EFFICACY OF PURIFIED AMINO ACIDS IN PRACTICAL DIETS FOR PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

By

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ABSTRACT

Shrimp growth and nitrogen loading is influenced by the quantity and quality of protein supplied to the diet. Protein is also the main cost component of the diet, and, therefore, feed formulations need to be cost effective. Feed formulations are moving towards using less fishmeal in diets and more plant-based protein sources, which are cheaper and more widely available. However, plant protein sources have some disadvantages and one of the major problems are unbalanced amino acid profile. To meet an amino acid requirement, a combination of different ingredients may be used, or the diet may be supplemented with crystalline amino acids (CAA). Formulations now are "nutrient-based" as shrimp have a requirement for amino acids rather than for protein which makes the determination of essential amino acid requirements important. Studies determining amino acid requirements have been variable and opinions differ on whether CAA are utilizable by shrimp due to their slow feeding habits. Other possible causes of this variation are unclear but there could be some environmental factors or differences in experimental design limitations. Lysine and methionine are often the most limiting amino acids in plant-based diets. Methionine is an amino acid that is often supplemented in shrimp diets when soybean meal is the main protein source which is known to be deficient in sulphur containing amino acids. Reported methionine requirement is quite high and studies are variable and often not repeatable. Before a methionine requirement can be determined a suitable source that produces a response needs to be identified. Consequently, the objective of the first study was to determine the efficacy of CAA in diets of Pacific White Shrimp. For this experiment, a basal diet was designed to have 30% crude

protein (CP) and 6% lipid. The CP was then decreased gradually to reach 28, 26, 24 and 22% CP in the first series. In the second series of diets, CAA was supplemented back to each of the lower CP diets to reach 30% CP of the basal diet. Limited statistical differences were found but, as the CP content of the diet decreases the percentage weight gain (PWG) and mean final weight (MFW) was significantly affected. None of the diets supplemented with CAA seemed to produce the same performance as that of the 30% CP diet. A second study was then formulated to determine the effect of protein level on CAA supplementation. A fishmeal-based reference diet was formulated, as in theory, fishmeal should be replete in essential amino acids required by this species. Two soybean meal-based diets were then formulated to reach 30 and 35% CP and each supplemented with essential amino acids in one set and methionine in another to reach the same level as a percent protein as that of the fishmeal reference diet. In terms of PWG, no significant differences existed among the diets, but in terms of protein retention efficiency (PRE), the fishmeal diets produced significantly higher performance compared to most other treatments. The last study looked at the efficacy of different methionine sources when supplemented to a soybean meal basal diet. Methionine (Met) was supplemented to reach 0.60% (by using DL-Met, coated Met, peptide Met and intact protein Met) of the diet from a basal diet containing 0.45%. However, none of the supplemented sources significantly improved performance of the shrimp leading to the conclusion that methionine may actually not be deficient in a soybean diet for Pacific White Shrimp. There is a clear need to find suitable sources that can be used for determining essential amino acid requirements, which may then also be used for diet supplementation in the industry. As per overall observation during the present study, it was not clear whether purified amino acids are effective in the diets of Pacific White shrimp or if a practical diet designed to be low in methionine is actually deficient.It may be necessary to complete further research to determine what the cause may be for

the poor utilization of CAA. Poor utilization is likely due to leaching but other factors such as pH of the diet, absorption from the gut and palatability may also be considered.

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CHAPTER I

GENERAL INTRODUCTION

About 70% of the fish and crustacean species produced in aquaculture are being fed directly and an estimated 68% of these species are dependent on the manufacturing of aquaculture feeds (Tacon and Metian 2015). In 2015, shrimp aquaculture was estimated to be the third largest consumer (6.18 million tonnes) of manufactured aquaculture feeds (Tacon and Metian 2015). This could be as the industry for shrimp production has shifted from an extensive to a more intensive production system. This leads to less food being available from the environment making formulated diets more important as well as the quality thereof (Shiau 1998). The total global shrimp feed production has been estimated to increase to 12 million tonnes by 2020 (Tacon *et al.* 2012).

Pacific white shrimp has been one of the most important culture species in the Americas due to it having a high economic value, fast growth rate and being adaptable to a wide range of salinities and temperatures (Cuzon *et al.* 2004, Roy and Davis 2010, Bondad-Reantaso *et al.* 2012). There has also been a rapid increase in production of this species in countries like China, Thailand, Indonesia and Vietnam after it was introduced from Latin America (FAO 2011). Production has grown to the point that these countries are now the main producers of this species. Given that feed cost normally makes up 50-60% of the production cost of a shrimp farm, (Millamena *et al.* 1998, Roy *et al.* 2009) optimizing the feed is critical to all operations. Therefore, feed formulations should consider nutritional requirements as well as economic efficiencies.

Protein is one of the major costs in formulated feeds (Shiau 1998, Kureshy and Davis 2002, Sookying and Davis 2012). Not only is the quantity of the protein in the diet important but also the quality of the protein as it can have an influence on the nitrogen loading of the culture system and cost of the feed (Samocha *et al.* 2004, Richard *et al.* 2010). This makes it crucial to understand the nitrogen requirements of important culture species such as *Litopenaeus vannamei* and therefore various studies have focused on the protein requirement of shrimp (Davis and Arnold 2000).

Protein sources that are of animal origin are considered the most desirable (Amaya *et al.* 2007). Especially marine protein sources due to their high palatability and it being a reliable source of essential amino acids, fatty acids, vitamins and minerals (Samocha et al. 2004, Forster and Dominy 2006, Sookying and Davis 2012). There are concerns with using these ingredients in terms of limited supply, variability in quality, safety issues and the likely increase in feed cost. The supply of fishmeal has been constant but the demand for fishmeal keeps increasing which will lead to an increase in the cost thereof (Samocha *et al*. 2004, Nunes *et al.* 2014). In 2007, shrimp was estimated to be the largest consumers of fishmeal in aquafeeds (Tacon *et al*. 2012) and this high level of shrimp production worldwide places pressure on the demand for fishmeal (Fox *et al.* 2004). Not only will the price for fishmeal increase as the demand for it increases but fishmeal has also been known to be able to contain some diseases such as bovine spongiform encephalopathy and may also be contaminated with polychlorinated biphenyls (Fox *et al*. 2004). There is also competition for fishmeal as it is used in other animal feeds and cost for fishmeal can fluctuate due to varying supplies. Therefore, there has been various studies focusing on the shift from using animal proteins such as fishmeal in diets to using alternative protein sources such as various plant protein sources (Samocha *et al*. 2004, Amaya *et al*. 2007, Roy *et al.* 2009).

Although protein is important, animals have a requirement for amino acids that are the building blocks of protein which are needed constantly for maintenance and growth (Shiau 1998). The quality of the protein is dependent on the amino acid profile and this is variable between different protein sources and even within the same protein source (Shiau 1998). Consideration of amino acid requirements also allows for formulations that can be more cost effective, as there is increased precision in the delivery of nutrients (Millamena *et al.* 1998).

Various soybean meals have become a common plant ingredient used and researched as a replacement for fishmeal due to their low price and consistent quality (Samocha *et al*. 2004, Alvarez *et al.* 2007, Fox *et al.* 2011, Qiu *et al.* 2018). This alternative plant protein source has some anti-nutritional factors or may be deficient in one or more essential amino acids. Together, these are limitations that needs to be addressed as limited studies report complete replacement of fishmeal by plant proteins (Cuzon et al. 2004, Fox et al. 2004). This makes it increasingly important to have proper feed formulations to prevail these problems and this often results in feed formulations being supplemented with lipids, phosphorus and amino acids to meet the nutritional requirement of the animal (Amaya *et al*. 2007, Fox *et al*. 2011). Therefore, to properly formulate a feed it is necessary to know the amino acid requirement of the animals which makes studies determining the essential amino acid requirement of an animal critical.

To meet the amino acid requirements of an animal the total amount of protein supplied in the diet can be increased. In addition, a combination of different protein sources with different amino acid profiles may be mixed or purified amino acids can be supplemented (NRC 2011). Formulating feeds on an amino acid rather than a protein basis, allows for the correct amino acid balance to be obtained. More precise formulations result in reduction of the feed costs, improved conversion of protein into growth as well as less nitrogen loss to the environment (Millamena *et*

al. 1997). Balancing levels of amino acids is critical as amino acids that are supplied in excess of the requirement will be used for energy (Nunes *et al*. 2014). When a diet is deficient in one or more essential amino acids, protein synthesis will be restricted, resulting in reduced growth.

Essential amino acid (EAA) requirements for some shrimp species have been reported and it is evident that the same amino acids that are essential for fish are also essential for the growth of shrimp (NRC 2011). Essential amino acids include lysine, methionine, threonine, arginine, phenylalanine, histidine, tryptophan, leucine, isoleucine, and valine. The requirement for these 10 amino acids is consistent across all species and was determined for *Penaeus japonicus* by using a radioactive acetate (Kanazawa and Teshima 1981, Teshima *et al.* 2002). In addition to EAA there are a number of interactions of non-essential amino acids. Tyrosine is synthesized from phenylalanine and cysteine is synthesized from methionine and therefore these amino acids are considered as being semi-essential or conditionally essential (NRC 2011). Shrimp may also have a potential dietary requirement for taurine (Shiau and Chou 1994, Yue *et al.* 2013) which have been shown to be conditionally essential for some marine fish and shrimp species (NRC 2011).

Precisely determining the dietary requirements for EAA is critical for the improvement of feed formulations. Determining the essential amino acid requirement for shrimp and finfish are often done by using crystalline amino acids (CAA). However, the use of CAA in the diets of shrimp has been proven to be difficult especially when compared to fish culture due to the feeding habits of the species (Fox *et al.* 1995). Shrimp are external masticators and feed are often subjected to the water for a longer time, leading to possible leaching of the water soluble crystalline amino acids (Cuzon et al. 2004, Fox et al. 2011). There have been some controversy concerning the efficacy of CAA compared to the use of intact protein amino acids in shrimp diets with some

studies reporting CAA being utilizable by shrimp and others not (Teshima *et al.* 1992, Fox *et al.* 1995, Xie *et al.* 2012).

With regards to essential amino acids in shrimp, there are few quantitative studies that would be considered classical quantified dose responses with few being verified across multiple laboratories. There are some studies looking at the lysine (Fox *et al*. 1995, Millamena *et al.* 1998, Richard *et al*. 2010, Xie *et al*. 2012), methionine (Millamena *et al.* 1996b, Fox *et al.* 2010, Fox *et al.* 2011, Lin *et al.* 2015, Façanha *et al.* 2016), arginine (Chen *et al.* 1992, Millamena *et al*. 1998), threonine (Millamena et al. 1997, Zhou *et al.* 2013), valine (Millamena *et al.* 1996a) and histidine, isoleucine, leucine, phenylalanine and tryptophan (Millamena *et al.* 1999) requirement of shrimp. The determined requirement for these amino acids vary between these studies and therefore there are still controversy over amino acid requirement for these species.

Methionine has an importance in structure and synthesis of substances such as metabolites, neurotransmitters, hormones and also acts as a precursor for molecules such as glutathionine peroxidase (Façanha *et al*. 2016). Given the importance of methionine and pacific white shrimp it is interesting to note that there is almost no agreement for a dietary requirement. Information is limiting for this species and quality of the studies done is questionable. Often these studies report a high methionine requirement (Façanha *et al.* 2016) and the result is that these amino acids are included at high levels in the diets by manufacturers which drives the use of higher levels of fishmeal or dietary supplements in the diets (Fox *et al*. 2011). Over supplementation of any ingredient or supplement leads to failure of the objective of the aquaculture industry, which is to develop cost effective and efficient feed formulations (Fox *et al.* 2006).

There is considerable disagreement in terms of dietary requirements as well as the efficacy of CAA used in shrimp feed. Therefore, the objective of this research was to evaluate the efficacy of CAA and different sources of methionine in the diets of Pacific white shrimp.

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CHAPTER II

UTILIZATION OF CRYSTALLINE AMINO ACIDS BY PACIFIC WHITE SHRIMP, *Litopenaeus vannamei*

Abstract

In view of the variability in recommendations for essential amino acid (EAA) and the use of amino acids supplements in shrimp diets, this study was designed to evaluate the efficacy of crystalline amino acids (CAA) in practical shrimp feeds. The basal diet was designed to contain 30% protein and 6% lipid. The primary protein source of the basal diet included: 5% fishmeal, 37% soybean meal, and 5% gelatine. The intact protein was incrementally reduced to produce diets with 28, 26, 24, and 22% protein. In a second series of diets, CAA were supplemented to the reduced protein diets to return the diets to 30% crude protein. Thus, producing a series of diets containing 30% crude protein with 2, 4, 6, and 8% CAA. The trial was conducted twice to confirm results. Replicates within treatments for both trials were variable, resulting in limited statistical differences. Intact crude protein significantly influenced, percentage weight gain (PWG) and mean final weight (MFW) and these parameters decreased with intact protein level of the diet. None of the diets with supplemented CAA appeared to have reached the same MFW or PWG as the basal diet (R^2-value (PWG), trial 1: 0.38, trial 2: 0.34, R^2-value, (MFW) trial 1: 0.44, trial 2: 0.38). Another trial was run using a fishmeal-based diet (30% CP), which in theory should be replete in all essential amino acids, and a soybean meal-based diet at two different protein levels (30 and 35% CP). The soybean meal-based diets were supplemented with essential CAA in one set and

only methionine in another to reach the same level as a percent protein, compared to a fishmeal-based diet. No significant differences were observed among the diets in terms of PWG. However, the fishmeal-based diet outperformed most of the other diets in terms of protein retention efficiency. Based on the results of these trials, it is questionable whether CAA are sufficiently utilized by shrimp.

KEYWORDS: crystalline amino acids, *Litopenaeus vannamei*, soybean meal, growth performance

1. Introduction

The quantity and quality of protein supplied in the diet has a major influence on shrimp growth as well as nitrogen loading and the cost of feed (Davis and Arnold 2000, Samocha *et al.* 2004, Zhou *et al*. 2013). Protein is also one of the main cost components of the diet and should be optimized. Less expensive protein sources are increasingly being used as alternatives for more expensive protein sources such as fishmeal to decrease the cost and improve the sustainability of the feed (Bureau and Encarnação 2006, Davis and Sookying 2009). This however increases the likelihood of amino acid deficiencies (Cuzon *et al*. 2004). Therefore, essential amino acid (EAA) requirements for popular aquaculture species such as the Pacific white shrimp (*Litopenaeus vannamei*) must be defined.

As fish and shrimp have a requirement for amino acids rather than protein (Masagounder *et al.* 2011), diet formulations should be developed based on amino acid requirements. Amino acids play a key role in both the structure and metabolism of living organisms (Zhou *et al*. 2013). Essential amino acid deficiencies can result in reduced growth and poor survival. In favor of improving the amino acid composition of the diet, a combination of different ingredients can be used, or the diet can be supplemented with synthetic amino acids.

Pacific white shrimp is an important aquaculture species as it is tolerant to a wide range of salinities, temperature, and has a good growth rate and is the dominant culture shrimp (Cuzon *et al.* 2004, Bondad-Reantaso *et al*. 2012). Few quality studies define EAA requirements of Pacific White shrimp (*L. vannamei*) and to date few studies confirm these requirements. It seems that results among studies determining amino acid requirements or the efficacy thereof are not repeatable and quite often conclusions are based on inappropriate statistical analysis such as regression on the means. Using crystalline amino acids (CAA) to determine the EAA requirement for finfish has been more successful due to quick consumption. However, for shrimp, it has been proven to be more difficult (Fox *et al*. 1995, Teshima *et al*. 2002). This is possibly due to slow feeding and external mastication of the feed which may result in leaching of purified water-soluble ingredients from the feed prior to consumption. Based on previous research, it is evident that the same ten amino acids that are essential for fish are essential for shrimp (Cowey and Forster 1971, Coloso and Cruz 1980, NRC 2011). These EAA are lysine, methionine, threonine, arginine, phenylalanine, histidine, tryptophan, leucine, isoleucine, and valine. Tyrosine, cysteine and taurine are conditionally or semi-essential amino acids (NRC 2011, Yue *et al*. 2013).

Several studies for shrimp have investigated the requirement of lysine (Fox *et al*. 1995, Millamena *et al.* 1998, Richard *et al*. 2010, Xie *et al*. 2012), methionine (Millamena *et al*. 1996b, Fox *et al.* 2010, Fox *et al*. 2011, Lin *et al*. 2015, Façanha *et al*. 2016), and other amino acids (Chen *et al*. 1992, Millamena *et al*. 1996a, Millamena *et al*. 1997, Teshima *et al.* 2002, Zhou *et al.* 2013). However, still controversy remains over the requirement and use of CAA by these species (Davis and Duan 2017). Given the need to precisely define requirements and confirm the use of supplements, the objective of this study was to determine the efficacy of CAA in diets of Pacific

white shrimp. The primary goal was to bring some clarification to the uncertainty of whether intact protein amino acids and CAA provide the same performance to shrimp.

2. Materials and methods

2.1 Experimental diets

To evaluate the efficacy of synthetic or CAA supplements, a series of 9 diets were designed (Table 1). The basal diet was formulated to contain 30% protein and 6% lipid using fishmeal, soybean meal, and gelatin as the primary protein sources. Intact protein was gradually reduced by decreasing all three of the protein sources to produce diets with 28, 26, 24, and 22% protein. However, the three protein sources remained in the same ratio toward each other in all the diets. Lipid levels were kept the same in all diets. In a parallel series of diets, CAAs were supplemented to the reduced protein diets to return the diets to 30% crude protein. Thus, producing a series of diets containing 30% crude protein (CP) with 2, 4, 6, and 8% CAAs. Proximate and amino acid (AA) composition of these diets are presented in Table 2. To confirm the results of the trial, the study was repeated using a second trial.

To evaluate the potential effect of dietary protein level on the response of CAA supplementation, a total of seven diets were formulated (Table 3). This included a fishmeal-based reference diet which was assumed to be replete in its amino acid profile and it contained 30% CP with 16.50% fishmeal as one of the primary protein sources. Two soy-based basal diets, potentially deficient in EAA were formulated to contain 30% and 35% CP. To each of these basal diets, the EAA profile was rebuilt to either mimic that of the fishmeal-based reference diet or to simply

return methionine and threonine, the most likely limiting amino acids, to that of the reference diet. These diets were used in trial 3.

Diets were made using standard laboratory procedures. A food mixer (Hobart Corporation, Troy, OH, USA) was used to fully mix the pre-ground dry ingredients. The oil was then added and left in the mixer until properly mixed. Nearly boiling water was then added to the diet to reach proper consistency needed for pelleting. A meat grinder with a 3-mm die was used to make pellets under pressure. The diets were then air dried at ≤ 50°C to reach a moisture content of 7.5 to 13% in an oven while using a fan for ventilation. Pellets were stored in plastic bags in a freezer after they were crumbled and sieved to a uniform size. Proximate composition (g $100g^{-1}$ sample "as-is") and amino acid profile (g $100g^{-1}$ sample "as-is") of the diets were analyzed at the University of Missouri Agricultural Experimental Station Chemical Laboratories (Columbia, MO, USA) and this can be seen in Table 2 for the diets of trials 1-2 and in Tables 4 for the diets of trial three.

2.2 Growth trial

For the first set of diets, two growth trials were conducted. Growth trial 1 consisted of nine treatments and four replicates per treatment and was run at the Auburn University E. W. Shell Fisheries Station, Auburn, AL. Five replicates per treatment were used for the second trial, which was run at Claude Peteet Mariculture Center in Gulf Shores, AL; in this trial the basal diet had only four replicates per treatment. Trial 3, using the second set of diets, was also run at the Claude Peteet Mariculture Center in Gulf Shores and had six replicates per treatment. A semi-closed recirculation system was used for running all trials; however, the system is in an environmentally-controlled building at Auburn University; whereas, the trials were run in a greenhouse in Gulf

Shores. Shrimp were reared in a nursery and fed a commercial diet (Zeigler PL Raceway Plus, 50% CP and 15% Crude fat, and later Shrimp starter diet, 55% CP and 15% Crude fat) until they were of a suitable size for stocking.

At stocking, juvenile shrimp were hand-sorted to a uniform size and then stocked at similar biomasses in each tank. The shrimp were stocked into 36 tanks at an initial weight of 0.22 – 0.27 g at 10 shrimp per 130-L aquarium at Auburn University. At Gulf Shores, 15 shrimp per 160-L tanks was stocked into 44 tanks at an initial weight of 0.28 – 0.32 g for trial 2. Shrimp for trial 3 using the second set of diets were stocked into 42 tanks at 15 shrimp per tank. Shrimp were not weighed at regular intervals as they are difficult to handle but they were counted weekly at Auburn University and every second week in Gulf Shores to adjust the feed input. A feed conversion ratio of 1.8 and a predicted growth of 0.25, 0.5, 0.8, 0.9, 1.1, 1.2 and 1.3 (for the seven weeks) was used at Auburn University, while 0.3, 0.6, for the first two weeks followed by 0.8 for the rest of the weeks was used in Gulf Shores for determining feed amounts fed per tank. This led to 0.64g being fed in the first week, 1.29g in the second week, 2.06g in the third week, 2.31g in the fourth week, 2.83g in the fifth week, 3.09g in the sixth week and 3.34 g in the seventh week for the first trial. For trial 2, 1.2g was fed in the first week, 2.3g in the second week, and 3.09g for all the following weeks until the trial was terminated after six weeks. Trial 3 was fed in the same way as trial 2 with 0.85g being fed the first week, 1.7g the second week and 3.09g the following weeks until termination. Trial 3 was run for eight weeks. At termination of each growth trial, the shrimp were counted and group weighed. Mean percentage weight gain (PWG), mean final weight (MFW), feed conversion ratio (FCR), and survival (%) were calculated.

Whole body protein was determined for shrimp in the third growth trial to allow the determination of protein retention efficiency (PRE). Protein was determined using Dumas method

with a Rapid N cube machine (Elementar Americas Inc. Mt. Laurel, NJ). The shrimp were dried in an oven at 95°C until constant weight after which they were ground. Pellets (two sample pellets from each tank) were made by a pellet press after 250 mg of sample was weighed into a small foil leaflet and this was balled up to be placed in the pellet press. Six aspartic acid pellets were also made for calibration of the Rapid N cube machine. After this preparation, the pellets were placed in the machine for crude protein determination of the sample (6.25 × N content).

2.3 Water quality

Water quality parameters, including dissolved oxygen, temperature, and salinity were measured twice daily using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, Ohio). pH was measured two times per week for trial one using pHTestr30 (Oakton Instrument, Vernon Hills, IL, USA) and twice daily for trials 2 and 3 by using the YSI 650 multi-parameter instrument. These measurements were done in the sump tank for the trial performed at Auburn University but was measured in the culture tank closest to the sump and the culture tank farthest away from the sump in the trials performed at Gulf Shores. Water samples were taken two times per week and total ammonia nitrogen (TAN) and nitrite was determined by using methods that are described by Solorzano (1969) and Spotte (1979), respectively, for trial on1. For trial 2 and 3, TAN was measured using an ion-selective electrode (Orion 4-star Plus pH/ISE, Thermo Fischer Scientific. Waltham, MA, USA). Nitrate and nitrite for trial 2 and 3 were measured by a colorimetric test kit (La Motte Chemicals, Chestertown, MD, USA). Water samples for these measurements was taken in the sump tank and frozen until analysis at Auburn University but analyzed the same day at Gulf Shores. All water quality data can be seen in table 5.

2.4 Calculations and Statistical analysis

Feed conversion ratio was calculated by feed offered/ (final weight-initial weight), while weight gain was calculated by (final weight-initial weight)/initial weight × 100%. Protein retention (%) was calculated by (final weight × final protein content) – (initial weight × initial protein content) × 100/protein offered.

Data were analysed using SAS (V9.3. SAS institute, Cary, NC, USA) and data for the growth trials for the first set of diets were analysed by using ANCOVA. This was done to determine if there were any significant differences among the treatments. For the second set of diets, one-way ANOVA as well as ANCOVA were used. Multi-comparison test, such as the Dunnet test for comparing all diets to the reference fishmeal diet and Tukey HSD, was used to identify differences among means. Survival data was analysed by using one-way ANOVA, ANCOVA as well as binomial but it did not make a difference in the significance.

3. Results

Growth performance of Pacific White Shrimp offered experimental diets from trial 1 and the repeated trial 2 are presented in table 6. Final mean weight (FMW) and percentage weight gain (PWG) were not significantly affected by supplementation of CAA ($P < 0.05$). As the CP content of the diet decreased, PWG, FMW and FCR and, therefore, the performance of the shrimp was significantly affected ($P < 0.05$) which is shown in Table 6 for both trial 1 and 2. Regression analysis showed an R^2-value for PWG in trial 1 of 0.30 ($y = 7.9392x^2 – 329.91x + 5140.9$) and in trial 2 of 0.34 ($y = -6.6237x^2 – 348.42x + 3059.6$). R^2-value for FMW in trial 1 was 0.45 ($y = 0.0084x^2 – 0.3285x + 7.8662$) and in trial 2 was 0.38 ($y = 0.0259x^2 – 1.231x + 19.069$). Regression results are shown in figure 1 and 2. Instead of the CAA supplemented diets giving the same

performance in terms of FMW and PWG as the 30% CP basal diet, the performance of the shrimp in these diets seemed to follow the intact protein content. None of the diets with supplemented CAA appeared to have reached the same FMW or PWG as the basal diet. This can be seen for PWG in Fig 1 (trial 1) and 2 (Trial 2). FMW and PWG was the highest for the 30% CP diet in trial 1 and 2 (FMW, trial 1: 5.46 g and trial 2: 5.56 g and PWG, trial 1: 2156% and trial 2: 1790%). The 22 + 8% showed the lowest PWG in trial 1 (1736%) but the diet showing the lowest FMW in trial 1 was the 24 + 6% diet (4.60 g). In trial 2 the 22% diet showed the lowest PWG and FMW (1355% and 4.41 g, respectively).

Poor FCR (2.13-2.55) was observed for trial 1 as shown in table 6. Whereas, trial 2 showed to have better FCR (1.41-1.80). The lowest FCR was observed for the 30% CP diet in both trials with trial 1 having a FCR of 2.13 and trial 2 of 1.41. Mean survival ranged from 82-97% in trial 1 and 92-97% in trial 2 but no significant differences ($P<0.05$) were observed in mean survival for either of the trials.

The third growth trial using the second set of diets was to determine whether an increased protein content will influence the efficacy of CAA supplementation in shrimp diets. Results for this trial can be seen in Table 7. Although some of the higher protein diets showed significantly better performance in terms of MFW and FCR than those of the diets with the lower protein content, no significant improvements ($P < 0.05$) in performance were shown in the diets supplemented with CAA as compared to the basal diet not supplemented with CAA. There were no significant differences ($P < 0.05$) for FMW, PWG or FCR among any of the 35% CP soy-based diets and the fishmeal diet. The 30% CP soy-based diet supplemented with all essential amino acids showed significantly lower ($P < 0.05$) FMW (6.61 g) and significantly higher ($P < 0.05$) FCR (1.54) compared to the fishmeal reference diet. The higher protein diets showed the same

performance as the reference fishmeal diet with no significant difference ($P < 0.05$) observed. The fishmeal reference diet showed the highest PRE (43.72%) and was significantly higher ($P < 0.05$) than some of the soy-based diets. The 30% CP soy-based diets supplemented with methionine and supplemented with all essential amino acids showed the lowest PRE (36.05 and 36.14%, respectively). The soy-based diets, however, had no significant differences ($P < 0.05$) for PRE between higher or lower crude protein content for supplementation of CAA or no supplementation. Survival for this trial was good and ranged from 88 – 97%.

4. Discussion

To produce an economical and nutritious feed for Pacific White shrimp, feed formulations must be continually improved. Protein is the most expensive components of the diet and feed formulators are moving towards including cheaper protein sources such as plant-based proteins and, therefore, moving away from the more expensive animal proteins like fishmeal. However, a higher inclusion of plant proteins causes some other issues, such as an unbalanced amino acid profile that needs to be addressed (Bulbul *et al.* 2015). Numerous studies have worked on determining an optimal CP requirement, but shrimp have a requirement for amino acids rather than protein. Thus, formulation of feed now pay more attention to the total level of EAA included in the diet and/or digestible EAA, which is known as "nutrient-based formulation" compared to "ingredient-based" formulation (Nunes *et al*. 2014). Supplementation of CAA to determine and meet the amino acid requirement of shrimp is increasingly important as the industry is moving towards diets with lower levels of fishmeal and more plant-based protein sources. However, some areas of the aquaculture sector have been slow to adopt utilizing CAA in formulated diets due to concerns of how efficiently these CAA are being utilized by the cultured animal (NRC 2011).

With regards to the published literature, EAA requirement determination with different shrimp species vary widely when using CAA. Due to these differences in amino acid requirements found among studies and the difficulties observed in some of these studies, it has raised the question of the efficacy of CAA in the diets of shrimp. An example is the variation of lysine requirement which was determined to be 4.93% (Xie *et al*. 2012), and 5.19% of CP for *P. vannamei* (Fox *et al*. 1995), 5.2% (Millamena *et al*. 1998) and 5.8% (Richard *et al*. 2010) of the CP for *P. monodon*, and 3.2-4.0% of the CP for *M. japonicus* (Teshima *et al*. 2002). Similarly, requirement levels for methionine has been estimated to be 1.3-1.6% of CP based on whole body protein for *M. japonicus* (Teshima *et al*. 2002), 2.9% (Richard *et al*. 2010) and 2.4% of the CP content for *P. monodon* (Millamena *et al*. 1996b). A basal diet containing wheat at 48.59% and soybean meal at 29.30% with a methionine content of 1.42% of the CP (0.44% of the diet) was reported to be deficient when compared to the pooled data when L-methionine, DL-methionine and 2-hydroxy-4-methylthio butanoic acid (HMTBA) was supplemented to the diet to reach 2.95-3.05% of the CP or 0.90-0.92% of the diet (Forster and Dominy 2006). However, the one-way ANOVA did not show any significant differences in MFW, growth and survival among the treatments, and, therefore, the basal diet may not have been deficient in methionine. Façanha *et al*. (2016) looked at methionine requirement of Pacific White shrimp reared under a green water system at three different densities (50, 75, 100 shrimp/m^2). Diets were formulated with a methionine content to vary from 0.48–0.94% of the diet. At two of the densities (50 and 75 shrimp/m^2), they did not observe a typical dose response and at the third density they observed a linear increase with no plateau in performance. They concluded that a dose-response was only observed at the higher density due to increased requirement for methionine and less nutrients available from the green

water as there is higher pressure on natural food sources. However, further investigation may be required to better answer why there has not been a typical dose-response at the lower densities.

Determining the amino acid requirement is considered easier in fish as compared to shrimp. Yet for certain fish species two to three fold differences in amino acid requirements have been observed (Bureau and Encarnação 2006). Differences between these fish studies were ascribed to differences in experimental design, fish size, diets, protocol and culture methods used (Bureau and Encarnação 2006). This could also explain the differences seen for EAA requirements among shrimp studies. It may also be considered that the statistical models selected, e.g. broken line regression, is not the most appropriate model for determining amino acid requirements and better consideration of the appropriate model may be required (Bureau and Encarnação 2006).

Limited data are available on EAA requirements for this species and the variation of requirement between studies as well as the differences in opinion of efficiency of CAA utilization by shrimp between different authors. Consequently, the objective of this research was to determine the efficacy of CAA in Pacific White shrimp diets. All the above studies have led to questions and concerns in terms of the efficacy of CAA in shrimp diets. Therefore, a 37.50% soybean meal, 5% gelatin and 5% fishmeal basal diet was developed that reached 30%CP. Decreasing the CP content gradually reaching 28, 26, 24, and 22% in the first series of diets while keeping the protein sources in the same ratio towards each other allowed us to supplement EAA in the form of CAA to reach the same AA levels as our 30% CP basal diet. All the supplemented diets are therefore expected to reach the same growth performance as that of the 30% CP basal diet if CAA are efficiently used by shrimp. However, in terms of shrimp growth and nutrient retention, the results indicated that there is no clear evidence that shrimp used CAAs. For trials one and two PWG and FMW decreased as the intact protein decreased and the supplementation of CAA did not improve the growth to the

same level as that of the 30% CP diet. PWG ranged from 1355% to 2156%, which should be enough to induce a deficiency in growth by the decrease in CP content and observe whether there is a response to CAA supplementation. Some studies have significant responses and deficiencies with lower growth performances having PWG as low as 66 – 100% starting with an initial bodyweight of 1.75g (Bulbul *et al*. 2015) and 84 – 151% with an initial bodyweight of 0.65g (Alam *et al.* 2004) for Kuruma shrimp where both studies ran for eight weeks. Pacific White shrimp in other studies had a PWG of 220 – 355% with an initial weight of 3 g (Niu *et al.* 2018), 516 – 683% with an initial weight of 0.4 g (Fox *et al*. 2010) and 1839 – 2147% with an initial weight of 0.52 g and ran for eight weeks (Xie *et al*. 2012) with all studies finding reportable responses. A study done with *Peneaeus monodon* reported a PWG of 293 – 560% with an initial weight of 0.05 g, using six replicates per treatment, and the trial run for 8 weeks which was sufficient for determining a threonine requirement (Millamena *et al*. 1997).

However, the variability within treatments for data in trials 1 and 2 resulted in poor regression coefficients making it difficult to draw clear conclusions. Regression coefficients for these results were R^2 = 0.39 and 0.34 for PWG looking at CAA supplementation for trials 1 and 2. Compared to Millamena *et al.* (1998) determining the lysine requirement, they observed a coefficient of 0.47 and when determining an arginine requirement, they observed a coefficient of 0.41 and was still able to determine a requirement. Millamena *et al*. (1996b) determined a methionine requirement while having a regression coefficient of 0.37. Both studies looked at *Penaeus monodon*. Therefore, this variation may occur across other studies also. PSE for our first trial was 0.16 for MFW, 0.36 for FCR, 3.92 for survival and 162 for PWG. Trial 2 had a PSE of 0.31 for MFW, 110.69 for PWG, 1.39 for survival and 0.11 for FCR. MFW and survival were very similar to those reported by Zhou *et al.* (2016) which was 0.22 and 5.35, respectively. However,

PWG and FCR were lower for their study, being 0.05 and 42.05, respectively. MFW had a PSE of 0.26 for Pacific White shrimp which is very similar to ours (Forster and Dominy 2006) and PSE for Pacific White shrimp in a methionine study was reported to be 0.12 for MFW and 80.6 for PWG by (Fox *et al*. 2011).

In terms of the differences between the use of CAA and intact protein amino acids there have been some studies. In a study by Xie *et al*. (2012), supplementing crystal L-lysine by increasing incrementally to shrimp diets, they concluded that shrimp were able to utilize crystalline L-lysine even though there was no diet without crystalline EAA or an intact protein reference. Similarly, other studies looking at an amino acid requirement which have an EAA mix supplemented to all experimental diets (with the testing amino acid being dropped and added in incremental quantities) and, therefore, there is no way of comparing the performance among diets where CAA have and have not been supplemented (Millamena *et al*. 1996b, Zhou *et al*. 2013, Xie *et al*. 2012, Millamena *et al*. 1998). The lysine requirement for Pacific White shrimp was determined by Fox *et al*. (1995). When lysine enriched wheat gluten (covalently bound lysine) was used in the diet, lysine requirement was estimated to be 4.49 ± 0.08 % of the dietary protein and 1.54 ± 1.60% of the diet. When lysine was supplied in the form of CAA, lysine requirement was estimated to be 5.19 ± 0.20 % of the protein and 1.75 ± 1.89% of the diet. They concluded that the apparent requirement of lysine as determined by CAA and covalently bound lysine was very similar (Fox *et al.* 1995) albeit the use of CAA resulted in a requirement that was 12% higher. They also reported that diets containing covalent supplemented lysine supported significantly higher individual growth rates of the shrimp as compared to diets containing crystalline lysine. Another study concluded that the crystalline lysine and methionine are effective supplements in the diets of Kuruma shrimp (Bulbul *et al.* 2015). In this trial a plant-based diet was improved by

the addition of lysine and methionine. The simple addition of fish solubles resulted in a better increase in final weight of the shrimp indicating this may be a response to consumption. It should be noted that weight gain ranged from 64 to 95%, which indicated very little tissue replacement to induce a deficiency.

In terms of the effect that CAAs have on the performance of fish, the vast majority of studies have found positive results. For example, studies conducted on Nile tilapia and channel catfish (Nguyen and Davis 2016), rainbow trout (Murai *et al.* 1987, Rodehutscord *et al.* 1995), Gibel carp (Hu *et al.* 2008) and Asian seabass (Williams *et al.* 2001), amino acids supplied as an intact source or in the crystalline amino acid form did not seem to affect performance of fish. On the other hand, there has been some studies that concluded that free or CAA are not used as effectively as intact protein amino acids in fish diets for catfish (Andrews *et al.* 1977, Zarate and Lovell 1997, Zarate *et al.* 1999) and carp (Yamada 1981, Nose *et al.* 1974), which compares to our results.

There may be a few reasons worth investigating as a possible cause for poor utilization of CAA in shrimp diets. These include leaching, decrease in diet pH, a problem with the palatability of the diet, a difference in the absorption between free and intact protein amino acids or whether the level of protein has an influence on the efficacy of amino acid supplementation. Leaching could be one of the possible causes for poor utilization of this amino acid source due to the slow feeding habits of shrimp and CAA being water soluble (Cuzon *et al*. 2004). Fox *et al.* (2011) found that poly-methionine and chelated methionine supplemented diets showed significantly lower methionine leaching loss when compared to a DL-methionine diet. However, they did state that loss as a percentage of the diet was low indicating that all the above amino acids supplements could be used. Alam *et al.* (2004, 2005) conducted studies to determine which binder and amount

of binder to use in kuruma shrimp diets. They concluded that coating crystalline methionine and lysine with carboxymethylcellulose or agar showed significantly higher results as compared to the basal diet without any supplementation or a diet with supplementation but no coating. Although improved growth or decreased leaching were found, the studies showed poor overall growth and survival. In our studies, gelatin was used in the first series of diets for trial 1 and 2 to improve binding and reduce leaching. In the second series of diets used in trial 3, Tic gum which is also hydroxypropyl methylcellulose, had been added to the diet for further binding in addition to the gelatin. This was to prevent further leaching of essential amino acids; yet in both cases there was no indication that the supplementation of CAA resulted in any benefits in terms of increased shrimp growth rates.

It was suggested that protein intake may be limiting a response; so, the following study evaluated both a 30% and 35% CP protein diet. Overall, the level of protein did not influence the response to CAA supplementation. The response in terms of growth may not be the best indicator of nutrient utilization, and protein retention may be a better measurement compared to final weight as protein retention is the main determinant of amino acid requirement (Bureau and Encarnação 2006). No significant difference was observed between protein retention comparing the 30 and 35% CP diets or the diets supplemented with CAA. The fishmeal diet did, however, show the highest protein retention compared to those of the other diets. This could be explained by fishmeal having a good amino acid balance or higher intake due to better palatability. However even though the basal diet was supplemented to have the same amino acids as a percentage of protein as the reference diet, none of the supplemented diets showed the same performance as that of the fishmeal diet. Similar to our results, a study on red drum where basal diets at 35 and 45% CP content were supplemented with a mixture of amino acids, the higher protein diet produced significantly higher

growth but there was no difference in performance whether amino acids were supplemented or not (Webb and Gatlin 2003). Richard *et al*. (2010) found that a high CP diet of 50% had significantly higher nitrogen gain as compared to a 15% CP diet. This difference between diet CP content is, however, high and, therefore, a difference would be expected. The 50% CP diet also showed significantly higher nitrogen gain when compared to the 34% CP diet. This study also found a significant interaction between the protein content and methionine level which differs from our study where no significant interaction was found. In a study done on Barramundi/Asian seabass where CAA was supplemented at a high protein level, it was found that amino acids from intact protein sources were twice as effective in improving FCR as compared to CAAs (Williams *et al*. 2001). On the other hand, at a lower protein level, CAA was utilized as effectively as compared to intact protein sources when used to supplement for meeting the essential amino acid requirements. In our study, even the lower CP content (30%) showed no improvement in performance when supplemented with CAA.

The pH of the diet may also be worth studying as a possible effect on shrimp performance in diets supplemented with CAA. Diets supplemented with CAA for *P. vannamei* had inferior growth and FCR when compared to a 28% shrimp protein control diet (Lim 1993). However, when the CAA diet was adjusted in pH to reach 8, they were able to enhance the growth results. The pH of the 30% intact CP diet in our study was found to be 5.82 and the pH of the 22+8% CP diet was found to be 4.80. Therefore, there was a pH unit change between the diet with no CAA supplementation and the diet with the highest level of CAA supplementation. Whether this was the cause for the deficient performance of diets supplemented with CAA is not clear. However, even the best performing diet in our study did not have a pH as high as 8 which was reported to give the best results as reported by (Lim 1993). Unpublished data in our laboratory has looked at

the direct addition of acid to shrimp diets as well as the use of hydrolysates both resulting in reduced pH of the diets with little effect on performance. Although pH cannot be ruled out, based on our experience it is unlikely the cause of poor performance.

Some studies have also looked at the absorption of free amino acids compared to amino acids obtained from intact dietary protein sources as this has also been theorized to be one of the probable causes for inefficient use of CAAs (Nunes *et al*. 2014). Purified amino acids may be absorbed quicker than those of intact protein amino acids (Schuhmacher *et al.* 1997, Ambardekar *et al.* 2009) or leave the stomach more rapidly than intact protein amino acids (Zarate *et al*. 1999). This difference could cause problems with nitrogen retention as amino acids supplemented are normally those needed for improving the rate of protein synthesis. These purified amino acids could end up as excess amino acids that are instead being catabolized. In a study done on rainbow trout, no differences were found between the rate of absorption of free amino acids compared to the absorption of intact protein amino acids (Murai *et al*. 1987). A study done at Auburn University on Pacific White shrimp also showed no asynchronous absorption with intact or free amino acids (Davis and Duan 2017). Another study where intact protein and the corresponding CAAs were administered orally in cannulated shrimp, the free amino acid concentration in the urine of the shrimp fed CAAs were significantly higher than those fed intact protein amino acids (Liou *et al.* 2005). They concluded that a diet containing free amino acids may result in a concentration of amino acids in the hemolymph that exceed maximum reabsorption concentration. This, however, leads to questions other than leaching being a problem of whether CAAs can be used by shrimp once ingested.

There has also been concern that CAA may be over supplemented and could lead to toxicity and depressed growth. When determining the lysine and arginine requirement of *P. monodon*, a

pronounced decrease in weight gain following an increase in weight gain as the amino acid supplied in the diet was increased was observed (Millamena *et al*. 1998). The author stated that it was unclear why this was happening but that it might be due to an imbalance when amino acids are supplied at a higher level than required. They observed the same trend in their study determining the methionine requirement (Millamena *et al*. 1996b). Zhou *et al*. (2013) reported an increase growth response in Pacific White shrimp as threonine was supplemented to the diets. Zhou *et al*. (2013) found that a 4.5:1 ratio of intact protein to protein from CAA can support adequate growth of shrimp; however, no comparison to that of an intact protein diet without supplementation was made. In our study, the diets containing 2% and 4% CAA had higher ratios of intact protein to protein from CAA (14:1 and 6.5:1, respectively) and none of these diets provided the same performance as those of the all intact protein diets although performance was not significantly less.

5. Conclusion

From the studies done it cannot be concluded whether CAAs are being used by shrimp or not. One of the possible reasons making a proper conclusion of the study difficult is the variation among replicates within treatments. The reason for the variation is unknown but is likely due to the quality or genetics of the PLs as similar problems were found across studies. Poor stability of the feed and leaching of amino acids could be another probable reason for variation within and between studies. Determining what conditions or factors that differ between the studiers and culture systems and, therefore, influence the difference between estimating the requirement of EAA for shrimp could be something that needs to be studied or better understood.

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Table 1 Diet formulations and chemical composition of test diets (g/100 g "as-is") used in trial 1 and 2

Ingredients	30%	28%	26%	24%	22%	28+2%	26+4%	24+6%	22+8%
Menhaden fishmeal[a]	5.00	4.59	4.19	3.79	3.39	4.59	4.19	3.79	3.39
Soybean meal[b]	37.50	34.39	31.39	28.39	25.39	34.39	31.39	28.39	25.39
Gelatin[c]	5.00	4.59	4.19	3.79	3.39	4.59	4.19	3.79	3.39
Menhaden fish oil[a]	3.42	3.54	3.65	3.76	3.87	3.54	3.65	3.76	3.87
Corn starch[e]	5.38	9.20	12.89	16.58	20.27	7.07	8.69	10.32	11.96
Whole wheat[f]	37.00	37.00	37.00	37.00	37.00	37.00	37.00	37.00	37.00
Lechitin[g]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol[e]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Mineral premix[h]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[i]	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride[k]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Stay-C (35% active)[j]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Ca-P dibasic[k]	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Arginine[k]	-	-	-	-	-	0.15	0.29	0.43	0.58
Histidine[k]	-	-	-	-	-	0.05	0.09	0.14	0.19
Isoleucine[e]	-	-	-	-	-	0.09	0.17	0.25	0.33
Leucine[e]	-	-	-	-	-	0.15	0.28	0.42	0.56
Lysine (76.6%)[o]	-	-	-	-	-	0.16	0.32	0.48	0.64
Methionine[m]	-	-	-	-	-	0.03	0.06	0.09	0.12
Phenylalanine[k]	-	-	-	-	-	0.09	0.18	0.26	0.35
Threonine[l]	-	-	-	-	-	0.08	0.15	0.22	0.29
Tryptophan[e]	-	-	-	-	-	0.02	0.05	0.07	0.10
Valine[n]	-	-	-	-	-	0.09	0.19	0.28	0.37
Glutamic acid[m]	-	-	-	-	-	0.61	1.21	1.81	2.39
Glycine[k]	-	-	-	-	-	0.61	1.21	1.81	2.39

[a]Omega Protein Inc., Houston, TX, USA.

[b]De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.

[c]Rousselot Inc., Mukwonago, WI, USA.

[e]MP Biomedicals Inc., Solon, OH, USA.

[f]Bob's red mill, Milwaukie, OR, USA.

[g]The Solae Company, St. Louis, MO, USA.

[h]Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.

[i]Vitamin premix (g/kg premix): Thiamin HCl, 4.95; Riboflavin, 3.83; Pyridoxine HCl, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.

[j]Stay-C® (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA.

[k]VWR Amresco, Suwanee, GA, USA.

[l]Spectrum®, Gardena, CA, USA.

[m]Alfa Aesar, Ward hill, MA, USA.

[n]United states biological, Swampscott, MA, USA.

[o]Aldrich-Sigma, St. Louis, MO, USA.

Table 2 Proximate composition and amino acid profiles (g/100g) of test diets presented in table 1.

	30%	28%	26%	24%	22%	28+2%	26+4%	24+6%	22+8%
Crude Protein	31.38	29.06	27.40	24.95	22.11	30.35	30.66	29.66	30.35
Crude fat	5.56	5.40	9.43	8.17	7.38	6.98	5.98	6.71	6.24
Crude fiber	3.57	3.34	3.45	3.20	3.10	3.52	3.40	3.18	3.04
Moisture	8.11	8.65	7.80	9.58	12.52	10.49	9.09	7.55	8.51
Ash	6.34	6.11	5.87	5.48	5.02	5.87	5.65	5.59	5.30
Arginine	2.05	1.91	1.73	1.62	1.41	1.93	1.97	1.86	1.93
Histidine	0.78	0.74	0.65	0.59	0.60	0.70	0.78	0.80	0.76
Isoleucine	1.20	1.13	1.06	0.97	0.84	1.15	1.18	1.16	1.15
Leucine	2.03	1.90	1.79	1.66	1.45	1.99	2.04	2.08	2.09
Lysine	1.74	1.62	1.49	1.36	1.23	1.65	1.76	1.76	1.76
Methionine	0.44	0.40	0.37	0.35	0.31	0.43	0.42	0.41	0.43
Phenylalanine	1.43	1.35	1.25	1.14	1.07	1.36	1.43	1.44	1.38
Threonine	1.03	0.93	0.88	0.81	0.71	1.02	1.00	1.00	1.05
Tryptophan	0.38	0.37	0.36	0.35	0.31	0.42	0.40	0.38	0.42
Valine	1.32	1.24	1.17	1.08	0.93	1.30	1.30	1.38	1.30
Alanine	1.54	1.43	1.33	1.25	1.06	1.41	1.32	1.10	1.15
Aspartic acid	2.77	2.47	2.32	2.14	1.73	2.53	2.28	2.09	2.03
Cysteine	0.38	0.35	0.34	0.31	0.27	0.35	0.33	0.32	0.30
Glutamic acid	5.51	5.15	4.95	4.65	4.07	5.62	5.95	6.33	6.58
Glycine	2.33	2.16	2.01	1.95	1.62	2.68	3.10	3.21	3.98
Hydroxylysine	0.20	0.19	0.14	0.14	0.20	0.15	0.20	0.18	0.15
Hydroxyproline	0.66	0.57	0.51	0.51	0.41	0.52	0.49	0.26	0.42

Ornithine	0.00	0.00	0.00	0.02	0.00	0.03	0.00	0.00	0.03
Proline	2.17	1.87	1.70	1.69	1.28	1.89	1.79	1.33	1.57
Serine	1.19	1.08	1.03	0.97	0.83	1.09	1.01	0.93	0.90
Taurine	0.20	0.21	0.22	0.22	0.21	0.20	0.19	0.20	0.20
Tyrosine	0.96	0.90	0.84	0.77	0.72	0.86	0.87	0.81	0.74
Total amino acids	30.31	27.97	26.14	24.55	21.38	29.28	29.81	29.03	30.32

Table 3 Diet formulation and chemical composition of fishmeal reference diet (RD FM), 30% CP soybean meal basal diet (BD 30), 30% CP basal diet supplemented with methionine (BD Met), 30% CP basal diet supplemented with EAA (BD 30+FMeaa), 35% CP soybean meal basal diet (BD 35), 35% CP basal diet supplemented with Met (BD 35 Met), 35% CP basal diet supplemented with EAA (BD 35+FMeaa), (g/100 g "as-is") used in trial 3

Ingredients (As-is %)	RD FM	BD 30	BD 30 Met	BD30+ FMeaa	BD 35	BD 35 Met	BD35+ FMeaa
Menhaden fishmeal	16.50	5.00	5.00	5.00	5.00	5.00	5.00
Soybean meal	32.05	38.80	38.80	38.80	54.30	54.30	54.30
Gelatin	-	5.00	5.00	5.00	5.00	5.00	5.00
Menhaden fish oil	2.77	3.90	3.90	3.90	3.94	3.94	3.94
Corn starch	6.22	3.65	3.65	3.59	1.51	1.52	1.51
Whole wheat	35.00	35.00	35.00	35.00	22.00	22.00	22.00
Lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol	0.10	0.10	0.10	0.10.	0.10	0.10	0.10
hydroxypropyl methylcellulose[a]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
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 chloride	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Stay-C (35% active)	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Ca-P dibasic	1.90	3.11	3.11	3.11	2.69	2.69	2.69
Arginine	-	-	-	-	-	-	-
Histidine	-	-	-	0.04	-	-	0.04
Isoleucine	-	-	-	0.06	-	-	0.02
Leucine	-	-	-	0.13	-	-	0.09

Lysine (76.6%)	-	-	-	0.24	-	-	0.06
Methionine	-	-	0.12	0.12	-	0.14	0.14
Phenylalanine	-	-	-	0.04	-	-	0.01
Threonine	-	-	0.10	0.10	-	0.07	0.07
Tryptophan	-	-	-	0.03	-	-	0.02
Valine	-	-	-	0.08	-	-	0.07
Glutamic acid	0.43	0.42	0.31	0.03	0.43	0.32	0.17
Glycine	0.43	0.42	0.31	0.03	0.43	0.32	0.17

[a]Tic gums, Belcamp, MD, USA.

*Ingredients came from the same sources as for the diets from objective 1

Table 4 Proximate composition and amino acid profiles (g/100g) of fishmeal reference diet (RD FM), 30% CP soybean meal basal diet (BD 30), 30% CP basal diet supplemented with methionine (BD Met), 30% CP basal diet supplemented with EAA (BD 30+FMeaa), 35% CP soybean meal basal diet (BD 35), 35% CP basal diet supplemented with Met (BD 35 Met), 35% CP basal diet supplemented with EAA (BD 35+FMeaa) diets in Table 2

	RD FM	BD 30	BD 30 Met	BD30+ FMeaa	BD 35	BD 35 Met	BD35+ FMeaa
Crude Protein	32.86	34.22	33.87	33.99	39.17	39.25	39.41
Crude fat	6.79	8.33	5.79	6.94	6.41	7.85	6.99
Crude fiber	3.62	4.20	4.57	3.76	4.77	4.01	4.08
Moisture	6.96	5.64	5.73	6.02	5.76	5.23	5.20
Ash	7.25	6.59	6.50	6.59	6.95	6.97	6.95
Arginine	1.96	2.12	2.17	2.19	2.58	2.58	2.61
Histidine	0.75	0.71	0.72	0.74	0.85	0.84	0.89
Isoleucine	1.42	1.33	1.35	1.38	1.58	1.56	1.61
Leucine	2.28	2.17	2.19	2.30	2.57	2.56	2.70
Lysine	1.92	1.75	1.77	1.97	2.14	2.15	2.30
Methionine	0.56	0.45	0.56	0.57	0.52	0.65	0.66
Phenylalanine	1.47	1.47	1.48	1.49	1.75	1.73	1.78
Threonine	1.12	1.04	1.15	1.18	1.28	1.36	1.40
Tryptophan	0.46	0.49	0.47	0.47	0.49	0.44	0.51
Valine	1.52	1.45	1.46	1.53	1.69	1.67	1.77
Alanine	1.50	1.64	1.65	1.63	1.87	1.85	1.89
Aspartic acid	2.87	2.85	2.89	2.93	3.59	3.57	3.64
Cysteine	0.41	0.40	0.40	0.41	0.47	0.46	0.48
Glutamic acid	6.00	6.22	6.03	5.80	6.70	6.67	6.68

Glycine	2.03	2.83	2.73	2.46	3.06	2.90	2.82
Hydroxylysine	0.10	0.15	0.14	0.12	0.14	0.14	0.14
Hydroxyproline	0.16	0.57	0.56	0.58	0.58	0.56	0.56
Lanthionine	0.02	0.00	0.00	0.00	0.00	0.03	0.02
Ornithine	0.02	0.03	0.03	0.02	0.03	0.03	0.03
Proline	1.76	2.37	2.35	2.38	2.50	2.44	2.52
Serine	1.08	1.13	1.12	1.20	1.34	1.42	1.49
Taurine	0.27	0.22	0.21	0.21	0.19	0.19	0.19
Tyrosine	1.02	0.95	0.99	0.99	1.16	1.19	1.18
Total amino acids	30.70	32.34	32.42	32.55	37.08	36.99	37.87

Table 5 Water quality data for trial 1 run at Auburn University and trial 2 and 3 run at Claude Peteet Mariculture Center

	Trial 1	Trial 2	Trial 3
Dissolved oxygen	6.11 ± 0.74	6.40 ± 0.35	7.05 ± 0.83
Salinity	7.25 ± 0.71	16.02 ± 1.28	12.73 ± 1.00
Temperature	28.24 ± 1.34	27.02 ± 1.57	27.66 ± 2.35
pH	7.47 ± 0.29	7.87 ± 0.13	7.85 ± 0.22
Nitrite	0.10 ± 0.07	0.24 ± 0.09	0.15 ± 0.14
TAN	0.08 ± 0.05	0.06 ± 0.06	0.01 ± 0.01

Table 6 Growth performance of *L. vannamei* fed experimental diets for seven weeks in trial 1 (IBW[e]: 0.25±0.02) and six weeks in trial 2 (IBW[e]: 0.30±0.01)

	Diet	MFW (g)	WG (%)[b]	FCR[a]	Survival (%)
Trial 1 (n= 4)					
	30%	5.46 ± 0.20	2156 ± 167	2.13 ± 0.10	87.50 ± 5.00
	28%	5.42 ± 0.65	2030 ± 298	2.20 ± 0.29	82.50 ± 5.00
	26%	4.87 ± 0.18	1899 ± 175	2.36 ± 0.08	97.50 ± 5.00
	24%	4.75 ± 0.17	1833 ± 218	2.48 ± 0.09	87.50 ± 12.58
	22%	4.73 ± 0.45	1803 ± 266	2.47 ± 0.27	92.50 ± 9.57
	28+2%	5.39 ± 0.54	2118 ± 320	2.23 ± 0.26	80.00 ± 8.16
	26+4%	4.94 ± 0.12	1961 ± 80	2.35 ± 0.07	90.00 ± 8.16
	24+6%	4.60 ± 0.29	1765 ± 170	2.55 ± 0.16	92.50 ± 9.57
	22+8%	4.62 ± 0.52	1736 ± 236	2.55 ± 0.31	92.50 ± 9.57
Model P-value		0.0005	0.0043	0.0015	0.1671
P-value Type III	AA	0.60	0.46	0.69	0.34
	CP level	<.0001	0.0005	0.0003	0.03
	AA*CP level	0.62	0.46	0.72	0.31
Trial 2 (n=5)					
	30%	5.56 ± 0.56	1790 ± 245	1.41 ± 0.15	96.67 ± 3.85
	28%	4.60 ± 0.29	1413 ± 130	1.72 ± 0.12	97.33 ± 5.96
	26%	4.63 ± 0.37	1462 ± 156	1.70 ± 0.14	97.33 ± 3.65
	24%	4.58 ± 0.64	1413 ± 226	1.75 ± 0.28	96.00 ± 3.65
	22%	4.41 ± 0.35	1355 ± 141	1.80 ± 0.17	97.33 ± 3.65
	28+2%	4.83 ± 0.40	1490 ± 97	1.63 ± 0.14	96.00 ± 5.96
	26+4%	4.89 ± 0.33	1561 ± 138	1.60 ± 0.11	92.00 ± 2.98
	24+6%	4.69 ± 0.11	1448 ± 87	1.68 ± 0.04	94.67 ± 5.58
	22+8%	4.49 ± 0.45	1412 ± 163	1.77 ± 0.21	97.33 ± 3.65
Model P-value		0.0073	0.0149	0.0133	0.4498
P-value Type III	AA	0.34	0.25	0.43	0.64
	CP level	0.003	0.0083	0.0042	0.55
	AA*CP level	0.37	0.27	0.48	0.54

[a]FCR: Feed conversion ratio = feed offered/ (final weight-initial weight)

[b]WG: Weight gain = (final weight-initial weight)/initial weight × 100%

[c]P-value for comparing amino acid supplementation with no amino acid supplementation

[d]P-value for the effect of CP level on the performance

[e]IBW: Initial body weight

Table 7 Growth performance of *L. vannamei* fed experimental diets for eight weeks at six replicates per treatment (IBW[5]: 0.22±0.02)

	MFW (g)[4]	WG (%)[2]	FCR[1]	Survival (%)	PRE (%)[3]
RD FM	7.65 ± 0.62[a]	3366 ± 183	1.33 ± 0.12[b]	94.44 ± 5.02	43.72 ± 4.79[a]
BD 30	7.00 ± 0.69[ab]	2985 ± 310	1.46 ± 0.15[ab]	87.78 ± 8.86	37.09 ± 4.66[ab]
BD 30 Met	6.77 ± 0.36[ab]	3047 ± 386	1.50 ± 0.09[ab]	91.11 ± 5.44	36.05 ± 3.77[b]
BD30+ FMeaa	6.61 ± 0.50[b]	2979 ± 299	1.54 ± 0.12[a]	96.67 ± 6.99	36.14 ± 4.03[b]
BD 35	7.45 ± 0.69[ab]	3303 ± 309	1.37 ± 0.13[ab]	88.89 ± 9.11	36.27 ± 3.30[b]
BD 35 Met	7.75 ± 0.36[a]	3241 ± 124	1.31 ± 0.06[b]	90.00 ± 6.99	36.20 ± 3.24[b]
BD35+ FMeaa	7.59 ± 0.47[ab]	3315 ± 234	1.33 ± 0.08[b]	90.00 ± 5.58	37.19 ± 3.00[ab]
Model P-value	0.0030	0.1410	0.0031	0.3848	0.9888
AA (Type III)	0.3651	0.8068	0.3726	0.3538	0.8286
CP level	<.0001	0.0063	<.0001	0.3686	0.9195
AA*CP level	0.3728	0.8051	0.3814	0.4162	0.8289
One-way ANOVA	0.0025	0.0667	0.0022	0.3180	0.0164
Type III SS p-value	0.0025	0.0667	0.0022	0.3180	0.0164

[1]FCR: Feed conversion ratio = feed offered/ (final weight-initial weight)

[2]WG: Weight gain = (final weight-initial weight)/initial weight × 100%

[3]PRE: Protein retention efficiency = (final weight × final protein content) - (initial weight × initial protein content) × 100/protein intake

[4]MFW: Mean final weight

[5]IBW: Initial body weight

Figure 1 Relationship between Percentage weight gain (y) and Crude protein content (x) for *L. vannamei* in trial 1. The regression line for CAA supplementation is described by $y = 7.9392x^2 – 329.91x + 5140.9$ ($R^2 = 0.39$). The regression line for no CAA supplementation is described by $y = 4.5736x^2 – 192.58x + 3822.8$ ($R^2 = 0.30$).

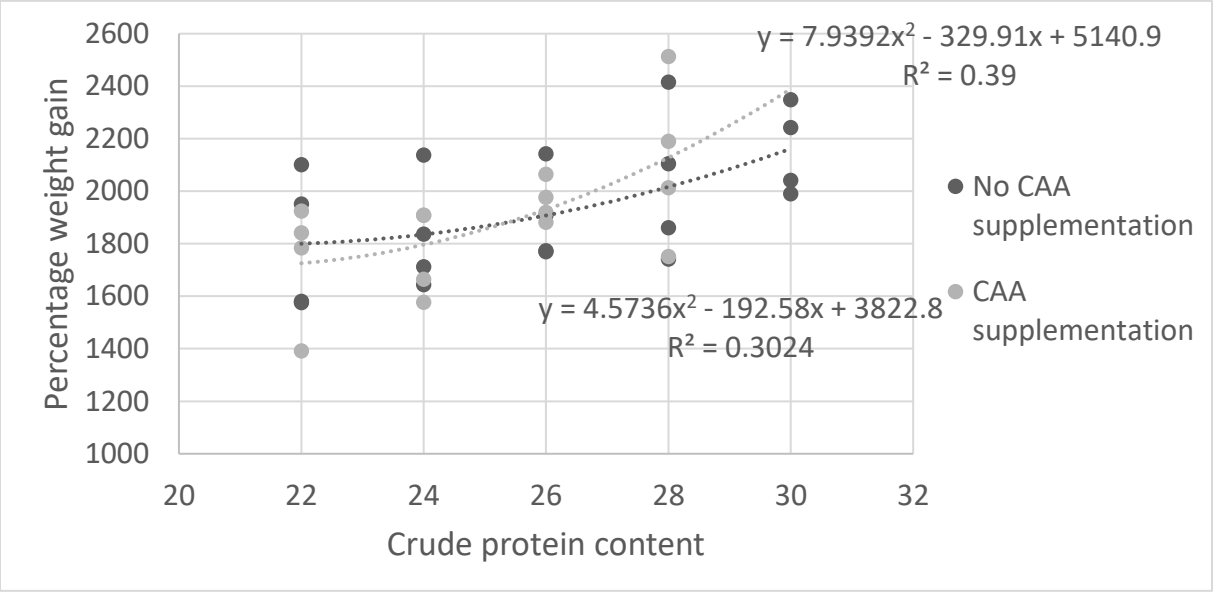
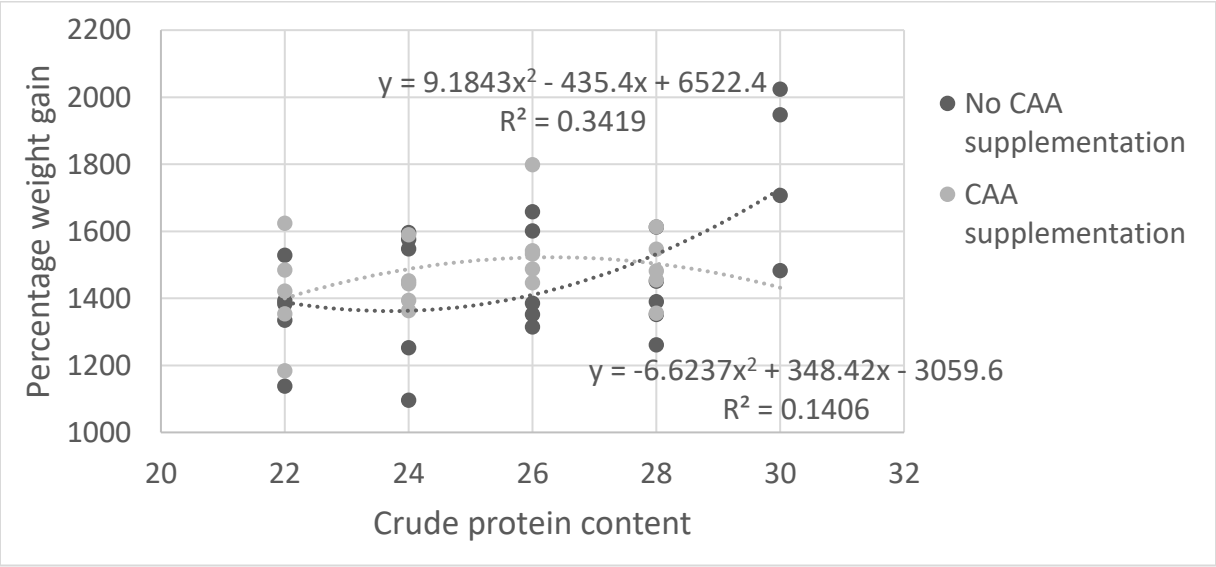


Figure 2 Relationship between Percentage weight gain (y) and Crude protein content (x) for *L. vannamei* in trial 2. The regression line for CAA supplementation is described by $y = -6.6237x^2 – 348.42x + 3059.6$ ($R^2 = 0.14$). The regression line for no CAA supplementation is described by $y = 9.1843x^2 – 435.4x + 6522.4$ ($R^2 = 0.34$).



CHAPTER III

UTILIZING DIFFERENT SOURCES OF METHIONINE IN PACIFIC WHITE SHRIMP, *Litopenaeus vannamei*, DIETS

Abstract

Methionine is an essential amino acid required for optimal growth for fish and shrimp. As the industry is moving towards using more plant-based protein and less fishmeal, formulators are focusing on refining amino acid requirements. There are some differences in terms of the methionine requirement for Pacific White shrimp. Therefore, this study was designed to look at different methionine sources supplemented to a basal diet that would be considered as deficient in methionine (0.45%) and total sulphur amino acids (TSA: meth + cys = 0.90%). The basal diet was designed to contain 34% CP and 8% lipid. To this basal diet, coated methionine (76% activity), DL-methionine, and peptide methionine (95%) were supplemented to reach 0.60% of the diet. A fifth diet was formulated to supplement methionine in the form of intact protein by using corn protein concentrate. This experiment was run twice with 10 shrimp per 150L tank (mean initial weight 0.55 ± 0.04) in the first trial and 30 shrimp per 150L tank in the second (mean initial weight 0.22 ± 0.01). Based on literature reports, the basal diet should be deficient in methionine but no significant improvement in growth performance was found when any of the methionine sources were supplemented. Also, no significant differences were found among the sources of methionine

supplemented to the diet. These results bring into question whether a methionine level of 0.45% or TSA of 0.90% is truly deficient for this species or if another reason explains why the methionine sources did not supply a response as expected.

1. Introduction

Ingredients that are of animal origin are considered the most suitable protein source for shrimp feed as they are more likely to meet nutritional requirements (Amaya *et al*. 2007). The supply of fishmeal and other animal proteins is in high demand for inclusion in many animal diets (Forster and Dominy 2006). Given the limited supply, a shift to using plant-based protein sources for inclusion in aquaculture diets has been made (Davis and Arnold 2000, Samocha *et al*. 2004, Achupallas *et al*. 2016).

When diets are formulated to include prominent levels of plant proteins, one or more amino acids could be deficient in the diet thus having an amino acid profile that is not balanced and this increases the need to know essential amino acid requirements for fish and shrimp (Teshima *et al*. 1992, Cuzon *et al*. 2004, Fox *et al*. 2004, Bureau and Encarnação 2006, Sookying *et al.* 2013). Soybean meal is a plant protein source that has been included in aquaculture diets of numerous species quite successfully (Alvarez *et al*. 2007, Sookying and Davis 2012, Sookying *et al*. 2013). Soybean meal, however, has the disadvantage of being limiting in sulphur amino acids. Methionine especially has commonly been stated to be the amino acid that requires supplementation when plant proteins are being included in the diet formulation. Methionine has an importance in structure and synthesis of substances, such as metabolites, neurotransmitters, and hormones, and also acts as a precursor for molecules such as glutathionine peroxidase (Façanha *et al*. 2016). Therefore, knowing a methionine requirement for Pacific White shrimp becomes important in feed formulations.

Unfortunately, determining the amino acid requirement has been proven to be more difficult for shrimp as compared to finfish due to rapid consumption of feed by fish as compared to external mastication of shrimp (Fox *et al*. 1995). Relative to requirements for other species, some authors have recommended quite high levels that are likely to be above the requirement. For

example, if methionine requirement is high and one choses to only use intact protein sources, it favors fishmeal in the diet formulation. In the case of methionine, few studies have determined the methionine requirement for shrimp. For *M. Japonicus*, the methionine requirement was estimated to be 1.3-1.6% of the CP which was derived by looking at the whole-body content of the species (Teshima *et al*. 2002). Requirement was determined to be 0.89% of the diet or 2.4% of the CP content (using broken-line analysis) for *P. monodon* by Millamena *et al*. (1996b) and 2.9% of the CP content by Richard *et al.* (2010). Lin *et al*. (2015) and Façanha *et al*. (2016) looked at the methionine requirement of *L. vannamei.* The NRC (NRC 2011) also reports requirements for methionine for Kuruma and Tiger shrimp at 0.7% but there is no requirement reported for Pacific White shrimp. For least cost formulation of shrimp diets, methionine, lysine and arginine are probably the most limiting and, therefore, important for proper feed formulation (Fox *et al*. 2006). If diet formulations for shrimp are to advance, a model for amino acid work in shrimp is needed. Given the inconsistency of results, the objective of this study was to determine the efficacy of four different methionine supplements: coated methionine, DL-methionine, methionine peptide, and intact protein methionine, in diets of Pacific white shrimp.

2. Materials and Methods

2.1 Experimental diets

Prior to diet formulation, the primary ingredients were analyzed for proximate composition. The diets were formulated to be iso-nitrogenous and iso-lipidic with all diets formulated to contain 35% crude protein (CP) and 8% total lipid on an as-is basis. Five diets were formulated with the basal diet not containing any methionine supplementation. Two different experiments with the same objective were run with slight differences in the ingredient composition

of the diets (Table 1). Proximate analysis of the diets are presented in table 2. Analysis of the diets confirmed that the basal diets contained 0.49 & 0.51% methionine and 0.41 & 0.46% cysteine. The first three test diets were produced by replacing glycine in the basal diet with various methionine sources to keep the diets isonitrogenous. A fifth diet was formulated to supplement methionine in the form of intact protein. Whereas diets 1-4 contained 4% poultry-by-product meal and 48% soybean meal as the primary protein sources, diet five contained 4% poultry-by-product meal, 33% soybean meal and 15% corn protein concentrate to reach the same methionine content as diet 2 to 4. Similarly, for the repeated experiment, diets 1-4 contained 6% poultry by-product meal and 49.50% soybean meal as the primary protein sources, diet five contained 6% poultry by-product meal, 32.50% soybean meal and 15.00% corn protein concentrate.

Diets were made using standard procedures used by the Aquatic Animal Nutrition Laboratory, School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University (AL, USA). Dried, pre-ground ingredients were mixed using a food mixer (Hobart, Troy, OH, USA). Once the dry ingredients were properly mixed, fish oil was added and mixed for another 15 minutes. For obtaining appropriate moisture and consistency for proper pelleting, boiling water was added to the mixture. A 3-mm die in a meat grinder was then used for passing the moist ingredient mix. Pellets were then placed in a fan ventilated oven with a temperature of <45 °C overnight to reach a moisture content of <10%. Before using the diets, they were ground and sieved to an appropriate size for shrimp. Diets were stored in a freezer at -20°C until used in the experiment. Proximate analysis was completed at University of Missouri and Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for trial 1 and at Ajinomoto Heartland for trial 2 (Table 2).

2.2 Growth trials

Five treatments were used in the growth trial with three replicates per diet for the first trial and five replicates per diet for the second trial except for the basal diet that only had four replicates. Juvenile shrimp were reared in a nursery system and offered commercial feeds (Zeigler PL Raceway Plus, 50% CP and 15% Crude fat, and later Shrimp starter diet, 55% CP and 15% Crude fat) until the shrimp reached an appropriate size. For trial one, the shrimp were stocked at 10 shrimp per 150-L tank (mean initial weight 0.55 ± 0.04). They were then stocked at 30 shrimp per 150-L tank in trial 2 (mean initial weight 0.22 ± 0.01). The shrimp were counted on a weekly basis for readjustment of the feed input. Feed inputs were determined by calculating feed requirements assuming a feed conversion ratio (FCR) of 1.8 and a predicted growth of 0.5, 0.8, 0.9, 1.0, 1.1 and 1.2 used over the six-week period for trial 1. Using the same FCR a predicted growth of 0.2, 0.4, 0.8, 0.8, 1 and 1.1, respectively, was used over the six-week growth period for trial 2. Feed was offered four times per day. At conclusion of the growth trial, the shrimp were counted, and group weighed. The final mean weight (FMW), FCR, percentage weight gain (PWG) and survival were calculated.

2.3 Water quality

Water quality parameters (dissolved oxygen (DO), temperature and salinity) were measured twice daily using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA). These were measured in the sump tank of the system. Other water quality parameters, such as pH, total ammonia-nitrogen (TAN) and nitrite, were measured twice every week. Water samples for TAN and nitrite was collected from the sump tank. Ammonia-nitrogen was measured by the method described by Solorzano (1969) and nitrite by Spotte (1979). Water quality parameters were kept as follow; for trial 1, DO was kept at 5.79 ± 0.28 mg/L, temperature at 28.2 ± 2.07°C, salinity

9.55 ± 2.13 ppt, pH at 7.24 ± 0.55, TAN at 0.06 ± 0.05 mg/L and nitrite at 0.01 ± 0.01 mg/L. DO was kept at 5.15 ± 1.14 mg/L, temperature at 29.77 ± 0.34 °C, salinity at 8.19 ± 0.78 ppt, pH at 7.12 ± 0.08, TAN at 0.17 ± 0.12 mg/L and nitrite at 0.08 ±0.04 mg/L for trial 2.

2.4 Calculations and Statistical analysis

Data were analysed using one-way ANOVA to determine if there were any significant differences among the treatments. This was done by using the SAS program (V9.3. SAS institute, Cary, NC, USA). Mean growth data was analysed for the assumptions of normality and homogeneity and the assumptions were not violated. Survival data was analysed using one-way ANOVA as well as binomial.

3. Results

The lowest FMW (6.54 g) and PWG (1122%) as well as the highest FCR (1.65) were for the shrimp that were fed the diet supplemented with methionine peptide as found in trial one. As seen in table 3, this was even lower than the basal diet (6.60g, 1064%, and 1.63, respectively) that has a lower methionine content compared to the methionine supplemented diets. The intact methionine source provided the better FCR of 1.53. However, there was no significant differences ($P < 0.05$) among any of the diets for any of these growth factors.

In contrast to the previous results, methionine peptide seemed to give the highest FMW (5.24 g), and PWG (2400%) compared to the basal diet in trial two. The highest FCR in this trial was observed for the intact protein methionine source at 1.93; whereas, the basal and peptide methionine showed the lowest mean FCR of 1.73. Similarly, there were no significant differences ($P < 0.05$) among the diets as seen in table 4. Survival was good for trial one ranging from 90-

100% and no significant differences found between treatments using one-way ANOVA or binomial analysis. Survival for trial 2 ranged from 80-92% and by using one-way ANOVA no significant differences was observed. However, survival of the intact protein methionine source was significantly lower (model P-value: 0.02) than the basal diet when analyzed by binomial analysis. Survival data is shown in table 4 and 5.

There was also no significant difference ($P < 0.05$) among methionine proximate analysis for tail muscle and hepatopancreas methionine (Table 7). However, the peptide methionine produced the highest methionine level in the hepatopancreas and the tail muscle (0.76 and 2.14g/100g "as-is", respectively).

4. Discussion

As feed formulation is becoming more "nutrient-based" (Nunes *et al.* 2014) and methionine is an essential amino acid for growth and maintenance of shrimp it becomes necessary to know its requirement. In a study conducted by Fox *et al.* (1995), they utilized defined diets to evaluate amino acid requirements in *P. vannamei.* In this work, wheat gluten was the primary protein source with the basal diet adjusted for select amino acids by adding crystalline lysine, arginine, methionine, histidine and threonine. Based on single deletion of each of the amino acids from the basal diets, they reported that the diet was limiting in methionine (0.35%), lysine (0.45%) or arginine (1.0%). They made the conclusion that vital amino acid requirement in terms of descending order in a wheat-based diet was lysine ≥ methionine ≥ arginine. This validates studying the methionine requirement for shrimp as it could possibly be an amino acid limiting growth in plant-based diets. Furthermore, soybean meal is by far the most available protein source used in feed formulations and is known to be limited in methionine, which further increases the need for determining the requirement (Fox *et al*. 2006, Yue *et al.* 2012).

To determine a methionine requirement, one must have a basal diet deficient in methionine and/or total sulphur amino acids and a supplement that can be added at graded levels that produces an appropriate biological response such as growth. This response should be repeatable and reasonably consistent across diet types and experimental protocols. However, reported methionine requirement varies widely between studies with the results questioned by some authors such as Davis and Duan (2017). For example, Niu *et al*. (2018) reported results using a 36% protein diet containing 20% fishmeal in the basal diet producing a diet with 0.6% methionine and 0.39 % cysteine resulting in a TSA content of 0.99% diet or 2.75% of the protein. This diet was then supplemented with increasing levels of two different sources of methionine. They reported that shrimp performance was decreased when the dietary methionine level was below 0.73% (1.98% of the protein) but also found no growth enhancement when the methionine level increased above 0.81% (2.20% of the protein) of the diets. As cysteine was limited (<40% of total sulphur amino acids) in these diets, it is probably more appropriate to view this as looking at a TSA requirement of 1.12% of the diet or 3.1% of the protein. Methionine to cysteine ratio should be 50:50 on a molar basis or 60:40 on a weight basis to save methionine as cysteine is being produced from methionine. Façanha *et al*. (2016) performed a study feeding increasing levels of methionine (0.48, 0.62 , 0.72 , 0.81 , and 0.94 g/100g dry diet) at three different stocking densities using a 36% CP feed containing 0.45 to 0.48 g cystine/ 100g dry weight diet. At two of the densities, they did not find a typical dose response but found a linear increase in the growth of the shrimp at the highest density (100 shrimp/m^2). They estimated that the methionine requirements needed to obtain maximum shrimp growth under green-water conditions was 0.72 to 0.81% of the diet and 1.99 to 2.30 as % protein, or as a TSA requirement of 1.19 to 1.28 g /100g dry diet or 3.28 and 3.63 as % protein. However, the question remains why the lower stocking densities did not show a typical

dose response and therefore why the response is not repeatable across all densities. Lin *et al.* (2015) completed a study on methionine requirement for three different sizes of shrimp using three different levels of dietary protein. They reported an increase in the percentage weight gain as the methionine level increased from 0.7 to 0.8 as a % of dry diet for small size shrimp. After this point as the methionine level increased the weight gain percentage decreased. However, in all the small and medium size groups there were no significant differences in percentage weight gain as the methionine level increased from 0.7 to 1.23. Limited differences were observed in the bigger size group with 0.71 giving the best results. By using regression analysis of the mean values, they concluded that there was a dose-response to methionine and presented a methionine requirement of 0.91% of the dry diet (2.28 as % of protein) or at TSA requirement of 1.39 (3.48 as % of protein) for the small sized shrimp (0.55g initial weight). Regression on the mean values of the replicates may not be the best approach for determining a requirement. Methionine requirement for the medium and large shrimp was determined to be less. In all three cases, in terms of growth there was very little difference between the basal diet and the best performing group and a clear dose response was not observed. Forster and Dominy (2006) reported that their basal diet containing 0.44% methionine and 0.48% cysteine was deficient in methionine for Pacific white shrimp when it was compared to the pooled results of this diet supplemented with three methionine sources. However, there was no significant difference found between the treatments when looking at one-way ANOVA for the growth data.

If methionine requirement is as high as reported by these studies, one would expect to see a response with at least one of the methionine sources used to supplement the basal diet (0.49-0.51%) in our study. Studies are often selective in what they present. For example, many of the studies defining a requirement, only find the basal diet “deficient” and the requirement is then

developed off of limited data. Some of these studies have reported regression analysis using means of the treatments instead of the replicates which may not be the most appropriate way to presenting these results. Broken-line regression may also not be the best way of determining an amino acid requirement when there are limited points below the requirement. From a biological point of view, a non-linear model may give a better approach (Bureau and Encarnação 2006). As studies are often in favor of a high methionine requirement it leads to forcing fishmeal into the diet or we may be over-supplementing methionine as various fishmeal replacement studies were successful despite the sulfur amino acids decreasing below that normally found in a fishmeal diet (Fox *et al.* 2011, Amaya *et al*. 2007, Sookying and Davis 2012). Over-supplementation of an amino acid could also have other negative effects such as excessive nitrogen excretion. Some studies also observed a marked decline in the growth once the level of the tested amino acid is supplemented above the estimated requirement (Millamena *et al*. 1996b, Millamena *et al.* 1998). This could be an indication of crystalline amino acid balance becoming problematic when over-supplemented. Not only are these factors that need to be considered when designing an amino acid requirement experiment but various other factors may also have an effect on the amino acid requirement such as the life stage of the shrimp as well as the rearing conditions (Façanha et al. 2016).

Before an amino acid requirement can be determined a suitable source that has been proven to be effective for supplementation has to be identified. Therefore, in this study, different methionine sources were tested for their potential as methionine supplements. In our work we used a practical diet using a small quantity of poultry meal, soybean meal and gelatin to produce a diet with 0.49 – 0.51% methionine and 0.41 – 0.46% cysteine. Each of the methionine sources (coated methionine, DL-methionine, peptide methionine and intact protein methionine) was supplemented to this basal diet to reach a level of 0.63-0.70% of the diet. It was stated by Fox *et al*. (1995) that

when determining the requirement for an amino acid, the diets should be isonitrogenous and the amino acid that is studied should be considerably deficient. In our study the basal diet should be deficient in methionine according to the reported requirement by other studies above, yet, supplementing methionine with four different sources did not improve any of the growth parameters significantly compared to that of the basal diet. In another study similar to our study, different methionine sources were supplemented to reach 0.5 to 0.6% of the diet (compared to a basal diet with 0.4%) but no depression in growth was observed for the basal diet that had no methionine supplementation, which lead to Fox *et al.* (2011) concluding that methionine in the basal diet was sufficient. They further concluded that it did not seem that supplementation of methionine to plant based diets is required. In their experiment the primary protein ingredients used in the diets included soybean meal, poultry meal and gelatin but no fishmeal which was intentionally done to lower the methionine content of the diet.

The question remains why some studies can determine a requirement and others not, but also why the studies are not repeatable. There could possibly be some family or genetic impacts that may influence the difference between studies. Determining the amino acid requirement for shrimp has been more difficult as compared to doing the same for fish. This could be caused by the feeding habits of shrimp. As shrimp masticate externally and have slow feeding habits, feed tend to stay in the water longer leaving time for amino acids to leach especially for the water soluble crystalline amino acids. This could be why sources such as DL-methionine, a crystalline amino acids (CAA) that is water soluble does not supply better growth but if this is the case then one would expect the intact protein source, coated methionine or peptide methionine to give some better results. Fox *et al*. (2011) observed significantly lower loss of methionine when supplemented as poly methionine and methionine in mineral chelated diets than the diets

supplemented with DL-methionine. DL-methionine however showed the highest apparent methionine digestibility but in terms of final weight there were no significant differences, therefore DL-methionine may have leached from the faeces during collection. There were also no significant differences in terms of growth performance between shrimp fed the different methionine diets and therefore no difference whether the methionine was supplemented as an intact or other source of methionine which agrees with the results that we have found in our study. A recent study reported that DL-met supplemented to a basal diet (6.3% Met and 2.3% Cys) up to the level of 6.9% of the diet (1.7% Cys) did not improve weight gain and feed utilization of Pacific white shrimp (Chen *et al.* 2018). Although it should be noted that this study also has a high methionine to cysteine ratio and therefore cysteine may have been limited in this study as well.

There are studies that are in favour of CAA use (Millamena *et al*. 1996b, Xie *et al.* 2012) but there are also studies that question the efficacy thereof. In a study done where Lysine was supplemented to *P. vannamei* diets in a covalent form (covalently attached to wheat gluten) compared to when it was supplied in the CAA form, shrimp showed significantly higher instantaneous growth rate at the determined requirement level (Fox *et al*. 1995). However, they also found that supplementation of CAA in small proportions to intact essential amino acids seems to be able to support growth of shrimp (Fox *et al.* 1995).

Peptide methionine may also have been expected to perform better compared to DL-methionine as it has a lower leaching rate. In our study, peptide methionine did perform better than DL-methionine in the second trial but not in the first although there were no significant differences. When comparing a peptide methionine product to DL-met in another study, it was reported that growth performance (%WG, MFW and somatic growth rate) at each level of supplementation was better for peptide methionine, although the difference was not significant (Niu *et al.* 2018).

Just like our study, there are some studies supplementing different sources of CAA or coated methionine to a basal diet lower in methionine and find no significant response for shrimp (Teshima *et al*. 1992, Forster and Dominy 2006) as well as for fish (Goff and Gatlin 2004, Yuan *et al.* 2011). Fox *et al.* (2004), found that a reduction in the DL-methionine supplemented to the diet from 0.3 to 0.1% improved the instantaneous growth rate of *L. vannamei* significantly.

Whether diet CP level can have an influence on determining the methionine requirement may also be considered. Richard *et al*. (2010) completing a study on *Penaeus monodon*, found that there was no significant effect of methionine supplemented with an essential amino acid blend to have the amino acid composition of shrimp whole body compared to a formulated diet where the methionine was formulated to be 30% deficient. The study was performed at three different protein levels and they did however find that there was a significant interaction between the level of protein and methionine for growth performance. They stated that this could be explained by the deficient methionine reducing growth and feed efficiency at the 34% CP and not at the lower (15%) or higher (53%) CP level. However, our diets were formulated to a 34-37% CP level and still the basal diet does not seem to be deficient in methionine.

In terms of tail muscle and hepatopancreas methionine level, there was no significant difference between the diets. However, another study found muscle protein content of shrimp fed a diet supplemented with peptide methionine to reach 0.87% was significantly higher compared to those fed the basal diet containing 0.60% methionine but no significant difference was found when DL-met was supplemented to the same level (Niu *et al.* 2018). At the lower levels of supplementation there was also no significant difference. They found that as the methionine content of the diet increased the muscle protein content also increased although not significantly.

Whole body methionine level was significantly lower in the basal diet (0.6%) as compared to DL-met supplemented at 0.73-0.82% and Met-Met supplemented at 0.64-0.87%.

In conclusion, it is clear from the few studies done on methionine requirement, that there is no consensus on the requirement and results do not seem to be repeatable. Often methionine requirement for this species is reported to be around 0.7-0.8% of the diet, yet we did not find a response when a basal diet of 0.49% methionine was supplemented to reach 0.69%. If this methionine content was truly deficient then one would expect to see an improved growth response with at least one of the methionine sources used in this study.

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Table 1 Diet formulation and chemical composition of test diets (g/100g) used for trial 1 and 2

	Trial 1					Trial 2				
	Basal	**Coated Met**	**DL-met**	**Met peptide**	**Intact prot**	**Basal**	**Coated Met**	**DL-met**	**Met peptide**	**Intact prot**
Poultry by-product meal[a]	4.00	4.00	4.00	4.00	4.00	6.00	6.00	6.00	6.00	6.00
Soybean meal[b]	48.00	48.00	48.00	48.00	33.00	49.50	49.50	49.50	49.50	32.50
Corn protein concentrate [c]	-	-	-	-	15.00	-	-	-	-	15.00
Gelatin[d]	5.00	5.00	5.00	5.00	-	5.00	5.00	5.00	5.00	-
Menhaden fish oil[e]	5.68	5.68	5.68	5.68	5.59	5.56	5.56	5.56	5.56	5.43
Lechitin[f]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol[g]	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Corn starch[g]	3.68	3.64	3.69	3.68	8.26	0.23	0.26	0.31	0.30	6.72
Whole wheat[h]	27.00	27.00	27.00	27.00	27.00	27.00	27.00	27.00	27.00	27.00
Mineral premix[i]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[j]	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride[k]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Stay C 35% active[l]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
CaP-dibasic[k]	2.80	2.80	2.80	2.80	3.00	2.80	2.80	2.80	2.80	3.10
Lysine[m]	-	-	-	-	0.47	-	-	-	-	0.57
Methionine[n]	-	0.20	0.15.	0.16	-	-	0.20	0.15	0.16	-
Glycine[k]	0.16	-	-	-	-	0.23	-	-	-	-

Results are expressed on an g/100g “as-is” basis

[a]Tyson Foods, Inc., Springdale, AR, USA

[b]De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.

[c]Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.

[d]Rousselot Inc., Mukwonago, WI, USA.

[e]Omega Protein Inc., Houston, TX, USA

[f]The Solae Company, St. Louis, MO, USA.

[g]MP Biomedicals Inc., Solon, OH, USA.

[h]Bob's red mill, Milwaukie, OR, USA.

[i]Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.

[j]Vitamin premix (g/kg premix): Thiamin HCl, 4.95; Riboflavin, 3.83; Pyridoxine HCl, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.

[k]VWR Amresco, Suwanee, GA, USA.

[l]Stay-C® (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA.

[m]Ajinomoto Heartland Onc, Chicago, IL, USA.[n]Alfa Aesar, Ward hill, MA, USA.

Table 2 Proximate analysis and amino acid profiles (g/100g) for the diets for trial 1 and 2

	Trial 1					Trial 2				
	Basal diet	**Coated Met**	**DL-met**	**Met peptide**	**Intact**	**Basal diet**	**Coated Met**	**DL-met**	**Met peptide**	**Intact**
Moisture	5.85	5.87	5.40	5.71	5.23	6.45	6.52	7.69	6.69	4.88
Crude Protein	34.56	34.89	34.91	34.81	35.87	35.75	36.91	36.61	37.09	35.43
Crude fat	9.64	10.07	10.12	8.77	9.22	nd	nd	nd	nd	nd
Crude fiber	3.68	3.88	3.91	3.45	3.37	nd	nd	nd	nd	nd
Ash	6.49	6.28	6.31	6.3	6.16	nd	nd	nd	nd	nd
Alanine	1.72	1.72	1.73	1.72	2.01	1.86	1.83	1.81	1.83	2.02
Arginine	2.30	2.32	2.33	2.34	1.88	2.52	2.42	2.40	2.43	1.89
Aspartic acid	3.06	3.08	3.11	3.07	2.84	3.44	3.32	3.29	3.32	2.93
Cysteine	0.41	0.42	0.42	0.42	0.53	0.46	0.41	0.42	0.42	0.51
Glutamic acid	6.10	6.16	6.19	6.17	7.12	6.28	6.22	6.15	6.22	6.83
Glycine	2.69	2.53	2.53	2.53	1.46	2.91	2.62	2.59	2.63	1.52
Histidine	0.74	0.74	0.75	0.74	0.78	0.81	0.77	0.77	0.77	0.78
Hydroxylysine	0.11	0.10	0.11	0.10	0.05	0.41	nd	nd	nd	nd
Hydroxyproline	0.65	0.64	0.65	0.66	0.12	0.61	nd	nd	nd	nd
Isoleucine	1.34	1.34	1.34	1.35	1.49	1.49	1.39	1.39	1.41	1.38
Lanthionine	0.03	0.02	0.01	0.04	0.02	nd	nd	nd	nd	nd
Leucine	2.31	2.32	2.34	2.33	3.63	2.48	2.41	2.39	2.41	3.44

Lysine	1.83	1.84	1.86	1.83	1.83	2.08	1.89	1.87	1.89	1.93
Methionine	**0.49**	**0.67**	**0.67**	**0.68**	**0.64**	**0.51**	**0.70**	**0.65**	**0.67**	**0.63**
Met+Cys	0.90	1.09	1.09	1.10	1.17	0.97	1.11	1.07	1.09	1.14
Phenylalanine	1.52	1.52	1.54	1.39	1.54	1.69	1.59	1.58	1.60	1.82
Proline	2.32	2.34	2.36	2.31	2.47	2.49	2.40	2.42	2.41	2.36
Serine	1.39	1.43	1.45	1.39	1.54	1.39	1.63	1.61	1.62	1.68
Taurine	0.22	0.22	0.22	0.22	0.23	0.21	nd	nd	nd	nd
Threonine	1.12	1.13	1.14	1.12	1.18	1.26	1.20	1.18	1.20	1.21
Tryptophan	0.38	0.38	0.37	0.38	0.36	0.35	0.38	0.37	0.40	0.40
Tyrosine	1.02	1.04	1.04	1.06	1.37	1.15	0.86	0.85	0.85	1.05
Valine	1.50	1.51	1.52	1.52	1.64	1.59	1.48	1.48	1.50	1.47

Results are expressed on an g/100g “as-is” basis

Proximate analysis done at University of Missouri and Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for trial 1 and at Ajinomoto Heartland for trial 2.

Table 3 Effects of different methionine sources on the growth performance of *L.* vannamei stocked at 10 shrimp per tank with three replicates per treatment from trial I (IBW: 0.55 ± 0.04) over a period of six weeks

	MFW	PWG	FCR	Survival
Basal diet	6.60	1064	1.63	100.00
Coated Met (76% activity)	6.85	1128	1.56	100.00
DL-met	6.60	1134	1.62	100.00
Peptide methionine (95%)	6.54	1122	1.65	96.67
Intact	6.99	1198	1.53	90.00
Model P-value	0.72	0.65	0.71	0.53
P-value (Type I & III SS)	0.72	0.65	0.71	0.53
PSE	0.34	5.09	0.17	1.43

IBW: initial body weight

MFW: mean final weight

PWG: percentage weight gain

FCR: feed conversion ratio

Pooled standard error (PSE) = $\sqrt{(Mean\ square\ error/n)}$

Table 4 Effects of different methionine sources on the growth performance of *L. vannamei* stocked at 30 shrimp per tank at five replicates per treatment from trial II (IBW: 0.22 ± 0.01) over a period of six weeks

	MFW	%WG	FCR	Survival
Basal diet	5.13	2186	1.73	91.67
Coated Met (76% activity)	5.11	2172	1.78	90.00
DL-met	5.11	2190	1.87	87.33
Peptide methionine (95%)	5.24	2400	1.73	90.00
Intact	4.77	2061	1.93	80.00
P-value	0.72	0.20	0.67	0.13
P-value (Type I & III SS)	0.72	0.20	0.67	0.13
PSE	0.37	7.28	0.25	1.33

IBW: initial body weight

MFW: mean final weight

PWG: percentage weight gain

FCR: feed conversion ratio

Pooled standard error (PSE) = $\sqrt{(Mean\ square\ error/n)}$

Table 5 Effects of different methionine sources on the Tail muscle and Hepatopancreas methionine content for *L. vannamei* stocked at 10 shrimp per tank with three replicates per treatment (IBW: 0.55 ± 0.04) over a period of six weeks in trial I (g/100g "as-is")

	Tail muscle	Hepatopancreas
Basal diet	2.10	0.74
Coated Met (76% activity)	2.11	0.67
DL-methionine	2.08	0.69
Peptide methionine	2.14	0.76
Intact	2.11	0.68
Model P-value	0.23	0.22
P-value (Type I & III SS)	0.23	0.22
PSE	0.08	0.11

IBW: initial body weight

CHAPTER IV

SUMMARY AND CONCLUSION

Shrimp is one of the major consumers of manufactured aquaculture feeds with global production of this species growing and production systems becoming more intensive. Feed cost normally makes up the biggest expense of a farming operation. Pacific white shrimp has been one of the major cultured species in the Americas and other countries in Asia. This makes having economical and nutritionally replete formulations important for successful farming of Pacific white shrimp. Protein is normally the most expensive ingredient used in feed formulations and the industry is moving away from animal protein sources such as fishmeal to using cheaper protein sources such as plant-based proteins. However, these proteins are often deficient in one or more essential amino acids (EAA) which makes studies defining the EAA requirement important.

. There are different ways to meet an EAA requirement of an animal but in general CAA are supplemented to a diet based on achieving the appropriate amino acid balance needed by the animal. Sufficient and quality research in terms of EAA requirements for Pacific White shrimp is still lacking in the industry. In general, published studies reporting amino acid requirements are variable, seldom repeatable and based on inaccurate statistical analysis. As EAA requirements are not well-defined reducing feed costs using inexpensive protein sources becomes complicated. The most widely used plant-based protein is solvent extracted soybean meal which is rich in lysine but very low in sulphur containing amino acids such as methionine. Hence, diets containing high levels

of this protein source are often supplemented with methionine to reach the predetermined requirement needed for the species. Hence, if we are to balance the amino acid profiles of our feeds we need to establish EAA requirement and thus validate the efficacy of amino acid supplements.

Therefore, the first study looked at the efficacy of CAA in Pacific white shrimp diets. EAA was supplemented at different levels in the form of CAA to a basal diet which was decreased in CP content. Growth performance of the shrimp however seemed to follow and respond to intact protein reduction rather than having improved growth due to CAA supplementation. This lead to more questions such as the effect of protein level on amino acid supplementation which was studied in a second set of diets. In these diets a fishmeal reference diet was formulated and two basal soybean meal diets at 30% and 35% CP content was supplemented with EAA in one set and methionine in another to reach the same level as a percent protein as that of the fishmeal reference diet. The CP content however did not seem to have an effect on the growth response of the shrimp to the CAA which was the same observation as for the first study.

The third study was designed based on the high level of methionine requirement (0.7-0.8% of the diet) reported in a limited number of studies for this species. Results are often not repeatable between studies and a methionine requirement cannot be determined if a suitable source for supplementation has not been identified. Thus, this study looked at supplementing different sources of methionine (DL-met, peptide met, coated met and intact protein met supplemented to 0.63-0.70% of the diet) to a soybean meal-based diet that should be deficient in methionine (0.49-0.51% of the diet) according to the various studies that have determined a methionine requirement for this species. However, none of the supplemented diets performed significantly better than the basal diet leading to the conclusion that the basal diet might not be deficient in methionine.

There are numerous reasons why these sources may not supply the desired result and one of the most commonly proposed theories is leaching of these sources from the diets especially of sources that are water-soluble such as CAA. Shrimp masticate externally and eat slowly compared to finfish. However other factors that may also cause this undesired effect are pH, palatability and absorption or use of these sources once it has been ingested.

These studies have indicated that CAA as a supplement may not be used as effectively by Pacific white shrimp as previously thought. Intact protein amino acids may produce better performance. Therefore, the results from these studies only raises more questions in terms of why some studies find results and other do not. There may be differences in the feed quality causing more leaching in some than in others. Our study also had considerable variation making proper conclusion difficult. The cause for the variation is also unknown but could be caused by the genetics of the post-larvae. On the other hand, in terms of methionine, it may actually not require supplementation in a soybean meal-based diet as the diet may not be deficient.

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