pigmentation (Mørkøre and Rørvik, 2001).

The composition of the diet may be important for the utilisation of carotenoids. A high fat content in the diet has a positive effect on absorption and retention of carotenoids (Choubert et al., 1991; Torrissen et al., 1990; Bjerkeng et al., 1997b, 1999; Clark et al., 2000; Hamre et al., 2004). The quality of the dietary fat may also be important. A high content of polyunsaturated fatty acids has a positive effect on carotenoid bioavailability (Waagbø et al., 1993; Bjerkeng et al., 1999, 2000; Regost et al., 2004; Rørå et al., 2005), whereas a high content of water-soluble dietary fibre has a negative impact on digestibility of carotenoids (Riedl et al., 1999). Antinutritional factors present in many vegetable feedstuffs have also been found to have negative effects on nutrient and carotenoid digestibility (Storebakken et al., 2000; Refstie et al., 2005). Furthermore, in the gilthead sea bream (*Sparus aurata*), the effect of ration level on nutrient digestibility was dependent on the composition of the diet. Ration level had no effect on nutrient digestibility when the diet was based on capelin meal but there was a negative effect of a higher ration level when the diets were made from brown fish meal and trash fish meal (Fernández et al., 1998). The bioavailability of carotenoids should therefore, be considered when formulating diets for rapidly growing salmon; possible negative effects of certain feed ingredients on carotenoid digestibility may not become evident until growth rates are approaching the upper limit. A basic understanding of how temperature, growth rate and diet composition interact to affect solubilization and uptake of carotenoids from the gut is vital to develop a pigment strategy for salmonid fishes that ensures a flesh carotenoid concentration above the market demand.

#### **8.4. Concluding remarks**

It is documented that carotenoids have beneficial effects on health and fecundity of fish and should be included in the diet to ensure maximum growth and welfare of the animal.

Based on the present knowledge, a diet concentration of at least 10 mg/kg seems adequate in most cases. However, the dietary recommendations for Atlantic salmon fry and juveniles are based mainly on studies done more than ten years ago and where the fish had been fed synthetic casein/gelatine based diets which may have resulted in lower growth rates compared to commercial diets. The digestibility of carotenoids is rather low and is negatively affected by feed intake. In fast growing fish, particularly juveniles, the bioavailability may be very low and the dietary concentration should perhaps be increased. The use of vegetable feed ingredients is becoming more popular and this can also interfere with utilisation of carotenoids in the diet. Currently, there is limited information on how the astaxanthin concentration in broodstock diets for Atlantic salmon affects fecundity and growth of fry and fingerlings. In rainbow trout, recent results suggest that the dietary concentration should be at least 12.5 mg/kg. In the seawater grow-out phase, the need for carotenoids is not defined by health and growth parameters but by the need to obtain a sufficient colouring of the flesh. There is currently work in progress on how to optimize the dietary pigment concentration in Atlantic salmon, whereas there is a lack of studies aimed to develop an optimal pigmentation strategy for rainbow trout.

## 8.5. References

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