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Investigating the appropriate mode of expressing lysine requirement of fish through non-linear mixed model analysis and multilevel analysis

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Abstract

Accurate estimates of lysine requirement are essential to fish feed formulation. However, controversy exists regarding the most appropriate mode to express lysine requirement. In the fish nutrition literature, essential amino acid (AA) requirement has been expressed as a percentage of diet, a percentage of dietary crude protein or a ratio to dietary digestible energy (DE). The controversy lies in the different assumptions regarding the effects of dietary protein and DE on lysine requirement. Non-linear mixed model analysis and multilevel analysis were carried out to investigate whether dietary protein or DE affected lysine requirement of fish. The non-linear mixed model analysis suggests that expressing lysine requirement as a percentage of dietary protein provides a better goodness of fit to the modelling dataset than expressing requirement as a fixed concentration of diet, which in turn is generally better than expressing requirement as a ratio to DE. Results from the multilevel analysis confirm that dietary protein content has a significant effect on lysine requirement, while DE does not. The findings of the present study could contribute to a better understanding of the underlying dietary factors that affect AA requirements of fish. The results of the present study could also be useful for developing nutritional guidelines and feed formulations for fish.

Key words: Requirements: Lysine: Protein: Energy: Fish

One of the fundamental aspects of animal nutrition and feeding is the accurate determination of the requirement of essential amino acids (EAA)[1]. Lysine is commonly the first limiting AA in fish feeds, especially when feeds are formulated with high levels of plant protein ingredients. This is because fish meals and animal protein ingredients are generally rich in lysine, whereas plant protein ingredients, especially cereal grain by-products, are poor in lysine content. As fish meals and animal protein ingredients are increasingly being substituted by plant protein ingredients in fish feeds, an accurate estimate of lysine requirement is not only critical to cost-effective feed formulation but also important to minimise environmental and ecological impact of aquaculture activities through nutritional strategies.

A better application of lysine requirement estimates in practical feed formulation faces several issues. First, estimates of lysine requirement for fish reported in the literature are highly variable. Moreover, one of the most controversial topics is the different modes of expressing EAA requirements in the literature[2]. Underlying this controversy are different assumptions regarding the effect of dietary composition (crude protein (CP) and digestible energy (DE)) on EAA requirements. Bureau & Encarnação[3] reviewed these different opinions: (1) EAA requirements can be expressed as a percentage of diet (% diet), which implies that EAA requirements are independent of dietary nutrient composition. This is commonly used by most fish nutritionists but difficult to justify because nutrient requirements are expected to be influenced by digestible nutrient compositions[2]. (2) EAA requirements can be expressed as a percentage of dietary CP (% protein), which assumes that EAA requirements are a function of dietary protein content. (3) EAA requirements are expressed in relation to DE as g/MJ DE, which assumes that EAA requirements depend on dietary DE content. In their review, Bureau & Encarnação[3] demonstrated that the expressing modes of EAA requirements have a significant impact on targets and recommendations of feed formulation.

Since the controversy arises from the different assumptions regarding the effects of CP and DE on EAA requirements, this issue can only be solved by elucidating whether CP or DE contents affect EAA requirement. Many studies have been published on lysine requirement of different fish species; however, investigations of the effect of dietary composition on lysine requirement have been scarce. In a quantitative

Abbreviations: % diet, percentage of diet; % protein, percentage of protein; AA, amino acid; BW, body weight; CP, crude protein; DE, digestible energy; EAA, essential amino acid; MBW, metabolic body weight; NRE, N retention efficiency.

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review, Hauler & Carter[4] investigated intra-species variation in lysine requirement by examining the order of difference in published values (the percentage difference between the highest value and the lowest value). The study of Hauler & Carter[4] suggests that it makes more sense to express lysine requirement as a ratio to DE than to express it as a percentage of dietary protein for fish. In recent years, several studies have examined the effect of dietary composition on lysine requirement. Encarnação *et al.*[5] observed that DE levels did not affect lysine requirement in rainbow trout, suggesting that it is not appropriate to express lysine requirement in relation to DE. On the other hand, an effect of CP on lysine requirement was observed in studies conducted with rainbow trout[6,7]; these studies might be construed as evidence to support the argument of expressing lysine requirement as % protein. At present, the controversy still remains and recent publication by the National Research Council[2] opted to continue to express AA requirement as % diet for want of a better alternative despite its significant limitations. It is therefore necessary to investigate the effect of dietary composition on lysine requirement in a systematic manner. Instead of examining these different factors one at a time through individual experiments, a meta-analytic mathematical modelling approach can be used to effectively integrate results from published studies. The body of literature data, especially the accelerated accumulation of published studies on different fish species during the past decades, provides a prime opportunity to use a modelling approach to delineate the effect of dietary factors, even though most of the studies in the literature did not specifically investigate the effect of dietary composition on lysine requirement. Furthermore, in a meta-analysis, it is important to account for the random effect of each study by using a mixed model approach instead of a fixed model approach, because each study represents a random sample of a larger population[8]. In the meantime, a meta-analysis can be viewed as a variant of multilevel analysis in which within-study and between-study effects are estimated[9].

Therefore, the objective of the present study was to conduct non-linear mixed model analysis and multilevel analysis to investigate whether dietary CP and DE contents affect lysine requirement, i.e. whether lysine requirement of fish should be expressed as % diet, % protein or g/MJ DE.

Materials and methods

Modelling dataset

A modelling dataset was established through a comprehensive literature review of studies on lysine requirement in fish. The modelling dataset included a total of forty-eight dose–response studies on lysine requirement of thirty-four fish species published in the literature[5,6,10–55]. Since the objective of the present study was to investigate whether dietary CP or DE levels affect the estimates of lysine requirement, the dietary contents of CP and DE had to be either reported or could be estimated from diet compositions reported in these dose–response studies; otherwise, studies were excluded from the modelling dataset. If necessary, DE contents were calculated when the gross energy content was reported and a digestibility coefficient of 85 % was assumed; otherwise, energetic values of 23·6, 39·5 and 17·2 kJ/g and digestibility values of 90, 95 and 70 % were assumed for protein, lipid and carbohydrate, respectively[2,4]. The dataset encompassed a wide variety of diet compositions and lysine levels (Table 1). Dietary protein levels ranged from 24 to 55·2 %, DE from 9·8 to 23 MJ/kg diet, and lysine from 0·2 to 7·0 % of diet. Weight gain is commonly used as a response criterion in dose–response studies of AA requirements, but increasingly protein gain is used as a response parameter as well[2]. Therefore, a sub-dataset was separated from the full dataset with protein gain as the response variable. The sub-dataset included twenty-eight studies that were conducted with twenty-one fish species. The sub-dataset is smaller than the full dataset due to two reasons: protein deposition had to be either reported in the studies or could be calculated based on the final and initial body compositions; protein deposition had to reach a plateau at the highest level of lysine tested in the experiments. Estimates of lysine requirement based on protein deposition may be higher than those based on weight gain[2]; therefore, a plateau may be reached at the highest tested level of lysine when using weight gain as a response parameter but not when using protein deposition as a response parameter in some studies. Dietary protein levels ranged from 31·6 to 51·2 %, DE from 11·4 to 23·0 MJ/kg diet, and lysine from 0·2 to 7·0 % of diet in the sub-dataset. Lysine deposition was not used as a response parameter in the present study because very few studies reported lysine deposition or lysine content in the fish body.

Table 1. Description of the datasets
(Mean, minimum and maximum values)

	IBW (g)	Protein (% diet)	DE (MJ/kg)	Lys (% diet)	Requirement by the broken-line model (% diet)
Full dataset*					
Mean	39·7	41·4	16·9	2·0	1·9
Minimum	0·04	24·0	9·8	0·2	1·1
Maximum	643	55·2	23·0	7·0	3·1
Sub-dataset†					
Mean	37·2	42·3	17·9	2·1	2·0
Minimum	0·7	31·6	11·4	0·2	1·2
Maximum	643	55·2	23·0	7·0	3·0

IBW, initial body weight; DE, digestible energy.
* Dataset with body-weight gain as the response criterion.
† Dataset with protein deposition as the response criterion.

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Non-linear mixed model analysis when dietary lysine concentration is expressed as percentage of diet, percentage of protein or g/MJ digestible energy

In the present study, four models commonly used to describe the nutrient requirement response of fish were fitted to the modelling datasets. These models are the broken-line model[56], the exponential model[22], the saturation kinetics model[57] and the four-parameter logistic model[58]. For the full dataset, the response variable was daily body-weight gain expressed as g/kg metabolic body weight (MBW, $BW^{0.8}$). For the sub-dataset, the response variable was N retention efficiency (NRE, %). The independent variable was lysine concentration expressed as % diet, % protein or g/MJ DE.

The broken-line model is described as follows:

$$y = L + U \times (R - x), \tag{1}$$

where y is the body-weight gain (g/kg MBW per d) or NRE (%); x, the lysine concentration (% diet, % protein or g/MJ DE); L, the ordinate of the breakpoint; R, the abscissa of the breakpoint; and U, the slope of the line for $x < R$. By definition, $(R - x)$ is zero when $x > R$.

The exponential model is described as follows:

$$y = a(1 - e^{-b(x-c)}), \tag{2}$$

where y is the body-weight gain (g/kg MBW per d) or NRE (%); x, the lysine concentration (% diet, % protein or g/MJ DE); a, the plateau of the curve (upper asymptote); b, the parameter characterising the steepness of the curve; and c, the intercept on the x-axis.

The four-parameter logistic model is described as follows:

$$y = \frac{a + (d(1+m) - a)e^{-kx}}{1 + me^{-kx}}, \tag{3}$$

where y is the body-weight gain (g/kg MBW/d) or NRE (%); x, the lysine concentration (% diet, % protein or g/MJ DE); a, the plateau of the curve (upper asymptote); d, the intercept on the y-axis; k, the scaling parameter that scales x; and m, the shaping parameter that locates the inflection point.

The saturation kinetics model is described as follows:

$$y = \frac{d(k_{0.5})^n + ax^n}{(k_{0.5})^n + x^n}, \tag{4}$$

where y is the body-weight gain (g/kg MBW per d) or NRE (%); x, the lysine concentration (% diet, % protein or g/MJ DE); a, the plateau of the curve (upper asymptote); d, the intercept on the y-axis; $k_{0.5}$, the concentration for $\frac{1}{2}$ of $(a + d)$; and n, the apparent kinetic order.

Data were analysed using the non-linear regression function of SAS software (version 9.1; SAS Institute). The mixed model procedure was used to consider the random effect of each study since the experiments represent a random sample of a larger population[8]. In the broken-line model, the random effect was added to parameters 'L' and 'U', which allowed accounting for between-study heterogeneity in maximum response and the steepness of response to AA intakes[59]. In the exponential model, the random effect was added to parameters 'a' and 'c', which allowed accounting for between-study heterogeneity in maximum response and the intercept on the x-axis[60]. Similarly, the random effect was added to parameters 'a' and 'd' in the four-parameter logistic model and the saturation kinetics model to allow accounting for between-study heterogeneity in maximum response and the intercept on the y-axis. Due to the limited size of the datasets, the random effect was not simultaneously added to more than two parameters to avoid undue model complexity and over-fitting of the data with an excess of random effects.

The goodness of fit of the models was evaluated by the corrected Akaike information criteria[61]. A smaller value indicates a better fit. The goodness of fit was compared for lysine concentration expressed as % diet, % protein or g/MJ DE. A better fit to the response curves was considered as a more appropriate way of expressing lysine requirement.

Multilevel analysis

A multilevel analysis was further conducted to investigate the effects of dietary CP and DE on lysine requirement. Meta-analysis can be viewed as a special case of multilevel analysis[9]. The following procedure for multilevel analysis was performed according to Hox[62]: (1) a baseline (null) model that included only the intercept was first computed (the baseline model assumed that lysine requirement should be expressed as % diet), (2) explanatory variables (CP and DE) were included in the model, and then (3) a χ^2 difference test was conducted to examine whether the model with explanatory variables had a significantly better fit than the baseline model. Subsequently, non-significant fixed-effect variables were dropped, and the multilevel analysis was re-run.

The multilevel meta-analysis model is a linear mixed-effect model that takes into account the random effect of each study, which can be written as follows:

$$y_j = \gamma_0 + \gamma_1 x_{1j} + \gamma_2 x_{2j} + u_j + e_j, \tag{5}$$

where y_j is the lysine requirement estimate (% diet) from the jth study; γ, the study-level fixed-effect regression coefficients; x, the fixed-effect explanatory variables (CP and DE); u_j, the study-level random error; e_j, the sampling error in the jth study.

In addition to dietary factors, biological factors such as body weight may also affect lysine requirement. Therefore, subsequent to dietary factors, a multilevel analysis was further carried out by adding body weight of fish as a fixed-effect explanatory variable to the multilevel model. Because the majority of studies in the modelling dataset were conducted with juvenile fish and the distribution of body weights was skewed, initial body weights of fish from each study were log-transformed to achieve a normal distribution.

It is known that mathematical models chosen to fit the response curve affect requirement estimates. Therefore, to ensure the comparability of the lysine requirement estimates across studies in the multilevel analysis, the estimates had to be obtained using the same dose–response model. In the present study, the broken-line model was chosen to obtain the requirement estimates for all studies.

The multilevel analysis was carried out using the linear mixed-effects procedure of SAS software (version 9.1; SAS Institute).

Results

Non-linear mixed model analysis when dietary lysine concentration is expressed as percentage of diet, percentage of protein, or g/MJ digestible energy

Tables 2 and 3 present the comparisons of the goodness of fit to the modelling dataset when lysine concentration was expressed as % diet, % protein or g/MJ DE for body-weight gain as the response variable, and for NRE as the response variable, respectively. The results suggest that irrespective of the response criteria, expressing lysine concentration as % protein gave the lowest corrected Akaike information criteria values, indicating the best fit to the modelling data. It appears that expressing lysine concentration as % protein provides a better goodness of fit to the modelling dataset than expressing it as % diet, which in turn is generally better than expressing it as g/MJ DE. The results also indicate that the best-fit mathematical model may differ depending on the assessed response variables. The saturation kinetics model was the best-fit model when the response variable was body-weight gain, whereas the exponential model was the best-fit model when the response variable was protein deposition.

Table 4 presents the estimates of lysine requirement by different mathematical models according to different expressing modes and response criteria. It appears that mathematical models greatly affect the estimates of lysine requirement. Irrespective of the mode of expressing lysine requirement or the response variable, the broken-line model yielded the lowest estimates. The highest estimates were dependent on the response variable: the exponential model yielded the highest estimates when the response variable was body-weight gain, whereas saturation kinetics yielded the highest estimates when the response variable was NRE. Differences between the highest and the lowest values across different mathematical models vary from 36 to 91 %. Response variables also affected the estimates of lysine requirement. In general, estimates were higher when using NRE as the response variable than those when using body-weight gain as the response variable. Moreover, the magnitude of difference between the results from two response variables was affected by mathematical models. When comparing the estimates obtained by using the two response criteria, the differences were relatively small (below 15 %) for the broken-line model and the exponential model, between 25 and 32 % for the logistic model, but as high as 50 % for the saturation kinetics model.

Table 2. Goodness of fit* when lysine concentration was expressed as % diet, % protein or g/MJ digestible energy (DE) and the response variable was expressed as body-weight gain (g/kg metabolic body weight per d)

Models	% Diet	% Protein	g/MJ DE
Broken-line	1134·9	1079·2	1210·4
Exponential	1137·0	1097·4	1121·4
Logistic	1059·4	988·1	1095·5
Saturation kinetics	1046·5	981·0	1066·1

* Selection criteria: corrected Akaike information criteria; smaller values are better.

Table 3. Goodness of fit* when lysine concentration was expressed as % diet, % protein, or g/MJ digestible energy (DE) and the response variable was expressed as nitrogen retention efficiency

Models	% Diet	% Protein	g/MJ DE
Broken-line	1438·6	1404·6	1496·9
Exponential	1385·4	1368·1	1385·1
Logistic	1394·3	1368·4	1419·0
Saturation kinetics	1398·7	1377·1	1418·0

* Selection criteria: corrected Akaike information criteria; smaller values are better.

Multilevel analysis

Table 5 presents the results from the multilevel analysis. The null model represents the assumption that lysine requirement should be expressed as % diet. This null model could not be rejected in favour of the alternative model with both CP and DE. In addition, the coefficient of the variable DE in the multilevel model with CP and DE was not statistically significant from zero. Therefore, the variable DE was dropped from the multilevel model, and this resulted in the alternative model with CP only. The mixed model analysis was then re-run on the alternative model with CP. Results from the χ^2 test on the deviance difference between the null model and the multilevel model with CP suggested that the null model could be rejected in favour of the multilevel model with CP, and the effect of CP was statistically significant ($P < 0{\cdot}05$). Multilevel analyses on the full dataset and the sub-dataset yielded the same results, suggesting that CP had a significant effect on lysine requirement irrespective of the response criteria (weight gain or protein deposition) in assessing lysine requirement. A further multilevel analysis was carried out with log-transformed initial body weights as a fixed effect being added onto the multilevel model with CP. The coefficient of the variable log BW in the resulted multilevel model was not statistically significant from zero. This lack of the effect from body weight was observed irrespective of whether weight gain or protein deposition was used as a response variable. Overall, results from the multilevel analysis suggest that CP had a significant effect on lysine requirement, whereas DE or BW did not appear to affect lysine requirement in the present study.

Discussion

Different modes of expressing AA requirement have been proposed and used in the fish nutrition literature, namely % diet, % protein or g/MJ DE. Controversy arises from the fact that each mode can find support on theoretical and experimental bases[2,3]. The present study analysed these modes of expression from a quantitative meta-analysis perspective. While it is difficult to investigate various factors in a single experiment, the meta-analysis provides an effective approach to integrate and synthesise experimental observations from

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Table 4. Estimates of lysine requirement by different modes of expression, response variables and mathematical models

Expression modes...	% Diet			% Protein			g/MJ DE		
Response variables	Weight gain	NRE	Difference (%)	Weight gain	NRE	Difference (%)	Weight gain	NRE	Difference (%)
Broken-line	1·66	1·70	2·4	4·55	4·84	6·4	0·89	1·01	13·5
Exponential	2·47	2·73	10·5	6·19	7·02	13·4	1·28	1·39	8·6
Logistic	1·79	2·37	32·4	4·64	5·81	25·2	1·06	1·40	32·1
Saturation kinetics	2·03	3·16	55·7	5·18	7·65	47·7	1·26	1·93	53·2

DE, digestible energy; NRE, N retention efficiency.

different studies. The present study carried out non-linear mixed model analysis and multilevel analysis to investigate the effects of dietary CP and DE on lysine requirement in order to identify the best way of expressing lysine requirement for fish.

Through the non-linear mixed model analysis, the present study found that the best goodness of fit to the modelling data was produced when lysine concentration was expressed as a percentage of dietary protein. The multilevel analysis further confirmed that dietary DE level did not affect lysine requirement, but CP content did; lysine requirement (% diet) increases with dietary protein level. Therefore, the results of the present study suggest that it is more appropriate to express lysine requirement as % protein than to express it as % diet or g/MJ DE. In fish nutrition, the limitations of the common practice of expressing AA requirement as a fixed concentration of diet have been recognised, but whether to express AA requirement as % protein or g/MJ DE remains difficult to resolve[2]. To relate AA requirement to DE content of the diet in fish nutrition is based on the assumption that DE determines feed intake (and consequently AA intake); therefore, dietary AA levels should be adjusted according to dietary DE levels to maintain the AA intake[2,22,63]. This mode of expression has also been found valid in pig nutrition based on the well-documented relationship between energy and protein supply on protein deposition[64–69]. In pigs, it has been well established that dietary energy supply affects AA requirement, but not the efficiency of AA utilisation; therefore, AA requirement is specific to a given energy level[67–71]. In poultry, recommendations on AA requirements can also be found as a ratio to dietary energy[72,73], but this mode of expression has been disputed by some studies[74,75]. Experimentation with rainbow trout has revealed that dietary DE levels affected the marginal lysine utilisation efficiency[5]. Similar effects of dietary energy content have also been observed on methionine and leucine utilisation in steers[76,77]. Results from these studies indicate that different farmed animals may differ in the relationship between energy supply and AA requirement/utilisation. The present study agrees with the experimental findings in fish that there is a lack of the effect of DE levels on lysine requirement and it is not appropriate to express lysine requirement as a ratio to DE[2,5].

In poultry, a number of studies have provided strong argument to relate AA requirement to dietary protein[74,78–81]. In fish, estimates of AA requirement expressed as % protein can also be found in the literature[82,83]. This mode of expression implies that dietary protein supply affects AA requirement, but does not affect the marginal AA utilisation efficiency. A few studies have investigated the effect of dietary protein on AA requirement or utilisation efficiency. Lysine utilisation appeared to be lower when rainbow trout were fed diets containing 35 % dietary protein compared with 55 % dietary protein, but the difference was not statistically significant[84]. A study conducted with Atlantic salmon fry also found a slight but non-significant increase in lysine retention efficiency by a high-protein diet[85]. Although not statistically significant, these results may be construed as evidence against expressing lysine requirement as % protein. Conversely, some studies observed that protein levels affected

Table 5. Results of the multilevel analysis

	Intercept		CP		DE or log BW		χ^2 test*		
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Deviance difference	df	*P*
Full dataset†									
Null model	1·860	< 0·0001	–	–	–	–	–	–	–
Multilevel model with CP and DE	0·853	0·014	0·033	< 0·001	− 0·021	0·291	1·1	2	0·577
Multilevel model with CP	0·692	0·024	0·028	< 0·001	–	–	5·9	1	0·015
Multilevel model with CP and log BW	0·715	0·023	0·028	< 0·001	− 0·014	0·617	0·9	2	0·638
Sub-dataset‡									
Null model	1·964	< 0·0001	–	–	–	–	–	–	–
Multilevel model with CP and DE	0·467	0·303	0·046	< 0·0001	− 0·025	0·231	3·5	2	0·174
Multilevel model with CP	0·207	0·604	0·041	0·0001	–	–	7·9	1	< 0·005
Multilevel model with CP and log BW	0·272	0·489	0·038	0·0005	0·054	0·141	5·3	2	0·071

CP, crude protein; DE, digestible energy; BW, body weight.
* χ^2 test on the deviance difference between the multilevel models and the null models.
† Dataset with body-weight gain as the response criterion.
‡ Dataset with protein deposition as the response criterion.

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lysine requirement, but not marginal lysine utilisation efficiency in rainbow trout fry[6,7]. The study of Nang Thu *et al.*[7] also observed that fish appear to regulate their feed consumption based on protein intake for potential protein deposition rather than energy intake, an observation that is in agreement with several other studies[5,86,87]. Results from these studies indicate that lysine requirement should be expressed as % protein. Therefore, it appears that there is conflicting experimental evidence regarding expressing lysine requirement as a percentage of dietary protein in the literature. The results from the present study lend support to the argument to relating AA requirement to dietary protein from a quantitative meta-analysis perspective. Future research is warranted to shed more light on this topic. In any case, one should heed the limitations associated with this mode of expression: the possible underestimate of AA requirement due to excessive protein and the lack of reflection of true protein composition by CP[2].

The present study disagrees in part with a previous quantitative review by Hauler & Carter[4], which suggested that it makes more sense to express lysine requirement as a ratio to DE than to express it as a percentage of dietary protein for fish. Differences exist between the present study and the study of Hauler & Carter[4]. The modelling dataset in the present study encompasses a broad number of studies and over half of these studies were published during the last decade, reflecting the latest information on lysine nutrition in fish. The present study also conducted separate analyses based on the response criteria of body-weight gain and protein deposition. It is known that different response criteria result in different AA requirement estimates[2]; therefore, separate analyses eliminate the confounding effect of response criteria. Furthermore, the present study employed two approaches (non-linear mixed model analysis and multilevel analysis) to quantitatively integrate and synthesise results from published studies, and the results from these two modelling approaches corroborate with each other. To account for the random effect of each study is particularly important for using a modelling approach to quantitatively integrate and synthesise results from different studies, because each study represents a random sample of a larger population[8]. This allows accounting for between-study heterogeneity[8]. In addition to dietary composition, other factors such as body size or life stage of fish, experimental condition and mathematical models used to derive the requirement may be associated with the variation of lysine requirements[2]. In the present study, mixed model analysis and multilevel analysis considered dietary factors as the fixed effect, and other factors as the random effect; these approaches effectively delineated the effect of dietary factors from other factors that contribute to the variation of lysine requirement estimates.

Mathematical models used in fitting the dose–response curve in requirement studies have been found to influence the estimates of nutrient requirement[2]. Indeed, there are considerable differences among lysine requirement estimates by the four non-linear models in the present study. Adding to this complexity is that estimates of lysine requirement also differ greatly according to the response criteria (body weight gain *v.* protein deposition). Estimates obtained from protein deposition were higher than those from body-weight gain, which agrees with literature findings[2,22]. This present study, however, further quantified the differences between the estimates obtained from different response variables and found these differences were dependent on different mathematical models: the differences were relatively small (below 15 %) for the broken-line model and the exponential model, between 25 and 32 % for the logistic model, but as high as 50 % for the saturation kinetics model. Irrespective of the mode of expressing lysine requirement or the response variable, the broken-line model yielded the lowest estimates. This is in agreement with literature reports that the broken-line model tends to underestimate nutrient requirement[88–90]. Furthermore, the present study established that the highest estimates were dependent on the response variable: the exponential model yielded the highest estimate when the response variable was body-weight gain, whereas saturation kinetics yielded the highest estimate when the response variable was NRE. The best-fit model is also dependent on response criteria. The present study identified that the saturation kinetics model was the best-fit model when the response variable was body-weight gain, whereas the exponential model was the best-fit model when the response variable was protein deposition efficiency. These results demonstrate the importance of specifying the chosen response criteria and mathematical models when reporting AA requirement estimates. Meaningful comparison of requirements from different studies can only be made on estimates produced by the same model. Consequently, in the present study, the multilevel analysis was carried out on lysine requirements estimated by the same model (the broken-line model) from different studies. Although the broken-line model may underestimate the actual requirement, it is inconsequential to the multilevel analysis. This is because the objective of the multilevel analysis was to test the dietary effect on lysine requirement on a comparable basis. The benefit of choosing the broken-line model is that it yields unequivocal requirement estimates as well as standard errors of the estimates, which are required by the multilevel analysis. In contrast, standard errors of requirement estimates are not produced by the other non-linear models. In the present study, results from the multilevel analysis and the non-linear mixed model analysis strongly corroborate with each other; results from both approaches support expressing lysine requirement as % protein for fish.

The present study focused on investigating dietary factors that affect lysine requirement. Other factors, such as biological factors (life stages or fish species), may also affect lysine requirement. Fish size could be an important factor in assessing AA utilisation and requirement. It is known that protein requirement decreases as fish grow, and thus it is likely that AA requirement also decreases with fish body weight[2]. However, so far, this has not been substantiated by direct experimental evidences. Most studies on lysine requirement have been carried out on juvenile fish, and there are very few data on fish of medium and large sizes. In the present study, the multilevel analysis was employed as an attempt to delineate the effect of body weight on lysine requirement. Results from the analysis appear to suggest a non-significant effect of body weight. The aforementioned lack of sufficient data on medium- and large-sized fish may have limited the scope of the analysis in the present study and consequently these results should be viewed as inconclusive. Differences among fish species might exist[2], although it has been argued that lysine requirement is relatively homogeneous across fish species[91]. However, the delineation and quantification of species effect are currently hindered by the limited data available on different fish species in the literature. Even though many studies have been conducted on lysine requirement, these studies spread over different species and thus the numbers of studies for each fish species are very limited. Since the objective of the present study was to find out the most appropriate mode of expressing lysine requirement, the pertinent issue is whether the most appropriate mode differs among fish species. Currently, there is no evidence to suggest that it differs. Therefore, in the present study, differences among species were ascribed to but not specifically differentiated from the between-study heterogeneity. The issues of fish species and life stages can be revisited in the future when there are a sufficient number of studies conducted with each fish species at different life stages to allow a proper quantitative evaluation.

The present study investigated the three modes of expressing EAA requirements commonly used by fish nutritionists. These modes are based on dietary inclusion levels. In practice, it is also possible to express lysine requirement as daily intake. Screening studies based on feed intake information would result in a significant reduction of sample size of the modelling datasets. Studies have to be excluded in the case where no information on feed intake was reported, where fish were fed to an excess or fixed amount but there was no report on whether the feeds were all consumed by fish or whether measures were taken to account for the feed wastage. Since the objective was to compare the three common methods of expressing lysine requirement and to investigate the underlying dietary factors that affect lysine requirement of fish, the option of expressing lysine requirement as daily intake was not assessed in the present meta-analysis.

It has been advocated that AA studies should move away from dose–response experiments to those striving to investigate the cause–effect relationship[1]. Increasingly, factorial models are being used to predict AA requirement[2]. The factorial approach is based on the information on maintenance requirement and AA deposition for body protein and its utilisation efficiency. A step further is to estimate AA requirements by mechanistic growth and nutrient partitioning models that take into account maintenance, deposition, inevitable and preferential AA metabolism[2,92]. EAA requirements can thus be dynamic and flexible based on the response of fish to a specific dietary composition to achieve a certain performance at a defined life stage. This would effectively incorporate both dietary factors and biological factors in estimating lysine requirement. In factorial and mechanistic models, AA requirements can be expressed as mg/d, mg/kg BW per d or mg/kg MBW per d, taking feed intake into account. Discussions on developing factorial or mechanistic models are beyond the scope of the present study. Nevertheless, to express AA requirement as % diet, % protein or g/MJ DE is simple, straightforward and will probably continue to be used in feed formulation, as one can find in the nutrient requirement tables published by the NRC[2]. The present study suggests that the current practice adopted by most fish nutritionists to express requirement as a fixed concentration of diet provides less goodness of fit than to express requirement as a percentage of dietary protein, but is generally better than to express requirement as a ratio to DE content.

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