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Methodological strategies for the determination of nutrient requirements in finfish

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SUMMARY – As the aquaculture production increases, based on a relatively low number of species with highly competitive but static or decreasing market prices, species diversification is becoming more important for the sustainability of Mediterranean Aquaculture. Since the main goal of aquaculture is to efficiently convert feeds into fish, studies addressed to define the nutritional requirements of candidate species are essential for the development of their culture techniques. A considerable amount of research has been devoted to study the nutritional requirements for the major cultivated finfish species. However, differences in requirements definition are frequently found in the literature not only for different but also for the same species. Thus, it is difficult to say whether this variation in the data obtained is simply due to methodological differences or it is the result of species-specific differences. Therefore, it is important to standardize experimental methods, accompanying all data with the experimental conditions employed when a new species is being studied. Comparison of different methodologies and experiences on different species are discussed in this study to better illustrate the possible strategies for the determination of qualitative and quantitative requirements in finfish.

Key words: Nutrition, methodology, finfish requirements, trial design.

RESUME – *"Stratégies méthodologiques pour la détermination des besoins nutritionnels chez les poissons". A mesure qu'augmente la production aquacole, basée sur un nombre relativement réduit d'espèces avec des prix de marché très compétitifs mais statiques ou à la baisse, la diversification des espèces devient plus importante pour la durabilité de l'Aquaculture Méditerranéenne. Etant donné que la finalité principale de l'aquaculture est de convertir efficacement des aliments en poisson, il est essentiel de faire des études visant à définir les besoins nutritionnels des espèces candidates pour développer leurs techniques de culture. Un grand nombre de recherches ont eu pour objet d'examiner les besoins nutritionnels pour les principales espèces cultivées de poissons. Cependant, des différences concernant la définition des besoins sont fréquemment rencontrées dans la littérature pas seulement pour des espèces différentes mais aussi pour la même espèce. Il est donc difficile de dire si cette variation des données obtenues est simplement due à des différences méthodologiques ou si elle est le résultat de différences spécifiques aux espèces. Il est par conséquent important de standardiser les méthodes expérimentales, et que toutes les données soient accompagnées par les conditions expérimentales employées lorsque l'on étudie une nouvelle espèce. La comparaison des diverses méthodologies et expériences sur différentes espèces est débattue dans cette étude afin de mieux illustrer les stratégies possibles pour la détermination des besoins qualitatifs et quantitatifs des poissons.*

Mots-clés : *Nutrition, méthodologie, besoins des poissons, conception des essais.*

Introduction

The studies on fish nutrition only began about forty years ago. Since then, significant advances have been made in all aspects of this subject. The number of species for aquaculture has been also dramatically increased. At the moment, nutrient requirements for most of the fish cultured species have not yet been defined and current recommendations are mostly based on data obtained with few selected fishes under intensive cultivation such as rainbow trout and channel catfish (Kaushik, 1995). For this reason, research studies on basic nutrition are needed for any candidate species of interest to aquaculture.

Recognizing the importance of fish nutrition research in aquaculture development and the differences found in the literature for different and the same species, it is necessary to standardized the applied methodology to determine the nutrient requirements. The present paper shows a review about nutrient requirements determination from the available literature, and suggest some practical recommendations including those defined in an EIFAC workshop held in Germany, in 1993.

The requirement for a particular nutrient could be defined both from a physiological or a practical point of view. Under the later, we can consider the *requirement for body maintenance* as the minimum rate of nutrient expenditure needed to keep the animal alive. For instance, we can consider the energy for maintenance as the energy needed to maintain the basal metabolism, plus the energy used for thermoregulation, plus the energy for involuntary activity such as body movement and muscular activity. But we can also define the *requirement for maximal growth,* where the relation fish-diet-feeding has an important effect in the determination of the quantitative needs. Besides, we can also talk about *requirement for least cost production*. Different works show that the requirements for maximal growth are always higher than the requirements for least cost production. Thus, de Silva *et al.* (1989) applying a second order polynomial relation to growth data compiled from different works on Tilapia, observed that the most economical dietary protein content was 28%, considerably less than the 34% protein level which supported maximum growth. Finally, it could be defined the *requirement for fish health*. The nutrient requirements determined for certain nutrients under optimal culture conditions increase when fish are exposed to unfavourable environmental conditions (poor water quality, stress, pathogens, ...). Thus for example, the definition of the requirement for vitamin C to improve immunological defences, is related to the production conditions applied.

Defining the methodology applied to the determination of requirements for different nutrient some general recommendations should be considered:

(i) A practical point of view to establish nutritional requirements is preferred.

(ii) If optimal culture conditions for the tested species have been established, the requirements should be assayed in such conditions. If not, it should be reviewed what it is known from this species in its natural conditions.

(iii) As far as possible, experimental conditions similar to those used at commercial scale should be used (feed preparation technique, water quality, photoperiod and fish stocking density).

(iv) Only one hypothesis tested per experiment is preferred.

(v) At least triplicate tanks of fish should be used per dietary treatment, as one tank of fish represents a single block observation.

(vi) To determine quantitative requirements it is important to consider different factors related to the species, such as age (larval to broodstock nutrition) and size, related to the medium, such as temperature, salinity, culture density, to the type of culture (extensive/intensive), related to the feeding strategy and feeding regime and finally, related to the feed, dietary energy content, nutrient availability in the ingredient source and interactions with other dietary nutrients or ingredients.

(vii) Complete feed ingredient descriptions should be provided, including International Feed Number (IFN), chemical composition and particle size, when reporting dietary formulations and the results of nutritional feeding trials. If a commercially prepared diet is used, the trade name and manufacturer should be indicated.

(viii) A standard diet should be used as a control in addition to any local diet also designed as a control. In most cases the use of different control diets among different authors makes comparison between them difficult.

(ix) According to the EIFAC workshop, a minimum of six dietary nutrient levels or treatments is recommended for nutrient requirement studies.

(x) Carcass analysis should be carried out at the beginning and at the end of the experiment.

(xi) An appropriate statistic analysis is always necessary.

(xii) Fish should be fed until "apparent satiation" instead of restricted feeding rates.

Cho *et al.* (1976) demonstrated that on restricted feed intake a 50-60% dietary protein level gave higher weight gain than 40%, whereas on *ad libitum* feeding the situation was inversed. According to

Dabrowski (1986) and other authors, the best way to obtain the higher growth rate its by using demand feeders, in this way a closer estimation of the *ad libitum* feeding is achieved. Once the maximum daily consumption is known, this amount of feed should be fed at different frequencies to determine which one gives the best growth rates. In many cases it is wrongly assumed that the selected feeding frequencies are able to promote the maximum growth rates.

Proteins and amino acids

Protein in fish is a main component constituent of tissue and organs, they are precursors of other nitrogen compounds (enzymes, hormones, slurry, neurotransmitters, cofactors, etc.) and constitute an important energy source. Thus, a consistent intake of protein or amino acids (AAs) is required, since they are continually used by the fish to build new proteins. Inadequate protein levels in the diet results in a reduction of growth and loss of weight. However, when an excess of protein is supplied in the diet, only part of it is used for protein synthesis and the remaining is transform into energy. According to Wilson and Halver (1986) and other authors, fish digest protein to obtain free amino acids, which are absorbed from the intestinal tract and used by various tissues to synthesize new protein. It has been generally assumed that apparent protein digestibility values are indicative of availability amino acids (Wilson *et al.*, 1981; Spyridakis *et al.*, 1988), but reasonable differences between apparent and true amino acids availability for certain feedstuffs can be found in some studies (Anderson *et al.*, 1992).

Reviews for optimum dietary protein requirement show some variations not only among different fish species but also for a single one (Luquet and Kaushik, 1981; Bowen, 1987); and it is difficult to say whether the variability in data obtained is due to methodological differences or really due to species-specific differences.

For the *qualitative determination* of essential amino acids (EAA), three different methods have been used: growth studies, ^{14}C labelling studies and enzymatic studies.

In the case of growth studies, diets deficient in one AA are used to feed the fish for a long period, while growth fish is monotorized. Then an initial test diet is necessary. For this purpose, different diets have been used based on casein, gelatine, zein and crystalline amino acid in different combinations. In the first studies, during the late 50's and the early 60's, Halver and co-workers found that whole egg protein gave the best results in chinook salmon (*Oncorhynchus tshawytscha*) being then adopted as the basal protein of the test diet. But frequently, test diets based in particular protein sources cause low feed intakes (i.e. Klein and Halver, 1970), related to their poor palatability or low fish growth response, making difficult to precise the requirements. Adequate control diets which are well accepted by the fish and able to maximize growth, such as those based in the muscle protein of the fish studied species, are always required to determine the requirements.

In labelling studies, fish is injected intraperitoneally with an adequate amount of a ^{14}C labelled substrate such as glucose or acetate. A significant amount of the ^{14}C will be incorporated into those amino acids that the organism can synthesize. After 6 days of normal feeding, fish are killed and homogenized and the protein isolated, amino acids separated by chromatography and radioactivity counted (Metailler *et al.*, 1973; Cowey and Walton, 1988). But this technique has the problem that some amino acids such as Trp, Asn and Gln are destroyed during the acid hydrolisis of proteins.

The enzymatic studies method was then used. With this technique those enzymes needed to synthesize Gln from Glu and Asn from Asp were found.

Using these methods the same 10 EAA have been found to be required for all the studied fish species.

When determining the qualitative requirements for essential amino acids it should be considered the sparing effect of certain AA (CYS/MET; TYR/PHE) and the antagonism between others (LYS/ARG; LYS/THR; LEU/ILE).

For the *quantitative determination* of EAA several methods have been used such as the dose-

response method, the AA oxidation studies, the plasma AA concentration studies, the accretion of AA in body tissues and studies about the rates of nitrogen retention/nitrogen deposition.

The dose-response method is the most commonly used and involves feeding of graded levels of one amino acid at a time, in a test diet containing either all crystalline amino acids or a mix of casein, gelatine and crystalline amino acids, or a semi-purified diet using an imbalanced protein (zein, corn, gluten) formulated in such way that the amino acid profile is identical to the test protein except for the amino acid being tested. The utilization of this technique present however two important disadvantages, long term growth studies are needed and there may be different ways of explaining the results.

The problems for comparison of trials results obtained by growth studies include:

(i) *Differences in the reference protein used*. As it was described above for the determination of qualitative requirements different protein sources in basal diets have been used including casein, gelatine, zein, corn gluten, fish meal, soybean meal and crystalline amino acids in different combinations. Keembiyehetty and Gatlin (1997) used semi-purified diets containing crystalline amino acids and lyophilized fish muscle to quantify the dietary threonine requirement of juvenile hybrid striped bass (*Morone chrysops* x *M. saxatilis*). For rainbow trout for instance, larger variations in lysine requirement have been reported in different works ranged from 1.03 to 2.80 % of diet or 4.2 to 6.1% of protein.

Also when fish meal is used in the diets is important to note that the protein and lipid content in the fish used to produce fish meal vary depending on fish species and season, being its freshness and processing temperature very important as well to determine its quality. This situation could be simplified by the use of standard control diets or a standardised reference fish meal. Recently, the Norsildmel Company in Norway produces a high quality reference fish meal from a specific raw material at a standardised process that can be used for experimental purposes.

(ii) *Reduced growth of fish fed free AA*. It has been shown in different species that fish can utilize more effectively larger amounts of free amino acids when the feeding frequency is high. Thus, the negative effects of feeding pure amino acids on dietary nitrogen utilization, due to the fast absorption and the wide variation of plasma amino acid concentration found with these diets can be reduced, providing a continuous supply of the amino acids to the tissues for protein synthesis (Tacon and Cowey, 1985; Cowey, 1988; Tibaldi and Lanari, 1991).

(iii) *Interactions among AA* (dietary imbalances of some AA with respect to another). For instance different authors have found a lysine-arginine antagonism in rainbow trout. Thus Kaushik *et al.* (1988) reported a slight growth depression in 100 g rainbow trout when 50% excess lysine was fed in a diet adequate in arginine.

(iv) *Subjective interpretation of dose-response curves*. The Broken-line model propose by Robbins *et al.* (1979) (Fig. 1.) has been extensively used to determine quantitative needs (Tibaldi and Lanari, 1991; Kim *et al.*, 1992; Lall *et al.*, 1994; Keembiyehetty and Gatlin, 1997). This model is based on the assumption that increasing levels of the tested nutrient will cause linear increases in growth up to a "break point" after which no further response is to be expected. But the response may be sometimes more curvilinear than linear, and then different models could be applied to interpret the results and different requirements obtained by the different models applied (Table 1; Figs 2, 3 and 4).

Different results can be also obtained if regression analysis are applied to different parameters. Thus, Keembiyehetty and Gatlin (1997) obtained estimations for threonine requirements of 9.7, 8.5 and 8.6 g/kg dry diet applying the broken-line model to weight gain, feed efficiency and protein to energy ratio (PER) results, respectively (Fig. 5).

(v)*Other nutrient dietary composition.* In the amino acid oxidation studies, it is assumed that when fish are fed an amino acid-deficient or imbalanced diet, the oxidation rate of such amino acid is low, and it gradually increases to be stabilized when the dietary level of this amino acid is meets the fish requirements. For instance, Lall *et al.* (1994) found that the arginine requirement in Atlantic salmon reared in sea water is 1.6% of dry matter (4.1% of protein) when a regression analysis is used between the percent of injected L-(U-^{14}C) arginine recovered as $^{14}CO_2$ versus dietary arginine level.

This value is similar to that determined by growth parameters. These methods may give variable results depending on the variable oxidation rates for the different EAA and by the different techniques of administration of the labelled AA. Besides, interactions between different AA and the time elapsed between meal and blood sampling may affect the results obtained (Kaushik, 1995).

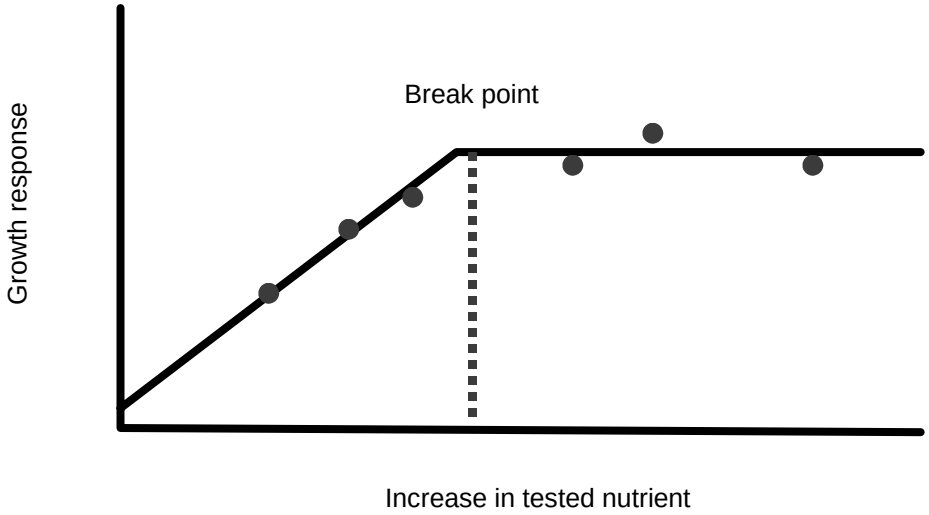

Fig. 1. Broken line model after Robins *et al.*, 1979.

Table 1. Diet formulation to determine protein requirement for 2-10 g *Pagrus pagrus* (Schuchard *et al.*, 1999)

Diet no. ingredient (% wet weight)	1	2	3	4	5	6
Fish meal	51.77	59.15	66.55	73.94	81.34	88.74
Fish oil	8.28	7.46	6.65	5.83	5.01	4.20
Raw starch	8.86	7.22	5.57	3.93	2.29	0.64
Gelatinized starch	26.59	21.67	16.73	11.80	6.86	1.92

Plasma AA concentration studies are based in the positive correlations between the levels of plasma or serum free essential amino acids and their respective levels in the diets reported for different species (Wilson *et al.*, 1978; Thebault, 1985). The requirement is considered to be met when the tested EAA accumulates in the plasma. High variability within replicates for each dietary treatment has been found as a disadvantage of this technique (Keembiyehetty and Gatlin, 1997).

In addition to these methods, different authors have tried to used the activities of catabolizing enzymes in liver and kidney to establish the amino acid requirements. However different studies (Walton *et al.*, 1986; Lall *et al.*, 1994) have shown that these activities vary considerably depend upon the nutritional status of animals. In this context, the postprandial peak of serum urea concentration has been proposed by Cho *et al.* (1992) and Tibaldi *et al.* (1994) to confirm the arginine requirement in rainbow trout and sea bass respectively, based in the observation that urea production from the arginase pathway is related to arginine intake.

The dietary protein requirement for fish is greatly influenced by the dietary protein-to-energy balance, by the amino acid composition and digestibility of the protein(s) tested levels (Table 2). Excess energy in the test diet may limit consumption since fish, like other animals, eat to meet their energy requirement. Despite most investigators use isoenergetic diets in terms of gross energy

contents to determine the protein requirements, the metabolizable energy of the various ingredients has not been determined for most fishes. Besides, comparisons of results from different authors is also complicate due to the differences in the physiological values used for calculating metabolizable energy in each study.

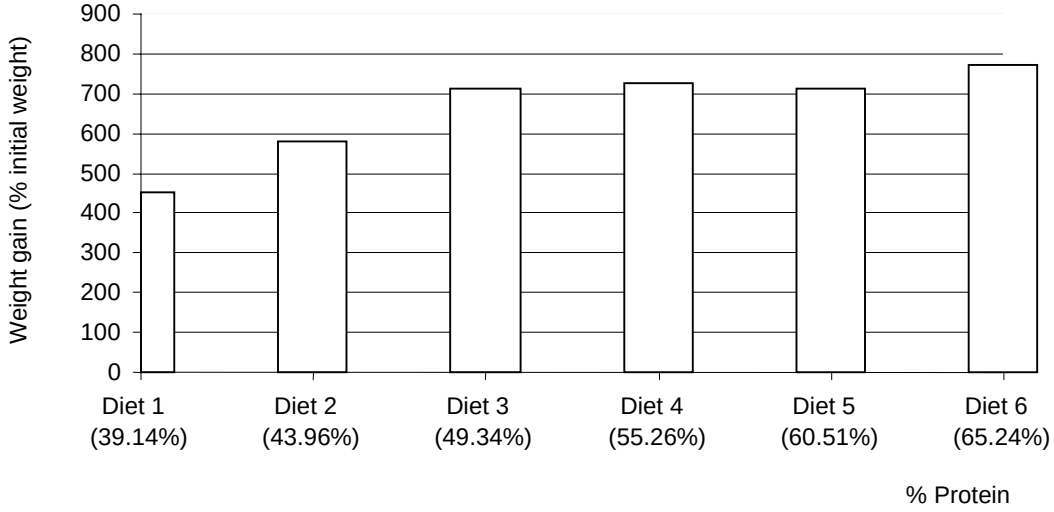

Fig. 2. Weight gain for 2-10 g *Pagrus pagrus* fed different dietary protein content (Schuchard *et al.*, 1999).

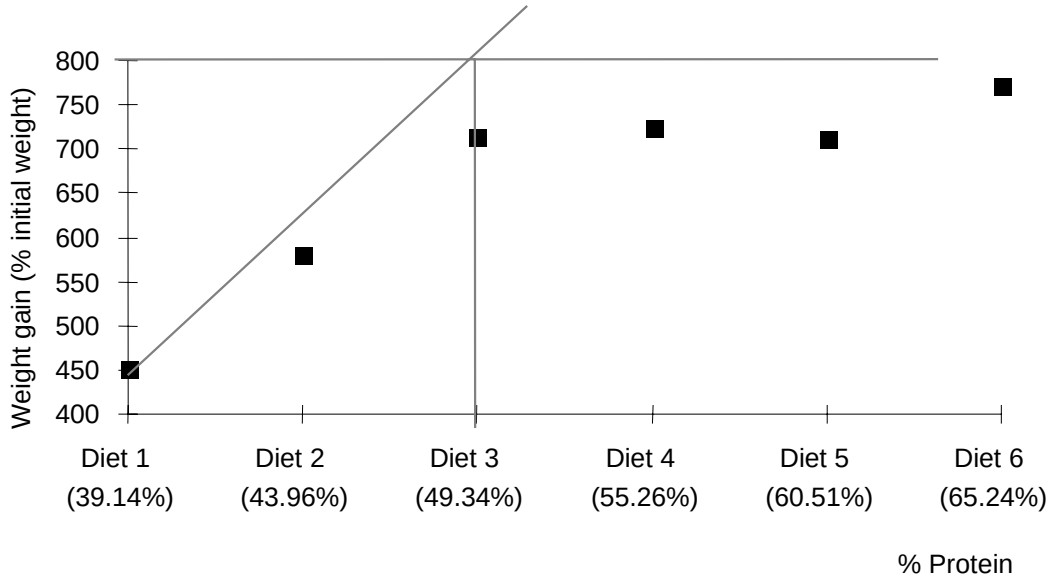

Fig. 3. Protein requirement for 2-10 g *Pagrus pagrus* by using weight gain data to apply the Broken-line model (Schuchard *et al.*, 1999).

Many investigators have demonstrated the important sparing effect of non-protein energy sources on the utilization of dietary protein. The utilization of dietary carbohydrate is known to vary among species, and it has been shown to spare protein in salmonids, plaice, turbot, channel catfish, sea bass, common carp and red sea bream. Lipids have also been shown to spare protein and enhance protein utilization in salmonids, common carp, channel catfish, turbot, red sea bream, gilthead sea bream, striped bass and *Tilapia aurea*.

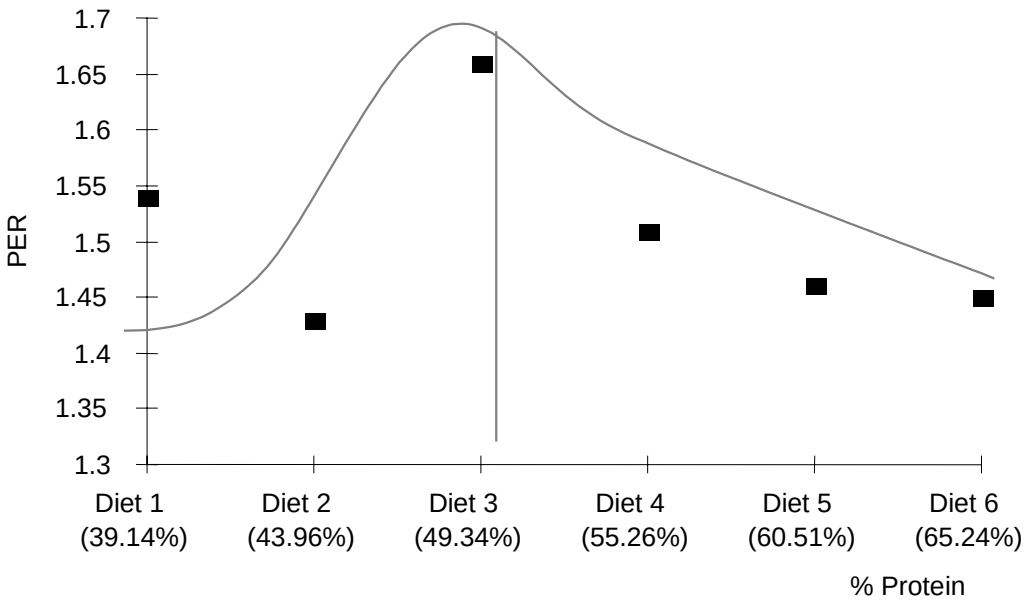

Fig. 4. Protein requirement for 2-10 g *Pagrus pagrus* by using data obtained for PER data (Schuchard *et al.*, 1999).

ame model for different parameters in Red drum (*Sciaenops ocellatus*)

Threonine requirement

Broken-line model

Weight gain	9.7 g/kg dry diet
Feed efficiency	8.5 g/kg dry diet
PER	8.6 g/kg dry diet

Fig. 5. Obtention of different values in the determination of quantitative requirements when different parameters are used (Keembiyehetty and Gatlin, 1997).

Table 2. Protein requirement for maximal growth reported for Tilapia

% protein	P/E (mg prot/kcal)	Fish (g)	Recalculated P/E[††]	Source
24	68.1	2.8	68.1	Shiau and Huang, 1989
30	95.3	1.6	108.0	Mazid *et al.*, 1979
34	108	7.5	129.6	Winfree and Stickney, 1981
40	116.6	0.5-1.0	116.6	Jauncey, 1982

[†]P/E = protein to energy ratio.
[††]P/E recalculated for the same metabolizable energy (protein 4.5 kcal/g; carbohydrate 3.49 kcal/g and lipid 8.5 kcal/g). From Shiau and Huang, 1989.

Recommendations

Young fish fed for a long period which allows a 5 to 10 fold increase in fish weight, should be used. Only high quality ingredients and several dietary protein levels should be tested. It is important to consider that the lipid content in fish meal used as protein source, will contain different levels of EFA and fat-soluble vitamins which will enhance growth and improve fish conversion. Lipid-extracted fish meal or fish muscle is recommended as reference protein. As a result from different works as in Mambrini and Kaushik (1995), the whole body EAA pattern of fish could be used in general as a good reference standard for the formulation of diets for new fish species.

Whenever it is possible different methodological approaches, to determine the requirements should be used.

Isoenergetic diets on a digestible or metabolizable energy basis, is preferable avoiding methods which involve fish or faeces handling for apparent digestibility measurements.

According to the EIFAC workshop (1993), the growth response of experimental fish should be monitored using a model such as specific growth rate or more preferably on the basis of the cube root of fish weight. Furthermore, nutrient requirement should be determined at the maximal possible rates of growth.

Energy

Studies about the energy requirements are scarce in marine fish, despite of the importance of P/E, P/DE and protein to lipid ratio (P/L) ratios in fish nutrition.

Energy supply (gross energy, GE) = digestible energy (DE) + fecal energy loss

DE intake = maintenance energy (Em) + retained energy (RE) + non-fecal energy loss

Metabolizable energy (ME) = DE – non-fecal energy loss

DE provides more information than dietary GE about the use of the energy intake. DE requirements have been shown to be considerably dependent on several parameters such as fish growth rate, amount of feed based on body weight, dietary factors (diet composition, ingredients used and processing methods) and water temperature. DE for maintenance (energy loss during starvation) is dependent on fish body weight and RE (mainly from protein and lipids) depends among others on the chemical for in which DE is absorbed. Rodehutscord and Pfeffer (1999), by pooling data from 20 different experiments with fast-growing rainbow trout, found that DE for maintenance was about 10-30% of the total energy requirement depending on the desired growth rate; these authors also observed that the DE for retention is highly dependent on the dietary fat content.

Part of the DE is lost by the non-fecal excretion of nitrogenous compounds, mainly ammonia and urea, and consequently, ME is not a constant proportion of the DE. This proportion depends on the quality and quantity of dietary protein and non-protein energy sources in the diet. For such reason, ME its difficult to estimate involving either the continuous monitoring of nitrogen excretion in water (Kaushik, 1980) or the use of metabolic chambers.

Methods

(i) *Energy balance methods.* It includes the determination of ME, DE for maintenance and RE by analysing whole body composition. DEm determination has been obtained measuring heat released from starved fish (basal metabolic rate). Results are variable within different works and are linearly dependent on body weight (Cho and Bureau, 1995). Thus, Ohta and Watanabe (1996) found that the energy requirement both for maintenance and maximum growth in rainbow trout slightly decreased with increase of fish body weight.

(ii) *Comparative carcass analysis methods*. These are long term feeding studies in which fish are fed with different dietary energy and the results depends on the duration of the experiment.

(iii) *Direct and indirect calorimetry*. These are short term but complicated studies to measure the non-utilized part of the energy intake. The metabolic energy cost of animals can be derived either by measuring immediately the heat production (direct calorimetry) or by measuring oxygen consumption and carbon dioxide production, along with urinary nitrogen excretion within a given time (indirect calorimetry).

(iv) *Factorial analysis.* This method includes the determination of energy need by rearing fish at different feeding levels with a nutritionally adequate diet.

(v) *Apparent digestibility indicator methods*. Comparative analysis of diets and faeces by the use of dietary internal (acid insoluble ash, fiber, ...) or external inert markers (mainly chromium or yttrium). Between the different methods that can be used for faeces collection those known as Guelph and Saint Peé systems give the more accurate results. Gross energy and digestibility energy values of dietary formulations should be measured directly; the use of standard calorific coefficients and assumed digestibility values may lead to serious error (Jobling, 1983). Even within given species fed a fixed dietary formulation, factors such as fish size, temperature and feeding regime may affect the apparent digestibility determination. It is therefore advisable to determine the digestibility coefficients simultaneously with the carrying out of growth experiments. However, if the aim is the preparation of practical diets, the digestibility can be estimated from the coefficients determined for each of the feed ingredients. When this method is used care must be taken that coefficients were obtained using the same fish species and that the quality and preparation of the feed ingredients were the same as those intended for use in the formulae.

Recommendations from the European Inland Fisheries Advisory Commission (EIFAC)

Provide data related to fish size and age, water temperature, life cycle stage, tank water current and water supply, photoperiod, water quality and stress as these are factors affecting energy requirements in fish.

Use DE to make a formula feed, and include digestibility estimations as a routine procedure in "growth trials" rather than relying on "previously published or perceived values" from other studies or fish species. For faecal collection the settling column ("Guelph" system) or rotating screen principle ("Saint Peé" system) may be recommended. The hand "Stripping" technique should be used with great care.

Metabolizable and net energy are recommended for the evaluation of complete diets as a measure of productivity. Retention of both nitrogen and energy is crucial to determine RE, net energy (NE), ME and DE. Open-circuit indirect calorimetry is recommended for the measurement of heat production.

Lipids and essential fatty acids

The importance of lipids in fish physiology is related with its different functions: dietary energy source, biomembrane structure and function maintenance, cellular membrane fluidity regulation, prostaglandin precursors, fat-soluble vitamins and carotenoid pigments carriers, hormones, etc.

Under a commercial point of view, lipid composition in fish flesh also affects the fillet texture and flavor. Interest in fish lipids has been also raised by the commercial importance of hepatic and muscular lipids in fish species, and its increased relevance for human nutrition. Results from different works support that fish body lipid composition is significantly affected by the dietary lipids.

Little is known concerning the detailed quantitative essential fatty acid (EFA) requirements in fish, which are affected by several factors such as fish age, dietary lipid levels, dietary α-tocopherol levels and other dietary antioxidants, dietary biotin levels, dietary ratios between several fatty acids such as eicosapentanoic/docosahexanoic EPA/DHA, n-3/n-6 ratio, dietary form in which they are fed (triglycerides/methyl esters/free fatty acids/phospholipids), dietary phosphoglyceride levels and environmental conditions.

Particular emphasis in the importance of the n-3/n-6 fatty acid ratio, and the effect of dietary lipids

and EFA on tissue histology and carcass fish quality are important fact being recently considered in this area.

In contrast with the AA qualitative requirements, qualitative requirements of fatty acids differ among the species studied, being particularly different for marine and freshwater fish (Watanabe, 1982). Nevertheless, it has been recently shown that probably most fish species have some requirement for polyunsaturated fatty acids (PUFA) from both n-6 and n-3 families (Sargent *et al.*, 1999; Izquierdo *et al.*, in press a). Four different methods have been used to determine the qualitative EFA requirements. Studies on fish *growth or survival response* to the presence of different dietary fatty acids are among the most commonly used. Lipid sources containing fatty acids esterified to triglycerides or phospholipids are always preferred over methyl or ethyl esters or free fatty acids, due to the lower digestive utilization or even toxicity of the later for some fish species or during specific periods of their life cycle (larval or juvenile stages for instance) (Izquierdo, 1996; Izquierdo *et al.*, in press a). If deficiency symptoms are to be observed, feeding periods longer than those required to find growth reductions should be held (Stickney and Hardy, 1989). Studies using *labelled fatty acids* as well as those determining the *enzymatic activity* of delta-desaturases, are conducted to determine the capacity of polyunsaturated fatty acid synthesis of each species and their ability of utilize dietary precursors of those fatty acids. In this type of studies, as well as in those involving *cell culture techniques*, *in vitro* studies should be combined with *in vivo* ones to confirm the hypothesis in alive fish under common culture conditions, since a particular fish species may show *in vitro* the ability to synthesize an essential fatty acid but under common culture conditions and to obtain high growth rates this ability is not high enough to cover the fish requirements.

Since quantitative requirements of essential fatty acids are species specific, studies must be conducted for each single new species for aquaculture. Different methods have been also used, commonly based in the growth and survival fish response to graded dietary levels of essential fatty acids or on the surge of deficiency symptoms or changes in the body lipid composition as a result of those diets. Feeding dietary lipids lacking EFA muscle water contents increase and both protein and particularly lipid contents decreased (Watanabe, 1982). The content of liver lipid is also increased, indicating a sing of fatty liver according to Watanabe *et al.* (1974) and other authors (Fig. 6). Reduction of dietary EFA levels under the required levels markedly reduces fish body EFA contents in both neutral and polar lipids, particularly quickly in some tissues with a great cell turnover such as gill or skin, but also affecting dramatically to other tissues such as neural, hepatic or muscular ones. Low dietary levels of eicosapentaenoic and docosahexaenoic fatty acids also reduce the fish resistance to stress (Montero *et al.*, 1998) and immunocompetence, increasing erytrocyte fragility and reducing alternative complement pathway activity as well as neutrophil activity.

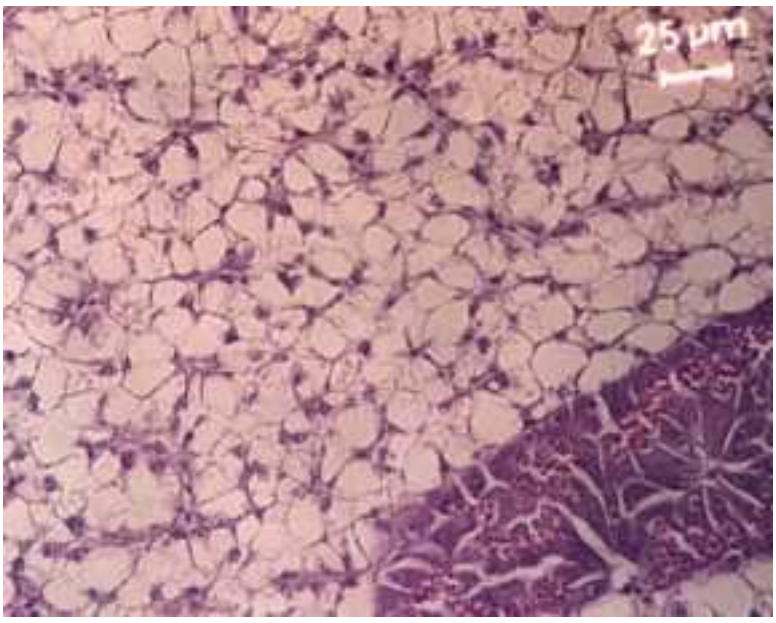

Fig. 6. Addition of soybean oil to gilthead sea bream diets significantly increased the liver content in 18: 2n-6 (Robaina *et al.*, 1995).

As digestion and transport to hepatic or peripheral tissues of the different molecular forms of fatty acids (triglycerides, phospholipids or free fatty acids) may be different during some periods of the life cycle (Izquierdo, 1996; Izquierdo *et al.*, in press b), if available, phospholipids, which frequently are better utilized, should be preferred over triglycerides and these ones over free fatty acids. Nevertheless, when trials are conducted in larval or early juvenile stages about 2% of lecithins containing polyunsaturated fatty acids should be included in the diet, since most fish species require these nutrients during these periods (Kanazawa *et al.*, 1989; Salhi *et al.*, 1995; Geurden *et al.*, 1997; Izquierdo *et al.*, in press b). Although early studies, used to defined the quantitative requirements based on the dietary contents of n-6 or n-3 PUFA or n-3 highly unsaturated fatty acid (HUFA), several studies during the last years have pointed out the particular importance of arachidonic, eicosapentaenoic and particularly docosahexaenoic acid for all fish species studied (Izquierdo, 1996; Sargent *et al.*, 1999; Izquierdo *et al.*, in press a). Therefore, requirements should be defined for each of these particular essential fatty acids or their precursors. Moreover, since there are biochemical, enzymatic and histological evidences of competitive interactions among these essential fatty acids, estimation of the dietary EFA requirements requires to consider not only their absolute dietary amounts but also the adequate proportions among them (Izquierdo *et al.*, in press a). For instance, Rodríguez *et al.* (1994), feeding gilthead sea bream larvae with rotifers containing a ratio of 2.5 EPA/DHA, obtained best growth when rotifers contained 55 g/kg n-3 HUFA on dry weight basis. But in a further experiment the same authors (Rodríguez *et al.*, 1994) found an improvement in larval growth when the EPA/DHA ratio in rotifers was reduced from 36 to 8, for a constant n-3 HUFA level of 30 g/kg. Finally, adequate levels of biotin, phosphorous, selenium and other nutrients which interfere with lipid metabolism as well as vitamin E and other antioxidant ingredients such as vitamin C, carotenoids, phospholipids or ethoxyquin, should be provided in the diets to promote the best utilization of dietary fatty acids.

Strategies for the study of the lipid requirement for marine fish larvae: *Particular case*

Although the most direct method to evaluate the EFA requirements in fish larvae should be to feed them with different levels of these nutrients these is particularly complicate since most fish larvae are unable to survive in formulated diets alone. To achieve this goal we must feed the larvae with enriched life prey or microdiets containing different levels of fatty acids, together with some non-EFA-content life food. Requirements are defined based in larval response in terms of: (i) growth rate (some species present strong effect, but some other not); (ii) survival rate and resistance to activity test (some species present strong effect, but some other not); and (iii) biochemical composition of the fish larvae.

Other additional and very useful methods are:

(i) *Study of the essential biochemical composition of the eggs*. Since, marine fish eggs should contain all the nutrients which are essential for embryonic and larval development up to the stage of yolk-sac absorption, their biochemical composition should give us some information about which nutrients are essential for such development. Thus, for example, in gilthead sea bream (*Sparus aurata*) and other sparids the major fatty acids in the total lipids of the eggs are DHA, palmitic acid, EPA and oleic acid, suggesting their importance for larval development.

(ii) *Study of the evolution of the fatty acid composition of the embryo during the development*. The evolution in the changes in the fatty acid composition during the embryo development should say us which of these fatty acids are preferentially preserved and are important for the larvae. In gilthead sea bream for example, during the first 3 days of larval development n-3 fatty acids like DHA, EPA and AA are preferentially preserved instead of saturated fatty acids or n-9 fatty acids which are used as energy source. Thus, DHA, EPA and AA, are regarded as essential or important for the development of the larvae.

(iii) *Study of the conservation or loss of fatty acids during larval development and during starvation and the feeding of the fish with several levels of these fatty acids*. Again for gilthead sea bream, after 5 days of starvation, some saturated fatty acids such as palmitic acid or monounsaturated fatty acids such as oleic acid are used to obtain energy while other fatty acids such as arachidonic acid and DHA are preferentially conserved. This biochemical strategy allows the larvae to preserve during the critical period of starvation, those fatty acids which are very important for the membrane construction and function.

As it happens when comparing requirements for other nutrients, determined quantitative EFA requirements sometimes differ among the different studies for a particular species (Izquierdo, 1996). These differences are particularly found during larval stages and can be related not only to differences in the culture conditions (i.e. the presence or not of different types of microalgae in different concentrations in the rearing tank) or larval fatty acid background (broodstock feeding or previous larval feeding) but also to the several dietary aspects which have been discussed above.

Recommendations

Bare in mind that probably most fish species have some requirement for arachidonic, eicosapentaenoic or docosahexaenoic acids. Therefore, requirements should be defined for each of these particular essential fatty acids or their precursors.

Presence of competitive interactions among these essential fatty acids requires to consider not only their absolute dietary amounts but also the adequate proportions among them.

Triglycerides or phospholipids should be always preferred as fatty acid sources.

Hypothesis formulated in *in vitro* studies should be confirmed in alive fish before its acceptation for practical purposes.

Since quantitative requirements of essential fatty acids are species specific, studies must be conducted for each single new species for aquaculture.

Lecithins (namely phospholipids mixtures with high quantities of phosphatidyl-choline, but also including other fractions) containing polyunsaturated fatty acids should be included in the diet.

Adequate levels of biotin, phosphorous, selenium and other nutrients which interfere with lipid metabolism as well as vitamin E and other antioxidant ingredients such as vitamin C, carotenoids, phospholipids or ethoxyquin, should be provided in the diets to promote the best utilization of dietary fatty acids.

Comparing the requirements obtain by several authors for larval stages, larval fatty acid background and culture conditions employed should be considered.

Dietary lipid quality and complete fatty acid analysis should always be especific, since rancidity or toxicity of some compounds could markedly interfere with the results.

Instead of limiting the information to fatty acid groups, complete fatty acid profiles of body lipids are preferred to provide information about the capacity of fatty acid desaturation and elongation of each species.

Vitamins

Fish performance and final production cost are easily effected by the dietary vitamin supplementation, so information about vitamin requirement in fish is important to optimize the production system. In the case of marine fish very little information is available although the requirements have been well estimated in salmonids.

The reported dietary vitamin requirements of fish is dependent upon the criteria used by researchers to estimate the requirement, ranging from low requirement levels for optimal growth to high requirement levels for maximum tissue storage or optimum disease resistance. Comparison between estimated vitamin requirements and the recommended feed levels are generally quite different. The supplemental levels of each vitamin in fish feeds are always higher than the required levels for the safety margin, in spite of the high price of these nutrients. Two main reasons could affect the differences: the availability of vitamins can be different depending upon the form of dietary input and also certain vitamins may be destroyed during feed manufacture by heat, moisture, alterations in pH, the presence of some metals, lipid oxidation... Destruction of vitamin C (ascorbic

acid) due to oxidation is one of the bigger problems in fish manufacture. Some vitamins are also lost during storage, thus feed should be used soon after pelleting. In case of crustacean feeds which are usually kept in a tank or pond for a long time, allowance should be made for the leaching of vitamins from pellets.

Vitamin deficiency symptoms usually appear earlier than that of minerals or fatty acids deficiency so shorter experiments are frequently conducted. According to Dabrowski (1986), it is important to note when the deficiency symptoms start to appear; younger fish are much more susceptible to lack of vitamins than are juveniles or adult fish. Symptoms of deficiency are manifested at different time depending on the species and fish size. In larval vitamin requirement studies it must be also taking into account the influence of parental feeding. Thus, Kinumaki *et al.* (1972) found that vitamin A and E levels in rainbow trout eggs depended upon the amount provided in the broodstock food. Vitamin D levels however, seemed to be more independent of parental nutrition.

Qualitative requirements for both liposoluble and hydrosoluble vitamins are determined using free-vitamin diets. For the *quantitative studies* dose-response methods based on fish growth, vitamin tissue storage levels, gross physiology – deficiencies, histopathology and specific vitamin-dependent enzyme activities are frequently used. Additional and very useful information is recently obtained through stress or immune responses studies. Interaction among different vitamins or vitamins and other nutrients must be also considered in studies to determine vitamin requirement.

For instance, a direct relationship between the requirement *of vitamin E and PUFA* content of the diets has been reported in few studies. It has been shown that addition of lipids to a diet is very effective in promoting protein utilization but this may be impaired with and increase of the dietary vitamin E requirement.

Deficiency signs of *Vit B_{12} and folic acid* produce anaemia in fish and the effect of combined deficiency have been reported to be additive. The metabolic interaction between both vitamins in fish it would appear that may be essentially the same as that in other domestic animals. According to Hilton (1989), Vit B_{12} would reduce the folic acid functionality by trapping folate as methyl donor for the homocystine for the synthesis of methionine.

In the case of Vit E and Vit C, it has been shown that ascorbic acid could have an sparing effect on tissue Vit E levels and the Vit E requirement of fish as both vitamins act to prevent lipid oxidation. The lack of effect of Vit E deficiency in presence of Vit C has been found in catfish (Gatlin *et al.*, 1986) and rainbow trout (Cowey *et al.*, 1983, 1984).

Vit D or its metabolites essential nutrients in fish diets, have been shown to act in the uptake of calcium from water-bone through the gills and stimulate the absorption of calcium from the intestinal mucosa. Sings of hypercalcaemia were observed after Vit D3 injection in fish (Swarup and Srivastav, 1984).

In studies to determine choline requirements, it is important to know that the level of this vitamin or other methyl donors in the diet can have an important effect (NRC, 1993). Thus, the potential interaction between choline and methionine must be accounted reliable estimate their requirements. For this purpose, the diet must be formulated to provided methionine at the minimum requirement level determined for the species to be evaluated. Craig and Gatlin (1996) estimated that choline requirement for juvenile red drum range from 330 to 676 mg choline/kg diet; data of weight gain and liver and plasma lipids obtained with a basal diet adjusted in methionine content with liophilized red drum muscle and amino acid premix where subjected to a broken-line regression analysis.

Recommendations

Dose response analysis using an adequate test diet is recommended. It is important to consider possible interactions, sparing effects, vitamin inhibitors and hypervitaminosis in studies with some specific vitamins.

Stable forms of vitamin C (ascorbate-2-sulphate, ascorbate-polyphosfate) are recommended, as

this vitamin is the most heat-labile vitamin in fish diets during the processing and storage of the feeds. However studies are needed to elucidate the availability of the different forms of this vitamin by different fish species.

Minerals

Knowledge about the mineral nutrition is one of the least advanced areas in fish nutrition. Despite it is known the important role of some of these nutrients in fish growth and health. One of the reasons for this lack is the difficulty added by the absorption of minerals by the fish from the surrounding waters making it difficult to properly control the dietary intake of the mineral being studied. This is a particular problem for studies of trace minerals, such as iron, zinc, manganese, copper and cobalt and for trials with salt water fish. For most fish species it is difficult to study the effects of calcium deficiency because calcium is actively absorbed from the water by the gills. Calcium serum concentration in rainbow trout for instance did not reflect the requirement level as determined by growth rate (Robinson *et al.*, 1986). Interactions between minerals also complicate the assessment of dietary requirements. Besides, its important to note as well that different meals contain different concentrations of minerals and that the use of this nutrients depend on their availability by the different fish species (Watanabe *et al.*, 1988; Sugiura *et al.*, 1998).

Purified diets (casein-gelatine) formulated with mix of certain amino acids and a mineral mix free of the tested mineral are frequently used for the *qualitative determinations*.

To determine *quantitative requirements*, dose-response methods based on fish growth and body composition, whole body mineral balance studies and methods based on tissue mineral retention or storage are used.

Care must be taken to obtain adequate fed intakes. Most studies about mineral requirements of fish have been conducted using purified diets, but semi-purified diets have been needed to obtain adequate feeding intake for some species as red drum (*Sciaenops ocellatus*), Gatlin *et al.* (1991). This authors used liophilized adult red drum muscle and egg white as protein sources to determine Zn requirement in this species. Mineral analysis at the end of the feeding trial showed that scale mineral and bone-zinc concentrations were significantly affected by dietary Zn concentration and were used together with growth parameters as the response criteria for the estimation of the dietary zinc requirement through the broken-linen regression analysis.

Different factors influence the mineral requirements of fish: fish size and growth rate, diet composition, availability of the mineral chemical form used and the mineral interactions with other dietary or water bone elements. Different requirement levels result from different works where different diets are used. Thus, for channel catfish (*Ictalurus punctatus*), Andrews *et al.* (1973) found a dietary calcium requirement of 1.5% of dry diet using anchovy meal as protein source which has low in calcium availability. After that, Robinson *et al.* (1986) found a lower requirement (0.45% dry diet) for the same species, using a purified diet with casein and different amounts of calcium sulfate with high availability of calcium. The latter author also used calcium-free water, as natural water usually contain appreciable amounts of dissolved calcium which can be utilized to meet part of the metabolic calcium requirement.

It has been reported that calcium and/or phosphorus level in the diet affects Zn bioavailability in different fish species. Porn-Ngam *et al.* (1993) found in rainbow trout a depletion in growth and Zn availability with an excess of dietary P. The proportion of P in the form of tricalcium phosphate contained in fish meal might also inhibit Zn availability (Satoh *et al.*, 1993). Also, presence of strong mineral binding agents, such as phytic acid, mainly in vegetable protein sources, affect mineral availability to a great extent, through the formation of insoluble phytates (mainly Zn - Ca - Mg – phytate) in the intestinal lumen resulting in lowered mineral availability.

Recommendations

Mineral requirements can be determined by regression analysis using the dose-response technique with dietary mineral levels ranging from suboptimal to possibly subtoxic levels.

Studies on comparative mineral availability should be carried out under identical conditions with fish on a suboptimal mineral supply status.

Gross requirement for minerals in fish is dependent upon numerous factors, including fish performance (requirement for a high growth rate), diet (semi-synthetic or practical diets are necessary for requirement studies), chemical binding form, interactions with other dietary elements, interactions with other water borne elements, and interactions with environmental conditions.

The dietary mineral requirements of fish are species specific, and so mineral requirements must be determined for individual fish species.

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