Development and Application of Non Marine Ingredients Based Diets for Pacific White Shrimp, *Litopenaeus vannamei*: Examining the Suitability of Alternative Lipid Sources

by

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Abstract

The Pacific white shrimp, *Litopenaeus vannamei*, is globally cultured due to its ability to tolerate and effectively grow and survive across a wide range of salinities, ease of reproduction, availability of domesticated strains and feeding habits. Following the worldwide trend of minimizing the usage of marine feed ingredients, the total substitution of marine protein sources for *L. vannamei* production diets using alternative, mainly plant based protein sources has been demonstrated. The next logical step is the substitution and/or reduction of the marine lipid sources from the diet. This goal has both advantages and disadvantages, as inappropriate substitutions could be problematic for the industry.

A series of experiments was conducted at different locations in the southern United States. These facilities included the Alabama Department of Conservation and Natural Resources, Marine Resources Division, Claude Peteet Mariculture Center in Gulf Shores, Alabama; the Alabama Fish Farming Center in Greensboro, Alabama; Greene Prairie Aquafarm in Boligee, Alabama; and the Texas Agrilife Research Mariculture Laboratory in Flour Bluff, Texas. Ponds, outdoor tanks and clear water laboratory tanks trials were carried out in parallel throughout the summers (May – September) of 2009 and 2010, with some of these studies representing one full commercial production cycle for *L. vannamei*.

Results from these studies demonstrated that formulated diets containing approximately 35% protein and 6 to 8% lipid with balanced amino acid and fatty acid profiles can be formulated using high levels of soybean meal as the primary protein source and alternative oils

and fats as lipid sources. Satisfactory production results were demonstrated when using soybean oil, flaxseed oil, palm oil and poultry grease in combination with marine fish oil, and stearin fish oil replacing marine fish oil. Performing a 90% replacement of marine fish oil showed no significant statistical decrease in production performance of *L. vannamei* as mean final weights, survival, FCR, and final standing crop.

Overall, results from these studies reveal that use of high levels of alternative oils and fats as main lipid sources in combination with marine fish oil in formulated diets for *L. vannamei* is viable as long as essential nutrients in diet are properly balanced to meet shrimp nutritional requirements.

This work summarizes recent research that validates the possibility of a diet free of marine ingredients and/or those with very limited inclusion levels.

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CHAPTER I

INTRODUCTION

The contribution of aquaculture to global production of seafood grew from 27 percent in 2000 to 41.3 percent in 2011, where 78% of the inland and 20% of marine fish production originated from aquaculture. The production of the Pacific white shrimp *Litopenaeus vannamei* dominates the marine species production and grew more than 13.5 times from 1999 to 2010 reaching 2.6 million tons (FAO, 2012). Albeit crustacean production represents only 9.6% (5.7 million tonnes) of the volume of species produced through aquaculture throughout the world, it accounts for 23.1% of the market revenue (FAO, 2010).

The Pacific white shrimp is an euryhaline species, and thus has a high tolerance to a wide variety of salinities (Bray et al., 1994; Roy et al., 2007). Hatchery production is fairly simple producing large quantities of resilient post-larvae (PLs) which are readily available in the market making it a very attractive species for large-scale commercial aquaculture.

One of the consequences of the aquaculture industry growth as a whole is an increased demand for feed and feed ingredients, mainly fish meal and fish oil (Sookying & Davis, 2011). This has subsequently enlarged the capture efforts of wild fish to produce meal and oil generating environmental concerns, pushing up the prices of those feed ingredients, and creating a competition with the industry aimed for human consumption (e.g. food, cosmetics, health, etc.). Studies conducted by Davis (2000); Davis et al. (2002); Samocha (2004); Browdy et al. (2006);

Roy et al. (2009); and Markey et al. (2010) have demonstrated the feasibility of a complete substitution of fish meal by one or a combination of alternative protein sources (e.g. soybean meal, poultry by-product meal, distiller's dried grains with solubles, pea meal, corn gluten meal) in production diets for *L. vannamei* with no adverse effects on performance. The substitution of fish meal by any alternative protein sources must not only account for the amino acid profile but shifts in fatty acids and other nutrients must also be adjusted. This is especially true of the essential fatty acids, which must be properly supplied if fish meal and other marine ingredients are removed.

In 2006 the aquaculture feed industry consumed 88% of the fish oil produced worldwide (Albert G. J. Tacon & Metian, 2008). As the fish oil supply is limited, the continued growth of the industry will require alternative cost-effective oil sources which are able to supply sufficient levels of essential fatty acids, possess acceptable stability and have good palatability (Francis, 2001; González-Félix et al., 2010). According to previous research by Lim, Ako, Brown, & Hahn (1997), Cheng & Hardy (2004), Browdy et al. (2006), Samocha et al. (2009) and González-Félix et al. (2010) a partial or total replacement of fish oil can be done if the essential highly unsaturated fatty acids (HUFA), such as arachidonic acid (ARA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are provided in sufficient quantity in the diet since this species has a limited capability to elongate and desaturate polyunsaturated fatty acids (PUFA) into HUFAs. The incorporation of 10% (of the total lipid) from marine fish oil (González-Félix et al., 2010) or an alternative source of those essential fatty acids (EFA), from the total oil in the diet, should supply enough HUFA for a good production performance of the species.

Based on the Oil World Annual 2008 report (Oil World, 2008), the major oils and fats produced in the world are palm oil, mainly from Indonesia and Malaysia in Southeastern Asia

with 42.4 million metric tons, representing 26.7% of the total production, while 37.7 million metric tons of soybean oil (23.7% of total production) are produced in North and South America. Fish and linseed or flaxseed oil are listed in 14th and 16th place, with a world production figure of 1.1 (0.7%) and 0.6 (0.4%) million metric tons, respectively. Hence, the availability and utilization of different oils, and costs related to the inclusion in the diet, are dependent on the location of the enterprise which is also influenced by market supply and demand.

Most seafood contains a high concentration of highly unsaturated fatty acids, mainly arachidonic acid (ARA, 20:4n-6), and the omega-3 fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), which are essential for a number of species, and a low concentration of saturated fatty acids (SFA), granting its most desired characteristic as a healthy product for human consumption (Simopoulos, 1991). The health benefits from seafood consumption have been disclosed for the first time in 1975 by Dyerberg, Bang, & Hjorne. Since then, several studies have reported the cardiovascular health benefits of the consumption of such omega-3 fatty acids. Such benefits include also the improvement of neuropsychological disorders (Sinclair et al., 2007), inflammation, obesity and asthma (Ford, 2005; Shore, 2008; Myers & Allen, 2011), obesity and allergic diseases (Hersoug & Linneberg, 2007), hostility and aggression (Hamazaki et al., 1996; Hamazaki & Hamazaki, 2008), cardiovascular diseases (Kerver et al., 2003; Hooper et al., 2006), cancer (Hooper et al., 2006), brain cognitive development (Innis, 2007; Wainwright, 2007), and a series of reviews on health benefits from the consumption of omega-3 HUFA (Simopoulos, 1991, 2002; Ruxton et al., 2004; Ford, 2005; Das, 2006; Hooper et al., 2006; Innis, 2007; Wainwright, 2007; Sinclair et al., 2007; Riediger et al., 2009; Ruxton & Derbyshire, 2009).

The culture of shrimp in low salinity waters (LSW) have been reported in the 1980s by Cawthorne and collaborators (1983) growing considerably in countries such as China, Thailand, Vietnam, Ecuador, Brazil, Mexico, and the United States since then (Roy & Davis, 2010). Despite the fact that the production in LSW is growing there are still challenges in rearing animals in less than ideal environments (Saoud et al., 2003; Roy et al., 2007b, 2007c; Roy et al., 2009), which depends on the variability of the LSW composition of anions (bicarbonate, carbonate, chloride, sulfate) and cations (calcium, magnesium, potassium, sodium) at physiologically appropriate ratios and concentrations (Saoud et al., 2003; Roy, 2006). Polar lipids, such as phospholipids, play an important role in osmoregulatory organs, such as the gill in aquatic organisms, maintaining its normal cell structure (Castell et al., 1989; Teshima et al., 1997) and functions (Teshima et al., 1997), improving the animals adaptations to low salinity environments (Gong et al., 2000); giving the fatty acid content in the diet an important role on the culture of these organisms.

Knowing of the benefits from seafood consumption due to its fatty acids profile, the aquaculture industry faces the challenge of delivering a product at a reasonable cost, along with all health benefits from the wild caught specimens at a steady supply. To the fatty acids health benefits we could also add the advantage of the possibility to control toxic compounds and diseases transmission from the final product.

Given the possible environmental, economic, and health benefits of using alternative oils, the objective of this research was to evaluate, under different field and laboratory conditions, the effect of several lipid sources on the production performance of *L. vannamei*. Specific objectives were included to identify the response of *L. vannamei* to different treatments:

1. Evaluate the production performance of *L. vannamei* to reduced levels of fish oil

when replaced by alternative lipid sources in practical diets under green and clear water conditions in different salinity levels and culture conditions;

2. Evaluate the shifts in fatty acid uptake by the shrimp on the edible portion (tail) due to various alternative lipid source on diet and culture conditions;
3. Evaluate the shifts in polar and non-polar lipids composition of osmoregulatory organ (gill) in response to various alternative lipid sources on diet under different culture conditions and salinities.

CHAPTER II

REPLACEMENT OF FISH OIL IN PLANT BASED DIETS FOR PACIFIC WHITE SHRIMP (*Litopenaeus vannamei*)

Abstract

Due to economic pressures from high fish oil prices as well as buyers and consumers requiring sustainable practices, the use of high levels of fish oils in aquafeeds is no longer desirable. The present study evaluated the replacement of marine fish oil (MFO) with alternative oils in a plant based diet. *Litopenaeus vannamei* juveniles (1.55 g) were stocked into 650 L circular tanks at 26 shrimp tank^{-1} and fed 13 experimental diets over a 58-day growth period. Diets were formulated with soybean oil (SO) as replacement for MFO at inclusion ratios of 100:0, 50:50, 40:60, 30:70, 20:80, and 10:90 as MFO:SO. The next series of diets were formulated to keep n-3/n-6 ratios close to the ratio attained at 50% MFO replacement while removing MFO. This was done by increasing linolenic acid content through the use of linseed oil (LO), resulting in the following MFO:SO:LO ratios, 40:53.4:6.6, 30:56.9:13.1, 20:60:20, 10:63.7:26.3. Three additional diets were evaluated which included a high LO (10:90 as MFO:LO) and a high soybean oil diet using a low linolenic acid soybean oil (LLSO) at a 10:90 ratio of MFO:LLSO. The final diet was a commercial diet which served as a reference. Results showed no statistically significant differences in final mean weight, growth, survival or FCR values of shrimp fed the various diets. Fatty acid (FA) profiles of tail muscle form shrimp fed the

various lipid sources in general conformed to the lipids of the feed. Shrimp fed Diet 11, with 19.81 mg of linolenic acid per gram of diet had the highest amount of this FA in tail muscle (5.61 mg g^{-1} wet tissue) and a relatively high n-3/n-6 ratio of 1.15, but at the same time, practically the lowest content of eicosapentaenoic (4.07 mg g^{-1} wet tissue) and docosahexaenoic (2.04 mg g^{-1} wet tissue) acid among the dietary treatments. This response is typical for animals that cannot elongate and desaturate polyunsaturated into highly unsaturated FA (HUFA). As shrimp production was not influenced by lipid source or n-3/n-6 ratio, clearly a range of lipids could be used to support growth. However, as the optimal dietary approach for humans is to consume preformed n-3 HUFA by eating seafood, it would be best for farmed shrimp to retain high levels of n-3 HUFA and high n-3/n-6 ratios as found in wild caught shrimp.

Introduction

According to the Food and Agriculture Organization of the United Nations (FAO, 2009), the world's production of captured and farmed shrimp is approximately 6 million tonnes. The exceptional increment in production over the past four decades has been primarily attributed to increased production from shrimp farming activities, since capture fisheries have been assumed to be at maximum sustainable yield for many years. In fact, more than 43% (2.6 million tonnes) of the world's total shrimp production was from farming. As world population continues to grow, a future increase in demand for seafood is expected. With the contribution from fisheries at the maximum sustainable level, aquaculture will be the only source for meeting the growing demand for seafood in general and for shrimp in particular.

In 2006 aquaculture production relied on fish oil for feeding species which consumed almost 22.7 million tonnes of aquafeeds containing about 835,000 tonnes of fish oil (Albert G. J. Tacon & Metian, 2008). Since global fish oil production in 2006 amounted 943,000 tonnes, it represents 88.5% of the total reported fish oil production for that year, at that time priced at more than US$750 per tonne (Decision News Media, 2006). Continued expansion of aquaculture will be possible only if cost-effective alternative sources of high quality oils are available to be used in aquafeeds. Vegetable oil use in aquafeeds without marine fish oil is often limited by the potential problems associated with insufficient levels of essential fatty acids (FA), anti-nutritional factors and poor palatability (Francis et al., 2001). However, several authors have reported that partial or total replacement of fish meal and fish oil with soybean meal and soy oil

had no adverse effect on growth performance (Davis & Arnold, 2000; Cheng & Hardy, 2004; Samocha et al., 2004). Nevertheless, to replace marine fish oils in commercial feeds, one must have a complete strategy that allows for the replacement of essential FA and enhance the palatability of the diet at the same time.

From an animal production standpoint, as we replace fish oil with alternative oils (*e.g*., soybean oil), we must ensure that we meet the animals' essential FA requirement. Partial replacement of fish oil in the grow-out period may be possible with little adverse effect on shrimp growth (Patnaik et al., 2006; Samocha et al., 2009). However, in the early growth period, growth and immunity may be compromised. In any case, substitution is likely to reduce the valuable long chain n-3 FA in shrimp tissues. It is also likely to introduce high levels of n-6 FA which are not appropriate from a human nutrition standpoint. Many Western diets are already excessively high in these FA, with n-3/n-6 ratios of 1/15 or lower (Simopoulos, 2002). Published data suggest this excessive amount of n-6 poly-unsaturated fatty acids (PUFA) promotes the pathogenesis of many diseases and, at the same time, describe the health benefits of n-3 highly-unsaturated fatty acids (HUFA) in preventing cardiovascular disease, cancer, inflammatory autoimmune diseases, and supporting brain development and function (Ruxton et al., 2004; Lands, 2008; Chapkin et al., 2009). Seafood is recommended in the diet to increase the n-3/n-6 ratio and promote human health; for that reason, it is critical to ensure that farmed shrimp retain high levels of n-3 HUFA and high n-3/n-6 ratios as found in wild caught shrimp.

Lipid content and the associated C18 PUFA, linoleic (18:2n-6) and linolenic (18:3n-3) acids, as well as n-3 and n-6 HUFA, eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and arachidonic acid (ARA, 20:4n-6) are required in diets for shrimp and other crustacean at levels between 5 and 10 g/kg (Akiyama, Dominy, & Lawrence, 1992;

González-Félix et al., 2002). This is an important consideration when replacing fish oil with other substitutes. In addition, a number of laboratory based lipid trials have demonstrated that the fatty acid profile of shrimp tissues parallels the shift in FA profiles of the diet (Deering, Fielder, & Hewitt, 1997; González-Félix et al., 2002; Glencross et al., 2002; González-Félix et al., 2003). Given that many plant-based feed ingredients contain a low n-3/n-6 ratio (Giovanni M. Turchini, Torstensen, & Ng, 2009), the challenge is to promote good shrimp performance while maintaining a high n-3/n-6 ratio ideal for human health. The purpose of this study was to evaluate the replacement of fish oil by soybean oil, as well as the combination of soybean with linseed oil for increasing the n-3/n-6 ratio in practical diets for Pacific white shrimp, *Litopenaeus vannamei*.

Materials and methods

Source of shrimp and experimental system

Research was conducted at the Texas AgriLife Research Mariculture Laboratory in Corpus Christi, TX, USA. Pacific white shrimp, *L. vannamei*, postlarvae were donated by Shrimp Improvement Systems (Islamorada, FL) and nursed for about 53 days. At the conclusion of the nursery phase, juvenile shrimp (1.55 ± 0.03 g) were hand sorted for uniform size and stocked into sixty-five partially shaded 650 L circular tanks (bottom area: 0.85 m^2) at 26 shrimp $tank^{-1}$ (40 m^{-3} or 31 m^{-2}). Group weight was recorded by tank and a one-way ANOVA was performed to ensure there were no statistically significant differences between treatments in mean shrimp weight at the time of stocking.

All tanks were covered with netting to prevent shrimp losses due to jumping. Each tank was provided with two air-stones having similar air flows of 8-10 L min^{-1}. Tanks were filled with

natural seawater and chlorinated (5 ppm); chlorine was allowed to dissipate prior to the start of the study. Culture water was circulated between all tanks at a rate of 1.9 L min^{-1} to provide one full turnover of water exchange every six hours. Naturally induced primary production was present and no external biofiltration was provided. Municipal freshwater was added to offset evaporative losses and to maintain salinity. Physiochemical parameters including pH, temperature, salinity and dissolved oxygen (DO) were measured twice daily in eight random tanks. Other water quality indicators including total ammonium-N (TAN, as NH_3+NH_4), nitrite-N (NO_2-N), nitrate-N (NO_3-N), and reactive phosphorus (PO_4-P) were measured once a week in eight representative tanks.

Experimental feeds and feeding

Diets were produced in the Nutrition laboratory at the Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, AL, USA. They were formulated to contain 35% protein using solvent extracted dehulled soybean meal and corn gluten meal as the primary protein sources, and different combinations of Menhaden fish oil (MFO) and soy and linseed oils to achieve 6% lipid content. Experimental diets were formulated with six levels of soybean oil (SO) as replacement for MFO at inclusion ratios of 100:0, 50:50, 40:60, 30:70, 20:80, and 10:90 as MFO:SO, and designated as Diets 1 through 6. Another set of diets was formulated to increase the n-3/n-6 ratios and maintain values close to the ratio attained at 50% MFO replacement, while removing MFO at the same time. This was done by increasing the content of linolenic acid with linseed oil (LO), in combination with SO for the replacement of fish oil at inclusion ratios of 40:53.4:6.6, 30:56.9:13.1, 20:60:20, and 10:63.7:26.3 as MFO:SO:LO; they were designated as Diets 7 through 10. Three additional diets were evaluated which included Diet 11 with a high LO

content and a MFO to LO ratio of 10:90, Diet 12 with a high soybean oil diet using a low linolenic acid soybean oil (LLSO) at a 10:90 ratio of MFO:LLSO. Finally, Diet 13 was a commercial diet (35% crude protein, 8% crude fat; Rangen, Buhl, ID, USA) which served as a reference. Feed formulations and ingredients are shown in Table 1. Samples of each diet were submitted to New Jersey Feed Laboratory, Inc. (Trenton, NJ, USA) for proximate analysis (Table 1); additional samples were frozen and stored at –20 ^{o}C for duplicate fatty acid analysis. Dietary treatments were randomly assigned using a double blind experimental design with five replicates per treatment. Initial rations were calculated assuming 100% survival, FCR of 1:1.4 and an estimated growth of 1.5 g week^{-1}. One tank from each treatment was equipped with a feed tray to estimate feed consumption. These same tanks were sampled weekly (group weight of five shrimp per tank). Information from the feed trays and the weekly sampling was used to adjust rations during the study.

Lipid and fatty acid analysis

At the end of the 58-day growth trial, the shrimp were harvested, weighed, and counted. Sub-samples of five frozen shrimp from each experimental tank were shipped to Auburn University for FA analysis. Before lipid analysis they were thawed and muscle from the five animals was pooled into a composite sample per tank, ground, and analyzed in duplicate for lipid and fatty acid composition. Experimental diets were analyzed for fatty acid composition as well. Lipids were extracted by the method of Folch et al. (1957) and quantified gravimetrically after drying under nitrogen. Total lipid content was expressed as percent of wet tissue.

FA were transesterified with boron trifluoride and fatty acid methyl esters (FAME) were analyzed with a Shimadzu GC-17A gas chromatograph (Shimadzu Scientific Instruments Inc.,

Portland, OR, USA) equipped with a capillary column (Omegawax 530, 30 m x 0.53 mm x 0.5 µm film thickness, Supelco 2-4019, Sigma-Aldrich, Oslo, Norway) using helium as the carrier gas and a flame-ionization detector as previously described (Quintero, Durland, Davis, & Dunham, 2009). FA were identified by comparison of retention times to those of known standards and they were quantified by using an internal standard, nonadecanoic acid methyl ester (C 19:0, Sigma-Aldrich, St. Louis, MO, USA); they were expressed as mg g^{-1} of diet or wet weight, and as percent of the total identified FAME.

Data analysis

Differences in final mean weights, growth, survival (arcsine transformed), and FCR, were analyzed using one-way ANOVA to determine if significant ($P < 0.05$) differences existed among treatment means. Duncan's multiple range test was used as the mean separation procedure. Statistical analyses were conducted using SAS statistical software version 9.1(SAS Institute Inc., Cary, NC, USA).

Results

Results of the daily and weekly water quality monitoring (Tables 2-3.) showed no statistically significant differences between treatments, and the observed values indicate that culture water parameters were within the suitable range for grow out of this species.

Mean final weights ranged from 15.9 to 17.1 g, survival from 90.4 to 99.1%, growth per week 1.76 to 1.91 g wk^{-1}, FCR 1.16 to 1.30, and final standing crop ranged from 0.592 to 0.658 kg m^{-3}. Harvest data are presented in Table 4, along with overall harvest means. One-way

ANOVA showed no significant differences ($P<0.05$) between treatments, including the commercial diet (Table 4).

Total lipid content of experimental diets was relatively constant, consequently they were considered isolipidic (Table 1), except for the commercial reference diet where total lipid content was higher (10.78%). The diets reflected the fatty acid profile of the oils used in their formulation. Diets with more menhaden oil (*e.g.*, diets 1, 13 and 2, respectively) had a higher content of long chained n-3 HUFA (Table 5), including EPA (4.65, 4.49, and 2.63 mg g^{-1} diet, respectively) and DHA (2.58, 1.98, 1.42 mg g^{-1} diet, respectively). They also contained the highest percentage of ARA (0.46, 0.48, 0.25 mg g^{-1} diet, respectively). Linolenic acid was higher in diets with more inclusion of linseed oil (diets 11, 10 and 9 with 19.81, 10.16, and 9: 7.29 mg g^{-1} diet, respectively).

After the 58-day feeding trial, total lipid content of shrimp muscle was not significantly affected by dietary treatment. However, fatty acid composition of shrimp muscle tissue reflected the fatty acid profile of the experimental diets fed (Table 6). The n-3 HUFA, DHA and EPA, were always significantly higher in tissues of shrimp fed diets with more menhaden oil (Diet 1: 7.89 mg EPA and 5.62 mg DHA g^{-1} wet tissue; Diet 13: 8.21 mg EPA and 5.30 mg DHA g^{-1} wet tissue; Diet 2: 7.54 mg EPA and 4.45 mg DHA g^{-1} wet tissue) (Fig 1.). Linolenic acid was significantly higher in muscle tissue of shrimp fed diets 11, 10, and 9 (5.61, 2.59, and 1.94 mg g^{-1} wet tissue, respectively), with the highest dietary content of linseed oil (Fig. 1). The highest n-3/n-6 ratio in shrimp muscle was observed in those animals fed diet 11 (1.31), followed by shrimp fed diets 1 (0.86) and 13 (0.71). For the rest of the dietary treatments, the n-3/n-6 ratios observed in shrimp tissue ranged from 0.15 to 0.49 (Table 6, Fig. 1).

Discussion

Due to both economic pressures from high fish oil prices and pressures from buyers and consumers requiring sustainable practices, the use of high levels of fish oils in aquafeeds is no longer desirable. In the current economic and social climate, feed mill manufacturers and producers are taking a pragmatic approach by looking into practices that will not only reduce feed production costs, but also improve their public image. The most logical approach to reducing the present dependence on fish oil is to increase the use of a combination of ingredients, including SO and soybean lecithin that will balance the formulations. Because any failure of diet formulations on an industry level can result in a negative response from the shrimp producers, it is important that nutrient limitations be identified. This requires that graded levels of nutrient supplementation be tested under semi-controlled production conditions, followed by testing of the resulting diets under pond production conditions. Hence, the present study was designed to evaluate the replacement of the majority of the marine fish oil with alternative oils. This included simple dilution with SO as well as using a mixture of SO and LO to improve the n-3/n-6 ratio.

Results of the present study showed no statistically significant differences in final mean weights, growth, survival or FCR values of shrimp fed diets with SO at different levels of MFO replacement, or diets with higher n-3/n-6 ratios using SO in combination with LO for replacement of MFO. Furthermore, in terms of production, results were equivalent to those of a 0.71 n-3/n-6 commercial diet, which was compared as a reference. The present study confirms that shrimp can be reared in an outdoor system where they have access to natural foods, using a plant based diet and successfully replace up to 90% of the marine oil using a variety of lipid sources. No indication of feed rejection or reduced growth with removal of fish oil and their

constituent n-3 HUFA was evident under this experimental conditions, even up to a replacement level of 90% of MFO.

Essential FA deficiencies have been induced in outdoor tank systems. Samocha et al. (2009) presumably induced an essential FA deficiency using the same outdoor system when shrimp were offered a plant based diet using soy and linseed oil with or without HUFA supplements from fermentation products. Studies conducted in clear water systems (Lim et al., 1997; Glencross & Smith, 2001; González-Félix et al., 2003) do report significant reduction in final weight of shrimp fed HUFA-deficient diets. In terms of growth and survival of shrimp in this study, increasing the n-3/n-6 ratio with the use of SO and LO to replace MFO showed no evident improvement, demonstrating that with appropriate diet formulations, the vast majority of the marine ingredients can be removed from shrimp feed. However, a source of HUFA is required even in systems where shrimp may have access to natural foods.

Fatty acid analysis confirmed that linolenic acid, although recognized as the precursor of n-3 HUFA in other organisms, has a very limited role as such in shrimp. Animals fed Diet 11, with 19.81 mg of linolenic acid per gram of diet, showed the highest level of this FA in shrimp tissue, 5.61 mg per gram of wet tissue, but at the same time, practically the lowest content of EPA (4.07 mg g^{-1} wet tissue) and DHA (2.04 mg g^{-1} wet tissue) among the treatments. Also, a relatively high n-3/n-6 ratio of 1.15 was observed in muscle of shrimp fed Diet 11, comparable to the one observed in those fed Diet 2 (n-3/n-6= 1.00) where 50% of MFO was replaced by SO, but shrimp fed Diet 2 showed almost double the amount of EPA (7.54 mg g^{-1} wet tissue) and DHA (4.45 mg g^{-1} wet tissue). This demonstrated the inability of shrimp to elongate and desaturate PUFA into HUFA which has been reported previously in other studies (Kanazawa, Teshima, & Ono, 1979; Kayama et al., 1980; Lim et al., 1997; González-Félix et al., 2003a). In

the case of humans α-linolenic acid is converted into EPA and DHA, and linoleic acid is converted into ARA. The enzyme delta-6-desaturase catalyzes the first rate-limiting step of both n-3 and n-6 HUFA synthesis. There is evidence that the synthesis of EPA from α-linolenic acid is limited, and DHA synthesis is even more limited, because of the common desaturation and elongation enzymes and the competition between the substrates for them (Brenna, 2002). An increase in EPA and DHA synthesis can be achieved by increasing dietary α-linolenic acid, but the optimal dietary approach for humans is to consume n-3 HUFA performed by eating seafood (Goyens et al., 2006).

We are then left with few options, and the social pressure and responsibility of finding alternative sources of high quality cost-effective oils, high in n-3 HUFA and n-3/n-6 ratios, to be used in aquafeeds. On the bright side, there is already some progress on this quest. For instance, products obtained by fermentation processes from heterotrophically grown algae have been reported to be a good source of nutrients and essential fatty acids for larval live food enrichment and for formulated broodstock diets of marine teleosts (Harel et al., 2002). In the case of shrimp, Patnaik et al. (2006) demonstrated that fish oil can be successfully replaced in diets for *L. vannamei* using spray-dried cells of *Schizochytrium* sp. and *Mortierella* sp. obtained by a proprietary commercial fermentation process. The same products, used for supplementation of DHA and ARA in practical diets for *L. vannamei*, showed that these alternative sources of HUFA are effective in promoting growth and survival of juvenile shrimp when raised in low salinity (González-Félix, Perez-Velazquez, & Quintero-Alvarez, 2009). These heterotrophically produced non-marine HUFA-rich products are in a preliminary stage of research as lipid sources in aquafeeds. Additional research on their effect at different life stages of *L. vannamei*, cultured under a variety of environmental conditions, would be helpful to fish oil replacement efforts, and

would be valuable in determining the economic viability of their large-scale use in shrimp aquafeeds. Another approach to improve n-3 HUFA in farmed animals has been the implementation of a finishing period using a fish oil based diet or finishing diet to restore the original fatty acid profile of farmed fish fillets for instance. However, the disadvantage is that it requires a considerable period of time, up to 16 weeks, to restore an optimal fatty acid profile for human consumption, and this approach still relies on the use of fish oil (Turchini, Francis, & De Silva, 2007). Finally, the use of genetically modified grain crop rich in n-3 HUFA, or the farming of transgenic fish with greater capability for n-3 HUFA biosynthesis are being explored and have produced exceptional results, but at the same time, it has attracted considerable negative publicity from consumers with respect to the use of this kind of biotechnology in food production (Turchini et al., 2009). All of the aforementioned approaches are valuable contributions in the quest for fish oil replacement, but the goal has not been met, yet.

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Table 1. Ingredient composition (g 100g^{-1} of feed) of experimental diets.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13
Soybean meal solvent extracted[a]	54.40	54.40	54.40	54.40	54.40	54.40	54.40	54.40	54.40	54.40	54.40	54.50	n/a[m]
Whole wheat[b]	28.38	28.38	28.38	28.38	28.38	28.38	28.38	28.38	28.38	28.38	28.38	27.81	
Corn gluten meal[c]	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	
Vitamin premix[d]	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	
Trace mineral premix[e]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	
Choline chloride[b]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	
Stay C 250 mg/kg (25%)[f]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	
CaP-dibasic[g]	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	
Lecithin (deoiled 53% lipid)[h]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
Cholesterol[i]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	
Menhaden fish oil[j]	4.57	2.29	1.83	1.37	0.91	0.46	1.83	1.37	0.91	0.46	0.46	0.46	
High linolenic acid soy oil[k]	-	2.29	2.74	3.20	3.66	4.11	2.44	2.60	2.75	2.91	-	-	
Linseed oil[l]	-	-	-	-	-	-	0.30	0.60	0.91	1.20	4.11	-	
Low linolenic acid soy oil[k]	-	-	-	-	-	-	-	-	-	-	-	4.11	
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
Estimated n-3/n-6 ratio	0.91	0.52	0.40	0.31	0.24	0.18	0.52	0.52	0.52	0.52	2.99	0.09	
Crude Protein (%)	37.40	36.50	37.20	37.70	37.60	37.80	35.30	36.10	37.10	38.70	37.70	37.00	36.20
Crude Fat (%)	7.06	6.97	7.24	7.40	7.51	7.39	6.95	6.68	7.00	7.01	7.11	7.33	10.78

[a] Faithway Feed Co., Guntersville, AL, USA.
[b] MP Biochemicals Inc., Solon, OH, USA.
[c] Grain Processing Corporation, Muscatine, IA, USA.
[d] 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.
[e] Trace mineral premix (g $100g^{-1}$): cobalt chloride 0.004, cupric sulphate pentahydrate 0.250, ferrous sulfate 4.0, magnesium sulfate heptahydrate 28.398, manganous sulphate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, filler 53.428.
[f] Stay C®, (L-ascorbyl-2-polyphosphate), Roche Vitamins Inc., Parsippany, NJ, USA.
[g] Fisher Scientific, Fair Lawn, NJ, USA.
[h] Solae Company, St. Louis, MO, USA.
[i] USB Biochemicals, Cleveland, OH, USA.
[j] Omega Protein, Inc. Reedville, VA, USA.
[k] American Soybean Association, St. Louis, MO, USA.
[l] Sigma-Aldrich Co., St. Louis, MO, USA.
[m] Complete formulation not available.

Table 2. Summary of daily water quality parameters during the 58-day nutrition study

		Temperature (^oC)	Salinity (ppt)	DO (mg L^{-1})	pH
AM	Mean	27.34	31.00	6.19	7.82
	Max	29.18	31.71	7.24	8.09
	Min	22.90	28.43	4.99	7.32
PM	Mean	28.97	31.02	6.23	7.90
	Max	30.95	31.78	7.30	8.22
	Min	25.09	29.70	5.31	7.59

Table 3. Summary of weekly water quality parameters during the 58-day nutrition study.

	TAN (mg L^{-1})	NO_2-N (mg L^{-1})	NO_3-N (mg L^{-1})	PO_4-P (mg L^{-1})
Mean	0.36	2.50	0.86	0.60
Max	1.54	6.30	4.21	1.80
Min	0.00*	0.00*	0.00*	0.00*

* Below detectable levels

Table 4. Harvest data collected after 58-day nutrition study

Treatment	Mean Weight (g)	Survival (%)	Growth (g wk^{-1})	FCR	Final standing crop (kg m^{-3})
Diet 1	16.90	92.32	1.88	1.24	0.624
Diet 2	16.65	96.16	1.85	1.19	0.640
Diet 3	16.61	93.84	1.85	1.24	0.620
Diet 4	16.62	99.05	1.85	1.16	0.658
Diet 5	16.18	94.62	1.80	1.26	0.613
Diet 6	16.42	98.08	1.83	1.18	0.644
Diet 7	16.23	98.46	1.81	1.19	0.639
Diet 8	15.90	93.08	1.76	1.30	0.592
Diet 9	17.08	90.40	1.91	1.25	0.613
Diet 10	16.02	96.92	1.78	1.24	0.622
Diet 11	16.33	96.16	1.82	1.22	0.627
Diet 12	16.22	96.92	1.80	1.22	0.628
Diet 13	16.78	96.16	1.87	1.19	0.645
P value (ANOVA)	0.304	0.964	0.314	0.926	0.937

Table 5. Fatty acid composition (mg g^{-1} diet and % of FAME) of experimental diets[1]

Selected FA	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13
mg g^{-1} diet													
16:0	10.09	8.98	8.95	8.19	8.07	7.63	9.04	7.82	7.18	7.93	6.17	8.53	12.27
18:0	1.90	2.16	2.28	2.18	2.33	2.32	2.28	2.07	2.00	2.32	1.80	2.44	2.65
18:1n-9	5.49	8.60	9.50	9.38	10.20	10.52	9.51	8.95	9.12	10.99	8.86	11.89	12.05
18:2n-6	11.90	19.78	21.05	21.77	21.97	21.77	20.50	19.90	20.57	23.15	16.00	25.95	12.93
18:3n-3	1.71	2.91	3.25	3.25	3.50	3.65	4.97	5.91	7.29	10.16	19.81	2.73	1.50
20:4n-6	0.46	0.25	0.20	0.14	0.11	0.07	0.21	0.15	0.10	0.05	0.06	0.05	0.48
20:5n-3	4.65	2.63	2.17	1.50	1.10	0.59	2.20	1.50	0.99	0.59	0.62	0.61	4.49
22:6n-3	2.58	1.42	1.19	0.86	0.62	0.33	1.26	0.86	0.56	0.33	0.35	0.33	1.98
Saturates[2]	15.69	13.05	12.85	11.50	11.17	10.36	12.93	11.00	9.92	10.71	8.42	11.44	18.70
Monounsaturates[3]	11.67	12.24	12.70	11.87	12.19	11.97	12.70	11.33	10.92	12.49	10.10	13.50	18.64
PUFA[4]	14.72	23.29	24.79	25.38	25.77	25.61	25.99	26.18	28.11	33.48	36.02	28.87	15.52
HUFA[5]	9.54	5.38	4.47	3.16	2.33	1.27	4.59	3.14	2.10	1.31	1.34	1.32	8.56
Total n-3[6]	10.73	8.03	7.49	6.24	5.71	4.85	9.34	8.89	9.29	11.41	21.12	3.98	9.53
Total n-6[7]	12.43	20.08	21.30	21.96	22.15	21.89	20.76	20.09	20.69	23.22	16.09	26.04	13.48
n-3/n-6	0.86	0.40	0.35	0.28	0.26	0.22	0.45	0.44	0.45	0.49	1.31	0.15	0.71
% of FAME													
16:0	19.54	16.63	16.33	15.76	15.68	15.51	16.08	15.12	14.08	13.67	11.04	15.48	19.98
18:0	3.68	4.00	4.16	4.19	4.52	4.71	4.05	4.01	3.93	4.00	3.23	4.43	4.31
18:1n-9	10.63	15.92	17.33	18.07	19.82	21.38	16.91	17.32	17.87	18.96	15.85	21.56	19.60
18:2n-6	23.06	36.69	38.43	41.96	42.70	44.24	36.48	38.55	40.27	39.91	28.65	47.08	21.03
18:3n-3	3.30	5.39	5.92	6.27	6.81	7.43	8.84	11.43	14.27	17.52	35.44	4.96	2.44
20:4n-6	0.90	0.46	0.37	0.28	0.22	0.14	0.37	0.28	0.19	0.09	0.10	0.10	0.79
20:5n-3	9.01	4.87	3.95	2.89	2.13	1.20	3.91	2.91	1.94	1.02	1.11	1.10	7.32
22:6n-3	5.00	2.62	2.17	1.65	1.20	0.67	2.25	1.66	1.10	0.56	0.62	0.60	3.24
Saturates[2]	30.38	24.17	23.43	22.14	21.71	21.05	23.00	21.29	19.45	18.47	15.07	20.76	30.45
Monounsaturates[3]	22.61	22.67	23.17	22.86	23.69	24.32	22.59	21.93	21.39	21.54	18.08	24.48	30.34
PUFA[4]	28.52	43.18	45.25	48.92	50.08	52.04	46.24	50.70	55.05	57.73	64.47	52.37	25.24
HUFA[5]	18.49	9.97	8.15	6.08	4.52	2.58	8.17	6.08	4.12	2.25	2.38	2.39	13.97
Total n-3[6]	20.80	14.87	13.67	12.02	11.09	9.85	16.60	17.21	18.19	19.69	37.79	7.22	15.54
Total n-6[7]	24.10	37.24	38.88	42.31	43.03	44.48	36.94	38.91	40.52	40.04	28.82	47.24	21.93

Table 6. Total lipid (%) and fatty acid composition (mg g^{-1} wet weight and % of FAME) of shrimp muscle tissue[8]

Selected FA	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13
Total lipid (%)	1.37^{a}	1.37^{a}	1.34^{a}	1.37^{a}	1.37^{a}	1.40^{a}	1.43^{a}	1.47^{a}	1.42^{a}	1.43^{a}	1.43^{a}	1.40^{a}	1.42^{a}
mg g^{-1} wet weight													
16:0	9.78^{a}	9.46^{ab}	8.96^{abc}	7.93^{cd}	8.70^{bcd}	8.46^{bcd}	8.45^{bcd}	8.27^{cd}	8.31^{cd}	8.07^{cd}	7.61^{d}	8.17^{cd}	9.93^{a}
18:0	6.01^{a}	6.58^{a}	6.31^{a}	5.93^{a}	6.38^{a}	6.38^{a}	5.93^{a}	5.90^{a}	6.09^{a}	6.27^{a}	5.89^{a}	6.17^{a}	6.11^{a}
18:1n-9	5.21^{d}	5.89^{bcd}	5.64^{bcd}	5.43^{cd}	5.99^{bcd}	6.41^{b}	5.42^{cd}	5.58^{cd}	5.79^{bcd}	6.06^{bc}	5.52^{cd}	6.12^{bc}	7.64^{a}
18:2n-6	7.59^{g}	10.78^{de}	11.03^{de}	11.17^{de}	13.07^{bc}	15.12^{a}	9.92^{ef}	10.78^{de}	12.16^{cd}	13.25^{bc}	9.26^{f}	14.32^{ab}	6.51^{g}
18:3n-3	0.48^{fg}	0.69^{efg}	0.71^{efg}	0.81^{efg}	0.97^{def}	1.18^{de}	1.00^{de}	1.40^{d}	1.94^{c}	2.59^{b}	5.61^{a}	0.81^{efg}	0.38^{g}
20:4n-6	1.60^{ab}	1.48^{bc}	1.38^{cd}	1.25^{defg}	1.34^{cde}	1.17^{efg}	1.30^{def}	1.28^{defg}	1.22^{defg}	1.15^{fg}	1.10^{g}	1.20^{defg}	1.74^{a}
20:5n-3	7.89^{a}	7.54^{ab}	6.84^{bc}	5.72^{de}	5.56^{e}	4.20^{f}	6.40^{cd}	5.79^{de}	5.21^{e}	4.16^{f}	4.07^{f}	4.19^{f}	8.21^{a}
22:6n-3	5.62^{a}	4.45^{b}	3.76^{c}	3.03^{de}	2.87^{de}	2.02^{f}	3.58^{c}	3.12^{d}	2.67^{e}	2.07^{f}	2.04^{f}	2.10^{f}	5.30^{a}
Saturates[2]	16.23^{a}	16.38^{a}	15.59^{ab}	14.12^{b}	15.37^{ab}	15.08^{ab}	14.71^{ab}	14.45^{ab}	14.66^{ab}	14.56^{ab}	13.86^{b}	14.59^{ab}	16.40^{a}
Monounsaturates[3]	9.46^{b}	9.19^{b}	8.58^{bc}	7.95^{c}	8.61^{bc}	8.84^{bc}	8.25^{bc}	8.26^{bc}	8.31^{bc}	8.41^{bc}	7.93^{c}	8.46^{bc}	11.37^{a}
PUFA[4]	11.38^{d}	15.42^{c}	15.58^{c}	15.69^{c}	18.20^{ab}	20.49^{a}	14.57^{c}	15.95^{bc}	18.02^{ab}	20.21^{a}	19.33^{a}	19.18^{a}	10.30^{d}
HUFA[5]	16.01^{a}	14.32^{b}	12.76^{c}	10.68^{de}	10.46^{e}	7.95^{f}	12.05^{cd}	10.93^{de}	9.75^{e}	7.92^{f}	7.72^{f}	8.04^{f}	16.20^{a}
Total n-3[6]	14.90^{a}	13.56^{ab}	12.13^{bcd}	10.28^{ef}	10.16^{ef}	8.05^{g}	11.84^{cde}	11.21^{def}	10.72^{def}	9.75^{f}	13.32^{abc}	7.68^{g}	14.81^{a}
Total n-6[7]	10.02^{gh}	13.61^{de}	13.82^{de}	13.83^{de}	16.05^{bc}	18.02^{a}	12.47^{de}	13.39^{de}	14.79^{cd}	16.03^{bc}	11.57^{fg}	17.26^{ab}	9.02^{h}
n-3/n-6	1.49	1.00	0.88	0.74	0.63	0.45	0.95	0.84	0.72	0.61	1.15	0.44	1.64
% of FAME	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13
16:0	18.44^{a}	17.12^{b}	17.07^{bc}	16.38^{de}	16.51^{de}	16.17^{e}	17.04^{bc}	16.67^{cd}	16.36^{de}	15.72^{f}	15.60^{f}	16.23^{de}	18.30^{a}
18:0	11.32^{d}	11.89^{c}	12.03^{abc}	12.22^{ab}	12.11^{abc}	12.18^{ab}	11.96^{bc}	11.90^{c}	12.02^{abc}	12.27^{a}	12.08^{abc}	12.28^{a}	11.26^{d}
18:1n-9	9.81^{g}	10.65^{f}	10.76^{f}	11.21^{de}	11.40^{d}	12.24^{b}	10.94^{ef}	11.26^{d}	11.42^{d}	11.90^{c}	11.28^{d}	12.17^{bc}	14.08^{a}
18:2n-6	14.29^{i}	19.53^{gh}	20.99^{f}	23.09^{e}	24.85^{c}	28.87^{a}	20.05^{g}	21.76^{f}	23.98^{d}	26.38^{b}	19.01^{h}	28.45^{a}	12.00^{j}
18:3n-3	0.91^{i}	1.24^{h}	1.35^{h}	1.67^{g}	1.85^{f}	2.24^{e}	2.02^{f}	2.84^{d}	3.84^{c}	5.12^{b}	11.50^{a}	1.60^{g}	0.71^{j}
20:4n-6	3.02^{b}	2.67^{c}	2.64^{c}	2.57^{c}	2.55^{cd}	2.24^{ef}	2.61^{c}	2.58^{c}	2.39^{de}	2.19^{f}	2.25^{ef}	2.39^{de}	3.22^{a}
20:5n-3	14.87^{a}	13.59^{b}	13.00^{c}	11.78^{d}	10.55^{e}	8.02^{f}	12.90^{c}	11.67^{d}	10.27^{e}	7.91^{f}	8.35^{f}	8.34^{f}	15.13^{a}
22:6n-3	10.60^{a}	8.04^{c}	7.17^{d}	6.26^{e}	5.45^{f}	3.86^{g}	7.22^{d}	6.29^{e}	5.24^{f}	3.96^{g}	4.15^{g}	4.17^{g}	9.75^{b}
Saturates[2]	30.60^{a}	29.64^{bcd}	29.71^{bc}	29.14^{cde}	29.17^{cde}	28.83^{efg}	29.66^{bc}	29.14^{cde}	28.88^{efg}	28.44^{fg}	28.38^{g}	29.02^{def}	30.22^{ab}
Monounsaturates[3]	17.81^{b}	16.60^{cde}	16.36^{ef}	16.42^{ef}	16.35^{ef}	16.89^{c}	16.65^{cde}	16.66^{cde}	16.39^{ef}	16.49^{def}	16.20^{f}	16.82^{cd}	20.95^{a}
PUFA[4]	21.43^{h}	27.91^{g}	29.64^{f}	32.41^{e}	34.61^{d}	39.11^{a}	29.42^{f}	32.18^{e}	35.54^{c}	39.96^{a}	39.63^{a}	38.14^{b}	18.99^{i}
HUFA[5]	30.15^{a}	25.85^{b}	24.29^{c}	22.03^{d}	19.87^{e}	15.17^{g}	24.27^{c}	22.02^{d}	19.19^{e}	15.11^{g}	15.78^{fg}	16.02^{f}	29.84^{a}
Total n-3[6]	28.07^{a}	24.47^{c}	23.07^{d}	21.22^{e}	19.31^{f}	15.37^{g}	23.86^{c}	22.60^{d}	21.12^{e}	18.81^{f}	27.27^{b}	15.29^{g}	27.27^{b}
Total n-6[7]	18.88^{i}	24.64^{g}	26.29^{f}	28.57^{d}	30.51^{c}	34.41^{a}	25.19^{g}	27.02^{e}	29.16^{d}	31.71^{b}	23.73^{h}	34.33^{a}	16.62^{j}

* Footnotes for tables 5 and 6

[1] Values represent averages of duplicate samples.
[2] Saturates: 14:0, 15:0, 16:0, 18:0, 20:0, 22:0. Internal standard, 19:0, was not considered.
[3] Monounsaturates: 15:1, 16:1 18:1, 20:1.
[4] PUFA: 16:2, 16:3, 18:2, 18:3, 20:2, 20:3, 22:2.
[5] HUFA: 18:4, 20:4, 20:5, 22:4, 22:5, 22:6.
[6] Total n-3: 18:3n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3.
[7] Total n-6: 18:2n-6, 20:3n-6, 20:4n-6.
[8] Values represent averages of duplicate samples per tank, and five tanks per dietary treatment.
Means within rows with the same letter are not significantly different (Duncan's alpha = 0.05).

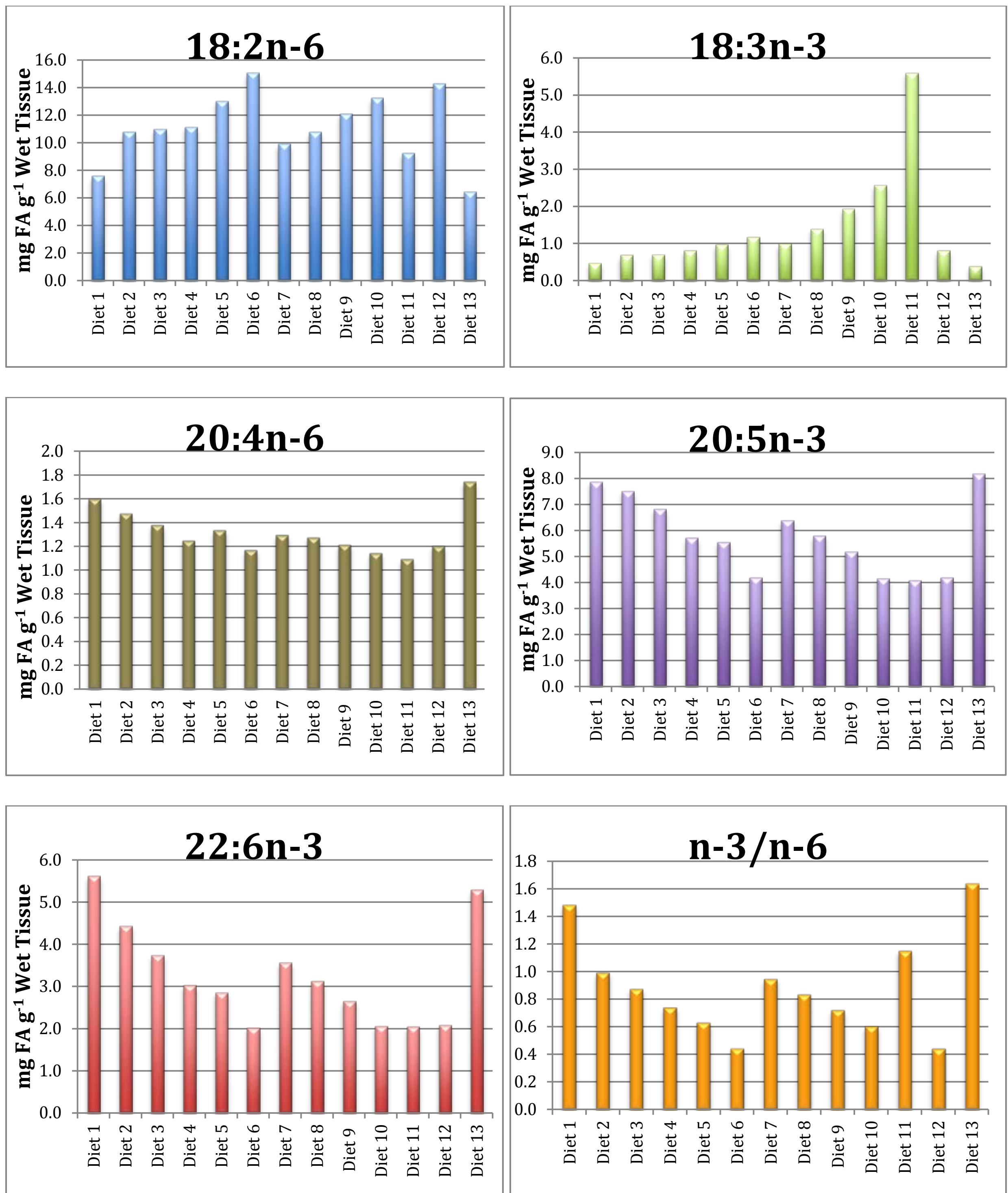

Figure 1. Linoleic (18:2-6), linolenic (18:3n-3), arachidonic (20:4n-6), EPA (20:5n-3), DHA (22:6n-3), and n-3/n-6 ratio in muscle of *L. vannamei* during the 58-day nutrition study. Values represent means of duplicate analysis for five replicate observations.

CHAPTER III

REPLACEMENT OF FISH OIL IN PLANT BASED DIETS FOR PACIFIC WHITE SHRIMP (*Litopenaeus vannamei*) IN PONDS AND GREEN WATER TANKS PRODUCTION

Abstract

The objective of this study is to demonstrate the feasibility of a partial substitution of menhaden fish oil by alternative lipid sources (soybean oil, poultry grease and flaxseed oil) in non-marine protein based shrimp (*Litopenaeus vannamei*) production diets (total of four diets) and its influence on fatty acid profile, and to a lesser extent to its final product flavor. Outdoor ponds and green water tanks were utilized to evaluate the response of the shrimp over the culture period. A 17-week pond production trial was conducted in sixteen, 0.1-ha ponds stocked at 38 shrimp m^{-2}. The four previously mentioned diets, a commercial reference diet and one treatment for which no feed was offered were evaluated in green water outdoor tanks (800 L) over a 12-week period. The tanks were stocked with juvenile shrimp (initial weight of 0.25 g) at a density of 30 shrimp per tank. The results for the pond study demonstrated that final production ranged between 5,070 and 5,363 kg ha^{-1}, mean final weights varied from 18.0 to 21.6 g, weekly growth ranged from 1.04 to 1.25 g, survival ranging from 65.6 to 75.4%, and FCR was between 1.37 and 1.45. There were no differences ($P \geq 0.05$) or notable trends in shrimp production variables among different test diets. Shrimp reared in the outdoor tanks confirmed these findings for the

experimental diets. Average final weights ranged between 13.8 and 14.8 g, weekly growth of 1.1 to 1.2 g, survival varied from 92.5 to 98.3%, FCR was between 1.05 and 1.11, and final standing crop ranged from 4,738 to 5,024 kg ha^{-1}. The results from the outdoor tank study also showed no statistical differences ($P \geq 0.05$) within the experimental diets. The commercial diet showed an improved performance of the shrimp, and the natural productivity treatment a restricted ability to grow after a certain size, respectively. The mean final weights were 15.7 and 3.2 g, weekly growth of 1.3 and 0.3 g, FCR of 0.93 and not determined and final standing crop of 5,401 and 741 kg ha^{-1}, respectively. The fatty acids profiles of edible tail muscle from shrimp reared on the various diets displayed a similar fatty acid profile as that of the diets. The percentage of menhaden fish oil kept in the diet was able to provide enough of the essential fatty acids, EPA, DPA and DHA, at similar levels where no significant statistical differences were found. In ponds the EPA of tissues ranged from 5.57 to 6.74 mg g^{-1} and in tanks from 4.74 to 8.35 mg g^{-1}, the DPA of tissues ranged from 0.35 to 0.40 mg g^{-1} in ponds and 0.47 to 0.71 mg g^{-1} in tanks, and the DHA of tissues ranged from 3.23 to 3.42 mg g^{-1} in ponds and 2.90 to 4.54 mg g^{-1} in tanks. The sensory test showed no statistical differences in texture, looks, aroma and flavor between the menhaden fish oil and soybean oil diets fed animals. These studies demonstrated that practical shrimp feeds containing non-marine protein ingredients and a percentage of fish oil replaced by alternative lipid sources had no negative impact on mean final weight, weekly growth, survival, FCR, final standing crop, fatty acids profile of the animals and organoleptic properties of *L. vannamei*.

Introduction

The aquaculture sector plays an important role as seafood supplier with production primarily occurring in developing countries. The most recent aquaculture and fisheries production data shows aquaculture being responsible for 41.3% of the seafood supply with 63.6 million tonnes produced and the fisheries capture of 90.4 million tonnes of seafood. The aquaculture sector grew from 32.4 million tonnes in the year 2000 to the actual 63.6 million tonnes, an 196.3% growth, where nearly 90% of the production takes place in Asia (FAO, 2012).

The modern Western diet, characterized by energy-dense, saturated fats and omega-6 fatty acids (n-6 FA) rich foods (Kerver, et al. 2003), is linked to several chronic diseases, such as cardiovascular diseases, obesity, diabetes, asthma, allergies (Ford, 2005; Hersoug & Linneberg, 2007; Shore, 2008; Myers & Allen, 2011), and chronic inflammatory diseases (Mokdad et al., 1999; Myers & Allen, 2011). The first evidence from the work of Dyerberg, Bang, & Hjorne (1975) of the health benefits to humans from seafood derived highly unsaturated fatty acids (HUFA) consumption changed the perspective on the eating habits of the Western civilization, giving a boost to the fisheries and aquaculture sector. Since then, an array of health benefits ranging from reduced aggression, brain cognitive development, respiratory and cardiovascular health attributed to seafood consumption has been extensively reported in the literature by work from researches such as Arts, Ackman, & Holub (2001), Chapkin, et al. (2009), Das (2006), Hamazaki, et al. (1996), Hamazaki & Hamazaki (2008), Myers & Allen (2011), Ruxton, et al. (2004), Simopoulos (1991), among several others.

The mild but distinctive flavor of shrimp with a texture described as tender and delicate (Erickson, et al. 2007), might be the reason for this sea creature to be ranked as the top 10 most consumed seafood in 2010 in the United States of America (NFI, 2012). Its distinctive flavor can unfortunately change according to variations in the environment and the animals dietary intake, such as, the salinity of the water (Huang,et al. 2004; Liang, et al. 2008), amino acid composition and concentration (Zayas, 1997; Grigorakis, 2007), pH of the muscle (Love, 1988; Grigorakis, 2007), nucleotides components (especially inosine monophosphate, IMP, and guanosine monophosphate, GMP) (Kuninaka, 1960; Hayashi, 1978; Liang et al., 2008), off-flavor compounds (geosmin and 2-methylisoborneol) (Farmer, et al. 2000), and lipids composition and concentration (Love, 1992)

The aquaculture sector has the ever increasing task of supplying the market with constant and healthy seafood product, considering that the wild stocks are being exploited to exhaustion and the consumer demand is at its highest level. Towards meeting this goal, the objective of this study is to demonstrate the feasibility of a partial substitution of menhaden fish oil by alternative lipid sources (soybean oil, poultry grease and flaxseed oil) in non-marine protein based shrimp (*Litopenaeus vannamei*) production diets and its influence on fatty acid profile, and to a lesser extent to its final product flavor.

Materials and methods

Source of shrimp and experimental system

Research was conducted at the Alabama Department of Conservation and Natural Resources Marine Resource Division, Claude Peteet Mariculture Center in Gulf Shores, Alabama, USA. Two growth trials were carried out in parallel which included a pond trial and

outdoor tank trial, during May through September 2010. Pacific white shrimp, *L. vannamei*, postlarvae were obtained from Shrimp Improvement Systems (Islamorada, FL) and nursed for about 2 weeks.

Pond Trial

A total of 16 lined with 1.52 mm thick high-density polyethylene lining (HDPE) ponds with approximately 0.1 ha in surface area (rectangular 46 x 20 m) and a 1.0 m average depth were used. Each pond was equipped with a 20-cm diameter standpipe, and a concrete catch basin (3.0 x 2.0 x 0.5 m); the bottom of each pond was covered with a 25 cm deep layer of sandy-loam soil, filled with brackish water from the Intra-costal Canal between Mobile and Perdido Bay filtered through a three foot 250 µm-mesh nylon filter sock (Micron Domestic Lace Mfg., Inc). Two weeks before stocking juvenile pacific white shrimp, the combination of inorganic liquid fertilizers, 32-0-0 mixed with 10-34-0, were applied in a rate of 1,697 ml and 303 ml, respectively, per pond which provided 5.73 kg of N and 1.03 kg of P_20_5 ha^{-1} in order to promote natural productivity in all ponds, and the second fertilization was reapplied at half of the initial rate when the Secchi disk reading was greater than 40 cm. Each pond was provided with a 1 hp paddlewheel aerator (Little John Aerator, Southern Machine Welding Inc. Quinton, Alabama, USA) (10 hp ha^{-1}). Additional aeration as required was provided using either 1 hp or 2 hp Aire-O2 aerators (Aire-O2, Aeration Industries International, Inc. Minneapolis, Minnesota, USA) to maintain dissolved oxygen above 3 mg L^{-1}. Brackish canal water was added to account for losses by evaporation. This study was a sustainable semi-intensive system with well-managed feeding, and minimal water exchanged.

At the conclusion of the nursery phase, juvenile shrimp (0.34 μg) were collected by dip nets, group weighed, and spread evenly into 16 buckets each representing one production pond.

Feeding strategy for the first four weeks was based on previous studies which assumed that PL feed on primary productivity. Thus, during this period, a small amount of feed was applied to promote natural productivity in pond. Thereafter, about sixty shrimp per pond were sampled each week using a 1.52 m cast nets (monofilament net, 1.22 m radius and 0.95cm opening). The sample was used to determine the average weight and observe the health of the shrimp. Four isonitrogenous and isolipidic diets (35% crude protein, 8% crude lipid) manufactured by Rangen Inc. (Buhl, Idaho, USA) with different lipid sources replacing a percentage of menhaden fish oil (menhaden fish oil, soybean oil, poultry grease, and flaxseed oil) were provided to the shrimp in the ponds (Table 1).

Physiochemical parameters including pH, temperature, salinity, and dissolved oxygen (DO) were measured three times daily at sunrise (0500 h - 0530 h), during the day (1500 h - 1530 h), and at night (2000 h - 2200 h), using a YSI 556 MPS meter. Secchi disk reading and TAN were monitored once weekly. Water samples were taken in all ponds at the depth of 80 cm and TAN was determined using a spectrophotometer (Spectronic 20 Genesys, Spectronic Instrument Inc. Rochester, NY, USA) following the Nesslerization method (APHA 1989).

The shrimp were harvested at the end of the 17-week study period. Feed was withdrawn 36 h before harvest in order to clear the shrimp gut. To harvest, about two thirds of the water was drained from ponds the night before harvest. The following day the remaining water was drained and the shrimp were pump harvested from the catch basin using a fish pump equipped with a 25-cm diameter suction pipe (Aqualife-Life pump, Magic Valley Heli-arc and Mfg, Twin Falls, Idaho, USA), and collected into the container truck. The shrimp were rinsed and weighed then

approximately 100 shrimp from each pond were randomly selected to measure individual weight. Mean final weight, weekly growth, final standing crop, percent survival, and FCR were determined.

Green Water Tank System Trial

An outdoor recirculating tank system consisting of a central reservoir (1,000 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) were utilized for this trial. A second 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 800 h and 1400 h to mimic a production pond setting. This resulted in a replacement of system water every few days, replenishing natural productivity. *L. vannamei* (0.25 ± 0.01 g, initial weight) were collected from the research ponds and size-sorted by hand. Juvenile shrimp were randomly selected and stocked at a rate of 30 shrimp per tank. Each tank and central reservoir were equipped 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.

Six treatments with four replicates were randomly assigned to the 24 tanks (750L). The same four experimental diets used in the production ponds, as well as a fifth commercial reference diet (Zeigler Shrimp Grower, 35% protein, 8% lipid) were utilized in the study (Table 1). One final treatment did not receive feed so that the natural productivity contribution could be assessed. At the conclusion of the 12 week growth trial shrimp were counted and group weighed. Mean final weight, weekly growth, survival, fed conversion ratio, and final standing crop were determined.

Experimental diets

Four experimental diets were formulated to contain soybean meal as the primary protein source with the inclusion of either 1.97% of menhaden fish oil, soybean oil, poultry grease, or flaxseed oil. The feed was top coated with 2.5% of menhaden fish oil to seal the pellet and supply essential fatty acids (EFA) (Table 1). The experimental diets were manufactured by Rangen Inc. (Buhl, Idaho, USA), under commercial feed manufacturing conditions as a sinking extruded pellet. All experimental diets contain approximately 35% protein and 8% lipid with lysine and methionine plus cysteine contents of 2% and 1.1% of the total protein, respectively (Table 1, Table 2). Dietary treatments were tested in production ponds and outdoor tank system.

For the trials, dietary treatments were randomly assigned using a double blind experimental design with four replicates per treatment. For the ponds trial rations were calculated assuming 70% survival, FCR of 1.2:1 and an estimated growth of 1.3 g $week^{-1}$; tanks trial rations were calculated assuming 100% survival, FCR 1.2:1 and predicted growth of 1.2 g $week^{-1}$.

Lipid and fatty acid analysis

At the end of the growth trials, the shrimp were harvested, weighed, and counted. Sub-samples of ten frozen shrimp from each experimental tank were shipped to Auburn University for fatty acids (FA) analysis. Before lipid analysis they were thawed and muscle from five animals was pooled into a composite sample per tank, ground, and analyzed in duplicate for lipid and fatty acid composition. Experimental diets were analyzed for fatty acid composition as well. Lipids were extracted by the method of Folch et al. (1957) and quantified gravimetrically after drying under nitrogen. Total lipid content was expressed as percent of wet tissue.

FA were trans-esterified with boron trifluoride (Appendix) and fatty acid methyl esters (FAME) were analyzed with a Shimadzu GC-17A gas chromatograph (Shimadzu Scientific Instruments Inc., Portland, OR, USA) equipped with a capillary column (Omegawax 530, 30 m x 0.53 mm x 0.5 µm film thickness, Supelco 2-4019, Sigma-Aldrich, Oslo, Norway) using helium as the carrier gas and a flame-ionization detector as previously described (Quintero et al., 2009). FA were identified by comparison of retention times to those of known standards, and they were quantified by using an internal standard, nonadecanoic acid methyl ester (C 19:0, Sigma-Aldrich, St. Louis, MO, USA); they were expressed as mg g^{-1} of diet or wet weight, and as percent of the total identified FAME.

Data analysis

Differences in final mean weights, weekly growth, survival, FCR, and final standing crop were analyzed using one-way ANOVA to determine if significant ($P < 0.05$) differences existed among treatment means. SNK's multiple range test was used as the mean separation procedure. Statistical analyses were conducted using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA).

Sensorial analysis

Shrimp samples

About thirty five shrimp (~500 g) from each of the four replicate of the menhaden fish oil and soybean oil treatments, treatments 1 and 2 respectively, where caught by cast net and placed temporarily in a large freezer (around -18˚C) at the research station, transported to Auburn

University on ice in an insulated chest, and then stored in a freezer (around -18˚C) until sensory test were made.

Sensory test

Reference preparation

References for organoleptic to test the shrimp characteristics in this study were similar to references recommended by Erickson, et al. (2007). Ocean/seawater and sweet solutions were changed every week, the old shrimp solution was changed every 3 weeks; the aftertaste solution and the three other solutions in the basic taste category were changed every 2 weeks. Jell-O, cheese, beef hot dog, surimi, pineapple were all kept in a refrigerator, removed just some minutes before starting a sensory test to make sure their textures were unchanged. Each described attribute was scored from 0-10 with aid of the references, found in Thi Le (2011).

Training panelists

Sensory characteristics of the shrimp were evaluated by 12 subjects (25-71 years old, 5 male: 7 female). Training consisted of five sessions over a 1-month period. In the first session, participants were informed of the study objective, the approach of sensory testing, and definitions used in the test. Then, the references were evaluated and concentrations and scoring scales discussed. In the second, third, and fourth sessions, panelists evaluated shrimp using the same references. But, all panelists used the same shrimp for evaluating each characteristic. For instance, one shrimp was cut into four small species and served for four participants for evaluating sweetness. The panelists would be expected to give a similar evaluation. Sheets containing descriptors of both raw and cooked shrimp were presented along with references.

Participants were provided a cup of distilled water and an empty cup to rinse their mouths and to spit out when necessary. In the fifth session, everything was set up and run as in an actual sensory test.

Descriptive analysis testing by trained panelists

Samples of frozen shrimp were put in a refrigerator at 4˚C one night before the day of sensory test. About 1 hour before testing, shrimp of each sample was deheaded and placed on three white paper plates that were marked clearly with the letters A, B, C to identify the samples. Shrimp cooking also was initiated 1 hour before evaluation was conducted. Shrimp were rinsed in cool, running water and then put into boiling water. Two minutes after all shrimp rose to the surface of water, they are removed and rinsed several times with cool water, deheaded and put on plates which are marked clearly with the letters A, B, C to identify samples.

Twelve panelists took turns in participating in sensory test at weekly intervals. In each test, there were six panelists. At first they were served with three plates that each contained three cooked shrimp. Then, they were provided three raw shrimp on three white plates.

The whole description of the standard references and ratings for the attributes can be found in Thi Le (2011); the main characteristics analyzed in raw shrimp samples were raw aroma (ocean/seawater, shrimp, and old shrimp), raw meat appearance (plumpness, and brown color), and raw shell appearance (darkness, stripe darkness, blotchiness, glossiness, and tail iridescence/rainbow color); cooked shrimp were analyzed for aroma (ocean/seawater, cooked shrimp, and old shrimp), appearance (red/orange color, brown color, blotchiness, and glossiness), taste (bitter, salty, sour, and sweet), mouthfeel (sliminess), texture (firmness, juiciness, chewiness, crispness, and fibrous), and aftertaste (iodine).

Sensory test statistical analysis

Differences in sensory tests were analyzed using one-way ANOVA to determine if significant ($P \leq 0.05$) differences existed among treatment means. Tukey's test was used as the mean separation procedure. Statistical analyses were conducted using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Experimental diets

Total lipid content of experimental diets was relatively constant; consequently they were considered isonitrogenous and isolipidic (Table 7). The diets reflected the fatty acid profile of the oils used in their formulation. Diet with more menhaden oil (diets 1 and 5) had a greater content of highly unsaturated acids (12.43, 7.39, 7.33, 7.84, and 16.26 mg g^{-1} diet, respectively), although diet 4 had a greater amount of total n-3 fatty acids due to flaxseed oil having high contents of linolenic acid (18:3n-3) (23.59 mg g^{-1} diet 4, versus 3.05 mg g^{-1} for diet 1, 4.77 mg g^{-1} for diet 2, 3.22 mg g^{-1} for diet 3, and 2.85 mg g^{-1} for diet 5). HUFAs like EPA (6.29, 3.80, 3.72, 3.98 and 6.53 mg g^{-1} diet, respectively), DPA (1.31, 0.79, 0.80, 0.86, and 1.86 mg g^{-1} diet, respectively) and DHA (3.47, 1.88, 2.03, 2.10 and 3.76 mg g^{-1} diet, respectively), were maintained at a proper level for shrimp growth on diets 1 through 4 by top coating the feed with 2.5% of menhaden fish oil. Arachidonic acid was greater on diet 5, the commercial diet (0.09, 0.07, 0.06, 0.09, and 0.19 mg g^{-1} diet, respectively for diets 1-5). Linolenic acid was greater on diet 2 with the inclusion of soybean oil (36.31 mg g^{-1} for diet 2, versus 28.82 mg g^{-1} for diet 1, 30.95 mg g^{-1} for diet 3, 30.22 mg g^{-1} for diet 4, and 24.57 mg g^{-1} for diet 5). The n-3/n-6 ratio

ranged from 0.34 to 1.04, with the lowest values on the soybean oil diet and the highest on the flaxseed oil diet, due mainly to their high linoleic and linolenic acid contents, respectively (Table 7).

Pond trial

Results of the daily and weekly water quality monitoring (Tables 2-3) showed no statistically significant differences between treatments, and the observed values indicate that culture water parameters were within the suitable range for grow out of this species.

Mean final weights ranged from 18.0 to 21.6 g, survival from 65.6 to 75.4%, growth per week 1.04 to 1.25 g wk^{-1}, FCR 1.37 to 1.45, and final standing crop from 5,070 to 5,363 kg ha^{-1}. Harvest data are presented in Table 4, along with overall harvest means. One-way ANOVA showed no significant differences (P<0.05) between treatments (Table 4).

After the 17-weeks feeding trial, total lipid content of shrimp muscle was not significantly affected by dietary treatment. However, fatty acid composition of shrimp muscle tissue reflected the fatty acid profile of the experimental diets fed. The use of 2.5 percent of menhaden fish oil to spray coat the diets kept almost all FAs at a similar level, mainly the DHA, EPA and HUFAs. The n-3 linolenic acid was greatest for animals from the flaxseed oil treatment (diet 4), reflecting the composition of the oilseed (2.31 mg g^{-1} wet tissue from treatment 4, versus 0.47 mg g^{-1} wet tissue from treatment 1, 0.66 mg g^{-1} wet tissue from treatment 2, and 0.75 mg g^{-1} wet tissue from treatment 3). The n-3/n-6 ratio found in shrimp muscle also reflected the dietary levels, with the lowest value found on the soybean oil treatment (1.52) and the highest value found on the flaxseed oil treatment (2.04) (Table 8, Figure 1).

Green Water Tank System Trial

Results of the daily and weekly water quality monitoring (Table 5) showed no statistically significant differences between treatments, and the observed values indicate that culture water parameters were within the suitable range for grow out of this species.

Mean final weights ranged from 3.2 to 15.7 g, survival from 73.3 to 98.3%, growth per week 0.3 to 1.3 g wk^{-1}, FCR 0.97 to 1.11, and final standing crop from 741 to 5,401 kg ha^{-1}. Harvest data are presented in Table 6, along with overall harvest means. One-way ANOVA showed significant differences between treatments with respect to mean final weight, weekly growth, survival, FCR, and final standing crop; means within columns with the same letter are not significantly different (SNK alpha = 0.05) (Table 6).

After the 12-weeks feeding trial, total lipid content of shrimp muscle was not significantly affected by dietary treatment, ranging from 1.22 to 1.45%. As expected, the fatty acid composition of shrimp muscle tissue reflected the fatty acid profile of the experimental diets fed. The use of menhaden fish oil to spray coat the diets kept almost all FAs at a similar level, mainly the DHA, EPA and other HUFA. These fatty acids were not statistical different from the animals that grew solely on natural productivity. The n-3 linolenic acid was higher on animals from the flaxseed oil treatment (diet 4), reflecting the composition of the oilseed (3.65 mg g^{-1} wet tissue from treatment 4, versus 0.41 mg g^{-1} wet tissue from treatment 1, 0.56 mg g^{-1} wet tissue from treatment 2, 0.54 mg g^{-1} wet tissue from treatment 3, 0.33 mg g^{-1} wet tissue from treatment 5, and 1.22 mg g^{-1} wet tissue from treatment 6). Arachdonic acid (ARA) was greater on treatment 4 (0.54 mg g^{-1} wet tissue), followed by treatment 6 (0.27 mg g^{-1} wet tissue), treatments 1 (0.10 mg g^{-1} wet tissue), 2 (0.12 mg g^{-1} wet tissue), 3 (0.10 mg g^{-1} wet tissue), and 5 (0.09 mg g^{-1} wet tissue) showed no statistical differences among themselves. The n-3/n-6 ratio found in

shrimp muscle also reflected the dietary levels, with the lowest value found on the soybean oil treatment (1.41) and the highest value found on the natural productivity treatment (4.37) (Table 9, Figure 1).

Sensorial analysis

The trained panelists who assayed the pond raised shrimp from the fish oil (treatment 1) and soybean oil (treatment 2) gave them similar sensory scores in all accounted attributes, so no significant statistical differences among treatments were found (Table 10); thus the panelists were not able to sensorialy distinguish between animals raised on fish oil or soybean oil diets. Figures 1 and 2, raw and cooked shrimp respectively, are visual representations of the results and graph lines are closely overlapping showing small differences among treatments.

Discussion

The reduction of marine feed ingredients is vital at the economic and social standpoint for the aquaculture industry. The combination of different feed ingredient sources is the best procedure to balance the nutritional content of the diets and assure that the organisms acquire all essential nutrients from the diet at the proper level for the species. In the case of lipids one must ensure that the EFA requirements are met. Reared *L. vannamei* under laboratory conditions (González-Félix at al., 2002) reported the EFA requirement as quite low. Studies by Samocha and colaborators (2009) conducted in outdoor tanks where natural foods are present demonstrated that in feed formulations without marine ingredients that a EFA deficiency will occur. In our previous work (Chapter 2) reported by González-Félix et al. (2010) an outdoor tank system was also used to demonstrate that the partial or total replacement of fish oil by other lipid

sources is feasible for proper growth of the animals, if a fraction of about 10% of fish oil or other sources of HUFA, mainly DHA, DPA and EPA, is added to the diet to provide those essential fatty acids.

Under laboratory conditions, modification of oils sources in diets is relatively simple; however; under commercial production conditions the ability to shift oil sources is more complicated particularly if the feed mill is making numerous products. For pelleted feeds, a limited amount of oil can be added at the mixer or more specifically the pre-pelleting mash can contain a finite level of oil as too much oil will interfere with the pelleting process. Similarly, a limited amount of oil can be added post pelleting to coat the pellet, which is commonly done to help seal the pellet and increase palatability. From a milling standpoint, using alternative oils in the mixer is the easiest point of modification and was the case for this research. In the present study, menhaden fish oil, soybean oil, poultry grease, and flaxseed oil were added to the diets at 2% inclusion with another 2.5% of menhaden fish oil sprayed on the diet to top coat it and provide the essential FAs.

Results of the studies showed no statistically significant differences in mean final weights, weekly growth, survival, FCR or final standing crop values of shrimp fed all four experimental diets with fish oil, plant oils or animal fat, under pond and tank conditions (Tables 4 and 6). Animals from the tank trial had an increased mean final weight, weekly growth, FCR and final standing crop values on the commercial diet treatment (Table 6) where the high inclusion of marine ingredients may have an increased palatability or nutrient differences in the formulations; and, animals reared solely on natural productivity presented the lowest performances on all parameters measured.

Samocha et al. (2009) induced essential FA deficiency in shrimp offering a plant based diet using soy and linseed oil without HUFA supplements, which was noted before by Lim et al. (1997), Glencross & Smith (2001) and González-Félix et al. (2003) as a significant reduction in final weight of shrimp fed HUFA-deficient diets, demonstrating the importance of a source of HUFA for shrimp production. The present study confirms that animals can grow to a certain size (3.2 g) relying on natural productivity (Table 6) being able to obtain all essential fatty acids from the natural food organisms (Table 9, Figure 1), thus as the animals grow they seem to be unable to filter out such small living organisms out of the water column, relying mainly on the exogenous feed provided by the producer.

The distinct fatty acids profile from each of the lipid sources supplied through the diet was reflected in the animal edible tissues (tail). For instance the soybean oil diet which has a high amount of total n-6 FAs (12.46 mg g^{-1} n-3 FAs and 36.51 mg g^{-1} n-6 FAs), shifted the n-3/n-6 ratio down to 0.34 in the diets, while the high n-3 FA flaxseed oil diet with 31.69 mg g^{-1} n-3 FAs and 30.49 mg g^{-1} n-6 FAs kept the n-3/n-6 ratio at 1.04 in the diets. The FA composition was, in the pond trial shrimp tail samples, 13.17 mg g^{-1} n-3 FAs and 8.67 mg g^{-1} n-6 FAs, with the n-3/n-6 ratio of 1.52; and 15.42 mg g^{-1} n-3 FAs and 7.58 mg g^{-1} n-6 FAs and a n-3/n-6 ratio of 2.04, respectively for soybean and flaxseed oil (Tables 7 and 8, Figure 1). The fatty acids analysis demonstrated the influence of the diet on the body composition of the animal reared, and the fact that there was no statistical differences in all treatments regarding essential FAs, mainly EPA and DHA, provides evident the ability of the animals to uptake and preserve those EFAs supplied through the diet, being it provided by the producer or by the ponds natural productivity (Tables 8 and 9, Figure 1).

Flaxseed oil is rich in n-3 FAs, being the most abundant the linolenic acid (18:3n-3) which is not one of the essential fatty acids for the *L. vannamei* shrimp, in the flaxseed diet its concentration reached 23.59 mg g^{-1} of the diet, while in the fish oil, soybean oil and poultry grease diets it was found at 3.05, 4.77 and 3.22 mg g^{-1} of the diets, respectively; the essential fatty acids such as EPA (20:5n-3), DPA (22:5n-3) and DHA (22:6n-3) were at a similar level in the soybean oil, poultry grease and flaxseed oil at 3.80, 0.79 and 1.88; 3.72, 0.80 and 2.03; and 3.98, 0.86 and 2.10 mg g^{-1} of EPA, DPA and DHA, respectively, for each lipid source (Table 7, Figure 1). The EFA were kept at optimal levels in the diet by the inclusion of menhaden fish oil in the formulation and some could be obtained from the natural productivity. The results on Tables 8, 9 and Figure 1 show no statistical differences for those FA in all treatments from the ponds and tanks trial.

Results found by the FA analysis of shrimp tissues from treatments with soybean oil, poultry grease and flaxseed oil attested to the evidence that the synthesis of EPA from α-linolenic acid was limited in shrimp; and, DPA and DHA synthesis even more limited because of the common desaturation and elongation enzymes and the competition between the substrates for them (Brenna, 2002); considerably the results from the flaxseed oil treatment where a large amount of linolenic acid was found in the diet and in the shrimp tissues without statistically increasing the amount of EPA, DPA and DHA when compared to the other treatments (Tables 7, 8 and 9, Figure 1).

Many consumers report a milder taste on shrimp aquacultured when compared to wild caught, which is possibly related to the diet and water salinity. Both treatments were reared in similar salinities (~12 ppt), thus the comparison of the diet influence on the sensorial attributes of the shrimp would be beneficial. Surprisingly after the sensorial analyses of the fish oil treatment,

high in HUFA and n-3 fatty acids, against an n-6 rich diet, soybean oil treatment, the panelists were unable to distinguish between the two oil sources, vamping the eagerness for the promotion of the usage of alternative oils on diets for shrimp production. Although, possible differences in aroma or basic taste might possibly occur due to variations in processing and cooking of shrimp for consumption, which can relate to consumer preferences and were not analyzed in this study.

Under the reported conditions, the alternative lipid sources; soybean oil, poultry grease, and flaxseed oil, have presented good production performances in a commercial setting, and typical results for both systems. The use of a non-marine protein based diet and the combination of alternative lipids with a percentage of fish oil, as a source of EFA, confirmed the results found in previous studies. The complete replacement of marine oil can be accomplished using lipid sources from genetically engineered organisms and/or supplemented with fermentation products as HUFA sources.

Unfortunately, the options available today in the market to replace the HUFA provided by marine oils for the aquaculture feed industry are scarce and cost prohibitive; thus, the industry is gradually evolving and investigating alternatives, such as algae production and reduced usage of fish oil in diets to supply the essential fatty acid EFA necessary for the production of the organisms and to satisfy the end consumer.

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Table 1. Ingredients composition (g $100g^{-1}$ of feed) of experimental diets.

	Diet 1[a]	Diet 2[a]	Diet 3[a]	Diet 4[a]	Diet 5[b]
Soybean meal (47% crude protein)	53.24	53.24	53.24	53.24	n/a[c]
Milo	19.03	19.03	19.03	19.03	
Corn distiller dried grain with solubles	6.68	6.68	6.68	6.68	
Poultry by-product meal (62% crude protein)	5.01	5.01	5.01	5.01	
Corn gluten meal	4.84	4.84	4.84	4.84	
Squid meal	0.50	0.50	0.50	0.50	
Menhaden fish oil (sprayed)	2.50	2.50	2.50	2.50	
Menhaden fish oil (mixed)	1.97	-	-	-	
Soybean oil (mixed)	-	1.97	-	-	
Poultry grease (mixed)	-	-	1.97	-	
Flaxseed oil (mixed)	-	-	-	1.97	
Soy lecithin	1.00	1.00	1.00	1.00	
Vitamin premix	0.33	0.33	0.33	0.33	
Trace mineral premix	0.09	0.09	0.09	0.09	
Stay C 350 mg/kg (35%)	0.02	0.02	0.02	0.02	
Calcium phosphate dibasic	3.14	3.14	3.14	3.14	
Copper sulfate	0.01	0.01	0.01	0.01	
Bentonite	1.50	1.50	1.50	1.50	
Mold inhibitor	0.15	0.15	0.15	0.15	
Total	100.00	100.00	100.00	100.00	
Protein (%)	**35.28**	**35.28**	**35.28**	**35.28**	**35.00**
Fat (%)	**8.04**	**8.04**	**8.04**	**8.04**	**7.00**

[a] Diets manufactured by Rangen Inc. Buhl, Idaho, USA.
[b] Commercial diet manufactured by Zeigler Bros, Inc. Gardners, Pennsylvania, USA.
[c] Complete formulation not available.

Table 2. Summary of daily water quality parameters for ponds trial during the 17-weeks nutrition study.

	Temperature (˚C)	Salinity (ppt)	DO (mg L^{-1})	pH
Mean	31.5	12.3	8.7	8.4
Max	37.2	23.3	26.8	10.3
Min	24.9	8.3	2.0	6.7

Table 3. Summary of weekly water quality parameters for ponds trial during the 17-weeks nutrition study.

	TAN (mg L^{-1})	Secchi disk reading (cm)
Mean	0.5	43.5
Max	5.0	115.0
Min	0.0[*]	15.0

[*] Below detectable levels

Table 4. Results after 17 weeks of pond trial, stocked 38,000 shrimp $pond^{-1}$, at 38 shrimp m^{-2} (38 shrimp m^{-3}) with 0.34 µg (wet weight) individuals (n=4).

Treatment	Mean Final Weight (g)	Weekly Growth (g)	Survival (%)	FCR	Final standing crop (kg ha^{-1})
1. Fish oil	18.9	1.09	74.0	1.40	5,254
2. Soybean oil	18.0	1.04	75.4	1.43	5,151
3. Poultry grease	21.6	1.25	65.6	1.45	5,070
4. Flaxseed oil	21.0	1.21	68.2	1.37	5,363
P-value	0.6557	0.6522	0.6743	0.8405	0.8182
PSE	4.01	0.23	11.28	0.12	424.32

Obs: Ponds with high mortalities due to plankton die-off were excluded from the results and statistical analysis.

Table 5. Summary of daily water quality parameters for tank trial during the 12-weeks nutrition study.

	Temperature (˚C)	Salinity (ppt)	DO (mg L^{-1})	pH	TAN[a] (mg L^{-1})
Mean	28.7	12.4	7.5	8.1	0.1
Max	33.7	17.9	13.1	9.0	0.7
Min	24.0	9.4	5.0	7.0	0.0[*]

[a] Total ammonia nitrogen (TAN) was measured twice a week.
[*] Below detectable levels.

Table 6. Results after 12 weeks of tank trial (green water system), stocked 30 shrimp $tank^{-1}$, at 35 shrimp m^{-2} (40 shrimp m^{-3}) with 0.25 g (wet weight) individuals (n=4).

Treatment	Mean Final Weight (g)	Weekly Growth (g)	Survival (%)	FCR	Final standing crop (kg ha^{-1})
1. Fish oil	14.7[b]	1.2[b]	96.7[a]	1.05[b]	5,024[b]
2. Soybean oil	13.8[b]	1.1[b]	97.5[a]	1.11[b]	4,738[b]
3. Poultry grease	14.8[b]	1.2[b]	92.5[a]	1.07[b]	4,835[b]
4. Flaxseed oil	14.0[b]	1.1[b]	98.3[a]	1.09[b]	4,819[b]
5. Com. feed	15.7[a]	1.3[a]	97.5[a]	0.97[a]	5,401[a]
6. Natural prod.	3.2[c]	0.3[c]	73.3[b]	n/a	741[c]
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
PSE	0.52	0.04	4.52	0.04	199.94

Obs: Tanks with high mortalities were excluded from the results and statistical analysis.

Table 7. Fatty acid composition (mg g^{-1} diet and % of FAME) of experimental diets[1] (n=4).

Selected FA	Treatment 1 Menhaden fish oil	Treatment 2 Soybean oil	Treatment 3 Poultry grease	Treatment 4 Flaxseed oil	Treatment 5 Commercial
Total lipid (%)	6.72	6.83	7.11	6.80	7.16
mg g^{-1} diet					
16:0	22.82	19.85	21.84	19.31	23.31
18:0	4.93	4.57	5.29	4.77	5.04
18:1n-9	22.52	21.20	26.30	23.02	22.90
18:2n-6	28.82	36.31	30.95	30.22	24.57
18:3n-3	3.05	4.77	3.22	23.59	2.85
20:4n-6	0.09	0.07	0.06	0.09	0.19
20:5n-3	6.29	3.80	3.72	3.98	6.53
22:5n-3	1.31	0.79	0.80	0.86	1.86
22:6n-3	3.47	1.88	2.03	2.10	3.76
Saturates[2]	36.60	31.05	33.28	30.44	35.53
Monounsaturates[3]	32.73	28.98	34.97	30.59	35.42
PUFA[4]	35.20	43.26	36.27	55.96	29.49
HUFA[5]	12.43	7.39	7.33	7.84	16.26
Total n-3[6]	15.92	12.46	10.91	31.69	18.11
Total n-6[7]	29.11	36.51	31.16	30.49	25.61
n3/n6 ratio	0.55	0.34	0.35	1.04	0.71
% of FAME					
16:0	19.51	17.93	19.53	15.47	19.98
18:0	4.21	4.13	4.73	3.82	4.31
18:1n-9	19.26	19.16	23.51	18.44	19.60
18:2n-6	24.64	32.81	27.64	24.23	21.03
18:3n-3	2.61	4.31	2.89	18.91	2.44
20:4n-6	0.08	0.06	0.05	0.08	0.79
20:5n-3	5.38	3.43	3.33	3.18	7.32
22:5n-3	1.12	0.69	0.73	0.71	2.09
22:6n-3	2.97	1.70	1.82	1.68	3.24
Saturates[2]	31.29	28.05	29.76	24.38	30.45
Monounsaturates[3]	27.99	26.18	31.27	24.50	30.34
PUFA[4]	30.10	39.09	32.42	44.85	25.24
HUFA[5]	10.63	6.68	6.56	6.27	13.97
Total n-3[6]	13.62	11.26	9.77	25.38	15.54
Total n-6[7]	24.89	32.98	27.84	24.44	21.93

Table 8. Total lipid (%) and fatty acid composition (mg g^{-1} wet weight and % of FAME) of shrimp muscle tissue for pond trial[8] (n=8).

Selected FA	Treatment 1 Menhaden fish oil	Treatment 2 Soybean oil	Treatment 3 Poultry grease	Treatment 4 Flaxseed oil
Total lipid (%)	1.55[a]	1.42[a]	1.49[a]	1.40[a]
mg g^{-1} wet weight				
16:0	8.58[a]	8.57[a]	8.39[a]	8.60[a]
18:0	5.56[a]	5.80[a]	5.49[a]	5.81[a]
18:1n-9	5.59[a]	5.57[a]	6.11[a]	5.40[a]
18:2n-6	6.58[a]	8.45[a]	7.34[a]	7.15[a]
18:3n-3	0.47[b]	0.66[b]	0.75[b]	2.31[a]
20:4n-6	0.09[a]	0.12[a]	0.13[a]	0.31[a]
20:5n-3	6.74[a]	6.62[a]	5.57[a]	6.58[a]
22:5n-3	0.40[a]	0.35[a]	0.39[a]	0.37[a]
22:6n-3	3.42[a]	3.29[a]	3.27[a]	3.23[a]
Saturates[2]	20.94[a]	21.74[a]	20.48[a]	21.81[a]
Monounsaturates[3]	8.12[a]	7.98[a]	8.40[a]	7.75[a]
PUFA[4]	9.61[a]	11.71[a]	10.50[a]	12.72[a]
HUFA[5]	10.78[a]	10.51[a]	9.50[a]	10.80[a]
Total n-3[6]	13.22[a]	13.17[a]	12.06[a]	15.42[a]
Total n-6[7]	6.77[a]	8.67[a]	7.59[a]	7.58[a]
n3/n6 ratio	1.95	1.52	1.59	2.04
% of FAME				
16:0	17.35[a]	16.52[b]	17.16[a]	16.27[b]
18:0	11.28[a]	11.20[a]	11.23[a]	10.96[a]
18:1n-9	11.26[b]	10.74[bc]	12.50[a]	10.21[c]
18:2n-6	13.26[c]	16.24[a]	15.02[b]	13.47[c]
18:3n-3	0.97[b]	1.27[b]	1.47[b]	4.32[a]
20:4n-6	0.19[b]	0.22[b]	0.26[b]	0.56[a]
20:5n-3	13.59[a]	12.74[b]	11.40[c]	12.34[b]
22:5n-3	0.81[a]	0.67[a]	0.80[a]	0.70[a]
22:6n-3	6.98[a]	6.32[a]	6.67[a]	6.13[a]
Saturates[2]	42.48[a]	41.90[a]	41.98[a]	41.17[a]
Monounsaturates[3]	16.33[b]	15.37[c]	17.18[a]	14.62[c]
PUFA[4]	19.34[c]	22.52[b]	21.44[b]	23.91[a]
HUFA[5]	21.85[a]	20.21[b]	19.40[b]	20.30[b]
Total n-3[6]	26.74[b]	25.35[c]	24.62[c]	29.00[a]
Total n-6[7]	13.66[c]	16.64[a]	15.52[b]	14.25[c]

Table 9. Total lipid (%) and fatty acid composition (mg g^{-1} wet weight and % of FAME) of shrimp muscle tissue for tank trial[9] (n=8).

Selected FA	Treatment 1 Menhaden fish oil	Treatment 2 Soybean oil	Treatment 3 Poultry grease	Treatment 4 Flaxseed oil	Treatment 5 Commercial	Treatment 6 Natural Productivity
Total lipid (%)	1.43[a]	1.45[a]	1.41[a]	1.22[a]	1.43[a]	1.34[a]
mg g^{-1} wet weight						
16:0	7.94[a]	6.79[a]	7.44[a]	10.96[a]	8.27[a]	5.89[a]
18:0	5.24[b]	4.66[b]	4.98[b]	7.62[a]	5.45[b]	4.02[b]
18:1n-9	5.49[ab]	5.12[ab]	6.01[ab]	7.57[a]	7.30[a]	3.62[b]
18:2n-6	6.69[b]	7.64[ab]	7.77[ab]	10.73[a]	5.92[bc]	2.89[c]
18:3n-3	0.41[b]	0.56[b]	0.54[b]	3.65[a]	0.33[b]	1.22[b]
20:4n-6	0.10[c]	0.12[c]	0.10[c]	0.54[a]	0.09[c]	0.27[b]
20:5n-3	7.34[a]	5.64[a]	5.71[a]	8.35[a]	7.16[a]	4.74[a]
22:5n-3	0.60[a]	0.49[a]	0.50[a]	0.71[a]	0.60[a]	0.47[a]
22:6n-3	3.91[a]	2.94[a]	2.96[a]	4.54[a]	4.16[a]	2.90[a]
Saturates[2]	19.54[a]	19.37[a]	19.87[a]	28.48[a]	20.52[a]	17.68[a]
Monounsaturates[3]	7.68[a]	6.77[a]	7.92[a]	10.18[a]	9.43[a]	5.99[a]
PUFA[4]	9.02[b]	10.19[b]	10.84[b]	17.67[a]	8.54[b]	10.11[b]
HUFA[5]	12.12[a]	9.34[a]	9.47[a]	14.43[a]	12.20[a]	8.92[a]
Total n-3[6]	13.88[a]	11.06[a]	11.47[a]	19.79[a]	14.21[a]	14.54[a]
Total n-6[7]	6.87[b]	7.85[b]	7.97[b]	11.42[a]	6.13[bc]	3.33[c]
n3/n6 ratio	2.02	1.41	1.44	1.73	2.32	4.37
% of FAME						
16:0	16.42[a]	14.80[b]	15.44[b]	15.37[b]	16.31[a]	13.49[c]
18:0	10.83[a]	10.14[a]	10.34[a]	10.75[a]	10.74[a]	9.26[b]
18:1n-9	11.36[bc]	11.22[bc]	12.51[b]	10.60[c]	14.40[a]	8.42[d]
18:2n-6	13.86[c]	16.60[a]	16.16[a]	15.04[b]	11.68[d]	6.69[e]
18:3n-3	0.85[de]	1.21[c]	1.12[cd]	5.17[a]	0.66[e]	2.81[b]
20:4n-6	0.20[d]	0.25[c]	0.22[cd]	0.77[a]	0.18[d]	0.61[b]
20:5n-3	15.16[a]	12.29[c]	11.90[c]	11.71[c]	14.11[b]	10.85[d]
22:5n-3	1.24[a]	1.06[bc]	1.06[bc]	0.99[c]	1.19[ab]	1.06[bc]
22:6n-3	8.08[a]	6.42[b]	6.18[b]	6.31[b]	8.21[a]	6.56[b]
Saturates[2]	40.41[a]	42.77[a]	41.32[a]	40.56[a]	40.49[a]	42.45[a]
Monounsaturates[3]	15.89[bc]	14.81[bcd]	16.48[b]	14.26[cd]	18.62[a]	13.81[d]
PUFA[4]	18.68[c]	22.05[b]	22.46[b]	25.00[a]	16.84[d]	23.43[ab]
HUFA[5]	25.02[a]	20.37[b]	19.74[b]	20.17[b]	24.05[a]	20.31[b]
Total n-3[6]	28.67[b]	24.11[c]	23.90[c]	27.69[b]	28.03[b]	33.30[a]
Total n-6[7]	14.22[c]	17.06[a]	16.57[ab]	16.03[b]	12.09[d]	7.70[e]

* Footnotes for tables 7, 8 and 9.

[1] Values represent averages of duplicate samples.
[2] Saturates: 14:0, 15:0, 16:0, 18:0, 20:0, 22:0. Internal standard, 19:0, was not considered.
[3] Monounsaturates: 15:1, 16:1 18:1, 20:1.
[4] PUFA: 16:2, 16:3, 18:2, 18:3, 20:2, 20:3, 22:2.
[5] HUFA: 18:4, 20:4, 20:5, 22:4, 22:5, 22:6.
[6] Total n-3: 18:3n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3.
[7] Total n-6: 18:2n-6, 20:3n-6, 20:4n-6.
[8] Values represent averages of duplicate samples per tank, and five tanks per dietary treatment. Means within rows with the same letter are not significantly different (SNK's alpha = 0.05).

Table 10. Sensory ratings of pond raised shrimp from fish oil and soybean oil treatments (treatments 1 and 2, respectively) (n=12).

Attribute class	Sensory attribute	Treatment 1	Treatment 2
		Fish oil	Soybean oil
Raw shrimp			
Aroma	Ocean/Seawater	6.40±1.06	6.43±1.80
	Shrimp	7.73±1.62	6.07±1.62
	Old shrimp	0.98±1.06	1.70±1.46
Raw meat appearance	Plumpness	7.03±1.20	7.17±1.36
	Brown color	3.00±1.25	2.87±1.51
Raw shell appearance	Darkness	6.70±1.19	4.93±2.09
	Stripe darkness	7.03±1.30	4.47±2.22
	Blotchiness	3.15±2.46	1.12±1.45
	Glossiness	7.27±1.27	6.93±1.44
	Tail iridescence	3.81±2.80	3.87±2.38
Cooked shrimp			
Raw aroma	Ocean/Seawater	6.40±0.99	5.87±1.19
	Shrimp	7.47±1.60	6.73±1.75
	Old shrimp	1.15±0.81	1.02±0.62
Appearance	Red/orange color	7.03±1.39	5.80±1.57
	Brown color	3.00±1.89	3.00±1.69
	Blotchiness	0.41±0.58	0.34±0.41
	Glossiness	7.03±1.20	6.73±1.03
Taste	Bitter	0.35±0.56	0.59±0.70
	Salty	2.87±1.36	2.73±1.49
	Sour	0.10±0.00	0.79±1.12
	Sweet	5.60±1.55	5.33±1.50
Mouthfeel	Sliminess	2.15±1.52	2.67±1.48
Texture	Firmness	6.73±0.80	6.33±0.62
	Juiciness	4.20±1.01	4.60±0.83
	Chewiness	5.40±1.40	4.87±0.83
	Crispness	5.13±0.99	5.07±0.80
	Fibrous	4.87±1.88	5.53±1.19
Aftertaste	Iodine	4.53±1.64	3.80±1.42

Obs: Rating scale ranged from 0 to 10, values expressed as means ± standard deviation. No statistical differences found among treatments ($P \leq 0.05$).

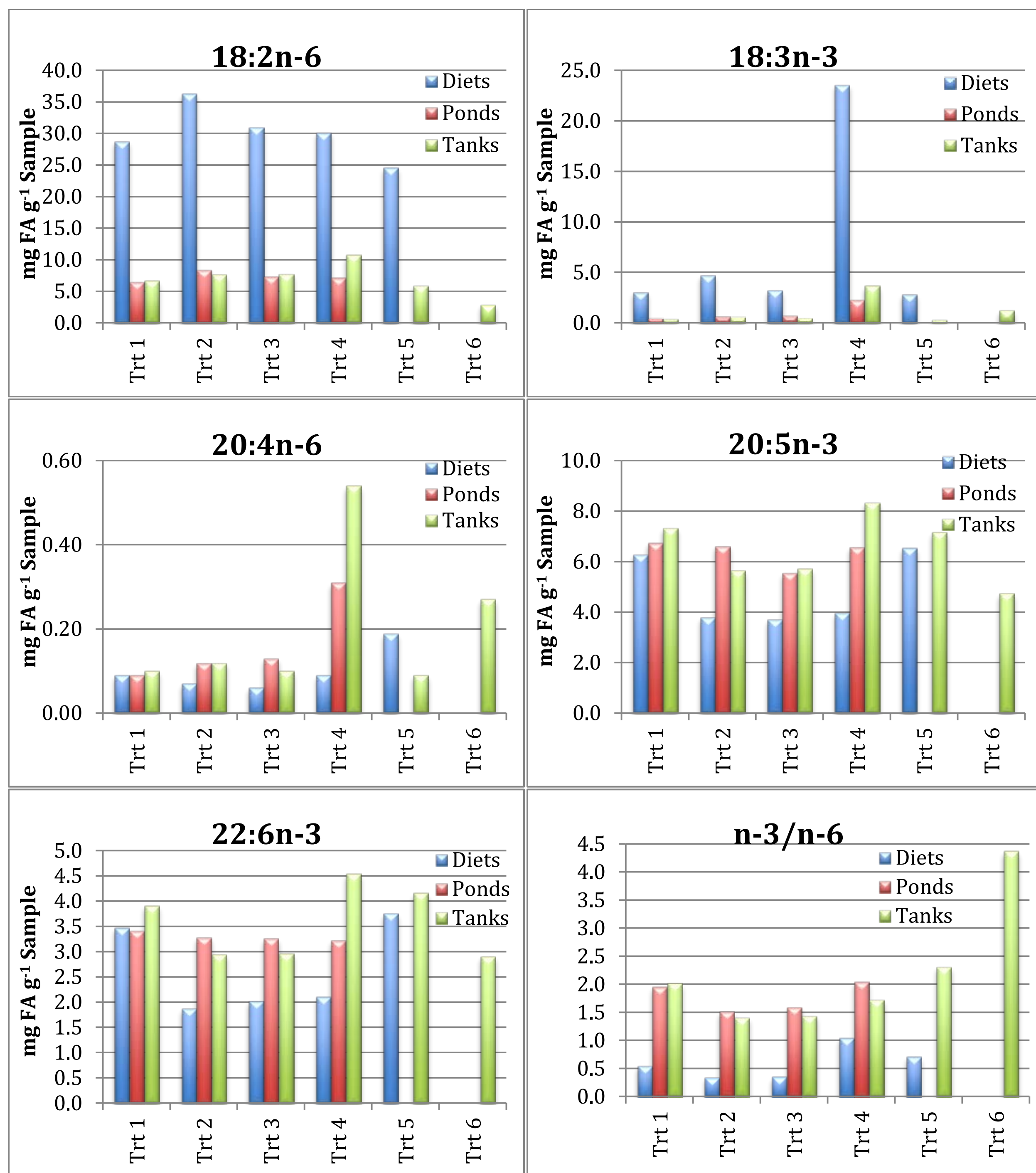

Figure 1. Linoleic (18:2n-6), linolenic (18:3n-3), arachidonic (20:4n-6), EPA (20:5n-3), DHA (22:6n-3), and n-3/n-6 ratio in muscle of *L. vannamei* during the 17-weeks nutrition study. Values for diets represent means of duplicate per sample, and values for trials represent means of duplicate analysis for four replicate observations.

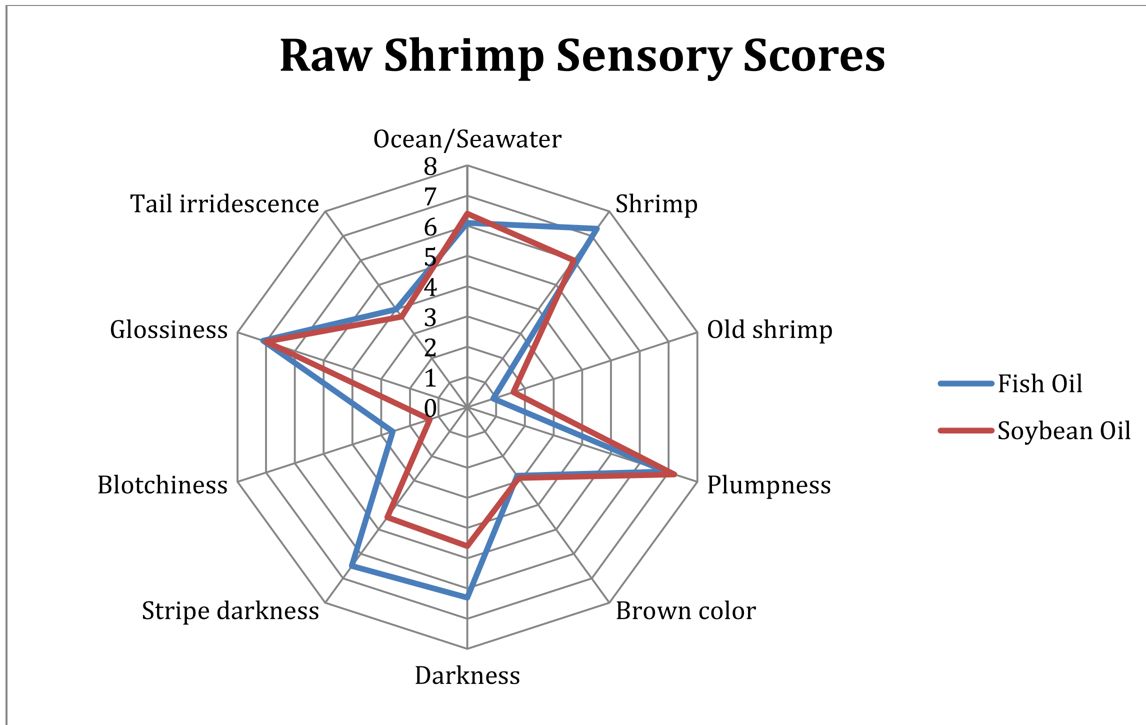

Figure 2. Mean values of sensory scores for the raw shrimp sensorial analysis under the fish oil and soybean oil treatments from ponds (treatments 1 and 2, respectively).

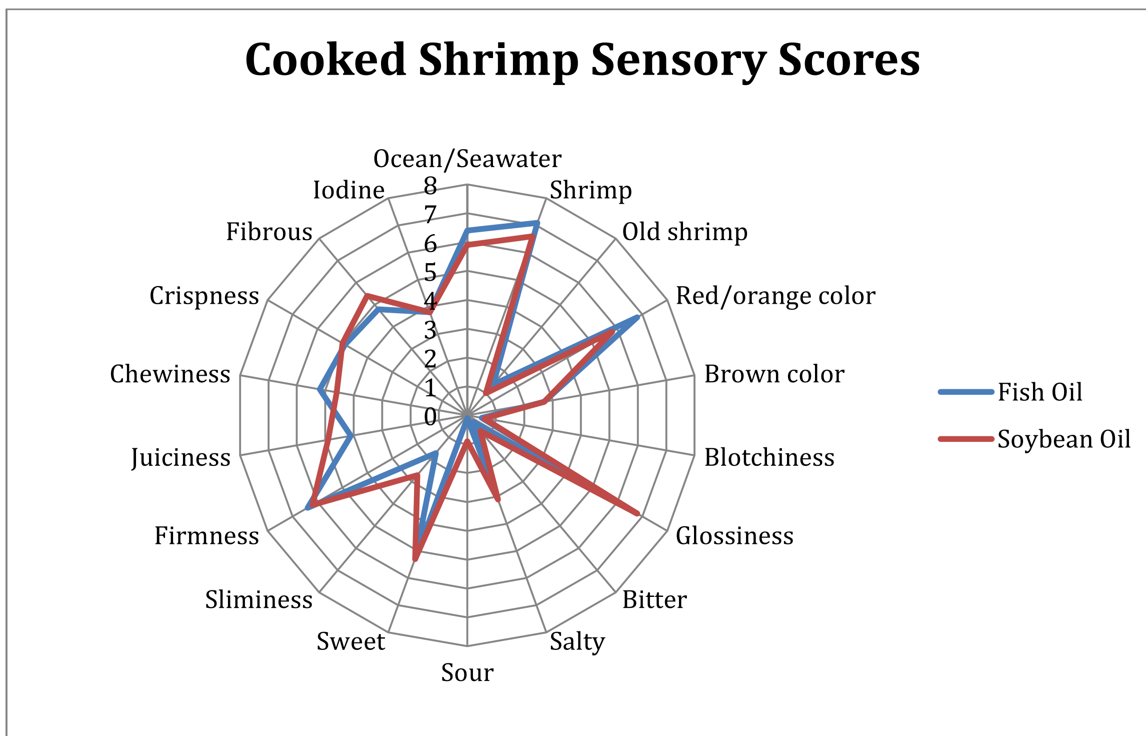

Figure 3. Mean values of sensory scores for the cooked shrimp sensorial analysis under the fish oil and soybean oil treatments from ponds (treatments 1 and 2, respectively) (n=12).

CHAPTER IV

REPLACEMENT OF FISH OIL IN PLANT BASED DIETS FOR PACIFIC WHITE SHRIMP (*Litopenaeus vannamei*) BY STEARINE FISH OIL OR PALM OIL

Abstract

The reduction on employment of marine ingredients in feeds for aquaculture is desirable to maintain the economical and environmental sustainability of the industry. The present study evaluates the replacement of menhaden fish oil (FO) with a variety of alternative oils both in a clear water system and outdoor green water system. The first trial evaluated a by-product of the purification of marine fish oil, called stearine fish oil (SFO), and palm oil (PO) in a brackish clear water system. The second trial evaluated SFO, PO, soybean oil (SO), flaxseed oil (FXO), and poultry grease (PG) in a low salinity green water system. A total of twelve non-marine protein based diets for juvenile Pacific white shrimp, *Litopenaeus vannamei*, were prepared with the inclusion of the aforementioned alternative lipid sources. In the clear water system trial, fifteen juvenile shrimp (0.29 g) were stocked into each of twenty four 340 L tanks and fed six different diets over an 8-week growth trial. Five diets were formulated with SFO as replacement for FO at inclusion ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 as FO:SFO, and another diet with PO replacing 90% of the FO. The results showed no statistically significant differences in mean final weight (g), weekly growth (g week^{-1}), survival (%) or final standing crop (kg ha^{-1}) values of shrimp fed the various diets. Fatty acid (FA) profiles of tail muscle form shrimp fed the

various lipid sources in general conformed to the lipids of the feed. Shrimp fed the PO diet (Diet 6), containing the lowest levels of EPA (20:5n-3), DPA (22:5n-3) and DHA (22:6n-3) per gram of diet (0.81, 0.26 and 0.69 mg, respectively), had high quantities of these FA in shrimp tail muscle, 3.34, 0.33 and 2.34 mg per gram of wet tissue respectively, when compared to the first five treatments. This response shows the resilience of those HUFAs to peroxidation and the preservation of those molecules in the organisms due to its biological relevance. A rapid assessment on the leaching of the aromatic amino acids phenylalanine, tyrosine, and tryptophan from commercial feed samples with or without top coating was done. The study in commercial shrimp feed with no extra top coating, fish oil top coating, or stearine fish oil top coating displayed a tendency in reducing the leaching of aromatic amino acids from commercial diets with the inclusion of fish oil and even further with stearine fish oil, albeit no significant statistical differences were found due to the wide range of results. The low salinity (2.0 ppt) green water system was stocked with twenty juvenile shrimp (0.92 g) per tank, into forty four 600 L tanks and fed nine different diets in eleven treatments over a 6-weeks growth trial. The diets previously run in the clear water trial with 100% FO, 100% SFO, and 90% PO were used along diets with 90% SO, or 90% FXO as replacement for FO, and four commercially produced diets supplemented with FO, SO, PG, or FXO at 2% of the diet. One treatment received half rations of the commercial fish oil diet, and a last treatment had to thrive on natural productivity present in the system. The results indicated statistically significant lower values for in mean final weight, weekly growth, and final standing crop values for shrimp in the natural productivity treatment (Treatment 17), when compared to the other treatments. Treatment 16, where half ration of fish oil commercial diet was provided had an improved FCR (0.69), although a poor final standing crop for treatments 16 and 13 (90% soybean oil) was noticed. Fatty acid (FA)

profiles from tail muscle form shrimp fed the various lipid sources in general conformed to the lipids of the feed. Compared to other treatments, addition of FXO (treatments 11 and 15) showed increased concentrations of linolenic acid (18:3n-3) in the diet (19.81 and 12.02 mg g^{-1} of diet, respectively) and the shrimp muscle tissues (5.36 and 3.41 mg g^{-1} of wet tissue, respectively). EPA was found at concentrations greater than 3.0 mg per gram of wet tissue in all treatments, and the lowest concentration of DHA found was 1.29 mg per gram of wet tissue. The results found from both experiments demonstrate the feasibility of replacement of marine fish oil by various alternative lipid sources in brackish and low salinity water conditions, and that the beneficial HUFAs are preserved in the edible tissues. Despite these findings, more research should be done under real commercial conditions.

Introduction

In the last three decades, the world food fish production of aquaculture has expanded by almost 12 times, at an average annual rate of 8.8 percent. World aquaculture production attained an all-time high in 2010, at 79 million tonnes, worth US$125 billion. Countries in Asia, such as, China, India, Viet Nam, Indonesia, Bangladesh, Thailand, Myanmar, the Philippines, and Japan, accounted for 89 percent of world aquaculture production by volume, with crustaceans representing 9.7 percent of the total Asian production (FAO, 2012).

In spite of the fact that the present aquaculture production numbers seem a big leap for the industry, the use of the earth's waters as a source for food and the surplus production from oceans and freshwater waterbodies has been essentially the same throughout human history. It has been a passive harvesting activity, which is a large contrast to what has happened with terrestrial food production where domestication and innovation have led to a significant increase in food production, supporting a growing population. Thus, aquaculture has supplied significant quantities of seafood only during the last few decades; the sector is still a very young industry (Asche, 2008).

Culture of the Pacific white shrimp, *Litopenaeus vannamei*, in low salinity waters (LSW) is usual in many countries (Roy & Davis, 2010). Due to environmental restrictions, land availability, and/or market demands. The producers are encouraged to find new locations for the production of this species which tolerates a wide salinity range (Roy et al. 2007). Despite the physiological challenges for the shrimp to thrive in LSW imposed by the ionic balance of the water (Roy, Davis, & Whitis, 2009). In 2010 the Pacific white shrimp production in China

reached 1 million tonnes with about half of its production done in low salinity waters (Valderrama & Anderson, 2011).

About forty six percent of the global aquaculture production (31.5 million tonnes) depended on nutrient inputs in the form of externally provided fresh feed items, farm-made feeds or commercially pelleted feeds by 2008 (FishStatPlus, 2011). Data from 1995 show that 75 percent of the shrimp production worldwide relied on commercial feeds using a total of 1.4 million tonnes of feed. By 2008, the use of commercial feed had expanded to 5.0 million tonnes with 95 percent of shrimp being produced using commercial feeds (A.G.J. Tacon, Hasan, & Metian, 2011).

Common feed ingredients used in aquaculture as protein sources are fishmeal, soybean meal, various oilseed cakes and meals; as carbohydrate sources, various cereals and cereal by-products; and lipids sources, fish oil and vegetable oils (Hasan, et al. 2007). Compound feeds are defined by the Food and Agriculture Organization of the United Nations (FAO, 2001) as "A mixture of products of vegetable or animal origin in their natural state, fresh or preserved, or products derived from the industrial processing thereof, or organic or inorganic substances, whether or not containing additives, for oral feeding in the form of a complete feed". These compound feeds are used for the production of freshwater and marine fish and crustaceans.

To meet the market demand for seafood from aquaculture, nutritionally complete feeds need to be available in the requisite quantities. Mostly, the availability and use of feed ingredients for aquaculture often focuses on fishmeal and fish oil resources; however, the sustainability of the aquaculture sector will probably be closely linked with the sustained supply of terrestrial animal and plant proteins, lipids and carbohydrates for feeds (FAO, 2012).

Each locality in the globe has its particularity regarding terrestrial or aquatic organisms produced due to geographical, environmental, cultural, and/or social factors. Asia, the largest seafood producer, produces large quantities of palm oil, while North and South America are the largest producers of soybean oil, and Chile, Ecuador and Peru, also in South America, are the biggest producers of fish oil (Silva, Francis, & Tacon, 2011).

In terms of world edible oil production, palm oil represents the largest component and is generally the cheapest product without genetic modification nor solvent extraction. Malaysia and Indonesia are the major global producers of this commodity, which by 2008 reached 42.4 million tonnes representing 26.7 percent of all vegetable and animal oils and fats produced worldwide. Fish oil is one of the least produced oils with 1.1 million tonnes produced globally, contributing to only 0.7 percent of the total oils and fats produced in that same year (Oil World, 2008; Gunstone, 2011). Half of the fatty acids (FA) present in palm oil are saturated, containing mainly palmitic acid (16:0) and the monounsaturated fatty acid oleic acid (18:1n-9). Palm oil is widely used in the food industry for its stability, being found in margarines, spray oils, canned fishes, soup and cake mixes, etc. (Aini & Yusoff, 2000).

The capture marine fisheries industry harvested 78.9 million tonnes of fish in 2011. About 23.2 million tonnes of fish, almost 30 percent of the world's capture fisheries, were destined to other uses than human consumption (FAO, 2012), implying the use in the pharmaceutical, chemical and cosmetic industry, feeding of cultured organisms, and production of fish meal and oil for animal feeds. The conversion of trash fish and by-catch fish into fish meal and fish oil is called 'reduction process', which renders about 20 percent of fish meal and 5 percent of fish oil (Silva et al., 2011). From such a process, another by-product called stearin or

stearine fish oil is obtained. The stearine fraction is less soluble and possesses a higher melting point due to the higher content of saturated acids (Gunstone & Herslöf, 2000).

Crustaceans in general are grazers, utilizing their foremost articulated appendages called maxilliped, and their mouthpieces to breakdown the feed pellets before ingestion leading to possible losses of key water-soluble nutrients due to leaching to the aquatic environment. Dietary ingredients such as amino acids, vitamins and minerals can rapidly leach from the feed particle (Yúfera et al., 2002); thus, the time that the feed sits in the aqueous enviroment before consumption by the animals is also an important factor related to nutrient losses. Coating agents such as ethyl cellulose (Goldblatt, Conklin, & Brown, 1980; Vervarcke et al., 2002), hydroxypropyl methyl cellulose (Vervarcke et al., 2002), and agar, pectins, gelatin, alginates, cereal gums, chitin, carrageenan, starches, etc. (Meyers & Zein-Eldin, 1972) have been researched to minimize nutrient and antibiotic leaching from feeds. Unfortunately, there are limited research data pertaining to oil top coating of feed pellets. Top coating is extensively used in the feed industry to reduce fines, improve attraction and seal feed pellets to reduce the oxidation of the nutrients.

To facilitate a better understanding of the use of alternative oils in aquaculture, the objective of this study was to demonstrate the feasibility of a partial or total substitution of menhaden fish oil by alternative lipid sources (stearine fish oil, palm oil, soybean oil, poultry grease, and flaxseed oil) in non-marine, protein-based shrimp (*Litopenaeus vannamei*) production diets under various rearing conditions, and its influence on the fatty acid profile of the diets and the uptake by the organisms. A rapid assessment of aromatic amino acid leaching from commercial feeds top coated with different oils was also performed to evaluate their anti-leaching properties.

Materials and methods

Source of shrimp and experimental system

The clear water tank trial research was conducted at the Alabama Department of Conservation and Natural Resources Marine Resource Division, Claude Peteet Mariculture Center in Gulf Shores, Alabama, USA, and the outdoor green water tank trial at Greene Prairie Aquafarm in Boligee, Alabama, USA. Both growth trials were carried out in parallel during the production period from May through September of 2010. Pacific white shrimp, *L. vannamei*, postlarvae were obtained from Shrimp Improvement Systems (Islamorada, FL) and nursed on 5,000 L tanks until reaching about 0.3 g for the clear water tanks and until 0.9 g for the outdoor green water tanks. All treatments were randomly assigned, water quality parameters (temperature, salinity, dissolved oxygen, and pH) were measured twice daily using a YSI 550A dissolved oxygen meter (YSI Inc., Yellow Springs, OH, USA), and two feedings per day at 800 h and 1600 h offered. Water samples for total ammonia nitrogen (TAN) were collected twice a week and TAN levels were determined using a spectrophotometer (Spectronic 20 Genesys, Spectronic Instrument Inc. Rochester, NY, USA) following the Nesslerization method (APHA 1989).

Clear Water Tank System Trial

Juvenile shrimp were obtained from the nursery system and selected by hand-sorting to a uniform size. Then juvenile shrimp (0.29±0.02 g, initial weight) were stocked into 24 square tanks (0.7 x 0.7 x 0.7 m, 340 L volume) in a recirculating system at a density of 15 shrimp per tank (30 shrimp m^{-2} or 43 shrimp m^{-3}). Six experimental diets were randomly assigned with four

replications (tanks) per treatment. Test diets were applied twice daily at 0800 and 1600 h for a 55-day experimental period. Feed input was pre-calculated on a weekly basis using an expected feed conversion ratio of 2:1 and a doubling in size until individual shrimp weighed one gram. Thereafter, a growth rate of 0.8 g per week was assumed. The animals were fed one of six isonitrogenous and isolipidic (35% crude protein, 8% crude lipid) diet with an increasing inclusion of stearine fish oil (0, 25, 50, 75, and 100%) and one diet with palm oil (90%) (Treatments 1 through 6, Table 1).

Each tank was covered with nylon netting and was continuously aerated with two air stones. Shrimp were counted every two weeks to readjust the daily feed input. At the conclusion of the 8-week growth trial, shrimp were counted and weighed as a group. Mean final weight (g), weekly growth (g $week^{-1}$), survival (%), estimated feed conversion ratio (FCR) and final standing crop (kg ha^{-1}) were determined.

Outdoor Green Water Tank System Trial

A series of forty-four circular tanks (0.8 m^2 bottom surface area, 600 L water volume) was set up adjacent to a commercial 1.6 hectare (4 acre) shrimp pond in west Alabama. The water source for the pond was low salinity groundwater with a salinity of 2.0 ppt. In addition, the farmer added K-Mag® and muriate of potash to raise potassium (K^+) and magnesium (Mg^{2+}) levels in the pond water to levels adequate for the culture of *L. vannamei* (0.92 ± 0.02 g, mean initial weight). Water from the pond was continuously pumped into the tanks and the overflow drained back into the pond *via* a central stand-pipe. Water was aerated using submersible diffusers (two per tank) connected to a ¼ hp regenerative blower. Twenty *L. vannamei* juveniles were stocked into each of forty tanks (11 treatments, 4 replicates). Shrimp were fed one of five

diets formulated at Auburn University (Auburn, Alabama, USA) (treatments 7 through 11), four commercial shrimp diets by Rangen, Inc. (Buhl, Idaho, USA) previously described in Chapter III (treatments 12 through 15); one treatment (treatment 16) received only half ration of one of the commercial diets (treatment 12), or a final treatment (17), that did not receive any feed, having to utilize the natural productivity. Feed input was pre-calculated on a weekly basis using an expected feed conversion ratio of 2:1 and a growth rate of 1.5 g per week.

Six weeks after the start of the experiment, all shrimp were harvested, counted and group weighed. Mean final weight (g), weekly growth (g $week^{-1}$), survival (%), estimated feed conversion ratio (FCR) and final standing crop (kg ha^{-1}) were determined.

Experimental diets

Clear Water Tank System Trial

Six experimental isonitrogenous and isolipidic diets (approximately 35% crude protein with 8% lipid) were formulated to contain soybean meal as the primary protein source. The first five diets were designed to replace menhaden fish oil by stearine fish oil in increments of 25% resulting in diets with 0, 25, 50, 75, and 100% substitution. A sixth diet with the replacement of 90% of menhaden fish oil by red palm oil (Swanson Health Products, Fargo, North Dakota, USA), also called unbleached palm oil (treatments 1-6, Table 1). Diets were prepared by mixing the ingredients in a mixer (Hobart, Troy, Ohio) for 30 minutes. Subsequently, hot water was added to the mixture until appropriate consistency for pelleting was obtained. Diets were then passed through a meat grinder and a 3-mm mesh sieve. Pellets were air dried (<50 ˚C) to a moisture content of less than 10%. After drying, pellets were crumbled, packed in sealed plastic bags and stored in a freezer until used.

Outdoor Green Water Tank System Trial

The outdoor trial used a series of diets (Table 2) that were also used on the clear water trial, Treatments 7, 8, 9 utilizing 100% fish oil, 100% stearine fish oil, 90:10 palm oil: fish oil; diets previously described on Chapter II, Treatments 10 with 90:10 soybean oil: fish oil and Treatment 11 with 90:10 flaxseed oil: fish oil; and, Treatments 12-16 utilizing diets commercially produced by Rangen Inc. (Buhl, Idaho, USA) and formulated to contain soybean meal as the primary protein source with the inclusion of either 2% of menhaden fish oil, soybean oil, poultry grease, or flaxseed oil, and top coated with 2.5% of menhaden fish oil to seal the pellet and supply essential fatty acids (EFA). Also, Treatment 16 (Table 6) received half ration of the fish oil commercial diet (Treatment 12, Table 2); and treatment 17 (NP) no feed provided, animals had to thrive on natural productivity.

Lipid and fatty acid analysis

At the end of the growth trials, the shrimp were harvested, weighed and counted. Harvested shrimp from each experimental tank were frozen and shipped to Auburn University for FA analysis. Before lipid analysis, they were thawed and muscle from ten shrimp were pooled into a composite sample per tank, ground, and analyzed in duplicate for lipid and fatty acid composition. Experimental diets were analyzed for fatty acid composition as well. Lipids were extracted by the method of Folch et al. (1957) and quantified gravimetrically after drying under nitrogen. Total lipid content was expressed as percent of wet tissue.

FA were transesterified with boron trifluoride and fatty acid methyl esters (FAME) were analyzed with a Shimadzu GC-17A gas chromatograph (Shimadzu Scientific Instruments Inc.,

Portland, OR, USA) equipped with a capillary column (Omegawax 530, 30 m x 0.53 mm x 0.5 µm film thickness, Supelco 2-4019, Sigma-Aldrich, Oslo, Norway) using helium as the carrier gas and a flame-ionization detector as previously described (Quintero et al., 2009). FAs were identified by comparison of retention times to those of known standards, and they were quantified by using an internal standard, nonadecaenoic acid methyl ester (C 19:0, Sigma-Aldrich, St. Louis, MO, USA); they were expressed as mg g^{-1} of diet or wet weight and as percent of the total identified FAME.

Aromatic amino acid leaching test

Commercial shrimp production feed manufactured by Zeigler (Zeigler Bros, Inc. Gardners, PA, USA) containing 35% crude protein and 7% crude lipid was divided in three treatments groups: plain commercial feed (PCF), menhaden fish oil (Omega Protein, Inc. Reedville, VA, USA) top coated feed (FOTC), and stearine fish oil (Omega Protein, Inc. Reedville, VA, USA) top coated feed (SOTC). The feed from the FOTC and SOTC were top coated with 3% of the respective oils, total of 10% crude lipid; the PCF diet only received the manufacturer top coating and no additional top coating, therefore having 7% crude lipid.

Five grams of feed from each treatment, in triplicate, was placed in a 600 mL beaker on a magnetic stirrer (~120 rpm) and 250 mL of deionized water (DI water) was added. A 10 mL syringe with an 1.6 µm glass fiber filter (Whatman Inc., Florham Park, NJ, USA) was used to take 1 mL samples at time 1, 2, 5, 15, 30, and 60 minutes. The standards consisted of a 0% aromatic amino acids (AAA) concentration blank sample (deionized water), and an 100% AAA concentration sample where five gram of commercial feed completely dissolved in 250 mL of DI water.

Aromatic amino acids content in samples were analyzed in a spectrophotometer for their characteristic light absorbance at different wavelengths; 257 nm for phenylalanine, 274 nm for tyrosine, and 280 nm for tryptophan.

Data analysis

Differences in final mean weights, weekly growth, survival, FCR, and final standing crop were analyzed using one-way ANOVA to determine if significant (P <0.05) differences existed among treatment means. SNK multiple comparison test was used as the mean separation procedure. Statistical analyses were conducted using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Experimental diets

Total crude protein and crude lipid content of experimental diets were relatively constant; consequently, they were considered isonitrogenous and isolipidic. Also, the diets reflected the fatty acid profile of the lipid sources used in their formulation (Table 7).

With the increasing inclusion (0, 25, 50, 75 and 100%) of stearine fish oil in substitution for menhaden fish oil in diets 1 through 5, the amounts of saturated, monounsaturated and polyunsaturated fatty acids followed an increasing pattern. Notably, with the increased inclusion of stearine fish oil, the concentration of stearic acid (18:0) also gradually increased from 4.88 to 6.04 mg g^{-1} of the diet. However, the 90% palm oil diet (treatments 6 and 9) had the higher amount of oleic acid (18:1n-9) (20.33 mg g^{-1} of the diet). Diet 5 with 100% of stearine fish oil had the highest content of saturated fatty acids (31.70 mg g^{-1} of diet), even more than diet 6 and

with 90% palm oil (30.78 mg g^{-1} of diet), although the latter had a much higher amount of monounsaturated fatty acid (21.34 mg g^{-1} of diet) than any other diet. Both diets formulated with flaxseed oil (diets 8 and 12) carried the highest concentrations of omega-3 FAs (21.12 and 16.14 mg g^{-1} of diet, respectively) due to large amounts of α-linolenic acid, 18:3n-3 (19.81 and 12.02 mg g^{-1} of diet, respectively), and diets with soybean oil (diets 7 and 10) had the highest amounts of omega-6 FAs (21.89 and 18.17 mg g^{-1} of diet, respectively) due to linoleic acid concentrations, 18:2n-6 (21.77 and 18.07 mg g^{-1} of diet, respectively) (Tables 7 and 8, Figures 1 and 2).

Arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), the HUFA essential for the optimum growth of the organisms, were found in concentrations ranging from 0.02 to 0.11 mg g^{-1} of the diet for ARA, 0.59 to 6.23 mg g^{-1} of the diet for EPA, 0.26 to 1.99 mg g^{-1} of the diet for DPA, and 0.33 to 4.50 mg g^{-1} of the diet for DHA. The lower levels of ARA were found in the palm oil diet (diet 6) and the highest levels in diets 2 and 3 with stearine fish oil (25 and 50% inclusion); EPA and DHA lowest concentrations were found in diet 7 with 90% inclusion of soybean oil and the highest concentration of EPA and DHA were found in diet 5 (100% stearine fish oil) and diet 3 (50% stearine fish oil), respectively. DPA concentration was the lowest in diet 6 (90% palm oil) and the highest in diet 2 (25% stearine fish oil). Diet 7 (90%SO) also showed the highest amount of omega-6s, and diet 8 (90%FXO) the highest amount of omega-3s, primarily, linolenic acid.

Diets 9, 10, 11 and 12, manufactured by Rangen Inc. (Buhl, Idaho, USA) under commercial conditions, were formulated to provide all essential fatty acids through top coating the feed with menhaden fish oil. The diets have been previously tried in commercial pond

production with brackish water (*vide* Chapter III), and were used in the low salinity green water tanks trial in Boligee, west Alabama.

Clear Water Tank System Trial

Daily and weekly water quality monitoring (Table 3) showed no statistically significant differences among treatments, and the observed values indicate that culture water parameters were within the suitable range for grow out of this species.

Mean final weights ranged from 5.5 to 6.1 g, survival from 85.0 to 96.7%, growth per week 0.7 to 0.8 g wk^{-1}, estimated FCR 1.53 to 1.86, and final standing crop from 1,108 to 1,339 kg ha^{-1}. Means harvest data are presented in Table 4. One-way ANOVA showed significant statistical differences ($P<0.05$) between treatments for FCR only.

At the end of the 8-week feeding trial, mean total lipid content of shrimp muscle was not significantly affected by dietary treatment, and mean fatty acid composition of shrimp muscle tissue reflected the FA profile of the diet.

The statistical analysis of the fatty acids profile of the shrimp tail tissue revealed no statistical differences among treatments 1 through 5, yet treatment 6 as statistically different from all other treatments on almost all FAME but palmitic acid (16:0), stearic acid (18:0), and the sum of the saturates, monounsaturates, and PUFAs (Table 8, Figures 1 and 3).

Outdoor Green Water Tank System Trial

Results of the daily and weekly water quality monitoring (Table 5) showed no statistically significant differences between treatments, and the observed values indicate that culture water parameters were within the suitable range for grow out of this species. When tanks

presented high total ammonia nitrogen (TAN) readings, a higher volume of water exchange in the whole system was performed.

The production performance data (Table 6) showed mean final weights ranging from 9.5 to 12.6 g, weekly growth between 1.2 and 1.7 g, survival of 90 to 100% of the animals, FCR of 0.69 up to 1.39, and final standing crop from 1,983 to 2,822 kg ha^{-1}. Treatments 7 through 16 showed no significant statistical differences among mean final weight and weekly growth, and no differences were found among all treatments on survival. The FCR from treatment 16, where animals received half rations of diet 12 (fish oil commercial), showed an improved FCR of 0.69. The worse final standing crop performances were found on treatments 10, 16 and 17 (2,310; 2,261; and 1,983 kg ha^{-1}, respectively). Animals reared solely on natural productivity, treatment 17, showed poor production performance when compared to the other treatments. Significant statistical differences were found in mean final weight (9.5 g), weekly growth (1.2 g) and final standing crop (1,983 kg ha^{-1}), although no statistical difference was found on survival (93.8%).

At the end of the 6-week feeding trial, total lipid content of shrimp muscle was not affected by dietary treatment, ranging from 1.34 up to 1.50%. Palmitic acid (16:0), stearic acid (18:0), and the sum of saturated FAs were similar for all 11 treatments. For the monounsaturated FAs, the palm oil treatment (treatment 9, 90%PO) had the highest amounts (9.08 mg g^{-1}) in the tissues as presented in Table 9 as oleic acid, 18:1n-9 (6.45 mg g^{-1}), and at the lower end, treatment 17 (natural productivity) had the smallest amount of oleic acid (3.15 mg g^{-1}) and, consequently, of monounsaturated FAs (6.20 mg g^{-1}). Animals fed diet containing 90% soybean oil had the highest amount of omega-6 (8.11 mg g^{-1}) and linoleic acid, 18:2n-6 (7.50 mg g^{-1}); and the 90% flaxseed oil diet the highest amounts of omega-3 (14.15 mg g^{-1}) and linolenic acid, 18:3n-3 (5.36 mg g^{-1}), yet also the highest amount of arachidonic acid, 20:4n-6 (0.66 mg g^{-1}).

Treatments 7, 100% fish oil, and 8, 100% stearine fish oil, showed the highest amounts of DHA (4.30 and 4.96 mg g^{-1}, respectively) and EPA (2.84 and 2.45 mg g^{-1}, respectively), with treatment 8 the highest in HUFAs (8.68 mg g^{-1}). DPA was higher on treatment 8 (100% stearine fish oil), at 0.57 mg g^{-1} of wet tissue. Treatment 17 also had significantly lower monounsaturated FAs, PUFAs, HUFAs, and total n-6 FAs (Table 9, Figures 2 and 4).

Aromatic amino acids

The magnetic stirrer was used to mimic the water movement in a production system and the feed manipulation by the animals. The results of the aromatic amino acids phenylalanine, tyrosine, and tryptophan leaching are presented on tables 10, 11 and 12.

The aromatic amino acid phenylalanine had leached an average of 8.1 percent from the commercial feed with no extra top coating at 1 minute, 7.1 percent of the fish oil top coated diet, and 5.5 of the stearine fish oil top coated feed. At 60 minutes the leaching had reached an average of 78.3, 75.2, and 70.0 percent, respectively. The reduction in leaching of all three aromatic amino acids followed the same trend with highest leaching levels found on the non-top coated feed, followed by the fish oil coating and the lowest leaching values seen with the waxy lipid coating by the stearine fish oil. Average leaching values at 60 minutes for tyrosine ranged from 50.1 percent in stearine fish oil up to 57.0 percent in non-coated feed, and averages for tryptophan ranged from 45.2 percent in stearine fish oil up to 51.4 percent in non-coated feed.

No significant statistical differences were found among the average leaching percentages among all three treatments for all aromatic amino acids assessed, although a nice decrease in aromatic amino acid leaching trend follows the addition of different top coating agents, as presented in Figure 5.

Discussion

The development of diets with low inclusion of marine ingredients, or better yet, marine-free ingredients diets is a big step to avoid the 'fish meal trap' described by Naylor et al. in the 2000s; hopefully, allowing the aquaculture industry to continue to expand. Currently, China and countries in Southeast Asia demonstrate the highest growth in the global aquaculture sector.

Studies previously presented in Chapter II (González-Félix et al., 2010) and Chapter III have demonstrated the feasibility of the partial replacement of fish oil by other lipid sources, such as soybean oil, poultry grease, and flaxseed oil. The two studies presented here followed the trend of growth trial performances from the previous studies with differing lipid sources (stearine fish oil and palm oil), even at low salinities that imposes some osmoregulatory stress on the animals, confirming the acceptance of the use of alternative lipid sources in shrimp reared under distinct production conditions.

In the clear water tank system, results (Table 4) indicate no significant statistical differences in terms of mean final weight, weekly growth, survival and final standing crop. Shrimp offered the palm oil diet were numerically smaller and had significantly higher FCR when compared to those of shrimp in the other treatments. Due to the eating habits of shrimp, FCR is an estimated value based on feed provided to the animals and weight gain as food intake is difficult to quantify. Consequently, these are relative values subject to variation from a variety of variables. The numerically low final weight and poor FCR indicate that subsequent studies should be conducted.

Palm oil has been used in diets for black tiger shrimp (Hajra et al., 1988) and research on the use of this lipid source has been done in tilapia (Al-Owafeir & Belal, 1996), *Machrobrachium rosenbergii* (Shin-ichi Teshima, 1998), bagrid catfish, *Mystus nemurus* (Ng et

al., 2000), African catfish (Lim et al., 2001, Ng et al., 2004), Atlantic salmon (Bell et al., 2002), red hybrid tilapia, *Oreochromis* sp. (Ng et al., 2003), black tiger shrimp (Kumaraguru vasagam et al., 2005), rainbow trout (Ng et al., 2010), among other fish and crustacean species with promising results. Palm oil also has the advantage of being a rich source of the antioxidants vitamin A (α and β-carotene), vitamin E (tocopherols and tocotrienols) (Ng, 2002), saturated and monounsaturated fatty acids, which gives this oil its characteristic for stability and resistance to oxidation. For the animals fed diets containing palm oil, the benefit from such antioxidants extends not just for the health of the animals but also for the prolonged shelf life of fresh and frozen products (Turchini et al., 2009). Also, as reported by Henderson & Sargent (1985), the saturated fatty acids (i.e. palmitic acid, 16:0) and monounsaturated fatty acids (i.e. oleic acid, 18:1n-9), main components of this oil, are the preferential substrate for energy production in the mitochondrial system of fish.

The choice of oils not commonly used in diets and containing peculiar fatty acid profiles such as the stearine fish oil and palm oil with good production results open doors to innovative options to the feed industry, mainly for Asian countries where most of the aquaculture production takes place today. Results with the use of stearine fish oil as an oil source are not surprising as it has a similar fatty acid profile to fish oil. Unlike fish oil this oil is solid at room temperature, due to its fatty acid profile, and consequently could have advantages on feed pellet stability and reduction of nutrients leaching. The experiment on aromatic amino acid leaching from commercial diets top coated with fish or stearine fish oil demonstrated, although not with significant statistical differences probably due to the high range of variance on the results, a reduction on leaching, complying with results from the only publication available on this lipid source for fish and shrimp by Ju et al. (2012).

As expected the diets with the highest inclusions of stearine fish oil and palm oil, diets 4,5, and 6, (Table 7) had higher levels of saturated fatty acids, thus the amount of HUFAs present in the diets were enough to supply the EFAs required for optimum growth of the animals. Furthermore, apparently the animals can elongate and desaturate those saturated fatty acids into monounsaturated and short chain PUFA (eighteen carbons or less), and/or allocate them to be used as energy source, preserving the PUFAs and HUFAs (Tables 8 and 9, Figures 1, 2, 3 and 4) to be used as specific biological components. According to Das (2006), EFA are important constituents of cell membranes conferring its fluidity and properties, thus influencing on the behavior of membrane bound enzymes and receptors. EFAs are also converted into eicosanoids acting as signaling molecules in inflammation or immunity responses and messengers in the central nervous system (Caterina & Basta, 2001), playing major roles in the brain, retina, liver, kidneys, adrenal glands and gonads (Das, 2006).

Non-marine protein-based diets commonly utilize lipid ingredients naturally low in HUFAs. Those diets, when formulated to have stearine fish oil or at least 10% of fish oil, from the total oil added to the diet, seem to prevent the EFAs deficiency reported by Lim et al. (1997), Glencross & Smith (2001), González-Félix et al. (2003), and Samocha et al. (2009).

The visual representation of selected fatty acids from the animals raised in the clear water system tank trial (Figure 3), shows a very similar FA uptake profile by the animals among the reference diet (100% fish oil) and the diets with increasing inclusion of stearine fish oil (25 to 100% stearine fish oil). For the diet with 90% palm oil, the graph skewed towards the 18 carbon mono and polyunsaturated FAs with about 200% of oleic, linoleic, and linolenic acids; and EPA and DHA concentrations dropped to 50% of the reference diet concentration in the animals.

Figure 4 presents the FA profiles of the shrimp reared in outdoor system exposed to natural foods. This figure shows a similar tendency from the previous graph, although in this trial 90% flaxseed oil was used, showing a high concentration of linolenic and arachidonic acids typical for this oilseed, uptaken by the shrimp, which due to its limited ability to elongate and desaturate PUFAs into HUFAs (Kanazawa, Teshima, & Ono, 1979; Kayama et al., 1980; Lim et al., 1997; González-Félix et al., 2003) stored it in its body or obtained from the natural feed source available in the system. Additionally, on the green water tank system (Table 6), an improved estimated FCR was shown on treatment 16, which received only half rations of diet 9, demonstrating the ability of the animals to also obtain nutrients from the natural productivity, proven on treatment 17, in which animals grew only up to 9.5 g, having lower weekly growth and final standing crop when compared to the other treatments. It should be noted that this level of natural foods is partially due to the system design which continually pumps water from a production pond thus replenishing natural foods on a continual basis.

Despite the lack of statistical differences seen on the aromatic amino acid leaching assessment, it is noticeable that the decrease in leaching by the top coating of the feed pellets and the variability of response with different oils. The standard deviation values of the different treatments at 60 minutes proffered that the top coating was not evenly distributed among the feed pellets, being the smallest variances found on the commercial diet (0.5 to 0.7), increasing the variance on the fish oil top coating (2.3 to 2.5), and reaching its highest values on the stearine fish oil top coated diets (7.6 to 10.6). Such variances can be related to the difficulty of evenly coating each feed pellet with the oils manually in a laboratory setup, increasing the difficulty level even more when dealing with the stearine fish oil, which is solid at room temperature and had to be slightly heated to freely run, and therefore be added to the diet. Hence, more research is

needed to draw a real conclusion on the performance of top coating of feed pellets with various agents.

The results obtained from both trials, in brackish and low salinity waters (14 and 2 ppt, respectively), confirms the feasibility of utilization of alternative oils in marine protein free diets, partially substituting fish oil, and that the animals can also obtain beneficial FAs and EFAs from the natural productivity, when available. All of the presented approaches are valuable contributions in the quest for fish oil replacement, but the goal has not been met, yet.

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Table 1. Ingredients composition (g $100g^{-1}$ of feed) of experimental diets.

Ingredients (as is basis)	Diet 1 100%FO	Diet 2 25%SFO	Diet 3 50%SFO	Diet 4 75%SFO	Diet 5 100%SFO	Diet 6 90%PO
Soybean meal solvent extracted[a]	54.70	54.70	54.70	54.70	54.70	54.70
Whole wheat[b]	26.50	26.50	26.50	26.50	26.50	26.50
Corn gluten meal[c]	6.00	6.00	6.00	6.00	6.00	6.00
Menhaden fish oil[d]	6.15	4.61	3.08	1.54	-	0.62
Stearine fish oil[d]	-	1.54	3.08	4.61	6.15	-
Palm oil[e]	-	-	-	-	-	5.54
Trace mineral premix[f]	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[g]	1.80	1.80	1.80	1.80	1.80	1.80
Stay C 250 mg kg^{-1} (25%)[h]	0.10	0.10	0.10	0.10	0.10	0.10
CaP-dibasic[i]	3.00	3.00	3.00	3.00	3.00	3.00
Choline chloride[b]	0.20	0.20	0.20	0.20	0.20	0.20
Lecithin (deoilded 53% lipid)[j]	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol – USB[k]	0.05	0.05	0.05	0.05	0.05	0.05
Crude Protein (%)	34.93	34.93	34.93	34.93	34.93	34.93
Crude Fat (%)	8.01	8.01	8.01	8.01	8.01	8.01

[a] Faithway Feed Co., Guntersville, AL, USA.
[b] Gold Medal, General Mills Inc., Minneapolis, Minnesota, USA.
[c] Grain Processing Corporation, Muscatine, IA, USA.
[d] Omega Protein, Inc. Reedville, VA, USA.
[e] Natural Red Palm Oil, Swanson Health Products. Fargo, ND, USA.
[f] Trace mineral premix (g $100g^{-1}$): cobalt chloride 0.004, cupric sulphate pentahydrate 0.250, ferrous sulfate 4.0, magnesium sulfate heptahydrate 28.398, manganous sulphate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, filler 53.428.
[g] 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.
[h] Stay C®, (L-ascorbyl-2-polyphosphate), Roche Vitamins Inc., Parsippany, NJ, USA.
[i] Fisher Scientific, Fair Lawn, NJ, USA.
[j] Solae Company, St. Louis, MO, USA.
[k] USB Biochemicals, Cleveland, OH, USA.

Table 2. Ingredients composition (g $100g^{-1}$ of feed) of experimental diets.

Ingredients (as is basis)	Diet 7 90%SO	Diet 8 90%FXO	Diet 9[z] FOC	Diet 10[z] SOC	Diet 11[z] PGC	Diet 12[z] FXC
Soybean meal solvent extracted[a]	54.40	54.40	53.24	53.24	53.24	53.24
Whole wheat[b]	28.38	28.38	-	-	-	-
Corn gluten meal[c]	6.00	6.00	4.84	4.84	4.84	4.84
Milo[z]	-	-	19.03	19.03	19.03	19.03
Corn distiller dried grain with solubles[z]	-	-	6.68	6.68	6.68	6.68
Squid meal[z]	-	-	0.50	0.50	0.50	0.50
Menhaden fish oil[z] (sprayed)	-	-	2.50	2.50	2.50	2.50
Menhaden fish oil[d] (mixed)	0.46	0.46	1.97	-	-	-
Soybean oil[e] (mixed)	4.11	-	-	1.97	-	-
Poultry grease[z] (mixed)	-	-	-	-	1.97	-
Flaxseed oil[f] (mixed)	-	4.11	-	-	-	1.97
Trace mineral premix[g]	0.50	0.50	0.09	0.09	0.09	0.09
Vitamin premix[h]	1.80	1.80	0.33	0.33	0.33	0.33
Stay C	0.10[i]	0.10[i]	0.02[j]	0.02[j]	0.02[j]	0.02[j]
CaP-dibasic[k]	3.00	3.00	3.14	3.14	3.14	3.14
Lecithin (deoilded 53% lipid)[l]	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol – USB[m]	0.05	0.05	n/a[z]	n/a[z]	n/a[z]	n/a[z]
Choline chloride[b]	0.20	0.20	n/a[z]	n/a[z]	n/a[z]	n/a[z]
Copper sulfate[z]	-	-	0.01	0.01	0.01	0.01
Bentonite[z]	-	-	1.50	1.50	1.50	1.50
Mold inhibitor[z]	-	-	0.15	0.15	0.15	0.15
Crude Protein (%)	35.20	35.20	35.28	35.28	35.28	35.28
Crude Fat (%)	7.99	7.99	8.04	8.04	8.04	8.04

[a] Faithway Feed Co., Guntersville, AL, USA.
[b] Gold Medal, General Mills Inc., Minneapolis, Minnesota, USA.
[c] Grain Processing Corporation, Muscatine, IA, USA.
[d] Omega Protein, Inc. Reedville, VA, USA.
[e] American Soybean Association, St. Louis, MO, USA.
[f] Archer-Daniels-Midland Co. – ADM, Decatur, Illinois, USA.
[g] 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.
[h] Trace mineral premix (g $100g^{-1}$): cobalt chloride 0.004, cupric sulphate pentahydrate 0.250, ferrous sulfate 4.0, magnesium sulfate heptahydrate 28.398, manganous sulphate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, filler 53.428.
[i] Stay C®, (L-ascorbyl-2-polyphosphate) 250 mg kg^{-1} (25%), Roche Vitamins Inc., Parsippany, NJ, USA.
[j] Stay C®, (L-ascorbyl-2-polyphosphate) 350 mg kg^{-1} (35%), Roche Vitamins Inc., Parsippany, NJ, USA.
[k] Fisher Scientific, Fair Lawn, NJ, USA.
[l] Solae Company, St. Louis, MO, USA.
[m] USB Biochemicals, Cleveland, OH, USA.
[z] Commercial diet manufactured by Zeigler Bros, Inc. Ingredients sources and/or amount undisclosed.

Table 3. Summary of the overall means for the water quality parameters for the clear water tanks trial during the 8-weeks nutrition study.

	Temperature (˚C)	Salinity (ppt)	DO (mg L^{-1})	pH	TAN[a] (mg L^{-1})
Mean	29.0	14.1	7.4	7.9	0.05
Max	32.8	15.9	9.9	8.3	0.41
Min	25.4	12.5	5.5	7.5	0.00[*]

[a] Total ammonia nitrogen (TAN) was measured twice a week.
[*] Below detectable levels

Table 4. Results after 8 weeks of clear tanks trial (clear water system), stocked 15 shrimp $tank^{-1}$, at 31 shrimp m^{-2} (44 shrimp m^{-3}) with 0.29 g (wet weight) individuals (n=4).

fish oil:alternative oil	Mean Final Weight (g)	Weekly Growth (g)	Survival (%)	FCR	Final standing crop (kg ha^{-1})
1. 100% Fish oil (Diet 1)	6.0 [a]	0.7 [a]	85.0 [a]	1.54 [a]	1,108 [a]
2. 25% Stearine oil (Diet 2)	6.1 [a]	0.7 [a]	96.7 [a]	1.53 [a]	1,262 [a]
3. 50% Stearine oil (Diet 3)	5.5 [a]	0.7 [a]	96.7 [a]	1.64 [ab]	1,146 [a]
4. 75% Stearine oil (Diet 4)	5.7 [a]	0.7 [a]	95.0 [a]	1.86 [b]	1,167 [a]
5. 100% Stearine oil (Diet 5)	5.8 [a]	0.8 [a]	93.3 [a]	1.58 [a]	1,339 [a]
6. 90% Palm oil (Diet 6)	5.5 [a]	0.7 [a]	96.7 [a]	1.84 [b]	1,145 [a]
P-value	0.1722	0.1563	0.1219	0.0247	0.4417
Pooled Standard Error (PSE)	0.1374	0.0169	1.3030	0.0331	35.6850

Means within columns with the same letter are not significantly different (SNK's alpha = 0.05).

Table 5. Summary of daily water quality parameters for the outdoor green water tanks trial during the 6-week nutrition study.

	Temperature (˚C)	Salinity (ppt)	DO (mg L^{-1})	pH	TAN[a] (mg L^{-1})
Mean	31.7	2.1	7.5	8.2	1.03
Max	37.7	2.1	13.4	9.5	3.48
Min	26.4	2.0	6.0	7.0	0.28

[a] Total ammonia nitrogen (TAN) was measured twice a week.

Table 6. Results after 6 weeks of outdoor tanks trial (green water system), stocked 20 shrimp $tank^{-1}$, at 25 shrimp m^{-2} (32 shrimp m^{-3}) with 0.92 g (wet weight) individuals (n=4).

	Mean Final Weight (g)	Weekly Growth (g)	Survival (%)	FCR	Final standing crop (kg ha^{-1})
7. 100% Fish oil (Diet 1)	11.1[a]	1.5[a]	97.5[a]	1.38[b]	2,467[abc]
8. 100% Stearine oil (Diet 5)	11.7[a]	1.5[a]	100.0[a]	1.30[b]	2,708[a]
9. 90% Palm oil (Diet 6)	11.1[a]	1.5[a]	100.0[a]	1.38[b]	2,536[abc]
10. 90% Soybean oil (Diet 7)	11.1[a]	1.5[a]	91.3[a]	1.39[b]	2,310[bcd]
11. 90% Flaxseed oil (Diet 8)	12.0[a]	1.6[a]	96.3[a]	1.27[b]	2,657[ab]
12. Fish oil commercial (Diet 9)	12.2[a]	1.6[a]	96.3[a]	1.24[b]	2,708[a]
13. Soybean oil commercial (Diet 10)	11.9[a]	1.6[a]	93.8[a]	1.28[b]	2,591[abc]
14. Poultry grease commercial (Diet 11)	12.3[a]	1.6[a]	96.3[a]	1.23[b]	2,733[a]
15. Flaxseed oil commercial (Diet 12)	12.6[a]	1.7[a]	96.3[a]	1.21[b]	2,822[a]
16. ½ ration fish oil commercial (Diet 9)	11.1[a]	1.5[a]	90.0[a]	0.69[a]	2,261[cd]
17. Natural productivity	9.5[b]	1.2[b]	93.8[a]	n/a[*]	1,983[d]
P-value	0.002	0.002	0.3614	<.0001	0.0005
PSE	0.138	0.020	0.857	0.017	35.491

[*] Treatment 11 was excluded from the statistical analysis for estimated FCR due to the impossibility to quantify the amount of feed available to the animal.

Means within columns with the same letter are not significantly different (SNK's alpha = 0.05).

Table 7. Fatty acid composition (mg g^{-1} diet and % of FAME) of experimental diets[1] (n=4).

Selected FA	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12
	100%FO	25%SFO	50%SFO	75%SFO	100%SFO	90%PO	90%SO	90%FXO	FOC	SOC	PGC	FXC
Total lipid (%)	7.16	7.26	7.55	6.97	7.06	6.77	7.39	7.11	6.12	6.83	7.11	6.80
mg g^{-1} diet												
16:0	10.04	11.10	12.43	14.31	15.83	21.16	7.63	6.17	11.64	9.88	11.05	9.84
18:0	2.00	2.12	2.53	2.75	3.08	3.12	2.32	1.80	2.51	2.28	2.68	2.43
18:1n-9	4.54	5.04	5.66	5.79	6.45	20.33	10.52	8.86	11.48	10.55	13.30	11.73
18:2n-6	8.25	10.70	11.09	11.96	12.97	15.25	21.77	16.00	14.69	18.07	15.64	15.41
18:3n-3	1.23	1.66	1.80	1.74	1.87	1.58	3.65	19.81	1.56	2.37	1.63	12.02
20:4n-6	0.07	0.11	0.11	0.09	0.08	0.02	0.07	0.06	0.05	0.04	0.03	0.05
20:5n-3	3.31	5.44	6.17	5.81	6.23	0.81	0.59	0.62	3.21	1.89	1.89	2.02
22:5n-3	1.13	1.99	1.81	1.85	1.62	0.26	0.41	0.49	1.31	0.79	0.80	0.86
22:6n-3	2.96	4.44	4.50	3.51	3.06	0.69	0.33	0.35	1.77	0.93	1.03	1.07
Saturates[2]	17.27	19.06	25.55	28.73	31.70	30.78	10.36	8.42	18.66	15.45	16.85	15.50
Monounsaturates[3]	10.18	11.48	13.09	13.40	14.84	21.34	11.97	10.10	16.69	14.42	17.70	15.58
PUFA[4]	10.82	14.42	15.47	16.32	17.81	17.17	25.61	36.02	17.95	21.53	18.34	28.52
HUFA[5]	7.77	12.22	13.24	11.66	11.63	1.94	1.27	1.34	6.34	3.68	3.72	3.99
Total n-3[6]	9.03	13.99	15.25	13.68	13.87	3.53	4.85	21.12	8.12	6.20	5.53	16.14
Total n-6[7]	8.38	10.90	11.35	12.19	13.22	15.29	21.89	16.09	14.84	18.17	15.75	15.54
n-3/n-6 ratio	1.08	1.28	1.34	1.12	1.05	0.23	0.22	1.31	0.55	0.34	0.35	1.04
% of FAME												
16:0	21.82	19.41	18.45	20.41	20.83	29.71	15.51	11.04	19.51	17.93	19.53	15.47
18:0	4.35	3.70	3.75	3.92	4.06	4.38	4.71	3.23	4.21	4.13	4.73	3.82
18:1n-9	9.87	8.81	8.40	8.25	8.49	28.53	21.38	15.85	19.26	19.16	23.51	18.44
18:2n-6	17.92	18.70	16.47	17.06	17.06	21.41	44.24	28.65	24.64	32.81	27.64	24.23
18:3n-3	2.67	2.90	2.68	2.49	2.46	2.21	7.43	35.44	2.61	4.31	2.89	18.91
20:4n-6	0.16	0.20	0.16	0.13	0.11	0.02	0.14	0.10	0.08	0.06	0.05	0.08
20:5n-3	7.19	9.51	9.17	8.29	8.21	1.14	1.20	1.11	5.38	3.43	3.33	3.18
22:5n-3	1.27	1.74	1.71	1.64	1.65	0.30	0.61	0.44	1.12	0.69	0.73	0.71
22:6n-3	6.42	7.76	6.68	5.00	4.03	0.96	0.67	0.62	2.97	1.70	1.82	1.68
Saturates[2]	37.53	33.34	37.94	40.98	41.71	43.22	21.05	15.07	31.29	28.05	29.76	24.38
Monounsaturates[3]	22.12	20.08	19.43	19.11	19.54	29.96	24.32	18.08	27.99	26.18	31.27	24.50
PUFA[4]	23.48	25.22	22.97	23.29	23.43	24.10	52.04	64.47	30.10	39.09	32.42	44.85
HUFA[5]	16.86	21.36	19.66	16.63	15.32	2.72	2.58	2.38	10.63	6.68	6.56	6.27
Total n-3[6]	19.59	24.46	22.64	19.51	18.27	4.96	9.85	37.79	13.62	11.26	9.77	25.38
Total n-6[7]	18.20	19.06	16.86	17.38	17.39	21.46	44.48	28.82	24.89	32.98	27.84	24.44

Table 8. Total lipid (%) and fatty acid composition (mg g^{-1} wet weight and % of FAME) of shrimp muscle tissue for the clear water trial[8] (n=8).

Selected FA	Treatment 1 100%FO	Treatment 2 25%SFO	Treatment 3 50%SFO	Treatment 4 75%SFO	Treatment 5 100%SFO	Treatment 6 90%PO
Total lipid (%)	1.50 [a]	1.63 [a]	1.63 [a]	1.66 [a]	1.54 [a]	1.63 [a]
mg g^{-1} wet weight						
16:0	9.55[a]	9.03[a]	10.29[a]	8.59[a]	8.34[a]	9.20[a]
18:0	5.91[a]	5.39[a]	6.04[a]	4.94[a]	4.88[a]	4.36[a]
18:1n-9	5.18[b]	4.65[b]	5.20[b]	4.26[b]	4.11[b]	9.43[a]
18:2n-6	6.86[b]	6.27[b]	7.05[b]	5.86[b]	5.64[b]	9.91[a]
18:3n-3	0.44[b]	0.39[b]	0.43[b]	0.35[b]	0.33[b]	0.66[a]
20:4n-6	0.14[a]	0.13[a]	0.14[a]	0.09[ab]	0.09[ab]	0.08[b]
20:5n-3	8.75[a]	7.98[a]	9.00[a]	7.40[a]	7.32[a]	3.34[b]
22:5n-3	0.57[a]	0.57[a]	0.75[a]	0.72[a]	0.79[a]	0.33[b]
22:6n-3	7.16[a]	6.28[ab]	6.63[ab]	4.99[ab]	4.27[bc]	2.34[c]
Saturates[2]	23.30[a]	21.88[a]	23.85[a]	20.28[a]	20.98[a]	19.78[a]
Monounsaturates[3]	8.42[a]	7.56[a]	8.50[a]	6.97[a]	6.71[a]	10.87[a]
PUFA[4]	8.96[a]	8.13[a]	9.21[a]	7.73[a]	7.54[a]	11.68[a]
HUFA[5]	16.78[a]	15.10[a]	16.69[a]	13.34[a]	12.60[a]	6.21[b]
Total n-3[6]	18.19[a]	16.35[a]	18.14[a]	14.62[a]	13.91[a]	7.35[b]
Total n-6[7]	7.07[b]	6.47[b]	7.27[b]	6.03[b]	5.82[b]	10.05[a]
n3/n6 ratio	2.57	2.53	2.50	2.42	2.39	0.73
% of FAME						
16:0	16.46[c]	17.08[bc]	17.62[b]	17.60[b]	17.39[b]	18.89[a]
18:0	10.21[a]	10.18[a]	10.34[a]	10.11[a]	10.16[a]	8.98[b]
18:1n-9	8.88[b]	8.77[b]	8.95[b]	8.74[b]	8.58[b]	19.40[a]
18:2n-6	11.79[b]	11.83[b]	12.03[b]	12.00[b]	11.74[b]	20.42[a]
18:3n-3	0.75[b]	0.73[b]	0.72[b]	0.71[b]	0.69[b]	1.37[a]
20:4n-6	0.24[a]	0.25[a]	0.23[a]	0.19[b]	0.18[b]	0.16[b]
20:5n-3	15.12[a]	15.06[a]	15.33[a]	15.15[a]	15.28[a]	6.89[b]
22:5n-3	0.99[e]	1.07[d]	1.29[c]	1.48[b]	1.65[a]	0.68[f]
22:6n-3	12.38[a]	11.84[ab]	11.33[b]	10.24[c]	8.92[d]	4.83[e]
Saturates[2]	41.13[a]	41.88[a]	41.21[a]	42.52[a]	44.00[a]	40.79[a]
Monounsaturates[3]	14.47[b]	14.29[b]	14.58[b]	14.31[b]	14.00[b]	22.35[a]
PUFA[4]	15.38[b]	15.36[b]	15.75[b]	15.83[b]	15.70[b]	24.05[a]
HUFA[5]	29.02[a]	28.48[ab]	28.46[ab]	27.34[bc]	26.30[c]	12.80[d]
Total n-3[6]	31.43[a]	30.83[a]	30.95[a]	29.97[ab]	29.03[b]	15.17[c]
Total n-6[7]	12.15[b]	12.22[b]	12.42[b]	12.36[b]	12.13[b]	20.71[a]

Table 9. Total lipid (%) and fatty acid composition (mg g^{-1} wet weight and % of FAME) of shrimp muscle tissue for outdoor tank trial[8] (n=8).

Selected FA	Trt 7 100%FO	Trt 8 100%SFO	Trt 9 90%PO	Trt 10 90%SO	Trt 11 90%FXO	Trt 12 FOC	Trt 13 SOC	Trt 14 PGC	Trt 15 FXC	Trt 16 ½ FOC	Trt 17 NP
Total lipid (%)	1.44[a]	1.45[a]	1.46[a]	1.50[a]	1.34[a]	1.35[a]	1.36[a]	1.40[a]	1.43[a]	1.36[a]	1.44[a]
mg g^{-1} wet weight											
16:0	8.31[a]	8.30[a]	9.47[a]	7.89[a]	8.72[a]	8.68[a]	8.64[a]	8.62[a]	8.56[a]	8.86[a]	7.53[a]
18:0	4.17[a]	4.10[a]	3.99[a]	4.22[a]	4.80[a]	4.19[a]	4.56[a]	4.23[a]	4.26[a]	4.30[a]	3.59[a]
18:1n-9	4.09[bcd]	3.66[de]	6.45[a]	3.93[cd]	4.43[bc]	4.63[bc]	4.46[bc]	4.79[b]	4.34[bcd]	4.28[bcd]	3.15[e]
18:2n-6	4.62[c]	4.43[c]	5.79[bc]	7.50[a]	5.81[bc]	5.40[bc]	6.16[ab]	5.65[bc]	5.39[bc]	4.73[bc]	2.95[d]
18:3n-3	2.14[c]	2.03[c]	2.28[c]	2.19[c]	5.36[a]	2.15[c]	2.60[bc]	2.47[bc]	3.41[b]	2.77[bc]	2.96[bc]
20:4n-6	0.37[b]	0.36[b]	0.34[b]	0.35[b]	0.66[a]	0.36[b]	0.41[b]	0.37[b]	0.47[b]	0.41[b]	0.41[b]
20:5n-3	4.30[ab]	4.96[a]	3.55[bc]	3.27[c]	3.62[bc]	4.54[ab]	4.21[abc]	3.74[bc]	3.86[bc]	4.13[abc]	3.29[c]
22:5n-3	0.43[c]	0.57[a]	0.43[c]	0.39[c]	0.44[c]	0.55[ab]	0.49[abc]	0.49[abc]	0.47[bc]	0.49[abc]	0.41[c]
22:6n-3	2.84[a]	2.45[ab]	1.67[de]	1.60[de]	1.61[de]	2.21[bc]	2.00[bcd]	1.65[de]	1.78[cde]	1.94[bcd]	1.29[e]
Saturates[2]	20.72[a]	20.54[a]	21.81[a]	20.36[a]	23.19[a]	21.79[a]	22.39[a]	21.74[a]	21.56[a]	22.26[a]	19.97[a]
Monounsaturates[3]	7.59[ab]	6.90[b]	9.08[a]	6.32[b]	7.30[ab]	7.57[ab]	7.29[ab]	7.79[ab]	7.16[b]	7.43[ab]	6.20[b]
PUFA[4]	10.14[b]	9.86[b]	11.74[b]	13.07[ab]	15.06[a]	11.13[b]	12.29[ab]	11.77[b]	12.32[ab]	11.22[b]	10.10[b]
HUFA[5]	8.28[ab]	8.68[a]	6.39[cd]	5.97[d]	6.74[bcd]	8.03[abc]	7.48[abcd]	6.64[bcd]	6.96[abcd]	7.38[abcd]	5.85[d]
Total n-3[6]	12.31[ab]	12.81[ab]	10.89[b]	10.16[b]	14.15[a]	12.41[ab]	12.19[ab]	11.34[ab]	12.43[ab]	12.37[ab]	11.42[ab]
Total n-6[7]	5.26[bcd]	5.05[cd]	6.42[bc]	8.11[a]	6.76[ab]	6.03[bc]	6.88[ab]	6.34[bc]	6.15[bc]	5.44[bc]	3.71[d]
n3/n6 ratio	2.34	2.54	1.70	1.25	2.09	2.06	1.77	1.79	2.02	2.27	3.08
% of FAME											
16:0	17.80[bc]	18.06[bc]	19.32[a]	17.28[d]	16.61[d]	17.90[bc]	17.44[bcd]	17.96[bc]	17.85[bc]	18.33[b]	17.73[bc]
18:0	8.93[ab]	8.91[ab]	8.12[c]	9.23[a]	9.23[a]	8.64[abc]	9.23[a]	8.82[ab]	8.87[ab]	8.91[ab]	8.47[bc]
18:1n-9	8.75[d]	7.96[ef]	13.14[a]	8.60[d]	8.51[de]	9.55[bc]	9.02[cd]	9.99[bc]	9.03[cd]	8.88[d]	7.44[f]
18:2n-6	9.90[d]	9.64[d]	11.80[bc]	16.39[a]	11.12[c]	11.14[c]	12.47[b]	11.79[bc]	11.21[c]	9.79[d]	6.94[e]
18:3n-3	4.58[de]	4.41[e]	4.66[de]	4.79[de]	10.26[a]	4.43[de]	5.26[cd]	5.15[cde]	7.08[b]	5.75[c]	7.03[b]
20:4n-6	0.79[c]	0.78[c]	0.70[c]	0.77[c]	1.26[a]	0.74[c]	0.83[bc]	0.78[c]	0.97[b]	0.85[bc]	0.97[b]
20:5n-3	9.20[b]	10.80[a]	7.25[de]	7.16[de]	6.94[e]	9.36[b]	8.54[bc]	7.82[cde]	8.06[cd]	8.56[bc]	7.76[cde]
22:5n-3	0.92[c]	1.25[a]	0.88[c]	0.85[c]	0.84[c]	1.13[b]	0.99[bc]	1.01[bc]	0.98[bc]	1.02[bc]	0.99[bc]
22:6n-3	6.08[a]	5.32[b]	3.41[efg]	3.50[e]	3.07[fg]	4.56[c]	4.04[d]	3.44[ef]	3.71[de]	4.02[d]	3.03[g]
Saturates[2]	44.34[c]	44.67[bc]	44.52[bc]	44.51[bc]	44.35[c]	44.87[bc]	45.23[bc]	45.33[bc]	44.90[bc]	46.13[b]	47.77[a]
Monounsaturates[3]	16.24[b]	15.00[cd]	18.50[a]	13.83[f]	13.96[ef]	15.62[cd]	14.75[d]	16.24[b]	14.92[cd]	15.37[cd]	14.63[de]
PUFA[4]	21.71[e]	21.46[e]	23.93[cd]	28.58[a]	28.79[a]	22.95[d]	24.85[bc]	24.55[bc]	25.67[b]	23.21[d]	23.82[cd]
HUFA[5]	17.71[ab]	18.87[a]	13.05[f]	13.08[f]	12.89[f]	16.55[b]	15.17[cd]	13.88[def]	14.51[cde]	15.29[c]	13.78[ef]
Total n-3[6]	26.34[bc]	27.87[a]	22.24[f]	22.26[f]	27.01[ab]	25.57[cd]	24.67[de]	23.68[e]	25.95[bcd]	25.61[cd]	26.94[ab]
Total n-6[7]	11.27[d]	10.99[d]	13.08[c]	17.73[a]	12.95[c]	12.44[c]	13.92[b]	13.23[bc]	12.77[c]	11.26[d]	8.73[e]

* Footnotes for tables 7, 8, and 9.

[1] Values represent averages of duplicate samples.
[2] Saturates: 14:0, 15:0, 16:0, 18:0, 20:0, 22:0. Internal standard, 19:0, was not considered.
[3] Monounsaturates: 15:1, 16:1 18:1, 20:1.
[4] PUFA: 16:2, 16:3, 18:2, 18:3, 20:2, 20:3, 22:2.
[5] HUFA: 18:4, 20:4, 20:5, 22:4, 22:5, 22:6.
[6] Total n-3: 18:3n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3.
[7] Total n-6: 18:2n-6, 20:3n-6, 20:4n-6.
[8] Values represent averages of duplicate samples per tank, and four tanks per dietary treatment. Means within rows with the same letter are not significantly different (SNK's alpha = 0.05).

Table 10. Leaching of the aromatic amino acid Phenylalanine, in percentage ± standard deviation, through time in commercial feed samples top coated with fish or stearine fish oil, compared to no top coated feed.

Top coating	1 min	2 min	5 min	15 min	30 min	60 min
No top coating	8.1 ± 1.2	13.2 ± 1.3	22.5 ± 0.7	40.8 ± 1.5	57.2 ± 0.9	78.3 ± 0.5
Fish oil	7.1 ± 1.6	13.1 ± 1.3	20.8 ± 1.9	38.7 ± 1.2	54.1 ± 0.6	75.2 ± 2.5
Stearine fish oil	5.5 ± 1.0	8.7 ± 1.5	18.8 ± 1.3	31.4 ± 7.4	52.2 ± 1.2	70.0 ± 10.6

Table 11. Leaching of the aromatic amino acid Tyrosine, in percentage ± standard deviation, through time in commercial feed samples top coated with fish or stearine fish oil, compared to no top coated feed.

Top coating	1 min	2 min	5 min	15 min	30 min	60 min
No top coating	5.4 ± 1.0	9.1 ± 1.0	15.4 ± 0.7	28.2 ± 1.2	39.9 ± 0.9	57.0 ± 0.7
Fish oil	4.5 ± 1.1	9.1 ± 1.0	14.1 ± 1.6	26.9 ± 0.9	37.6 ± 0.5	53.9 ± 2.3
Stearine fish oil	3.8 ± 0.8	5.9 ± 1.4	12.9 ± 1.0	21.5 ± 4.8	36.0 ± 0.7	50.1 ± 8.2

Table 12. Leaching of the aromatic amino acid Tryptophan, in percentage ± standard deviation, through time in commercial feed samples top coated with fish or stearine fish oil, compared to no top coated feed.

Top coating	1 min	2 min	5 min	15 min	30 min	60 min
No top coating	4.7 ± 1.0	8.0 ± 1.1	13.6 ± 0.8	25.2 ± 1.3	35.7 ± 0.9	51.4 ± 0.7
Fish oil	3.8 ± 1.0	8.2 ± 0.9	12.4 ± 1.6	24.1 ± 0.7	33.5 ± 0.5	48.5 ± 2.3
Stearine fish oil	3.4 ± 0.8	5.1 ± 1.5	11.4 ± 1.1	19.0 ± 4.0	32.0 ± 0.6	45.2 ± 7.6

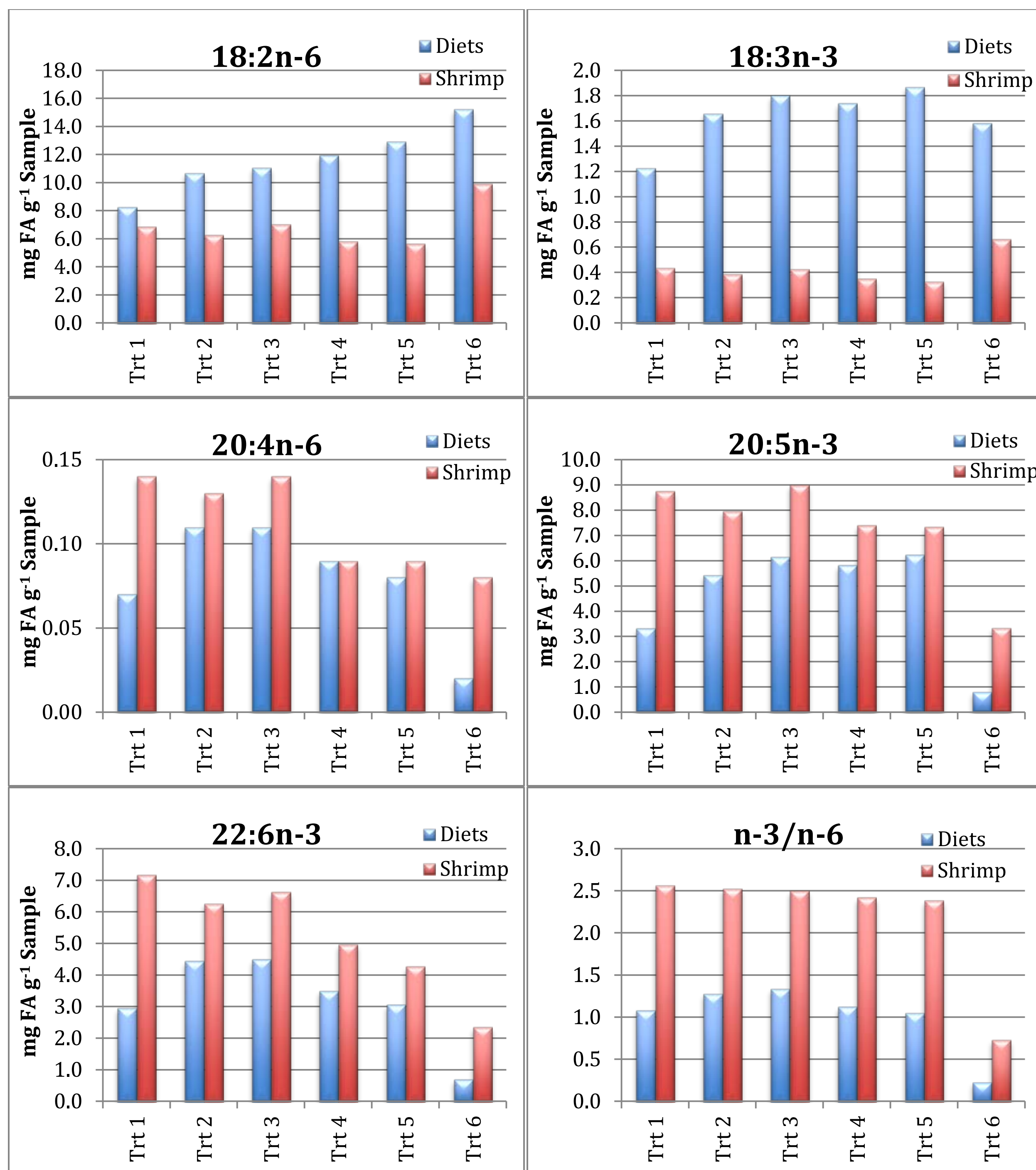

Figure 4. Linoleic (18:2-6), linolenic (18:3n-3), arachidonic (20:4n-6), EPA (20:5n-3), DHA (22:6n-3), and n-3/n-6 ratio in muscle of *L. vannamei* during the 8-weeks nutrition study in the clear water tanks. Values for diets represent means of duplicate per sample, and values for trials represent means of duplicate analysis for four replicate observations.

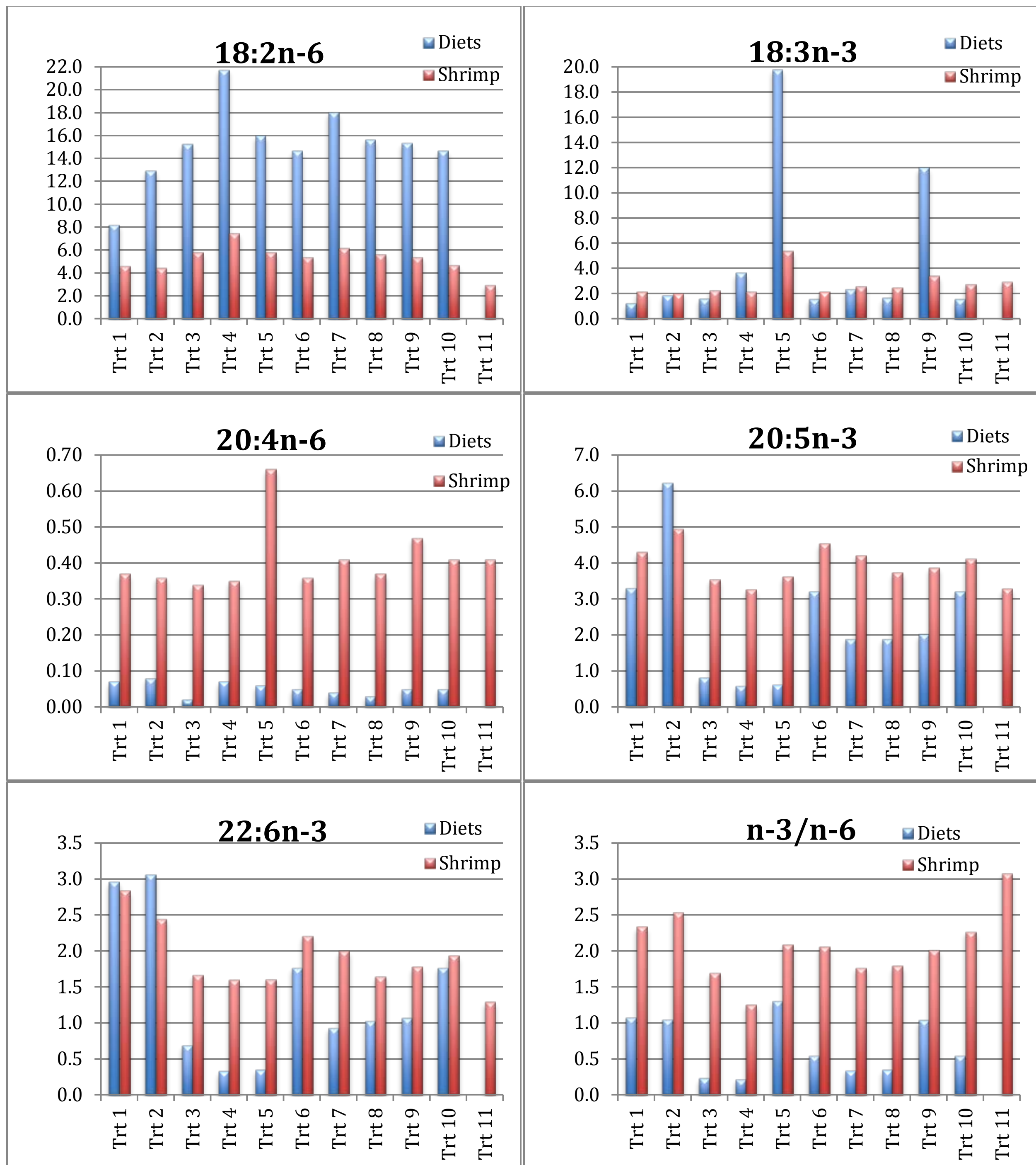

Figure 2. Linoleic (18:2-6), linolenic (18:3n-3), arachidonic (20:4n-6), EPA (20:5n-3), DHA (22:6n-3), and n-3/n-6 ratio in muscle of *L. vannamei* during the 6-weeks nutrition study in the outdoor tanks. Values for diets represent means of duplicate per sample, and values for trials represent means of duplicate analysis for four replicate observations.

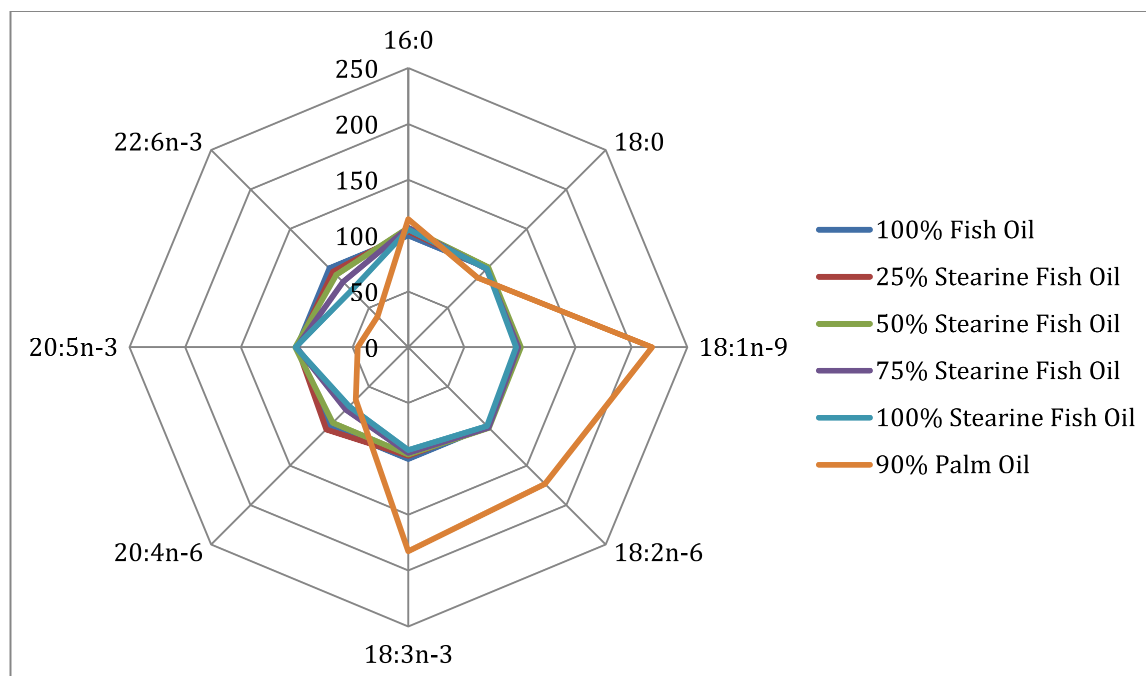

Figure 3. Percentage of the fatty acids, palmitic (16:0), stearic (18:0), oleic (18:1n-9), linoleic (18:2n-6), linolenic (18:3n-3), arachidonic (20:4n-6), EPA (20:5n-3), and DHA (22:6n-3) in muscle of *L. vannamei* during the clear water tank trial. Test diets fatty acids composition in percentage were calculated considering the menhaden fish oil diet, reference diet, profile as having 100% of the FAME.

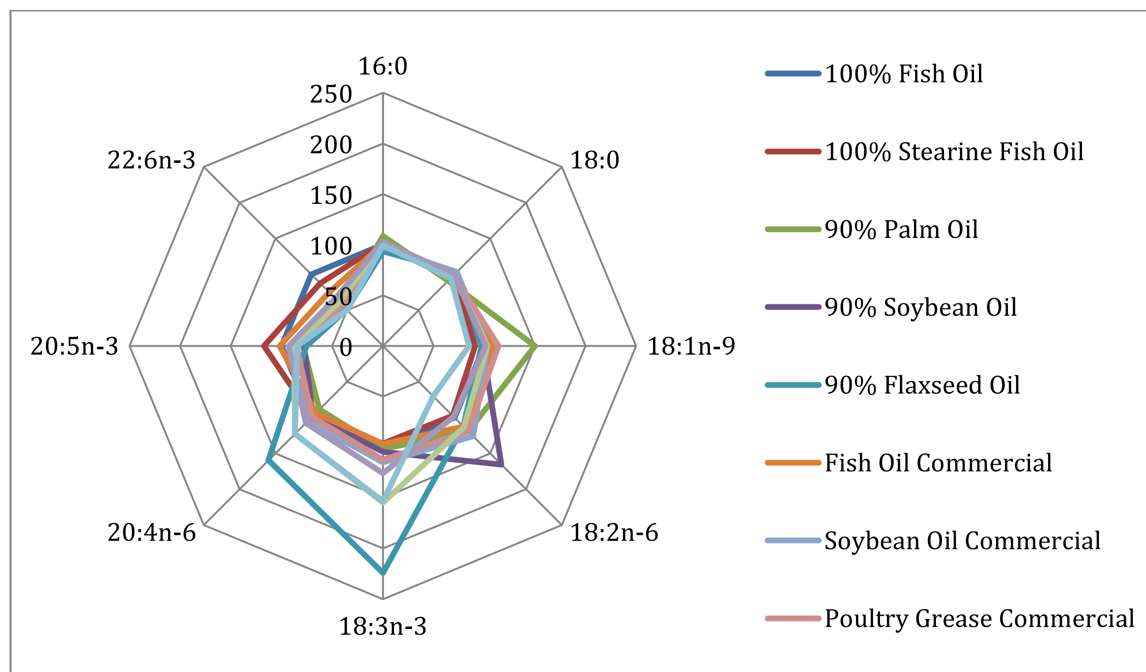

Figure 4. Percentage of the fatty acids, palmitic (16:0), stearic (18:0), oleic (18:1n-9), linoleic (18:2n-6), linolenic (18:3n-3), arachidonic (20:4n-6), EPA (20:5n-3), and DHA (22:6n-3) in muscle of *L. vannamei* during the low salinity outdoor tank trial. Test diets fatty acids composition in percentage were calculated considering the menhaden fish oil diet, reference diet, profile as having 100% of the FAME.

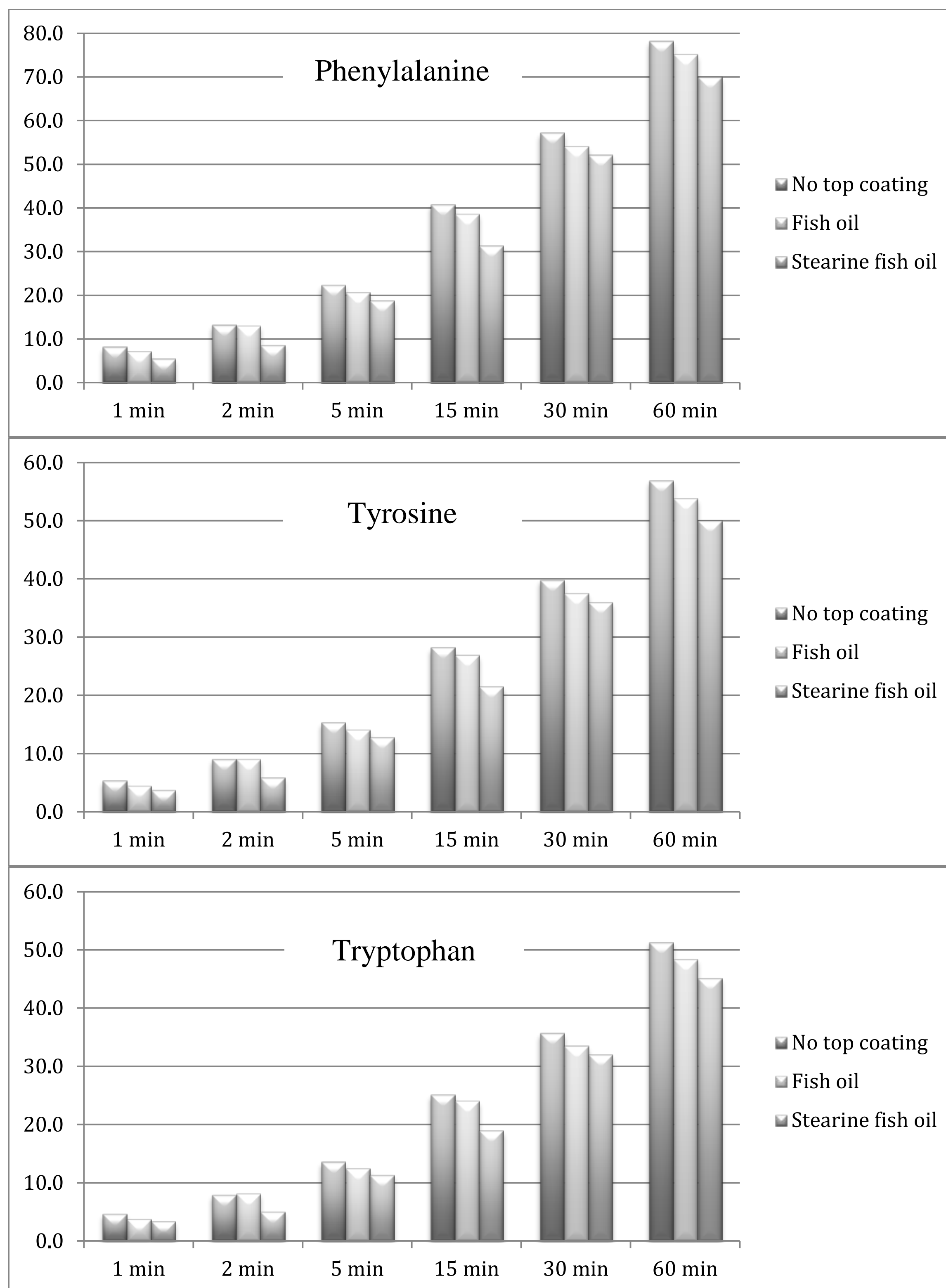

Figure 5. Leaching of the aromatic amino acid Phenylalanine, Tyrosine, and Tryptophan, in percentage, through time in commercial feed samples top coated with various oils.

CHAPTER V

REPLACEMENT OF FISH OIL IN PLANT BASED DIETS FOR PACIFIC WHITE SHRIMP (*Litopenaeus vannamei*) AND FATTY ACIDS UPTAKE UNDER DIFFERENT SALINITIES

Abstract

Environmental, social and economic pressures for a more sustainable aquaculture are driving shrimp farmers inland, further away from seawater sources. Simultaneously, high marine feed ingredients prices provide a push towards the use of alternative ingredients which in turn stimulates a vast field for research. The euryhaline characteristic of *Litopenaeus vannamei* has shown its great potential for inland low salinity water culture, which requires osmoregulatory modifications in the animal, mainly in the osmoregulatory organs (i.e. gills). Given that fluidity of the membranes could influence osmoregulatory capacity, there is considerable interest in the consequences of shifting the lipid sources. Menhaden fish oil, palm oil, soybean oil, and flaxseed oil were used in this study to demonstrate the feasibility of the partial replacement (90%) of marine oil source. Although problems with water quality did occur randomly in some experimental systems, results were quite encouraging. The fatty acids profile of the shrimp gills mostly reflected the dietary fatty acids profile, and the effects of salinity and the interaction of the diet with the salinity did not give clear results. Given the limited amount of information on

the correlation of fatty acid deposition in gill tissues to osmoregulation in different salinities further studies should be carried out to look at more sensitive indicators for osmoregulation.

Introduction

Shrimp culture in inland areas has been conducted in countries in Southeastern Asia since the 1990s in low salinity waters (LSW) ranging from 2 to 5 ppt salinity (Boyd & Thunjai, 2003). In countries like China, Ecuador, Thailand, Vietnam, Brazil, Mexico, and the United States, shrimp production is moving inland (Roy & Davis, 2010) farther from the coastal areas and consequently the sea water sources due to social and environmental factors, with China having close to half of its production conducted in LSW (Valderrama & Anderson, 2011). Shrimp cultured in LSW, with an imbalanced ionic content can suffer from osmotic stress, leading to poor production results and in some cases high mortalities rates (Boyd, 2002). Such an issue was approached by Gong et al. (2004), who claimed the macro- and micro-nutrient composition of the feed for low salinity rearing of shrimp could minimize or even nullify the osmoregulatory stress. In addition to the aforementioned nutrients, lipids play a critical role in membrane fluidity and consequently could influence osmoregulatory capacity of the shrimp.

Lipids are important dietary components, required in specific amounts for different species. The three main functions of lipids in organisms are: in their relatively reduced state, used for energy storage, principally as the non-polar (or neutral) lipids triacylglycerol and steryl esters, in lipid droplets, also used as fatty acids and sterol components storage needed for membrane biogenesis; components of cellular membranes and forming organelles, as polar lipids (phospholipids), also responsible for budding, tabulation, fission and fusion, essential on cell division, reproduction and intracellular membrane trafficking; and, acting as messenger in signaling and molecular recognition processes (van Meer et al., 2008).

According to Morris et al. (1982) and Haines (1994), living cells are capable of modulating permeability by opening and closing pores in the membrane which in a long term, after the pores close, will depend on the fatty acid composition of the cell membrane (Morris et al., 1982; Hurtado et al., 2007), decreasing with the increasing number of carbons composing its fatty acids (Paula et al., 1996), and also by the number and position of the double bonds in the molecules (Haines, 1994). Thus, the fatty acids (FA) deposition modifications on the gill will also have an influence on ions flux through the cell membranes and osmoregulation of the organism.

The literature available on shifts in lipid class and fatty acid composition in response to environmental osmoregulatory stressors in Atlantic cod, *Gadus morhua* (Staurnes et al., 1994), eel (*Anguilla anguilla*), sea bass (*Dicentrarchus labrax*) (Zwingelstein et al., 1998); euryhaline crabs, such as *Eriocheir sinensis* and *Carcinus maenas* (Chapelle et al., 1982; Chapelle & Zwingelstein, 1984; Zwingelstein et al., 1998), *Brachionus plicatilis* (Frolov et al., 1991), *Chasmagnathus granulata* (Luvizotto-Santos et al., 2003); and shrimp *Farfantepenaeus paulensis* (Lemos et al., 2001) determined to some degree of lipid mobilization on and during osmotic adaptation. Unfortunately, the data are diverse and sometimes contradictory.

The purpose of this study was to evaluate the partial (90%) substitution of fish oil in shrimp diets by various lipid sources (palm oil, soybean oil, or flaxseed oil) with distinct fatty acids profiles and the influence on lipid profiles of the shrimp's osmoregulatory organs (gill).

Materials and methods

Source of shrimp and experimental system

The grow-out trial was conducted at the Alabama Fish Farming Center in Greensboro, Alabama, USA, during the months of June and July of 2010. Pacific white shrimp, *L. vannamei*, post-larvae were obtained from Shrimp Improvement Systems (Islamorada, FL) and nursed until reaching about 0.5 g. All treatments were randomly assigned, water quality parameters (temperature, salinity, dissolved oxygen, and pH) were measured twice daily using a YSI 550A dissolved oxygen meter (YSI Inc., Yellow Springs, OH, USA), and two feedings of one of the four practical diets per day at 800 h and 1600 h were offered. Water samples for total ammonia nitrogen (TAN) were collected twice a week and TAN levels were determined using a spectrophotometer (Spectronic 20 Genesys, Spectronic Instrument Inc. Rochester, NY, USA) following the Nesslerization method (APHA 1989).

Aquarium Trial

Juvenile shrimp were obtained from the nursery system and were selected by hand-sorting to a uniform size. Then juvenile shrimp (0.49±0.03 g, initial weight) were stocked at a density of 12 shrimp per aquarium into 32 aquaria (57 L volume), each group of 4 aquaria with treatments randomly assigned comprised a recirculating system with a solids collection sump and a biofilter (400 L total per system). Tap water was used to fill each system and was dechlorinated with 1 g of sodium thiosulfate. Each individual system was raised to either 2 ppt or 32 ppt salinity using reconstituted seawater (Crystal Sea Marinemix, Marinemix Enterprises International, Inc., Baltimore, MD, USA), and each aquarium equipped with an airstone and supplied with aeration via a ¼ hp regenerative blower.

Eight treatments at two different salinities (2 and 32 ppt) were fed one of four experimental diets randomly assigned with four replications (tanks) per treatment. Test diets

were applied twice daily at 0800 and 1600 h for a 35-day experimental period. Feed input was pre-calculated on a weekly basis using an expected feed conversion ratio of 1.75:1 and a doubling in size until individual shrimp weighed one gram. Thereafter, a growth rate of 1.0 g per week was assumed. The animals were fed an isonitrogenous and isolipidic (35% crude protein, 8% crude lipid) diet with 100% menhaden fish oil, or one of three diets with 90% replacement of menhaden fish oil by palm oil, soybean oil, or flaxseed oil (Table 1).

Shrimp were counted every week to readjust the daily feed input. At the conclusion of the 35-day growth trial, shrimp were counted and weighed individually. Mean final weight (g), weekly growth (g $week^{-1}$), survival (%), and estimated feed conversion ratio (FCR) were determined.

Experimental diets

Four experimental isonitrogenous and isolipidic diets (approximately 35% crude protein with 8% lipid) were formulated to contain soybean meal as the primary protein source. The reference diet had 100% of menhaden fish oil, the next 3 diets had a replacement of 90% of menhaden fish oil by palm oil, soybean oil, or flaxseed oil (Diets 1-4, Table 1). Diets were prepared by mixing the ingredients in a mixer (Hobart, Troy, Ohio) for 30 minutes. Subsequently, hot water was added to the mixture until appropriate consistency for pelleting was obtained. Diets were then passed through a meat grinder and a 3-mm mesh sieve. Pellets were air dried (<50˚C) to a moisture content of less than 10%. After drying, pellets were crumbled, packed in sealed plastic bags and stored in a freezer until use.

Lipid and fatty acid analysis

At the end of the growth trials, the shrimp were harvested, weighed, and counted. Harvested shrimp were frozen from each experimental tank and shipped to Auburn University for fatty acids analysis. Before lipid analysis, they were thawed and gills from the animals were pooled into a composite sample per tank, ground, and analyzed in duplicate for total lipid and polar/non-polar lipid composition. Experimental diets were analyzed for fatty acid composition as well. Lipids were extracted by the method of Folch et al. (1957) and quantified gravimetrically after drying under nitrogen. Total lipid content was expressed as percent of wet tissue.

Fatty acids were separated into polar and non-polar fractions using 500 mg silica cartridges for solid phase extraction (Whatman Inc., Clifton, NJ, USA) followed by transesterification with boron trifluoride, then fatty acid methyl esters (FAME) were analyzed with a Shimadzu GC-17A gas chromatograph (Shimadzu Scientific Instruments Inc., Portland, OR, USA) equipped with a capillary column (Omegawax 530, 30 m x 0.53 mm x 0.5 µm film thickness, Supelco 2-4019, Sigma-Aldrich, Oslo, Norway) using helium as the carrier gas and a flame-ionization detector as previously described (Quintero, Durland, Davis, & Dunham, 2009). Fatty acids were identified by comparison of retention times and expressed as percent of the total identified FAME.

Data analysis

Differences in final mean weights, weekly growth, survival, and estimated FCR were analyzed using one-way ANOVA to determine if significant (P <0.05) differences existed among treatment means. SNK multiple range test was used as the mean separation procedure. The fatty acids were analyzed by two-way ANOVA for diet, salinity and their interactions. Statistical

analyses were conducted using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Results of the daily water quality monitoring (Table 2) for temperature, salinity, dissolved oxygen (DO), and pH showed parameters within the suitable range for grow out of this species; however, spikes in total ammonia nitrogen (TAN) and nitrite (NO_2) in low salinity water, diets 3 and 4 (90% soybean oil and 90% flaxseed oil), resulted in low survival (Table 3). As soon as the high levels of nitrogenous wastes were detected, water exchange was performed (Table 2).

In the low salinity treatments, the mean final weights ranged from 2.4 to 3.2 g, weekly growth from 0.5 to 0.7 g, survival from 61.1 to 79.2 percent, and estimated FCR of 3.1 up to 4.3. In the high salinity treatments, the mean final weights ranged from 1.3 to 1.9 g, weekly growth from 0.2 to 0.4 g, survival from 87.5 to 95.8 percent, and estimated FCR from 7.1 up to 11.2. Harvest data with overall harvest means presented in Table 3 showed significant statistical differences in mean final weight, weekly growth, survival and FCR according to the one-way ANOVA ($P<0.05$) test among treatments and production conditions (low or high salinity).

Total protein and lipid content of experimental diets were relatively constant; consequently, they were considered isonitrogenous and isolipidic (Table 1). The diets reflected the fatty acid profile of the oils used in their formulation. Diets with more menhaden oil (diet 1) had a higher content of long chained n-3 HUFA (Table 4): EPA (20:5n-3) 3.31 mg g-1 diet, DPA (22:5n-3) 1.13 mg g-1 diet, and DHA (22:6n-3) 2.96 mg g-1 diet. Palm oil diet (diet 2) had higher concentrations of palmitic acid (16:0) 21.16 mg g^{-1} diet and oleic acid (18:1n-9) 20.33 mg

g^{-1} diet. Diet with the inclusion of soybean oil had a higher amount of linoleic acid (18:2n-6) at 21.77 mg g^{-1} diet. And, linolenic acid (18:3n-3) was higher in the diet with inclusion of flaxseed oil (diet 4) at 19.81 mg g-1 diet.

Total lipid content of shrimp gill, from wet tissue, was not significantly ($P \geq 0.05$) affected by dietary treatment after the 35-day feeding trial. Fatty acid composition of shrimp gill tissue reflected the fatty acid profile of the experimental diets (Tables 4 and 5). Wet tissue FAME analysis showed that n-3 HUFA, DHA, DPA and EPA were always significantly higher in gills of shrimp fed diets with more menhaden oil, Treatment 1: 19.22% EPA, 0.88% DPA and 9.50% DHA and Treatment 5: 18.45% EPA, 0.93% DPA and 9.11% DHA, in the polar fraction; Treatment 1: 16.76% EPA, 0.96% DPA and 9.19% DHA and Treatment 5: 11.84% EPA, 0.90% DPA and 6.40 percent DHA, in the non-polar fraction (Table 5, Figures 3, 4 and 5). Oleic acid (18:1n-9) was significantly higher in shrimp fed diets with palm oil, Treatment 2 and 6, 15.11 and 19.46 percent for the polar fraction, respectively, and 19.25 and 21.32% for the non-polar fraction, respectively. The soybean oil diet (Treatments 3 and 7) displayed significantly greater concentrations of linoleic acid (18:2n-6) in the polar fraction, 24.79 and 23.18% for Treatments 3 and 7, respectively, and non-polar fraction, 29.00 and 23.76% for each treatment respectively; unexpectedly, the polar fraction of Treatment 6 (soybean oil) had a significantly high concentration of the fatty acid, 22.69%. The flaxseed oil diet provided to animals in Treatments 4 and 8 had high levels of α-linolenic acid, 10.86 and 10.97%, respectively, in the polar fraction and 14.73 and 12.00%, respectively, in the non-polar fraction; and, although the feed did not present higher levels of arachidonic acid (20:4n-6), 0.06 mg g^{-1} diet, than the other diets, it had a significantly statistical higher percentage in the polar fraction 2.47 and 3.12% for Treatments 3

and 4, respectively, and 2.60 and 2.95% in the non-polar fraction for the respective treatments (Tables 4 and 5, Figures 2, 4 and 5).

Knowing that there are significant statistical differences among diets, as stated in the last paragraph, the salinity and the interaction between different diets and salinities were analyzed using two-way ANOVA (Table 6). The polar fraction of the FAME palmitic acid (P=0.0144), stearic acid (P=0.0091), arachidonic acid (P<.0001) and DPA (P=0.0315) showed significant statistical differences under the two salinities, while the interaction effect between diet and salinity was only shown on linoleic acid (P=0.0343), arachidonic acid (P<.0001), and EPA (P=0.0269). The non-polar fraction of the FAME displayed a very different result for the two salinities with only arachidonic acid not being influenced (P=0.1451); palmitic acid (P<.0001), stearic acid (P<.0001), oleic acid (P=0.0014), linoleic acid (P=0.0161), α-linolenic acid (P=0.0043), EPA (P=0.0015), DPA (P=0.0028) and DHA (P=0.0006) showed significant statistical differences. The interaction effect on the non-polar fraction was shown in the palmitic acid (P=0.0050), oleic acid (P=0.0233), α-linolenic acid (P=0.0066), arachidonic acid (P=0.0024), EPA (P<.0001), and DHA (P=0.0001).

Discussion

Production of the Pacific white shrimp, *Litopenaeus vannamei*, in low salinity inland waters has advantages and disadvantages. The advantages being the cost of land, preservation of mangroves and coastal areas, and the proximity to the consumer market, while some disadvantages are the osmoregulatory costs for the animals which may affect their growth performance and the necessity of addition of key minerals, to some well water sources that do not have proper mineral balance.

L. vannamei, a euryhaline aquatic species, has to actively osmoregulate to survive in a wide range of salinities. Studies on the brown shrimp *Crangon crangon* showed that a considerable portion of energy is required to maintain its hemolymph composition (Hagerman & Uglow, 1982). Organisms have to undergo metabolic reorganizations and alterations to meet the energy demand associated with the shifts in the environmental salinities, utilizing primarily proteins and lipids as energy sources (Tseng & Hwang, 2008). Mayzaud & Conover (1988) demonstrated in zooplankton the allocation of energy from protein and/or lipid related to environmental changes, showing that lipid is the preferred substrate when energy demands are high.

Studies conducted by Hansen et al. (1992, 1995), Crockett (1999), Nordgarden et al. (2002), Hansen & Grosell (2004), and Sangiao-Alvarellos et al. (2003, 2005) on the role of membrane lipids, non-esterified fatty acids and triacylglycerols in the plasma, transferases, dehydrogenases, and enzymes in the gill, liver, and muscle, in osmoregulation, show results somewhat contradictory and inconclusive.

In addition to physiological studies some authors such as Gong et al. (2004) manipulated the feed formulation for shrimp in an attempt to energetically and ionically compensate for the ionic losses and variances of the environmental surroundings, with some improvement in the growth and survival of the animals. Albeit the comparison of results was across two different farms which does not exclude the site interaction on the results. Research conducted by Roy et al. (2007) on mineral supplementation of shrimp diets reared in 4 ppt salinity waters utilizing purified forms of potassium, magnesium and sodium chloride in practical diets found no significant improvement in growth and survival, although on a follow-up trial with magnesium and sodium chloride protected by a coating agent and potassium in an amino acid complex

demonstrated that the supplementation of potassium as an amino complex does help improve growth of the species in low salinity waters.

Technical problems in some of the recirculating systems resulted in poor water quality (Table 2) and low survival of cultured organisms (Table3). Such issues were corrected as soon as noticed; the remaining animals were reared until the end of the experiment and sampled for tissue analysis.

Growth results from the experiments were lower than typically observed for this species under similar conditions. Shrimp reared in low salinity water outperformed shrimp reared in high salinity (Table 3), agreeing with results from experiments done by Bray et al. (1994) where animals reared in 5 and 15 ppt salinity presented better growth results than the ones reared in 25, 35 and 49 ppt salinity waters.

The primary purpose of this trial was to examine the influence of the diet, salinity, and the interaction of both components on the osmoregulatory organs of *L. vannamei*, and relate it to the possibility of shifts in fatty acids deposition by the organisms in such organs to control or mitigate the ionic flux to and from the environment. The fatty acids profile of the osmoregulatory organs from the shrimp (Table 5) primarily reflected the fatty acids profile from the diets, although the different salinities and the interaction between salinity and diet (Table 6) did not prove to have a direct effect on the deposition of fatty acids in the gill tissues under some circumstances; for example, the polar fraction of linoleic acid showed no significant statistical differences under the different salinities but showed a significant interaction among diet and salinity, and DPA showed significant statistical differences in both fractions under the diferent salinities treatments although no significant interaction took place indicating an effect. The

correlation in both fractions of fatty acids and the different salinities is easily visualized on Figures 1 through 5.

In Figure 1 is noticeable the reduction of palmitic acid (16:0) in both fractions, polar and non-polar, of fatty acids in animals reared in high salinity, indicating the consumption of this fatty acid to supply energy for osmoregulation. The exact opposite occurred with stearic acid (18:0), as apparently it was consumed as energy source by the low salinity reared animals. All of the other mono, poly and highly-unsaturated fatty acids analyzed Figures 1, 2 and 3, did not show any trends other than a reflection of the dietary lipid source, which can be also be visualized in Figures 4 and 5.

Research regarding osmoregulatory stress and shifts in lipid class and fatty acid composition in the gills of fish and crustaceans, unfortunately, focus on a few specific lipids and/or fatty acids, mostly accounting for those molecules as energy supply for ionic regulation. Osmoregulatory stress caused by cold water shock was examined in Atlantic cod by Staurnes *et al*. (1994). Increases in blood plasma cortisol and glucose concentrations, and gill Na-K-ATPase were observed, albeit no differences in gill lipid class or fatty acid composition were found.

The work conducted by Zwingelstein *et al*. (1998) on eel (*Anguilla anguilla*), sea bass (*Dicentrarchus labrax*), trout (*Salmo gairdnerii*), crab from the North sea (*Eriocheir sinensis*), and crab from the Mediterranean sea (*Carcinus maenas*), focused on the effects of the environmental salinity and temperature shifts on radiolabeled serine exchange for the phosphatidylserine synthesis, its decarboxylation to provide phosphatidylethanolamine, and its subsequent *N*-methylation to phosphatidylcholine, showing that these animals undergo changes in their gills, muscle, liver or hepatopancreas, to adapt to the new environment. Apparently, phosphatidylserine can activate protein kinases that play key roles in membrane regulation by

regulating the majority of cellular pathways, phosphorylation of proteins (production of ATP), and transmembrane signaling (Manning *et al.*, 2002).

Studies by Luvizotto-Santos *et al*. (2003) on wild caught estuarine crab *Chasmagnathus granulate* found that the hepatopancreas lipids in animals exposed to fresh water (0 ppt salinity) or high salinity waters (40 ppt) were not mobilized during osmotic stress regulation, although the gill and muscle total lipids were significantly lower in crabs subjected to hypo-osmotic stress than those subjected to the hyper-osmotic stress or maintained at the control salinity (20 ppt). The salinity differences considered were related to the annual seasonal cycles, *i.e.* winter and summer, which also affects the natural food availability and subsequently the dietary lipid class composition, which was not assessed or even considered in the study.

The early post-larval (PL) stages of the shrimp *Farfantepenaeus paulensis* develops in estuarine areas until the adoption of benthic habits, rendering its tolerance to low salinity waters. Lemos *et al*. (2001) studied the development of these animals from PL-VI to PL-XV in different salinities (5, 15, 25, and 34 ppt) reporting the best salinities for the aquaculture of the early stages of this species between 15 and 25 ppt, due to reduction in growth at 34 ppt.

The production of shrimp in low as well as high salinity waters is viable and widespread in the world. It can be carried out under different salinities with the use of alternative lipids with good production results. Due to the scarce amount of information and the complexity of the interaction of gill membrane lipids and osmoregulation, related to the ionic balance of the surrounding environment, more research on its interactions should be done. The fatty acids profile of the shrimp gills mostly reflected the dietary fatty acids profile, and the effects of salinity and the interaction of the diet with the salinity did not give clear results. Given the limited amount of information on the correlation of fatty acid deposition in gill tissues to

osmoregulation in different salinities, further studies should be carried out to look at more sensitive indicators for osmoregulation.

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Table 1. Ingredients composition (g $100g^{-1}$ of feed) of experimental diets.

	Diet 1 FO	Diet 2 PO	Diet 3 SO	Diet 4 FXO
Soybean meal solvent extracted[a]	54.70	54.70	54.40	54.40
Whole wheat[b]	26.50	26.50	28.38	28.38
Corn gluten meal[c]	6.00	6.00	6.00	6.00
Menhaden fish oil[d]	6.15	0.62	0.46	0.46
Palm oil[e]	-	5.54	-	-
Soybean oil[f]	-	-	4.11	-
Flaxseed oil[g]	-	-	-	4.11
Trace mineral premix[h]	0.50	0.50	0.50	0.50
Vitamin premix[i]	1.80	1.80	1.80	1.80
Stay C 250 mg kg^{-1} (25%)[j]	0.10	0.10	0.10	0.10
CaP-dibasic[k]	3.00	3.00	3.00	3.00
Choline chloride[b]	0.20	0.20	0.20	0.20
Lecithin (deoilded 53% lipid)[l]	1.00	1.00	1.00	1.00
Cholesterol – USB[m]	0.05	0.05	0.05	0.05
Crude Protein (%)	34.93	34.93	35.20	35.20
Crude Fat (%)	8.01	8.01	7.99	7.99

[a] Faithway Feed Co., Guntersville, AL, USA.
[b] Gold Medal, General Mills Inc., Minneapolis, Minnesota, USA.
[c] Grain Processing Corporation, Muscatine, IA, USA.
[d] Omega Protein, Inc. Reedville, VA, USA.
[e] Natural Red Palm Oil, Swanson Health Products. Fargo, ND, USA.
[f] American Soybean Association, St. Louis, MO, USA.
[g] Archer-Daniels-Midland Co. – ADM, Decatur, Illinois, USA.
[h] Trace mineral premix (g $100g^{-1}$): cobalt chloride 0.004, cupric sulphate pentahydrate 0.250, ferrous sulfate 4.0, magnesium sulfate heptahydrate 28.398, manganous sulphate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, filler 53.428.
[i] 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.
[j] Stay C®, (L-ascorbyl-2-polyphosphate), Roche Vitamins Inc., Parsippany, NJ, USA.
[k] Fisher Scientific, Fair Lawn, NJ, USA.
[l] Solae Company, St. Louis, MO, USA.
[m] USB Biochemicals, Cleveland, OH, USA.

Table 2. Summary of daily water quality parameters for the clear water aquarium trial during the 35-day nutrition study.

	Temperature (˚C)	Salinity (ppt)	DO (mg L^{-1})	pH	TAN[a] (mg L^{-1})	NO_2[a] (mg L^{-1})
100% Fish oil (Diet 1)	27.8 ± 0.9	2.2 ± 0.1	6.4 ± 0.7	7.7 ± 0.2	1.3 ± 0.9	0.7 ± 0.4
90% Palm oil (Diet 2)	27.8 ± 0.7	2.3 ± 0.1	6.7 ± 0.4	7.7 ± 0.2	1.7 ± 0.4	2.3 ± 1.1[b]
90% Soybean oil (Diet 3)	27.6 ± 0.8	2.4 ± 0.1	6.7 ± 0.4	7.6 ± 0.3	1.2 ± 0.4	2.9 ± 1.7[b]
90% Flaxseed oil (Diet 4)	27.5 ± 0.8	2.3 ± 0.1	6.8 ± 0.5	7.8 ± 0.2	0.9 ± 0.7	2.6 ± 2.4[b]
100% Fish oil (Diet 1)	27.6 ± 0.9	31.0 ± 1.1	5.6 ± 0.4	7.9 ± 0.1	1.4 ± 0.9	0.1 ± 0.1
90% Palm oil (Diet 2)	27.4 ± 0.9	31.1 ± 1.0	5.8 ± 0.3	8.0 ± 0.2	1.8 ± 1.8	0.1 ± 0.1 [b]
90% Soybean oil (Diet 3)	27.5 ± 0.8	31.3 ± 1.0	5.4 ± 0.5	8.0 ± 1.0	1.9 ± 0.9	0.2 ± 0.2
90% Flaxseed oil (Diet 4)	27.7 ± 0.9	31.9 ± 1.0	5.6 ± 0.4	8.0 ± 0.2	0.9 ± 0.9	1.4 ± 1.9[b]

[a] Total ammonia nitrogen (TAN) and nitrite (NO_2) were measured twice a week.

[b] Aquariums where water exchanges were performed when high levels of TAN and/or NO_2 were detected.

Table 3. Results after 35-day of aquarium trial (clear water system), stocked 15 shrimp tank^{-1}, at 31 shrimp m^{-2} (44 shrimp m^{-3}) with 0.29 g (wet weight) individuals, in two different salinities (2 or 32 ppt) (n=4).

fish oil:alternative oil	Mean Final Weight (g)	Weekly Growth (g)[1]	Survival (%)	FCR
100% Fish oil (Diet 1) 2 ppt	3.2 [a]	0.7 [a]	75.0 [bc]	3.1 [a]
90% Palm oil (Diet 2) 2 ppt	2.5 [bc]	0.5 [bc]	79.2 [ab]	4.3 [a]
90% Soybean oil (Diet 3) 2 ppt	2.4 [bc]	0.5 [bc]	22.9 *	4.2 [a]
90% Flaxseed oil (Diet 4) 2 ppt	3.1 [ab]	0.7 [ab]	61.1 [c]	3.3 [a]
100% Fish oil (Diet 1) 32 ppt	1.6 [de]	0.3 [d]	87.5 [ab]	8.0 [b]
90% Palm oil (Diet 2) 32 ppt	1.3 [e]	0.2 [d]	87.5 [ab]	11.2 [c]
90% Soybean oil (Diet 3) 32 ppt	1.4 [de]	0.2 [d]	93.8 [a]	9.7 [bc]
90% Flaxseed oil (Diet 4) 32 ppt	1.9 [cd]	0.4 [cd]	95.8 [a]	7.1 [b]
P-value	<.0001	<.0001	0.0077	<.0001
PSE	0.0721	0.0182	2.1342	0.3189

* Due to high mortality treatment was excluded from statistical analysis for survival.
[1]Weekly growth calculated after shrimp reached an average weight of 1 g.
Means within columns with the same letter are not significantly different (SNK alpha = 0.05).

Table 4. Fatty acid composition (mg g^{-1} diet and % of FAME) of experimental diets[1] (n=4).

Selected FA	Diet 1 FO	Diet 2 PO	Diet 3 SO	Diet 4 FXO
Total lipid (%)	7.16	6.77	7.39	7.11
mg g^{-1} diet				
16:0	10.04	21.16	7.63	6.17
18:0	2.00	3.12	2.32	1.80
18:1n-9	4.54	20.33	10.52	8.86
18:2n-6	8.25	15.25	21.77	16.00
18:3n-3	1.23	1.58	3.65	19.81
20:4n-6	0.07	0.02	0.07	0.06
20:5n-3	3.31	0.81	0.59	0.62
22:5n-3	1.13	0.26	0.41	0.49
22:6n-3	2.96	0.69	0.33	0.35
Saturates[2]	17.27	30.78	10.36	8.42
Monounsaturates[3]	10.18	21.34	11.97	10.10
PUFA[4]	10.82	17.17	25.61	36.02
HUFA[5]	7.77	1.94	1.27	1.34
Total n-3[6]	9.03	3.53	4.85	21.12
Total n-6[7]	8.38	15.29	21.89	16.09
n-3/n-6 ratio	1.08	0.23	0.22	1.31
% of FAME				
16:0	21.82	29.71	15.51	11.04
18:0	4.35	4.38	4.71	3.23
18:1n-9	9.87	28.53	21.38	15.85
18:2n-6	17.92	21.41	44.24	28.65
18:3n-3	2.67	2.21	7.43	35.44
20:4n-6	0.16	0.02	0.14	0.10
20:5n-3	7.19	1.14	1.20	1.11
22:5n-3	1.27	0.30	0.61	0.44
22:6n-3	6.42	0.96	0.67	0.62
Saturates[2]	37.53	43.22	21.05	15.07
Monounsaturates[3]	22.12	29.96	24.32	18.08
PUFA[4]	23.48	24.10	52.04	64.47
HUFA[5]	16.86	2.72	2.58	2.38
Total n-3[6]	19.59	4.96	9.85	37.79
Total n-6[7]	18.20	21.46	44.48	28.82

Table 5. Total lipid (%), polar, and non-polar fractions of the fatty acid composition (% of FAME) of shrimp gill tissue from aquarium trial[8] (n=4).

	Low salinity (2 ppt)				High salinity (32 ppt)			
Selected FA **% of FAME**	Trt 1 FO Polar	Trt 2 PO Polar	Trt 3 SO Polar	Trt 4 FXO Polar	Trt 5 FO Polar	Trt 6 PO Polar	Trt 7 SO Polar	Trt 8 FXO Polar
Total lipid (%)	1.64 ^{a}	1.23 ^{a}	1.33 ^{a}	1.51 ^{a}	1.61 ^{a}	1.29 ^{a}	1.46 ^{a}	1.43 ^{a}
16:0	15.74 ^{a}	14.67 ^{a}	13.27 ^{b}	11.63 ^{c}	15.32 ^{a}	15.15 ^{a}	11.20 ^{c}	11.43 ^{c}
18:0	13.95 ^{b}	11.18 ^{c}	15.76 ^{ab}	14.24 ^{b}	15.20 ^{ab}	12.59 ^{c}	16.66 ^{a}	14.96 ^{ab}
18:1n-9	7.51 ^{b}	15.11 ^{a}	9.50 ^{b}	8.20 ^{b}	8.38 ^{b}	19.46 ^{a}	8.48 ^{b}	9.54 ^{b}
18:2n-6	11.60 ^{d}	16.55 ^{b}	24.79 ^{a}	15.11 ^{c}	12.92 ^{d}	22.69 ^{a}	23.18 ^{a}	16.61 ^{c}
18:3n-3	0.79 ^{b}	5.27 ^{b}	1.77 ^{b}	10.86 ^{a}	0.92 ^{b}	1.29 ^{b}	1.73 ^{b}	10.97 ^{a}
20:4n-6	0.32 ^{c}	0.30 ^{d}	0.37 ^{c}	2.47 ^{b}	0.30 ^{c}	0.16 ^{d}	0.36 ^{c}	3.12 ^{a}
20:5n-3	19.22 ^{a}	10.14 ^{d}	11.96 ^{c}	12.95 ^{bc}	18.45 ^{a}	10.00 ^{d}	13.60 ^{b}	12.55 ^{bc}
22:5n-3	0.88 ^{a}	0.60 ^{c}	0.59 ^{c}	0.67 ^{bc}	0.93 ^{a}	0.79 ^{ab}	0.65 ^{bc}	0.65 ^{bc}
22:6n-3	9.50 ^{a}	3.91 ^{bc}	5.24 ^{bc}	6.21 ^{b}	9.11 ^{a}	4.78 ^{c}	5.92 ^{b}	6.02 ^{b}
Saturates[2]	31.09 ^{ab}	30.31 ^{bcd}	29.98 ^{abc}	27.03 ^{d}	31.81 ^{a}	28.45 ^{bcd}	28.80 ^{bcd}	27.23 ^{cd}
Monounsaturates[3]	14.98 ^{b}	18.66 ^{a}	13.07 ^{c}	11.65 ^{c}	14.51 ^{b}	22.93 ^{a}	11.91 ^{c}	12.63 ^{c}
PUFA[4]	23.76 ^{c}	35.35 ^{b}	38.46 ^{a}	38.69 ^{a}	24.63 ^{c}	32.54 ^{b}	38.27 ^{a}	37.39 ^{a}
HUFA[5]	30.17 ^{a}	15.69 ^{d}	18.50 ^{c}	22.63 ^{b}	29.05 ^{a}	16.08 ^{d}	21.02 ^{b}	22.75 ^{b}
Total n-3[6]	34.12 ^{a}	23.89 ^{d}	23.60 ^{c}	35.36 ^{a}	33.15 ^{a}	19.21 ^{d}	25.96 ^{b}	32.86 ^{a}
Total n-6[7]	12.07 ^{d}	19.94 ^{b}	25.23 ^{a}	17.77 ^{c}	13.35 ^{d}	22.90 ^{a}	23.62 ^{a}	19.84 ^{b}
% of FAME	Trt 1 FO Non-Polar	Trt 2 PO Non-Polar	Trt 3 SO Non-Polar	Trt 4 FXO Non-Polar	Trt 5 FO Non-Polar	Trt 6 PO Non-Polar	Trt 7 SO Non-Polar	Trt 8 FXO Non-Polar
16:0	15.82 ^{c}	17.20 ^{c}	17.19 ^{c}	13.14 ^{d}	20.34 ^{b}	21.29 ^{a}	13.55 ^{c}	14.92 ^{cd}
18:0	13.81 ^{cd}	12.39 ^{d}	18.68 ^{ab}	15.06 ^{bcd}	18.64 ^{ab}	14.53 ^{bc}	15.56 ^{a}	16.47 ^{bc}
18:1n-9	9.12 ^{d}	19.25 ^{b}	13.06 ^{c}	11.76 ^{c}	10.15 ^{d}	21.32 ^{a}	9.43 ^{c}	13.59 ^{c}
18:2n-6	14.68 ^{d}	22.14 ^{c}	29.00 ^{a}	19.48 ^{c}	13.76 ^{d}	20.22 ^{c}	23.76 ^{b}	18.91 ^{c}
18:3n-3	0.97 ^{c}	2.62 ^{c}	2.02 ^{c}	14.73 ^{a}	1.22 ^{c}	1.57 ^{c}	1.67 ^{c}	12.00 ^{b}
20:4n-6	0.40 ^{c}	0.18 ^{c}	0.36 ^{c}	2.60 ^{b}	0.35 ^{c}	0.18 ^{c}	0.31 ^{c}	2.95 ^{a}
20:5n-3	16.76 ^{a}	6.59 ^{c}	6.27 ^{c}	7.83 ^{c}	11.84 ^{b}	5.09 ^{c}	5.35 ^{c}	7.72 ^{c}
22:5n-3	0.96 ^{a}	0.57 ^{b}	0.41 ^{c}	0.47 ^{bc}	0.90 ^{a}	0.46 ^{bc}	0.42 ^{c}	0.38 ^{c}
22:6n-3	9.19 ^{a}	3.17 ^{cd}	2.83 ^{cd}	3.92 ^{c}	6.40 ^{b}	2.35 ^{d}	3.79 ^{cd}	3.50 ^{cd}
Saturates[2]	32.05 ^{bc}	32.71 ^{bc}	37.96 ^{ab}	29.55 ^{c}	42.07 ^{a}	38.43 ^{a}	31.14 ^{a}	32.85 ^{bc}
Monounsaturates[3]	17.65 ^{c}	23.29 ^{b}	16.15 ^{cd}	14.92 ^{d}	16.52 ^{cd}	24.82 ^{a}	16.13 ^{d}	16.51 ^{cd}
PUFA[4]	22.64 ^{de}	29.23 ^{c}	35.85 ^{b}	40.08 ^{a}	20.01 ^{e}	25.90 ^{d}	40.29 ^{bc}	34.38 ^{b}
HUFA[5]	27.67 ^{a}	14.77 ^{c}	10.04 ^{d}	15.46 ^{c}	21.39 ^{b}	10.85 ^{d}	12.45 ^{d}	16.27 ^{c}
Total n-3[6]	33.10 ^{a}	19.70 ^{c}	15.02 ^{d}	31.54 ^{a}	24.42 ^{b}	13.13 ^{e}	26.98 ^{d}	26.69 ^{b}
Total n-6[7]	15.24 ^{d}	22.58 ^{c}	29.39 ^{a}	22.30 ^{c}	14.25 ^{d}	20.51 ^{c}	24.06 ^{b}	21.93 ^{c}

* Footnotes for tables 4 and 5.
[1] Values represent averages of single samples.
[2] Saturates: 14:0, 15:0, 16:0, 18:0, 20:0, 22:0.
[3] Monounsaturates: 15:1, 16:1 18:1, 20:1.
[4] PUFA: 16:2, 16:3, 18:2, 18:3, 20:2, 20:3, 22:2.
[5] HUFA: 18:4, 20:4, 20:5, 22:4, 22:5, 22:6.
[6] Total n-3: 18:3n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3.
[7] Total n-6: 18:2n-6, 20:3n-6, 20:4n-6.
[8] Values represent averages of single samples per tank, and four tanks per dietary treatment. Means within rows with the same letter are not significantly different (SNK alpha = 0.05).

Table 6. Two-way ANOVA results for main fatty acids from gill samples analyzed ($P \leq 0.05$), fed four different diets (FO, PO, SO, or FXO) under two different salinities (2 or 32 ppt). Differences due to main effects and their interactions are indicated (GLM Procedure, Type III Sum of Squares). Values with no statistical significant differences are shown in bold font.

FAME		**Diet**		**Salinity**		**Interaction**	
		Polar	*Non-Polar*	*Polar*	*Non-Polar*	*Polar*	*Non-Polar*
Palmitic Acid	16:0	<.0001	<.0001	0.0144	<.0001	**0.2642**	0.0050
Stearic Acid	18:0	<.0001	<.0001	0.0091	<.0001	**0.8949**	**0.1586**
Oleic Acid	18:1n-9	<.0001	<.0001	**0.6213**	0.0014	**0.0960**	0.0233
Linoleic Acid	18:2n-6	<.0001	<.0001	**0.0589**	0.0161	0.0343	**0.5272**
α-Linolenic Acid	18:3n-3	<.0001	<.0001	**0.7466**	0.0043	**0.9785**	0.0066
Arachidonic Acid	20:4n-6	<.0001	<.0001	<.0001	**0.1451**	<.0001	0.0024
Eicosapentaenoic Acid EPA	20:5n-3	<.0001	<.0001	**0.8963**	0.0015	0.0269	<.0001
Docosapentaenoic Acid DPA	22:5n-3	<.0001	<.0001	0.0315	0.0028	**0.1384**	**0.1097**
Docosahexaenoic Acid DHA	22:6n-3	<.0001	<.0001	**0.7661**	0.0006	**0.1991**	0.0001

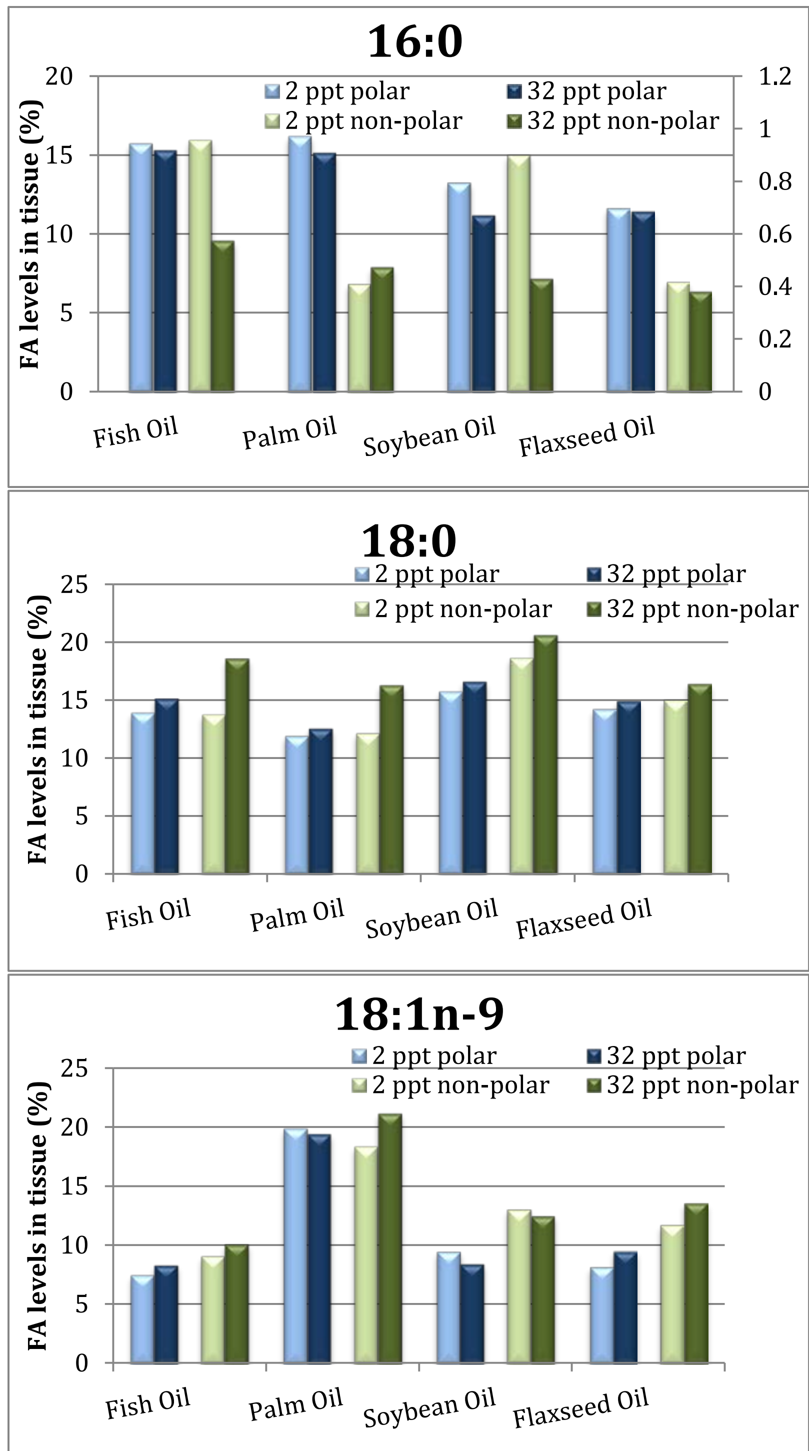

Figure 5. Polar and non-polar fractions of saturated (16:0, 18:0) and monounsaturated (18:1n-9) fatty acids in gill tissue of shrimp raised on diets with various lipid sources (Menhaden fish oil, palm oil, soybean oil and flaxseed oil) under low (2 ppt) and high (32 ppt) salinity. In the16:0 graph, the values for the non-polar fatty acid levels in tissues, as percentage, are in the secondary axis.

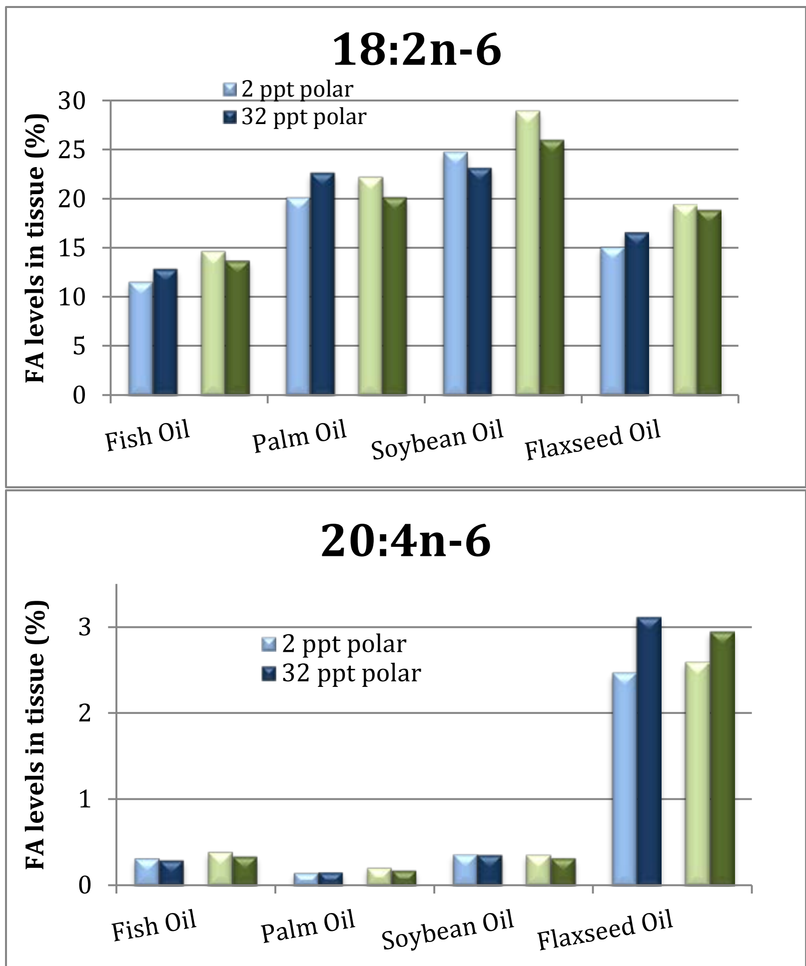

Figure 6. Polar and non-polar fractions of polyunsaturated (18:2n-6) and highly unsaturated (20:4n-6) omega-6 fatty acids in gill tissue of shrimp raised on diets with various lipid sources (Menhaden fish oil, palm oil, soybean oil and flaxseed oil) under low (2 ppt) and high (32 ppt) salinity.

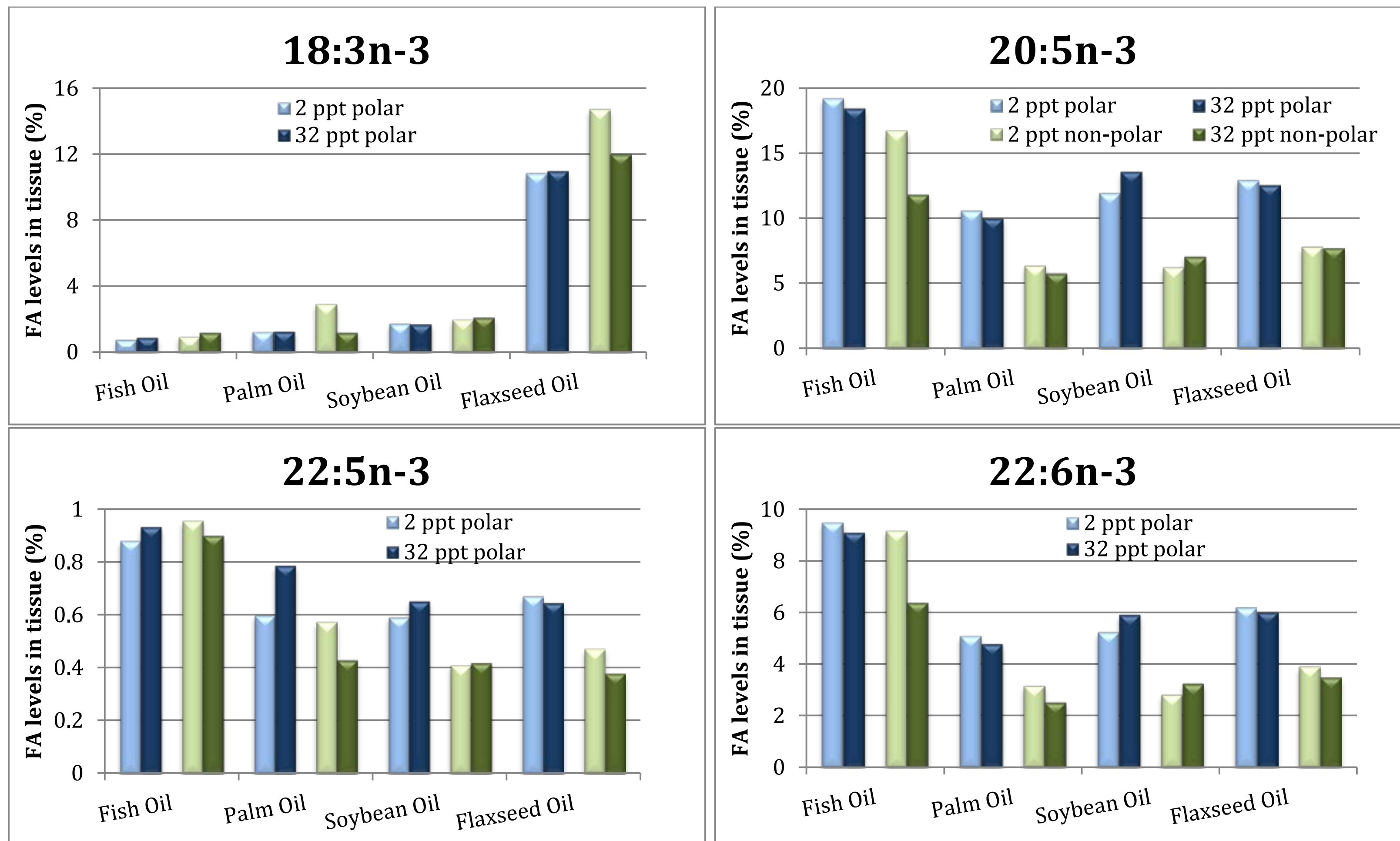

Figure 7. Polar and non-polar fractions of polyunsaturated (18:3n-3) and highly unsaturated (20:5n-3, 22:5n-3, 22:6n-3) omega-3 fatty acids in gill tissue of shrimp raised on diets with various lipid sources (Menhaden fish oil, palm oil, soybean oil and flaxseed oil) under low (2 ppt) and high (32 ppt) salinity.

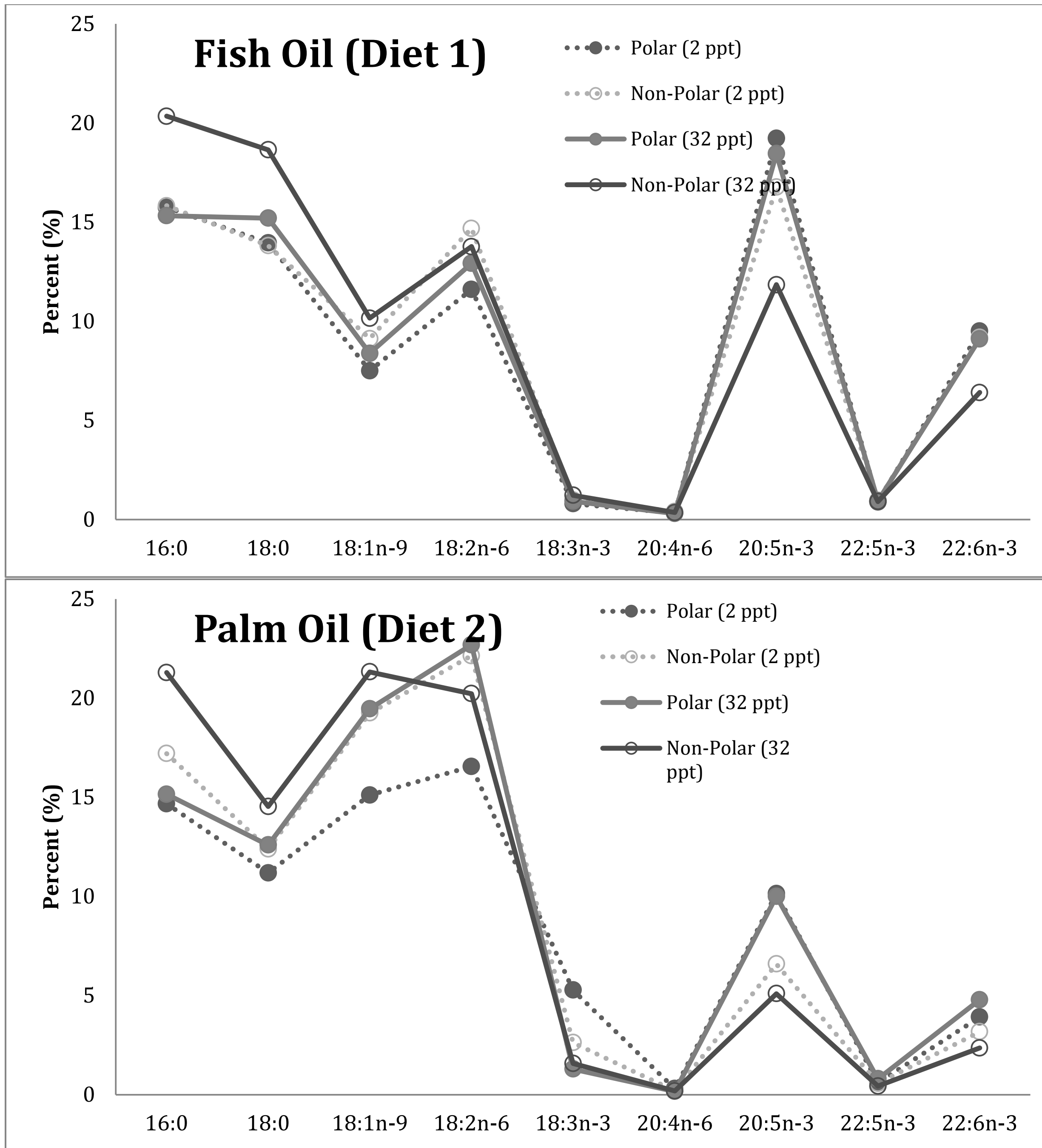

Figure 8. Major fatty acids analyzed: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n-9) linoleic acid (18:2n-6), **α-**linolenic acid (18:3n-3), arachidonic acid (20:4n-6), EPA (20:5n-3), DPA (22:5n-3) and DHA (22:6n-3), in percentage, from *Litopenaeus vannamei* gills reared in clear water under different salinities (2 or 32 ppt) and fed different lipid sources Fish Oil (Diet 1) or Palm Oil (Diet 2).

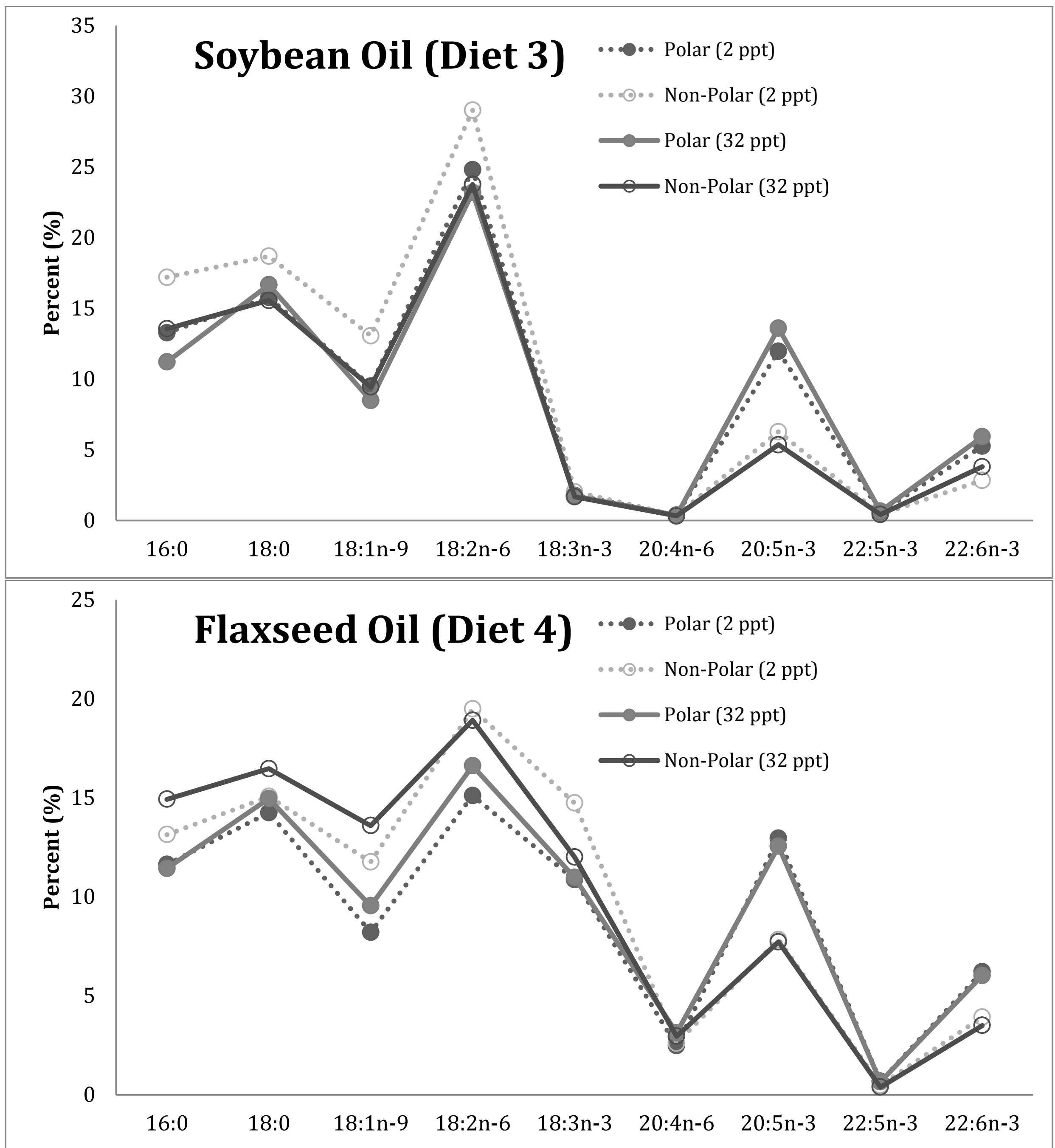

Figure 9. Major fatty acids analyzed: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n-9) linoleic acid (18:2n-6), α-linolenic acid (18:3n-3), arachidonic acid (20:4n-6), EPA (20:5n-3), DPA (22:5n-3) and DHA (22:6n-3), in percentage, from *Litopenaeus vannamei* gills reared in clear water under different salinities (2 or 32 ppt) and fed different lipid sources Soybean Oil (Diet 3) or Flaxseed Oil (Diet 4).

Summary & Conclusions

Aquaculture is evolving at a fast pace to keep up with the ever expanding consumer market demand. Concurrently, the protein and lipids sources to feed the produced organisms, traditionally from fish meal and fish oil, is becoming scarce, soaring the prices of formulated feed to a point where it becomes unsuitable to the aquaculture industry. In order to keep the sustainability of the aquaculture activity, alternative ingredients for feed has to be extensively researched for each target species. The studies designed and carried out through this dissertation were aimed at furthering the knowledge on alternative lipid sources in the Pacific white shrimp, *Litopenaeus vannamei*, feed, and the uptake and incorporation of the fatty acids from these lipid sources into the animal body. These studies have improved our understanding of the biology and physiology of *L. vannamei* as it relates to fatty acids uptake and the provision of essential fatty acids through the diets.

The replacement of marine fish oil by vegetable oils (soybean oil, low linolenic acid soybean oil, or safflower oil, which is also named flaxseed oil), regarding as well the omega-3 and omega-6 fatty acids ratios were examined in the second chapter of this dissertation. *Litopenaeus vannamei* has limited ability to elongate and desaturate fatty acid with molecules larger than 18 atoms of carbon, giving the highly unsaturated fatty acids an essential fatty acids status for this species, being required to be supplied at a minimal level through the diet. Furthermore, some authors have also suggested that the dietary n-3/n-6 fatty acid ratio could influence the uptake of fatty acids by the animals compromising its rearing if not adjusted. Based

on our study, the vegetable oils tested can be used to replace up to 90 percent of marine fish oil with no adverse effect on the shrimp production results in rearing systems where the animals have access to natural foods, and the n-3/n-6 ratio in the diets seemed not exert any influence on the growout results as well.

From the knowledge acquired from the previous study that the essential fatty acids requirements for this species can be met if a small percentage of marine fish oil is left in the diet, in chapter 3, commercially produced diets with soybean oil, poultry grease, or flaxseed oil as lipid sources and a percentage of marine fish oil were tried in a pond system and green water tank system to confirm the feasibility of its usage in a commercial-like scale. The production results were not affected by dietary treatment and, as expected, the fatty acids profile of the animals reflected the dietary fatty acids profile. The contribution as food source from the natural productivity was also demonstrated with animals presenting limited growth (3.2 g, mean final weight) at the end of 12 weeks in the green water tank system. According to the literature, the dietary composition and the salinity of the water in which shrimp are raised influences on the flavor of the final product, thereof a sensorial analysis of animals fed fish oil or soybean oil was conducted with trained panelists, resulting in no differences in flavor, texture or appearance among the treatments, which is a positive acknowledgment for the aquaculture industry.

In the fourth chapter, lipid sources with peculiar fatty acids profiles, such as stearine fish oil and palm oil, both composed mainly of saturated fatty acids and monounsaturated fatty acids were examined in a brackish clear water system (14.1 ppt) for 8 weeks, with no significant statistical differences, in production results, from the menhaden fish oil (reference) diet. Stearine fish oil, a by-product from the filtration of marine fish oil, and palm oil, the most produced vegetable oil worldwide, are very stable and resistant to peroxidation, being the last one a rich

source of vitamin A and E, powerful natural antioxidants. In this same chapter (chapter 4), another trial comprised of the diets formulated to have 100% menhaden fish oil, 100% stearine fish oil, and 90% palm oil from the previous experiment; along with diets containing 90% soybean oil, and 90% flaxseed oil, utilized on chapter 2; and, the commercial diets with fish oil, soybean oil, poultry grease, and flaxseed oil, tested on chapter 3, also, with one treatment receiving a ½ ration of the commercial fish oil diet, and another fed only on natural productivity, were all examined over a 6 weeks period in a low salinity (2.0 ppt) green water system with similar promising production results from the aforementioned experiments. Due to the stability and peroxidation resistance of the stearine fish oil, an evaluation of its anti-leaching properties as feed pellet top coat was performed, comparing a regular commercial shrimp diet without top coating, fish oil top coating, or stearine fish oil top coating. This study indicated a reduction in aromatic amino acid leaching from the diets with stearine fish oil and would warrant the applicability of this waxy product in the animal feed industry.

The 5th chapter of this dissertation examines the shifts in polar and non-polar lipid composition of the shrimp gills due to osmoregulation at low and high salinity, 2 and 32 ppt respectively. Diets formulated to have 100% menhaden fish oil or 90% palm oil, both tested in chapter 4; and diets containing 90% soybean oil, or 90% flaxseed oil (chapter 2), were investigated under the different salinities. The fatty acids profile of the gills reflected the one from the diets, not being influenced by the salinity or the interaction between salinity and diet. Some technical issues related to water quality occurred randomly during the trial, although results are quite encouraging.

The studies conducted herein have improved the knowledge on the feasibility of alternative lipid sources in diets for the Pacific white shrimp, *Litopenaeus vannamei*. However,

there is still a need for further examination of non-marine fish lipid sources with higher levels of highly unsaturated fatty acids, and the applicability of such lipid sources for the commercial feed industry. The emancipation of the aquaculture feed industry from marine fish feed ingredients will represent a big step in the evolution of the sector as a whole, and a big boost for the growth of the industry worldwide.

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Appendix

Total lipid and fatty acids extraction:

Lipid extraction, saponification, trans-esterification, and inclusion of internal standard procedure:

1. Wash, dry and weight tubes;

2. Weight sample, around 2 g (for feed add water ~50% of the dry weight);

3. Add 34 mL of Chloroform/Methanol (2:1);

4. Homogenize;

5. Filter in glass fiber pre-filters into pre-weighted tubes (screw caps);

6. Bring up to 40 mL of chloroform/methanol (2:1);

7. Add 8 mL of 0.74% KCℓ solution;

8. Flush with N_2, shake and store overnight in refrigerator for phases separation.

9. Remove upper phase with pipette and kitassato (vacuum);

10. Evaporate the remaining with N_2;

11. Weight flasks (for total lipids);

12. Add 100 µL of internal standard in each flask (0.1 g in 5 mL of hexane).

*The amount of nonadecanoic acid methyl ester (C19:0) weighed to prepare the internal standard has to be written down to be used on the calculations from the GC readings;

13. Add 1 mL of 0.5N KOH in MeOH, cap and place in 70˚C waterbath for 20 min;

14. Add 1 mL of BF_3 in MeOH (14% BF_3), flush with N_2, place in 70˚C water bath for 40 min;

15. Cool down, add 2 mL of hexane and 2 mL of saturated NaCℓ solution, vortex for 1 min;

16. Pipette hexane layer, flow through glass pipette with Na_2SO_4 into small test tube;

17. Evaporate the hexane with N_2;

18. From excel spreadsheet calculate how much hexane to add to reach a 10 mg lipid/mL of hexane concentration;

19. Place the sample in an injection vial and keep in -80˚C until injected into the GC.

Total lipid and polar/non-polar fatty acids fractions extraction:

Lipid extraction, separation of polar and non-polar lipid fraction, saponification and trans-esterification procedure:

1. Wash, dry and weight tubes;
2. Weight sample, around 0.2-0.5 g (for feed add water ~50% of the dry weight);
3. Add 6 mL of Chloroform/Methanol (2:1);
4. Homogenize;
5. Filter through sintered glass filter using syringe filter, put into pre-weighted tubes (screw caps). Rinse syringe with Chloroform/Methanol (2:1);
6. Bring up to 10 mL of chloroform/methanol (2:1);
7. Add 2 mL of D.I. water;
8. Flush with N_2, shake and store overnight in refrigerator for phases separation.
9. Remove upper phase with pipette and wash lower phase with fresh upper phase three times (2 mL) by gently allowing it to flow down the side of the test tube;
10. Evaporate the remaining with N_2;
11. Add 1 mL of methanol to make one phase. Add 0.5 g of Na_2SO_4 and decant. This is your lower phase. Weigh new set of clean/dry test tubes/samples (record tube weights);
12. Transfer lower phase to dried, pre-weighed test tube. Evaporate chloroform under N_2. Weight flasks (for total lipids);
13. Wet weighed sample with 1-2 mL chloroform. Wet silica cartridge with 1-2 mL of chloroform;
14. Load the lipid extract (20-100 mg) onto the silica cartridge with a 10 mL glass syringe;

15. Let the sample (chloroform + lipid) load by gravity, then use air pump to get the rest of the sample and remove cartridge (discard the solution that went through the cartridge);
16. In a clean set of 15 mL screw cap tubes, elute the non-polar (neutral) fraction with 3 mL (2 times) of hexane:diethyl ether (1:1) utilizing the syringe and air pump;
17. Into a different screw cap tube, elute the polar phase with 5 mL of chloroform:methanol (1:1) followed by 5 mL straight methanol, utilizing the syringe and air pump;
18. Evaporate under N_2 at 40°C;
19. Add 1 mL of 0.5N KOH in MeOH, cap and place in 70˚C waterbath for 20 min;
20. Add 1 mL of BF_3 in MeOH (14% BF_3), flush with N_2, place in 70˚C water bath for 45 min;
21. Cool down, add 2 mL of hexane and 2 mL of saturated NaCℓ solution, vortex for 1 min;
22. Pipette hexane layer, flow through glass pipette with Na_2SO_4 into small test tube;
23. Evaporate the hexane with N_2;
24. Add 500 µL of hexane to sample;
25. Place the sample in an injection vial with inserts and keep in -80˚C until injected into the GC.

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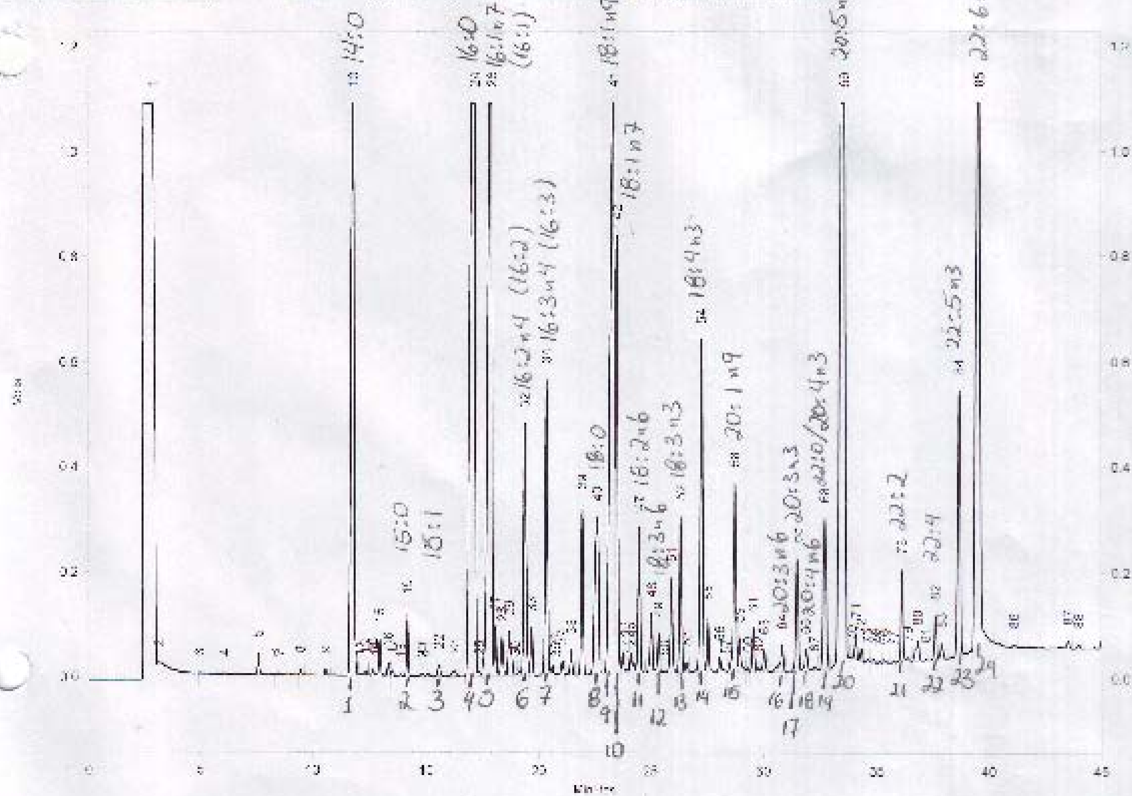

FID Results

Pk #	Retention Time	Area	Area %
1	2.901	35131942	19.53
2	3.121	514150	0.29
3	4.961	8772	0.00
4	6.095	9376	0.01
5	7.596	177634	0.10
6	8.368	12607	0.01
7	8.529	18642	0.01
8	9.17	49314	0.03
9	10.591	62431	0.03
10	11.861	10396942	5.78
11	12.091	22715	0.01
12	12.356	50701	0.03
13	12.658	60511	0.03
14	12.773	25609	0.01
15	12.971	369049	0.21
16	13.481	132130	0.07
17	13.531	52169	0.03
18	13.591	17840	0.01
19	14.206	725389	0.40
20	14.839	43999	0.02
21	15.076	42150	0.02