Optimization Of Plant Based Diets For Pacific White Shrimp

(*Litopenaeus vannamei*)

by

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ABSTRACT

Supplies of marine ingredients are finite and their prices are high, fluctuating and the price is expected to continue to rise. The aquaculture industry has long recognized that the viable utilization of plant feedstuffs formulated in aquafeeds is essential for the sustainable development of aquaculture. Soybean meal is regarded as economical and nutritious feedstuffs with moderate crude protein content and a reasonably balanced amino acid profile, which can function as the primary protein source in practical shrimp feeds. To facilitate the continued development of plant based feed formulations, a series of studies were conducted to determine the impact of utilizing high level of soybean meal on feed formulations. Traditional sources of soybean meal have certain characteristics including the presence of several antinutritional factors and a high carbohydrate concentration which may limit the quantity that can be used in the aquafeeds. New strains of selectively bred non-genetically modified (non-GM) soybeans can reduce level of trypsin inhibitors, oligosaccharides, and/or enhance protein levels. The first study evaluated the efficacy of six new varieties of soybean meal in practical feed formulations by evaluating the biological response of shrimp in terms of growth and in vivo digestibility in high soy feed formulations. Results of this study demonstrated that new lines of soybean could be used to improve growth and digestibility coefficients in shrimp feed and the commercialization of nutritionally improved soybean should be encouraged. Soybean meal is an inexpensive ingredient. To help reduce the shrimp feed cost, soybean meal can be replaced with other less expensive ingredients such as Dried Distillers Grains with Solubles (DDGS) from sorghum (S-

DDGS). Furthermore, the use of pelleted or extruded feeds may result in shifts in performance as the processing conditions are considerably different. Hence, the second component of this research was to evaluate the biological response to practical diets containing grade level of S-DDGS in extruded and pelleted shrimp feeds. Results of this study revealed that S-DDGS can be included in practical diets without negative effects on growth, survival, and feed conversation ratio (FCR). Hence the use of S-DDGS (up to 40%) should be encouraged as an alternative protein in shrimp feed formulations. As fishmeal is replaced with plant based protein sources, there are a number of nutrients that will change, including minerals such as copper, zinc and iron etc. Copper is essential for the survival of all organisms, including shrimp. Three trials in this study were conducted to evaluate growth and tissues response to two copper sources (copper sulfate pentahydrate and tri-basic copper chloride) for *L. vannamei* in a practical feed formulation. Results in this study demonstrated that tri-basic copper chloride (TBCC) was a safe, effective and highly available source of copper in shrimp diet formulations for *L. vannamei*.

Overall, results from these studies reveal that the use of new varieties of soybean meal should be encouraged for use in shrimp feed formulations. Meanwhile, it also indicated the use of high level of soybean meal as main protein source in combination with S-DDGS in formulated diets formulation as long as essential nutrients in diets are properly balanced to meet shrimp nutritional requirements. In addition, TBCC could be used as alternative copper source in shrimp diet formulations for *L. vannamei*.

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CHAPTER I

INTRODUCTION

Pacific white shrimp (*Litopenaeus vannamei*) is native to the eastern Pacific Ocean, from the Mexican state of Sonora to as far south as northern Peru. Currently, it is one of the most prevalent cultured species and accounts for over 66% shrimp aquaculture production, which totaled 3.49 million metric tonnes globally with a worldwide value over $14 billion in 2009 (FAO, 2011). Reasons for this increased production include the capacity of *L. vannamei* for rapid growth, good survival in high-density culture, and disease tolerance for intensive grow-out production (Williams et al., 1996; Ponce-Palafox et al., 1997).

Maintaining the growth of the shrimp industry depends in large part on having adequate supplies of high-quality feed. Current feed formulations rely on fishmeal and fish oil as primary nutrient sources. The predicted supplies of these key ingredients are clearly inadequate to support the demand, and levels used in feeds will have to be reduced (NRC, 2011). In addition, the costs of fishmeal and fish oil have increased over time due to increased demand, limitations of availability, and growing social and environmental concerns regarding wild fish extraction practices (Tacon and Metian, 2008). Hence, the aquaculture industry has long recognized the need to reduce the quantities of marine feed ingredient in aquaculture diets.

Soybean meal is considered a reliable and cost-effective protein source for shrimp feeds. The reasons for this are the high protein content, high digestibility, relatively well-balanced amino acid profile, reasonable price, and steady supply (Amaya et al., 2007a, b; Baker 2000; Davis and Arnold, 2000). Among the common ingredients that have been investigated as fishmeal replacements, soybean meal has generally been successfully incorporated into shrimp formulations (Akiyama et al., 1989; Álvarez et al., 2007; Davis and Arnold 2000; Forster et al.,

2003; Hardy, 1999; Lawrence et al., 1986; Lim and Dominy, 1990, 1991, 1992; Mendoza-Alfaro et al., 2001; Paripatananont et al., 2001; Piedad-Pacual et al., 1990; Sookying and Davis, 2011, 2012). The protein digestibility value was found to be higher in soybean protein (96.4%) than that in marine animal meals such as fish meal (80.7%), shrimp meal (74.6%), and squid meal (79.7%) for *L. vannamei* (Akiyama et al., 1989). However, Lim and Dominy reported (1990) the weight gain of shrimp significantly decreased as levels of dietary soybean meal increased to 42% or higher, and the 70% soybean meal diet was utilized very poorly by the shrimp. Commodity soybean meal has a number of anti-nutritional factors that limit its inclusion in feed formulations (Liener, 2000). However, new strains of selectively-bred, non-genetically modified (non-GM) soybeans have reduced levels of oligosaccharides, lecithin, trypsin inhibitors, and/or enhanced levels of protein, which may support better growth, improved digestion, and afford higher substitutions in formulations for marine shrimp.

Digestibility data are very important for the formulation of suitable feeds. Nutrient digestibility information is indispensable for *L. vannamei* to improve the accuracy of a diet in formulation. Determining digestibility of food and feeds in animals requires collection of fecal material. Direct and indirect methods are used to collect feces. The indirect method of digestibility determination is widely used with most species of farmed fish and shrimp. This method relies on the collection of a representative sample of feces that is free of uneaten feed particles and the use of a nontoxic, inert, indigestible digestion indicator, such as chromic oxide or yttrium oxide. The term “Apparent digestibility coefficients” (ADC) is used to acknowledge the fact that values obtained using either the direct or indirect method are not corrected for endogenous gut losses (NRC, 2011). The indirect method of digestibility determination is widely used with most species of farmed fish and shrimp. This method relies on the collection of a

representative sample of feces that is free of uneaten feed particles and the use of a nontoxic, inert, ingestible digestion indicator, such as chromic oxide or yttrium oxide, added to feed (NRC, 2011). Apparent digestibility coefficients could be used to select ingredients that optimize the nutritional value and cost of formulated diets and also provide estimates of nutrient availability in foods. Apparent digestibility coefficients for dry matter, protein, lipid, energy, phosphorus, amino acids and chitin have been determined for *L. vannamei* (Akiyama et al., 1989; Brunson et al., 1997; Clark et al., 1993; Cruz-Suárez et al., 2009; Smith et al., 1985; Yang et al., 2009). According to the available research, there is lack of information on apparent digestibility coefficients for new varieties of soybean meal for *L. vannamei.*

Distiller's dried grains with solubles (DDGS) is also a potential protein source for shrimp feed due to its low price and consistent supply as a co-product of the bio-ethanol production which is expected to increase rapidly in the next decade. In 2001, the United States produced about 3.1 million metric tonnes of DDGS. The production of DDGS has increased rapidly from 16.4 million metric tonnes in 2006 to 35.3 million tonnes in 2010 (Renewable Fuels Association 2010). Therefore, DDGS has become a promising protein ingredient due to its low cost and abundant supply. The nutrient composition of DDGS contains crude protein, fat, ash, acid detergent fiber, and neutral detergent fiber ranging from 26.0-31.7%, 9.1-14.1%, 3.7-8.1%, 11.4-20.8%, and 33.1-43.9%, on dry matter basis, respectively (Cromwell et al., 1993). However, the protein quality of DDGS is poor because of the low level of some essential amino acid contents, particularly lysine (Liu and Rosentrater, 2012). Spiehs et al. (2002) reported that DDGS contained about 30% crude protein from 119 samples for 10 essential amino acids and that the average values of lysine, methionine, tryptophan, threonine, arginine, histidine, phenylalanine,

isoleucine, leucine, and valine were 0.85, 0.55, 0.25, 1.13, 1.20, 0.76, 1.47, 1.12, 3.55, and 1.50%, respectively.

Feed ingredients are selected and combined based on their nutritional content, cost and how they affect the physical characteristics of pellet. Feed manufacturing is the physical process of forming feed ingredient mixture into particles used to feed shrimp. Most commercial feeds are manufactured as pellets using cooked extrusion, compression pelleting, or cold extrusion processes (NRC, 2011). Pelleting is by far the most popular method of producing crustacean feeds due to its technological and economic advantages (Lovell, 1989, 1990; MacGrath, 1976). The advantages of pelleted feed include less bridging in bins, dust and feed waste, increased bulk density, nutrient density and nutrient availability, reduced ingredient segregation, decreased microbiological activity and improved palatability. While many processing technologies result in an agglomerated feed, only a few have sufficient energy inputs to ensure microbiological safety of feed. Feed safety is a major factor in choosing extrusion methods over traditional pelleting methods (Riaz, 2009).

Extruded pellets have advantages over steam pellets for they generally have superior water stability and floating properties, which allow direct determination of feed consumption (Stickney, 1979). Robinette (1977) reported that the extrusion processing has much greater levels of heat, moisture, and pressure than steam pelleting. These higher levels may increase the bioavailability of carbohydrate and destroy heat labile anti-nutrients. Compared to steam pelleting, extrusion processing can also damage nutrients such as ascorbic acid (Hilton et al., 1977; Lovell and Lim, 1978). Hilton et al. (1981) indicated that extruded pellets increased the gastric emptying time of trout which was probably responsible for the reduced total feed consumption and weight gain but may have improved feed efficiency. In the rainbow trout

(*Salmo gairdneri* R.) extrusion processing appeared to increase carbohydrate digestion and absorption, increase liver, body weight and percent liver glycogen content as compared with steam pelleting (Hilton et al., 1981). Although both systems have advantages, there is a lack of studies that evaluate the biological response of shrimp to typical practical diet that are processed using extrusion or pelleting technologies.

As fishmeal is substituted with alternative plant based protein sources, there are a number of nutrients that will change, including minerals. As compared to most other nutrient groups, information concerning mineral nutrition of shrimp is limited. Conducting research on mineral nutrition of aquatic species is relatively difficult. Problems associated with quantification of mineral requirements include identification of the potential contribution of minerals from the water, leaching of mineral from the diet prior to consumption, and availability of suitable test diets that have a low concentration of the targeted mineral (NRC, 2011). The metabolism of various minerals by aquatic organisms is influenced not only by dietary concentrations but also by the concentration and relative composition of dissolved ions in the aquatic medium, which may influence the organism's osmoregulation, ion regulation, and acid-base balance (Moyle and Cech, 2000).

Copper (Cu) is an essential element for all organisms including fish (Watanabe et al., 1997; Lorentzen et al., 1998). It functions in hematopoiesis and in numerous copper dependent enzymes including lysyl oxidase, cytochrome *C* oxidase (CCO), superoxide dismutase (SOD), ferroxidase, and tyrosinase (O'Dell, 1976). It is also important as a part of antioxidant enzymes (Lorentzen et al., 1998). Crustaceans utilize hemocyanin as the oxygen-carrying pigment. This copper-containing pigment has an analogous role to hemoglobin in red-blooded animals (Lovell, 1989).

Knowledge of bioavailability of supplemental copper sources plays a vital role in selection of a copper source in feed production (Miles et al., 1998; Spears et al., 2004; Luo et al., 2005). Copper sulfate (CuSO4) is the most common form of copper used in feeds for growth promotion. Chelated forms of various elements have also been found to be effective for some aquatic animals (Paripatananont and Lovell, 1995, 1997; Apines-Amar et al., 2004). Chelated minerals are widely utilized in the livestock and poultry industries; however, research concerning these compounds with respect to aquatic species such as fish has been very limited (Apines-Amar et al., 2003, 2004). Tri-basic copper chloride (TBCC) is a more concentrated form of copper than copper sulfate (58% vs 25% Cu). Because it has low hygroscopicity and is insoluble in neutral water, it should be a less reactive and less destructive form of copper when combined with vitamins in diets (Cromwell et al., 1998). Cromwell et al. (1998) indicated that TBCC was as effective as copper sulfate to improve growth for weanling pigs. Luo et al. (2005) reported that TBCC was a safer product and was more available than copper sulfate for broilers, and similar results were found in steers (Spears et al., 2004). According to the available research, there is a lack of information for evaluating the growth performance and bioavailability of two copper sources [copper sulfate pentahydrate ($CuSO_4$) and tri-basic copper chloride (TBCC)] for *L. vannamei*.

The long-term goal of this study is to use new varieties of soybean meal and Dried Distillers Grains with Solubles (DDGS) from sorghum (S-DDGS) as an alternative protein source in shrimp feed formulation to improve the sustainable development of aquaculture. To further optimize plant-based feeds, trace mineral supplements may also need to be optimized. Three specific objectives were included as follows:

1. Determine growth performance and digestibility coefficients for protein, energy and dry matter for new varieties of soybean meal in Pacific white shrimp juvenile *L. vannamei*.

2. Evaluate the effect of processing methods (extrusion and pelleting) on physical and nutritional characteristics of shrimp feed with different levels of S-DDGS.

3. Evaluate the growth performance, tissues response and bioavailability of two copper sources (copper sulfate pentahydrate and tri-basic copper chloride) for *L. vannamei* in practical feed formulations.

CHAPTER II

USE OF NEW SOYBEAN VARIETIES IN PRACTICAL DIETS FOR PACIFIC WHITE SHRIMP, *Litopenaeus vannamei*

Abstract

This study was designed to evaluate the efficacy of eight sources (designated A-H) of soybean meal (SBM) that included six new non-genetically modified soy varieties in practical feed formulation for *Litopenaeus vannamei*, using both growth and digestibility trials. A soybean meal-based reference diet was formulated by using conventional soybean meal (527 g kg^{-1} diet), which was then replaced on an iso-nitrogenous basis with various other experimental soybean meals. In a 6-week growth trial, shrimp in four replicate tanks per dietary treatment (10 shrimp/tank, initial weight 0.52 ± 0.04 g) were cultured in a recirculating system. There were no significant differences with respects to percent weight gain and survival across all dietary treatments; however, final weights and FCR were lower in shrimp offered diet 3. Apparent digestibility coefficients for the eight (A–H) different soybean meals were determined in *L. vannamei* for dry matter (ADMD), gross energy (ADE) and crude protein (ADP) using 10 g kg^{-1} chromic oxide as inert marker with 70:30 replacement techniques. Coefficients ranged from 71.3%–88.3%, 76.6%–91.3%, and 93.6%–99.8%, for ADMD, ADE, and ADP, respectively. Improved digestibility values were observed in soybean C, which was characterized by crude protein (471 g kg^{-1}), crude fat (97 g kg^{-1}), low cooking temperature (180 °C), higher nitrogen solubility index (689 g kg^{-1}), and protein dispersibility index (619 g kg^{-1}). This indicates that new lines of soybean meal can be used to improve digestibility coefficients in shrimp feeds.

1. Introduction

The use of cost-effective feed formulations for *L. vannamei* is critical to improving profit margins by reducing feed cost. The selection of appropriate feed ingredients should target not only cost reduction but also improving the nutritional quality of feeds and reducing metabolic waste while meeting all nutrient requirements of the rapidly growing shrimp. Ingredient digestibility is the measurement of the proportion of energy and nutrients that an animal can obtain from a particular ingredient through its digestive and absorptive processes (Glencross et al., 2007). Apparent digestibility coefficients can be used to select ingredients that optimize the nutritional value and cost of formulated diets and also provide estimates of nutrient availability in feeds. Therefore, the nutrient digestibility data of diets and feed ingredients are of utmost importance to nutritionists and feed formulators to optimize nutritional value and the cost of diets (Smith et al., 2007).

Although several studies of nutrient digestibility for shrimp feed have been documented (Akiyama et al., 1989; Brunson et al., 1997; Cruz-Suárez et al., 2009; Davis et al., 1993; Nieto-López et al., 2011; Smith et al., 2007, 1985; Terrazas-Fierro et al., 2010; Yang et al., 2009), information on digestibility coefficients for novel feed ingredients is indispensable to accuracy in dietary formulations as these ingredients become commercially available. Among the common ingredients that have been investigated as fishmeal replacements, soybean meal has generally been successfully incorporated into shrimp formulations (Akiyama, 1989; Álvarez et al., 2007; Amaya et al., 2007; Davis and Arnold, 2000; Lawrence et al., 1986; Lim and Dominy, 1990; Mendoza-Alfaro et al., 2001; Sookying and Davis, 2011, 2012; Zhu et al., 2013). Because of its steady supply, price and amino acid composition, soybean meal is one of the primary protein sources used today in animal feeds (Baker, 2000). Nevertheless, the nutritional value of

conventional soybean meal is often lower compared to that of fishmeal for penaeid shrimp (Zaldivar, 2002). Commodity soybean meal has a number of anti-nutritional factors that limit its inclusion in feed formulations (Liener, 2000). However, new strains of selectively-bred non-genetically modified (non-GM) soybeans can have reduced levels of oligosaccharides, lectins, trypsin inhibitors and/or enhanced levels of protein that may afford higher substitutions in formulations for marine shrimp. As explained before, digestibility coefficients for new varieties of soybean meal in practical diets for *L. vannamei* are presently unavailable as is an assessment of the biological responses of penaeid shrimp to these novel feed ingredients. Therefore, the objective of the present study was to determine growth performance and digestibility coefficients for protein, energy and dry matter for new varieties of soybean meal in Pacific white shrimp juvenile *L. vannamei*.

2. Materials and Methods

Eight sources of soybean meal, including six new varieties of non-GM soybean meal were obtained for the evaluation of their potential as an ingredient in aquaculture feeds for *L. vannamei*. These ingredients were characterized and then used in two experiments, including both growth and digestibility trials. Commodity soybean meal A was obtained from Faithway Feed Co., LLC, Guntersville, AL. The non-GM soybean meals were donated by Navita Premium Feed Ingredients (NPFI), West Des Moines, IA, USA. These were genetically unique, patented non-GM soy cultivars that contained increased levels of protein and amino acids and reduced levels of some anti-nutritional factors. Beans of different cultivars (B–H) were produced in Iowa, Illinois, Indiana and Maryland, USA, and made into meals by conventional processing methods.

A complete chemical characterization of each meal is provided in Table 3 for dry matter, moisture, fiber, fat, crude protein and ash (Eurofins Scientific, Inc. through NPFI).

2.1. Growth trial

Eight plant-based diets (Table 1) using soybean meals as the primary protein source were utilized to evaluate the biological response of shrimp to the various dietary treatments. The growth trial was conducted with juvenile shrimp reared over a 6-week period in a low salinity, indoor recirculating culture system. Each test diet was offered to four replicate tanks of shrimp.

Research was conducted at the E.W. Shell Fisheries Research Station (EWS), Auburn, AL, USA. Pacific white shrimp, *L. vannamei*, post larvae were obtained from Shrimp Improvement Systems (Islamorada, FL). At the conclusion of the nursery phase, juvenile shrimp (0.52 ± 0.04 g) were hand-sorted for uniform size and stocked into 40 aquaria (60 L) at a density of 10 shrimp $tank^{-1}$.

To minimize shrimp losses due to jumping, each aquarium was covered with a plastic plate. Each tank was provided with one air-stone. Tanks were filled with reconstituted seawater, and culture water was circulated throughout the system at a rate of 3.6 L min^{-1} to provide one full turnover of water exchange approximately every hour. Water temperature, dissolved oxygen (DO) and salinity were monitored twice daily (0830 and 1630) using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH) and were maintained within acceptable levels for *L. vannamei* at 28.28 ± 1.55 °C, 6.42 ± 0.41 mg L^{-1} and 3.99 ± 1.01 g L^{-1}, respectively.

Experimental diets for the growth trial were prepared at the Aquatic Animal Nutrition Laboratory of the School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University (Auburn, AL, USA), using standard procedures for the laboratory production of shrimp feeds to

contain 360 g kg^{-1} protein and 80 g kg^{-1} lipid. Pre-ground dry ingredients and oil were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 10–15 min. Hot water was then blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted by using a meat grinder with a 3-mm die, air dried (< 50˚C) to a moisture content of 8–10%. After drying, pellets were crumbled, packed in sealed plastic bags and stored in a freezer (-20˚C) until use. Dietary treatments were randomly assigned, and each experiment was conducted using a double blind experimental design. The protein value for the diets was confirmed, ranging from 342 to 357 g kg^{-1} diet on an as is basis.

Shrimp were fed four times daily. Based on historic results, feed inputs were determined assuming a weight gain of 0.8 g per week and feed conversion ratio (FCR) of 2. Shrimp were counted each week to adjust rations for mortality. At the conclusion of the 6-week growth trial, shrimp were counted and group-weighed. Mean final weight, final biomass, percent survival, and feed conversion ratio were determined.

2.2. Digestibility trial

Apparent digestibility coefficients for dry matter, protein and energy in eight sources of soybean meal were determined by using chromic oxide (Cr_2O_3, 10 g kg^{-1}) as an inert marker. The reference diet (Table 2) was formulated to contain 280 g kg^{-1} crude protein and 80 g kg^{-1} lipid. The test diets (70:30 mixture of reference diet and test ingredient), which contained 322–391 g kg^{-1} protein and 80 g kg^{-1} lipid, were produced using the previously mentioned technique. Proximate composition, amino acid, and carbohydrate profiles of eight sources of soybean meal are shown in Table 3.

Four replicate groups of 10 shrimp (~ 8.8 g mean weight) were stocked in a closed recirculating system consisting of sixteen 60-L plastic tanks, biological filter, reservoir, circulation pump, and supplemental aeration. Shrimp were allowed to acclimate to each diet for 3 d. before starting the collection of feces. Feces were collected for 5–7 d. Prior to each feeding the tanks were cleaned by siphoning. The shrimp were then offered an excess of feed. One hour after offering the feed, feces were collected by siphoning through a 500 µm mesh screen. Shrimp were offered five feedings per day. To ensure the fecal strands were from the current day's feed, feces obtained after the first feeding were discarded. Collected feces were rinsed with distilled water, sealed in plastic containers and frozen (-20 °C). Samples from four replicate tanks were kept separate and frozen until analyzed. Samples were dried by placing each sample in an oven at 105 °C until a constant weight was obtained. Gross energy of diets and fecal samples were analyzed with a semi micro-bomb calorimeter (Model 1425, Parr Instrument Co., Moline, IL, USA). Chromic oxide concentrations were determined by the method of McGinns and Kasting (1964) in which, after a colorimetric reaction, absorbance was read on a spectrophotometer (Model 4001/4, Thermo Spectronic Genesys 20, Thermo Fisher Scientific, FL,) at 540 nm. Protein was determined by micro-Kjeldahl analysis (Ma and Zuazago, 1942). All sample analyses were conducted in triplicate. The ADC of energy and dry matter of each diet and the ADC of energy for the feed ingredients were determined using standard formulas (Cho et al., 1982). The ingredient ADC of energy was adjusted according to Forster (1999).

The apparent digestibility coefficients for dry matter (ADMD), protein (ADP) and energy (ADE) were calculated according to Maynard and Loosli (1969) and Hardy and Barrows (2002), as follows:

$$\text{ADMD (\%)} = 100 - \left[100 \times \left(\frac{\%\ Cr_2O_3 \text{ in feed}}{\%\ Cr_2O_3 \text{ in feces}}\right)\right]$$

$$\text{ADP and ADE (\%)} = 100 - \left[100 \times \left(\frac{\%\ Cr_2O_3 \text{ in feed}}{\%\ Cr_2O_3 \text{ in feces}} \times \frac{\%\ \text{nutrient feces}}{\%\ \text{nutrient feed}}\right)\right]$$

2.3. Statistical analysis

Statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC, USA). Data was analyzed by using one-way analysis of variance to determine if significant differences existed among treatment means. Duncan's multiple range tests were used to identify significant differences between treatment means. Correlation and regression analyses were used to explore possible interactions of chemical properties of the soybean meals with digestibility values. All statistical tests were considered significant at $P < 0.05$.

3. Results

The overall means for water quality variables observed over a 6-week trial were maintained within suitable ranges for growth of this species. Water temperature ranged from 24.60 to 31.70 °C with an average of 28.28 ± 1.55 °C, salinity varied from 2.5 to 5.8 g L^{-1} with an average of 3.99 ± 1.01 g L^{-1}, and dissolved oxygen ranged from 5.50 to 7.20 mg L^{-1} with an average of 6.42 ± 0.41 mg L^{-1}. Results from the growth trial reveal that final biomass, final mean weight, percent weight gain, FCR and survival were typical for this system (Table 4). Final biomass ranged from 29.2 to 38.7 g, mean final weight from 3.9 to 4.4 g, survival from 72.5 to 97.5 %, FCR 2.1 to 2.8, and percent weight gain from 599.0 to 771.9%. In regards to survival and percent weight gain there was no significant difference among any of the treatments. There

was minor difference (P = 0.037) between diet 3 and other diets, and no significant differences among other diets on final mean weight and FCR.

Apparent digestibility coefficients for the reference diet, test diets, and ingredients are presented in Table 5. Determined coefficients for the experimental diets ranged from 71.95% to 77.06% for ADMD, 88.95% to 92.97% for ADE, and 77.57% to 82.14% for ADP (Table 5). Interestingly, ADMD, ADP, ADE coefficients in the reference diet (RD) were significantly lower than reference diet with soybean meal C (RDC) and reference diet with soybean meal H (RDH).

Apparent dry matter digestibility coefficients for the test ingredients ranged from 71.26% to 88.26% (Table 5). Significant differences ($P < 0.05$) in ADMD of the test ingredients were observed. For the high-fiber (56 g kg^{-1}) ingredient B, ADMD was lowest and significantly so as compared to ADMD values for soybean meal A, C and H. A tendency was observed for ADMD values to be lower in ingredients with high fiber content. Apparent crude protein digestibility coefficients in the ingredients ranged from 93.64% to 99.79% (Table 5) and that of meal C was highest among all ingredients tested, being significantly higher than ADP of meals A, D and E. The ADE coefficients in test ingredients ranged from 76.62% to 91.34% (Table 5). The ADE coefficient of soybean meal B was lowest among all tested ingredients and was significantly lower than ADE of meals A, C and H.

4. Discussion

Dietary protein plays a vital role affecting growth performance and feed cost (Hu et al., 2008). Consequently, highly digestible protein sources would make it easier for shrimp to assimilate protein and presumably grow faster than shrimp fed marginally digestible feed

ingredients. Similarly, higher protein availability in shrimp feeds would facilitate higher protein deposition into tissues and decrease excretion of nitrogen metabolites into the environment. Because the proportion of nutrients that are absorbed in a form readily utilizable by shrimp is affected by, among other things, anti-nutrients, it is possible that selectively bred soybean varieties with reduced levels of anti-nutritional factors may afford an increased bioavailability of nutrients to shrimp. This would make possible a higher inclusion level in feeds than conventional commodity soybean meal which, in turn, could reduce the overall cost of shrimp feed formulations.

In the present study only minor differences in final mean weight and final biomass and no significant differences in percent weight gain and survival were observed. This indicates that all diets containing the various sources of soybean meal were able to support reasonable growth and survival of shrimp over a 6-week culture period. There were significant differences in mean final weights (Table 4), with diet 3 producing the smallest shrimp (3.4 g) and diet 4 the largest (4.4 g) shrimp. It is interesting to note the poorest growth and highest survival rate was observed in shrimp fed diet 3, which utilized meal C. This meal also had the highest digestibility values (ADP 99.8%) as compared to other meals. These results confirm that digestibility and growth do not always go hand in hand. The improved growth performance of shrimp maintained on diets 4 using ingredient D, may be due to the reduced level of trypsin inhibitors (52,100 TIU vs 19,500 TIU in meal C vs D, respectively) and relatively lower levels of oligosaccharides (58.5 g kg^{-1} vs 50.7 g kg^{-1} combined raffinose and stachyose in the meal C and D, respectively), which may have influenced growth. These results indicate that the composition of soybean meal can influence growth and consequently the development of soybean lines with modified nutrient profiles is warranted.

The apparent dry matter digestibility coefficients provide a measure of the total quantity of an ingredient that is digested and absorbed. Because not all the components of a feedstuff are digested equally, ADMD coefficients, by definition provide an estimate of the quantity of indigestible materials present in a feed ingredient (Brunson et al., 1997). The ADMD coefficients for different soybean meal sources in this study with *L. vannamei* ranged from 71.26% to 88.26%. In contrast, Akiyama et al. (1989) reported an ADMD value for soybean meal of only 55.9% for the same species using nutritionally incomplete diets. More in agreement with results from the present study, Divakaran et al. (2000) reported ADMD values for de-hulled, solvent-extracted, toasted soybean meal as ranging from 61.2% to 84.7%. More recently, Cruz-Suárez et al. (2009) also obtained ADMD values with *L. vannamei* for four different soybean products ranging from 82.7% to 91.7%. Values for ADMD obtained in the present study are in general agreement with those observed in previous studies. Slight differences in these values may be due, in part, to differences in the size of shrimp utilized, specific environmental/experimental conditions and/or differences in ingredients/diets manufacturing process. An important observation of the current study is that the ADMD of feed consumed by shrimp tended to decrease as fiber and ash content of the test ingredient increased. Accordingly, the ADMD coefficient in meal B (high fiber) was significantly lower than ADMD values for meals A, C and H.

In contrast with dry matter digestibility values, those for apparent crude protein digestibility were relatively higher (93.64–99.79%) for the test ingredients. The ADP values from soybean meal by *L. vannamei* are similar to those reported for the same species by Akiyama et al. (1989; 89.9%–96.4%), Ezquerra et al. (1997; 91.0%), Ezquerra et al. (1998; 85.9%), Divakaran et al. (2000; 91%–102.2%), Siccardi et al. (2006; 96.9%), Cruz-Suárez et al.

(2009; 93–96%), Liu et al. (2013; 92.3%), and Zhu et al. (2013; 78.8–89.8%). Observed differences in ADP among experimental meals could be attributed to several factors including processing, presence of trypsin inhibitors as well as differences in nitrogen solubility indices (NSI), and protein dispersibility indices (PDI). Trypsin inhibitors, which constitute approximately 6% of the total protein of soybeans (Kakade et al., 1973), have generally been known to cause growth depression and pancreatic hypertrophy in numerous experimental animals (Lim and Akiyama 1991). Negative effects on protein digestibility have been attributed to trypsin inhibitors due to their ability to bind to digestive enzymes (Francis et al., 2001). However, out of all shrimp species cultivated in the world only very few like the Indian white shrimp, *Penaeus indicus*, have been reported to exhibit digestive sensitivity to soy-derived protease inhibitors (Osmondi, 2005).

To elucidate such interactions in the present study, correlation analysis followed by regression analysis was utilized. We observed that ADP values had a direct relationship with the presence of trypsin inhibitor inhibitors ($P < 0.05$) as well as with measured NSI ($P < 0.1$) and PDI ($P < 0.3$) values (Table 6). PDI measures the dispersibility of proteins in water and is generally used as a quality parameter of protein denaturation upon thermal processing of soybean (Qin et al. 1996). PDI is often used to characterize the protein quality of raw materials. PDI has been demonstrated to be a simple and effective procedure in assessing the quality of heat-treated soybeans (Hsu and Satter, 1995). Batal et al. (2000) suggested that PDI is a more consistent and sensitive indicator of minimum adequate heat processing of soybean meal than urease index or protein in KOH. For example, a SBM containing low urease (0.3 or below) and high PDI (40 to 45 %) may indicate that the sample is high quality because it has been adequately heat processed, but not over-processed (Căpriţă et al., 2010). The PDI values for meals B, E, and F subjected to

cooking temperature of 320 ˚C were much lower than those for meals C and D with processing temperature of 180 ˚C. In this study, the trend of PDI values, which decreased with increased temperature, is similar to those reported for soybean meal by Qin et al. (1996). The processing temperature (e.g., cooking and drying) is of particular importance, and the occurrence of protease inhibitors can be attributed to insufficient heat during processing (Swick, 2000; Genovese et al., 2006). Heat treatment of soybean meal can reduce levels of some anti-nutrients (e.g., protease inhibitors, chitosan inhibitors, lectins, goitrogens, anti-vitamins, glycinin and β-conglycin). However, significant levels of heat-resistant anti-nutritional factors may remain in conventional soybean meal (e.g., saponins, lipase inhibitors, tannins, phyto-estrogens, oligosaccharides, antigens and phytate) (Francis et al., 2001).

In contrast to simple processing, marker-assisted soy breeding programs can selectively discriminate among varieties that have high levels of either heat-labile or heat resistant anti-nutrients such that the resulting meals – produced in the conventional manner – have less of these noxious compounds. Trypsin inhibitor values for meals B, E, and F subjected to cooking temperature of 320 ˚C were much lower than those for meals C and D with processing temperature of 180 ˚C, corroborating the heat-labile nature of these inhibitors. With regard to the response to trypsin inhibitor, there was a poor correlation with ADP and ADE values (Fig.1). As this experiment was not designed to look specifically at trypsin inhibitor, it is not surprising that other factors may affect the results. For example, the sources and processing of the soy are not controlled. Furthermore, the level of trypsin inhibitor reported is that of the ingredient and not the final diet. Hence, one has to realize that multiple factors are at play. The high digestibility of diet 3 could be due to other factors. For example, as this source was processed at lower

temperatures, it also has the highest PDI value. Given that PDI values are often correlated with digestibility, this may be the overriding factor.

Based on the results from Table 5, differences in energy digestibility among ingredients were similar to differences in ADMD value. Both ADMD and ADE coefficients were significantly ($P < 0.05$) lower for meal B than for meals A, C and H. The lowest ADE value was found to be for meal B which, excluding meal E, subsequently had the highest fiber contents (Table 3, 56 g kg^{-1}) compared to other ingredients. This observation is in agreement with the report by Brunson et al. (1997), in which energy digestibility of plant products used for aquatic animals was also found to be inversely related to the fiber content of the materials and in some species to starch content. Fiber could potentially contribute to the gross energy of some animals, but monogastric organisms such as shrimp are unable to digest it. Because the energy in fiber is typically unavailable for cultured marine organisms, ADE values of a diet tend to decrease as fiber content increases (Lech and Reigh, 2012).

To summarize the present experiments, there were no significant differences in survival or percent weight gain among treatments; albeit, there were minor differences in final weight in the growth trial. In the digestibility trial, meal C displayed the highest ADMD and ADP values compared to the other experimental meals, and also exhibited a significantly higher ADP value than conventional soybean meal A. Thus, meal C may hold a high potential as a shrimp feed ingredient. In general, the selection of new lines of soybean on biochemical properties is promising and could lead to improvements in the nutritional value of soybean meals for shrimp and other species.

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Table 1 Ingredient compositions (g kg^{-1} of feed) of experimental diets used in a 6-week growth trial. All diets were developed to contain 360 g kg^{-1} protein and 80 g kg^{-1} lipid. Diets were designed to use various soybean meals produced from different varieties of soybeans on an equal protein inclusion level.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8
Source	A[1]	B[2]	C[2]	D[2]	E[2]	F[2]	G[2]	H[3]
Soybean meal	527	550	592	572	520	490	468	448
Menhaden fish meal [4]	60	60	60	60	60	60	60	60
Menhaden Fish Oil [4]	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7
Corn Gluten meal [5]	80	80	80	80	80	80	80	80
Soy oil [7]	59	22.9	-	4.9	27.9	36.3	26.4	61.2
Corn Starch [6]	79.8	92.9	73.8	88.9	117.9	139.5	171.4	156.6
Whole wheat [8]	130	130	130	130	130	130	130	130
Mineral premix [9]	5	5	5	5	5	5	5	5
Vitamin premix [10]	20	20	20	20	20	20	20	20
Stay C 250 mg kg^{-1} [11]	1	1	1	1	1	1	1	1
CaP-diebasic [8]	25	25	25	25	25	25	25	25
Lecithin, soy [12]	10	10	10	10	10	10	10	10
Cholesterol[8]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

[1] Faithway Feed Co., Guntersville, AL, USA.
[2] Navita Premium Feed Ingredients, Inc., West Des Moines, Iowa, USA
[3] Navita 3010 (high protein low oligosaccharides meal). Rose Acre Farms, Seymour, IN, USA
[4] Omega Protein Inc., Reedville, VA, USA.
[5] Grain Processing Corporation, Muscatine, IA, USA.
[6] Empyreal 75. Cargill corn milling North America, Blair Nb, USA
[7] ADM Co., ProPlex™-DY, Decatur, IL, USA.
[8] MP Biochemicals Inc., Solon, OH, USA.
[9] Trace mineral premix without Mg (g kg^{-1}): cobalt chloride 0.04, cupric sulphate pentahydrate 2.50, ferrous sulfate 40, manganous sulphate monohydrate 6.50, potassium iodide 0.67, sodium selenite 0.10, zinc sulfate heptahydrate 131.93, filler 818.26.
[10] Vitamin premix (g kg^{-1}): thiamin HCl 0.5, riboflavin 3.0, pyridoxine HCl 1.0, DL Ca-pantothenate 5.0, nicotinic acid 5.0.
[11] Stay C®, (L-ascorbyl-2-polyphosphate), Roche Vitamins Inc., Parsippany, NJ, USA.
[12] Enhance D-97, Solae Company, St. Louis, MO, USA

Table 2 Formulation of the reference diet for determination of digestibility coefficients in *Litopenaeus vannamei*

Ingredients	Amount (g kg^{-1} diet dry weight)
Menhaden fish meal[1]	100.0
Soybean meal[2]	310.0
Menhaden fish oil[1]	41.2
Whole wheat[3]	496.6
Trace mineral premix[4]	5.0
Vitamin premix w/o choline[5]	18.0
Choline cloride[6]	2.0
Stay C 250 mg kg^{-1} [7]	0.7
Chromic oxide[6]	10.0
Lecithin[8]	15.0
Cholesterol[3]	1.5

[1] Omega Protein Inc., Reedville, VA, USA.
[2] Faithway Feed Co., Guntersville, AL, USA.
[3] MP Biochemicals Inc., Solon, OH, USA.
[4] Trace mineral premix without Mg (g kg^{-1}): cobalt chloride 0.04, cupric sulphate pentahydrate 2.50, ferrous sulfate 40, manganous sulphate monohydrate 6.50, potassium iodide 0.67, sodium selenite 0.10, zinc sulfate heptahydrate 131.93, filler 818.26.
[5] Vitamin premix (g kg^{-1}): thiamin HCl 0.5, riboflavin 3.0, pyridoxine HCl 1.0, DL Ca-pantothenate 5.0, nicotinic acid 5.0.
[6] Fisher Scientific, Fair lawn, NJ, USA
[7] Stay C®, (L-ascorbyl-2-polyphosphate 350 g kg^{-1}), Roche Vitamins Inc., Parsippany, NJ, USA.
[8] Enhance D-97, The Solae Company, St. Louis, MO, USA

Table 3 Proximate composition, amino acid and carbohydrate profiles of the experimental ingredients[1]

Composition (g kg^{-1})	A	B	C	D	E	F	G	H
Cooking Temp (°C)	-	320	180	180	320	320	285	-
Moisture	111	55	102	105	64	64	48	-
Crude Protein	544	483	471	489	515	547	562	561
Crude Fat	16	70	97	92	65	53	76	-
Crude Fiber	41	56	51	54	56	42	33	26
Acid Detergent Fiber	45	59	56	61	60	50	38	-
Netural Detergent Fiber	73	89	98	92	92	75	57	-
Phosphorus	8	5	5	6	6	6	6	7.3
Calcium	3	3	3	2	4	3	2	-
Trypsin inhibitor (TIU)[3]	-	5700	52100	19500	5700	5000	25400	-
NSI [2]	168	194	689	590	131	121	326	-
PDI [3]	183	75	619	585	71	72	287	-
Amino acids								
Alanine	23.7	21.7	20.5	21.1	22.0	22.6	24.1	22.9
Arginine	39.6	36.0	34.3	36.6	38.0	41.1	41.7	41.9
Aspartic acid	59.9	56.0	53.0	54.8	56.8	60.8	64.3	63.4
Cysteine	7.0	7.2	7.0	6.7	6.8	7.3	8.0	8.1
Glutamic acid	96.2	86.9	82.4	86.0	87.7	94.1	99.3	103.5
Glycine	23.1	20.8	13.3	20.4	21.3	22.2	23.3	-
Histidine	15.1	12.3	11.7	12.6	13.0	13.5	14.2	14.5
Hydroxylysine	1.4	0.2	0.2	0.2	0.2	0.2	0.2	-
Isoleucine	25.7	23.6	22.5	22.3	23.6	25.0	25.9	25.1
Leucine	42.2	37.5	35.5	37.2	38.5	40.5	42.9	42.7
Lysine	34.9	31.2	30.2	30.8	31.3	32.6	35.3	34.5
Methionine	7.4	7.1	6.8	6.7	7.1	7.3	7.8	7.6
Ornithine	0.6	0.3	0.2	0.2	0.3	0.3	0.3	-
Phenylalanine	28.7	24.7	23.4	24.1	25.1	26.4	27.9	28.3
Proline	26.0	24.0	22.7	24.1	24.7	26.4	28.1	27.6
Serine	23.0	20.3	19.5	21.6	21.4	22.2	25.2	24.1
Threonine	21.4	17.9	17.1	18.2	18.6	18.9	20.9	20.9
Tryptophan	7.9	6.6	6.0	7.8	6.5	7.0	7.3	7.0
Tyrosine	20.5	16.8	16.5	17.2	17.6	17.9	19.6	20.4
Valine	27.1	24.8	23.4	23.6	24.9	26.4	27.0	26.3
Carbohydrates								
Maltose	3.5	2.8	1.6	1.9	1.2	2.7	2.5	-
Raffinose	10.5	9.3	9.8	6.7	1.6	2.3	8.2	-
Stachyose	60.7	49.9	48.7	44.0	6.8	14.2	59.8	-
Sucrose	86.5	79.9	79.1	74.1	85.5	76.6	61.9	-

[1] Ingredients were analyzed by Eurofins Scientific, Inc. Nutrition Analysis Center, Des Moines, IA 50321. Ingredient A is traditional soybean meal, Ingredient B-H are new varieties soybean meal.
[2] NSI nitrogen solubility index.
[3] PDI protein dispersibility index.

Table 4 Response of juvenile *L. vannamei* (0.52 g initial weight) offered a plant based diet using various types of soybean meal over a 6-week culture period[1]

Diet	Final Biomass (g)	Final mean weight (g)	Weight gain (%)	FCR	Survival (%)
1	37.8^{ab}	4.3^{a}	698.1	2.2^{b}	87.5
2	30.7^{c}	4.0^{a}	668.4	2.4^{b}	77.5
3	33.0^{abc}	3.4^{b}	603.1	2.8^{a}	97.5
4	38.7^{a}	4.4^{a}	771.9	2.1^{b}	87.5
5	29.2^{c}	4.1^{a}	686.9	2.4^{b}	72.5
6	31.6^{bc}	3.9^{ab}	599.0	2.5^{ab}	82.5
7	29.9^{c}	4.1^{a}	689.3	2.3^{b}	72.5
8	32.3^{abc}	4.2^{a}	738.1	2.3^{b}	77.5
PSE[2]	2.05	0.20	48.27	0.14	5.95
P-value	0.0216	0.041	0.207	0.037	0.082

[1]Mean of quadruplicate. Base on Duncan test, Number within the same column with different superscript are significant different (P <0.05).
[2] Pooled Standard Error.

Table 5 Apparent dry matter digestibility (ADMD), apparent protein digestibility (ADP), and apparent energy digestibility (ADE) in a trial with shrimp offered a reference diet (RD) or test dies of the RD (70%) and one of eight sources of soybean meal (30%)[1].

	Test diet			Ingredient		
	ADMD-D	ADP-D	ADE-D	ADMD-I	ADP-I	ADE-I
Reference diet	72.2±0.6^{c}	89.0±1.0^{c}	77.9±0.6^{b}			
RD-A	76.1±1.5^{ab}	90.9±1.6^{b}	82.0±1.0^{a}	85.2±4.9^{ab}	93.8±3.9^{b}	90.4±3.0^{a}
RD-B	72.0±1.6^{c}	92.3±1.9^{ab}	77.6±1.3^{b}	71.3±5.3^{c}	96.9±4.6^{ab}	76.6±4.6^{b}
RD-C	77.1±0.7^{a}	93.0±0.5^{a}	82.1±0.4^{a}	88.3±2.2^{a}	99.8±1.3^{a}	90.6±1.1^{a}
RD-D	73.2±2.3^{bc}	91.0±0.9^{b}	79.6±2.0^{ab}	75.4±7.6^{bc}	94.2±2.3^{b}	83.2±6.4^{ab}
RD-E	73.6±4.1^{bc}	90.9±1.6^{b}	79.1±3.4^{b}	76.8±13.6^{abc}	93.6±3.9^{b}	82.2±12.3^{ab}
RD-F	74.8±2.3^{abc}	91.9±0.9^{ab}	80.2±2.0^{ab}	80.9±7.6^{abc}	95.7±2.1^{ab}	85.4±6.5^{ab}
RD-G	73.2±1.9^{bc}	91.8±0.6^{ab}	78.9±1.6^{b}	75.3±6.4^{bc}	95.6±1.3^{ab}	81.4±5.4^{ab}
RD-H	76.2±2.0^{ab}	92.7±0.8^{ab}	82.1±1.7^{a}	85.5±6.6^{ab}	97.7±1.9^{ab}	91.3±5.4^{a}
PSE[2]	0.35	0.20	0.29	1.31	0.52	1.12
P-value	0.013	0.002	0.003	0.035	0.075	0.030

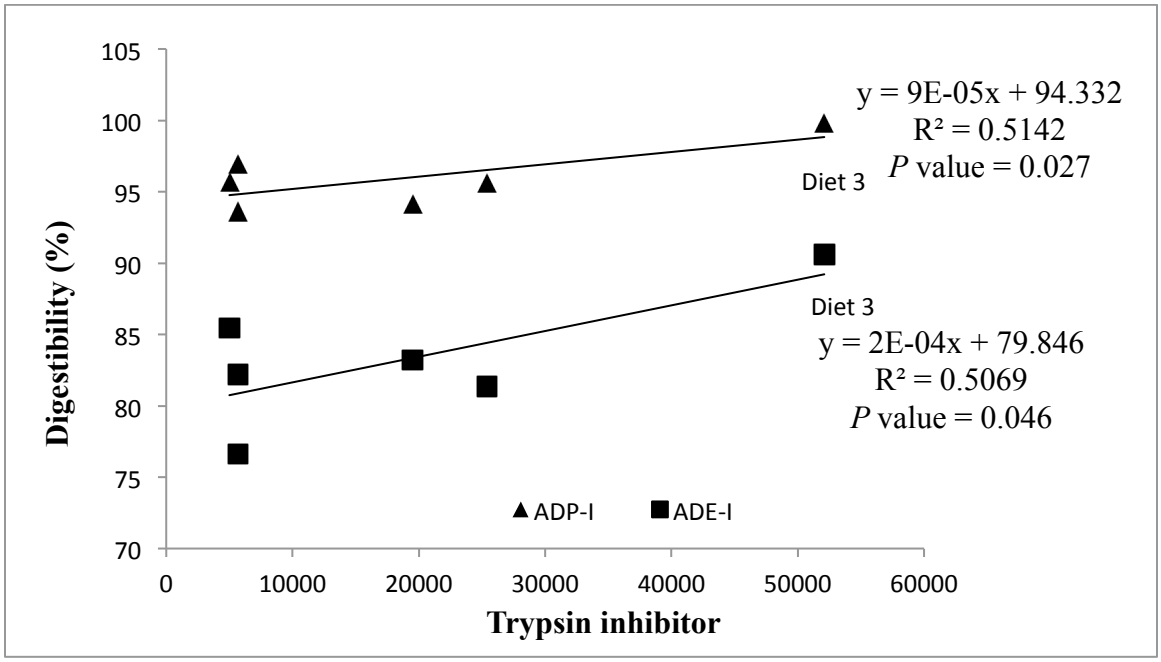
[1] Mean of quadruplicate. Base on Duncan test, Number within the same column with different superscript are significant different ($P < 0.05$).
[2] Pooled Standard Error.

Table 6 *P*-values from correlation analysis of apparent digestibility of dry matter (ADMD), energy (ADE), and crude protein (ADP) with the chemical characteristics of the ingredient.

	P- Value		
	ADMD	ADE	ADP
Cooking Temp	0.155	0.096	0.297
Moisture	0.025	0.009	0.974
Protein (DB[1])	0.988	0.815	0.106
Oil (DB)	0.562	0.480	0.092
Crude Fiber (DB)	0.518	0.435	0.729
Acid Detergent Fiber (DB)	0.518	0.469	0.778
Neutral Detergent Fiber (DB)	0.716	0.853	0.342
Trypsin inhibitor (TIU[2])[3]	0.031	0.046	0.027
NSI	0.324	0.301	0.091
PDI	0.238	0.178	0.202
Phosphorus (DB)	0.298	0.136	0.043
Calcium (DB)	0.806	0.973	0.734
Sucrose	0.256	0.306	0.616
Maltose	0.911	0.817	0.614
Stachyose	0.765	0.675	0.444
Raffinose	0.452	0.469	0.204

[1] DB = Dry matter basis.
[2] TIU = trypsin inhibitor units as defined by Kakade et al. (1969).

Figure 1 Relationship between ADCs of crude protein and energy versus trypsin inhibitor for differences soybean meal.



CHAPTER III

UTILIZATION OF SORGHUM CO-PRODUCT (S-DDGS) IN AQUATIC ANIMAL FEED PRODUCTION FOR PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

Abstract

Increasing cost of fish meal and declining price of shrimp has necessitated the search for alternative sources of protein, which is the most expensive aquatic feed ingredient. This study examined the use of distiller dried grain with solubles (DDGS) from sorghum (S-DDGS) in the production of sinking shrimp feed. Five shrimp feeds were formulated with increasing percentages of S-DDGS (0 g kg^{-1}, 100 g kg^{-1}, 200 g kg^{-1}, 300 g kg^{-1} and 400 g kg^{-1}) and then extruded and pelleted. Various physical properties of feeds were significantly influenced by S-DDGS inclusion. Specific mechanical energy (SME) generally increased with increasing levels of S-DDGS in the formulation. Bulk density of extruded and pelleted feed varied from 0.53 – 0.58 g cm^{-3} and 0.61 – 0.65 g cm^{-3}, respectively. With the exception of the extruded feed with the highest level of S-DDGS (88% sink) all feeds were characterized as close to 100% sinking. Pellet durability index (PDI) of extruded and pelleted feed did not show any particular pattern in the unmodified test, whereas both demonstrated an increasing trend, up to 20 g kg^{-1} and 30 g kg^{-1} DDGS for the extruded and pelleted feed, respectively in the modified test. The proximate content of the feeds shifted as S-DDGS levels increased, particularly with respect to crude fiber and starch. Degree of gelatinization generally increased with S-DDGS, and extruded feeds were better gelatinized than pelleted feeds. Water stability for extruded and pelleted feed ranged from 76.2–91.57% and 80.46–85.04%, respectively, and was significantly influenced by duration in water and level of S-DDGS. The five extruded and pelleted feeds were then evaluated in two

growth trials. In the first trial, these treatments were assigned amongst 40 tanks (60 L) with four replications per treatment for 9 weeks. In the second trial, the same treatments were assigned amongst 60 tanks (60 L) with six replications per treatment for 6 weeks. In both trials, juvenile shrimp (initial weight 0.36–0.38g) were stocked at a density of 10 shrimps per tank. In both growth trials there were no significant differences in final mean weight and survival of the shrimp due to the level of S-DDGS within extruded or pelleted feeds. Based on pooled data, extruded feeds produced significantly larger shrimp and lower feed conversion ratio in trial 1; however, pelleted feeds produced significantly larger shrimp and lower feed conversion ratio in trial 2. Under the reported conditions there were limited differences in performance of the shrimp due to processing or level of S-DDGS inclusion of the shrimp feed.

1. Introduction

The number of countries engaged in aquaculture production of shrimp has increased from 33 in 1984 to 62 in 2001 (Menon and Paul, 2001). Shrimp culture is the major aquaculture industry in many countries, accounting for approximately 3.49 million metric tonnes of the global production with a worldwide value over 14 billion dollars in 2009 (FAO, 2011). Shrimp is the most consumed seafood in the United States. The projection is that demand will continue to increase in the US and abroad (Johnson, 2003). Presently, increasing demand has driven up the cost of protein sources such as fish meal (FM), leading to increased production cost of raising shrimp, while shrimp prices generally continue to decline due to depressed market or overproduction (Amaya et al., 2007). Increasing feed costs is often attributed to the cost and inclusion level of fish meal (El-Sayed, 1999; Garza deYta et al., 2012; Hardy, 2006;). There is a concerted ongoing effort to replace expensive protein sources such as fish meal with other less expensive protein sources in shrimp feed production (Amaya et al., 2007; Forster and Dominy, 2006; Garza et al., 2012). Some studies have explored the use of plant ingredients for replacing expensive animal sources of ingredients (Davis and Arnold, 2000; Gatlin et al., 2007). Soy protein in the form of defatted soybean meal or soy protein concentrate has been used to replace fish meal with comparable results (Paripatananont, et al., 2001; Sookying and Davis, 2012). Clemente and Cahoo (2009) reported that the price of soybean has increased 65% in one decade from $158.3 per metric tonnes in June 1999 to $445.2 per metric tonnes in June 2009. This has drawn attention to other sources of plant protein such as sorghum DDGS, a co-product of ethanol production from sorghum.

Grain sorghum is commonly used as an ingredient in diets for livestock, such as poultry, cattle and swine, and has been studied widely for these applications (Cohen and Tanksley, 1973;

Huck et al., 1998; Kyarisiima et al., 2004; Madacsi et al., 1988). Distillers dried grains with solubles (DDGS) is a co-product of the ethanol industry that contains moderate quantities of protein that could serve as replacement for fish meal in aquatic feed. There has been an increase in the number and capacity of ethanol plants where sorghum DDGS is a major co-product. Sorghum DDGS compared to DDGS from other grains has a relatively higher level of protein (30.3%), fat (12.5%) and fiber (10.7%). The use of plant protein as replacement for animal protein (primarily fish meal) in aquatic feed is often limited by lower digestibility values, insufficient essential amino acids and possible palatability issues. However, they remain the most economically viable alternative for use in aquatic feed (Davis and Arnold, 2000).

Garza deYta et al. (2012) reported that DDGS had a similar effect on survival and growth of redclaw crayfish (*Cherax quadricarinatus)* as poultry by-product meal or fish meal. Thompson et al. (2006) completely replaced fish meal with a combination of DDGS and soybean meal in redclaw crayfish feed and found no significant difference in feed conversion rate, survival or total yield. Gatlin et al. (2007) documented the use of various plant protein sources, namely soybean, canola, barley, corn, cottonseed, peas/lupins and wheat, in production of aquafeed and expressed the pros and cons of different ingredients. Conventional DDGS contains 28-32% protein and a relatively high in fiber content, which limits its use in aquafeeds (Gatlin et al. 2007)

With regards to commercial feed formulations, feed ingredients are selected and combined based on their nutritional content, cost and how they affect the physical characteristics of pellet. Feed manufacturing is the physical process of forming feed ingredient mixture into particles used to feed shrimp. Commercial feeds are manufactured as pellets using cooked extrusion, compression pelleting, or cold extrusion processes (NRC, 2011). Pelleting is the most

popular method of producing crustacean feeds due to its technological and economic advantages (Lovell, 1990, 1989). The advantages of pelleted feed include less bridging in bins, dust and feed waste, increased bulk density, nutrient density and nutrient availability, reduced ingredient segregation, decreased microbiological activity and improved palatability. While many processing technologies result in an agglomerated feed, only a few have sufficient energy inputs to ensure microbiological safety of feed. Feed safety is a major factor in choosing extrusion methods over traditional pelleting methods (Riaz, 2009). Extruded pellets have advantages over steam pellets for they generally have superior water stability, and in the case of floating fish feeds this property allows direct determination of feed consumption (Stickney, 1979). Robinette (1977) reported that the extrusion processing has much greater levels of heat, moisture and pressure than steam pelleting. Due to higher temperature, moisture and pressure in extrusion processing, it may increase the bioavailability of carbohydrate and destroy heat labile antinutrients. Compared to steam pelleting, extrusion processing can also damage nutrients such as ascorbic acid (Hilton et al., 1977; Lovell and Lim, 1978). Hilton et al. (1981) indicated that extruded pellets increased gastric emptying time of trout which was probably responsible for the reduced total feed consumption and weight gain but may have improved feed efficiency. In the rainbow trout, extrusion processing appeared to increase carbohydrate digestion and absorption, increased liver: body weight and percent liver glycogen content as compared with steam pelleting (Hilton et al. 1981). Although both methods to produce pellets have advantages, there are few studies to determine if there are actual biological advantages associated with either process. According to available research, there is a lack of studies evaluating biological response to typical practical diet produced under various processing technologies.

Utilization of S-DDGS in shrimp feed will be an important value-added application. However, use of this co-product in aquatic feed needs to be thoroughly investigated from processing, nutritional and functional perspective, especially with respect to shrimp production. This study was designed to evaluate the effect of processing method (extrusion and pelleting) on physical and nutritional characteristics of shrimp feed made from different levels of S-DDGS.

2. Materials and Methods

S-DDGS was obtained from Prairie Horizons Agri-Energy (Phillipsburg, Kansas, USA). Other ingredients used for formulating the shrimp feed diets included menhaden fish meal (Omega Proteins, Houston, Texas, USA), solvent extracted soybean meal (Kansas State University Feedmill, Manhattan, Kansas, USA), wheat gluten, wheat starch, menhaden oil, and Stay C (Lortscher Agri Service, Bern, Kansas, USA), Lecithin (Solae, St. Louis, Missouri USA); and minerals and vitamins premix (Rangen, Buhl, Idaho USA).

2.1. Feed production using extrusion and pelleting methods

The basal diet (Table 1) was formulated as a typical practical diet for shrimp containing 35% protein and 8% lipid. This diet was modified to contain 100, 200, 300 or 400 g kg^{-1} of sorghum distillers dried grain with solubles (S-DDGS) while keeping formulations isonitrogenous relative to the basal diet. The S-DDGS replaced solvent extracted soybean meal, whole wheat and menhaden fish oil while maintaining equivalent levels of protein and lipid. Additionally, DL methionine was supplemented to maintain minimal levels of methionine (Table 1).

A pilot-scale single screw extruder (X-20, Wenger Manufacturing, Sabetha, Kansas, USA) was used for producing extruded diets. The ingredients were mixed in a ribbon blender and fed into the pre-conditioner using a volumetric feed system, the equivalent of which for this production was 184 kg hr^{-1}. Steam and water were added in the pre-conditioner at the rate of 5.5–7.8 kg hr^{-1} and 21–25 kg hr^{-1}, respectively. Steam injection rate in the extruder was approximately 26 kg hr^{-1}. This lead to an approximate in-barrel moisture of 30.0-31.8% (wet basis). The barrel temperature and screw profilw are shown in Figure 1. The extruder was operated at a screw speed of 307 rpm. A die with 6 circular openings of approximately 3.10 mm diameter each was used, and product was cut with a knife operating at 997 rpm. Post-extrusion the feed was dried in a double pass dryer/cooler system (Series 4800, Wenger Manufacturing, Sabetha, KS, USA) with a retention time of 17.45 min at approximately 104.4°C and a cooling time of 4.45 min. They were subsequently packed into 3-ply paper sacks for cool dry storage at ambient temperature (25 ± 2°C) before further analysis.

The total specific mechanical energy (SME) applied to the whole process was calculated based on motor loads and screw speeds, in relation to the mass flow rate and power rating of the motor, described by the following equation 1 (Karkle et al. 2012):

$$SME\,(kJ.kg^{-1}) = \frac{\left(\frac{\tau - \tau_0}{100}\right) \times \frac{N}{N_r} \times P_r}{\dot{m}} \qquad (1)$$

where, τ is the % torque; τ_0 is the no load % torque (24% for 200–400 rpm); N is the extruder screw speed (307 rpm); N_r is the rated screw speed (508 rpm); P_r is the rated motor power (37.29 kW); and $\dot{m}$ is the mass flow rate or throughput.

A pilot-scale pellet mill (Master Model 1000HD, CPM, Crawfordsville, Indiana) was used for producing compression pelleted diets. Prior to pelleting, 1% calcium lignosulfonate (Ameribond 2X, LignoTech USA, Rothschild, Wisconsin, USA) was added to the mash feed in a double shaft paddle mixer (Forberg, Model F500, Ontario, Canada). The mash feed was then transferred to a holding bin above the pelleting system. The mash feed was fed into the pelleting system at a dry rate of approximately 500 kg hr^{-1}. It was then conditioned with a retention time of approximately 30 s and to a temperature of 85°C in a cylindrical steam conditioner (Custom built: dimension 30 x 91 cm, Bliss Industries, Ponca City, Oklahoma) prior to pelleting. Pelleting was accomplished using a ring-die pellet mill (CPM, Master Model 1000HD, Crawfordsville, Indiana) equipped with a pellet die having the effective dimensions of 0.24 x 3.18 cm, die opening and land length, respectively. Pelleted feed was cooled in a double pass horizontal perforated bed cooler (Custom built, Wenger Manufacturing, Sabetha, KS, USA) prior to being sacked off in 50 lb 3-ply paper sacks.

2.2. Feed physical characterization

Bulk density, Radial expansion ratio and Pellets durability index (PDI)

In order to determine bulk density (BD) for each feed, a liter cup was filled with pellets and weighed. Bulk density was determined as mass per unit volume in duplicate. Expansion ratio was determined by using a digital caliper to measure the diameter of the pellets after processing. Measurements were done in 10 replications per treatment. Expansion ratio was calculated as the square of the mean diameter of pellet, d_p divided by the square of the diameter of die hole, d_d (Eq. 2):

$$Expansion\ Ratio\ (ER) = \frac{d_p^2}{d_d^2} \quad (2)$$

Pellet durability index (PDI) was determined using an adapted version of the ASAE standard method S269.4 (ASAE 1997). Approximately 500 g of finished diets from each treatment was placed into two separate compartments of a custom-made tumbling equipment. One compartment had 5 metal pieces (nuts) to simulate a harsher handling environment, while the other did not have any. The tumbler was operated for 10 minutes before emptying the compartments. The tumbled samples were sieved through a US standard sieve No. 270 (0.053 mm) to separate the unbroken feed, which was reweighed. PDI was calculated as follows,

$$PDI\ (\%) = \frac{m_f}{m_i} \times 100 \quad (3)$$

where m_f is the mass of unbroken feed after tumbling and m_i is the initial mass. PDI was calculated for feed in both the compartment with metal pieces (modified method) and the one without (unmodified method).

2.3. Sinking property

Fifty pellets were picked randomly from each treatment and dropped into water in a one liter beaker at room temperature (≈ 25°C). The number of floating pellets was counted after one minute to obtain the total number of pellets that sank. All experiments were replicated at least twice, and sinking percentage (S) was determined as follows,

$$S\ (\%) = \frac{N_s}{50} \times 100 \quad (4)$$

where N_s is the number of sinking pellets.

2.4. Water Stability

Water stability of the pellets was determined by slight modification to Jayaram and Shetty (1981) method. Five grams of pellets were weighed into 100 ml beakers filled with water. Beakers were placed in a water bath operated at 28°C (Aquacop, 1978) for 1, 3, 5 and 7 hrs. Water was drained after each time period, and the beaker and feed left were dried in an oven (Thelco Lab Oven, Precision Scientific Chicago, Il, USA) at 105°C until constant weight. Water stability was calculated as the difference between mass of beaker (with feed) before and after water immersion divided by the initial mass of the feed.

2.5. Degree of Gelatinization

Extruded and milled pellets were ground in a kitchen blender (Osterizer Galaxie Cycle Blend, USA). Water was added to the powdered flakes at a ratio of 2:1, moisture to dry. After thorough mixing in a beaker with glass paddle, they were left over night to equilibrate. Approximately 20–30 mg was scooped into a stainless steel high volume pan and closed with a lid with O-ring insertion. Samples were placed into DSC (Q200, TA Instrument, Delaware, USA) cell compartment and heated by ramping the temperature at a rate of 10°C min^{-1} from 10–130°C. The process was also repeated for the raw ingredients mix used for producing the pellets. Integration of the endothermic peaks of thermograms from both the raw flour and pellets were performed on Universal Analysis 2000 software (version 4.7A, TA Instruments Waters-LLC, USA). The degree of gelatinization was calculated using the following expression in equation 5:

$$Degree\ of\ Gelatinization\ (DG) = \left(\frac{\Delta H_{raw\ Ingredients} - \Delta H_{pellet}}{\Delta H_{raw\ ingredients}}\right) X\ 100\% \quad (5)$$

2.6. Growth trials

Dietary treatments were tested in an indoor tank system, located at the E.W. Shell Fisheries Research Station, Auburn, Alabama. Post-larval *L. vannamei* were obtained from GMSB Shrimp Hatchery (Summerland Key, Florida) and maintained in a 220-L polyethylene nursery tank connected to a biological filter. During the first week PL were intermittently offered *artemia nauplii* (200 nauplii per shrimp) and offered a commercial feed daily using automatic feeders. Food size was increased as the PL grew with the final feed being a commercial 35% protein diet (Rangen Inc, Buhl, Idaho USA).

Juvenile shrimp were obtained from the nursery system and selected by hand sorting to a uniform size. In trial 1, juvenile shrimp (0.35 ± 0.032 g) were stocked into 40 tanks (60 L), which were part of a recirculating system at a density of 10 shrimp per tank with four replications per treatment over a 63-day growth trial. In trial 2, juvenile shrimp (0.38 ± 0.02 g, initial weight) were also stocked into 60 tanks (60 L), which were part of a recirculating system at a density of 10 shrimp per tank with six replicates per treatment over a 42-day growth trial.

Daily feed inputs were calculated, based upon an expected weight gain of 0.8 g per week and an expected feed conversion ratio of 1.8:1. Dissolved oxygen, temperature, and pH were measured twice a day using a YSI 556 MPS meter (Yellow Spring Instrument Co., Yellow Springs, OH, USA). Total ammonia-nitrogen was measured once per week using the Orion ammonia electrode probe (Thermo Fisher Scientific Inc., Waltham, MA, USA). At the conclusion of each growth trial, the shrimp were counted and group weighed. Final mean weight, final biomass, percent survival, and feed conversion ratio (FCR) were determined.

2.7. Statistical analysis

Data were analyzed by using a one-way analysis of variance to determine significant ($P < 0.05$) differences among treatment means. When significant differences were identified, Duncun multiple range test was used to determine significant differences between treatment means. Additionally, data from growth trial were analyzed by using two-way analysis of variance to determine significant ($P < 0.05$) difference for two different processing methods. The statistical analyses were performed using SAS software package (SAS Institute Inc., Cary, NC USA).

3. Results and Discussion

3.1. Proximate composition

The proximate composition of S-DDGS is presented in Table 2. The level of protein is typical for DDGS but the fiber content is high and there is a minimal amount of starch, which was depleted during the prior fermentation process. Proximate compositions of the extruded and pelleted feed diets are presented in Table 3. Analysis of variance showed that the effect of S-DDGS and processing methods was significant ($P <0.05$) on the variation observed in all the chemical constituent of the feeds. Protein ranged from 33.6 – 35.5% and fat from 7.77 to 8.75% across the levels of S-DDGS inclusion, indicating a reasonable replacement strategy. Fiber levels of the feed increased as S-DDGS increased. The effect of fiber is shown on SME (Fig. 2), with increase seen with increased level of DDGS, which was due to increased frictional/viscous effect of fiber flow in the extruder. Ash content was in the range of 6.97–7.50%, which would be expected as the ash contents of the replacement ingredients were similar. Percent starch decreased with increased level of S-DDGS in feed, which can be traced back to the low level in S-DDGS (Table 1) and the decrease in whole wheat that was reduced due to the need to make

room in the formulations. Moisture content of the pelleted feed was approximately 8.35%, while that of extruded feeds ranged from 4.15–9.45% which is probably due to variations in the drying.

3.2. Physical characteristics of the feeds

The SME input during extrusion of S-DDGS based aquatic feeds are shown in Figure 2. It ranged from 48.75 – 55.47 $kJ.kg^{-1}$. There was a gradual increase in SME as S-DDGS was included in the feed, which might be due to increases in frictional force of the melt viscosity with higher fiber content. Rosentrater et al. (2009) asserted that SME is a function of product composition. Their study showed a curvilinear trend in SME of extruded feed with increase in DDGS level from 20 to 30%, temperature from 100 to 150°C and protein from 3 to 32.5%.

Bulk density of extruded and pelleted feed was 0.53–0.58 gcm^{-3} and 0.61–0.65 gcm^{-3}, respectively (Fig. 3). There was no particular trend shown for bulk density of extruded feed, while pelleted feeds showed a decreasing trend with increase in S-DDGS level. However, there was no significant difference between samples within each processing method, but slightly lower density was observed for extruded diets compared with the pelleted diets. This can be attributed to the structural development, which favors a slightly more porous product in extrusion processing. Extrusion is characterized as using higher mechanical and thermal energy input, which favors expansion, whereas pellet milling imparts higher compaction forces, which produce a feed with higher mass per unit volume than in extrusion. Chevanan et al. (2007) observed a significant increase in bulk density for DDGS-based aquatic feed with adjusted protein content for all samples to 28%. Bulk density is a function of the feed component and processing conditions. A slight change in moisture content could result in significant change in density,

given other conditions as stable. The variation observed in extruded aquatic feed in this study could be due to slight changes in extrusion processing conditions.

The radial expansion ratio of the extruded and pelleted feeds is shown in Figure 4, and it varied from 1.022–1.089 and 0.996–1.010, respectively. There was no particular trend shown with respect to the level of S-DDGS. The expansion observed in extruded feeds was quite minimal and could have resulted primarily from die swell. Pelleted feeds showed no expansion whatsoever, which is typical of pelleting method (Briggs et al., 1999).

The PDI is a major quality index for feeds. It is usually performed to test the strength of feed and to determine the level of possible disintegration during handling such as packaging and transportation. PDI from the modified test for extruded and pelleted feed varied from 89.4–96.3% and 94.1–96.2%, respectively (Fig. 5a). However, PDI for the unmodified extruded and pelleted feed ranged from 94.9–97.9% and 96.88–97.5%, respectively (Fig. 5b). Modified method, irrespective of processing, resulted in less durability compared with the unmodified method for the obvious reason of having additional impact from the nuts included during testing. PDI of extruded diets was not substantially different from that of pelleted diets, except for the control diets, which had lower PDI (89.4%) than extruded. The lower PDI for extruded diets could be attributed to greater porosity and less densely packed feed ingredients. PDI obtained from unmodified method did not differ substantially with S-DDGS addition, irrespective of the processing method. PDI from modified method, however, showed a gradual increase with increased level of S-DDGS (up to 200 g kg^{-1} for pelleted and 300 g kg^{-1} for extruded) in the diet for both extruded and pelleted feeds, before it declined slightly at 40% inclusion. Kannadhason et al. (2007) had reported an increase in PDI for S-DDGS based aquatic feed for increased level of S-DDGS from 20 to 40%. Rosentrater et al. (2009) showed a curvilinear effect of S-DDGS

addition on aquatic feed PDI in their study. When increase in S-DDGS level from 20 to 25% led to an increased PDI, however, a further increase to 30% caused a decrease in PDI measured. Pellets' composition and heat treatment play important roles in structural development of feed. What is surprising is the effect of starch on PDI in this study. It was expected that starch would act as a binder and a decrease in starch content (higher S-DDGS level – Table 1 & 3) would lead to a lower PDI. However, the interaction of other ingredients of the diet, such as external lipids (fish oil), protein type and increased fiber content, could be responsible for the PDI pattern shown by the different processing methods (Chevanan et al. 2007; Kannadhason et al. 2007).

3.3. Sinking percentage

Diets produced by pellet milling were all sinking feed, indicating very densely packed products. This was also confirmed by the higher bulk density of the pelleted diets compared to the extruded diets (Fig. 3). All extruded feeds showed sinking characteristics for all treatments except for the one with 40% S-DDGS that had a sink rate of 88% (Table 4). This treatment also had the lowest bulk density. However, when the bulk density of the feeds from the two methods was compared with that of water (1 g cm^{-3}), it was expected that all the feeds should float. The porous nature of the feeds allowed them to absorb water for the first few minutes after being poured into water, which increased their overall density above that of water thereby making them sink.

3.4. Degree of gelatinization

The data for degree of gelatinization are shown in Table 5. DG for pelleted diet ranged from 13.7 – 52.7%. For extruded diets, it ranged from 11.8–85.6%. Generally, a decreased level

of gelatinization was observed as S-DDGS as the formulation increased. Extruded samples showed higher gelatinization than pelleted feeds, which could be a result of more energy input in the extruder than in the pellet mill. Since the extrusion and pelleting conditions were constant during processing, it was expected that gelatinization would remain the same for the different formulations. However, this was not the case. This result was due to decreased starch (more S-DDGS and less whole wheat) and increased fiber level in the formulation. That is to say, there was less starch available for gelatinization. Soluble fiber (especially in ground form as is the case in S-DDGS) is reported to have a binding effect, thus reducing contact area for other constituent of the diets such as starch (Loar II and Corzo, 2011).

3.5. Water stability

Pellet feed water stability (WS) is an important quality attribute as it measures the feed's ability to withstand disintegration and nutrient leaching while in water before consumption by aquatic animals. The water stability for the extruded and milled pellets ranged from 76.2–91.57% and 80.46–85.04%, respectively, factoring the effect of soaking time and S-DDGS level (Table 6A,B). In general, there were limited significant differences with a diet across time from 1 hr to 6 hr. However, there were some differences within a given time across dietary inclusion levels of S-DDGS. Obaldo et al. (2002) used three different methods for pellets water stability and reported a decreasing trend in mass retention after 6 hours of soaking in water. Jayaram and Shetty (1981) also reported a decrease in water stability of two new pelleted fish feed with time (90.58 to 70.26% and 85.52 to 82.25%) from 1hr to 7hr. Lim and Cuzon (1994) in a review paper presented dry matter data (82.5–88.0%) for feeds after 8 hours of soaking using different

types of binders. The range of WS reported by these authors for experimental feeds were within the range reported in this study.

3.6. Growth trial

The two laboratory trials were conducted without any noticeable problems with water quality or disease occurrence. Water quality parameters were within suitable ranges for the culture of this species. Water quality parameters were as follows: trial 1 temperature, 27.68 ± 0.99°C; DO, 6.28 ± 0.40 mg L^{-1}; salinity, 10.91 ± 3.56 g L^{-1}; trial 2 temperature, 30.34 ± 1.03°C; DO, 5.45 ± 0.23mg L^{-1}; salinity, 19.22 ± 0.52 g L^{-1}; and TAN, 0.045 ± 0.0.15 mg L^{-1}.

For growth trial 1, results indicated that there were no significant differences in final mean weight, biomass, survival, percentage weight gain and feed conversion ratio (FCR) of *L. vannamei* fed with diets containing increasing percentages of S-DDGS for extruded or pelleted feed (Table 7a). For the extruded diet, mean final weight ranged between 4.48 and 4.99 g, with 200 g kg^{-1} S-DDGS treatment having the largest mean final weight; biomass ranged from 36.9 and 43.6 g $tank^{-1}$; FCR ranged between 2.83 and 3.02; survival ranged from 82.5 to 92.5%; and percentage weight gain ranged between 1153.3 and 1260.9%. For the milled pelleted diet, mean final weight ranged between 4.18 and 4.50 g; biomass ranged from 31.3 and 36.4 g $tank^{-1}$; FCR ranged between 3.10 and 3.26; survival ranged from 75.0 to 85.0%; and percentage weight gain ranged between 1060.1 and 1196.7%.

For growth trial 2, results indicated that there were no significant differences in final mean weight, biomass, survival and percentage weight gain of *L. vannamei* fed with diets containing increasing percentages of S-DDGS for extruded or pelleted (Table 7b). For the extruded diet, there were no significant differences between treatments; however, for pelleted

diet, FCR of 200 g kg^{-1} S-DDGS sorghum treatment was significant lower than FCR of 200 g kg^{-1} S-DDGS sorghum treatment. For the extruded diets, mean final weight ranged between 4.02 and 4.69 g; biomass ranged from 36.42 and 43.62 g $tank^{-1}$; FCR ranged between 1.82 and 2.19; survival ranged from 90.0 to 93.3%; and percentage weight gain ranged between 971.75.29 and 1171.84%. For the milled pelleted diets, mean final weight ranged between 4.19 and 5.15 g; biomass ranged from 35.82 and 43.83 g $tank^{-1}$; FCR ranged between 1.66 and 2.07; survival ranged from 76.67 to 90.00%; and percentage weight gain ranged between 1002.8 and 1280.8%.

For growth trial 1, based on pooled data, extruded feeds produced significantly larger shrimp and lower feed conversion. However, for growth trial 2, based on pooled data, extruded feeds produced significantly higher survival but not larger shrimp or lower FCR. The use of sorghum S-DDGS up to 400 g kg^{-1} of the diet resulted in acceptable performance, which was comparable to the control diet without any S-DDGS.

Due to the increased supply and low price of DDGS, there is considerable interest in the use of DDGS in aquaculture diets. Recent research has shown that corn DDGS can be used as an ingredient in a number of species including rainbow trout (Cheng and Hardy, 2004), channel catfish (Li et al., 2010, 2011; Lim et al., 2009; Robinson and Li, 2008; Tidwell et al., 1990; Webster et al., 1991, 1992, 1993), sunshine bass (Thompson et al., 2008), tilapia (Coyle et al., 2004; Lim et al., 2007; Schaeffer et al., 2009; Shelby et al., 2008; Wu et al., 1994, 1996, 1997) and Pacific white shrimp as well (Lawrence et al., 2011; Roy et al., 2009). Currently, no studies are available for S-DDGS in feed for *L. vannamei*. However, the results in this study demonstrated that inclusion of 400 g kg^{-1} S-DDGS in feed has negligible effects on shrimp performance.

This result is in agreement with the finding of Robinson and Li (2008) and Lim et al. (2009) who showed diet inclusion rates of 20–40% DDGS was successfully used in feed for channel catfish. Lim et al. (2007) demonstrated 20% of DDGS could be included in the diet of Nile tilapia as a replacement of a combination of soybean meal and corn meal without affecting the overall growth performance. Wu et al. (1996) evaluated the growth response of Nile tilapia fry fed all-plant protein diets containing 32, 36 and 40% crude protein, incorporation of 16–49% DDGS resulted in good weight gain (WG), feed efficiency ratio (FER) and protein efficiency ratio (PER). Tidwell et al. (1990) and Webster et al. (1991) found that 40 and 35% DDGS, respectively, can be used in catfish diets containing 8–10% fish meal as substitutes for the combination of soybean meal (SBM) and corn meal (CM) on an equal protein basis without requiring lysine supplementation. Webster et al. (1991) showed a diet containing 70% DDGS appeared to be deficient in lysine because supplementation of lysine at a level to meet lysine requirement improved the growth of catfish. However, the characteristics of DDGS sorghum include moderate protein and low lysine and methionine.

The results in our study showed no trend of decreasing growth with increasing levels of S-DDGS as both lysine and methionine, the two most limiting amino acids were likely replete. This result was different from Lim et al. (2007), who found that diets containing 8% fish meal had increasing dietary levels of DDGS to 40% without the addition of lysine significantly reduced WG and PER relative to those obtained with diets containing lower DDGS levels (0, 10 and 20%). Another study from Lim et al. (2009) suggest that with lysine supplementation to a level equal to that of the control diet, at least 40% of DDGS can be included in the diet of juvenile channel catfish as a replacement of a combination of soybean meal and corn meal without affecting the growth performance, feed utilization efficacy and survival. Lim et al.

(2009) could not ascertain whether supplemental lysine can be omitted from 10-40% DDGS catfish diet containing 8% menhaden fish meal because diets without lysine supplementation were included in their study. The negative growth from Lim et al. (2007) study may be due to lysine deficiency in the diets. In contrast, results from the current study indicated the growth was not significantly affected S-DDGS level up to 40% even without lysine supplementation although all diets containing S-DDGS were supplemented with methionine.

Limited research was available for comparing the extrusion and pelleting of feed for aquatic species. The results in trial 1 were similar to those of Hilton et al (1981), who indicated that extruded pellets have greater weight gain and feed efficiency in rainbow trout. Vens-Cappell (1984) also showed that the superior conversion of the extruded feed as compared with pelleted feed is due to its higher content of digestible energy. The results in trial 2 were in agreement with Booth et al. (2002, who) indicated that feed intake, weight gain and specific growth rate of sliver perch fed steam-pelleted diets were greater than those of fish fed extruded diets. However, extruded or pelleted diets in both growth trials had limited significant effect on shrimp performance.

4. Conclusion

Based on the results of the present study, S-DDGS can be included in practical diets for the Pacific white shrimp, without negative effects on growth, survival and FCR. Although some performance differences were observed between extruded or pelleted feed, these were not consistent across the two trials. The physico-chemical properties of shrimp feed, such as SME, bulk density, expansion ratio of feed, PDI, degree of gelatinization and water stability, were significantly influenced by the level of inclusion of S-DDGS. The modified PDI of both pelleted

and extruded feeds generally increased up to 200 g kg^{-1} inclusion and then leveled off. Degree of gelatinization generally decreased with increasing S-DDGS inclusion for extruded or pelleted. As expected, some shifts in processing parameters were observed as the formulations were changed. However, all the feeds were reasonable physical characteristics and supported good performance of the shrimp. Based on the observed results, the use of S-DDGS products should be encouraged as an alternative protein source in shrimp feed formulations.

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Table 1 Composition (g kg^{-1} as is basis) of five practical diets formulated with different levels of distillers dried grains with solubles from sorghum (S-DDGS) as a partial replacement for solvent extracted soybean meal and whole wheat.

S-DDGS level	0	100	200	300	400
Menhaden fishmeal[1]	50.0	50. 0	50.0	50.0	50.0
Soybean meal solvent extracted[2]	550.0	491.0	433.0	375.0	317.0
DDGS Sorghum[3]	0.0	100.0	200.0	300.0	400.0
Menhaden Fish Oil[4]	55.7	48.6	41.4	34.2	27.0
Whole wheat[4]	304.2	270.1	235.0	199.9	164.9
Mineral premix[5]	1.9	1.9	1.9	1.9	1.9
Vitamin premix[5]	3.3	3.3	3.3	3.3	3.3
Choline chloride[4]	2.0	2.0	2.0	20	2.0
Stay C 250 mg/kg (25% active)[4]	1.0	1.0	1.0	1.0	1.0
Calcium phosphate dibasic[4]	22.0	22.0	22.0	22.0	22.0
Lecithin[6]	10.0	10.0	10.0	10.0	10.0
Methionine–DL[4]		0.20	0.45	0.70	0.95

[1] Omega Proteins, Houston, TX, USA

[2] Kansas State University Feedmill, Manhattan, KS, USA

[3] Prairie Horizons Agri-Energy, Phillipsburg, KS, USA

[4] Lortschre Agri Service, Bern, KS, USA

[5]Rangen, Buhl, ID, USA

[6] Solae, St. Louis, MI, USA

Table 2 Proximate composition of the sorghum based distillers dried grains.

Nutrient	g kg^{-1}	Amino Acid	g kg^{-1}
Dry matter	911.2	Alanine	26.26
Protein	339.2	Arginine	5.44
Lipid	87.6	Aspartate	20.51
Crude Fiber	102.1	Cysteine	5.02
Ash	44.1	Glutamate	57.10
Phosphorus	9.0	Glycine	11.25
Starch	23.9	Histidine	7.30
		Isoleucine	14.52
		Leucine	38.06
		Lysine	10.07
		Methionine	4.67
		Phenylalanine	16.63
		Serine	14.66
		Threonine	10.50
		Tyrosine	10.07
		Valine	15.28
		Total	267.33

Table 3 Proximate composition of extruded and pelleted S-DDGS based aquatic feeds (g kg^{-1}).

S-DDGS level	Protein	Fat	Crude Fiber	Ash	Starch	Moisture
	Extruded Feed					
0	355	85	20	75	169	41
100	353	85	24	75	151	42
200	342	78	26	73	135	77
300	336	67	31	70	113	95
400	355	79	35	73	106	42
	Pelleted Feed					
0	341	78	21	72	156	82
100	340	81	24	72	140	84
200	340	83	28	73	126	79
300	339	85	33	72	117	88
400	336	88	36	71	101	84

Table 4 Sinking characteristics of extruded and pelleted S-DDGS based aquatic feeds.

S-DDGS Level, g kg^{-1}	Sink %	
	Extruded Pellets	Milled Pellets
0	100	100
100	98	100
200	98	100
300	100	100
400	88	100

Table 5 Degree of gelatinization (DG) of extruded and pelleted S-DDGS based aquatic feeds

	Level S-DDGS, g kg^{-1}	Temperature at Gelatinization Peak, °C	Enthalpy, J/°C	DG, %
Raw Ingredient	0	70.18	0.986	-
	100	70.45	1.096	-
	200	70.33	0.784	-
	300	70.85	0.695	-
	400	70.75	0.642	-
Pelleted	0	70.03	0.467	52.66
	100	74.27	0.597	45.53
	200	69.72	0.661	15.68
	300	69.63	0.599	13.74
	400	70.16	0.522	18.73
Extruded	0	72.24	0.142	85.59
	100	80.12	0.241	77.99
	200	61.28	0.277	64.62
	300	59.41	0.519	25.35
	400	65.29	0.566	11.84

Table 6 Water stability property of extruded and pelleted S-DDGS based aquatic feeds

A: Extruded Feed

S-DDGS level, %	3 hr	5 hr	7 hr
0	88.02 (a)	87.84 (a)	91.57 (a)
10	85.53 (ab)	85.54 (a)	85.78 (b)
20	82.93 (bc)	81.87 (bc)	81.09 (c)
30	81.62 (c)	80.71 (c)	76.20 (c)
40	85.66 (ab)	85.09 (ab)	86.22 (a)
P-value	0.016	0.014	0.0001

B: Pelleted Feed

S-DDGS level, %	3 hr	5 hr	7 hr
0	81.83 (a)	80.46 (a)	81.17 (a)
10	82.94 (b)	83.15 (a)	82.64 (a)
20	84.16 (c)	83.32 (a)	83.06 (a)
30	83.89 (bc)	83.18 (a)	82.61 (a)
40	83.64 (bc)	83.25 (a)	82.04 (a)
P-value	0.014	0.199	0.052

Different letters in bracket in the same column for each feed type are significantly different at $P<0.05$ (tested effect of DDGS level); the same letter in the same row are not significantly different $P>0.05$ (tested effect of soaking time).

Table 7a Response of juvenile *L. vannamei* (0.35 ± 0.032 g) to the test diets after a 63-day growth trial. Diets were either extruded (E) or pelleted (P).

S-DDGS, g kg^{-1}	Mean Weight (g)	Final Biomass (g)	FCR	Survival (%)	Weight Gain (%)
Extruded					
0	4.59	37.40	2.99	82.50	1226.08
100	4.48	36.90	3.02	82.50	1175.50
200	4.99	43.60	2.72	87.50	1190.63
300	4.69	43.40	2.87	92.50	1260.90
400	4.82	42.00	2.83	87.50	1153.29
P value	0.6011	0.0350	0.6296	0.2959	0.5258
Pelleted					
0	4.50	36.40	3.10	82.50	1196.70
100	4.28	33.60	3.22	80.00	1171.10
200	4.22	35.60	3.24	85.00	1062.60
300	4.43	34.70	3.10	80.00	1163.80
400	4.18	31.30	3.26	75.00	1060.10
P value	0.9045	0.5410	0.9668	0.8938	0.7167
Pooled Data					
Extruded	4.71	40.67	2.89	86.50	1201.28
Pelleted	4.32	34.35	3.18	80.50	1130.90
P value	0.0135	0.0001	0.0106	0.0842	1.8226

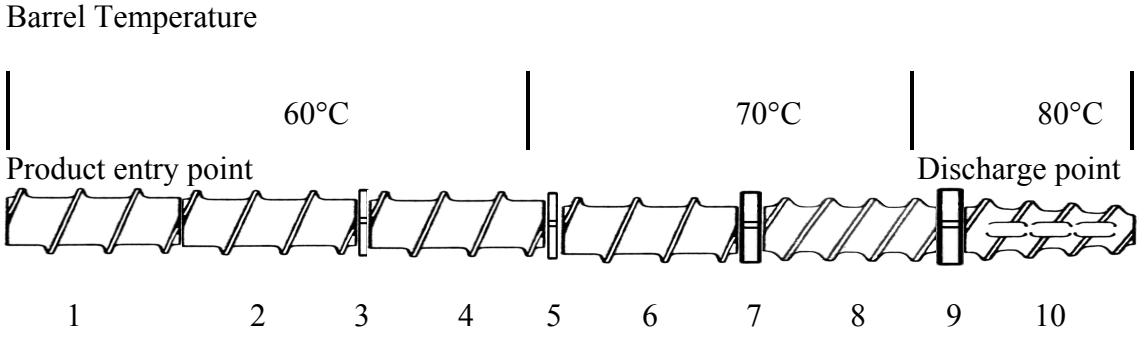
Based on Student-Newman-Keuls Test, no significant differences ($P > 0.05$) were found among treatment means (n = 4). FCR, feed conversion ratio = feed offered per shrimp/ weight gain per shrimp.

Table 7 b Response of juvenile *L. vannamei* (0.38 ± 0.02 g) to the test diets after a 42-day growth trial (Trial 2). Diets were either extruded (E) or pelleted (P).

S-DDGS, g kg^{-1}	Mean Weight (g)	Final Biomass (g)	FCR	Survival (%)	Weight Gain (%)
Extruded					
0	4.14	37.10	2.12	90.00	971.80
100	4.02	36.42	2.19	90.00	974.40
200	4.66	42.78	1.84	91.67	1171.80
300	4.69	43.62	1.82	93.33	1174.80
400	4.33	40.98	1.99	93.33	1077.10
P value	0.0802	0.0822	0.0592	0.9549	0.0971
Pelleted					
0	4.86	37.56	1.78^{ab}	78.00	1177.30
100	4.99	39.00	1.71^{ab}	78.00	1247.20
200	5.15	43.83	1.66^{b}	85.00	1280.80
300	4.68	35.82	1.84^{ab}	76.67	1130.40
400	4.19	37.62	2.07^{a}	90.00	1002.80
P value	0.0587	0.3391	0.0469	0.1699	0.0579
Pooled data					
Extruded	4.37	40.18	1.99	91.67	1073.98
Pelleted	4.77	38.76	1.81	81.53	1167.69
P value	0.0169	0.2629	0.02	0.0005	0.0881

Based on Student-Newman-Keuls Test, no significant differences ($P > 0.05$) were found among treatment means (n = 6).
FCR, feed conversion ratio = feed offered per shrimp/ weight gain per shrimp.

Figure 1 Schematic diagram of single screw elements for X-20 extruder with barrel temperature profile.



1-2 =single flight screws; 3=small steamlock; 4=single flight screw; 5=small steamlock; 6=single flight screw; 7=medium steamlock; 8=double flight screw; 9=large steamlock and 10=double cut cone.

Figure 2 Specific mechanical energy input during extrusion of S-DDGS based aquatic feeds.

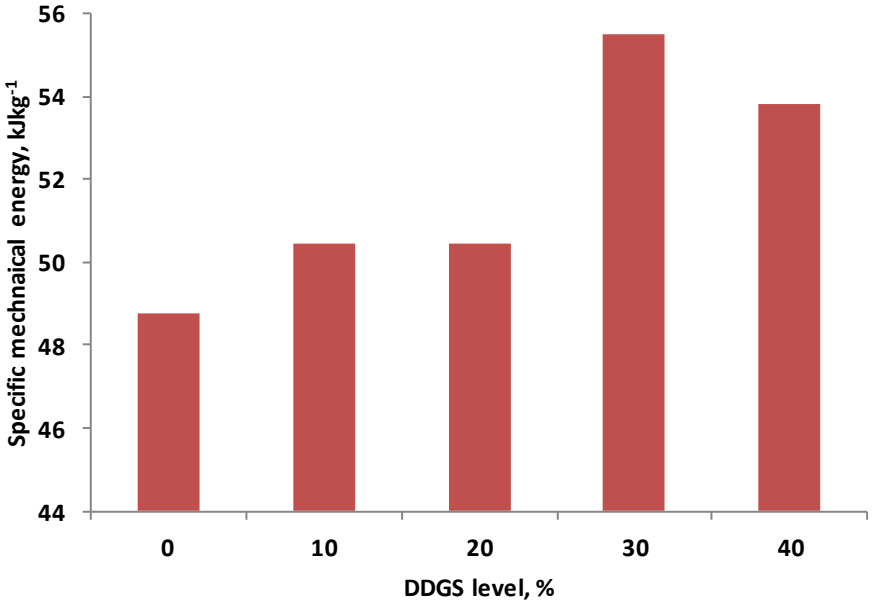


Figure 3 Bulk density of extruded and pelleted S-DDGS based aquatic feeds.

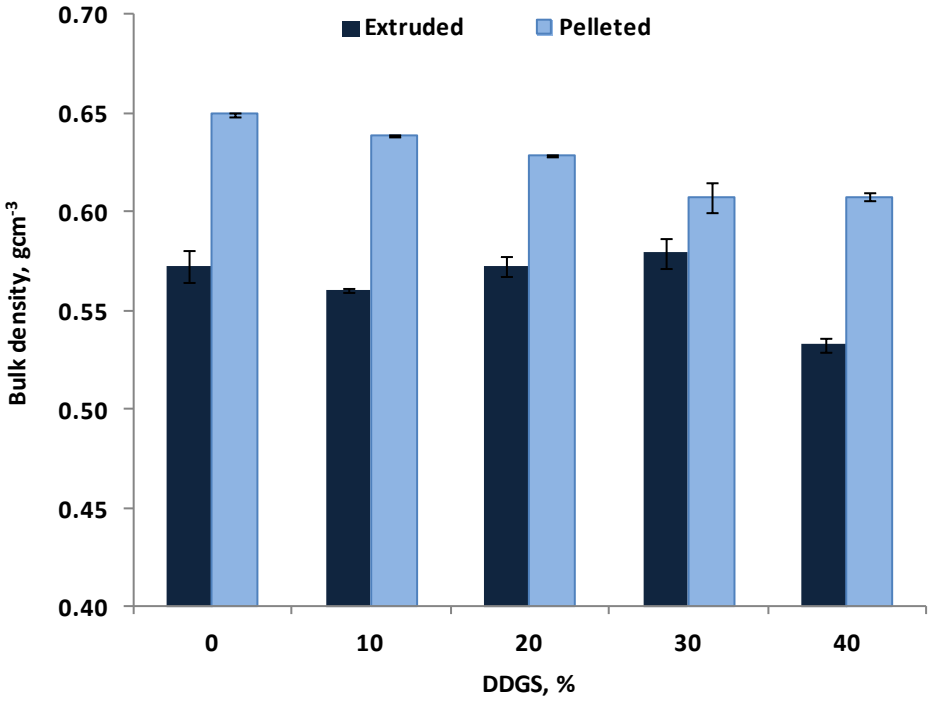


Figure 4 Expansion ratio of extruded and pelleted S-DDGS based aquatic feeds.

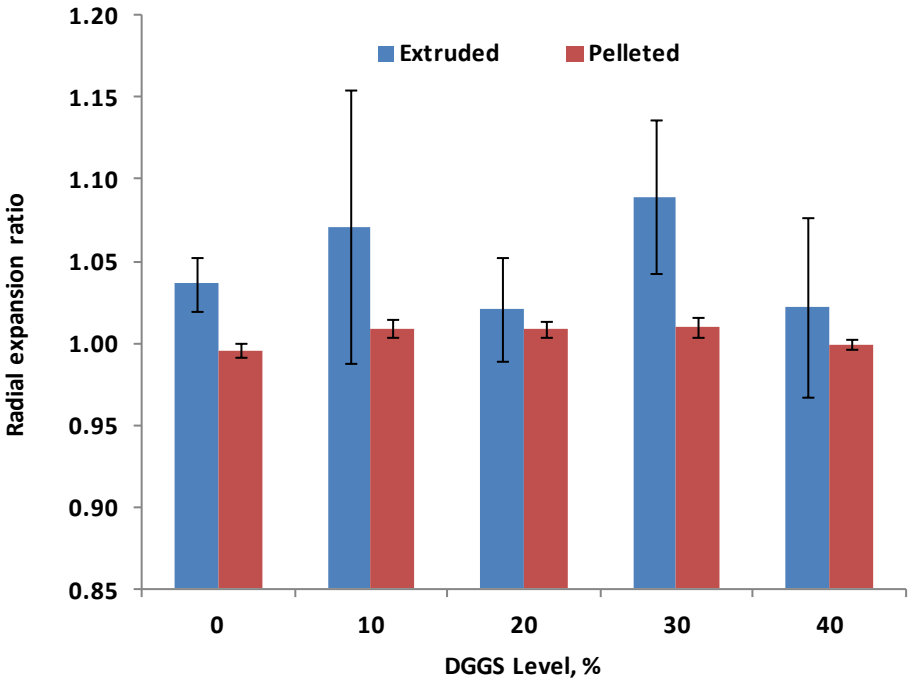
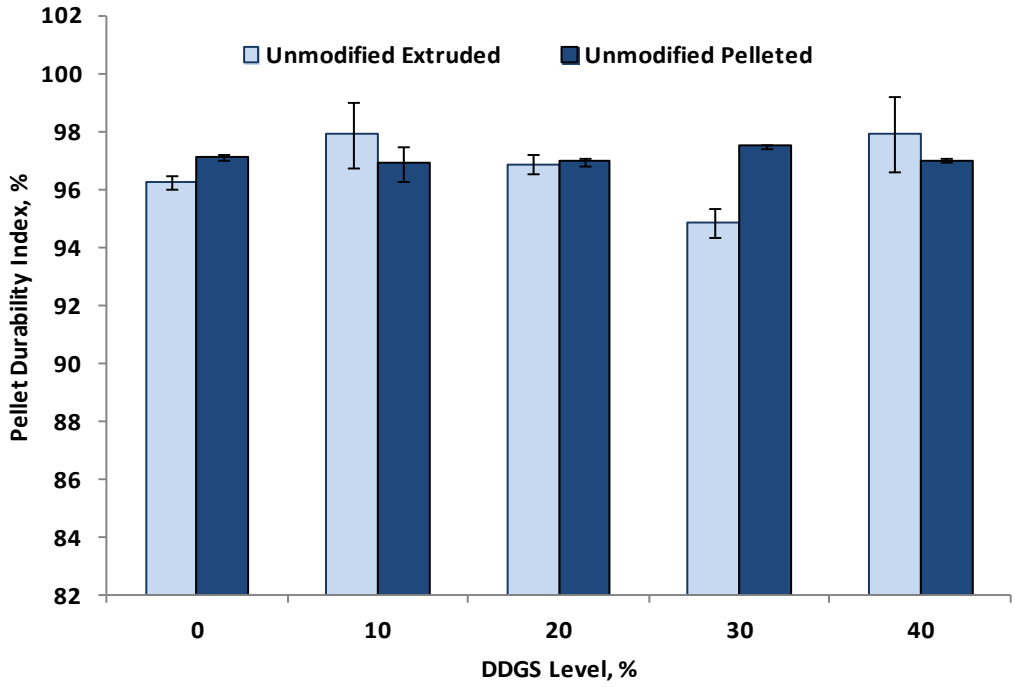
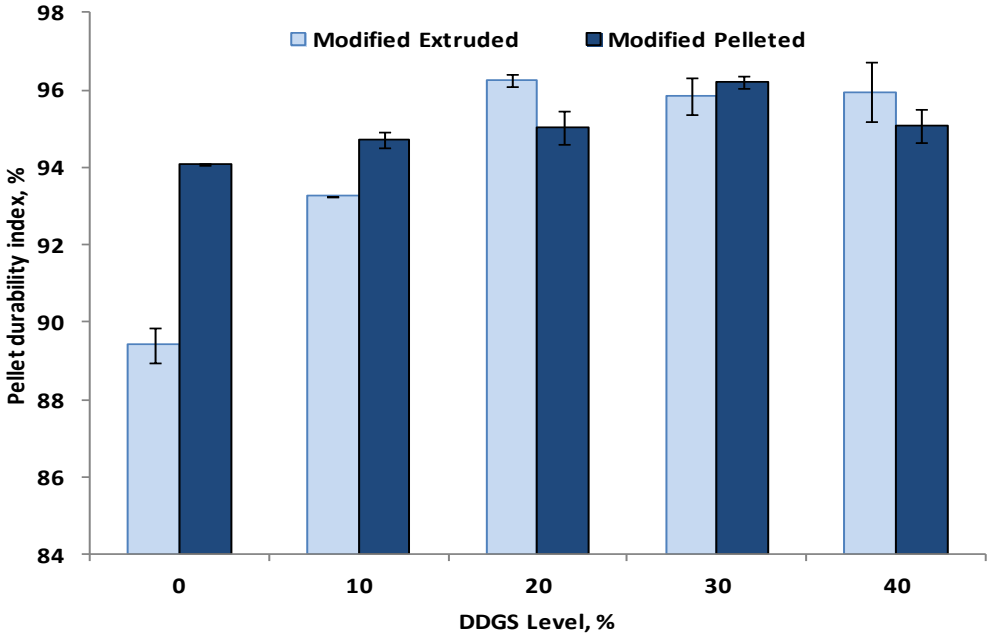


Figure 5 Pellet durability index of S-DDGS based extruded and pelleted diets using both (a) unmodified and (b) modified (more rigorous, with metal pieces) testing methods.





CHAPTER IV

COMPARATIVE EVALUATION OF COPPER SULFATE AND TRIBASIC COPPER CHLORIDE ON GROWTH PERFORMANCE AND TISSUE RESPONSE IN PACIFIC WHITE SHRIMP *Litopenaeus vannamei* FED PRACTICAL DIETS

Abstract

Three trials were conducted to evaluate the response of Pacific white shrimp *Litopenaeus vannamei* to dietary copper sulfate containing 25.45% copper (Cu) and tri-basic copper chloride (TBCC) containing 58.81% Cu. The basal diet used in each trial was primarily composed of fishmeal, soybean meal, corn starch, whole wheat and corn gluten meal, and was formulated to contain Cu about 10 mg Cu kg^{-1}. In trial 1, two sets of diets, one supplemented with copper sulfate (6, 12, and 24 mg Cu kg^{-1}) and the other with TBCC (6, 12, and 24 mg Cu kg^{-1}) were conducted in clear water recirculating system at a salinity of 21.01‰, with four replications per treatment of shrimp (initial mean weight 0.31 ± 0.01 g) for 8-weeks. In trial 2, shrimp (initial mean weight 0.39 ± 0.01 g) was fed the same six diets as trial 1 for a period of 6 weeks at a salinity of 15.51% in an outdoor green water recirculating system. In trial 3, six diets containing supplemented graded levels of dietary copper (0, 5, 10, 20, 40, and 60 mg Cu kg^{-1}) from TBCC were fed to juvenile shrimp (initial mean weight 0.28 g) at a salinity of 19.00%, with four replications per treatment for 8-week in a clear water recirculating system. At the conclusion of the trials, final mean weight, percent weight gain, final biomass, feed conversion ratio (FCR), and survival were determined. There were no significant effects from copper supplemental levels or source on final mean weight, final biomass, FCR, and survival in all three trials. However, in trial 3, there was a trend for positive response of percent weight gain to copper supplementation

with TBCC. Copper analysis of select tissues (carapace, hepatopancreas, and hemolymph) of shrimp from trial 1 and 2 indicated some significant difference among the treatments. In trial 1 and 2, when the tissues' concentrations are pooled across dietary levels for each copper source, copper levels of the tissues from shrimp fed TBCC were significantly lower than those of shrimp fed diets supplemented with copper sulfate. In trial 3, the concentration of copper in the carapace, hemolymph, and hepatopancreas in general increased with increasing TBCC supplementation. Linear regression of $\log_{10}$ transformed select tissues copper concentration on analyzed copper intake resulted in a slope ratio estimate for bioavailability of copper from TBCC that was lower to copper sulfate. The results of the present study demonstrate that TBCC is an effective copper source. Given the lower solubility of TBCC, it may be a more appropriate copper source for shrimp feeds.

1. Introduction

Copper (Cu) is an essential trace element for all living organisms (Burgess et al., 1999; Cervantes and Gutiérrez-Corona, 1994; Davis and Gatlin, 1996; Lall, 2002; Lorentzen et al., 1998; Shiau and Bai, 2009; Underwood and Suttle, 1999; Watanabe et al., 1997). Copper was first shown to be essential for growth and hemoglobin production in rats (Hart et al., 1928). Copper is important for animals as it is involved in the activity of functions in numerous copper dependent enzymes such as lysyl oxidase, cytochrome *c* oxidase (CCO), ferroxidase, tyrosinase and superoxide dismutase (SOD) (O'Dell, 1976). In addition, copper proteins and chelates also have metabolic roles (Watanabe et al., 1997). Copper is of particular importance in malacostraca crustacean where hemocyanin – an analogue of red-blooded animals' hemoglobin – is utilized as the oxygen-carrying pigment (Lovell, 1989). Rao and Anjaneyulu (2008) and Rao et al. (2008) provided strong evidence that copper is essential for both molting and reproduction in Pacific white shrimp (*Litopenaeus vannamei*). Depledge (1989) documented that 40% of the whole-body copper load in shrimp on a fresh weight basis is found in hemocyanin. This suggests a considerable increase in the physiological demand for copper by crustaceans above that required by vertebrates (NRC, 2011). Several studies have documented the dietary copper requirement in fish (Gatlin and Wilson, 1986; Lin et al., 2008; Tan et al., 2011). The dietary copper requirement of Pacific white shrimp (*L. vannamei*), tiger shrimp (*Penaeus monodon*), and fleshy prawn (*Fenneropenaeus chinensis*) has been reported as 16–32 mg kg^{-1}, 10–30 mg kg^{-1}, and 25 mg kg^{-1}, respectively (Davis et al., 1993; Lee and Shiau, 2002; Wang et al., 1997).

Knowledge of the bioavailability of supplemental copper sources is critical for the selection of a copper source in feed production (Luo et al., 2005; Miles et al., 1998; Spears et al., 2004). Sources of copper differ in their ability to be metabolized and utilized by cattle and

broilers, especially in the presence of copper antagonists (Arias and Koutsos, 2006; Kegley and Spears, 1994). Copper sulfate ($CuSO_4$) is the most common form of copper used in feeds for growth promotion. Tri-basic copper chloride (TBCC) is a more concentrated form of copper than copper sulfate (58% vs 25% Cu). Compared to copper sulfate, TBCC has good characteristics such as low hygroscopicity, very low chemical reactivity, and low activity in catalyzing the destruction of certain vitamins and other organic compounds when concentrated in premixes or diets (Cromwell et al., 1998; Luo et al., 2005; Miles et al., 1998). Cromwell et al. (1998) indicated that TBCC was as effective as copper sulfate to improve growth for weanling pigs. Shao et al. (2010) reported that TBCC was more bioavailable to crucian carp (*Carassius auratus gibelio*) than copper sulfate. Luo et al. (2005) reported that TBCC was a safer product and was more available than copper sulfate for broilers, and similar results were found in steers (Spears et al., 2004). Cheng et al. (2008) documented that TBCC is of similar availability based on performance, carcass characteristics and fat metabolism in lambs. Bharadwaj et al. (2014) suggested that chelated copper is safe, effective and highly available source of copper in *L. vannamei*. However, there is presently no information available on the use of TBCC in shrimps.

In the present study, three trials were conducted to evaluate the growth performance and bioavailability of two copper sources - copper sulfate and TBCC for *L. vannamei*. Two trials were conducted in two different types of recirculating systems (clear water or green water) using the same diets to evaluate the effects of different copper sources and levels. The third trial was designed to determine the shrimp's response to graded dietary copper levels from TBCC.

2. Materials and Methods

2.1. Experimental Design and Diets

In trial 1 and 2, tri-basic copper chloride (Micronutrients, Div. of Heritage Technologies, LCC, Indianapolis, IN, USA) and copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$; Sigma Chemical Co., St. Louis, MO, USA), were added to a basal diet (containing 10 mg Cu kg^{-1}, mainly from soybean meal, fish meal, whole wheat, and corn gluten meal) at 6, 12, and 24 mg Cu kg^{-1} (Table 1). In trial 3, the basal diet was formulated to contain 10 mg Cu kg^{-1}. This diet was then supplemented with 0, 5, 10, 20, 40, and 60 mg Cu kg^{-1} from TBCC (Table 2). Therefore, the total analyzed dietary copper concentrations were 10, 15, 19, 29, 48, and 66 mg Cu kg^{-1}(Table 3).

Research test diets (360 g kg^{-1} protein, 80 g kg^{-1} lipid) were prepared in the Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University (Auburn, AL, USA) using standard procedures for the laboratory production of shrimp feeds. Pre-ground dry ingredients and oil were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 10–15 min. Hot water was then blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 3-mm die, air dried (< 50 $^{\circ}$C) to a moisture content of 8–10%. After drying, pellets were crumbled, packed in sealed plastic bags and stored in a freezer until needed.

Post larval shrimp, *L. vannamei,* were obtained from Shrimp Improvement Systems (Islamorada, Florida, USA) and nursed for two weeks in an indoor recirculating system. All trials were conducted at the Claude Peteet Mariculture Center in Gulf Shores, Alabama, USA.

2.2. Growth trial 1 (*Clear water system*)

Juvenile shrimp were obtained from the nursery system and selected by hand sorting to a uniform size. Juvenile shrimp (initial mean weight 0.31 ± 0.01 g) were stocked at a density of 15 shrimp per tank into a recirculating system containing 24 square tanks (0.6 x 0.6 x 0.6 m, 216 L volume). The recirculating system consisted of a biological filter, reservoir, supplemental aeration and a circulation pump. Make up water was renewed twice per week at a rate of 40% of the system volume at each exchange.

Six dietary treatments containing increasing level of dietary copper supplements (6, 12 and 24 mg kg^{-1}) from $CuSO_4$ containing 25.45% copper or TBCC containing 58.81% copper were randomly assigned among 24 tanks with four replications per treatment (Table 1). Test diets were offered four times daily at 0800, 1000, 1400, and 1600 h for an 8-week period. Feed inputs were pre-calculated on a weekly basis using an expected feed conversion ratio of 2:1 and a doubling in size until individual shrimp weighs one gram. Thereafter, a growth rate of 0.8 gram per week was assumed. Each tank was covered with netting and was continuously aerated with two air stones. Shrimp were counted every week to adjust feed input based on the mortality. At the conclusion of the 8-week growth trial, shrimp were counted and group weighed. Final biomass, final mean weight, FCR and survival were determined.

2.2. Growth trial 2 (*Green water system*)

The outdoor tank system was a semi-closed recirculating system consisting of a central reservoir (800 L) with a biological filter, a 0.33 hp sump pump, and 24 circular polyethylene tanks (0.85 m height x 1.22 m upper diameter, 1.04 m lower diameter). Another sump pump was

used to move water from one of the production ponds to the central reservoir at a rate of 8 L min^{-1} during the study period between 0800 h and 1400 h to mimic a production pond setting.

Juvenile *L. vannamei* (initial weight 0.39 ± 0.01 g) were collected from the nursery tank. Juvenile shrimp were randomly selected and stocked at a density of 40 shrimp m^{-2}. Each tank and central reservoir was provided with two air stones connected to a 0.5 hp regenerative blower (Sweetwater Aquaculture Inc., Lapwai, ID, USA) to supply aeration. All tanks were covered by netting to prevent the shrimp from jumping out.

Shrimps were offered the same six diets as trial 1 four times per day at 0800, 1000, 1400 and 1600 h. Daily feed input was calculated based upon an expected growth of 1.3 g wk^{-1} and an estimated FCR of 1.2. At the conclusion of the 6-week growth trial, shrimp were counted and group-weighed. Final mean weight, final biomass, survival, and FCR were determined.

2.3. Growth trial 3 (*clear water system*)

Six dietary treatments containing increasing levels of supplemental dietary copper (0, 5, 10, 20, 40 and 60 mg kg^{-1}) from TBCC containing 58.81% copper were randomly assigned among 24 tanks with four replications per treatment (Table 2). The experimental diets were conducted in the same recirculating system as the trial 1; therefore, the daily management was the same as previously described for trial 1.

2.4. Water quality monitoring

In all trials, dissolved oxygen (DO), temperature, pH, and salinity were monitored twice daily in two of the rearing tanks at 0800h and 1600h using YSI Professional Plus meter (Yellow Spring Instrument Co., Yellow Spring, OH, USA). Total ammonia nitrogen was measured once

weekly. Total ammonia nitrogen was analyzed with an Orion ammonia electrode probe (Thermo Fisher Scientific Inc., Waltham, MA, USA). Water samples were collected every week and immediately frozen for subsequent copper analysis. All glassware in this analysis was carefully washed with 1:1 nitric acid solution and rinsed with deionized water. Copper samples were filtered through Whatman paper No. 42. Samples were measured by using the Hach Porphyrin Method (Hach Chemical Company, Loveland, CO, USA) to develop a copper-induced color. The color was evaluated with an Aquamate 9423 AQA 2000E standard spectrophotometer (Thermo scientific, England).

2.5. Sample collection and analytical methods

At the conclusion of the growth trial, shrimps were group-weighed per tank. After weighing, hemolymph samples from all shrimps per tank were collected utilizing 250 µl capillary tubes to pierce the abdominal segment near carapace. After bleeding, the shrimp and hemolymph samples were immediately frozen for subsequent mineral analyses. The individual shrimp were weighed, and then the heart, carapace and hepatopancreas were removed and weighed. The heart and hepatopancreatic indices [wet organ weight (mg)/ wet shrimp weight (g)] were determined for 30 shrimp per treatment.

Frozen shrimp were rinsed with deionized water, and then the carapace and hepatopancreas were dissected from 6–8 shrimp per tank and oven dried at 90 ˚C to a constant weight. Hepatopancreas, carapace, and hemolymph samples were wet-ashed, and copper was analyzed by atomic absorption spectrophotometry according to procedures described by Association of Official Analytical Chemists (AOAC).

2.6. Statistical analysis

Statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC, USA). Data from trial 1 and 2 were analyzed using two-way analysis of variance to determine if significant differences existed among treatment means. The Student-Newman-Keuls multiple comparison tests were used to identify significant differences between treatments means (Steel and Torrie, 1980). Outliers in copper tissues data were deleted using the method of Leys et al. (2013). Data from trial 3 were analyzed using one-way ANOVA. All statistical tests were considered significant at $P < 0.05$.

Multiple linear regression equations were calculated by least squares using the GLM procedure of SAS. Relative bioavailability values were determined using copper sulfate as the standard source by slope ratio comparisons from multiple linear regression (Littell, et al., 1997; Littell, et al., 1995). Slope ratios and their standard errors were estimated using the method of error propagation as described by Littell et al. (1995). Tissues copper concentrations exhibited variance heterogeneity and were subjected to $\log_{10}$ transformation prior to analysis. Differences among sources were determined by differences in their respective regression coefficients.

3. Results

3.1. Water quality

In trial 1, water quality parameters (mean ± standard deviation) for the following water quality were observed: temperature, 27.36 ± 1.45°C; pH, 7.86 ± 0.09; salinity, 21.01 ± 1.01 ‰; DO, 6.28 ± 0.47 mg L^{-1}; and total ammonia nitrogen (TAN), 0.07 ± 0.08 mg L^{-1}. Water quality parameters for trial 2 were monitored: temperature, 28.09 ± 2.19°C; pH, 8.04 ± 0.15; salinity, 15.51 ± 3.12 ‰; DO, 6.69 ± 0.37 mg L^{-1}; and TAN 0.03 ± 0.02 mg L^{-1}. In trial 3, water quality

parameters were observed: temperature 27.40 ± 1.48°C; pH, 7.63 ± 0.10; salinity, 19.00 ± 0.60 ‰, and DO, 6.19 ± 0.31 mg L^{-1} and TAN 0.04 ± 0.02 mg L^{-1}. The copper concentrations of water samples from all three trials remained below 2 µg L^{-1} during the culture period. Water quality conditions in the trials were suitable for the normal growth and survival of this species.

3.2. Growth performance

In trial 1, final mean weight of the shrimp ranged from 6.75 to 7.42 g, final biomass was between 98.5 and 102.0 g, percent weight gain ranged from 2033.0 to 2281.1%, FCR varied from 1.63 to 1.75, and survival ranged between 90.0 and 95.0% (Table 4). In trial 2, final mean weight of shrimp ranged from 7.87 to 8.45 g, final biomass was between 234.1 and 253.5 g, percent weight gain ranged from 1944.2 to 2087.1%, FCR varied from 0.97 to 1.05, and survival ranged between 99.2 and 100.0%. In trial 3, final mean weight of shrimp ranged from 8.47 to 9.08 g, final biomass was between 111.1 and 127.2 g, percent weight gain ranged from 2903.2 to 3127.8%, FCR varied from 1.31 to 1.44, and survival ranged between 86.7 and 100.0% (Table 4). There were no significant differences with regards to the effect of copper level or source on the shrimp's final mean weight, final biomass, FCR, and survival in any of the three trials. The two-way ANOVA indicates that growth performance was not significantly affected by the sources of copper. In trial 2 there was a slightly significant interaction between the copper source and dietary level: shrimp fed the diet supplemented with 24 mg kg^{-1} of copper sulfate (C34) had significantly higher percent weight gain than shrimp fed the diet supplemented with 12 mg kg^{-1} of copper sulfate (C22). However, this was not seen in the shrimps fed TBCC supplemented feeds. In trial 3, there was a positive correlation between percent weight gain and increasing copper supplementation with TBCC.

3.3. Tissue Copper Concentration

Dietary copper levels influenced tissues copper concentrations (Table 5). In trial 1, no significant difference occurred in carapace copper concentrations for either source of copper. The hepatopancreas and hemolymph copper concentrations in treatment with copper sulfate (C34) were significantly higher than the treatment C22. The same diets were utilized in both clear water tanks (Trial 1) and green water tanks (Trial 2). The results for hepatopancreas copper concentrations in both trials were very similar. In trial 2, the carapace copper concentrations of shrimp offered diets with copper sulfate (C34) had significantly higher levels than other treatments. In trial 2, the hemolymph copper concentrations were not significantly different among treatments utilizing either copper sulfate or TBCC. However, copper levels of the hemolymph from shrimp reared on diets with copper sulfate were significantly higher than those reared on TBCC. In trials 1 and 2, when the data were pooled across supplemental levels, the tissues' copper concentrations of the carapace, hepatopancreas and hemolymph for shrimp reared on TBCC were significantly lower than those of shrimp reared on diets with copper sulfate (Fig. 1.). In trial 3, the copper content of analyzed tissues (carapace, hepatopancreas and hemolymph) generally increased with supplementation (Table 5). The carapace copper concentrations of shrimp maintained on diets T50 and T70 were significant higher than that of shrimp reared on T20. The copper concentration of the hepatopancreas of shrimp reared on diets T50 and T70 were significantly higher than that of shrimp reared on the other treatments. The hemolymph copper concentrations of shrimp maintained on T30, T50, and T70 diets were significantly higher than other treatments.

Dietary copper sources and levels on copper concentrations of heart index and hepatopancreas index (Table 5). In trial 1, no significant difference occurred in heart index for either source of copper. Heart index ranged from 1.68 to 1.89 for shrimp fed diets containing 21 mg total analyzed Cu kg^{-1} diet to 45 mg total analyzed Cu kg^{-1} diet. There was significantly difference in hepatopancreas index between copper sources. Hepatopancreas index ranged from 50.1 for shrimp fed the diet containing 28 mg total analyzed Cu kg^{-1} diet from copper sulfate to 57.4 for shrimp fed the diet containing 27 mg total analyzed Cu kg^{-1} from TBCC.

3.4 Estimate relative bioavailability of copper sources

Regression equations of $\log_{10}$ transformed tissues copper concentration on analyzed copper intake are presented in Table 6. Linear regression of carapace, hepatopancreas and hemolymph copper concentration on analyzed copper levels indicated that TBCC and copper sulfate had similar bioavailabilities. When the response to copper sulfate was set at 100.0%, in trial 1 relative bioavailability values (RBV) of TBCC were 85.8%, 77.8% and 93.0%. In trial 2 relative bioavailability values (RBV) of TBCC were 86.1%, 78.2% and 90.8%, based on carapace, hepatopancreas and hemolymph, respectively.

4. Discussion

The dietary essentiality of copper has been reported for fresh water species such as Atlantic salmon (*Salmo salar*), common carp (*Cyprinus carpio*), tilapia (*Oreochromis* spp.), channel catfish (*Ictalurus punctatus*), yellow catfish (*Pelteobagrus fulvidraco*), rainbow trout (*Oncorhynchus mykiss*), grass carp (*Ctenopharyngodon idella*), blunt snout bream (*Megalobrama amblycephala*) and marine species such as grouper (*Epinephelus* spp.), tiger

shrimp (*P. monodon*), fleshy prawn (*F. chinensis*), Pacific white shrimp (*L. vannamei*) using the traditional copper source – copper sulfate (NRC, 2011; Shao et al., 2012; Tan et al., 2011; Tang et al., 2013). Tri-basic copper chloride as a potential copper source used in the commercial feed industry should be studied for bioavailability as well as safety. There are no studies with shrimp that have compared or demonstrated difference in availability from TBCC or copper sulfate. However, there are studies that demonstrate it to be a more bioavailable source than copper sulfate in pigs (Cromwell et al., 1998), broilers (Luo et al., 2005), steers (Spears et al., 2004) and crucian carp (Shao et al., 2010).

Unlike terrestrial animals, aquatic animals may be able to utilize, to some extent, mineral dissolved in the water to meet physiological requirements (Davis and Gatlin, 1996). Several studies demonstrated that water-borne copper could influence growth, survival, and tissues of shrimp (Djangmah and Grove, 1970; Frías-Espericueta et al., 2008; Li et al., 2005, 2007; Manyin and Rowe, 2009). As anthropogenic inputs of copper can influence environmental levels and consequently copper intake, it is important to evaluate levels in the rearing water. In the present study, the copper concentrations in the brackish water used in the three trials remained below 2 μg L^{-1} and mostly below detection levels during the culture period. These values were similar to Fabricand et al. (1962) who reported 2.2 μg L^{-1} of total copper in all sampled areas of the Atlantic and Pacific Ocean. Slowey and Hood (1971) documented that copper concentration in Gulf of Mexico waters range from 0.12 to 4.9 μg L^{-1} in the depth range from 10 to 300 m. The water copper concentration in this study was low (< 2 μg L^{-1}), while the system exchanged water at a rate of approximately 80% of total volume per week. Therefore, the problem associated with the potential contribution of mineral from the water should be negligible.

Across all three trials, the growth performance of *L. vannamei* was not significantly affected by dietary copper level or source. A limited response in terms of growth was also observed in rainbow trout (Knox et al., 1982, 1984), channel catfish (Gatlin and Wilson, 1986), and abalone (Ogino and Yang, 1980; Wang, et al., 2009). Davis et al. (1993) reported that weight gain of Pacific white shrimp increased in response to copper supplementation up to 32 mg Cu kg^{-1} (34 mg total Cu kg^{-1} diet). In the present study, the percent weight gain of shrimp did not increase with copper supplementation from copper sulfate in trial 1 and trial 2. However, there was a general increase in percent weight gain in shrimp offered diets with increasing copper supplementation from TBCC in trials 2 and trial 3. Interestingly, in other studies with *L. vannamei*, Davis et al. (1993) did not document any significant differences for weight gain among treatments from 18 mg total calculated Cu kg^{-1} diet to 66 mg total calculated Cu kg^{-1} diet from copper sulfate. Bharadwaj et al. (2014) reported no significant difference for final mean weight and growth rate per week after observing the treatment of 9 mg total analyzed Cu kg^{-1} diet to 135 mg total analyzed Cu kg^{-1} diet from copper sulfate. The results within our research trials are in agreement with Davis et al. (1993) and Bharadwaj et al. (2014), as there was no significant difference for shrimp growth performance from the treatment 24 mg total analyzed Cu kg^{-1} to 42 total analyzed Cu kg^{-1} diet from copper sulfate in trial 1 and trial 2.

A comparison of the growth data of trial 1 and trial 2 (Table 4) indicated that the shrimp growth performance in trial 2 (green water system, 6-week) was much better than in trial 1 (clear water system, 8-week) using the same diets with the same source of shrimp with similar initial weight (0.32 g in trial 1 vs 0.39 g in trial 2) and temperature. Shrimp often grow faster and survive well in the green water system due to the presence of natural foods such as algae. Based on the growth results in both trials 1 and 2 (Table 4), the shrimp growth performances from diets

with copper sulfate were similar to those of shrimp maintained on diets with TBCC either in clear water system or green water system. Similarly, trials 1 and 3 can be compared as they were also conducted on equivalent conditions. In this case, shrimp in trial 3 exhibited higher final biomass, final mean weight, percent weight gain and lower FCR than shrimp in trial 1. These results indicate that TBCC is an effective copper source as compared to copper sulfate for *L. vannamei*.

Bharadwaj et al. (2014) conducted a similar study with *L. vannamei* in which graded levels of dietary copper from copper sulfate (9–430 mg total analyzed Cu kg^{-1}) or chelated copper (30–96 mg total analyzed Cu kg^{-1}) were evaluated in a semi-purified diet. Juvenile shrimp (initial weight 0.39 g) were reared at 29.5 ± 0.3 °C with a reported weight gain from 8.36–9.74 g and percent weight gain at 2143.2–2494.9% fed by dietary copper from copper sulfate or chelated sources. These results were slightly higher than the results in trial 2 (initial weight 0.39 g, Temp 28.09 ± 2.19 °C) with gained shrimp weight at 7.87–8.45 g and weight gain at 1944–2087% from copper sulfate or TBCC for the same culture period (6-week). The slight difference in growth performance might be influenced by the temperature, as the temperature in the study by Bharadwaj et al. (2014) was higher than the temperature in the present study with similar levels of protein (35%–36%) and 8% lipid. However, these growth results from *L. vannamei* appear to be greater than those reported for *P. monodon* (Lee and Shiau 2002). Lee and Shiau (2002) reported that grass shrimp *P. monodon* (initial weight 0.29 g, Temp 26–28 °C) exhibited low percent weight gain of 65.8–207.5% over an 8-week period when fed purified diets with seven copper levels from copper sulfate (0.92–152.80 total analyzed mg Cu kg^{-1} diet). Therefore, the high growth response of *L. vannamei* might better represent current commercial growth rates of genetically improved stocks of this species.

To quantify mineral requirements in shrimp or fish, growth may not be a good or sufficient indicator of nutrient status; in such cases, the element analyses of tissues should be followed (Baker, 1986; Cowey, 1992; Davis and Gatlin, 1996). Several studies demonstrated that the carapace, hepatopancreas, and hemolymph of crustacean were a significant portion of the body burden of copper (Davis et al., 1993; Depledge, 1989; Icely and Nott, 1980; Johnston and Barber, 1969; White and Rainbow, 1985). In other studies with cattle (Nockels et al., 1993), ewes (Hatfield et al., 2001; Pal et al., 2010), pigs (Apgar et al., 1995), rats (Du et al., 1996), steers (Spear et al., 2004), and rainbow trout (Apines-Amar et al., 2004), tissue concentrations of copper were higher or similar for organic compared to inorganic sources. The cellular processes for isolating copper from general circulation in the hepatopancreas ~~begin~~ isare only beginning to be understood. Very little is currently known about the roles of other tissues such as gill, kidney, intestine, and integument for copper regulation (Ahearn et al., 2004). However, Spears et al. (2004) documented that TBCC and copper sulfate were similar in their ability to increase copper status in copper-depleted steers fed a diet low in molybdenum.

In trials 1 and 2, it is interesting to note that when the selected tissues (carapace, hepatopancreas and hemolymph) concentrations are pooled across dietary levels for each copper source, copper levels of the tissues from shrimp fed TBCC were significantly lower than those of shrimp fed diets supplemented with copper sulfate (Fig. 1). This result is similar to Zhang et al. (2009), who reported that smaller quantities of copper levels in liver were gained using TBCC compared to feeding with copper sulfate for broilers. Meanwhile, Miles et al. (1998) reported that copper sulfate had a greater percentage of larger particles than TBCC and TBCC had a better uniformity of particle size. For this reason, shrimp might have better copper absorption of TBCC than copper sulfate.

In trial 3, the carapace, hemolymph, and hepatopancreas copper contents increased with dietary TBCC levels, suggesting that TBCC also could accumulate excess copper in tissues as well as copper sulfate. This trend was also reported by Davis et al. (1993), albeit hepatopancreas copper concentrations were much higher than those seen in *L. vannamei.* Hepatopancreas copper contents in trial 3 were 16.4–33.0 μg g^{-1} in shrimp fed with 10–66 total analyzed mg Cu kg^{-1} diet from TBCC. This amount was lower than found by Davis et al (1993) who reported that 30.1–170.0 μg g^{-1} fed with 2–130 total calculated mg Cu kg^{-1} diet from copper sulfate. This might indicate that metabolism of the two forms of Cu are different. This species has less copper accumulation in the tissues from TBCC as compared to copper sulfate.

The term "bioavailability" is defined as the degree to which an ingested nutrient in a particular source is absorbed in a form that can be utilized in metabolism by the animal (Ammerman, et al., 1995). In the present study, it indicated that the tissues value of relative bioavailability for TBCC was lower than copper sulfate. This result is not consistent with other studies (Luo, et al., 2005; Miles, et al., 1998; Shao, et al., 2010). Shao et al. (2010) determined the relative bioavailability values of TBCC were 118%, 131% and 173% relative to copper sulfate (100%) for crucian carp, *C. auratus gibelio.* Miles et al. (1998) obtained a value of 106% for TBCC compared to 100% copper sulfate based on liver copper concentration of chicks. Luo et al. (2005) showed that the linear regression of $\log_{10}$ transformed liver copper concentration on added total copper intake resulted in the slope ratio estimate of 109.0%, 108.4% and 106.9% respectively, for bioavailability of copper from TBCC compared with 100 for that in copper sulfate. However, it should be kept in mind that bioavailability is not an inherent characteristic of a specific source of any mineral element (Fairweather-Tait, 1987). It is an experimentally determined value that is dependent on conditions during a specific test situation. Therefore, there

is no single correct value to assign to any particular source of an element, although this is a common perception among researchers and the feed industry (Cao, et al., 2000).

In conclusion, the growth performance results demonstrated that TBCC was available as a copper source as well as copper sulfate to shrimp from diets. Analysis of the tissues showed that TBCC resulted in lower levels of accumulation of copper in the tested tissues. The study showed that TBCC was a safe, effective and available source of copper in the Pacific white shrimp *L. vannamei*.

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Table 1 Composition of six practical diets formulated with increasing percentages of dietary copper 5, 10 and 20 mg kg^{-1} from $CuSO_4$ containing 254.5 g kg^{-1} copper or TBCC containing 588.1 g kg^{-1} copper. The diets were formulated to be isonitrogenous at 360 g kg^{-1} protein and 80 g kg^{-1} lipid

Ingredients (As is basis g kg^{-1})	C16	C22	C34	T16	T22	T34
Fish meal[1]	50.0	50.0	50.0	50.0	50.0	50.0
Soybean meal[2]	483.5	483.5	483.5	483.5	483.5	483.5
Menhaden Fish Oil[1]	57.9	57.9	57.9	57.9	57.9	57.9
Corn Starch[3]	36.0	36.0	36.0	36.0	36.0	36.0
Whole wheat[4]	250.1	250.1	250.1	250.1	250.1	250.1
Mineral premix Cu-free[5]	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin premix[6]	18.0	18.0	18.0	18.0	18.0	18.0
Choline chloride[4]	2.0	2.0	2.0	2.0	2.0	2.0
Stay C[7]	1.0	1.0	1.0	1.0	1.0	1.0
CaP-diebasic[8]	25.0	25.0	25.0	25.0	25.0	25.0
Lecithin[9]	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol[10]	0.5	0.5	0.5	0.5	0.5	0.5
Empyreal 75 CGM[11]	60.0	60.0	60.0	60.0	60.0	60.0
Cellulose[12]	0.976	0.952	0.905	0.990	0.980	0.959
$CuSO_4 \cdot 5H_2O$	0.024	0.048	0.095			
TBCC				0.010	0.020	0.041
Proximate composition						
Crude protein	368.3	358.9	359.7	365.6	359.4	357.2
Moisture	80.1	91.9	86.8	74.2	90.6	92.4
Lipid	84.8	81.4	82.9	85.0	83.2	83.4
Ash	72.1	70.7	70.3	71.6	71.2	70.7

[1] Omega Protein Inc., Reedville, VA, USA.
[2] Faithway Feed Co., Guntersville, AL, USA.
[3] Grain Processing Corporation, Muscatine, IA, USA.
[4] MP Biochemicals Inc., Solon, OH, USA.

[5] Trace mineral premix Cu free (g kg^{-1}): cobalt chloride 0.04, ferrous sulfate 40.0, magnesium sulfate heptahydrate 283.98, manganous sulphate monohydrate 6.50, potassium iodide 0.67, sodium selenite 0.10, zinc sulfate heptahydrate 131.93, filler 534.28.
[6] Vitamin premix (g kg^{-1}): thiamin HCl 0.5, riboflavin 3.0, pyridoxine HCl 1.0, DL Ca-pantothenate 5.0, nicotinic acid 5.0.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35%), Roche Vitamins Inc., Parsippany, NJ, USA.
[8] Fisher Scientific, Fair Lawn, NJ, USA.
[9] Solae Company, St. Louis, MO, USA.
[10] USB Biochemicals, Cleveland, OH, USA.
[11]Cargill Corn Milling, Cargill, Inc., Blair, NE, USA
[12] Sigma Chemical Co., St. Louis, MO, USA

Table 2 Composition of six practical diets formulated with different dietary copper level (0, 5, 10, 20, 40 and 60 mg kg^{-1}) from TBCC containing 588.1 g kg^{-1} copper. The diets were formulated to be isonitrogenous at 360 g kg^{-1} protein and 80 g kg^{-1} lipid.

Ingredients (As is basis)	T10	T15	T20	T30	T50	T70
Fish Meal[1]	45.0	45.0	45.0	45.0	45.0	45.0
Soybean meal[2]	495.5	495.5	495.5	495.5	495.5	495.5
Menhaden Fish Oil[1]	58.4	58.4	58.4	58.4	58.4	58.4
Corn Starch[3]	32.0	32.0	32.0	32.0	32.0	32.0
Whole wheat[4]	250.1	250.1	250.1	250.1	250.1	250.1
Trace Mineral premix Cu-free[5]	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin premix[6]	18.0	18.0	18.0	18.0	18.0	18.0
Choline cloride[4]	2.0	2.0	2.0	2.0	2.0	2.0
Stay C[7]	1.0	1.0	1.0	1.0	1.0	1.0
CaP-diebasic[8]	25.0	25.0	25.0	25.0	25.0	25.0
Lecethin[9]	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol[10]	0.5	0.5	0.5	0.5	0.5	0.5
Empyreal 75 CGM[11]	56.5	56.5	56.5	56.5	56.5	56.5
Cellulose[12]	1.000	0.992	0.983	0.966	0.932	0.898
TBCC	0.000	0.009	0.017	0.034	0.068	0.102
Proximate composition (g kg^{-1})						
Crude protein	356.0	340.0	339.0	361.0	354.0	357.0
Moisture	65.7	78.9	73.3	78.0	76.2	68.6
Lipid	91.7	86.9	86.2	85.3	86.1	89.2
Ash	70.2	69.8	71.9	67.4	70.0	68.2

[1] Omega Protein Inc., Reedville, VA, USA.
[2] Faithway Feed Co., Guntersville, AL, USA.
[3] Grain Processing Corporation, Muscatine, IA, USA.
[4] MP Biochemicals Inc., Solon, OH, USA.
[5] Trace mineral premix Cu free (g kg^{-1}): cobalt chloride 0.04, ferrous sulfate 40.0, magnesium sulfate heptahydrate 283.98, manganous sulphate monohydrate 6.50, potassium iodide 0.67, sodium selenite 0.10, zinc sulfate heptahydrate 131.93, filler 534.28.
[6] Vitamin premix (g kg^{-1}): thiamin HCl 0.5, riboflavin 3.0, pyridoxine HCl 1.0, DL Ca-pantothenate 5.0, nicotinic acid 5.0.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35%), Roche Vitamins Inc., Parsippany, NJ, USA.
[8] Fisher Scientific, Fair Lawn, NJ, USA.
[9] Solae Company, St. Louis, MO, USA.
[10] USB Biochemicals, Cleveland, OH, USA.

[11]Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[12] Sigma Chemical Co., St. Louis, MO, USA.

Table 3 The treatments in three trials used to evaluate the efficacy of different sources of copper in Pacific white *shrimp Litopenaeus vannamei.*

Copper Source	Trial	Diet	Supplemental Cu (mg kg^{-1})	Dietary Cu (mg kg^{-1})[1]	Analyzed Cu (mg kg^{-1})[2]
Copper Sulfate ($CuSO_4 \cdot 5\ H_2O$)	1& 2	C16	6	16	24
	1& 2	C22	12	22	28
	1& 2	C34	24	34	42
Tribasic Copper Chloride (TBCC)	1& 2	T16	6	16	21
	1& 2	T22	12	22	27
	1& 2	T34	24	34	45
TBCC	3	T10	0	10	10
	3	T15	5	15	15
	3	T20	10	20	19
	3	T30	20	30	29
	3	T50	40	50	48
	3	T70	60	70	66

[1] The dietary copper concentrations containing either copper source were calculated in the diet formulation.

[2] The diets containing the level of either copper source were analyzed for copper.

Table 4 Effect of dietary copper sources and levels on performance of *Litopenaeus vannamei.*

Trial	Diet	Analyzed Cu (mg kg^{-1})	Final Biomass (g)	Final Weight (g)	Weight Gain (%)[1]	FCR[2]	Survival (%)
Trial 1	C16	24	101.8	7.12	2181.5	1.69	95.0
	C22	28	102.0	7.42	2281.1	1.63	90.0
	C34	42	91.2	6.75	2033.0	1.70	90.0
	T16	21	98.5	6.93	2053.4	1.75	95.0
	T22	27	100.4	7.17	2206.3	1.69	93.3
	T34	45	101.2	6.99	2142.1	1.73	95.0
	PSE[3]		10.54	0.09	34.78	0.02	1.44
	Two-way ANOVA						
	Model		0.6977	0.4521	0.3373	0.4969	0.7845
	Source		0.7030	0.7169	0.6582	0.6601	0.3484
	Level		0.6133	0.2059	0.1789	0.2441	0.6242
	Source × Level		0.4116	0.5214	0.3645	0.5358	0.7740
Trial 2	C16	24	253.5	8.45	2087.1^{ab}	0.97	100.0
	C22	28	234.1	7.87	1944.2^{b}	1.05	99.2
	C34	42	249.3	8.38	2084.4^{a}	0.98	99.2
	T16	21	243.6	8.12	1957.6^{ab}	1.01	100.0
	T22	27	243.0	8.16	2018.6^{ab}	1.01	99.2
	T34	45	246.8	8.30	2056.6^{ab}	0.99	99.2
	PSE[3]		8.91	0.01	13.48	0.01	0.28
	Two-way ANOVA						
	Model		0.0991	0.0661	0.0215	0.1408	0.8424
	Source		0.7555	0.7321	0.3226	1.0000	1.0000
	Level		0.0650	0.0561	0.0488	0.0392	0.3874
	Source × Level		0.1327	0.0817	0.0229	0.4241	1.0000
Trial 3	T10	10	123.0	8.47	2903.2	1.41	96.7
	T15	15	127.2	8.48	2906.3	1.44	100.0
	T20	19	120.2	8.70	2960.1	1.31	91.7
	T30	29	111.1	8.54	2954.8	1.40	86.7
	T50	48	124.7	8.77	3002.6	1.36	95.0
	T70	66	121.4	9.08	3127.8	1.32	90.0
	PSE[3]		2.56	0.11	49.09	0.01	1.74
	P-value		0.5694	0.6104	0.7879	0.6524	0.3130

Values are means of four replicates. Means within columns with the same letter are not significant different ($P > 0.05$) based on analysis of variance followed by Student Newman-Keuls multiple range test.

[1] Weight gain (%) = (Final weight – Initial weight)/Initial weight × 100%.

[2] Feed conversion ratio (FCR) = Feed intake/(Final weight – Initial weight).

[3] Pooled Standard Error.

Table 5 Effects of dietary copper sources and levels on copper concentrations of carapace, hepatopancreas, hemolymph, heart index and hepatopancreas index in *Litopenaeus vannamei*.

Trial	Diet	Analyzed Cu (mg kg^{-1})	Carapace (μg g^{-1})	Hepatopancreas (μg g^{-1})	Hemolymph (μg g^{-1})	Heart index[1]	Hepatopancreas Index[2]
Trial 1	C16	24	15.3	17.2^{b}	160.0^{b}	1.89	52.5^{abc}
	C22	28	15.8	16.5^{b}	151.6^{b}	1.68	50.1^{c}
	C34	42	16.3	34.1^{a}	185.8^{a}	1.70	51.2^{bc}
	T16	21	13.1	14.4^{b}	139.8^{b}	1.77	56.1^{ab}
	T22	27	10.3	13.2^{b}	137.7^{b}	1.80	57.4^{a}
	T34	45	12.1	13.2^{b}	149.3^{b}	1.85	56.0^{ab}
	PSE[3]		1.6	1.3	7.0	0.13	0.5
	Two-way ANOVA *P-value*						
	Model		0.1141	0.0001	0.0025	0.5088	0.0012
	Source		0.0082	0.0001	0.0013	0.5022	0.0001
	Level		0.7449	0.0001	0.0129	0.5825	0.8755
	Source × Level		0.6124	0.0001	0.2792	0.2538	0.4431
Trial 2	C16	24	19.4^{b}	26.8^{b}	101.9^{a}		
	C22	28	19.4^{b}	34.0^{b}	106.5^{a}		
	C34	42	31.1^{a}	52.6^{a}	113.5^{a}		
	T16	21	18.6^{b}	21.2^{b}	83.3^{b}		
	T22	27	20.2^{b}	18.1^{b}	82.0^{b}		
	T34	45	17.1^{b}	17.6^{b}	85.3^{b}		
	PSE[3]		2.5	4.5	3.8		
	Two-way ANOVA *P-value*						
	Model		0.0134	0.0013	0.0001		
	Source		0.0416	0.0002	0.0001		
	Level		0.1309	0.0917	0.1987		
	Source × Level		0.0192	0.0277	0.4526		

Values are means of four replicates. Means within columns with the same letter are not significant different ($P > 0.05$) based on analysis of variance followed by Student Newman-Keuls multiple range test.

[1] The heart index= wet heart (mg)/wet weight shrimp (g)

[2] The hepatopancreas index= wet hepatopancreas (mg)/wet weight shrimp (g)

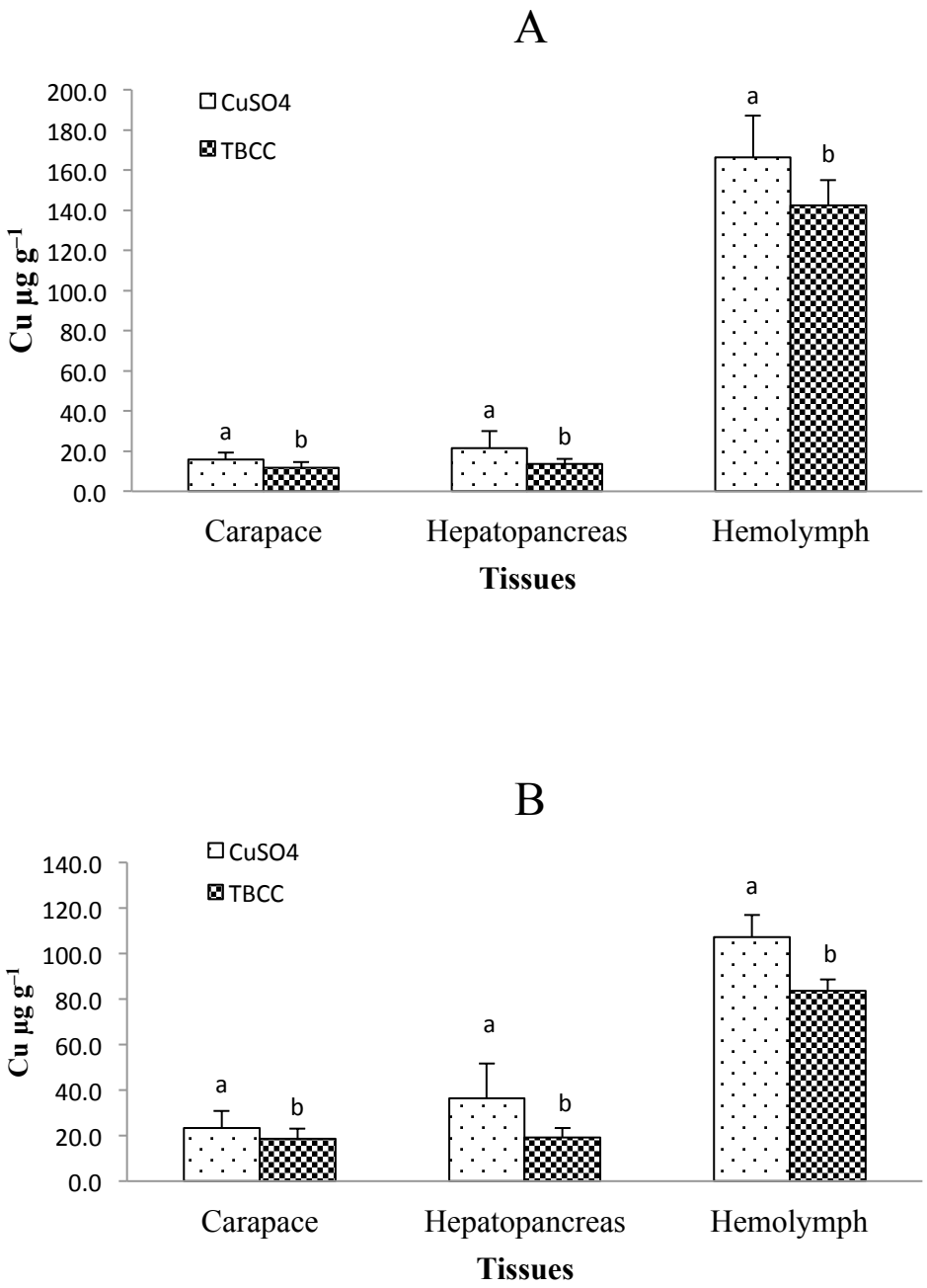
[3] Pooled Standard Error

Table 6 Relative bioavailability values (RBV) of Cu based on multiple linear regression of $\log_{10}$ Cu concentrations in carapace, hepatopancreas and hemolymph of shrimp on dietary analyzed Cu during the culture period.

Trial	Dependent variable	Cu sources	Slope[1] ± SE	RBV%[2]
Trial 1	Carapace Cu[3]	CuSO4	0.032 ± 0.0031	100
		TBCC	0.028 ± 0.0031	85.8
	Hepatopancreas Cu[4]	CuSO4	0.038 ± 0.0031	100
		TBCC	0.030 ± 0.0029	77.8
	Hemolymph Cu[5]	CuSO4	0.058 ± 0.0055	100
		TBCC	0.054 ± 0.0054	93
Trial 2	Carapace Cu[6]	CuSO4	0.037 ± 0.0034	100
		TBCC	0.032 ± 0.0033	86.1
	Hepatopancreas Cu[7]	CuSO4	0.044 ± 0.0037	100
		TBCC	0.035 ± 0.0036	78.2
	Hemolymph Cu[8]	CuSO4	0.054 ± 0.0051	100
		TBCC	0.049 ± 0.0050	90.8

[1] Slopes of the fitted regression lines.
[2] RBV%: relative bioavailability values of copper from copper sulfate and TBCC were estimated by slope ratio model, based on linear regression of $\log_{10}$ copper concentration in carapace, hepatopancreas and hemolymph on daily analyzed copper level, using copper sulfate as the standard source.
[3] Intercept=0.15, R^2=0.80, *P*=0.0001
[4] Intercept=0.14, R^2=0.85, *P*=0.0001
[5] Intercept=0.25, R^2=0.83, *P*=0.0001
[6] Intercept=0.16, R^2=0.82, *P*=0.0001
[7] Intercept=0.15, R^2=0.84, *P*=0.0001
[8] Intercept=0.26, R^2=0.81, *P*=0.0001

Figure 1 Effects of dietary copper sources with levels pooled, on carapace, hepatopancreas, and hemolymph in *Litopenaeus vannamei* in trial 1 (A) and trial 2 (B).



CHAPTER V

SUMMARY AND CONCLUSIONS

Continued rapid growth and intensification of aquaculture production depends upon the development of sustainable feed formulations to support the long-term expansion of the industry. . One of the primary challenges facing the feed industry is to identify economically viable and environmentally friendly alternative ingredients to replace fishmeal and fish oil in feed formulations. Many researchers have focused on the goal of increasing the use of plant-based ingredients and reducing the use of marine ingredients in shrimp feed formulations. Toward this goal, high levels of soybean meal can be used as major protein sources in shrimp formulations. Soybean meal is considered an economical and nutritious feedstuffs with moderate protein content, high digestibility, relatively well-balanced amino acid profile, and steady supply with a reasonable price. The overall goal of this study was to advance our understanding of plant-based feed formulations through a better knowledge of various sources and nutrients that may be limiting. More specifically, the goal was to evaluate new varieties of soybean meal to determine if the efficacy of Dried Distillers Grains with Soluble (DDGS) from sorghum (S-DDGS) as an alternative protein source in shrimp feed formulation would improve the sustainable development of aquaculture. To further optimize plant-based feeds, trace mineral supplements may also be needed; hence, Cu supplements were also evaluated.

Commodity soybean meal has a number of anti-nutritional factors, which limit its inclusion in feed formulations. New strains of selectively bred, non-genetically modified (non-GM) soybeans can have reduced levels of oligosaccharides, lectins, trypsin inhibitors and/or enhanced levels of protein, which may afford fewer problems and allow for higher substitutions

in formulations for marine shrimp. Based on this study, there were no significant differences in survival or percent weight gain among treatments using a range of new soy lines. In the digestibility trial, soybean meal C displayed the highest ADMD and ADP values compared to the other experimental meals and also exhibited a significant higher ADP value than conventional soybean meal A. This finding indicates that new lines of soybean meal can be used to improve digestibility coefficients in shrimp feeds.

DDGS is a by-product of ethanol production that is a potential protein source for shrimp feed due to its lower feed cost. Based on the results of the present study, S-DDGS can be included in practical diets at the inclusion rate up to 40% for the Pacific white shrimp, without negative effects on growth, survival and FCR. Although some performance differences were observed between extruded or pelleted feed, these were not consistent across the two trials. As expected, some shifts in processing parameters were observed as the formulations were changed. However, all the feeds produced were reasonable in terms of the physical characteristics and supported good performance of the shrimp. Thus, the use of S-DDGS products should be encouraged as an alternative protein source in shrimp feed formulations.

The use of plant-based sources in feeds may result in the reduced availability of trace minerals, such as copper, due to the presence of the antagonists in plant meals. The effect of supplemental two copper sources (copper sulfate pentahydrate and tri-basic copper chloride) in the practical diets for *L. vannamei* was evaluated on growth performance and tissues' responses. Based on the results of this study, there was no significant effect of copper supplemental level and source on final mean weight, final biomass, FCR, and survival in three growth trials. Shrimp growth performance across three trials did not respond to dietary copper supplements. This study

showed that TBCC was a safe, effective and available source of copper in the Pacific white shrimp *L. vannamei*.

These studies have indicated that new varieties of soybean should be pursued and that ethanol byproducts such as S-DDGS can be used effectively in practical diets for *L. vannamei*. The soybean meal C may hold a high potential as a shrimp feed ingredient. Thus, its use can be encouraged in shrimp feed. The S-DDGS can be included in practical diets and generally increased up to 40% for extruded or pelleted feed without negative growth performance and with good physical characteristics. This study also improved the knowledge of copper sources in shrimp diets. The use of TBCC should be encouraged as an alternative copper source in shrimp feed formulation.

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