Estimation of feed level of methionine by *Litopenaeus vannamei* (Boone) using covalently-attached and crystalline sources in low-protein semi-purified diets

Joe M. Fox[1], Addison L. Lawrence[1], Susmita Patnaik[1], Ian Forster[2], Zhi Yong Ju[2] and Warren Dominy[2]

Texas A&M University-Corpus Christi, Corpus Christi, TX, USA

Oceanic Institute, Waimanalo, HW, USA

E-mail: Joe.Fox@tamucc.edu

Abstract

Synthetic sources of methionine are commonly used to supplement plant protein-based aquaculture feeds. A study was conducted to identify requirement of methionine for juvenile (0.41 g ± 0.02) Pacific white shrimp (*Litopenaeus vannamei*) fed low-protein (20% CP) diets formulated to be nutritionally-adequate in essential amino acids with the exception of methionine. Graded levels of methionine were prepared by supplementing a semi-purified basal diet with either soybean meal covalently enriched with methionine or with crystalline L-methionine. Covalent diets contained 0.298, 0.362, 0.473, 0.617, 0.766 and 0.958% methionine, whereas crystalline treatments contained 0.298, 0.384, 0.481, 0.576 and 0.748% methionine. Leaching trials with experimental feeds indicated increased, but minimal, leaching of methionine with increased dietary inclusion level and by time for both diet types. Leaching trials with requirement feeds indicated increased, but minimal, leaching of methionine over 60 min. Growth trials showed no significant difference (P>0.05) in survival of shrimp fed either type of diet during the 28-day feeding trial. Percentage weight gain of shrimp fed the covalently-attached methionine ranged from 541 - 643%. Growth of shrimp decreased when fed diets containing covalent methionine levels above 0.617%, suggesting possible inhibition (I_m = 0.55% dietary methionine). Percentage weight gain of shrimp fed the crystalline diets ranged from 541 - 683%. Broken-line analysis estimated a methionine requirement level of 0.74% using crystalline methionine supplementation. Leaching trials with requirement feeds indicated increased, but minimal, leaching of methionine over 60 min.

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Although 60% of the global fish meal market is associated with aquaculture (Jackson, 2009), there exists a clear and demonstrable need to reduce its use in feeds. This need derives from its often poor market availability and high economic-environmental cost (Larsen 2002). The ability to replace fish meal in commercial production feeds for shrimp has been demonstrated using grain-based protein sources (Davis and Arnold 2000; Forster et al. 2003; Samocha et al. 2004; Amaya et al. 2007) and could help mitigate increasing demand. In a recent study, Fox et al. (2010) demonstrated the potential use of mineral chelates of methionine in grain-based shrimp feeds and that an appropriate feed level of this amino acid was less than 0.4% (as-fed) under experimental conditions. It is likely that additional reductions in demand could also result from the establishment of high contributory levels of natural productivity in grow-out ponds or by supplementation of diets with artificial sources (e.g., crystalline methionine). Plant proteins and their concentrates are of interest as fish meal replacements due to their typically lower cost and improved potential for sustainable production. However, many of these ingredients (e.g., soybean meal) can contain relatively low levels of essential amino acids (Conklin 2003). Use of simple crystalline sources (e.g., D-L- or L-methionine, etc.) is problematic due to difficulties associated with quantification of ingestion, leaching of EAA from the feed pellet and variable uptake rate (Fox et al. 1995a).

A common ingredient used in commercial feeds is soybean meal, a plant protein source first-limiting in methionine, but representing an approximately 25% decrease in cost of dietary protein relative to fish meal (Pike, 2006). Its use in feeds as a primary source of protein often requires supplementation with synthetic forms of this essential amino acid (e.g., L-methionine, D-L methionine or analogs) (Forster and Dominy, 2006). This is at least partially due to difficulties in formulating basal diets deficient/low in only one amino acid. Use of simple crystalline sources (e.g., D-L- or L-methionine, etc.) is problematic due to difficulties associated with quantification of ingestion, leaching of EAA from the feed pellet and rapid uptake rate (Fox et al. 1995a).

Covalent attachment of amino acids to purified proteins represents another option for supplementation of shrimp feeds, albeit at the research level. Fox et al. (1995b) estimated and compared requirement for lysine by juvenile *Litopenaeus vannamei* using covalent attachment to

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wheat gluten and crystalline sources. Results indicated slightly higher requirement in crystalline diets. The objectives of our study were: 1) to quantify leaching of methionine from semi-purified diets supplemented with graded levels of methionine by covalent attachment to soybean meal or as crystalline sources and 2) to estimate an appropriate feed level for methionine by shrimp fed low protein diets using these two different diet types. Information regarding an appropriate level of supplementation of methionine is required for least-cost formulation of feeds, especially considering the growing number of ingredients having potential for replacement.

Materials and Methods

Study locations and sources of animals

This study was conducted at the Texas AgriLife Research Mariculture Laboratory (Port Aransas, Texas, USA). Postlarval shrimp (*L. vannamei*) were obtained from Shrimp Improvement Systems, Inc., (Islamorada, Florida, USA) and reared to an initial weight of 0.41 ± 0.02 g for stocking.

Leaching trial

Leaching trials were conducted on treatment diets containing graded levels of methionine provided in both covalent and crystalline form (Table 1). Five feed pellets of approximately similar weight were obtained for each diet. For each diet, these were weighed and place into 1.5-mL centrifuge tubes to which 1.2 mL of distilled water was added. Pellets were submerged for 5 min and 60 min without agitation, followed by collection and transfer of 0.5 mL supernatant to a centrifuge tube. The supernatant was then vacuum-dried and analyzed for methionine content using reverse-phase pre-column derivatization amino acid analysis via HPLC (Beckman Gold™ HPLC System, Fullerton, California, USA) (Rozan et al. 2000).

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Table 1. Pre-mixture composition (% dry wt. of diet) used to formulate defined diets for estimation of methionine requirement

Ingredients	Methionine-deficient	Covalently-attached	L-methionine-enriched
SBM-90[c]	22.22	0.00	22.22
SBM-90, covalent-Met[f]	0.0	22.22	0.0
Squid muscle meal[c]	6.00	6.00	6.00
Cholesterol[c]	0.20	0.20	0.20
Diatomaceous earth[d]	2.28	2.28	2.28
Chromic oxide[b]	1.00	1.00	1.00
KCl[b]	2.51	2.51	2.51
L-methionine[b]	0.00	0.00	0.99
MgO, feed grade[b]	1.57	1.57	1.57
Menhaden oil[e]	2.66	2.66	2.66
Soybean oil[c]	0.44	0.44	0.44
Phospholipid-69%[c]	4.00	4.00	4.00
Dicalcium phosphate[d]	5.73	5.73	5.73
Sodium HMP[b]	1.00	1.00	1.00
Vitamin-mineral premix[c]	0.46	0.46	0.46
Ascorbic acid, Stay-C[g]	0.04	0.04	0.04
Wheat starch[d]	42.43	42.43	41.44
Analyzed composition			
Crude protein	19.63	19.75	20.06
Crude lipid	7.21	7.25	7.14
Marine lipid (formulated)	3.00	3.00	3.00
Total ash	17.05	16.63	16.91
Methionine	0.30	0.96	0.75
Methionine + cysteine	0.61	1.25	1.04

[a]NutraSweet-Kelco, Chicago, IL, USA, [b]Sigma-Aldrich Chemical, St. Louis, MO, USA, [c]Ziegler Bros., Gardners, PA, USA, [d]MP Biochemicals, Cleveland, OH, USA, [e]Omega Protein Corporation, Inc., Houston, TX, USA, [f]Prepared at Oceanic Institute, Waimanalo, HW, USA, [g]Roche Vitamins Inc., Parsippany, NJ, USA.

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Growth trial

Quantification of dietary requirement of methionine was undertaken using diets in which graded levels of methionine were achieved by either covalent attachment to high-protein-content soybean meal (90% crude protein, CP) (SBM-90), or by direct addition of L-methionine to a basal mix (Table 1). Three basal diets were prepared: methionine-deficient (0.30%, as-fed), covalently- enriched (1.02%); and L-methionine-enriched (0.80%). Basal diets were similar with respect to crude protein (~20%, as-fed), crude lipid (7.82%), marine lipid (3.00%) and ash (17.60%) and were replete with respect to EAA other than methionine. Low-protein level feeds were used due to high frequency of feeding (15 times daily) and level of attractants (6% squid muscle meal). Consumption of treatment diets was similar irrespective of methionine concentration. Alginate (2.00%, as-fed) and sodium hexametaphosphate (1.00%) were added to all diets as binders. Methionine was attached to SBM90 using a carbodiimide reaction (Voutsinas and Nakai, 1996). Higher methionine content in experimental covalent diets was achieved by increasing the proportion of the enriched diet (1.31% methionine) and decreasing that of the deficient (0.29% methionine) (Table 1). Graded levels of methionine were achieved in the crystalline diets by similar means (i.e., various proportions of methionine-deficient and methionine-enriched diets). Dietary level of cysteine was similar for all diets (0.16%, as-fed).

Diets were prepared by homogenizing dry ingredients, with the exception of binders, in a V-mixer for 30 min. Methionine-deficient dry ingredients were mixed with enriched ingredients (for either covalent or crystalline diets) in specific proportions to achieve the following methionine concentrations: 0.294, 0.380, 0.480, 0.610, 0.830 and 1.016 g/100 g (final diet concentration) for the covalent diets and 0.294, 0.380, 0.480, 0.610, 0.830 g/100g for crystalline diets. Afterwards, 400 mL of hot water and binders were blended into dry mixes and re-mixed to an appropriate consistency for formation of pellets. The moist mash was then passed through a Hobart A200 meat grinder (Hobart Corporation, Troy, New Jersey, USA) containing a 3 mm die. Moist, uncut pellets were air dried (< 50 C) in a convection dryer to a moisture content of 8% and crumbled to achieve appropriate particle size. All experimental feeds were analyzed for methionine concentration (Table 2) using the previous method.

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Table 2. Comparison of analyzed methionine concentration of experimental diets

Diet type	Formulated methionine concentration (% of diet)	Analyzed methionine concentration (% of diet)
Covalently-attached	0.29	0.30
	0.38	0.36
	0.48	0.47
	0.61	0.62
	0.80	0.77
	1.02	0.96
L-met-supplemented	0.29	0.30
	0.38	0.38
	0.48	0.48
	0.61	0.58
	0.80	0.75
Cysteine, covalent diets	0.16	0.31 (analyzed)
Cysteine, crystalline diets	0.16	0.31 (analyzed)

Shrimp used in feeding trials (0.41 ± 0.02 g) were separated into two groups, according to diet type, and fed a maintenance ration of either the covalent or crystalline feed containing highest methionine levels for a period of two weeks prior to stocking. At the start of the trial, seven shrimp were placed into 20-L rectangular-bottomed tanks (n = 9 tanks/treatment, total = 99 tanks) at a density of 54 g/m^2. All tanks used in the study were connected to a common recirculating aquaculture system (87 MT total volume) to which 6% was added daily to compensate for loss of water from evaporation and salinity maintenance. System water exchange rate was maintained at 1,200% per day per tank and within parameters appropriate for the culture of *L. vannamei* (30 ± 1 C, 30 ± 1 salinity, pH 8.1 ± 0.1, TAN < 1.0 mg/L). Daily treatment feed rates were determined using a standard laboratory feeding curve in which feed was administered on an ad libitum basis 15 times daily to each tank using wheel-type automated feeders. Uneaten feed, feces, exuviae and dead shrimp were removed from tanks at the beginning of each day of the trial. The feeding trial was terminated after 28 d.

At the conclusion of the feeding trial, shrimp in each replicate tank were enumerated and weighed as a group. Dependent variable used to evaluate performance of treatment feeds included percent survival, percent weight gain, total biomass gain and estimated feed conversion ratio (FCR). one-way ANOVA (α = 0.05) was used to analyze results for each diet type by treatment level of methionine. Significant differences ($\underline{P}$<0.05) in means of dependent variables

by diet type and methionine level ($P<0.05$) were separated by Tukey's HSD test. Methionine feed level was estimated for shrimp fed each diet type using a one-slope broken-line regression model (Robbins et al. 2006). All statistical analyses were undertaken using SAS ver. 9 (Statistical Analytical Systems Institute, Cary, NC, USA).

Results

Leaching trial

Methionine loss from the basal diet (0.30%, no supplemental methionine) was undetectable (Table 3). For covalent diets leaching appeared to increase only in terms of time of submergence. Methionine loss ranged from 0.014 - 0.024% of the diet and, at most, represented 3.8% of total feed methionine. For crystalline diets, loss of methionine appeared to increase slightly with time of submergence and dietary supplementation (Table 3). As with covalent diets, overall loss was minimal (0.003 - 0.022% of the diet) with the highest being 2.93% of feed methionine. In general, leaching from crystalline feeds appeared to be lower than that of covalent treatments.

Table 3. Leaching of methionine from treatment diets

Diet type	Formulated methionine concentration (% of diet)	Methionine leaching after 5 min (% of diet)	Methionine leaching after 60 min (% of diet)
Covalent	0.294	nd[a]	nd
	0.380	Nd	0.017
	0.480	Nd	0.021
	0.610	0.017	0.024
	0.803	0.015	0.023
	1.016	0.014	0.017
Crystalline	0.294	nd	nd
	0.380	0.003	0.004
	0.480	0.008	0.006
	0.610	0.011	0.016
	0.803	0.011	0.022

[a]nd = no loss detected

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Growth trial

Survival of shrimp fed covalent diets was similar (P = 0.407) and ranged from 92.9 - 100.0% (Table 4). Percent weight gain increased from 541% in the basal diet to a high of 643% in the 0.62% methionine treatment (Table 4, Fig. 1). Increases in dietary methionine level, beyond 0.62% resulted in decreased percent weight gain to a low of 514%. Treatment means were fitted to an inhibition model and generated the following equation, y = 15,580*(1-e(-0.20*x))*(e(-26,591x/15,580)), where y = percent weight gain and x = dietary concentration of methionine (g/kg). Inhibition was estimated at 0.55% of diet.

Table 4. Mean survival, percent weight gain, total individual weight gain, biomass density, and growth rate of shrimp fed covalent and crystalline diets containing various levels of methionine

Diet Type	Methionine Conc. (% of diet)	Survival (%)	Percent weight gain	Individual wt. gain (g)	Final biomass density (g/m^3)	Growth rate (g/wk)
Covalent	0.30	98.2 ± 5.05	540.87 ± 16.85^{a,d}	2.23 ± 0.26^{a,b}	193.3 ± 22.8^{a,b}	0.56 ± 0.03^{a,b}
	0.36	100.0 ± 0.00	595.57 ± 10.99^{a,c}	2.45 ± 0.07^{a}	201.5 ± 6.9^{a,b}	0.61 ± 0.01^{a,b}
	0.47	98.2 ± 5.05	643.39 ± 12.22^{c}	2.52 ± 0.16^{a}	200.4 ± 15.5^{a,b}	0.63 ± 0.01^{a,b}
	0.62	98.2 ± 5.05	640.20 ± 23.08^{b,c}	2.61 ± 0.21^{a}	203.3 ± 20.2^{a}	0.65 ± 0.02^{a}
	0.77	92.9 ± 4.08	581.60 ± 9.60^{a,b}	2.42 ± 0.13^{a}	186.1 ± 24.6^{a,b}	0.60 ± 0.01^{a}
	0.96	98.2 ± 5.05	516.56 ± 16.85^{d}	2.14 ± 0.17^{b}	174.2 ± 16.5^{b}	0.58 ± 0.02^{a}
Crystalline	0.30	98.2 ± 5.05	540.87 ± 16.85^{a}	2.23±0.26^{a}	193.3±22.8^{a}	0.56±0.07^{a}
	0.38	100.00 ± 0.00	572.27 ± 20.02^{a}	2.30±0.12^{a,b}	175.2±20.6^{a}	0.58±0.03^{a}
	0.48	98.20 ± 5.05	608.05 ± 12.22^{a,b}	2.55±0.20^{a,b}	190.6±19.2^{a}	0.64±0.05^{a}
	0.58	94.64 ± 2.79	671.85 ± 20.51^{b}	2.64±0.23^{ab}	193.3±22.8^{b}	0.66±0.06^{a,b}
	0.75	98.20 ± 5.05	683.39 ± 31.50^{b}	2.72±0.27^{b}	193.3±22.8^{b}	0.68±0.07^{b}

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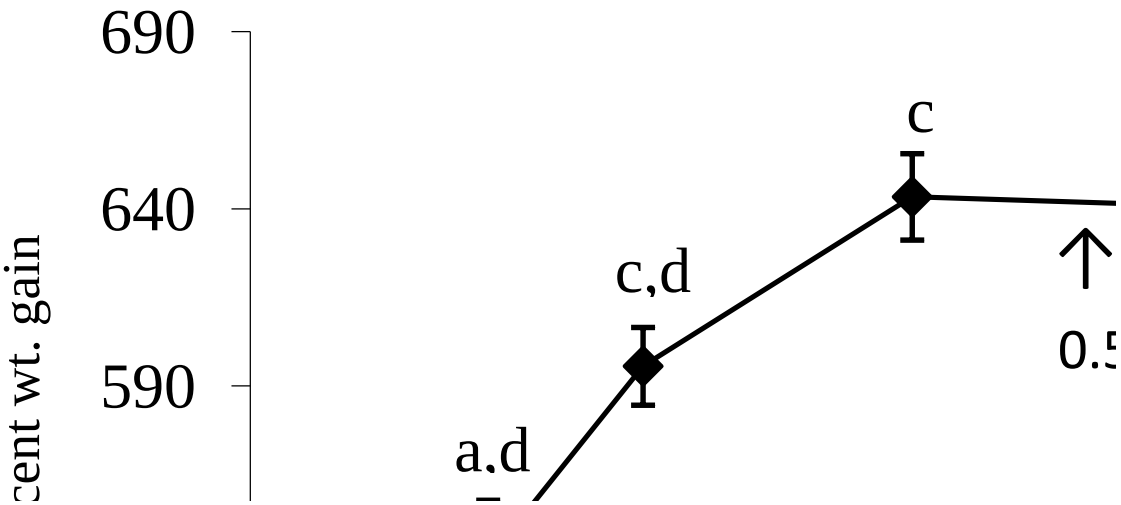

Figure 1. Percent weight gain of shrimp fed diets enriched with various levels of covalent methionine. Values represent means ± *SEM*. Means having common superscripts are not significantly different ($P>0.05$)

Survival of shrimp fed crystalline diets was similar (P = 0.433) and not significantly different from shrimp fed the covalent diets (P = 0.433) (Table 4). Weight gain varied from 540 - 683% and steadily increased with dietary level of methionine up to 0.481%, after which it reached a plateau (Fig. 2). Use of the one-slope broken-line model yielded an estimated methionine requirement level of 0.739% (3.7% of protein) derived from the following equation: Y = 683 + [-467(0.62 - X)].

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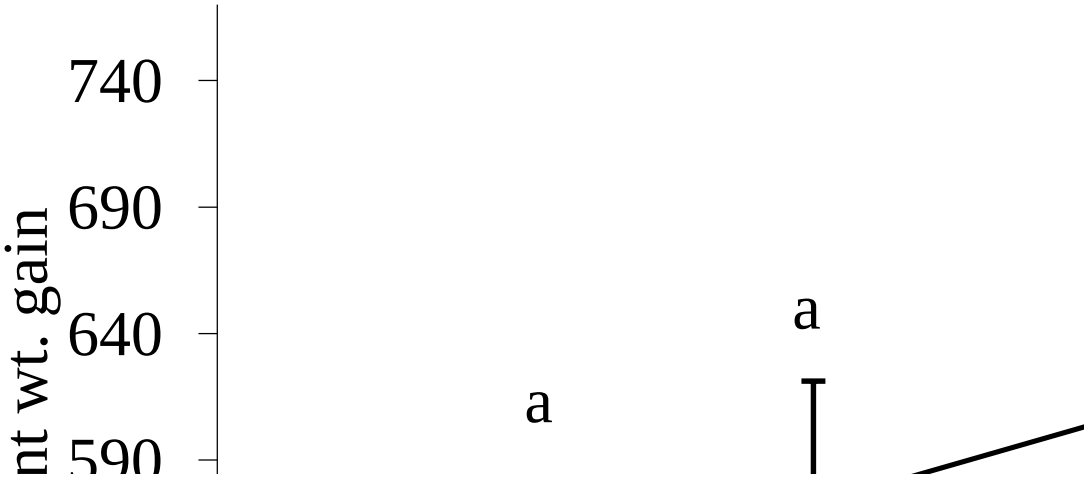

Figure 2. Percent weight gain of shrimp fed diets enriched with various levels of crystalline methionine. Values represent means ± *SEM*. Means having common superscripts are not significantly different (P>0.05)

Discussion

Leaching trials

Water-soluble organic molecules (e.g., crystalline or free amino acids) in aquaculture feeds leach into the water column over time. This observation was initially made for free amino acid-enriched diets fed to the kuruma prawn, *Marsupenaeus japonicus* (Deshimaru and Kuroki 1974a,b; Deshimaru 1976). In contrast, use of CAA has not presented problems in fin fish nutrition due to relatively complete (and rapid) consumption of feed pellets. Because shrimp are continuous feeders, it is postulated that nutrient requirement studies using growth as a performance index should provide feeds on at least a semi-continuous basis. In a study of lysine requirement by *L. vannamei*, (Fox et al. (1995b) showed that shrimp fed crystalline and covalent lysine diets elicited similar growth response. In that study shrimp were fed 15 times daily using automated feeders. The effect of methionine leaching on results in the present study was minimal due to similar high feeding frequency.

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In a 6-wk feeding trial with *M. japonicus*, Alam et al. (2005) examined the effects of coated or non-coated methionine and/or lysine supplemented to a soy protein (SPI)-based diet. Shrimp fed the diets supplemented with coated methionine or lysine tended to have higher weight gain compared to shrimp fed the diet without supplementation of the amino acids. The juveniles fed the diet containing both coated methionine and lysine showed significantly higher growth performance than those for which uncoated amino acids were used, implying reduced leaching by coated pellets. Recent research conducted at our laboratory has shown that complexing of methionine with minerals (i.e., chelate methionine) significantly reduces leaching when compared to DL-methionine. The present study confirmed similarity of leaching rates of methionine in both types of diets and further showed that overall leaching rates were relatively low (undetectable - 0.024%) for all treatment levels. For this reason, the effect of methionine leaching on growth trial results (below) was considered minimal, especially considering that shrimp were fed 15 times daily. As expected, leaching of methionine increased with time and dietary concentration for both diet types. Although the means of supplementation of methionine to experimental feeds appeared to have no effect on leaching, it does not suggest that feeds containing crystalline methionine do not require different binding strategies or that frequent feeding (e.g., 15 times daily) is recommended for commercial culture situations.

Growth trials

Results from growth trials using covalent and crystalline methionine diets were somewhat inconclusive given the apparent inhibition identified with covalent treatment (inhibition at 0.55% of the diet). This should not be interpreted as a "requirement" level for methionine as statistical analyses used all treatment means. Examination of the percent weight gain response for the first four treatment levels of methionine (Fig. 3) shows an apparent inflection approximating 0.50% of the diet, but cannot be estimated by broken-line analysis. Broken-line analysis of growth response to crystalline enrichment estimated a methionine requirement 0.74% of the diet. It is possible that this response would be different had an additional treatment level higher than 0.75% been used. Survival of all shrimp was high under all experimental conditions, indicating that dietary levels of methionine as low as 0.30% are capable of supporting growth and survival over a four-wk period in the absence of natural productivity.

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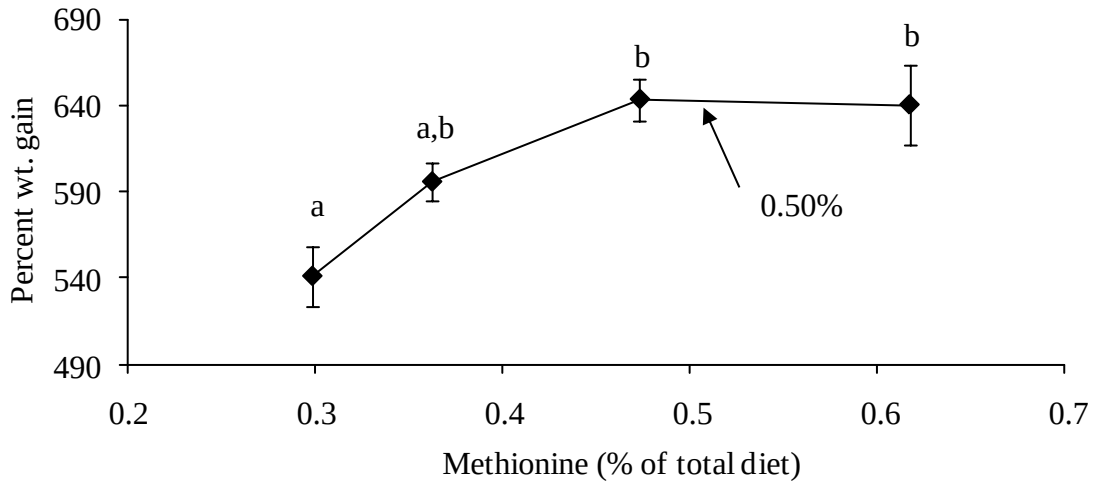

Figure 3. Percent weight gain of shrimp fed diets enriched with various levels of covalent methionine (first four treatment means only. Values represent means ± SEM. Means having common superscripts are not significantly different (P>0.05)

The efficacy of different methionine sources in feeds was evaluated by Forster and Dominy (2006) for Pacific white shrimp, *Litopenaeus vannamei*. Their study used a methionine-deficient diet (30% CP) formulated with high inclusion levels of de-hulled, solvent-extracted soybean meal (i.e., no fish meal) to yield a basal methionine concentration of 0.45%, as-fed. Test diets were manufactured in which L-methionine, DL-methionine, and 2-hydroxy-4-methylthiobutanoic acid (HMTBA) were supplemented at the 0.5% level (equivalent to 1.5% of total amino acids). Cysteine concentration was maintained constant among their diets at 0.5%. Results showed that final weight and growth rate of shrimp fed the basal control diet were significantly lower than those of shrimp fed the methionine-supplemented diets, indicating that, under experimental conditions, a dietary level of 0.45% methionine was deficient. Results also indicated that L-methionine, DL-methionine, and HMTBA could be used to meet the methionine requirement of this species. The results of this study compare favorably with those of the present in that a need for supplementation above the 0.45% level was shown.

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Millamena et al. (1996) determined an apparent methionine requirement of 0.89% of the diet for postlarval *Penaeus monodon*. This level was somewhat higher than inclusion levels suggested by the present study (0.74%). In general, results from the present study using covalently-attached diets were comparable to those of Millamena et al. (1996) in which feeds contained a much higher level of protein (37 vs. 20%) and high levels of crystalline amino acids. Their feeds were also pre-coated with carboxymethylcellulose (CMC) to reduce leaching and κ-carrageenan to improve water stability of pellets. Differences between these two studies could have been associated with age of animal used (postlarvae vs. juvenile), species (*P. monodon* vs. L. vannamei); feeding frequency (three times daily vs. 15 times per day) or binder (CMC vs. alginate and sodium hexametaphosphate). Considering that leaching of methionine was minimal from crystalline diets used in the present study, the higher requirement requires further investigation, but could have been related to form of crystalline methionine used. In a study by Sveier et al. (2001) with Atlantic salmon (*Salmo salar* L.), it was shown that inclusion of protein-bound methionine yielded superior protein utilization over that of either L-, D- or DL-methionine. In terms of protein productive value, D-methionine was more efficient than that of L-methionine. Millamena et al. (1996) did not indicate the form of methionine used in their study. These results conflict with those of Forster and Dominy (2003) who showed that either L-methionine or DL-methionine are suitable for supplementation of methionine-deficient diets fed to *L. vannamei*.

A recent study conducted by Fox et al. (2010) using plant protein-based feeds fed to *L. vannamei* showed no significant difference in weight gain by shrimp offered diets containing $\geq$ 0.41% methionine. This could have been related to the higher level of cysteine (0.45%) used in the basal diet of the former. The total sulfur-containing amino acid (TSAA) content of their diet was 0.86%. This approximates the TSSA level estimated by the present study for covalently-attached diets of 0.80%. Results from Fox et al. (2010) are substantially lower than those of the present study when using crystalline sources of methionine (0.41 vs. 0.74% of diet). When compared on a TSAA basis, the difference is also apparent. Total sulfur-containing amino acid requirements have been determined for various species of finfish and have generally ranged between 2 and 4% of dietary CP, with methionine accounting for 60% of the requirement on a weight basis or 50%

on a molar basis (Cai and Burtle 1996; Ruchimat et al. 1997; Schwarz et al. 1998; Twibell et al. 2000; Mai et al. 2006; Yan et al. 2007).

To our knowledge, there has been no definitive study on digestibility of methionine covalently-attached to soy protein concentrate; however, the carbodiimide reaction used in the present study was similar to that used by Fox et al. (1995a,b) to attach lysine to wheat gluten. They demonstrated similar apparent lysine digestibility of enriched wheat gluten to that of unenriched. Voutsinas and Nakai (2006) subjected methionine-enriched soy protein hydrolysate to *in vitro* pepsin-pancreatin digestion and reported high levels of digestibility of bound amino acids. The carbodiimide reaction increased molecular weight of the enriched soy product with no selective amino acid binding. Therefore, it is unlikely that selective binding occurred via the carbodiimide reaction in the present study. This is substantiated by the relatively similar growth rates of shrimp fed diet enriched by the two methodologies.

Questions remain regarding the apparent higher estimated requirement level of methionine using crystalline sources. This could have been associated with loss of crystalline methionine to the water column as a result of manipulation of the feed pellet by shrimp. Because leaching coefficients were similar and low for both diet types, it is not likely that losses occurred as a result of simple submergence of pellets. As with the present study, leaching trials are often conducted under static conditions, without similar manipulation of the feed pellet.

Are estimates of dietary requirement for methionine, therefore influenced by means of enrichment? Fox et al. (1995b) showed an apparent lysine requirement of 1.50% using carbodiimide attachment of lysine to wheat gluten, whereas that by crystalline addition was 1.73%. In the present study, treatment levels of methionine in crystalline diets were not high enough to definitively pinpoint a requirement level. This contrasts with results from the covalently-attached data, in which a distinct inflection point was observed. This could indicate that the covalent attachment method is more efficient in meeting the requirement, but should be cautiously considered, given that percent weight gain was higher for the crystalline-fed shrimp than the covalent at the highest inclusion levels (0.75 and 0.77%, respectively).

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As shown in Fig. 1, weight gain of shrimp fed covalent-methionine diets decreased beyond the 0.62% treatment level. This response was unexpected, but clearly suggests that high levels of methionine-enriched soy protein hydrolysate cannot be used for methionine requirement research. Without either analysis of binding of methionine soy protein or in-vivo methionine digestibility of treatment diets, it is difficult to attribute this response to any single physiological mechanism. The inhibition modeling applied to data simply estimates at what dietary concentration on-set of growth-related problems initiate, not why. Previous use of the carbodiimide reaction to attach amino acids to proteins has not indicated problems with binding or digestibility (Voutsinas and Nakai 2006; Fox et al. 1995a). However, if hydrolysate products are not adequately purified post-attachment of amino acids and the dietary inclusion level of the "new" product is high, it is conceivable that either a toxicity issue or lowering of gut pH could occur.

It must be emphasized that a biological requirement level of 2.50 - 3.70% methionine as a percentage of dietary protein is not necessarily appropriate for commercial shrimp production feeds. The diet of shrimp in an outdoor pond includes both extrinsic (e.g., feed) as well as intrinsic (e.g., natural productivity) sources of nutrition. For this reason, commercial feed formulae are seldom restricted by methionine. In a study by Richard et al. (2009) on methionine requirement by *P. monodon*, it was shown that maintenance levels of methionine approximated 0.11 g/kg body weight per day for nitrogen maintenance. This suggests that growth of shrimp, in the presence of adequate levels of natural productivity (e.g., pond environment), might not be limited by feed level of methionine. It is therefore likely that a requirement level of 2.50 - 3.70% of total protein has more relevancy to research situations in which levels of extrinsic nutrition are low. Regardless, methionine requirement is likely spared to a certain degree by dietary cysteine (as above). Unfortunately, very little if any research has addressed ability of cysteine to spare methionine requirement by marine penaeid shrimp let alone an appropriate ratio of cystine to methionine. This research topic should be investigated in light of development and use of fish meal replacement feeds.

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