Current Status of Amino Acid Requirement Research with Marine Penaeid Shrimp

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Abstract

Most of the cost for production of commercial production feeds used in marine penaeid shrimp culture can be attributed to the provision of dietary protein. Because protein is comprised of amino acid residues, it is surprising that relatively little research has addressed the nutritional requirements of shrimp for essential amino acids. This is especially critical for the continuation of efforts to reduce fish meal use in commercial production feeds. Requirement data, in combination with that for protein and energy digestibility, will allow for the manufacture of more accurate least-cost production feeds for *Litopenaeus vannamei* and other species. This paper provides historical results of essential amino acid requirement research on marine penaeid shrimp, comments on methodological approaches and reports on current preliminary efforts to determine apparent requirement for methionine by *L. vannamei*.

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Basis for Conducting Research

Knowledge of essential amino acid (EAA) requirement in marine penaeid shrimp (shrimp) is critical for the least-cost optimization of commercial feed formulae. Without this information, as well as that of nutrient digestibility coefficients, cost-efficiency of feed ingredients can only be estimated. Unlike the formulation of traditional agricultural feeds, those for shrimp are not done so on the basis of EAA availability (e.g., digestibility or some other index of quality). Thus, knowledge of EAA requirement is only partially useful. Similarly, quantification of EAA concentration in feeds is not helpful unless accompanied by some index of bioavailability.

Qualitative Requirements for EAA

The first step toward quantification of requirements for EAA by any animal, aquatic or terrestrial, has been qualitative confirmation of essentiality (i.e, which amino acids are essential). Research in this area has been undertaken for several species of shrimp: *Farfantepenaeus aztecus* (Shewbart *et al.*,, 1972); *Palaeomon serratus* (Cowey and Forster, 1971); *Marsupenaeus japonicus* (Kanazawa and Teshima, 1981); and *Penaeus monodon* (Colosso and Cruz, 1980). These studies used radio-labeled carbon compounds to show essentiality for lysine, arginine, methionine, threonine, histidine, isoleucine, leucine, valine, phenylalanine, and tryptophan. Other amino acids considered to have a sparing effect on EAA requirement are cystine (biosynthesized from serine and methionine) for methionine and tyrosine (hydroxylated form of phenylalanine) for phenylalanine. Methionine, lysine and arginine are probably most limiting to least-cost optimization of commercial feed formulae. Slight variations to this hierarchy are to be expected in areas where frequent substitution of protein-dense feed ingredients occurs.

Historical Perspective

One of the first studies to document the need for research on amino acid essentiality in shrimp was that of Cowey and Forster (1971), who investigated EAA requirements of the prawn, *Palaeomon serratus*. In that same year, Lee (1971) studied protein utilization related to growth of *Penaeus monodon*. In 1972, a series of culture experiments was undertaken by Deshimaru and Shigeno to examine the amino acid composition of artificially composed feeds for *Marsupenaeus japonicus*. They found that diets having an amino acid pattern similar to that of short-necked clams and shrimp showed the highest feed efficiency. Their recommendation was that shrimp feeds should contain a similar amino acid profile to that of shrimp. This somewhat confirmed the prediction of Phillips and Brockway (1956) that dietary proteins should have similar EAA composition to that of the animal being studied. In 1972, the qualitative requirement for EAA by *Farfantepenaeus aztecus* was determined by Shewbart *et al.,* (1972). This was later confirmed for *M. japonicus* by Kanazawa and Teshima (1981), and *P. monodon* by Coloso and Cruz (1980).

Research continued during the mid-late 1970s focused on developing a purified diet for *M. japonicus* using highly digestible sources of protein and crystalline amino acids (CAA). Much of this work was conducted in Japan by Deshimaru and Kuroki (Deshimaru and Kuroki 1974a, 1974b, 1975; Deshimaru 1975, 1976). One conclusion of their work was that supplementation of diets with

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crystalline amino acids (CAA) resulted in poor growth and survival of shrimp. Increase in the rate of protein to amino acids in the protein source improved both growth and feed intake and lowered mortality (Deshimaru and Kuroki, 1975). In a subsequent study, Deshimaru (1976) showed that poor performance of diets highly supplemented with amino acids was probably due to rapid absorption and uncoordinated assimilation in tissues (Deshimaru, 1976). Herein lay a substantial bottleneck to EAA requirement research: how to develop a purified standard reference diet for EAA research composed entirely of protein-bound amino acids without causing substantial compounding effects (i.e., without varying the concentration or availability of other nutrients required for growth).

Few additional advancements in EAA requirement research for shrimp occurred in the 1980s until the determination by Teshima *et al.,* (1986) that small amounts of CAA could be used to supplement intact protein in research diets fed to *M. japonicus* larvae. Supplementation of casein with crystalline L-arginine improved its nutritive value compared to that of a live food control. In a second trial, the authors substituted one-half of the dietary protein (i.e., casein) with a CAA mixture whose amino acid composition approximated that of whole body shrimp larvae. The diet containing the CAA/casein mixture yielded similar or better growth than that of the live food control. In a subsequent study, Teshima and Kanazawa (1988) showed that growth of *M. japonicus* fed a soy protein-based diet was improved by supplementation with a methionine enriched soybean plastein, but not with the same diet supplemented with crystalline methionine.
Substantial interest in shrimp requirements for EAA resumed in the 1990s with the quantification of arginine requirement by *Penaeus monodon* (Chen *et al.*,, 1992) and supplementation of *M. japonicus* diets with methionine-enriched plastein (Teshima *et al.*,, 1992). To avoid previously identified problems with utilization of CAA, Chen *et al.,* (1992) fed *P. monodon* juveniles a basal diet containing casein to which pure microencapsulated (cellulose acetate phthalate) arginine was supplemented at various levels. Optimal growth was identified by broken line analysis of weight gain to be 5.47% of dietary protein. This was the first paper to quantify an EAA requirement by marine penaeid shrimp.

In 1990, researchers at the Texas Agricultural Experiment Station, Shrimp Mariculture Project (Port Aransas, Texas, USA) developed a semi-purified diets for determination of lysine requirement by *L. vannamei* (Table 1). This research was made possible by the use of an intact protein (e.g., wheat gluten) and an enriched analog. Wheat gluten is naturally low in lysine content (1.3%, d.m.). Previous research by Li-Chan *et al.,* (1979) and Fox *et al.,* (1994) showed that lysine concentration in wheat gluten could be enriched up to 6-8-fold using a carbodiimide reaction. Because wheat gluten comprised a high percentage of the reference diet (approx. 25%, d.m.), it was also necessary to insure that apparent lysine digestibilities of the two forms was similar. Different proportions of enriched and unriched wheat gluten were prepared to provide lysine at concentrations ranging from 3.43 – 6.57% of dietary protein. This study also determined lysine requirement in 35% CP CAA, 45% CP intact protein and 45% CP CAA diets.

Research on EAA requirements of shrimp further expanded in the late 1990s with landmark studies with *P. monodon* by Millamena and colleagues. Their investigations determined requirements for methionine, valine, threonine, lysine, arginine, histidine, isoleucine, leucine, phenylalanine and tryptophan (Table 2). Most of their work was conducted with 20 – 50 mg juveniles over an eight-

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week period. Requirement levels for EAA were lower than their respective concentrations in shrimp muscle tissue for methionine and valine, but not for threonine. Total sulfur-containing amino acid requirement was estimated at 3.5% of dietary protein in a feed containing 0.41% cystine (Millamena *et al.*,, 1996).

Table 1. Ingredient composition (% of diet) of lysine requirement diets containing 35% protein

Ingredients[1]	Experimental diets (% LYS)					
Percent of diet	1.20	1.30	1.50	1.70	1.90	2.30
Percent of protein	3.43	3.71	4.29	4.86	5.43	6.57
Covalent-specific[2]						
Wheat gluten	24.69	22.94	19.44	15.94	12.43	5.43
Lysine-gluten	0.30	2.03	5.49	8.96	12.43	19.36
Fish meal	20.00	20.00	20.00	20.00	20.00	20.00
Wheat starch	37.86	37.89	37.93	37.96	38.00	38.07
Crystalline-specific[2]						
Wheat gluten	24.97	24.85	24.62	24.38	24.15	23.68
L-Lysine HCl	0.02	0.15	0.40	0.65	0.91	1.41
Fish meal	20.00	20.00	20.00	20.00	20.00	20.00
Wheat starch	37.87	37.86	37.84	37.83	37.80	37.77

[1]All diets contained the following ingredients (percentage of total diet): menhaden oil (2.53%), cholesterol (0.25%), lecithin (0.50%), cellulose (2.81%), diatomaceous earth (4.15%), ascorbic acid (0.50%), vitamin premix (2.00%; Akiyama, 1986), mineral premix (4.00%; Akiyama, 1986), L-arginine HCl (0.25%), and L-methionine HCl (0.15%).
[2]Ingredients are specific to their specific diet type.

Table 2. Apparent essential amino acid requirement by *Penaeus monodon*

EAA	Weight Gain (%)	Survival (%)	Requirement (% diet)	Requirement (% protein)
methionine[1]	578-779	63-88	0.89	2.40
total sulfur AA[1]	578-799	63-88	1.30	3.50
valine[2]			1.35	3.40
threonine[3]	293-560	53-78	1.40	3.50
lysine[4]	672-1,581	57-80	2.08	5.20
arginine[4]	678-1,017	70-93	1.85	5.07
histidine[5]	393-800	77-85	0.80	2.20
isoleucine[5]	431-739	68-80	1.01	2.70
leucine[5]	992-1,592	78-90	1.70	4.30
phenylalanine[5]	507-777	65-78	1.40	3.70
tryptophan[5]	629-818	73-83	0.20	0.50

[1]Millamena *et al.,* (1996)
[2]Millamena *et al.,* (1996)
[3]Millamena *et al.,* (1997)
[4]Millamena *et al.,* (1998)
[5]Millamena *et al.,* (1999)

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Until 2004, no EAA requirement had been determined for *M. japonicus* using broken-line analysis (i.e., dose response) as with either *P. monodon* (Chen *et al.,* (1992); Millamena *et al.,*, 1996, 1997, 1998, 1999) or *L. vannamei* (Fox *et al.,*, 1995). Previous research had shown that *M. japonicus* poorly utilized CAA and that determination of EAA would also require some means of reducing dry matter loss from pellets (i.e., leaching). To reduce leaching losses, Alam *et al.,* (2004) fed *M. japonicus* graded levels of crystalline arginine coated with carboxymethylcellulose (CMC). Broken-line analysis of percentage weight gain showed an apparent arginine requirement of 2.66% of diet (5.32% dietary protein). In their recommendations for optimal dietary value of arginine, Alam *et al.,* (2004) corrected for dry matter loss to arrive at slightly lower estimated values.

Current Status of EAA Requirement Research

Recent EAA requirement research conducted on marine penaeid shrimp has largely been limited due to a general tendency of industry to accept currently-used dietary inclusion levels in shrimp production feeds; however, this paradigm is rapidly changing. The recent emphasis on environmental sustainability of shrimp production systems, especially with respect to use of fish meal, has increased interest in development of plant protein-based feeds. Plant sources of protein are often of limited use in aquaculture feeds due to low EAA content. For example, common soybean meal is replete in lysine, but not in methionine; hence, if soybean meal is used as the sole source of protein in a shrimp feed, the formulation will not be nutritionally-complete. In contrast, corn and wheat byproducts are rich in methionine, but limiting with respect to lysine. Plant sources of protein are also substantially limiting with respect to essential HUFA (e.g., arachidonic, docosohexaenoic and eicosopentaenoic acids) and typically contain anti-nutritional factors (e.g., phytic acid).

One potential means of improving nutritional value of shrimp feeds containing plant proteins is by supplementation or enrichment with synthetic amino acids (CAA or others) and is particularly desirous with respect to methionine. Apart from D-L methionine and a methionine-enriched plastein (Teshima *et al.,*, 1992), there are several commercial varieties or forms of methionine which have recently become available to the feed industry, including a hydroxy analog of methionine, 2-hydroxy-4-methylthiobarbituric acid (HMTBA), and binary-bound mineral chelates of methionine (Geisen, personal communication). In the past, the major problem with use of synthetic methionine (i.e., D-L form), as with all CAA, has been its potential loss (i.e., leaching) from pellets once submerged in the aquatic environment. Recent preliminary research with HMTBA has demonstrated growth response of rainbow trout, *Oncorhyncus mykiss* (Chen *et al.,*, 2003). More recent research with shrimp, *L. vannamei*, and the common carp, *Cyprinus carpio*, has demonstrated adequate bioavailability of this compound (Vasquez-Añón and Giesen (2004).

In order for soybean meal and other plant protein sources to replace fish meal in commercial shrimp production feeds, use of synthetic methionine is considered critical; however, an even more fundamental issue should be the determination of apparent requirement for methionine and total sulfur-containing amino acids (methionine plus cystine) by shrimp. To date, methionine requirement has been determined for only one species of shrimp, *P. monodon* (Millamena *et al.,*, 1996). In this study, graded levels of dietary methionine (0.72-1.12%) were achieved by adding pre-coated (CMC) crystalline amino acids to a basal diet. Feed pellets were also coated with CMC, corn starch and k-carrageenan to reduce leaching. Although pellets were tested for "water stability," no data was

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presented. Furthermore, neither apparent digestibility of methionine nor feed consumption rate was confirmed for each dietary treatment.

Research by Samocha *et al.,* (2004) has shown that 100% replacement of fish meal with non-marine protein and oil sources was possible under semi-commercial pond conditions. Fox *et al.,* (2004) showed under controlled experimental conditions that a reduction in fish meal content of research diets from 25 to 12.5% was possible without use of novel feed ingredients. In the latter study, growth of shrimp fed the 12.5% fish meal feed was similar to those fed the 25% feed. Both studies should be considered preliminary in nature due to a lack of information regarding apparent requirement for methionine and total sulfur containing amino acids.

Recommended Approach to Determining EAA Requirement

In order to determine apparent requirements for essential amino acids by shrimp, it is recommended that a comprehensive stepwise approach be taken: 1) identify artificial sources of the EAA in question (e.g., methionine); 2) evaluate these compounds for solubility of the EAA; 3) develop an otherwise nutritionally complete basal feed formulation for requirement research which minimizes the need for supplementation with other synthetic EAA and contains an appropriate digestible protein:energy ratio (e.g., 26 mg CP: kJ); 4) formulate feeds with graded levels of the EAA; 5) prepare these feeds as digestibility diets (i.e., containing 0.5 – 1.0 % chromic oxide for determination of *in–vivo* apparent dry matter, protein and EAA digestibility; 6) confirm actual proximate analysis and EAA content of these feeds; 7) conduct water stability trials to determine temporal loss (0.0 – 1.0 hr) of EAA from feed pellets at the planned feeding trial salinity; 8) prepare actual feeding trial feeds based upon the previous; 9) confirm actual proximate nutrient and EAA concentration of feeding trial feeds; and 10) conduct feeding trial. These guidelines emphasize the need to characterize and standardize availability of EAA in experimental diets. Based upon early research conducted with *M. japonicus* and *P. monodon* (see above), most studies have indicated that feeds containing EAA bound to protein are less likely to be lost from feeds than those supplied in crystalline form.

It is also important that the experimental design of EAA requirement studies be carefully considered. Typically five to seven dietary treatments are utilized in order to provide sufficient spread in the anticipated growth response to allow proper analysis of the dose response. Optimally, the median dietary treatment level should be one that approximates the requirement level of the EAA in question. In broken-line regression analysis, this arrangement would provide a minimum of two data points below and in excess of the apparent requirement. In some cases it could be prudent to undertake a preliminary feeding trial with widely-spaced EAA treatment levels in order to estimate a final range of treatments. There is no clear optimum regarding level of replication within dietary treatments; however, ability to statistically identify growth effects increases proportionally to number of treatments due to a reduction in variance of means within treatments.

If possible, shrimp used in EAA requirement research should be from the same population and derived from high health stocks. They should be obtained as postlarvae and reared under physiochemical criteria similar to that of the anticipated feeding trial. The size (wet weight) of shrimp used in EAA requirement feeding trials should be small enough to exhibit rapid growth yet of

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adequate size to be considered relevant to the commercial industry (typically 0.2 - 1 g initial weight). –Juvenile shrimp should be pre-sorted for size uniformity and stocked at equal numbers and similar biomass loads. Stocking density should be suitable to allow good growth rates and not reduce growth due to density effects when the shrimp are large. Typically, juvenile shrimp reared in clear water systems will double there weight each week until they are about 1 g of weight. Thereafter they typically display weight gain rates in excess of 0.9 g/wk. In an eight-week feeding trial, a shrimp having an initial weight of 1.0 g could achieve a final weight in excess of 8 g.

Treatment diets should be offered to shrimp on a frequent, yet similar basis. This allows for more rapid weight gain of shrimp receiving diets replete in EAA and could reduce potential for leaching of artificial sources of CAA (Yamada *et al.*,, 1981). Fox (1995) offered treatment diets to shrimp on a continuous basis (14 feeding per day) using compartmentalized automated feeders. Results obtained with CAA lysine supplementation were similar to those using protein-bound lysine for 45% CP feeds (4.67% of protein), whereas, an elevated apparent requirement for lysine was shown using CAA lysine (4.49 vs. 5.19% of protein).

A variety of performance indicators are used to provide data for determining dietary requirement for EAA. These include growth (%), biomass gain (%) and weight gain (%). Instantaneous growth rate (IGR) is often reported to normalizes weight gain into an expression of daily weight increment, thus allowing for better comparison of results from similar trials having slightly different durations:

$$GR\ (\%) = [((\ln (W_f / W_i)) * 100] / Td$$

where W_f = final mean weight of shrimp in replicate tank, W_i = initial weight of shrimp in replicate tank, and T_d = total days of feeding trial. Although this transformation is commonly reported, it's utility is very limited and should only be used for animals of similar sizes and similar growth trials. As previously mentioned, most studies determining EAA requirement use broken-line regression analysis of data. This analysis identifies the "break-point" in the one-slope growth curve yielding the best weight gain. Additional indices of performance that could be considered for determination of EAA requirements include various feed and nutrient efficiency indicators: feed conversion ratio, feed efficiency, protein efficiency ratio or net protein utilization. As nutrient requirements are dependent on daily intake feed inputs and conversion efficiencies should be carefully reported.

Methionine Supplementation-A Case Study

Preliminary research was conducted at Texas A&M University-Corpus Christi (Corpus Christi, TX) and Auburn University (Auburn, AL) in which DL-methionine, polymerized methionine and mineral chelates of methionine (Novus International, Inc., St. Louis, MO) were separately added to a soybean protein-based (i.e., low methionine) basal feed. This research was conducted to 1) identify a water-stable source of methionine; 2) determine *in-vivo* methionine digestibility from various synthetic sources; and 3) evaluate apparent methionine requirement by *L. vannamei* using graded levels of synthetic water-stable methionine added to a plant protein-based semi-purified feed.

Leaching trial

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A leaching trial was conducted using digestibility feeds (see subsequent section) containing DL-methionine, polymerized methionine and various mineral chelates of methionine analogue (Ca, Cu, Mg, Mn and Zn; Mintrex^{TM} methionine; Novus International, St. Louis, Missouri, USA). Feeds were submerged for a set time period (45 min) under three different salinity treatments (0, 15 and 30 ppt). Feeds were dried in a drying oven at 60C for 24 hr to obtain a constant weight. Approximately 10 g of dry feed was weighed and placed into each of three 125 mL erlenmeyer flasks. A total of 50 mL of deionized water (0 ppt salinity treatment) was then added to each flask and flasks were gently swirled to disperse feed pellet and cause them to submerge. This step was repeated for two other salinity treatments (15 and 30 ppt, prepared using an artificial sea salt mixture). Additional flasks were also prepared in a similar manner to provide for a 3 * 3 factorial design (time * salinity) for 30 and 45 min samples (total of 9 flasks per dietary treatment). Flasks were then placed on an innOva^{TM} platform shaker (60 rpm; New Brunswick Scientific, Edison, New Jersey, USA) at room temperature (28 C). After 45 min, a flask was removed from the shaker and, using an automatic pipette, 1.00 mL of sample removed and filtered through a 0.45 μm membrane filter into a small acid-washed vial. This step was repeated for each of the remaining vials at their appropriate sample times. Leachate samples were analyzed for free methionine using Waters HPLC.

Digestibility trial

Sub-adult shrimp were stocked at a rate of 15 shrimp per tank (rectangular, plastic, 200 L total volume, 140 L water volume, 0.19 m^2 bottom surface area). Acclimation to experimental conditions was accomplished by *ad libitum* feeding of shrimp for a period of one week prior to experimentation. The experimental system was maintained within the following physiochemical parameters: 29-31 C, 25-27 ppt salinity, >75% saturation, >1,200% turnover per tank per day. All effluent from tanks was sequentially processed through parallel 25 μm bag filtration (Model FV1 housing (Aquatic Ecosystems, Apopka, FL), pressurized counterflow biofilter/degasser (Model DG1T, Aquatic Ecosystems, Apopka, FL), Smart^{TM} UV disinfection unit (Model EU40, Aquatic Ecosystems, Apopka, FL) and in-line heat exchanger (Model DE6115, Aquatic Ecosystems, Apopka, FL). Basal and experimental diets were formulated to contain 35% CP, 6% lipid, with an E:P of 11.4 (Table 3). As shown, no methionine was added to the basal diet and methionine supplementation in experimental diets ranged from 0.12 – 0.20%.

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Table 3. Ingredient composition (g/100 g dry wt. of diet) of defined diets used to evaluate apparent digestibility of various methionine sources.

Ingredients	Basal	DL	Mn	Ca	Cu	Zn	Polymer
Soybean meal	66.70	66.70	66.70	66.70	66.70	66.70	66.70
Menhaden fish oil	4.60	4.60	4.60	4.60	4.60	4.60	4.60
Wheat starch	22.43	22.33	22.31	22.31	22.31	22.31	22.32
Vitamin pre-mix	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Stay C 250	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Calcium phosphate	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Soy Lecithin	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Methionine source	0.000	0.100	0.116	0.120	0.119	0.120	0.106
Chromic oxide	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Formulated content							
Crude protein	35.08	35.18	35.19	35.19	35.19	35.19	35.19
Lysine	5.68	5.67	5.67	5.67	5.67	5.67	5.67
Methionine + cystine	2.87	3.14	3.15	3.15	3.15	3.15	3.15
Methionine	1.35	1.63	1.63	1.63	1.63	1.63	1.63
Arginine	6.43	6.41	6.41	6.41	6.41	6.41	6.41

Four replicate tanks were used for each dietary treatment (1 control and five treatments, 24 total) and evenly distributed within the experimental system. Prior to initiation of experimentation, dead shrimp, excess feed and feces were removed from tanks. The initial feeding was accomplished by time-course addition of 2.0 g of feed to each tank. After 60 min, tanks were vacuum-cleaned and fed a second ration of 2.0 g of feed. After 30 min, uneaten feed and feces were removed from tanks and shrimp were allowed to digest feed and produce feces for a period of 60 min. After this period, feces were collected in each tank by siphoning onto a mesh screen and rinsing with deionized water. Samples were stored at -80 C for subsequent lyophilization and analyis. Feeding of experimental diets and fecal collection continued over a four-day period until approximately 1.0 g of dry weight feces was collected.

At the end of each day of experimentation, shrimp were fed a final ration of 2.0 g feed. The next morning, tanks were cleaned as above and an experimental protocol similar to the previous day was undertaken. Lyophilized samples of feed and feces were subjected to determination of chromic oxide (McGinnis and Kasting, 1954), protein-N (Leco N Analyzer, Leco Corporation, St. Joseph, MI) and methionine concentration (HPCL, Waters, Milford, CT). Apparent digestibility coefficients (ADC) for the test and reference diets were calculated by the following equation (Pond *et al.,* 1995):

$$ADC\ (\%) = 100 - \frac{\%\ \text{indicator in diet}}{\%\ \text{indicator in feces}} * \frac{\%\ \text{nutrient in feces}}{\%\ \text{nutrient in diet}} \times 100$$

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where indicator is chromic oxide and nutrient is dry matter, protein, or methionine. Differences in apparent digestibilities were determined by one-way ANOVA. In cases where differences were identified among treatments, a *post-hoc* Student Neuman Keuhls analysis was performed using SPSS, Version 12.0. Results from this analysis were used to evaluate sources of methionine (from the five treatments) most similar to the basal control diet in terms of dry matter, protein and methionine digestibility.

Methionine Growth Trial

A series of preliminary trials were conducted to determine the TSA requirement of juvenile shrimp, all trials were conducted using similar methods hence only the final growth trial will be described in detail. Postlarval *L. vannamei* were obtained from a commercial shrimp hatchery and reared to a mean weight of 0.50 ±0.05 g at which point they were stocked into an outdoor recirculating aquaculture system for the growth trial. Postlarval holding and growth trial facilities were located at Auburn University's Claude Pettit Marine Laboratory in Gulf Breeze, Alabama, USA.
Synthetic methionine (Mintrex-Cu, Novus International, St. Louis, Missouri, USA) was added to a basal soybean-based basal mix to achieve five treatment levels of methionine (Table 4). All experimental diets were isocaloric, isonitrogenous, and possessed similar consumption and binding characteristics. Dry feed ingredients were mixed for a period of 30 min after which marine oils, binder and water were added and further mixed to produce a moist feed mash. This uncooked mash was then extruded into feed strands using a Hobart A-200 meat chopper with 2.0 mm dye. Feed strands were dried in a forced-air convection oven to achieve a moisture content approximating 8% (w:w). Feed strands were then ground using a household coffee grinder and sieved to achieve a particle size of 3-4 mm (length) x 2 mm (diameter). Experimental feeds were stored at -20C until use.

Table 4. Ingredient composition of plant-based methionine requirement diets

		DL		Cu			
Ingredient	Basal	0.05	0.10	0.04	0.08	0.12	0.16
Poultry meal	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Soybean meal	44.60	44.60	44.60	44.60	44.60	44.60	44.60
Menhaden fish oil	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Wheat starch	34.13	34.13	34.13	34.12	34.11	34.10	34.09
Trace mineral premix	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Choline cloride	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Stay C 250 mg/kg	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Dicalcium phosphate	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Lecithin	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Methionine source	0.00	0.05	0.10	0.04	0.08	0.12	0.16
Gelatin	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Cholesterol	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

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Shrimp were stocked into 1,000 L square-bottomed fiberglass tanks at a density of 60 shrimp per tank (60/m^2 bottom surface area). Eight tanks were assigned to each dietary treatment and the feeding trial lasted for ten weeks. Shrimp within treatments were offered similar rations of their respective experimental feed using automated wheel-type feeders. Uneaten feed and feces and dead shrimp were removed on a daily basis from experimental tanks. Throughout the duration of the feeding trial, culture system water was monitored for temperature, salinity and pH (daily) and total ammonia-nitrogen (Solarzano, 1969), nitrite-nitrogen (Strickland and Parsons, 1972) and nitrate-nitrogen (Mullin and Riley, 1955).

Results of Leaching Trials

Results showed significantly greater leaching at 0 ppt for all feeds (P<0.0001) and from the DL-methionine supplemented feed (P<0.0001), regardless of salinity (Table 5). A significant interaction in terms of leaching between salinity and diet was also shown (P<0.0001). This indicates that any of the methionine supplements, with the exception of DL-methionine, could be used for subsequent determination of apparent methionine requirement.

Table 5. Mean loss of methionine (%) from experimental diets containing various methionine sources, by salinity, after 45 min submergence

	Loss from feed (%)			Loss compared to basal (%)		
	0 ppt	15 ppt	30 ppt	0 ppt	15 ppt	30 ppt
Basal	0.40	0.28	0.35	na	na	na
Poly-MET	0.15	0.08	0.06	-0.25	-0.20	-0.29
Ca-MET	0.14	0.09	0.06	-0.26	-0.19	-0.29
Cu-MET	0.19	0.10	0.10	-0.21	-0.18	-0.25
Mn-MET	0.21	0.08	0.13	-0.19	-0.20	-0.22
Zn-MET	0.11	0.08	0.07	-0.29	-0.20	-0.28
DL-MET	3.04	1.99	2.45	2.64	1.71	2.10

Results of Digestibility Trials

Results from digestibility trials are presented in Table 6 and show that despite differences in apparent dry matter digestibility of diets (ADMD), protein digestibility was similar for all diets (P>0.0500). Apparent methionine digestibility of feeds was ranked in the following order DL-MET = Ca-MET $\geq$ basal feed = Cu-MET = Mn-MET = POLY-MET = Zn-MET. These results indicate that because of similarity in apparent methionine digestibility any of the methionine supplements, with the exception

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of DL-methionine, could be used to supplement methionine requirement diets. They also suggest that high apparent digestibility of the DL-MET feed was due to leaching of DL-methionine from either feed or feces.

Table 6. Apparent dry matter, protein and methionine digestibility (% ± s.e.m.) of various methionine sources

Diet	ADMD	APD	AMETD
Basal	57.17 ± 3.15[b,1]	67.18 ± 3.51	63.57 ± 7.17[b,1]
Poly-MET	52.35 ± 4.56[b]	61.38 ± 8.13	59.03 ± 12.02[b]
Ca-MET	54.47 ±.3.41[b]	61.80 ± 3.63	64.83 ± 2.13[a,b]
Cu-MET	56.29 ±.5.73[b]	65.57 ± 7.80	61.88 ± 6.48[b]
Mn-MET	65.81 ±.3.62[a]	63.06 ± 0.55	61.44 ± 1.35[b]
Zn-MET	59.77 ±.4.21[a,b]	64.46 ± 2.10	57.77 ± 8.54[b]
DL-MET	58.25 ±.9.09[a,b]	67.79 ± 6.40	84.29 ± 7.89[a]

[1]Means with similar superscripts are not significantly different (P>0.0500).

Results of Preliminary Methionine Growth Trial

In order to elucidate the dietary requirement for total sulfur amino acids, a series of preliminary trials was conducted. The first feeding trial utilized a 36% CP diet with 8% lipid using a poultry byproduct (25% diet) and soybean (25.5% diet) meal basal diet. The basal diet contained a total sulfur amino acid content of 2.8% of the protein. In this experiment the mean initial weight was 0.24g (4.5 % CV) and after five weeks of feeding, shrimp had grown in excess of 1,000% (weight gain); however, there were no significant differences in final weight between treatments with varying levels of DL methionine. Because DL methionine might not have been delivered, we chose to evaluate the leaching (see previous section) and evaluate alternative sources of methionine (e.g., chelated forms of methionine and a polymer of methionine).

To evaluate whether these forms were acceptable to shrimp, a second preliminary growth trial was conducted using a simple practical diet containing 53.2% soybean meal, 6% poultry meal, 4% menhaden fish oil, 31.53% wheat starch, 2.57% vitamin/ mineral premix, and 0.2% cholesterol. The basal diet was formulated to contain 32% protein, 6% lipid and a total sulfur amino acid content of 2.88% protein (1.33% methionine). These diets were then supplemented with adequate levels of synthetic methionine to bring the TSAA content to 3% of the protein. Juvenile shrimp (0.24 g initial weight) were reared over a seven week period reaching a final mean weight of 3.97 (basal), 3.82 (DL-M), 3.97 (Cu-M) and 3.99 g (Polymer-M). Survival ranged from 85% to 95% with the polymer yielding lowest survival. Although percent weight gain was in excess of 1,500%, there were no statistical differences among treatments, indicating that any of the forms were suitable for further research.

Based on results from the aforementioned trials a final growth trial conducted. All experimental diets (Table 4) were formulated to contain 32% CP, 6% lipid and 11.9 kcal/100 g protein. Source of methionine and TSAA content (% protein) of treatment diets was as follows: basal (2.62%), DL-methionine 0.05 (2.77%), DL-methionine 0.10 (2.93%), Cu-methionine (2.71, 2.81, 2.91 and 3.01%).

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The results of this trial are presented in Table 7 and show that no significant differences in survival (86.67 – 96.67%) or percent weight gain, biomass gain or FCR were shown. These results indicate that the lowest dietary inclusion level of methionine (0.40% of the diet, 1.26% of protein) was adequate to support in excess of 760% weight gain of juvenile *L. vannamei*. Similarity of growth and feed performance indices is likely the result of all treatment diets possessing adequate amounts of available methionine or TSA. This implies that the apparent requirement for methionine could be less than 0.4% of diet (1.26% of protein or TSA 2.62% of protein). Future research will focus on reducing methionine content of the basal diet. These results are substantially different from those of Millamena *et al.,* (1996) for *P. monodon* in which apparent methionine requirement was reported as 0.89% of diet or 2.40% of protein. Results from the present study are surprising considering the primary source of protein in the growth study was soybean meal. Soybean meal has an apparent protein digestibility of 89.9% vs. 99.1 and 97.3% for casein and gelatin, the natural protein sources used in Millamena *et al.,* (1996). Other major differences in the methodology between the present study and that of Millamena *et al.,* (1996) are the obvious species difference and the initial weight of shrimp used: 680 mg vs. 20 mg, respectively. Also, Millamena *et al.,* (1996) used a CAA mixture supplement casein and gelatin in order to achieve similarity between the basal feed amino acid composition and that of shrimp muscle tissue. The ratio of CAA to natural or intact protein was very high, comprised 54% of dietary amino acid-nitrogen. The apparent total sulfur-containing amino acid (SAA) requirement reported by Millamena *et al.,* (3.5%) was higher than that of carp (3.1%; Nose, 1979), Japanese eel (3.2%; National Resources Council, 1983), Mozambique tilapia (3.2%; Jackson and Capper, 1982), channel catfish (2.34%; Harding *et al.,*, 1977), and rainbow trout (2.7%; Ogino, 1980), and red drum (3.03%; Moon and Gatlin, 1991). The present study predicted an apparent SAA requirement less than 2.62% for *L. vannamei*. These results in apparent SAA in aquatic organisms could be associated with species and age (Baker, 1977), dietary protein source, level of CAA (Woodham and Deans, 1975) and experimental design (Robbins *et al.,*, 1979; Baker, 1986).

Table 7. Mean results from preliminary methionine growth trial

Diet	Initial Weight (g)	Final Weight (g)	Survival (%)	Weight Gain (%)	FCR
Basal	0.67	5.78	83.33	860.7	1.96
DL- 0.05	0.67	5.73	96.67	862.7	1.91
DL- 0.1	0.66	5.89	90.00	893.5	1.91
MCU 0.04	0.68	5.72	95.00	843.0	1.95
MCU 0.08	0.67	5.80	86.67	872.2	2.04
MCU 0.12	0.68	5.75	91.67	846.6	1.96
PSE	0.013	0.1338	2.96	19.92	0.061
P-value	0.8443	0.9499	0.1872	0.5315	0.7375

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