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Application of meta-analysis towards understanding the effect of adding a methionine hydroxy analogue in the diet on growth performance and feed utilization of fish and shrimp

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Abstract

Methionine hydroxy analogue (MHA) has been widely used and shows positive effects on growth in poultry, swine, ruminant and aquatic animals. Nevertheless, the utilization efficiency of methionine hydroxy analogue remains controversial considering the wide variation in effects across studies, feeding parameters and environmental culture conditions. Meta-analysis can quantify the effect of adding MHA on animal performance. Here, we analysed the effect of MHA-supplemented diets on the final weight (FW), per cent weight gain (WG), protein efficiency ratio (PER), feed conversion ratio (FCR) and feed efficiency (FE) in common, diverse aquaculture taxa. To conduct the meta-analyses, twenty-three published studies were included that accounted for 249 effect sizes estimated across eight fish and one shrimp species. The effect size (measured as the standardized mean difference; Hedges' g) of response parameters between MHA level in a diet formulation and an MHA-less control condition was calculated. Based on these results, adding MHA in the diet can significantly improve FW, WG, PER, and FE and decrease FCR for fish rather than shrimp. Using meta-regression analysis, there was a significant quadratic linear relationship between MHA addition and effect size for FW ($P = 0.002$ for MHA, $P = 0.042$ for MHA^2), and significant linear relationships between MHA addition and effect size for WG ($P = 0.0005$) and FCR ($P = 0.002$). There was no significant relationship, linear or non-linear, between the MHA addition and effect size for FE ($P = 0.985$) and PER ($P = 0.461$). In all, when properly dosed in diets, MHA can significantly improve aquaculture production for fish.

Key words: feed utilization, fish, growth performance, meta-analysis, methionine hydroxy analogue, shrimp.

Introduction

The global supply of fishmeal has reached a plateau (FAO 2018), causing higher prices and lower availability for aquafeed producers around the world. Less expensive protein sources, especially plant proteins that provide a steady supply and availability of large quantities, have grown in popularity within the aquafeed industry for more than a decade. Considerable effort has gone into the development and validation of high soya feed formulations for shrimp and fish (Cheng *et al.* 2018; Novriadi *et al.* 2018; Qiu *et al.* 2018). Methionine (Met) is usually the first limiting amino acid which plays an important role for many fish and shrimp diets, especially those containing higher levels of plant protein sources (Mai *et al.* 2006; Espe *et al.* 2008). Consequently, the supplementation of formulated aquaculture feeds with crystalline amino acids (CAAs) has also become a common practice to fulfil the nutritional requirements of essential amino acids (EAAs) for multiple species (Yuan *et al.* 2011). However, several studies have shown that plant protein diets supplemented with CAAs are not as efficient as diets with protein-bound amino acids, possibly due to the high potential for leaching of CAA (Fox *et al.* 2006; Gu *et al.* 2013) or the difference between CAAs and intact proteins in the intestinal uptake and metabolism of AAs (Schuhmacher *et al.* 1997; Ambardekar *et al.* 2009).

Therefore, there is considerable interest in products with high utilization efficiency and lower leaching loss to replace the traditional CAAs.

Various methods, such as coating, encapsulation, microencapsulation or polymerization, have been used to reduce the leaching losses and absorption rate for fish and shrimp diets (Villamar & Langdon 1993; Zhou *et al.* 2007; Gu *et al.* 2013), determine reference dietary amino acid patterns for aquatic animals (Millamena *et al.* 1996; Alam *et al.* 2002) and retard their absorption from the intestine (Alam *et al.* 2004). Niu *et al.* (2015) demonstrated that microcapsules could regulate methionine release and stimulate the synchronous adsorption of other amino acids to synthesize proteins. The enzymatic oligomerization of α-amino acid esters for a wide array of proteases and amino acid esters has also been studied extensively (Schwab *et al.* 2012). Oligo-methionine is prepared by the papain-catalysed oligomerization of L-methionine ethyl ester (Jost *et al.* 1980) and has been shown to have nearly the same or better supplementary effects as free methionine on growth performance in rats (Chiji *et al.* 1990; Kasai *et al.* 1996), Pacific white shrimp (Gu *et al.* 2013), turbot (M. Gu, W.B. Zhang, N. Bai, K.S. Mai & W. Xu, unpublished data) and yellow croaker (Ma *et al.* 2016). However, both products have significantly increased the cost of the diets, especially for oligo-methionine-supplemented feed.

An analogue of methionine, named 2-hydroxy-4-(methylthio) butanoic acid (MHA) (84% Ca salt), is much more efficient and cheaper than other alternatives, such as crystalline methionine (Ma *et al.* 2013), coated methionine and oligo-methionine. It has been shown to be effective in meeting methionine requirements and is widely used in the production of diverse animals, such as livestock and aquatic species (Cheng *et al.* 2003; Forster & Dominy 2006; Zhao *et al.* 2010; Browdy *et al.* 2012; Ma *et al.* 2013; Hu *et al.* 2015). Although the literature is still contentious regarding the utility of MHA supplements to improve the growth performance and feed utilization for aquaculture animals, some recent narrative reviews have summarized the effect of MHA in aquaculture (NRC 2011; Nunes *et al.* 2014). Though they are of certain value in summarizing literature and identifying research gaps, there are some limitations in these past analytic narratives. For example, they misinterpreted the research findings, focused too much on statistical difference instead of biological significance (i.e. vote counting) and failed to examine the major moderators involved in previous studies. Quantitative syntheses (i.e. meta-analyses) offer powerful alternatives to narrative reviews considering that they provide insights as to the magnitude of intervention effects (Glass 1976). For example, several recent meta-analyses have compared the biological efficiency of DL-methionine with methionine-hydroxy-analogue-free-acid in broiler chickens (Kratzer & Littell 2006; Vazquez-Anon *et al.* 2006; Sauer *et al.* 2008). Moreover, Feng *et al.* (2018) used meta-analysis to evaluate the productivity response to methionine supplementation in cows and define the relationship between metabolizable Met intake and production. Despite the growing interest in quantitative syntheses in the broader literature (De Ridder & Lensvelt-Mulders 2018; Hossain *et al.* 2019; Zych *et al.* 2019), meta-analysis is still relatively rare in aquaculture (Novriadi 2017; Wadsworth *et al.* 2018).

As a functional and economical source of Met, MHA has been the primary focus for Met supplementation in the aquaculture industry (Nunes *et al.* 2014). The main chemical difference between MHA and DL-methionine is that MHA has a hydroxyl group on the alpha carbon, while DL-methionine has an amino group that resembles commonly used organic acids, such as lactic acid or fumaric acid. In organisms, MHA can be converted to L-Met in the peroxisomes within the liver and kidney, while D-Met is processed with a mitochondrial enzyme in most tissues once absorbed (Baker 2006). A wide range of bioavailability of MHA in comparison with DL-Met estimates have been reported using different meta-analytical methods in broilers, pigs and cows (Jansman *et al.* 2003; Vazquez-Anon *et al.* 2006; Sauer *et al.* 2008; Vedenov & Pesti 2010). Nevertheless, the utilization efficiency of MHA in fish and shrimp diets remains controversial. The recent National Research Council (NRC) stressed that MHA can be used as a source of methionine by fish; however, MHA is not available or efficacious, like DL-Met or L-Met, on an equimolar basis (NRC 2011). Nunes *et al.* (2014) rebutted the NRC (2011), which omitted some publications where the relative biological efficiency of MHA for poultry and aquaculture species was higher than the range reported by the NRC. In our study, all of the publication data were screened systematically using PRISMA guidelines (Fig. 1).

Knight *et al.* (2006) demonstrated that the differences in feed intake or consumption are not due to the inefficiency of conversion of MHA to DL-Met that led to the differences in performance between MHA and DL-Met. The result of meta-analysis to evaluate the bio-efficiency can also vary with the different statistical models and criteria used in select publications (Agostini *et al.* 2015). Thus, in the present study, we used meta-analysis to quantify the effects of MHA inclusion on the growth performance (final weight, per cent weight gain and protein efficiency ratio) and feed utilization (feed conversation ratio or feed efficiency) for fish and shrimp instead of estimating the biological efficiency of MHA in comparison with DL-Met for aquaculture animals. We hypothesized that supplementation of MHA in the diet would have a positive effect on fish and shrimp growth performance and feed utilization. Additionally, we hypothesized that there would be interspecific differences in the effect of MHA supplementation in the diet.

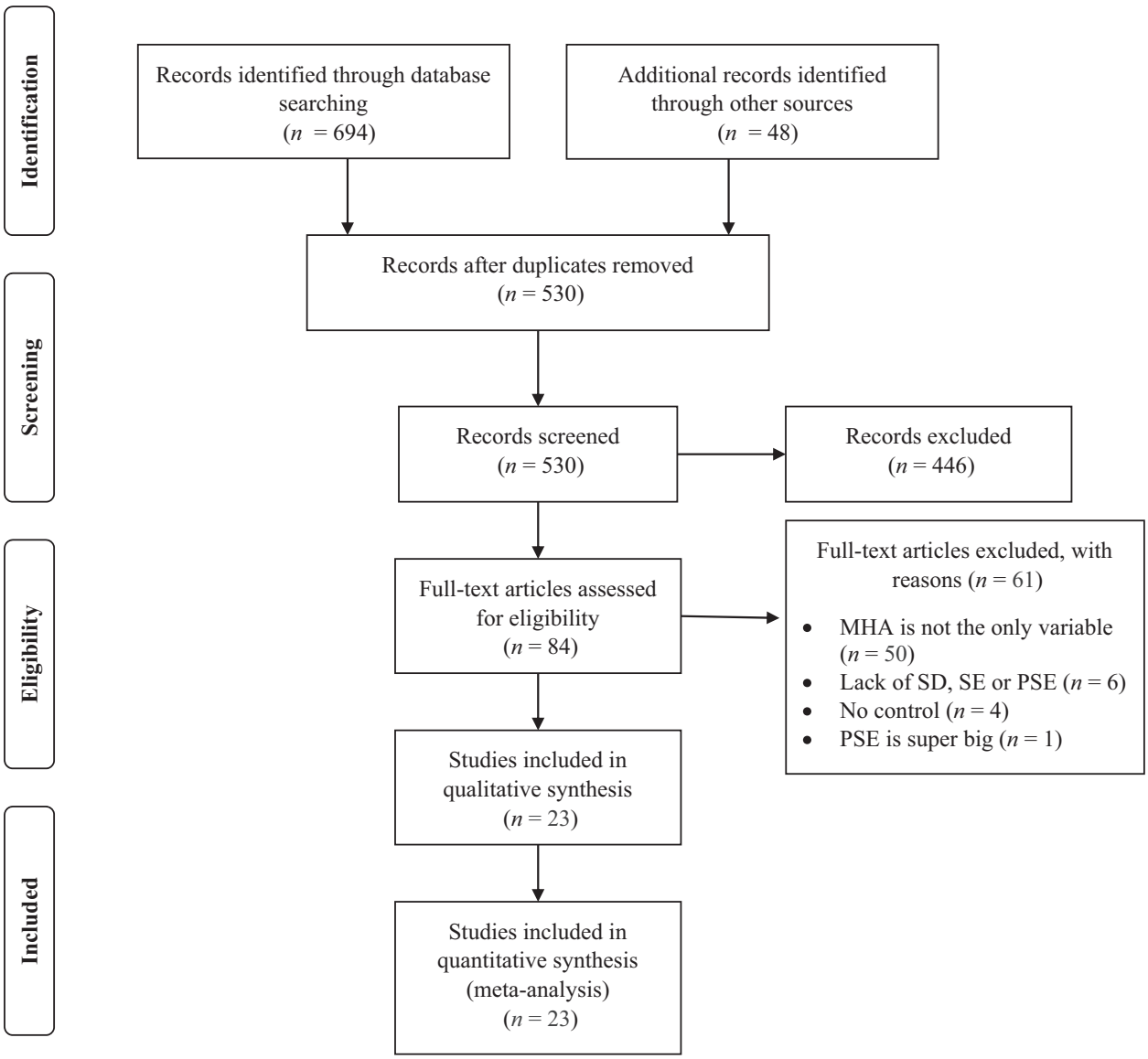

Figure 1 Flow chart for search results and selection details based on Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

Method

Search method

Relevant literature was gathered from Web of Science, Google Scholar and China National Knowledge Infrastructure (CNKI) to identify articles published from 1986 (MHA was first used in aquaculture in 1978, and the first published report included in this analysis was published in 1986) to June 2018 with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (see http://www.prisma-statement.org/statement.htm; Fig. 1).

Keywords included a combination of the following terms: 'methionine hydroxy analogue and fish', 'methionine hydroxy analogue and shrimp', 'MHA and fish', 'MHA and shrimp', '2-hydroxy-4-(methylthio) butanoic acid and fish' and '2-hydroxy-4-(methylthio) butanoic acid and shrimp'. Additional studies were included based on previous experiences with the literature and the reference lists of newly discovered and relevant publications. Data gleaned from each study included mean response, sample size and some measurement of treatment error (e.g. standard deviation (SD), standard error (SE) or pooled standard error (PSE)). Data were extracted from tables, when available, or from graphs using the tool available at http://datathief.org/.

The evaluation of growth performance in fish or shrimp associated with MHA supplements in the diet was based on

changes in final weight, per cent weight gain and/or protein efficiency ratio. The evaluation of MHA-mediated feed utilization effects was based on feed conversion ratio (FCR) or feed efficiency (FE). A number of moderating variables were considered to explain variation in the meta-analysis, when available, including the concentration of fishmeal in the basal diet, methionine (Met) concentration in the basal diet, sample size, duration of an experiment, focal species, type of methionine hydroxy analogue (MHA, MHA salt, or Alimet) and the concentration of methionine hydroxy analogue (MHA) added to the diet. To be included in this study, a study needed to (i) focus on fish or shrimp, (ii) include diets that manipulated only one variable associated with MHA or without other main ingredient changes and (iii) conduct controlled experiments that assessed the growth performance and provided sufficient details for calculating an effect size and confidence intervals, namely treatment mean responses and some estimate of error (e.g. SD or SE). Some articles provided a pooled standard error (PSE) as group mean error. PSE is the approximately the average SE for all the groups. In the absence of any other information, we used the same PSE for each group in a study. SE was converted to SD using the following formula: SD = SE * sqrt (replicate number). Almost all the different doses of MHA tested in the papers were collected. However, some high doses of MHA that were used to determine its safety in feed were omitted based on methionine requirements of relevant species.

Primary response calculations are provided below.

$$\begin{aligned}&\text{Per cent weight gain (WG) (\%)}\\&= 100 \times \left[(\text{final weight (g)} - \text{initial weight (g)})\right.\\&\quad\left./\text{initial weight (g)}\right]\end{aligned}$$

$$\begin{aligned}&\text{Protein efficiency ratio (PER)}\\&= \text{body weight gain (g)/protein intake (g)}\end{aligned}$$

$$\text{Feed conversionratio (FCR)} = \text{feed given (g)/weight gain (g)}$$

$$\begin{aligned}\text{Feed efficiency (FE)} = {} & (\text{final weight (g)} - \text{initial weight (g)})\\ & /\text{feed offered (g)}\end{aligned}$$

Studies without controls or that did not provide estimates of error were not considered in later analyses. Studies fulfilling the above criteria were considered eligible to be included in this study.

Data collection

The following types of data were gleaned from each study, including sample size, number of treatments, focal species, MHA supplement concentration in each diet, final weight (FW), WG, PER, FCR, and FE for each treatment and control group, type of MHA, study duration and concentration of fishmeal added to each treatment. Due to small sample sizes, species were grouped into broader categories (e.g. Jian carp and grass carp both belong to carp). The complete data set included 249 effect sizes (n = 54 for FW, n = 67 for WG, n = 43 for PER, n = 52 for FCR, n = 33 for FE) from 23 studies that included eight different fish species including carp (Shen *et al.* 2007; Jia 2010; Li 2010; Xiao *et al.* 2011; Shan *et al.* 2015; Pan *et al.* 2016), channel catfish (Zhao *et al.* 2017), red drum (Goff & Gatlin 2004), sea bass (Keembiyehetty & Gatlin III 1995; Keembiyehetty & Gatlin 1997; Kelly 2005; Kelly *et al.* 2006; Savolainen & Gatlin III 2010; Zhang *et al.* 2017), rainbow trout (Poston 1986; Cheng *et al.* 2003), Nile tilapia (Zhao *et al.* 2010), large yellow croaker (Ma *et al.* 2016), and turbot (Ma *et al.* 2013; Hu *et al.* 2015), and one shrimp species, the Pacific white shrimp (Forster & Dominy 2006; Browdy *et al.* 2012; Chen *et al.* 2018).

Data accessibility

R code, literature search and data are as available in the Supporting Information.

Data analyses

Effect size calculations

Hedges' g served as the effect size because it corrects for differences in sampling effort among studies and adjusts for small sample sizes and was calculated using the following formula: $g = \frac{Mean_T - mean_C}{\sqrt{\frac{(n_1-1)SD_1^2+(n_2-2)SD_2^2}{n_1+n_2-2}}} * \left(1 - \frac{3}{4*(n_1+n_2)-9}\right)$, where mean_T is the mean of the treatment group, mean_C is the mean of control group, n_1 and n_2 are two sample sizes, and SD_1^2 and SD_2^2 are the estimated population variance of both groups (Hedges & Olkin 2014).

Mean Hedges' g effect sizes, 95% confidence intervals (95% CI) and 95% prediction intervals (95% PI) were calculated for each primary response variable (WG, FW, PER, FCR and FE). A positive Hedges' g value (lower 95% CI >0) indicates that the performance of fish or shrimp improved with the presence of MHA supplement in the diet. A negative Hedges' g value (upper 95% CI <0) indicates that the performance of fish or shrimp declined with the presence of an MHA supplement in the diet. Hedges' g is also interpreted in a similar way to Cohen's d (Cohen 1977) that suggested using the following criteria when interpreting the magnitude of standardized mean difference effect sizes: small effect = 0.2, medium effect = 0.5 and large effect = 0.8. Considering that MHA supplements to

diets (active level: 0.034–1.346%) are continuous variables, meta-regression was used to determine whether a significant relationship existed between MHA supplements with FW, WG, PER, FCR and FE. Lastly, heterogeneity estimates, such as I^2 and the chi-squared statistic (Q), were calculated for all study groups. Q is distributed as a chi-square statistic with number of studies minus one degrees of freedom (Gavaghan *et al.* 2000), which has sufficient power to test heterogeneity if the number of studies is large (Higgins *et al.* 2003).

Leave-one-out sensitivity analyses were used to see how the among-study heterogeneity (I^2) varied with the removal of a single study from each meta-analysis. Funnel plots and Tweedie's non-parametric trim and fill approach were implemented to assess publication bias.

Moderator tests

Given the heterogeneity in experimental methods, focal taxa and diet formulations across the studies used in this meta-analysis, a random effects model was used. A mixed-effects model was used for the moderator tests given that subgroup responses were hypothesized to vary across species due to the addition of MHA in the diet.

All statistical analyses were performed using the *metafor* package in R version 3.3.0.

Results

Overall effect size summary

All the papers' data set details and meta-analysis outcomes were shown in Tables 1 and 2, respectively. Results' tables accessibility: Forest plot without and with different concentrations of methionine hydroxy analogue (MHA; active level: 0.034–1.346%) added as a continuous moderator for corresponding parameters with 95% confidence limits for each study were listed in Figures S1–S5. MHA-supplemented diets of fish or shrimp showed significant positive effects on FW (mean effect = 3.02, 95% CI = 2.14, 3.89; $P < 0.0001$; $n = 54$; Fig. S1), WG (mean effect = 3.81; 95% CI = 2.78, 4.83; $P < 0.0001$; $n = 67$; Fig. S2), PER (mean effect = 2.95; 95% CI = 2.06, 3.84; $P < 0.0001$; $n = 43$; Fig. S3) and FE (mean effect = 3.12; 95% CI = 1.51, 4.74; $P < 0.0001$; $n = 34$; Fig. S5), and significant negative effects on FCR (mean effect = −1.50; 95% CI = −2.07, −0.93; $P < 0.0001$; $n = 52$; Fig. S4). Thus, MHA in the diet has large positive benefits to a variety of important aquaculture production parameters based on the overall effect size. Heterogeneity estimates were high ($I^2 > 98\%$) based on the leave-one-out sensitivity analyses showing that no particular study accounted for a majority of the across study variation in effect sizes. Moreover, including different focal species as a moderator in our overall model had little effect on heterogeneity ($I^2 = 98.95\%$ for FW, $I^2 = 99.28\%$ for WG, $I^2 = 98.57\%$ for PER, $I^2 = 97.78\%$ for FCR and $I^2 = 99.43\%$ for FE).

Publication bias results from funnel plot showed that there is publication bias for all the effect sizes of FW, WG, PER, FCR and FE (Figs S6–S10). Thus, trim and fill was used to estimate the number of missing studies that might exist in this meta-analysis. Summary of adjusting publication bias results using trim and fill method was shown in Table S1. The trim and fill method estimated 14 missing studies on the left side of the funnel plot for FW. Incorporation of these randomly created studies still resulted in a significant effect of MHA supplementation in the diet (adjusted mean effect = 1.24; 95% CI = 1.15, 1.33; $P < 0.0001$; fail-safe $n = 68$). The trim and fill method estimated 13 missing studies on the left side of the funnel plot for WG and incorporation of these randomly created studies still showed a significant effect of MHA in the diet (adjusted mean effect = 1.64; 95% CI = 1.55, 1.72; $P < 0.0001$; fail-safe $n = 81$). The trim and fill method estimated 17 missing studies on the left side of the funnel plot for PER and incorporation of these randomly created studies which showed a significant effect (adjusted mean effect = 1.05; 95% CI = 0.95, 1.15; $P < 0.0001$; fail-safe $n = 60$). The trim and fill method estimated 8 missing studies on the right side of the funnel plot for FCR and incorporation of these randomly created studies still led to a significant MHA effect (adjusted mean effect = −0.81; 95% CI = −0.89, −0.73; $P < 0.0001$; fail-safe $n = 60$). The trim and fill method estimated 12 missing studies on the left side of the funnel plot for FE and incorporation of these randomly created studies still resulted in a significant MHA effect (adjusted mean effect = 0.73; 95% CI = 0.63, 0.84; $P < 0.0001$; fail-safe $n = 46$).

The role of moderators (species) on effect size estimate

Species information from each study was recorded to enable categorization of those species to Chordata (fish) or crustacean (shrimp; Figs 2–6). Not surprisingly, there is a statistically significant difference in effect size of FW, WG, PER, FCR and FE (all the $P < 0.0001$) between Chordata (fish) and crustacean (shrimp). The overall effect size for PER is similar to the effect size for all the fish species since there is no data set collected from the shrimp species for PER. MHA-supplemented diets of fish showed large positive effects on FW (mean effect = 3.23, 95% CI = 2.30, 4.17; $P < 0.0001$; $n = 47$; Fig. 1), WG (mean effect = 4.04, 95% CI = 2.99, 5.08; $P < 0.0001$; $n = 61$; Fig. 2) and FE (mean effect = 3.21; 95% CI = 1.54, 4.88; $P < 0.0001$; $n = 32$; Fig. 5), and negative effects on FCR (mean effect = −1.54; 95% CI = −2.19, −0.90; $P < 0.0001$; $n = 43$; Fig. 4). MHA-supplemented diets of shrimp showed no significant effects on FW (mean effect = 1.58,

Table 1 Summary of details of the studies used in this meta-analysis

Species	Primary response criteria	Data set number	Dietary met (% dry diet)	Met activity (%)	MHA type	MHA inclusion level (%)	FM %	Reference
Pacific white shrimp *Litopenaeus vannamei*	FCR	1*2[a]	0.59% Met + 0.58% Cys (basal)	84	HMTBa	0.1%	7.5%	Browdy *et al.* (2012)
		1*2[a]	0.49%% Met 0.61% Cys (basal)	84	HMTBa	0.2%	0	
	FW, FE	1	0.46% Met + 0.5% Cys (basal)	84	HMTBa	0.6%	None	Forster and Dominy (2006)
	FW, WG, FCR	3	0.63% Met + 0.24% Cys (basal)	84	HMTBa	0.04, 0.07, 0.1%	15%	Chen *et al.* (2018)
Turbot *Scophthalmus maximus*	PER, FE	1	0.6% Met + 0.41% Cys (basal)	84	HMTBa	1%	23.6%	Hu *et al.* (2015)
	FW, PER, FE	5	0.59% Met (basal)	84	HMTBa	0.3, 0.6, 0.9, 1.2, 1.5%	23.6%	Ma *et al.* (2013)
Nile tilapia *Oreochromis niloticus GIFT strain*	FW, PER, FCR	1	2.1% Met + 1.6% Cys	88	MHA	0.19%	None	Zhao *et al.* (2010)
Red drum	FE, PER	1	Unknown	84	MHTBa	0.59%	13.09%	Goff and Gatlin III (2004)
Sciaenops ocellatus	FE	1	Unknown	84	MHTBa	0.59%	11.84%	
	FE	1	Unknown	88	MHA	0.59%	11.84%	
Channel catfish *Ictalurus punctatus*	FW, WG, FCR, PER	1	0.42% Met + 0.38% Cys (basal)	88	MHA	0.17%	1%	Zhao *et al.* (2017)
		1	0.42% Met + 0.38% Cys (basal)	84	HMTBa	0.18%	1%	
Rainbow trout	FW, FCR	1	1% Met (calculated)	88	MHA	0.06%	24.6%	Cheng *et al.* (2003)
Oncorhynchus mykiss		1	1% Met (calculated)	88	MHA	0.13%	16.4%	
		1	1% Met (calculated)	88	MHA	0.2%	8.2%	
		1	1% Met (calculated)	88	MHA	0.28%	0	
		5	0.92, 0.97, 1.02. 1.07, 1.12% Met	88	MHA	0.055, 0.11, 0.165, 0.22, 0.275%	17.5%	
Rainbow trout	WG	3	2.24, 2.75, 3.78% TSAA	88	MHA	0.2, 0.4, 0.8%	0	Poston (1986)
Salmu garrdnnuri		3	2.68, 3.65, 5.58% TSAA	84	HMTBa	0.375, 0.75, 1.5%	0	
	WG, FCR	4	2.26, 2.97, 3.69, 4.48% TSAA	84	HMTBa	0.3%, 0.6%, 0.9, 1.2%	0	

Table 1 (continued)

Species	Primary response criteria	Data set number	Dietary met (% dry diet)	Met activity (%)	MHA type	MHA inclusion level (%)	FM %	Reference
Hybrid striped bass *Murone chrysops* × *M. saxatilis*	WG, FE, PER	1	0.38% Met + 0.13% Cys (basal)	88	MHA	0.58%	13.06%	Keembiyehetty and Gatlin III (1995)
	WG, FE, PER	1	1.12% TSAA	84	MHA	0.3%	14.25%	Keembiyehetty and Gatlin (1997)
	WG, FE, PER	1[b]	1% TSAA	88	MHA	0.67%	12.4%	Kelly *et al.* (2006)
		1[b]	1% TSAA	84	HMTBa	0.67%	12.4%	
		2[c]	1%, 1.25% TSAA	88	MHA	0.49%, 0.74%	12.4%	
	WG, FE, PER	1[d]	1% TSAA	88	MHA	0.67%	12.4%	Kelly (2005)
		1[d]	1% TSAA	84	HMTBa	0.67%	12.4%	
		2[d]	1%, 1.25% TSAA	88	MHA	0.49%, 0.74%	12.4%	
	WG, FE, PER	1	1.1% TSAA	84	HMTBa	0.57%	15%	Savolainen and Gatlin III (2010)
Japanese sea bass *Lateolabrax japonicus*	FW, WG, FCR	4	1.09% Met + 0.8% Cys (basal)	84	HMTBA	0.24, 0.48, 0.71, 0.95%	20%	Zhang *et al.* (2017)
Gibel carp *Carassius auratus gibeilo*	FW, WG, FCR	5	0.52% Met + 0.38% Cys (basal)	84	HMTBa	0.095, 0.19, 0.285, 0.38, 0.475%	10%	Jia (2010)
	FW, WG, FCR, PER	5	0.37% Met + 0.36% Cys (basal)	84	HMTBa	0.119, 0.238, 0.357, 0.476, 0.595%	3.5%	
Common carp *Cyprinus carpio*	FW, WG, FCR, PER	3	0.96, 1.02, 1.07% TSAA	88	MHA	0.12, 0.18, 0.24%	3%	Li (2010)
		3	1.39, 1.43, 1.47% TSAA	88	MHA	0.514, 0.564, 0.614%	5%	
Jian carp *Cyprinus carpio var.* Jian	WG, FCR, PER	1	0.58% Met (basal)	88	MHA	0.11%	3%	Shan *et al.* (2015)
		1	0.58% Met (basal)	84	HMTBa	0.12%	3%	
	FW, WG, FE, PER	5	0.69% Met + 0.79% Cys (basal)	88	MHA	0.51, 0.76, 1.02, 1.27, 1.53%	7.5%	Xiao *et al.* (2011)
	WG, FCR, PER	4	0.831, 0.868, 0.906, 0.944% TSAA	84	HMTBa	0.045, 0.09, 0.135, 0.18%	0%	Shen *et al.* (2007)
Grass carp *Ctenopharyngodon idella*	FW, WG, FE	5	0.4% Met + 0.48% Cys (basal)	88	MHA	0.24, 0.44, 0.64, 0.85, 1.05%	3%	Pan *et al.* (2016)
Large yellow croaker *Larimichthys crocea*	WG, FE, PER	2	0.93% Met + 0.38% Cys (basal)	84	HMTBA	0.25, 0.75%	31.8%	Ma *et al.* (2016)

(a) There are two culture periods including 72 and 96 days. (b–d) Mean that this study culture period is 10, 6 4 weeks, respectively.
FCR, feed conversion ratio; FE, feed efficiency; FW, final weight; PER, protein efficiency ratio; TSAA, total sulphur amino acid; WG, weight gain.

Table 2 Effect size calculation outcomes for final weight, weight gain, protein efficiency ratio, feed conversion ratio and feed efficiency

	Final weight			Weight gain			Protein efficiency ratio			Feed conversion ratio			Feed efficiency		
	k	Hedges' *g* value	C.L.	*k*	Hedges' *g* value	C.L.	*k*	Hedges' *g* value	C.L.	*k*	Hedges' *g* value	C.L.	*k*	Hedges' *g* value	C.L.
All species	54	3.02	2.14–3.89	67	3.81	2.78–4.83	43	2.95	2.06–3.84	52	−1.5	−2.07 to −0.93	33	3.12	1.51–4.74
Subgroups															
All fish species	47	3.23	2.30–4.17	61	4.04	2.99–5.08	43	2.95	2.06–3.84	42	−1.54	−2.19 to −0.90	32	3.21	1.54–4.88
Litopenaeus vannamei	7	1.58	−0.83 to 3.98	6	1.19	−1.36 to 3.74				10	−1.33	−2.63 to −0.02	1	0.98	−8.32 to 10.29
Each species															
Sea bass	4	1.64	−1.27 to 4.51	15	1.74	0.11 to 3.37	11	1.94	0.35 to 3.53	4	−0.54	−2.68 to 1.61	11	3.87	1.06 to 6.68
Carp	26	3.93	2.79 to 5.07	32	3.36	2.25 to 4.48	22	2.69	1.57 to 3.80	22	−1.49	−2.41 to −0.58	10	0.68	−2.18 to 3.54
Catfish	2	1.73	−2.34 to 5.8	2	1.80	−2.63 to 6.23	2	1.26	−2.38 to 4.90	2	−1.91	−4.94 to 1.13			
Rainbow trout	9	0.64	−1.27 to 2.56	10	10.42	8.39 to 12.44				13	−1.78	−2.97 to −0.58			
Nile tilapia	1	1.94	−3.18 to 7.69				1	2.23	−2.92 to 7.39	1	−2.85	−7.14 to 1.45			
Turbot	5	6.48	3.84 to 9.12				6	5.33	3.20 to 7.47				6	4.76	1.06 to 8.47
Large yellow croaker				2	2.49	−1.93 to 6.91							2	0.90	−5.49 to 7.29
Red drum							1	9.10	3.32 to 14.88				3	8.39	2.75 to 14.03

C.L., 95% confidence limits (lower and upper); *k*, sample size (no. of comparisons).

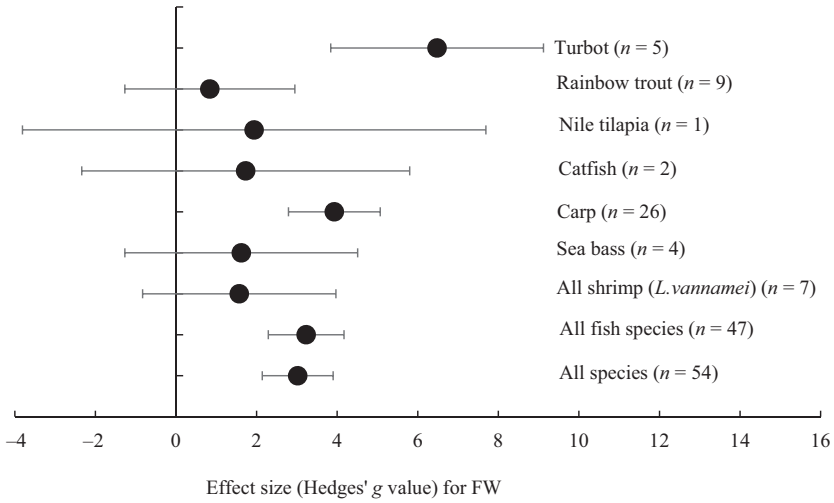

Figure 2 Effect size averages and 95% confidence intervals for all species, targeted subgroups (fish or shrimp) or each species on final weight (FW) from the primary meta-analysis. *N* denotes the number of measurements used to calculate each group effect size. Meta-analysis considers error across and within studies. If $n = 1$, the effect size's mean and error are reported in a paper. If $n > 1$, the effect size's mean and error are reported across studies or within a paper.

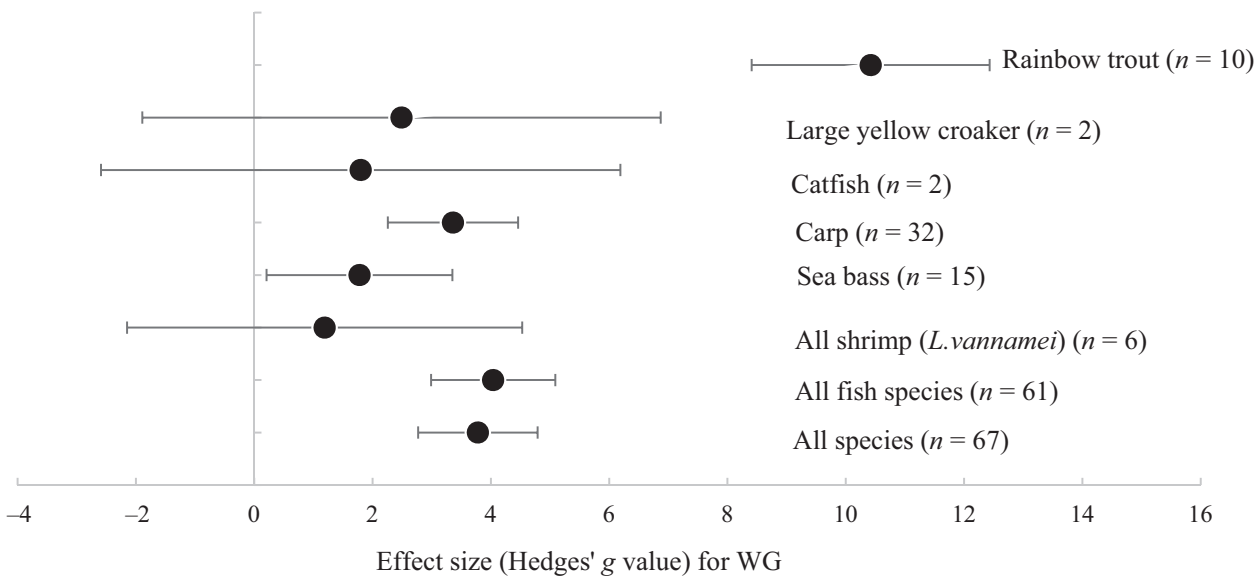

Figure 3 Effect size averages and 95% confidence intervals for all species, targeted subgroups (fish or shrimp) or each species on weight gain (WG) from the primary meta-analysis. *N* denotes the number of measurements used to calculate each group effect size. Meta-analysis considers error across and within studies. If $n = 1$, the effect size's mean and error are reported in a paper. If $n > 1$, the effect size's mean and error are reported across studies or within a paper.

95% CI = −0.83, 3.98; $P = 0.1986$; $n = 7$; Fig. 1), FE (mean effect = 0.98; 95% CI = −8.32, 10.29, $P = 0.8325$; $n = 1$; Fig. 5), WG (mean effect = 1.19, 95% CI = −1.36, 3.74; $P = 0.4852$; $n = 6$; Fig. 2) and a minor effect on FCR (mean effect = −1.33; 95% CI = −2.63, −0.02; $P < 0.0473$; $n = 10$; Fig. 4). Moreover, the effect sizes of Chordata (fish) are significantly larger than that in crustacean (shrimp) for all parameters.

Subsequently, we analysed the effect of species on all five variables, and FW, WG, PER, FCR and FE yielded significant results (Figs 2–6). Given the significant result for FW, WG, PER, FCR and FE ($P < 0.0001$ for QM: test of moderators), we performed a multiple comparison. The result showed that the effect sizes of FW for carp and turbot were positively affected by the addition of MHA in diets (Fig. 2). The effect sizes of WG for sea bass, carp and rainbow trout

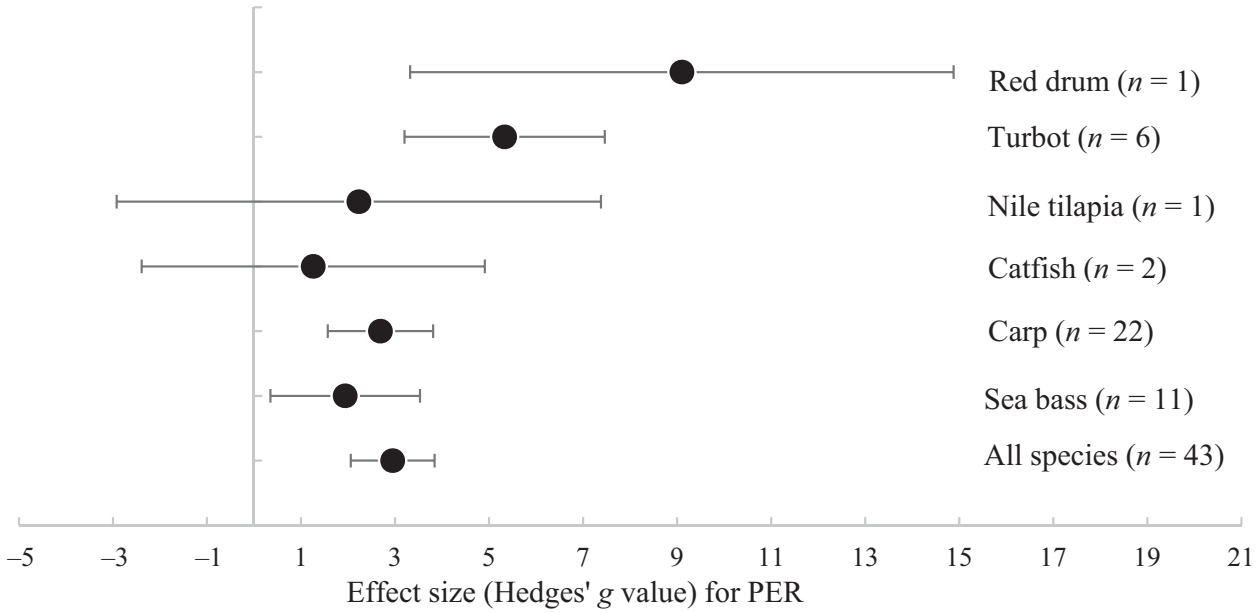

Figure 4 Effect size averages and 95% confidence intervals for all species, targeted subgroups (fish or shrimp) or each species on protein efficiency ratio (PER) from the primary meta-analysis. *N* denotes the number of measurements used to calculate each group effect size. Meta-analysis considers error across and within studies. If $n = 1$, the effect size's mean and error are reported in a paper. If $n > 1$, the effect size's mean and error are reported across studies or within a paper.

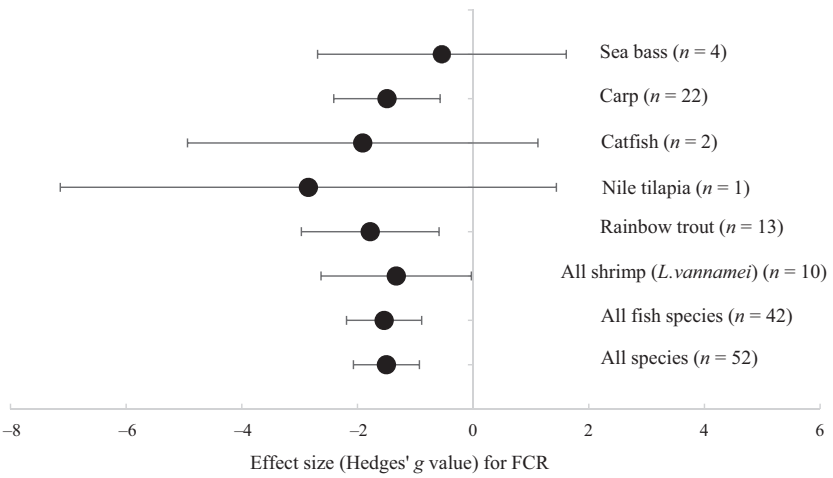

Figure 5 Effect size averages and 95% confidence intervals for all species, targeted subgroups (fish or shrimp) or each species on feed conversion ratio (FCR) from the primary meta-analysis. *N* denotes the number of measurements used to calculate each group effect size. Meta-analysis considers error across and within studies. If $n = 1$, the effect size's mean and error are reported in a paper. If $n > 1$, the effect size's mean and error are reported across studies or within a paper.

were positively affected by the addition of MHA in diets (Fig. 3). The effect sizes of PER for carp, bass, red drum and turbot were positively affected by adding MHA to diets (Fig. 4). The effect sizes of FCR for carp and rainbow trout were negatively affected by adding MHA to diets (Fig. 5). The effect sizes of FE for sea bass, red drum and turbot were positively affected by adding MHA in the diet (Fig. 6). The multiple comparison results for other species (large yellow croaker, carp and the Pacific white shrimp) were not significant.

Meta-regression analysis

Methionine hydroxy supplements in 23 studies ranged from 0.03 to 1.53% (active level: 0.034–1.346%). When considering the influence of MHA additions to fish or

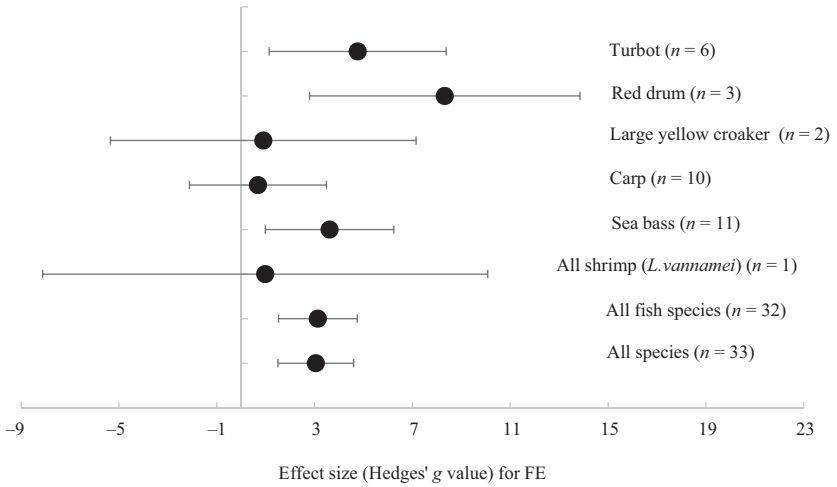

Figure 6 Effect size averages and 95% confidence intervals for all species, targeted subgroups (fish or shrimp) or each species on feed efficiency (FE) from the primary meta-analysis. *N* denotes the number of measurements used to calculate each group effect size. Meta-analysis considers error across and within studies. If $n = 1$, the effect size's mean and error are reported in a paper. If $n > 1$, the effect size's mean and error are reported across studies or within a paper.

shrimp diets, there was a significant quadratic linear relationship between MHA addition and effect size for FW ($P = 0.002$ for MHA, $P = 0.042$ for MHA^2) and a linear relationship between MHA addition and effect size for WG ($P = 0.0005$) and FCR ($P = 0.002$; Fig. 7). There was no significant relationship, linear or non-linear, between the MHA addition and effect size for FE ($P = 0.985$) and PER ($P = 0.461$; Fig. 7).

Discussion

The efficacy of MHA has been evaluated in a number of past projects with varying results (Browdy *et al.* 2012; Ma *et al.* 2013; Hu *et al.* 2015). The application of a meta-analysis gives us an opportunity to quantify the effect of MHA-supplemented diets across a several species and determine the effects on growth performance and feed utilization of fish and shrimp. The overall effect size (measured as Hedges' *g*) between MHA inclusion level in the diet formulation and a control condition was 3.02 for FW, 3.81 for WG, 2.95 for PER, −1.50 for FCR and 3.12 for FE findings (Figs S1–S5). In our present study, there was a publication bias based on funnel plot analysis for all the effect size measurements (Figs S6–S10). The data for FW, WG, PER and FE collected from this present study shifted the funnel plot to the right side instead of showing symmetry on both sides because adding MHA always showed positive effects on growth performance and feed utilization for animals. However, all the effects following the trim and fill method were still significant even though all the total effect sizes for FW, WG, PER, FCR and FE are considerably reduced from that estimated by the original data. Thus, we demonstrated that publication bias did not affect this present meta-analysis conclusion. This is also the first quantitative synthesis to demonstrate that MHA significantly improved the growth performance and feed utilization for fish and shrimp in aquatic feeds.

In this analysis, species was used as a categorical variable to determine its effect as a potential moderator. In total, there are a total of 23 publications, including 20 papers for eight species of fish (carp, catfish, red drum, sea bass, rainbow trout, tilapia, large yellow croaker and turbot) and three papers on one shrimp species (Pacific white shrimp), which reflect the current utilization of MHA focusing on commercial value species. In general, shrimp should grow faster than the fish in terms of weight gain percentage compared with their initial weight. Surprisingly, there are statistically significant improvement in the effect size between Chordata (fish) in FW, WG, PER, FCR and FE (Figs 2–6) rather than crustacean (shrimp) which indicate that adding MHA in the fish diets has improved growth and efficiency compared with the shrimp diets. Another factor to be cognizant of is the difference in variability between the fish and shrimp assessment groups, in which shrimp had higher variation due to having less studies. Therefore, the magnitude of each effect size may have been underestimated to account for this variance inflation and inclusion of more studies could possibly show a greater positive effect comparable to fish.

In general, shrimp have more difficulty utilizing crystalline amino acid as compared to fish, which is supported by this study. Poor utilization could be due to leaching as well as asynchronous absorption. For fish, it has been demonstrated that the absorption rate of crystalline amino

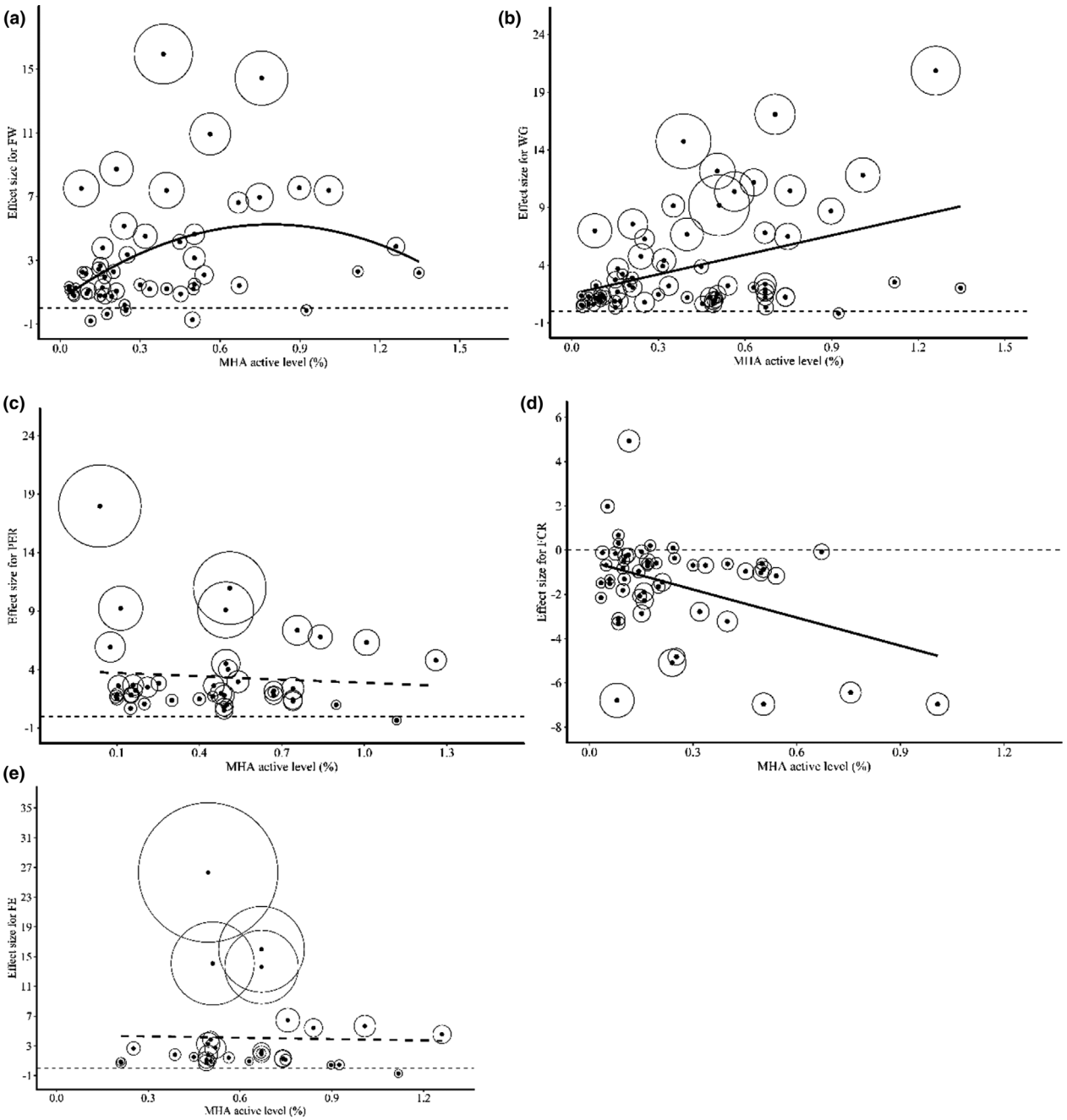

Figure 7 Random-effect model regression of inclusion of adding different concentrations of methionine hydroxy (MHA) on the effect size of final weight (FW), weight gain (WG), protein efficiency ratio (PER), feed conversion ratio (FCR) and feed efficiency (FE). The meta-regression result indicated that a significant quadratic linear relationship between effect size for FW ($P = 0.002$ for MHA, $P = 0.042$ for MHA2) (a) and linear relationship for effect size for WG ($P = 0.0005$) (b) and FCR ($P = 0.002$) with supplemented MHA (active level: 0.034–1.346%) (d). There was no significant relationship between effect size for PER ($P = 0.461$) (c) and FE ($P = 0.985$) (e) supplemented with MHA (activity level: 0.034 to 1.346%). The centre of circle is the mean effect size. The diameter of circles represented the 95% CI of the effect size.

acid is faster than intact protein, which needs to be digested into small peptides or amino acids before absorption (Rønnestad *et al.* 2000), therefore leading to an asynchronous absorption of crystalline amino acids. Though, Davis and Duan (2017) reported that the CAA should be available for metabolism as there was no indication of asynchronous absorption of supplemented AAs in the absorption and clearance patterns of AAs in shrimp haemolymph.

However, the uptake mechanism of MHA is different with DL-Met (Richards *et al.* 2005) and its conversion to L-met by transamination has also been ubiquitous demonstrated in poultry (Martín-Venegas *et al.* 2011). However, there is limited information on the metabolic conversion in *in vivo* or *in vitro* for aquatic species, which need to be further studied. Moreover, as benthic feeders, shrimp break down feed pellets before ingestion instead of swallowing the whole pellet like fish, which resulted in further leaching losses of key soluble nutrients (Soller *et al.* 2019).

The results also showed that the effect of adding MHA for FW, WG, PRE, FCR and FE was significantly associated with different species. The effect size of final weight was greater in carp and turbot, and the effect sizes of weight gain were significantly higher in carp, sea bass and rainbow trout. Besides, we also evaluated PER to reflect the effect of MHA addition on protein utilization for the animals, which should show the same trend with growth performance. The results showed that carp, bass, red drum and turbot have significantly higher PER effect sizes. In general, the significant difference in species on growth performance and feed utilization is consistent. Almost all the species reached higher FW, WG and PER, which leads to lower FCR and higher FE. In the study of Goff and Gatlin III (2004), the pooled SE for WG is high (2484.5); thus, WG data were omitted but FE and PER data were still collected for this analysis, which led to red drum showing greater FE and PER instead of WG. Furthermore, those species with significantly FW, WG and PER may grow faster compared with other species, especially under the conditions to evaluate Met requirements. Additionally, the differences in absorption dynamics (Knight & Dibner 1984), the activity of specific enzymes converting to L-methionine (Knight & Dibner 1984; Dibner & Ivey 1992) and transport (Dibner 2003; Lobley *et al.* 2006) in different species may also contribute to the different results in biological efficiency.

No significant differences were found for the other species among the effect of different species analysed. One explanation is the small sample size and high variability across the studies for some species like catfish, tilapia, shrimp and large yellow croaker. When including different species as a moderator in our overall model, heterogeneity was reduced. A high degree of the heterogeneity still remained after we included the species as a moderator in this model. This indicates that there are other unaccounted variables that may also affect the relationship between growth performance and feed utilization with MHA inclusion in the diet. Thus, potential and reasonable moderators still need to be analysed, like type of MHA.

Meta-regression analysis displayed that there was an increasing trend in effect size of growth performance and feed utilization as the active level of MHA increased. Most importantly, the corresponding effect size results varied with MHA active levels were more obvious using meta-regression (Fig. 7) when compared with the second series of forest plots [Figs S1–S5 (b)] albeit we did not focus on the trend. There are some effect sizes <0.2 at both ends of the meta-regression graph, which also cannot be discerned by the naked eye. This indicates that not all the studies' data set showed significant growth advantages on the effect of adding MHA in fish or shrimp diets. Reviewing said studies could help identify possible reasons for poor utilization of MHA.

First, meeting the Met requirement by formulating the dietary inclusion levels of MHA was on the basal of considering the context of Met levels in the basal diet. On the one side, the Met levels in the basal diets are varied especially in low FM diets replaced by high plant protein sources. Any positive effects produced from MHA cannot be got if the basal diets with sufficient Met whatever forms of Met we supply. Thus, the cost of the formulation could be reduced by formulating on Met basis across corresponding species to avoid under or over formulate the feed with MHA. Most studies with high effect sizes were conducted below the Met or TSAA requirement. Hence, the growth performance of fish and shrimp increased with the inclusion of increasing MHA in the diets as exemplified by Ma *et al.* (2013). In this study, the researchers added graded levels of MHA in the same basal diet, which produced obvious improvements. Hossain *et al.* (2007) investigated the effects of various organic acids including MHA to improve phosphorus utilization and absorption in diets. Hence, the Met requirement was not considered; thus, the study was omitted, whereas other studies for which poor effect sizes were observed may have been conducted for other reasons. One examples of effect sizes for final weight that were <0.2 include Cheng *et al.* (2003)'s first experiment. As indicated by the authors, the main reason is that lysine levels were low in MHA-supplemented diets, which suggests that amino acid balance is also important for the optimal growth of animals. Meanwhile, there also exist sparing effects of cystine for methionine, which could replace about 50% of the total sulphur amino acid (TSAA) requirement of fish or shrimp on an equal molar basis (Harding *et al.* 1977; Moon & Gatlin III 1991; Kim *et al.* 1992; Goff & Gatlin III 2004). The TSAA requirement can be met either with Met supplemented singly or the proper ratio of Met to Cys in fish or shrimp diets (Ahmed *et al.* 2003), which need to be under consideration when we supplement MHA in the diets as well as the balance of amino acids.

In addition, some small effect sizes for growth performance still can be seen when supplemented with high levels of MHA. Methionine often leads to poor performance when the dietary concentrations are five times or more

above the required level (Baker 2006), which has been demonstrated to affect the absorption and utilization of other amino acids (Mai *et al.* 2006). Consequently, the experiments with safety assessments of MHA in the diet were removed from the data set for this analysis as we sought to evaluate the effects of MHA under normal conditions. For example, some data from Hu *et al.* (2015) were omitted because of the high levels of MHA used to determine the safety of turbot. In other cases, for example Cheng *et al.* (2003) and Pan *et al.* (2016), the requirement for methionine was not defined prior to testing which may have led to poor performance and hence a poor effect size (<0.2). Thus, consideration should be given to the biological and experimental factors when interpreting results.

Although we did not present the results of FM levels as moderators, the effect of MHA is also highly interrelated with the FM levels in basal diets, presumably due to the high FM level of methionine. The primary reason to add MHA in diets is to satisfy the Met requirement of animals to achieve maximum growth and/or other performance measures. As exemplified by Cheng *et al.* (2003), in some species reducing the fishmeal level results in undefined nutritional deficiencies, palatability and/or digestibility shifts may overshadow a response to MHA.

In conclusion, this meta-analysis demonstrated that adding MHA has a significant positive effect on the growth performance and feed utilization for fish rather than shrimp. However, results also vary among fish species. The correct supplementation of MHA in fish and shrimp feeds represents an opportunity to reduce the feed costs in the face of the volatile commodity market of protein sources.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Forest plot of mean effect sizes without (a) or with (b) different concentrations of methionine hydroxy analogue (MHA; activity level: 0.034–1.346%) added as a continuous moderator (grey part in the graph) for final weight (FW) with 95% confidence limits for each study.

Figure S2. Forest plot of mean effect sizes without (a) or with (b) different concentrations of methionine hydroxy analogue (MHA) (activity level: 0.034–1.346%) added as a continuous moderator (grey part in the graph) for weight gain (WG) with 95% confidence limits for each study.

Figure S3. Forest plot of mean effect sizes without (a) or with (b) different concentrations of methionine hydroxy analogue (MHA) (activity level: 0.034–1.346%) added as a continuous moderator (grey part in the graph) for protein retention efficiency (PRE) with 95% confidence limits for each study were listed below, respectively.

Figure S4. Forest plot of mean effect sizes without (a) or with (b) different concentrations of methionine hydroxy analogue (MHA) (activity level: 0.034–1.346%) added as a continuous moderator (grey part in the graph) for feed conversation ratio (FCR) with 95% confidence limits for each study.

Figure S5. Forest plot of mean effect sizes without (a) or with (b) different concentrations of methionine hydroxy analogue (MHA) (activity level: 0.034–1.346%) added as a continuous moderator (grey part in the graph) for feed efficiency (FE) with 95% confidence limits for each authors with multiple publications included in this meta-analysis have year of publication listed.

Figure S6. Evidence of publication (reporting) bias. (a) Funnel plot of standardized mean difference of final weight (FW); in the absence of bias the points should resemble a symmetrical inverted funnel. (b) Funnel plot showing the additional missing studies imputed by trim and fill in white; the white vertical line indicates the possible summary if the theoretical missing studies were to be included.

Figure S7. Evidence of publication (reporting) bias. (a) Funnel plot of standardized mean difference of weight gain (WG); in the absence of bias the points should resemble a symmetrical inverted funnel. (b) Funnel plot showing the additional missing studies imputed by trim and fill in white; the white vertical line indicates the possible summary if the theoretical missing studies were to be included.

Figure S8. Evidence of publication (reporting) bias. (a) Funnel plot of standardized mean difference of protein retention efficiency (PRE); in the absence of bias the points should resemble a symmetrical inverted funnel. (b) Funnel plot showing the additional missing studies imputed by trim and fill in white; the white vertical line indicates the possible summary if the theoretical missing studies were to be included.

Figure S9. Evidence of publication (reporting) bias. (a) Funnel plot of standardized mean difference of feed conversion ratio (FCR); in the absence of bias the points should resemble a symmetrical inverted funnel. (b) Funnel plot showing the additional missing studies imputed by trim and fill in white; the white vertical line indicates the possible summary if the theoretical missing studies were to be included.

Figure S10. Evidence of publication (reporting) bias. (a) Funnel plot of standardized mean difference of feed efficiency (FE); in the absence of bias the points should resemble a symmetrical inverted funnel. (b) Funnel plot showing the additional missing studies imputed by trim and fill in white; the white vertical line indicates the possible summary if the theoretical missing studies were to be included.

Table S1. Summary of testing and adjusted publication bias results after trim and fill was applied.