Final Report
Project No.
Development of Genetic Markers for Improving Soy Protein Utilization by Shrimp- Phase II

Background

The 2013 SAA sponsored project found that full-sib families grew at different rates when fed a high soy diet and the alleles of single nucleotide polymorphisms (SNPs) had different frequencies in the fast and slow growing families. The goals of this research project were to confirm that family differences in growth exist when the shrimp are fed a high soy protein diet, to confirm that growth on a high soy protein diet is heritable, to evaluate whether the SNPs found to influence growth in the 2013 project also had a significant effect on growth in this trial and to evaluate whether a marker assisted selection program will result in faster genetic improvement than a traditional family selection program.

Aquaculture feeds, especially those for shrimp, continue to utilize a major share of the global production of fishmeal. In 2009 aquaculture feeds consumed 63% of the fishmeal produced. Crustacean feeds consumed 26% of the fishmeal used in aquaculture (Tacon and Metian 2008; Chamberlain, 2011; Olsen 2011). The production of fishmeal has remained relatively stable for the last decade while aquaculture grew 97% and increased its consumption of the total world fishmeal supply from 33% to 73% (Blezinger *et al*., 2015). Continued use of high levels of fishmeal is not sustainable (Kristofferson and Anderson 2006; Worm *et al*., 2006; Deutsch *et al*. 2007; Olsen and Hassan 2012;Watson and Pauly 2013). Some assert that the increase in demand for fishmeal by aquaculture will lead to the depletion of wild fisheries stocks (Naylor *et al*., 1998). Aquaculture cannot continue to rely on stocks of wild-caught fish, a number of which are classified as fully exploited, over exploited or depleted (Naylor *et al*., 2000; FAO, 2010, 2012; NRC, 1997; Natale *et al*. 2013).

Research with rainbow trout has shown that genetic variation exists for utilization of plant protein. A strain of rainbow trout developed by the USDA-ARS for improved utilization of plant-based diets was compared to two other strains of trout. The selected ARS strain outgrew the other two strains on the plant-based feed and the selected ARS strain was found to grow better on the plant-based feed than the fishmeal feed (Overturf *et al*., 2013). In another study Pierce *et al*. (2008) concluded that substantial genetic variation exists in a commercial Kamloops strain of rainbow trout for utilizing plant-based diets containing soybean meal and oil. A similar genotype-diet interaction was observed in juvenile rainbow trout fed fish meal or high plant protein diets (Dupont-Nivet *et al*. 2009). Studies have also indicated family related differences in response to dietary plant protein inclusion in the European sea bass, a carnivorous marine fish species (Le Boucher *et al*. 2010; Geay *et al*. 2011).

Recent evidence that suggests strain there are strain dependent differences in degree of sensitivity to dietary soybean meal in rainbow trout. Some strains exhibited lower levels of gut inflammation when fed higher levels of dietary soybean meal (Venold *et al*. 2012). Several studies have shown that shrimp can tolerate high levels of dietary soy protein without loss in

performance although there is no information on histological alterations in the hepatopancreas (Amaya *et al*. 2007; Sookying and Davis 2011; Sookying *et al*. 2013).

In earlier SAA-supported research conducted by IAI in 2013, we observed differences between families in their ability to utilize soybean meal. The research was conducted in two phases, an 8-week trial that evaluated 20 families followed by a 6-week trial that evaluated the top five performing families. In the first trial 20 families were fed either a fishmeal based diet or a fishmeal free diet that contained approximately 65% soybean meal. Shrimp fed the high soy diet (14.89 g) performed significantly better than shrimp fed the fish meal diet (11.94 g) across all families. Analysis of performance results indicated that diet and family significantly affected weight ($p<0.001$). The differences between families in their response to the high soy diet suggested a higher tolerance to soy in some families. The second trial demonstrated that the top five families from the first trial performed equally well on both fishmeal and high soy diets.

A second IAI funded trial was conducted with a different line of the Pacific white shrimp after the completion of the 2013 SAA sponsored trial. In the second trial there was a significant difference in final weight ($P<.01$) between families. The average final weight of the families ranged from 9.5 g. to 12.2 g.

The objective of this research was to refine a selection program that will result in the development of shrimp lines with enhanced growth when fed a ration that contains no fishmeal.

Methods

Twenty-one families were produced from broodstock maintained in the IAI breeding program located in Kauai. The goal was to produce 10 families from parental families that grew the fastest on the 2013 soy trial and produce 10 families from parental families that grew the slowest on the 2013 soy trial. However, due to the spawning of the females and differential livability 10 families were produced from high growth x high growth matings, 1 family from a high growth x medium growth mating, 3 families from low growth x medium growth matings and 7 families from low growth x low growth matings. From fertilization through hatching each family was kept in individual 40 L liter aquaria. After hatching and until the families reached the PL10 stage, approximately 21 days post spawning, they were maintained in individual 100 L tanks. At PL10 life stage 500 PLs per family were transferred to a 780 liter net cage. The cages for all 21 families were located within the same 8.5 meter diameter tank. Growing the animals in cages within one tank minimized the environmental differences between families prior to the start of the trial. From PL10 to 3 grams, the shrimp were fed a standard fishmeal-based diet at a rate approximately equal to 7% of the biomass. When average weight was 3.0 grams was reached all animals were marked with a colored elastomer tag on various abdominal parts to identify the family. After marking with the elastomers the animals were transferred to 700 L round fiberglass tanks with automatic feeders and self-cleaning bottoms. A total of 36 tanks were used for the 21 families. Seventy-five shrimp were placed in each tank. The families were distributed in the tanks using an incomplete block design so both the family differences and the diet differences could be estimated. Each block consisted of three adjacent tanks. Each diet was randomly assigned to one of the tanks in each block. For one week after the transfer to the 700 L

tanks all shrimp were fed a standard diet with fishmeal to allow them to overcome the handling stress and to acclimate to the experimental tanks before starting the study.

Three different diets were fed in the trial. The diets fed were a fishmeal based diet (FMC), a soy protein diet with 5% krill meal (HIS) and a soy protein diet with 10% krill meal (HIK). The diets were formulated to contain approximately 40% crude protein and 8.5% fat (Table 1). The diets were balanced for protein, energy, amino acids, fatty acids, phospholipids, cholesterol, attractants, vitamins and minerals and supplied the nutrient requirements for this species. Soybean meal and other plant proteins were the primary replacement for fishmeal. The fishmeal diet (FM) contained approximately 35% fishmeal and is considered to be representative of inclusion levels currently found in commercial shrimp feeds. The fishmeal diet also contained soybean meal and wheat. The high soy diet (HIS) and high soy with extra krill meal (HIK) was devoid of fish meal and all of the fish meal was substituted with soybean meal. Sinking diets (2 mm pellets) were produced at the IAI feed laboratory in Kauai. The diets were subjected to proximate and nutrient analysis to confirm their composition. Each diet was fed to 12 tanks. Throughout the feeding trial, the diets were fed according to a standard feeding table using automatic belt feeders.

A random sample of animals from each tank was weighed bi-weekly. The amount of feed fed was adjusted according to the estimated biomass based on the average sample weight of each tank. Every morning and evening the water temperature and dissolved oxygen for each tank was recorded.

The trial was terminated at 6 weeks when the average weight was 14 grams. At the termination of the trial each animal was individually weighed. The family and sex was recorded along with the weight. A tissue sample was taken from a selected group of animals fed the soy protein diets for DNA analysis. Tissue samples were collected from the 10 largest males, the 10 smallest males, the 10 largest females and the 10 smallest females from the 5 fastest growing families and the 5 slowest growing families to validate the usefulness of the SNPs found in the 2013 trial to improve soy protein utilization.

The tissue samples from the selected sample of animals were analyzed for the presence of the SNP alleles found to be associated with the utilization of soy protein in the 2013 trial. The candidate SNP sequences were formatted to Fluidigm submission standards and undergone design evaluation. Assays that pass in silico design QC were authorized for synthesis by Fluidigm Corporation (San Diego). Genomic DNA (gDNA) was extracted via 96-well column plate method. Specific Template Amplification (STA) was performed on each sample of gDNA to maximize assay consistency. The sample DNA and SNP assays were loaded onto a “chip”; an Integrated Fluidic Circuit (IFC) that accommodates 96 assays by 95 samples capturing 9,120 discreet data points. Samples were processed against all SNPs using Fluidigm EPI hardware. Assays were scored based on fluorescence intensity analyses populated by assay for all samples on each IFC. The SNP assays that amplified well were scored for genotype for each sample. The SNP genotypes were compared with the phenotype to confirm the validity of the predicted trait-associated markers.

At the end of the trial 5 samples were randomly selected from each treatment and fixed in Davidson's Alcohol Formalin Acetic Acid Fixative by injecting the solution in living shrimp with 27 gauge needle (Lightner 1996; Tran *et al*. 2013). The Davidson's fixative was injected laterally into the hepatopancreas and regions anterior and posterior to the hepatopancreas and into the abdomen of the shrimp. Approximately 10% of a shrimp's weight by volume was injected into each shrimp. Following injection shrimp were bisected and immersed in Davidson's solution for 72 h following which they were stored in 70% alcohol prior to shipment to the University of Arizona for evaluation. The University of Arizona conducted a histopathological evaluation of hepatopancreas tissue from the animals from the different treatments. The hepatopancreas samples were processed according to conventional techniques for paraffin embedding and sectioning. Paraffin sections were stained with Mayer-Bennet's hematoxylin/eosin/phloxine (H&E) and examined microscopically. The following were examined:

1. Qualitative evaluation of the activity of "R-cells" in the hepatopancreas. Evaluation of lipid storage as lipid vacuoles/droplets within the cytoplasm of R-cells using a subjective non-quantitative scale from L0 (lowest) to L4 (highest).
2. General evaluation of the hepatopancreas to determine the presence of any pathologies such inflammation (also referred to as hemocytic congestion), sloughing/blebbing of tubule epithelial cells or bacterial infection.

Performance and feed conversion were analyzed by GLM ANOVA for differences between diets, families and blocks. The individual body weight were analyzed by general linear model (GLM) analysis of variance (ANOVA) to evaluate the differences between diets, families, sexes.

Results

When the trial was terminated all shrimp were weighed individually. Analysis of the final individual weights concluded that the difference between blocks, diet and families were highly significant. The average weight of the shrimp fed FMC was 13.4 g which was significantly smaller than those fed HIS and HIK. The shrimp fed the HIS and HIK diets weighed 14.2 g on both diets. The average weight of the families ranged from 11.5 g to 16.2 g. The difference in final weight between the families was highly significant. The diet x family interaction was significant because the families changed ranking on the different diets. The two slowest growing families ranked the same on the three different diets. The remaining families changed rank when fed the different diets. However, the 5 fastest growing families on each diet were the same but the ranking of the families on each diet was different.

A sample of shrimp from each tank was weighed every two weeks. There were no significant differences between the diets in the growth in any of the two week periods. However, the total growth for the shrimp fed FMC was significantly lower than the total growth for the shrimp fed the HIS and HIK diets.

There were no significant differences in the feed consumption between the diets for any of the periods or the total feed consumption.

The feed conversion for the complete trial was 1.40 for FMC, 1.39 for HIS and 1.50 for HIK. These differences were not significant.

The DNA of 403 shrimp was analyzed to determine if the alleles of 16 single nucleotide polymorphisms (SNPs) that appeared to influence growth in the first trial had a significant effect on the growth in this trial. The accuracy of prediction, the R^2, was .16 when all 16 SNPs were used to predict weight. The accuracy of predicting weight using the family information was .48. Combining the SNP data with the family information increased the accuracy of selection to .50. The accuracy of predicting weight using the 7 SNPs with the greatest effect and the family information was .48.

The broad sense heritability for weight of the shrimp fed HIS and HIK was .34. The data structure did not allow the calculation of the narrow sense heritability.

Results from the histopathological evaluation of hepatopancreas tissue showed that R-cell activity was normal for the shrimp examined. No pathogens were detected and there was no indication of septic hepatopancreatic necrosis (SHN) or vibriosis in the hepatopancreas of shrimp examined. No atrophy of hepatopancreatic tubules was observed and there was also no evidence of intertubular hemocytic congestion. These findings suggest that feeding of high dietary levels of soybean meal did not cause any histopathological abnormalities in the hepatopancreas.

Conclusions

1. The shrimp in this trial grew faster on the HIS and HIK diets than the shrimp fed the FMC diet. The faster growth rate on the HIS diet than the FMC diet confirms the growth rate difference in the first study.
2. There are family differences in the growth on the high soy diets.
3. The ranking of the families for final weight is different on a standard fish meal and high soy diets.
4. The fastest genetic improvement for growth on a high soy diet will be achieved when the shrimp are fed a high soy diet.
5. The genetic improvement in growth will be similar for a traditional family selection program and a marker assisted selection program.

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Table 1. Composition of experimental diets fed to Pacific white shrimp *L. vannamei*

Ingredients, % of diet	**FM [1]**	**HIS [1]**	**HIK[1]**
Fishmeal, Menhaden	21.30		
Soybean Meal, 48%	35.00	64.43	60.17
Wheat	36.02	23.00	23.62
Krill Meal	3.00	5.16	9
Lecithin	1.3	1.9	1.41
Monocalcium Phosphate	0.687	1.64	1.83
Vitamin/Mineral Premix*	0.25	0.25	0.25
Fish Oil	2.33	3.02	3.52
Cholesterol	0.114	0.19	0.174
DL-Methionine		0.104	0.019
L-Lysine		0.105	0.003
Proximate and Nutrient Composition, % of diet			
Moisture	6.21	8.57	8.17
Crude Protein	40.60	39.90	40.1
Crude Fat	8.45	8.73	8.83
Ash	8.08	6.55	6.61
Crude Fiber	1.79	2.83	2.60
Arginine	2.43	2.66	2.71
Lysine	2.35	2.30	2.42
Methionine	0.73	0.57	0.62
Histidine	0.97	1.01	1.04
Threonine	1.49	1.33	1.54
Cholesterol	0.20	0.15	0.24

* Vitamin/Mineral Premix contained the following per kg of premix: Vitamin A – 3,500,000 IU, Vitamin D3 – 1,500,000 IU, Vitamin E – 75g, Vitamin K3 – 15g, Vitamin B1 – 12.5g, Vitamin B2 – 10 g, Vitamin B6 – 12.5g, Vitamin B12 – 0.01g, Niacin – 50g, Pantothenic Acid – 40g, Biotin – 0.5g, Folic Acid 5 g, Vitamin C – 100 g, Copper – 12.5g, Iron – 15 g, Manganese – 15g, Iodine – 0.5 g, Cobalt 0.1 g, Zinc – 50 g, Selenium – 0.175 g.

[1] FM, HIS and HIK denote fishmeal, high soymeal and high soy and krill diets respectively.

Table 2. Means and standard errors for final weight.

Diet	Mean	Std. Error
FMC	13.4	0.08
HIS	14.2	0.09
HIK	14.2	0.08

Table 3. Means and standard errors for growth in each period for each diet.

Diet	Growth Weeks 0-2	Growth Weeks 2-4	Growth Weeks 4-6	Total Growth
FMC	3.3±0.12	2.7±0.22	3.7±0.18	9.7±0.2
HIS	3.0±0.17	3.2±0.17	4.2±0.25	10.4±0.28
HIK	3.2±0.17	3.0±0.24	4.2±0.16	10.4±0.29

Table 4. Effect of Block and Diet on Growth in *Litopenaeus vannamei* fed either a fishmeal based or high soy diet.

		Mean Square			
Source of Variation	Degrees of Freedom	Growth Weeks 0-2	Growth Weeks 2-4	Growth Weeks 4-6	Total Growth
Block	11	0.210	0.462	0.695	1.413*
Diet	2	0.351	0.812	1.261	2.083*
Residual	22	0.321	0.555	0.369	0.500

*P< .05

Table 5. Means and standard errors for feed consumption in each period for each diet.

Diet	Feed Weeks 0-2	Feed Weeks 2-4	Feed Weeks 4-6	Total Feed
FMC	238.6±5.85	369.7±6.54	366.3±6.53	974.6±16.19
HIS	246.8±7.29	359.1±13.78	357.4±13.68	963.3±34.08
HIK	246.3±10.19	370.9±13.22	368.6±13.37	985.9±34.86

Table 6. Effect of Block and Diet on Feed Consumption in *Litopenaeus vannamei* fed either a fishmeal based or high soy diet.

		Mean Square			
Source of Variation	Degrees of Freedom	Feed 0-2 weeks	Feed 2-4 weeks	Feed 4-6 weeks	Total Feed
Block	11	959.1	1628.0	1610.7	11514.8
Diet	2	257.9	512.3	419.6	1531.9
Residual	22	667.3	1631.3	1645.5	10077.2

Table 7. Effect of Block and Diet on Feed Conversion in *Litopenaeus vannamei* fed either a fishmeal based or high soy diet.

Source of Variation	Degrees of Freedom	Mean Square
Block	11	0.144
Diet	2	0.046
Residual	22	0.096

Table 8. Effect of Block and Diet on Final Weight in *Litopenaeus vannamei* fed either a fishmeal based or high soy diet.

Source of Variation	Degrees of Freedom	Mean Square
Block	11	82.800***
Diet	2	126.46***
Sex	1	7.91
Family	20	137.85***
Diet x Family	40	5.58*
Residual	2323	3.91

*P<.05
***P<.001