

# Adequately Defining the Amino Acid Requirements of Fish: The Case Example of Lysine

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## Abstract

A critical review of the literature highlights significant discrepancies in the estimates and modes of expression of amino acid requirements. Using lysine as a case example, this paper highlights some of these discrepancies and potential limitations of current approaches.

Published estimates of lysine requirements for rainbow trout varied from 1.3 – 2.9 % of the diet and NRC (1993) estimated lysine requirement at 1.8% of the diet. Results from recent studies and detailed data analysis suggest that lysine requirement to maximize weight gain is about 2.3% of the diet in rainbow trout, where requirement to maximize protein gain of this species appears to be closer to 2.7% of the diet. These estimates are significantly higher and appear more appropriate (robust) than the lysine requirement proposed by NRC (1993).

Different modes of expression of lysine requirement are used, often interchangeably, in the literature. It is important to understand that these different modes of expression are based on different assumptions and that the use of different modes of expression will result in dramatically different recommendations, especially since aquaculture feeds are formulated to widely different nutritional specifications (protein, energy, etc.). Studies suggest that expressing lysine requirement as a function of digestible energy or protein contents of the diet is not appropriate. Studies have also indicated that “newer” approaches of estimating amino acid requirements (e.g., factorial amino acid requirement, ideal protein concept), widely used in poultry and swine nutrition, may have significant pitfalls when used in fish nutrition.

## Introduction

Formulated aquaculture feeds are among the most expensive animal feeds on the market. Part of the high cost of aquaculture feeds is attributable to the use of high levels of expensive ingredients (e.g. fish meal, fish oil). In order to improve cost-effectiveness of feeds, fish meal is increasingly being replaced by more economical protein sources. This increased reliance on proteins with poorer amino acid profiles is bringing about the need to pay greater attention to the essential amino acids (EAA) requirements of fish.

Lysine is the first limiting EAA in many protein sources used in fish feeds (corn gluten meal, feather meal, etc.) and lysine-rich ingredients (fish meal, blood meal) are often expensive. Accurate definition of lysine requirements for cultured fish species is important to improve cost-effectiveness of feeds. A review of the literature reveals very significant gaps and idiosyncrasies in our understanding of amino acid nutrition of fish. Differences in the modes of expression used, as well as, in the estimates of amino acid requirements amongst studies, are extensive.

Recent studies on lysine utilization and requirement of rainbow trout have yielded very interesting results and have highlighted significant limitations or pitfalls associated with popular mode of expression of amino acid requirements (e.g. % of the diet, g/MJ DE, % protein) or methods of estimating amino acid requirements (e.g., factorial amino acid requirement, ideal protein concept).

## Variability of Lysine Requirement Estimates

The EAA requirements for several species of fish have been quantified over the past 40 years (Wilson, 2002). A number of empirical methods have been applied to establish the quantitative dietary EAA requirements of fish. Of the various methods used, most quantitative EAA requirements have been established by dose-response studies. Several reviews have identified the considerable variation in the estimates of AA requirements for fish (Tacon & Cowey 1985; Wilson, 1989; Cowey, 1994; Hauler & Carter 2001a). The values obtained indicate large variations not only in the estimates of requirements of different species but also a wide variation for estimates within species. The variation reported in requirement values between and within species is at least two-fold, and in some cases, even approaches three-fold.

Published estimates of lysine requirements for rainbow trout varied as widely (1.3 – 2.9 % of the diet; 3.7 – 7.3 % CP). It is unlikely that the biological variability within a single fish species could be responsible for the great variability in estimates. Careful analysis of the different requirement studies reveals a wide variation in experimental designs and conditions between the different laboratories/experiments (Table 1). Variations in AA estimates in dose-response experiments are typically regarded as the result of differences in methodology and interpretations between laboratories (Kim *et al.*, 1992a; Wilson, 1993; Cowey, 1994; Hauler and Carter, 2001a&b). Experimental design and conditions, such as fish size, culture protocols, model used, and basal diets composition differ significantly between laboratories/experiments, and can affect

requirement estimates. Some of the discrepancies observed may also be attributed to different overall levels of performance by the fish. In many studies, growth rates achieved with the semi-purified diets used appear much lower than what would have been achievable with a practical diet.

Table 1. Differences in experimental methodology adopted in lysine requirement studies for rainbow trout.

Author	IB W (g)	Feeding Regime	Diet CP %	Diet Lipid %	Diet Lysine levels	Lys range % Diet	Thermal Growth Coefficient (TGC)	Response Variable	Model	Estimated Lysine Requirement
Ketola, 1983	1	Pair-feeding	47	12	5	0.5-2.9	0.12	Weight gain	ANOVA	2.9% of diet 6.1% of CP
Walton <i>et al.</i> , , 1984	7	Satiety	45	17	7	1.0-2.6	0.17	Weight gain	Broken Line	1.9% diet 4.3 % CP
Lanari <i>et al.</i> , , 1991	107	Fixed Ration	40	n/a	n/a	n/a	n/a	Weight gain	Broken Line	2.2 % diet 5.41 % CP
Kim <i>et al.</i> , , 1992	14	Satiety	35	10	8	0.7-1.6	0.20	Weight gain	Broken Line	1.3% of diet 3.7 % CP
Pfeffer <i>et al.</i> , , 1992	17	Satiety	47	15	8	1.5-3.0	0.13	Protein gain	Polynomial	1.8 % diet 3.7 % CP
Rodehutschord <i>et al.</i> , , 1997	51	Satiety	32	28	21	0.5-5.8	0.23	Weight gain	Exponential	2.3 % of diet 7.3 % of CP
Encarnaç�o <i>et al.</i> , , 2004	24	Satiety	40	11 -24	6	1.2-2.4	0.22	Weight gain	Exponential	2.3 % of diet 5.8 % of CP

n/a – Not available

There is need to better understand the factors responsible for the wide variation and improve our understanding of requirements. Recent studies critically examining the effect of some “experimental” factors (e.g., data analysis procedure, parameter selection, diet composition) on estimates of lysine requirement of rainbow trout have yielded very interesting results and valuable lessons. They also indicated that there are significant limitations or pitfalls associated with popular mode of expression of amino acid requirements (e.g. % of the diet, g/MJ DE, % protein) or methods of estimating amino acid requirements (e.g., factorial amino acid requirement, ideal protein concept).

### **Impact of Data Analysis Procedure (Mathematical Model Selection)**

Early studies on lysine requirement estimated required levels by applying a range test (ANOVA) to dose-response data (e.g. Ketola, 1983). This approach has limited value, since it does not indicate accurately the level of nutrient needed to maximize the growth response. In dose-response studies, fitting response curves to the experimental data has been suggested as the preferred way of evaluation if the number of dietary levels is sufficient and if there is a sufficiently wide range in dietary supply (from highly deficient to a level definitely not promoting further increment) (Cowey, 1992; Rodehutsord & Pack, 1999). However, fitting response curves has been handled quite differently and several non-linear models as well as a linear (broken line) approach have been utilized.

### **Broken Line Model**

The broken line model has, by far, been the most popular model used in fish nutrition research. This linear model assumes that a growing animal will respond linearly to supplementation of a limiting indispensable nutrient until the exact requirement is met, after which no further (represented by a horizontal line (slope = 0) (Figure 1 A)), or negative, response will be observed.

The general equation of the broken line model is:

$$Y = f + 0.5 g (x - h - |x - h|)$$

Where  $Y$  is the response criteria (body weight gain g/fish),  $x$  is the independent variable (lysine concentration in g/kg diet)  $f$  is the ordinate,  $h$  abscissa of the breakpoint,  $g$  slope of the line for  $x < h$ .

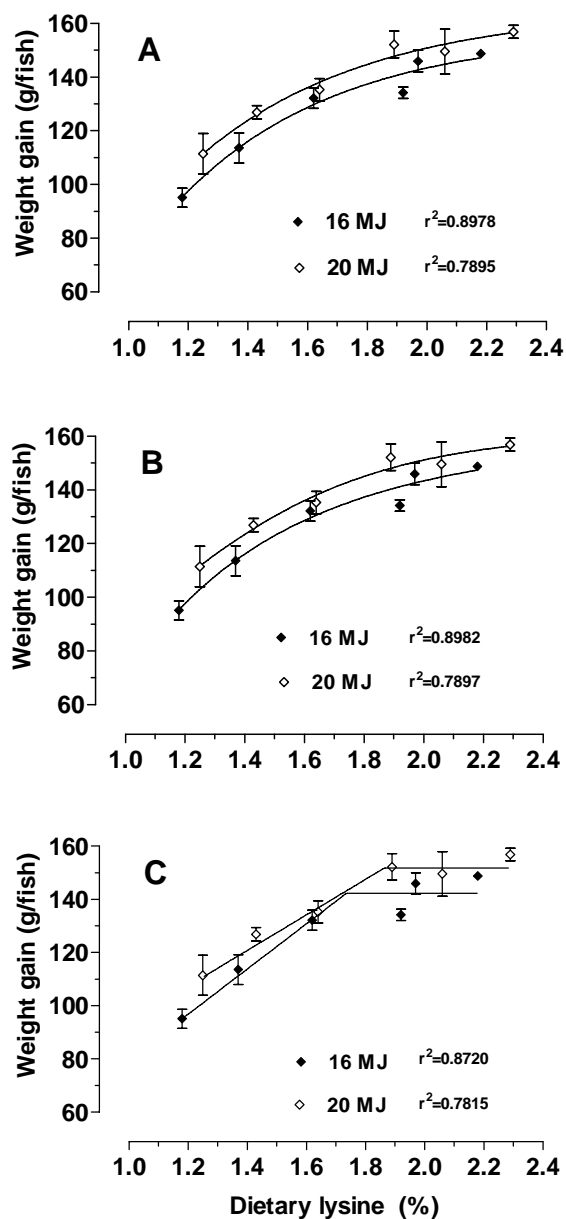


Figure 1. Models for the interpretation of dose-response experiments. A- Broken line, B- Exponential model. C- Sigmoidal model. Source: Encarnação *et al.*, (2004)

This model is fitted by obtaining the least squares estimates of  $f$  and  $g$  for several values of  $x$  using ordinary least squares technique. The maximum likelihood estimate of  $h$  is taken as the value of  $h$  which maximizes the model sum of squares (Robbins *et al.*, 1979).

For several amino acids, intake and weight gain are apparently linearly related, and the broken line regression became the model of choice for requirement studies, mainly because one single point could be objectively defined as “the requirement” which simplified the interpretation of response curves.

### Non-linear Models

Non-linear models are based on the biological principle often referred to as “law of diminishing returns”. Of all non-linear approaches, exponential (Rodehustcord, *et al.*, 1995a, 1997) and the sigmoidal (Mercer *et al.*, 1989; Gahl *et al.*, 1991) are the ones that have been more widely used in requirement studies.

For the exponential model, the response to increasing nutrient intake is not linear but follows the law of diminishing returns, which can be defined as the decreasing increment in gain as equal increments of nutrient are added to the diet near maximum gain (Gahl, *et al.*, 1991). At highly deficient levels, the first units of supplementation of the limiting amino acid will result in a significant increase in performance (linear response). The effects will diminish progressively with further increased levels of supplementation (the slope of the curve decreases progressively), until no further increase in performance is achieved. The estimate level of requirement from non-linear curves is generally calculated as the level of the AA required to achieve 95 % of maximum performance (weight gain or protein gain).

The exponential model (Figure 1 B) derived from Rodehustcord *et al.*, (1995) is:

$$Y = Y_{max} (1 - e^{-b(x - c)})$$

Where  $Y_{max}$  is the plateau of the curve (upper asymptote),  $b$  is the parameter describing the steepness of the curve and  $c$  is  $x$  at  $y = 0$ .

The sigmoidal response model comprises 4 parameters to describe the sigmoidal shape of the response curve (Mercer *et al.*, 1989). As defined by Mercer *et al.*, (1989), the different sections of this nutrient response curve are threshold, deficient, adequate and optimal. An inhibition constant was then included for the development of Saturated Kinetic Model.

The sigmoidal model (Figure 1 C) derived from Gahl *et al.*, (1991) is:

$$Y = \frac{Y_{max} + [d(1 + m) - Y_{max}] e^{-kx}}{1 + m e^{-kx}}$$

Where  $Y_{max}$  is the plateau of the curve (upper asymptote),  $d$  intercept of  $y$  axis,  $k$  scaling parameter that scales  $x$ , and  $m$  shaping parameter that locates the inflection point.

A non-linear approach model has been suggested as the most appropriate to describe the response from a biological background (Mercer *et al.*, 1989; Gahl, *et al.*, 1991; Cowey, 1994; NRC, 1993; Rodehutsord & Pack 1999). Use of different models, however, must take into consideration the experimental design, namely the range of the concentration of the nutrient under test. In cases where a very wide range in dietary concentration (concentration varying from extremely deficient up to concentration where the response is clearly inhibited) the Saturation Kinetics Model (SKM) with inhibition appears the suitable approach (Rodehutsord & Pack 1999). This is, however, not the case for most of the EAA requirement studies published so far. Extremely high concentrations of the studied AA are rarely included in the experimental design.

The sigmoidal approach generally appears superior to the exponential approach in requirement studies where the concentration of the studied AA varies from severely deficient to levels high above the optimum level, since it better describes the linear or near linear part of the ascending section of the response curve (Rodehutsord & Pack 1999). However, the more restricted the range of the tested AA is, the more it will be justified to use the simple, easy to interpret exponential approach (Rodehutsord & Pack 1999). This is of particular interest in studies where the AA concentrations of dietary ingredients do not allow the formulation of diets severely deficient in the EAA studied.

A re-evaluation of EAA requirements for rainbow trout using different mathematical approaches, exponential model (Rodehutsord, *et al.*, 1995a), four parameter logistic function (Gahl, *et al.*, 1991), saturation kinetics model (Mercer *et al.*, 1989), and the broken line model (adaptation of Robbins *et al.*, 1979), demonstrated that requirement derived from these experiments were considerably different depending on the model used (Rodehutsord & Pack 1999). The use of the broken line model resulted in the lowest requirement values for the AAs analyzed, while the requirements obtained using the exponential model were the highest.

In a recent lysine requirement study, Encarnação *et al.*, (2004) observed that dietary lysine requirement estimates obtained using the broken line approach resulted in lower requirement value (1.8 % of diet) compared to an exponential and four-parameter logistic equation models (2.3 % of diet) (Figure 2). The estimated requirement value of 2.3 % of diet was in good agreement with that of Rodehutsord, *et al.*, (1997) who used a similar dietary and data analysis model. These studies indicate that requirement values proposed by NRC (1993) for rainbow trout (1.8 % of diet) are significantly below what is required to maximize weight gains. This is mainly a result of the fact that broken-line analysis advocated by NRC (1993) does not very faithfully represent the response of the animal to increasing levels of this amino acid. The intercept of the two linear functions is clearly below the lysine level maximizing weight gain (Figure 3).



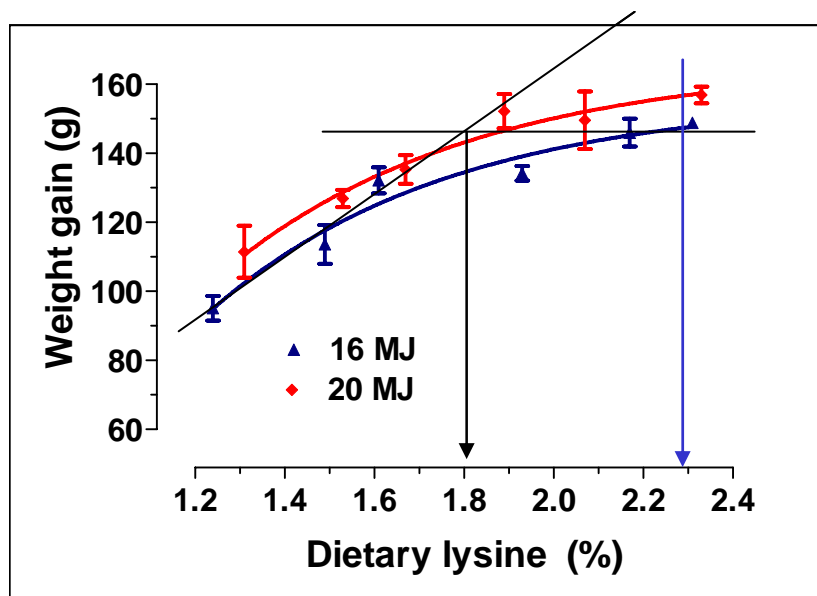


Figure 2 – Live weight gain response to dietary lysine levels at two diet energy levels (16 and 20 MJ DE/kg) after 12 week of dietary treatment. Values are mean weight gain per fish  $\pm$  SD. Using an exponential model, requirement for achieving 95% of maximum performance was 2.3 % diet for the 16 MJ and 20 MJ DE diets. Using the broken model, requirement was estimated at 1.8% of the diet for both types of diets. Data: Encarnaç o *et al.*, (2004)

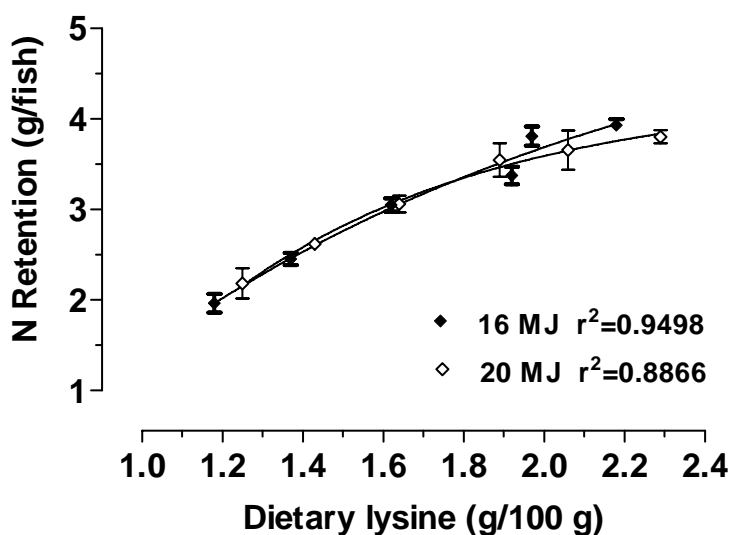


Figure 3 - Nitrogen retention in response to increasing dietary lysine level at two digestible energy levels (16 and 20 MJ). Using an exponential model, requirement for achieving 95% of maximum N retention was 2.7 % diet for the 16 MJ and 20 MJ DE diets.

Source: Encarnaç o *et al.*, (2004)

Supporting experimental evidence for the lysine requirement estimates of Encarnaç o *et al.*, (2004) and Rodehutschord *et al.*, (1997) is provided in Table 2. Bureau *et al.*, (unpublished) observed that a corn gluten meal-based diet (49% corn gluten meal, 18% fish meal) formulated to meet NRC (1993) lysine requirement for rainbow trout (1.8% lysine) supported significantly a lower growth rate (expressed as thermal-unit growth coefficient) and feed efficiency than a fish meal based diet containing 3.2% lysine (Table 2, depletion period). Simply supplementing the corn gluten meal based diet with 0.5% lysine HCL significantly improved growth rate and feed efficiency of the fish to values comparable to those obtained with the fish meal-based diet (Table 2, repletion period). More evidence is provided by Bureau and Cho (2000) who observed that a high quality diet, containing corn gluten meal and fish meal as the main protein sources containing 2.1% lysine performed significantly less well than fish fed diets formulated with the same ingredients but containing 2.5 to 2.9% lysine (Table 3).

Table. 2 - Performance of rainbow trout fed diets with different lysine levels during a depletion period (week 1 to 12) and a repletion period (week 13 to 16) (data from Bureau *et al.*, , unpublished).

	<b>Fish meal-based diet with 3.2% lysine</b>	<b>Corn gluten meal-based diet with 1.8% lysine</b>
<b>Part 1 (weeks 1 to 12)</b>		
Thermal-unit growth coefficient <sup>1</sup>	0.261 a	0.209 b
Feed efficiency, gain/feed	1.19 a	0.94 b
<b>Repletion trial</b>		
	<b>Fish meal-based diet with 3.2% lysine</b>	<b>Corn gluten meal-based diet+ 0.5% Lysine HCl 2.3% lysine</b>
<b>Part 2 (weeks 13 to 16)</b>		
Thermal-unit growth coefficient <sup>1</sup>	0.260 a	0.275 a
Feed efficiency, gain/feed	1.07 a	1.11 a

<sup>1</sup>Thermal-unit growth coefficient (TGC) = (Final body weight)<sup>1/3</sup> - (Initial body weight)<sup>1/3</sup> /  $\Sigma$  (Temperature ( C) x Days)

Table 3. Growth performance of Atlantic salmon fed soybean meal and corn gluten meal–based diets for 24 weeks.

Ingredients	Diet					
	1	2	3	4	5	6
Fish meal, herring, 68% CP	20	20	20	20	56	38
Corn gluten meal, 60% CP	10	20	30	40	-	20
Soybean meal, 48% CP	35	23	12	-	-	-
Blood cell meal, spray-dried	5	5	5	5	5	5
<b>Composition</b>						
Digestible protein (DP), %	39.4	39.8	40.6	40.9	40.7	40.9
Digestible energy (DE), MJ/kg	19.6	19.9	20.2	20.5	19.9	20.2
DP:DE, g/MJ	20.0	20.0	20.0	20.0	20.4	20.2
Lysine, %	3.1	2.7	2.5	2.1	3.6	2.9
<b>Performance</b>						
Initial body weight, g/fish	5.3	5.3	5.3	5.3	5.3	5.4
Final body weight, g/fish	82.8*	87.9	86.8	81.0*	85.8	87.8
Feed efficiency, gain: feed	1.16*	1.24	1.22	1.16*	1.21	1.24

\* Significantly different from control diet (Diet 6).

Source: Bureau and Cho (2000)

### Impact of Response Parameter Selection

Live weight gain is commonly used as the main (and often only) response criterion in essential amino acid studies. However, evidence suggests that protein deposition may be a more robust parameter. Animals will seek to eat sufficient of a nutritionally balanced food/feed to try to follow a genetically determined growth path (Oldham *et al.*, 1997), where maximal protein accretion and associated carcass lean growth rate determine nutrient requirements for growth and composition of growth (Schinkel and de Lange, 1996). Thus, and since, protein deposition is the main determinant of amino acid requirements, information on the requirement level should ideally be based in relation to protein or lysine retention, as proposed by Rodehutsord *et al.*, (1997) Susenbeth *et al.*, (1999), and Hauler & Carter, (2001b) .

Evidence from the studies of Encarnaç o *et al.*, (2004), Rodehutsord *et al.*, (1997), and Rodehutsord and Pack (1999) suggest that lysine requirement to maximize weight gain is about 2.2 to 2.3% of the diet where requirement to maximize protein gain appears to be closer to 2.7% of the diet. Encarnaç o *et al.*, (2004) observed that fish fed diets with about 2.2% lysine were fatter than fish fed diets with 2.7% lysine. Recent experimental evidence (Wang *et al.*, , unpublished) indicated that body protein concentration of rainbow trout increases steadily up to 2.7 or 2.8% lysine in the diet. The impact of the increase in protein accretion at high dietary lysine concentration (e.g. 2.7%) on flesh quality and pigmentation, or marketable product yield (e.g. fillet yield) remains to be explored. However, studies on broiler chicken have suggested significantly better breast meat yield in animal receiving diets with higher lysine levels than that required to maximize weight gain (Rezaei *et al.*, 2004).

## Appropriate Mode of Expressing EAA Requirements

A survey of the fish nutrition literature indicates that a variety of modes of expression have been adopted to present EAA requirements. These different modes of expression have been used interchangeably in scientific reviews. There are significant differences in opinion as to the most appropriate mode of expressing AA and this issue has been debated in various publications over the past several years (Wilson, 1985; Cowey, 1994; Rodehutsord *et al.*, 1997; Encarnação & Bureau, 2001).

Amino acid requirements of fish may be expressed as diet concentration or percentage of diet (% diet), per unit of DE (g/MJ DE) or as a proportion of diet protein content (g/16 g N) (Table 4). Traditionally, EAA requirements of fish and other animals have been expressed as % of diet (NRC, 1993). This approach implies that diet composition does not influence EAA requirement. Pfeffer *et al.*, (1992) and Rodehutsord *et al.*, (1995a, 1997, 1999), based on the evidence derived from other monogastric species (swine and poultry), hypothesized that EAA requirements of fish should be expressed per unit of diet digestible energy (g/MJ DE). These researchers suggested that dietary DE content determines feed intake of the fish, and that high-energy diets will require a higher EAA concentration to compensate for a lower feed intake by the fish.

Table 4. Lysine requirement of salmonid fish according to different schools of thinking or modes of expression.

References	Requirement
NRC (1993)	1.8 % diet
Encarnação and Bureau (2001)*	1.2 g/MJ digestible energy
Mambrini and Guillaume(1999)	4.8 % protein (g/ 16 g N)

\*Interpretation of NRC (1993) requirement based on Rodehutsord *et al.*, (1997) recommendation to express requirement in relation to digestible energy content of the diet.

In contrast, Cowey and Cho (1993) and Mambrini and Guillaume (1999) suggested that EAA requirements should be expressed as a proportion of dietary protein. Cowey and Cho (1993) assumed that efficiency of utilization of the first limiting dietary EAA decreases with increasing protein level. At higher levels of protein, or higher protein: energy ratios, more AA are catabolized to supply energy, and the first limiting dietary EAA may not be spared at the expense of NEAA or other non-limiting EAA. This assumption seems to imply that the AAs catabolized for energy need to be in same balance as needed for maintenance and protein production.

EAA requirements established by dose response studies have been frequently expressed as percentage of dietary protein. In such studies, to avoid deficiencies and to ensure that the tested EAA is the first limiting at all tested levels, the remaining EAA (and NEAA) are supplied in excess of their requirements. This relative oversupply of dietary AAs should result in an underestimation of AA requirements when expressed as percentage of dietary protein. Moreover,

the use of diets with excessive protein for a particular life-stage of a fish species is likely to have underestimated requirements expressed as percentage of dietary protein (Hauler & Carter, 2001a). Expressing EAA requirements as % of protein, would nevertheless, make sense in the context of a low protein feed formulated based on the ideal protein concept where each EAA is equally limiting to protein accretion. It also requires that requirements for both balanced protein and the AA composition of ideal protein is established.

These different modes of expression adopted in the literature affect how the information on EAA requirements is used when formulating practical cost-effective feeds and can result in diametrically different recommendations (Table 5). Individual EAA levels deemed adequate in the diet may be very different depending on the mode of expression adopted, the composition of the diet formulated, and the AA composition of the ingredients used in the formulation. The magnitude of these differences is non-negligible given the cost of lysine or lysine-rich ingredients (fish meal, blood meal). More importantly, these dramatically different recommendations cast doubt over the true benefit of considerable investment of research dollars for amino acid requirement studies of various species. A great deal of effort, and resources, is invested in very accurately determining the “minimum” amount of a given amino acid required to maximize performance. It is, consequently, rather disturbing to note that this very precise information, when used in practice, results in dramatically different recommendations, depending on which mode of expression is used. This “unacceptable” situation is due to the fact that most studies have focused on requirement for individual EAA but very few studies have attempted to integrate this knowledge and apply it within a practical context. It is also mainly due to the fact that the impact of diet composition on amino acid utilization and requirement of fish (and other animals!) has been poorly studied and remains poorly understood.

Table 5. Dietary lysine level expected to meet requirement of rainbow trout fed different feed formulae according to different schools of thinking or modes of expression of requirement.

<b>Composition</b>	<b>Starter</b>	<b>Grower</b>	<b>High energy</b>
Crude Protein, %	50	42	45
Lipids, %	12	18	25
Digestible energy, MJ/kg	17	17	20
<b>School of thought**</b>	<b>Lysine level deemed adequate (g/kg feed)</b>		
1) % diet	18	18	18
2) g/MJ DE	20	20	24
3) % Protein	24	20	22
<b>High-Low, % difference</b>	<b>32</b>	<b>13</b>	<b>33</b>

\*\* See Table 4

Expression of EAA requirements in relation to DE content of the diet (g/MJ DE) is also based on the assumption that the energy content in the diet will determine the level of feed intake by the fish, and, consequently have a direct effect on EAA intake. This assumption is derived from evidence in the literature showing that when offered diets with various DE levels; fish appear to adjust their feed intake to maintain a particular (daily) energy intake (Jobling & Wandsvik, 1983; Boujard & Medale, 1994; Kaushik & Medale, 1994; Yamamoto *et al.*, 2000a; Yamamoto *et al.*, 2002; Yamamoto *et al.*, 2005). However, Encarnaç o *et al.*, (2004) obtained very similar estimates of lysine requirement (2.28 vs 2.33 % diet) with diets with different dietary DE levels (16 vs 20 MJ DE). The observations of Encarnaç o *et al.*, (2004) suggest that expressing EAA requirements in relation to DE content of the diet is not entirely appropriate. Fish fed high DE diets achieved higher weight gain and similar protein deposition at lower levels of feed intake as compared to fish fed the low DE diets (Figures 2 and 3). The lower feed intake observed with increasing diet DE density was offset by a higher efficiency of utilizing lysine for body protein deposition, especially at marginally deficient levels of lysine intake (Figure 4). Subsequent studies, however, suggest that this “lysine-sparing” effect is dependent on the dietary DE source used (Encarnaç o *et al.*, 2006). Fatty acids appear to be more effective in improving lysine and protein deposition when compared to other energy-yielding nutrients, such as amino acids (Encarnaç o *et al.*, 2006). This further supports the conclusion that expressing EAA requirements in relation to diet DE content is probably not adequate. Results from Encarnaç o *et al.*, (2004), however, suggest that fish regulate their feed consumption to achieve a certain target protein deposition, rather than adjust their feed intake to maintain a particular DE intake. In pigs, several studies have showed that lysine utilization for protein retention is independent of energy intake, when lysine intake is below the level necessary to meet protein retention capacity (Susenbeth 1995, Susenbeth *et al.*, 1999, Mohn *et al.*, 2000). As shown by Moehn *et al.*, (2000), energy and lysine had independent effects on body protein deposition. Energy intake did not affect protein deposition (and lysine deposition) when lysine intake was limiting and vice versa. Encarnaç o, *et al.*, (2004) showed that even at marginal levels of intake, efficiency of lysine utilization is affected by the DE level in the diet in rainbow trout. This finding suggest that regulation of lysine utilization in fish is different from pigs and poultry, in which AA catabolism appears to play a much smaller role in supplying energy than carbohydrates and lipids. Furthermore, energy concentration and lipid levels in pig and poultry diets are much lower than in fish diets. Therefore, caution is warranted when transposing concepts or aspects of AA utilization in other monogastric animals to fish species.

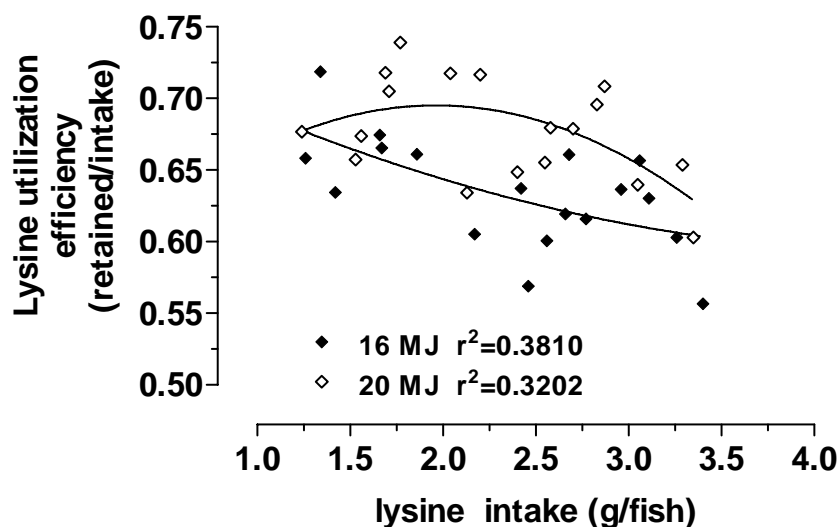


Figure 4 - Effect of lysine and energy intake on efficiency of lysine utilization. Efficiency was calculated as: g lysine retained per fish / g lysine intake per fish. Each point represents the mean per fish (n = 20) for each tank. Response is described by the equations:  $Y = 0.7516 - 0.0689x + 0.00745x^2$  for 16 MJ diets, and  $Y = 0.5625 + 0.1352x - 0.0345x^2$  for 20 MJ diets. Source: Encarnaç o *et al.*, (2004).

Essential AA requirements established by dose response studies have been very frequently expressed in relation to dietary protein on the assumptions that EAA requirements are dependent on the protein content of the diet, and that a certain balance between amino acids need to be respected (Cowey & Cho, 1993). Recent studies by Abboudi *et al.*, (2002) and Webb & Gatlin (2002), reported that lysine requirement (% diet) of Atlantic salmon fry (Abboudi *et al.*, 2002), and red drum (Webb & Gatlin, 2002), was independent of dietary protein level (% diet). These results suggest that expressing EAA requirements as a % of protein is not totally appropriate. In the study of Webb & Gatlin (2002), similar estimates of lysine requirement for red drum (1.5 % diet), were obtained using diets with different dietary protein levels (35 % and 45 % CP). Evidence generated by Encarnaç o *et al.*, (2006) corroborates these findings. Requirement studies with rainbow trout, using similar dietary models but differing in the dietary protein level (32 % CP; Rodehutschord *et al.*, (1997), and 40 % CP; Encarnaç o *et al.*, (2004)), achieved similar growth rates and similar estimates of lysine requirement, expressed as diet concentration (2.3 % of diet). Dramatically different estimates of requirements (7.3 g/16 g N; Rodehutschord *et al.*, (1997) vs. 5.8 g / 16 g N) are obtained when lysine requirements are expressed in relation to protein content of the diet, thus suggesting that expressing lysine requirements as a % of protein is generally not appropriate.

Expressing AA requirements as a function of protein content of the diet implies an assumption that catabolism of limiting EAA is dependent on overall AA catabolism (Cowey & Cho, 1993). At higher levels of protein, or higher digestible protein to digestible energy (DP/DE) ratios, more

amino acids are catabolized to be used as energy sources, and it is assumed that the limiting dietary EAA may not be spared at the expense of other non-limiting EAA. Results from Encarnaç o *et al.*, (2006) showed that increasing dietary DP level with different AAs had no effect on protein gain or efficiency of lysine utilization at marginal levels of lysine intake. This indicates that the catabolism of excess AA in the diet does not affect the inevitable catabolism of the limiting EAA. This finding is pretty meaningful since some protein sources with imperfect amino acid profile (e.g. feather meal) may actually be economical DE sources, especially in a context of high fish and plant oil prices, such as the one seen recently (e.g. fish oil and soya oil price = USD \$600-900/metric ton in July 2006) or for species with poor tolerance to high lipid or carbohydrate levels (e.g. marine carnivorous fish species). Expression of EAA requirement in relation to protein may result in a “need” for amino acid supplementation in diets formulated to high protein (and DE) levels with “imbalanced” economical protein sources.

Expressing EAA requirements as % of protein should, nevertheless, make sense in the context of a low protein feed formulated, based on the ideal protein concept, where each EAA is equally limiting to protein accretion.

### **Ideal Protein Concept**

According to the ideal protein concept, optimum dietary essential amino acid quantities are considered as proportions relative to total EAAs, rather than as proportions relative to the whole diet (Wang and Fuller, 1989). The optimum dietary AA pattern for an animal can be defined as the ideal ratio among dietary AAs required for maintenance and production, which produces maximum N retention for a given N intake. Once a pattern of EAA has been identified as being representative of the ideal dietary pattern, one AA is estimated quantitatively by dose response, while the remainders are predicted from the pattern. Ideal amino acid patterns are usually stated as the ratio of each of the 10 EAA to lysine, which is given the arbitrary value of 100.

Although the ideal protein concept has been used in the formulation of feeds for pigs and chickens for several years, there has been relatively little research regarding ideal EAA patterns for fish. Studies have confirmed the applicability of the method proposed by Wang and Fuller (1989) to fish (Green & Hardy, 2002a&b; Rollin *et al.*, 2003a). These alternate methods may be useful as guidelines to establish an EAA profile to formulate practical or experimental diets for fish species whose EAA requirements are partially unknown. They fail, however, to consider possible effects of the physiological status, diet composition and culture conditions on requirement estimates (Tibaldi & Kaushik, 2002).

The methodology of Wang and Fuller (1989) assumes that EAA are utilized with similar efficiencies and that maintenance requirements for all EAA are similar. Results for swine, chickens and rat suggest that most EAA are used with a similar efficiency, except for methionine and cysteine, and threonine (Moehn, 2004). In fish, however, recent studies suggest that it is possible that different EAA are not utilized with the same efficiency (Rodehutsord *et al.*, 1995b; Rollin *et al.*, , 2003a; Ronnestad *et al.*, 2001; Conceiç o, *et al.*, 2003a).



The ideal protein concept assumes that efficiency of utilization of the first limiting dietary EAA decreases with increasing protein level. At higher levels of protein, or higher protein:energy ratios, more AA are catabolized to supply energy, and the first limiting dietary EAA is assumed not be spared at the expense of NEAA or other non-limiting EAA. This assumption also implies that the AAs catabolized for energy need to be in same balance as needed for maintenance and protein production, and this is probably not true. The use of the ideal amino acid concept would call for a “need” to “balance” the EAA composition of feeds formulated to high protein contents (e.g. to cost-effectively increase DE level of the feed) using protein sources with “imperfect EAA profiles”. The need to “balance” the EAA content of the feed, at high protein levels, is still a matter of debate.

Studies have suggested that “imbalances” in the amino acid profile of the diet may depress feed intake and reduce efficiency of utilization of EAA. Yamamoto *et al.*, (2000b) showed that when allowed self-selection, rainbow trout showed preference for diets with balanced EAA profiles, regardless of the dietary protein level. In poultry most of the experiments with imbalanced mixtures of EAA reported a depression in food intake but showed no reduction in utilization of the first EAA when growth rate is regressed on AA intake (Boorman & Ellis, 1996). A reduction in feed intake of fish fed diets with imbalanced EAA profiles has also been reported in fish (Fauconneau, 1988, Mambrini & Kaushik, 1994, Davis & Morris, 1997, Green *et al.*, 2002a). The theoretical mechanism by which feed intake is reduced when imbalanced diets are fed, has been described by Harper *et al.*, (1964). These workers suggested that plasma AAs stimulate the synthesis or inhibit the breakdown of proteins in the liver which implies a decreased supply of the limiting EAA in tissues. Consequently, the free amino acid pattern of plasma and tissue becomes imbalanced which is detected by the appetite regulating system in the brain. As a consequence, feed intake is reduced and this reduction on feed intake is at the origin of the growth depression effect of amino acid imbalances. Encarnaç o *et al.*, (2006) observed that imbalance levels of certain amino acids (pyruvate precursors) seemed to affect fish performance by reducing feed intake; however, these amino acids only induces negative impacts in growth performance when the diet was deficient in lysine. Rainbow trout had a higher tolerance for an excess of TCA cycle intermediates precursors of (glu, gln, asp). The deleterious effects of these “imbalances” appear, however, to be highly on dependent on the types and levels of amino acids in excess. Most studies have been conducted either with diets containing high levels of gelatin vs. diets with high levels of casein, or through the use of synthetic (dispensable) amino acid supplements. These dietary manipulations are not realistic since there are clearly not representative of the types of “amino acid imbalance” observed under practical conditions. Preliminary observations suggest that practical (realistic) amino acid imbalances created by high levels of feather meal or gelatin had no effect on feed intake, growth performance of rainbow trout (Elharoun and Bureau, 2006).

## Crystalline Amino Acids: Potential Limitations?

Concurrent to the increase utilization of more economical protein sources, crystalline amino acids are becoming increasingly used to meet AA requirement of the fish and are, as such, becoming key components of cost-effective fish feed formulae. The efficiency with which dietary free AAs are used by fish differs among published reports. Several studies observed that crystalline AAs were utilized as efficiently as those of intact protein origin in meeting EAA requirements of fish (Murai *et al.*, 1987; Kim *et al.*, 1987; Kim *et al.*, 1991; Rodehutschord *et al.*, 1995b; Williams *et al.*, 2001). Murai *et al.*, (1987) observed that free AAs from either casein or a crystalline AAs diet, were absorbed at similar rates, in a pattern reflecting dietary AA profile with the exception of some EAA. Efficiency of crystalline AA utilization is apparently dependent on dietary protein level, being lower at higher protein diets and higher for low protein diets (Williams *et al.*, 2001).

Conversely, studies with rainbow trout (Yamada, *et al.*, 1981b; Murai *et al.*, 1987; Schuhmacher *et al.*, 1993; Schuhmacher *et al.*, 1997), carp (Yamada *et al.*, 1981a), catfish (Zarate & Lovell, 1997), and Atlantic salmon (Sveier *et al.*, 2001) have indicated that free AAs do not appear to be utilized as effectively as the AAs in intact protein. Diets that contain high levels (> 20%) of free AAs have proven to be less effective in promoting growth of fish than diets that contain only intact protein (Cowey, 1994). Elharoun and Bureau (2004) have observed that digestible lysine from blood meal was significantly more bioavailable, i.e. more efficiently utilized, at low levels, than free lysine.

These results may be the result of faster absorption rate for free lysine compared to protein-derived lysine. Free lysine has been shown to have a shorter residence time in the stomach compared to protein-bound lysine. Several studies with terrestrial (Rolls *et al.*, 1972; Butts *et al.*, 1993; Schumacher *et al.*, 1997) and aquatic animals (Yamada *et al.*, 1981; Kaushik and Dabrowski, 1983; Murai *et al.*, 1987; Cowey and Walton, 1988) have shown that free amino acids are absorbed much more rapidly than protein bound amino acids. Working with channel catfish, Zarate *et al.*, (1999) observed that the ratio between lysine and digestion indicator ( $\text{Cr}_2\text{O}_3$ ) in the stomach remained fairly constant in fish fed a diet in which lysine was mainly provided by soybean meal, whereas it dropped significantly in fish fed a diet in which 50% of the lysine originated from L-lysine HCl. Zarate *et al.*, (1999), concluded that free lysine leaves the stomach more rapidly than protein-bound lysine. Tantikitti and March (1995) studied plasma amino acid profiles in the systemic circulation of rainbow trout acclimated to diets containing different protein sources, with and without supplementation with free amino acids. Plasma concentrations of lysine, methionine, and isoleucine increased more rapidly after feeding a diet supplemented with free amino acids than fish fed the basal diet. This faster absorption may result in temporary higher tissue concentration of the amino acid provided in the free form and resulting in greater proportion of this amino acid being catabolized (Batterham, 1984; Schumacher *et al.*, 1997). Batterham and Morrison (1981), Batterham (1984), Tantikitti and March (1995), and Zarate *et al.*, (1999) provide evidence of better metabolic utilization of lysine in animals fed more frequently. Reducing the solubility and absorption rate of free amino acids

using coating or encapsulation techniques (Chen *et al.*, 1992; Teshima *et al.*, 1990, 1992; Fox *et al.*, 1995; Segovia-Quintero *et al.*, 2004) reportedly improves the efficiency of utilization of these amino acids. The efficiency of utilization of free amino acids appears to be a dynamic function on how dietary and physiological factors interact. These observations do raise some question as to the accuracy and applicability of EAA requirement estimates obtained from dose response studies with free amino acids. It appears advisable to use slightly higher “safety margin” when meeting a significant proportion of the requirement for an amino acid using free amino acids, compared to a highly digestible protein-bound amino acid source.

### **Use and Limitations of Factorial Models of Amino Acid Requirements**

A currently popular method to estimate EAA requirement is to mathematically represent the main biological processes that determine EAA utilization for body protein deposition (PD). Factorial models that distinguish needs for maintenance and growth have been used to estimate EAA requirements in poultry (Baker, 1991) in swine (Moughan, 1989; Susenbeth, 1995), and in fish (Hauler & Carter, 2000b).

The simplest factorial approach assumes constant lysine retention per unit of live weight gain or protein deposition, a constant efficiency of lysine utilization and the addition of obligatory lysine loss (maintenance). Lysine requirement (g/animal/day) can then be calculated by multiplying protein retention by lysine concentration in the protein retained and dividing by a constant efficiency of utilization (Susenbeth, 1995). Dietary lysine concentration can be calculated from predicted feed intake or feed conversion ratio.

The efficiency, with which each absorbed EAA is retained, however, is controversial, as is the question of whether this efficiency decreases or remains constant as amino acid intakes above maintenance increase. Several studies focusing on EAA accretion over a wide range of dosing levels have generally reported results that suggest that EAA is a linear function of EAA intake (Susenbeth, 1995; Adeola, 1995; Baker, 1991, Baker *et al.*, 1996; Edwards *et al.*, 1997; Edwards *et al.*, 1999).

The curve-fitting procedures of Gahl *et al.*, (1991, 1995), however, indicated a decrease in efficiency of utilization as limiting EAA intakes increased above maintenance (diminishing returns). Studies in which efficiencies of EAA utilization have been compared suggest that the efficiencies are different for each EAA (Fuller, 1994; Adeola, 1995; Gahl *et al.*, 1997). Accurate measurement of efficiencies, however, present some complicated problems because they involve both growth and consumption data (Baker, 1986). The variation in body weight range (among experimental animals), or time period, over which EAA utilization/requirements is studied, is an important aspect that should be taken in consideration (Mohn *et al.*, 2000; Mohn *et al.*, 2004). Any function describing response to a nutrient includes variation between individuals in at least some of its parameters (Fuller & Garthwaite, 1993). Due to variability in amino acid requirements for maximum PD between individual animals or due to changes over time in feed intake and maximum PD, the linear plateau relationship that applies to one animal and over a

short time (e.g. 1 day) period will transform into a non linear relationship in a group of animals, in particular when responses are measured over extended periods. The constant marginal efficiency of EAA utilization will automatically change into a non-linear marginal efficiency, if utilization efficiency is measured over multiple days, simply because dietary EAA intake (of individual animals within a group) can be below and above requirements for maximum PD for part of the multiple experimental period within each experimental treatments/dietary AA levels. Due to the inter-animal variation, the mean efficiency of nutrient utilization determined in groups of animals will be lower than that determined in individual animals, especially when nutrient intake levels are close to those of requirements for maximum performance (Baker, 1986).

Hauler & Carter (2001a), through a review of published dose-response studies, observed that similar to studies in pigs (Susenbeth, 1995, Adeola, 1995) and poultry (Baker, 1991, Baker *et al.*, 1996, Edwards *et al.*, 1999), in fish, the marginal efficiency of lysine utilization for live weight gain appeared to be constant. In a study with Atlantic salmon, it was established that lysine utilization for lysine gain (lysine retention / lysine intake) was constant despite differences in dietary lysine concentration and in the levels of lysine intake by the fish (Hauler & Carter 2001b). Based on this observation Hauler & Carter (2001b) suggested that lysine utilization remains constant at marginal lysine intakes over different dietary formulations and life-stages. Consequently, lysine requirement (allowance) for Atlantic salmon could be appropriately estimated by a factorial approach, assuming constant efficiency of lysine utilization and accounting for obligatory lysine loss (maintenance) (Hauler & Carter 2001b).

The results of Encarnaç o *et al.*, (2004; 2006), however, provide evidence that, at marginal levels of intake the efficiency of lysine utilization for PD is not constant, but it is affected by dietary lysine concentration, and DE level when supplied by lipids, but not other DE sources. Studies also showed that the source of dietary lysine (protein bound vs. free lysine) (Zarate and Lovell, 1997; Elharoun and Bureau, 2004) and the level of protein in the diet (Rodehutsord *et al.*, 2000) can affect lysine utilization for PD.

The assumption used in current factorial model, i.e. constant lysine retention per unit of live weight gain or protein deposition, constant lysine content of deposited body protein, constant marginal efficiency of lysine utilization for lysine retention (when intake is below requirement for maximum lysine retention), and constant of obligatory lysine loss (maintenance requirements), are probably not always realistic. Current factorial models increasingly appear too simplistic. They estimate EAA requirement “*en vases clos*”, i.e. by generating independent estimates for each EAA, and assuming that there are no interactions between EAA and other nutrients (e.g. fatty acids, glucose), assuming constant efficiency of utilization of amino acids regardless of physiological state, and assuming that “feed intake” or “energy requirement” or “feed conversion ratio” are “determinant” that are “independent” from diet composition, nutrient intake, interaction between nutrients, changes in target protein and lipid deposition or physiological state, etc.

The current factorial models are useful but are neither rational nor robust enough to adequately describe and predict EAA requirements under a wide variety of conditions. There is a need to develop more rational and mechanistic approaches of representing nutrient (including EAA) utilization in fish. More work needs to be carried out to better understand the impacts of dietary, environmental, and endogenous factors on amino acid utilization and accretion in fish. Future models should represent explicitly the intake and metabolic processing of nutrients by the animal, and would have the necessary complexity to accommodate and/or represent interactions between factors and the effects of endogenous factors.

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