# Fact or Fiction: Methionine Requirement for Pacific White Shrimp *Litopenaeus vannamei*

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## Abstract

Considerable effort has been invested into the development and validation of alternative feed formulations for shrimp. Based on the PI's experience with the transfer of this technology to feed manufactures, the primary constraint is a poor understanding or a lack of defined studies that pinpoint the methionine or total sulfur amino acid (methionine + cysteine) requirement of shrimp. Many feed manufactures have the methionine requirement set relatively high which favors the inclusion of expensive fishmeal vs that of non-marine protein sources. Given current economic and social concerns, this is not a sustainable approach. There are several publications evaluating methionine supplements to Pacific white shrimp feeds. Yet these papers do not provide a clear definition of the requirement, which is a major constraint to feed manufactures acceptance of low fishmeal feed formulations. One theory often presented, is that crystalline amino acids are absorbed and circulate in an asynchronous patter to those from intact proteins. Based on current research we have demonstrated that the uptake of amino acids in shrimp corresponds to the digestive physiology of the animal. In that, shrimp are semi-continuous feeders that process and digest foodstuffs very quickly. From the initiation of feeding, an upswing in amino acids in the hemolymph was apparent within 10 minutes confirming very quick processing and digestion of nutrients. The clearance of amino acids was also relatively fast with amino acid levels returning to overnight fasting levels within 60 minutes of fasting. This cycle of nutrient cycling corresponds to the semi-continuous feeding habits of shrimp. Based on both absorption and clearance patterns of the amino acids, there was no indication of a synchronous absorption of supplemented amino acids; hence, crystalline amino acids should be available for metabolism. Hence, if we can produce a deficient diet, we should be able to induce a classic dose response. Across numerous growth trials, published and unpublished, the response of juvenile shrimp to a range of methionine sources have been evaluated such sources include Dl-methionine, Micro-encapsulated methionine, coated methionine, synthetic peptides and various chelated forms. Yet, within the published literature there is little consistency of responses or any definitive studies. Many of these studies present contradictory or inconsistent data and hence do not provide strong evidence for a definitive requirement. Within our laboratory, across numerous independent growth trials, increasing the level of methionine as a supplement or using corn protein concentrate as an intact protein source, increasing methionine levels of the diet produced very little evidence of growth enhancement or no consistency of the response. In this authors opinion, inconsistent responses are due to other factors and the methionine requirement for this species is

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quite low and may not be a limiting amino acid in many commercial feed formulations. The alternative hypothesis, is that many of our purified forms are not available to shrimp or that test diets and systems are not appropriate. Clearly, there is a need to better understand amino acid metabolism in shrimp and define limiting amino acids as well as techniques to consistently define amino acid requirements.

Keywords: Methionine, Requirement, shrimp

## Introduction

Pacific white shrimp, *Litopenaeus vannamei*, is regarded as the primary cultured shrimp species worldwide and a high value commodity with a production of around 4.5 million metric tons in 2016 (Anderson 2016). As shrimp consumption is expected to continue to increase, it is vital to develop sustainable alternative ingredients in the shrimp diets to support the expansion of the shrimp industry (Achupallas *et al.* 2016b, Achupallas *et al.* 2016a). From a nutritional standpoint, the popularity of Pacific white shrimp is due to their adaptability to a range of diets, tolerance of plant-based feeds and ability to utilize natural productivity. This species is very tolerant of diets with a range of fishmeal levels including fishmeal-free diets (Morris *et al.* 2011, Roy *et al.* 2009, Samocha *et al.* 2004).

Using proper replacement strategies (Davis 2007), several studies have demonstrated that fishmeal and marine oil levels can be reduced or eliminated from growout diets of the Pacific white shrimp. Currently, a mixture of cheaper, high-quality plant proteins (e.g. solvent-extracted soybean meal, specialized soy protein, corn protein concentrate, distillers grain solubles, pea meal) or terrestrial animal protein sources (e.g. poultry by-product meal, meat and bone meal, blood meal) can be used to successfully replace fishmeal in shrimp feeds without compromising growth or survival (Sookying *et al.* 2013). However, this can only be accomplished if shifts in nutrient requirements such as essential amino acids (particularly methionine), energy content of the diet, essential fatty acids as well as minerals such as phosphorus are accounted for (Davis 2007). Despite the preponderance of information on alternative feed formulations (see Sookying *et al*. (2013) for a review), a wide spread acceptance of low fishmeal feeds by the aquaculture industry in general, and the shrimp industry is still lacking. Although fishmeal levels in the Americas has been reduced over the years, the level of fishmeal in shrimp feeds in Asia is much higher i.e. 20% or greater of the diet (personal observation). It is well known that fishmeal supplies will not increase as most fisheries are beyond sustainable limits. A portion of this demand will be met with fisheries byproducts that are currently underutilized as well as that from processing aquaculture products. Hence, if aquaculture is to expand, the industry must move away from fishmeal as a primary protein source, particularly in production diets.

From a feed formulation standpoint, one must ask, why do we find ourselves in such a situation? First, there are very few quality studies defining the essential amino acid requirements for this species. The NRC reports a dietary methionine requirement of 0.7% and total sulfur amino acid (TSAA, methionine + cysteine) requirement of 1% for both the Kuruma and Tiger shrimp but do not report one for the Pacific white shrimp (NRC 2011). Fox *et al.* (2011) reported on a series of studies designed to evaluate digestibility, leaching and the methionine requirement of the Pacific white shrimp. Although this research confirmed digestibility of a range of methionine supplements, they were unable to demonstrate a methionine deficiency. Forster and Dominy (2006) evaluated three methionine sources supplemented to a basal diet. Albeit they reported, a deficiency in the basal diet which contained 0.45% methionine. This statement was based on a contrast in which all supplemented data was pooled. However, the authors also report ANOVA results were > 0.1 for the reported growth data. More recently, Façanha *et al.* (2016) reported on results for a methionine trial at three densities in outdoor tanks. They observed no dose response at two of the densities and a "linear" increase in weight gain in the third, with the requirement estimated at 0.72 (1.19% TSAA) to 0.81 (1.28 TSAA) using a 36% protein diet. In this author's opinion, this is quite odd as the response should not be affected by density and there was no clear performance plateau in the shrimp for which a response was seen. In another recent publication, Lin *et al.* (2015) reported on three experiments with three different sizes of shrimp. They found almost no difference in weight gain across all experiments and treatments. Yet using regression analysis presumably of the mean values, a "dose response" to methionine was reported which estimated a methionine requirement of 0.91% diet or 2.28% of the protein in a diet with 0.5% cysteine. Once again, this is very weak data, which may not have been analyzed using the most appropriate methods (i.e. regression on treatment means), the requirement estimate is much higher than that reported for other species, and the results were not repeatable across size classes.

The definition of a requirement is further complicated by trade journal publications which have printed a range of articles which may be misinterpreted. For example, one recent article presents a survey of essential amino acids (EAA) levels in commercial feeds. The authors indicated that the average TSAA level found in feeds for Pacific white shrimp was 3.36 % of the protein. They further supported a recommendation of extremely high levels of methionine and other EAA. This report indirectly advocates for high levels of marine protein sources as that is the only way to reach such high level of methionine and TSA. However, these numbers clearly conflict with whole-body levels, cysteine replacement value, as well as levels published for other species. Therefore, these numbers very likely misrepresent requirements, and such reports are not doing the industry any good. With the exception of lysine (Fox *et al.* 1995), there are few classical requirement studies to refute these claims. We would suggest that a concerted effort and systematic, rational evaluation of limiting AA must be initiated in an effort to define requirement in the Pacific white shrimp through a classic approach. Such data would allow feed formulators to have the confidence to exchange protein sources based on AA profiles and cost.

With the support of a range of funding sources my laboratory has systematically developed soy-based feed formulations in shrimps (Sookying *et al.* 2013). Across numerous trial with intact protein source, we have demonstrated no response to substitutions, which have driven the methionine and total sulfur amino acid levels to very low levels, without a decrease in growth or survival. Recently our laboratory has expended considerable effort into looking at limiting amino acids in shrimp feed. The following summaries recent experimental data attempting to elucidate the methionine requirement and evaluate the efficacy of crystalline amino acids.

## Methods

### *Diet preparation*

The basal diets used in the various trials were designed to contain 34% protein and 9% lipid using primarily soybean meal and fishmeal or poultry by product meal as the primary protein sources (Table 1, 2, 3, and 4). With the exception of methionine, the basal and test diets were formulated to meet the nutritional requirements of the Pacific white shrimp. The test diets were prepared in the feed laboratory of Auburn University, Auburn, AL, USA using standard practices. In short, pre-ground dry ingredients and oil were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Hot water was then blended into the mixture to attain a consistency appropriate for pelleting. Each diet was pressure pelleted using a meat grinder and a 3 mm die. After pelleting, diets were dried to a moisture content of 8-10% and stored at 4 C.

*Experimental system*

For all the trials, Pacific white shrimp were stocked into 75-L tanks which were a component of a 2,500-L indoor recirculation system (reservoir, bead filter, fluidized biological filter and circulation pumps and heater). Each diet was offered to shrimp in three to five replicate aquaria for the length of the trial. Water temperature was maintained at around 28 C using a submerged 3,600-W heater (Aquatic Eco-Systems Inc., Apopka, Florida, USA). Dissolved oxygen was maintained near saturation using air stones in each aquarium and the sump tank using a common airline connected to a regenerative blower. Dissolved oxygen and water temperature were measured twice a day using a YSI-55 digital oxygen/temperature meter (YSI corporation, Yellow Springs, Ohio, USA) while pH, TAN and Nitrite-N measured once per week.

*Free amino acids (AA) of hemolymph*

To determine levels of free amino acids (AA) in the hemolymph of shrimp, replicate groups of four shrimp were utilized for each collection point. Shrimp at a given time point were netted and placed in an ice slurry to anesthetize the shrimp, after which hemolymph was collected from the abdominal segments using a syringe and 25 gauge needle.

For this work a 1 ml syringe was preloaded with 0.5 ml of an anti-coagulant and used to collect approximately 0.5 ml of hemolymph. To maintain semi-quantitative data, trial one recorded the volume whereas in the following trials, the weight of the anticoagulant and hemolymph were determined gravimetrically. The anticoagulant solution (Liu *et al* 2004) contains 30 mM Sodium Citrate Tribasic Dihydrate (Sigma S4641); 0.34 M Sodium Chloride (NaCl); 10 mM EDTA – Ethylene Diamine Tetraacetic Acid (Sigma, E9884); in de-ionized (DI) water. This is made by weigh 4.4115 g sodium citrate tribasic dehydrate, 9.945 g sodium chloride, 1.461 g EDTA, and dissolve in 500 ml DI water. To obtain free amino samples of the hemolymph, the collected hemolymph was centrifuged and decanted. The blood samples from shrimp in a single aquarium were pooled resulting in 2 to 3 samples/treatment each with blood from 3-4 shrimp.

Three trials were conducted with the Basal Diet (BD) as well as the BD with select AA. For each trial, the shrimp were preconditioned to the diets for four days. The prior evening the tanks were cleaned to remove any food sources. The next day and prior to feeding a group of shrimp were bled to determine fasted levels of free AA, the remaining shrimp were offered food for predetermined time periods and then the feed was withdrawn and shrimp fasted.

*Growth Trials*

A series of growth trials were carried out using juvenile shrimp. Diets were offered using a standard feeding protocol with 4 feedings per day. Diets were offered to shrimp at a suitable level based on historic performance as well as observed consumption of the feed. At the conclusion of the growth trials, the shrimp were weighed, enumerated, and frozen for subsequent analysis.

*Biochemical analysis*

Samples of hemolymph and shrimp were freeze until required. Tissue samples requiring analysis were sent to University of Missouri laboratory.

*Statistical analysis.*

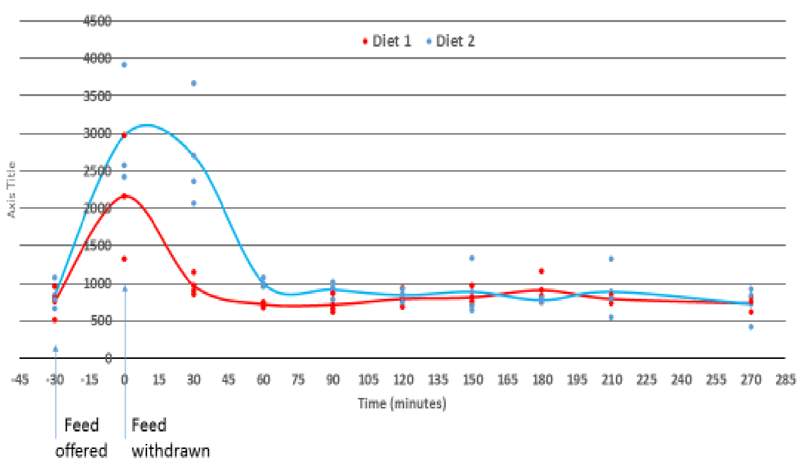
All data was analyzed using SAS (V9.4. SAS Institute, Cary, NC, USA). Data from growth trials were analyzed using one-way analysis of variance to determine significant ($P < 0.05$) differences among the treatment means followed by Student-Neuman-Keuls multiple range test to distinguish significant differences between treatment means.

**Results and Discussion**

Due to the limitation of available data on methionine requirements for shrimp, initial work concentrated on confirming amino acid levels in the hemolymph of shrimp. A small component of this data is presented which demonstrates the upswings after feeding and the clearance of amino acids once the shrimp were fasted (Figures 1 – 3). We have evaluated both long term (up to 4.5 hours after feeding) as well as short term (while feeding).

In the first trial presented in this summary, the shrimp were fasted overnight to allow the identification of basal levels of AA. After overnight fasting the shrimp were sampled (-30 minutes), then provided feed for 30 minutes after which another sample was taken (0 minutes) and the post feeding levels of AA determined (0 to 270 minutes). As most AA followed a similar pattern, the sum of AA in the hemolymph is presented in Figure 1. It is clear that all AA were near their peak after feeding and the level declined within 60 minutes to near fasting levels. Albeit, AA levels were followed over 270 minutes for which there was no secondary peaks for methionine or the other supplemented amino acids (lysine, arginine or taurine).

Figure 1. Shrimp (7 g) were offered one feeding over a 30 minute period after fasting overnight. Total AA levels (*ug*/ml) of hemolymph is presented for Diets 1 (Basal) and Diet 2 ( Basal +Lys, Meth, Arg, Taur).



To better define the absorption process and to evaluate if there may be an asynchronous peak during absorption, a second trial was conducted, which simply looked at the uptake of AA over a 1 hr period (Figure 2). Again, the shrimp were fasted overnight and their hemolymph sampled to determine basal levels (-60 minutes). The shrimp were then offered feed for a total of 60 minutes during which time intermittent samples were taken. This data is presented for methionine (Figure 2), albeit the trend was similar for all amino acids. Both the supplemented basal (Diet 1) and DL-methionine supplemented diets (Diet 3) were followed. Although, the level of AA is consistently higher in the supplemented diet, the pattern for AA uptake is similar with a clear upswing in AA levels in 10-15 minutes after the initiation of feeding. This is quite different from that of fish species for which digestion and absorption is delayed several hours primarily due to holding of food in the stomach.

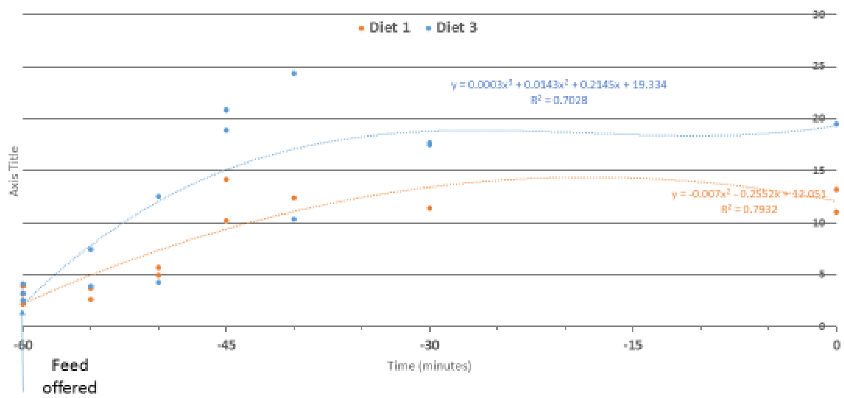



Figure 2. Shrimp (30.6 g) were offered one feeding after fasting overnight. Methionine levels (*ug*/ml) of hemolymph over a 60 minute period when feed is available to the shrimp is presented for Diets 1 (Basal) and 3 (Basal + DL methionine).

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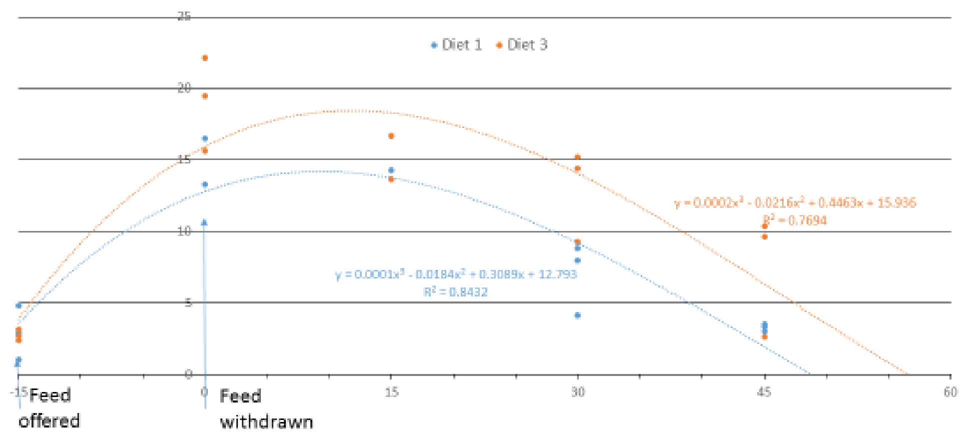
The third trial focused on the reduction of AA levels post feeding identify if there are different patterns that would indicate asynchronous absorption. For this trial, the shrimp were fasted overnight and a basal AA level determined (-15 minutes) the shrimp were then allowed to have feed for 15 minutes at which time a sample was taken (0 minutes) and the feed withdrawn. Samples were collected after 15, 30 and 45 minutes. To confirm the basal levels, an additional sample was taken after ~1.5 hr (data not presented). The methionine levels of the hemolymph are presented in Figures 3 with other amino acids following a similar pattern. Once again, the supplemented diet has higher levels of AA but there was no clear asynchronous uptake or clearance from the hemolymph.

Figure 3. Methionine levels (ug/ml) levels of hemolymph from shrimp (16.6 g) fed Diets 1 (Basal) and Diet 3 (Basal + DL methionine). Shrimp were sampled after overnight fasting (T-15) and offered feed for 15 minutes (T-15 to T0) then fasted over a 60 minute period.



The presented data, clearly demonstrates that AA in shrimp is much quicker than that of fish species with AA appearing in the hemolymph within 10 to 15 minutes. This is primarily due to the rapid and continual processing of food by shrimp. Based on the results of these studies there is no evidence that shrimp cannot utilize free AA or that there is asynchronously absorbed.

Presently there are few studies published on methionine requirement of the Pacific white shrimp. The few studies dealing with methionine supplements have failed to establish what would be considered a strong and convincing methionine requirement for this species. Hence, the second component of the research was designed to evaluate the efficacy of various methionine sources as well as attempt to identify a dietary requirement. Table 1a presents a series of diets which were supplemented with a range of methionine sources including: DL-methionine, coated methionine, as well as a modified diet using intact proteins. Proximate and AA composition of the test diets are presented in Table 1b with the basal diet analyzing at 0.49% methionine (0.41 cysteine) and the remaining diets at 0.64 to 0.68% methionine. These diets were offered to 0.5 g shrimp over a 42-day growth trial after which the AA profile of the shrimps tail muscle and hepatopancreas were evaluated. Growth and survival were very good with over 1000% weight gain observed with good FCR and survival (Table 1c). However, there were no differences in the growth, feed or survival rates observed. To determine if there were shifts in tissue composition, AA levels of the shrimp muscle and hepatopancreas were determined. There were no differences or trends seeing in methionine levels of these tissues. These results indicated that there were no differences in deposition across all treatments.

Table 1a. Composition (g/100g as is) of test diets.

| | Basal | Coated Meth (76%) | DL-Met | Intact protein |
|---|---|---|---|---|
| Poultry by product meal | 4.00 | 4.00 | 4.00 | 4.00 |
| Soybean meal | 48.0 | 48.00 | 48.00 | 33.00 |
| Corn protein concentrate | 0.00 | 0.00 | 0.00 | 15.00 |
| Gelatin | 5.00 | 5.00 | 5.00 | 0.00 |
| Menhaden fish oil | 5.68 | 5.68 | 5.68 | 5.59 |
| Lecithin | 1.00 | 1.00 | 1.00 | 1.00 |
| Cholesterol | 0.08 | 0.08 | 0.08 | 0.08 |
| Corn Starch | 3.68 | 3.64 | 3.69 | 8.26 |
| Whole wheat | 27.00 | 27.00 | 27.00 | 27.00 |
| Mineral premix | 0.50 | 0.50 | 0.50 | 0.50 |
| Vitamin premix | 1.80 | 1.80 | 1.80 | 1.80 |
| Choline chloride | 0.20 | 0.20 | 0.20 | 0.20 |
| Stay C 35% active | 0.10 | 0.10 | 0.10 | 0.10 |
| CaP-dibasic | 2.80 | 2.80 | 2.80 | 3.00 |
| Lysine | | | | 0.47 |
| Methionine sources | | 0.20 | 0.15 | 0.00 |
| Glycine | 0.16 | | | |

Table 1b. Analysis of test diets.

| | Basal | Coated Meth | DL-Met | Intact protein |
|---|---|---|---|---|
| Crude Protein | 34.56 | 34.89 | 34.91 | 35.87 |
| Moisture | 5.85 | 5.87 | 5.4 | 5.23 |
| Crude Fat | 9.64 | 10.07 | 10.12 | 9.22 |
| Crude Fiber | 3.68 | 3.88 | 3.91 | 3.37 |
| Ash | 6.49 | 6.28 | 6.31 | 6.16 |
| Amino Acids | | | | |
| Alanine | 1.72 | 1.72 | 1.73 | 2.01 |
| Arginine | 2.30 | 2.32 | 2.33 | 1.88 |
| Aspartic Acid | 3.06 | 3.08 | 3.11 | 2.84 |
| Cysteine | 0.41 | 0.42 | 0.42 | 0.53 |
| Glutamic Acid | 6.10 | 6.16 | 6.19 | 7.12 |
| Glycine | 2.69 | 2.53 | 2.53 | 1.46 |
| Histidine | 0.74 | 0.74 | 0.75 | 0.78 |
| Hydroxy-lysine | 0.11 | 0.10 | 0.11 | 0.05 |
| Hydroxy-proline | 0.65 | 0.64 | 0.65 | 0.12 |
| Isoleucine | 1.34 | 1.34 | 1.34 | 1.49 |
| Lanthionine | 0.03 | 0.02 | 0.01 | 0.02 |
| Leucine | 2.31 | 2.32 | 2.34 | 3.63 |
| Lysine | 1.83 | 1.84 | 1.86 | 1.83 |
| **Methionine** | **0.49** | **0.67** | **0.67** | **0.64** |
| Phenylalanine | 1.52 | 1.52 | 1.54 | 1.85 |
| Proline | 2.32 | 2.34 | 2.36 | 2.47 |
| Serine | 1.39 | 1.43 | 1.45 | 1.54 |
| Taurine | 0.22 | 0.22 | 0.22 | 0.23 |
| Threonine | 1.12 | 1.13 | 1.14 | 1.18 |
| Tryptophan | 0.38 | 0.38 | 0.37 | 0.36 |
| Tyrosine | 1.02 | 1.04 | 1.04 | 1.37 |
| Valine | 1.50 | 1.51 | 1.52 | 1.64 |
| Sum AA | 33.25 | 33.47 | 33.68 | 35.04 |

Table 1c. Response of juvenile shrimp (0.55 ± 0.04 g) to various test diets over a 42-day growth trial. Means of three replicate groups of shrimp.

| Diet | Final Biomass (g) | Mean Weight (g) | Weight Gain (g) | Percent Gain | FCR | Survival (%) |
|---|---|---|---|---|---|---|
| Basal | 65.99 | 6.60 | 6.03 | 1064.3 | 1.63 | 100.0 |
| Coated | 68.49 | 6.85 | 6.29 | 1127.9 | 1.56 | 100.0 |
| DL-Met | 66.04 | 6.60 | 6.07 | 1134.4 | 1.62 | 100.0 |
| Intact | 63.58 | 6.99 | 6.45 | 1197.6 | 1.53 | 90.0 |
| P value | 0.9533 | 0.7205 | 0.704 | 0.6533 | 0.217 | 0.5251 |
| PSE | 5.179 | 0.2384 | 0.2622 | 59.77 | 0.7118 | 4.71 |

To determine if we could induce a response to graded levels of methionine the first dose response trial utilized both DL-methionine and a microencapsulated methionine (Table 2a). This trial was repeated twice with the data from the second trial reported. The analysis for proximate and AA composition of the test diets is presented in Table 2b. The methionine levels ranging from 0.54% (0.42% cysteine) in the basal diet to 0.83% methionine for the DL-methionine supplemented diet and 0.81% methionine for the microencapsulated methionine diet. Diet 10 was designed as an intact protein diet utilizing corn protein concentrate which increased the methionine to 0.62% of the diet.

At the conclusion of a 42-day growth trial (Table 2c) the shrimp grew from 0.21 g to a maximum weight of 6.26g. Survival and growth were very good in this trial and there was a large increase in weight gain or tissue replacement (2467 to 2832%). However, the response was not typical of a dose response study. That is to say there was no increase in weight gain corresponding to increases in methionine levels. Proximate composition and AA profile of the whole shrimp were also evaluated for this trial with a slight decrease in the level of protein in the shrimp and significant differences in methionine levels of the whole shrimp. In this trial, the basal diet resulted in a significant depression in whole body tissue levels that was lower than most other diets. Based on regression analysis, there is a general increase as the level of methionine increased from 0.54% of the diet, albeit not a very strong response ($R^2$ = 0.54). This may indicate a slight deficiency of the basal diet.

Table 2a. Composition (g/100g as is) of test diets

| | | DL- Methionine | | | | Micro-encapsulated methionine | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Poultry by product meal | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 |
| Soybean meal | 47.20 | 47.20 | 47.20 | 47.20 | 47.20 | 47.20 | 47.20 | 47.20 | 47.20 | 47.20 |
| Menhaden Fish Oil | 5.70 | 5.70 | 5.70 | 5.70 | 5.70 | 5.70 | 5.70 | 5.70 | 5.70 | 5.55 |
| Corn Starch | 2.83 | 2.83 | 2.83 | 2.83 | 2.83 | 2.78 | 2.73 | 2.63 | 2.53 | 2.08 |
| Whole wheat | 27.09 | 27.09 | 27.09 | 27.09 | 27.09 | 27.09 | 27.09 | 27.09 | 27.09 | 27.09 |
| Trace Mineral premix | 0.50 | 0.50 | 0.50 | 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 | 1.80 | 1.80 | 1.80 |
| Choline chloride | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Stay C | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| CaP-dibasic | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 |
| Lecithin | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Cholesterol | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 |
| Corn protein isolate | | | | | | | | | | 7.90 |
| DL-Methionine | | 0.05 | 0.10 | 0.20 | 0.30 | | | | | |
| Meth. Micro-encapsulated | | | | | | 0.10 | 0.20 | 0.40 | 0.60 | |
| Gelatin | 7.00 | 7.00 | 7.00 | 7.00 | 7.00 | 7.00 | 7.00 | 7.00 | 7.00 | |
| Glycine | 0.30 | 0.25 | 0.20 | 0.10 | | 0.25 | 0.20 | 0.10 | | 0.30 |

Table 2b. Proximate and amino acid (AA) composition of test diets (g/100g as is)

| Exp1615 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Crude Protein | 37.76 | 38.05 | 37.97 | 37.54 | 37.5 | 38.03 | 37.84 | 37.93 | 38.24 | 37.68 |
| Moisture | 5.13 | 6.48 | 6.47 | 7.41 | 7.42 | 6.65 | 7.07 | 6.61 | 6.76 | 5.89 |
| Crude Fat | 9.32 | 8.62 | 8.86 | 8.79 | 8.64 | 9.22 | 9.29 | 10.03 | 9.21 | 9.58 |
| Crude Fiber | 3.12 | 3.72 | 3.7 | 3.71 | 3.54 | 3.83 | 3.77 | 3.72 | 3.72 | 3.76 |
| Ash | 5.91 | 5.83 | 5.75 | 5.72 | 5.72 | 5.75 | 5.74 | 5.72 | 5.79 | 5.84 |
| Amino Acid | | | | | | | | | | |
| Alanine | 1.91 | 1.94 | 1.88 | 1.92 | 1.93 | 1.94 | 1.93 | 1.93 | 1.95 | 1.92 |
| Arginine | 2.45 | 2.56 | 2.58 | 2.54 | 2.55 | 2.54 | 2.57 | 2.57 | 2.59 | 2.23 |
| Aspartic Acid | 3.21 | 3.33 | 3.28 | 3.29 | 3.29 | 3.33 | 3.31 | 3.33 | 3.36 | 3.32 |
| Cysteine | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.43 | 0.42 | 0.43 | 0.42 | 0.53 |
| Glutamic Acid | 6.39 | 6.5 | 6.41 | 6.42 | 6.44 | 6.5 | 6.46 | 6.49 | 6.55 | 7.22 |
| Glycine | 3.29 | 3.26 | 3.08 | 3.11 | 3.06 | 3.26 | 3.21 | 3.12 | 3.04 | 1.95 |
| Histidine | 0.76 | 0.78 | 0.77 | 0.77 | 0.77 | 0.78 | 0.78 | 0.78 | 0.79 | 0.86 |
| Hydroxy-lysine | 0.12 | 0.14 | 0.13 | 0.14 | 0.14 | 0.13 | 0.13 | 0.13 | 0.13 | 0.07 |
| Hydroxy-proline | 0.86 | 0.87 | 0.83 | 0.95 | 0.97 | 0.86 | 0.87 | 0.87 | 0.88 | 0.16 |
| Isoleucine | 1.38 | 1.43 | 1.42 | 1.4 | 1.39 | 1.42 | 1.4 | 1.4 | 1.43 | 1.61 |
| Lanthionine | 0.01 | 0.04 | 0.02 | 0.03 | 0.03 | 0.03 | 0.02 | 0.02 | 0.02 | 0.06 |
| Leucine | 2.39 | 2.45 | 2.45 | 2.41 | 2.4 | 2.44 | 2.42 | 2.43 | 2.46 | 3.34 |
| Lysine | 1.92 | 1.98 | 1.96 | 1.95 | 1.95 | 1.98 | 1.97 | 1.97 | 1.99 | 1.81 |
| Methionine | 0.54 | 0.6 | 0.63 | 0.73 | 0.83 | 0.61 | 0.63 | 0.75 | 0.81 | 0.62 |
| Phenylalanine | 1.58 | 1.62 | 1.63 | 1.6 | 1.6 | 1.61 | 1.61 | 1.62 | 1.63 | 1.89 |
| Proline | 2.66 | 2.53 | 2.56 | 2.58 | 2.6 | 2.57 | 2.62 | 2.62 | 2.65 | 2.28 |
| Serine | 1.51 | 1.53 | 1.46 | 1.51 | 1.51 | 1.55 | 1.51 | 1.53 | 1.53 | 1.59 |
| Taurine | 0.16 | 0.19 | 0.23 | 0.22 | 0.21 | 0.21 | 0.22 | 0.21 | 0.21 | 0.21 |
| Threonine | 1.17 | 1.2 | 1.18 | 1.18 | 1.19 | 1.2 | 1.19 | 1.2 | 1.2 | 1.29 |
| Tryptophan | 0.41 | 0.4 | 0.37 | 0.36 | 0.37 | 0.38 | 0.38 | 0.38 | 0.37 | 0.42 |
| Tyrosine | 0.99 | 1.1 | 1.12 | 1.09 | 1.09 | 1.08 | 1.11 | 1.11 | 1.11 | 1.38 |
| Valine | 1.57 | 1.61 | 1.6 | 1.59 | 1.58 | 1.6 | 1.59 | 1.59 | 1.63 | 1.75 |

| Sum AA | 35.7 | 36.48 | 36.01 | 36.21 | 36.32 | 36.45 | 36.35 | 36.48 | 36.75 | 36.51 |
|---|---|---|---|---|---|---|---|---|---|---|

Table 2c. Response of juvenile shrimp (0.21 ± 0.017 g) to various test diets over a 42-day growth trial. Means of four replicate groups of shrimp. These diets were evaluated in two trials, results of the second trial are presented. Means within the same column with different letters are significantly different based on Student-Newman-Kuels test. Dunnett's T-test did not indicate that any of the values within a column were different from that of the basal diet.

| Trt. | Diet | Final No. | Final Biomass (g) | Mean Weight (g) | Weight Gain g) | Weight Gain % | FCR | Percent Survival |
|---|---|---|---|---|---|---|---|---|
| 1 | Basal | 15.0 | 85.70 | 5.71ab | 5.49ab | 2520.5 | 1.39ab | 100.0 |
| 2 | DL 0.05 | 15.3 | 81.70 | 5.36b | 5.15b | 2473.7 | 1.48a | 101.7 |
| 3 | DL 0.1 | 15.3 | 87.05 | 5.71ab | 5.50ab | 2591.3 | 1.39ab | 101.7 |
| 4 | DL 0.2 | 14.8 | 85.65 | 5.80ab | 5.59ab | 2582.6 | 1.37ab | 98.3 |
| 5 | DL 0.3 | 15.0 | 89.85 | 5.99ab | 5.77ab | 2623.9 | 1.32ab | 100.0 |
| 6 | Micro 0.1 | 15.0 | 85.80 | 5.72ab | 5.51ab | 2580.9 | 1.39ab | 100.0 |
| 7 | Micro 0.2 | 14.8 | 87.65 | 5.94ab | 5.72ab | 2696.3 | 1.34ab | 98.3 |
| 8 | Micro 0.4 | 15.5 | 84.10 | 5.43b | 5.22b | 2467.0 | 1.46a | 103.3 |
| 9 | Micro 0.6 | 14.8 | 86.30 | 5.85ab | 5.64ab | 2665.0 | 1.35ab | 98.3 |
| 10 | Intact Protein | 15.0 | 93.85 | 6.26a | 6.04a | 2832.4 | 1.26b | 100.0 |
| P-value | | 0.3984 | 0.1303 | 0.0180 | 0.0169 | 0.7155 | 0.0193 | 0.3984 |
| PSE | | 0.2261 | 2.584 | 0.160 | 0.1584 | 129.98 | 0.0395 | 1.507 |

The last growth trial the basal diet (Table 3a) was similar to that used in other trials and would be expected to have a similar level of methionine. The first series of diets were formulated with increasing levels of corn protein concentrate (0, 5, 10, 15, 20 and 25%) as a substitute for soybean meal allowing for an incremental increase in dietary methionine. In the second set of diets, the basal diet was supplemented with high levels of DL-methionine (0, 0.1, 0.2, 0.4, 0.6%). These diets were then offered to juvenile shrimp (0.85g) over a 42-day growth trial. In this trial, the shrimp offered the diets supplemented with DL-methionine and the highest level of corn protein concentrate were significantly smaller than the shrimp offered the basal diet. Again, there was not trend in the data in terms of improved growth due to methionine supplementations whether it be from intact protein or DL-methionine. Once again there is no consistency to the data, which points to the basal diet not being deficient or marginally deficient at best.

Table 3a. Composition (g/100g as is) of test diets

| | **Basal** | **CP5** | **CP10** | **CP15** | **CP20** | **CP25** | **M-0.1** | **M-0.2** | **M-0.4** | **M-0.6** |
|---|---|---|---|---|---|---|---|---|---|---|
| Menhaden fishmeal | 4.50 | 4.50 | 4.50 | 4.50 | 4.50 | 4.50 | 4.50 | 4.50 | 4.50 | 4.50 |
| Soybean meal | 46.10 | 39.58 | 33.06 | 26.55 | 20.04 | 13.51 | 46.10 | 46.10 | 46.10 | 46.10 |
| Corn protein concentrate | | 5.00 | 10.00 | 15.00 | 20.00 | 25.00 | | | | |
| Gelatin | 5.00 | 4.00 | 3.00 | 2.00 | 1.00 | | 5.00 | 5.00 | 5.00 | 5.00 |
| Menhaden fish oil | 5.43 | 5.49 | 5.55 | 5.61 | 5.67 | 5.73 | 5.43 | 5.43 | 5.43 | 5.43 |
| Lecithin | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Cholesterol | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 |
| Corn Starch | 3.69 | 6.15 | 8.61 | 11.06 | 13.51 | 15.98 | 3.69 | 3.69 | 3.69 | 3.69 |
| Whole wheat | 27.00 | 27.00 | 27.00 | 27.00 | 27.00 | 27.00 | 27.00 | 27.00 | 27.00 | 27.00 |
| Mineral premix | 0.50 | 0.50 | 0.50 | 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 | 1.80 | 1.80 | 1.80 |
| Choline chloride | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Stay C | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| CaP-dibasic | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Arginine | | | 0.17 | 0.35 | 0.53 | 0.70 | | | | |
| Lysine | | | 0.07 | 0.24 | 0.42 | 0.59 | | | | |
| DL-Methionine | | | | | | | 0.10 | 0.20 | 0.40 | 0.60 |
| Threonine | 0.19 | 0.20 | 0.21 | 0.22 | 0.24 | 0.25 | 0.19 | 0.19 | 0.19 | 0.19 |
| Glutamic acid | 1.41 | 1.40 | 1.15 | 0.79 | 0.41 | 0.06 | 1.31 | 1.21 | 1.01 | 0.81 |

Table 3b Response of juvenile shrimp (0.85 g) to various test diets over a 42-day growth trial. Means of four replicate groups of shrimp is presented. Means with different superscripts within a column are significantly different based on Student-Newman-Kuels test. Means with a * indicates a significant difference in the response as compared to that of the basal diet.

| | Final Biomass (g) | Mean Weight (g) | Weight Gain (g) | Weight Gain (%) | FCR | Percent Survival |
|---|---|---|---|---|---|---|
| Basal | 53.8 | 5.98 | 5.14 | 611.3 | 1.92 | 90 |
| CP5 | 44.89 | 4.99 | 4.14 | 483.7 | 2.46 | 90 |
| CP10 | 42.87 | 4.72 | 3.89 | 470.3 | 2.58 | 90 |
| CP15 | 52.23 | 5.51 | 4.63 | 533.2 | 2.16 | 95 |
| CP20 | 46.49 | 5.41 | 4.59 | 565.4 | 2.17 | 87.5 |
| CP25 | 35.44* | 4.65* | 3.79* | 440.5* | 2.71 | 77.5 |
| M-0.1 | 41.33 | 4.44* | 3.61* | 432.1* | 2.75* | 92.5 |
| M-0.2 | 38.48 | 4.66* | 3.82* | 455.0* | 2.57* | 82.5 |
| M-0.4 | 45.78 | 5.72 | 4.86 | 566.2 | 2.04 | 80 |
| M-0.6 | 52.34 | 5.6 | 4.72 | 539.3 | 2.08 | 93.33 |
| P-value | 0.047 | 0.0162 | 0.017 | 0.0256 | 0.0330 | 0.5178 |
| PSE | 4.065 | 0.3218 | 0.3212 | 38.83 | 0.1955 | 6.165 |

## Conclusion

Research to date in our laboratory has demonstrated that the uptake of amino acids in shrimp corresponds to the digestive physiology of the animal, in that shrimp are semi-continuous feeders that process and digest foodstuffs very quickly. From the initiation of feeding, an upswing in amino acids in the hemolymph was apparent. The clearance of amino acids was also relatively fast with amino acid levels returning to overnight fasting levels within 60 minutes. This cycle of nutrient corresponds to the semi-continuous feeding habits of shrimp. Based on both absorption and clearance patterns of the amino acids, there was no indication of a synchronous absorption of supplemented amino acids.

Results from a series of growth trials conducted with a range of supplements as well as intact protein sources producing a range of dietary methionine levels yet consistent and persuasive data was not obtained. Across four independent growth trials, increasing the level of methionine as a pure supplement or using corn protein concentrate as an intact protein source, produced very little evidence of growth enhancement. Clearly, some of the issue is variation inherent in shrimp trials. This leads to three possible conclusions: 1) the basal diet is replete or only marginally deficient in methionine and TSAA, making the methionine supplementation ineffective and the identification of a deficiency more problematic, 2) another amino acid is first-limiting, thereby also rendering methionine supplementation ineffective, or 3) crystalline methionine does not work in shrimp feeds, though this is contradicted by the work conducted with lysine in which crystalline and intact lysine both produced a dietary response. Given the number of attempts as well as reports in the literature of poor responses to methionine levels once can only conclude that the deficiency is at or below that of our basal diets (0.54 methionine and 0.42% cysteine). This data does not correspond to reports in the literature which indicate a higher methionine requirement. It is this authors opinion, that we need a method and diet that will consistently and repeatedly induce a deficiency. Until this has been developed there will be no agreement on a dietary requirement.

Unfortunately, unless we can demonstrate a dose response to methionine (or other AA) levels, feed formulators are going to continue to over-formulate diets with fishmeal and will limit the use of soybean meal. Hence, we propose to take a step back and develop a test diet with crystalline amino acids to evaluate the ranking of limiting amino acids. This approach will provide the foundation for a systematic evaluation of EAA requirements for the Pacific white shrimp.

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