Project Number:	1830-352-0501-G
Project Title:	NMR Metabolomics Investigation of FIGLU as a Biomarker of Nutritional Stress in Red Drum (Sciaenops Ocellatus) Fed Soy-Based Diets
Organization:	South Carolina Department of Natural Resources
Principal Investigator Name:	Dr. Michael Denson

Project Status - What key activities were undertaken and what were the key accomplishments during the life of this project? Please use this field to clearly and concisely report on project progress. The information included should reflect quantifiable results (expand upon the KPIs) that can be used to evaluate and measure project success. Technical reports, no longer than 4 pages, may be included in this section.

This research project consisted of a twelve-week feeding trial on juvenile red drum to investigate a specific metabolite, formimino-glutamate (FIGLU) as potential metabolic marker of nutritional stress in fish fed diets with high contents (60%) of U.S. soybean meal as the protein source in fishmeal-free feeds. Following a 7-week conditioning period during which juvenile red drum were fed a fishmeal-based soybean meal-free diet fish with an average weight of 61.3 g were sorted into the 24-tank experimental aquaculture system at the Hollings Marine Laboratory, Charleston, SC. Fish density was 25 fish/tank, with the exception of tanks 21-24 which received 30 fish each, to provide extra fish in case of mortalities during the course of the trial. Experimental temperature in the tanks was 25°C.

During the course of 12 weeks, the feeding trial investigated four fishmeal-free high soybean meal (60%) experimental feeds manufactured by our partners at USFWS, along with four control diets to monitor performance. A total of 8 diets were tested in this study (see feed formulations in Table 1):

- Natural reference diet (cut squid, shrimp, fish; positive control for growth performance));
- Soybean meal (SBM) 60%, unsupplemented (negative control for performance); identical to previous SAA trials conducted in our lab;
- Fishmeal control (also used as conditioning diet) (positive control replacing all SBM with fishmeal (FM) on a digestible protein basis);
- Supplemented SBM 60% (SBM60 diet supplemented with all the supplements our partners at USFWS typically add to plant-based feeds for other species;
- **Vitamin B12 5X** (SBM 60% diet supplemented with 5X the general vitamin B12 requirements for red drum reported in the literature);
- **Folate 5X** (SBM 60% diet supplemented with 5X the general folate requirements for red drum reported in the literature);
- **Methionine 2X** (SBM 60% diet supplemented with 2X the general methionine requirements for red drum reported in the literature)
- **5X B12/ 5X folate/ 2X Methionine combination diet** (SBM 60% diet supplemented with 5X B12, 5X folate, and 2X methionine).

Tissue samples (liver, intestine, heart, and muscle), in addition to plasma were collected for subsequent NMR-based metabolomics analysis upon fish anesthetization at three different time points: initial sampling (T0) at the end of the conditioning period and before the start of the feeding trial; midpoint sampling (T6) conducted 6 weeks after the beginning of the feeding trial; final sampling (T12) at the end of the 12-week feeding trial. Growth and performance metrics were recorded. Based on performance metrics (SGR, weight gain %, FCR) the natural reference diet outperformed all experimental feeds, as expected; the fishmeal control diet was the second best-performing diet. No significant differences in performance were detected among the soy-based diets regardless of supplementation (see Table 2). Feed conversion ratios of 1.0 or below suggests that all experimental diets performed well, and this is exceptional for feeds containing high levels of soybean meal. The initial (T0) tissue sampling was performed on 12 fish, and 72 fish per time point were sampled at T6 and T12 for a total number of samples of 156 per tissue (780 samples over 5 tissues, including plasma). Liver, muscle and heart tissue samples from the three different time points have been homogenized for subsequent extraction; however, NMR-based metabolomics analysis focused on the liver tissue. As indicated in the proposal, the additional tissues sampled will be analyzed on an as-

needed basis depending on the results obtained from analysis of the liver samples. Liver tissues from the T0 and T6 were extracted and the polar extracts analyzed by NMR spectroscopy. Unfortunately, due to an instrument malfunction of the NMR spectrometer in connection with hurricane Florence, our tissue analysis by NMR spectroscopy has been delayed. In the meantime, other tissues are being processed for subsequent extraction. Results from multivariate statistical analysis, specifically PCA performed on the processed NMR spectra will be provided in an addendum final report.

The putative biomarker of nutritional deficiencies, FIGLU was not detected by NMR in fish fed the natural diet (consistent with our previous observations in other trials), however, FIGLU was detected in all soy-based diets investigated in this study, and interestingly also in the liver of fish that were fed the fishmeal-based experimental diet, which contained significant amounts of wheat flour (~41%). This is an interesting finding which supports the hypothesis that FIGLU is not a mere marker of soybean meal consumption in fish. Relative levels of FIGLU in liver samples at the different time points are currently being measured to evaluate the effect of vitamin B12, folate and/or methionine supplementation as possible mitigation approaches. If our proposed supplementations prove to be able to significantly reduce FIGLU levels and improve the overall metabolic fingerprint of juvenile red drum as judged based on the comparison with the two best performing reference diets, new supplementation protocols can be developed that can be adopted by fish nutritionists to produce new alternative feeds containing at least 60% soybean meal-derived protein for red drum and possibly other fish species. A logical next step would be to investigate this biomarker in other fish species to determine its general applicability.

Did this project meet the intended Key Performance Indicators (KPIs)? List each KPI and describe progress made (or not made) toward addressing it, including metrics where appropriate.

1) Analysis of metabolomics measurements, correlation with growth outcomes and strong validation of the putative biomarker, FIGLU within the 1-year mark.
   Analysis of metabolomics measurements has been delayed due to instrument malfunction in connection with tropical systems. However, NMR metabolomics analysis for two of the three time points (T0 and T6) has been completed
2) Adoption by feed mill nutritionists of mitigation strategies for nutritional stress based on our putative biomarker through nutritional supplementation of specific micronutrients within 1 year from the end of the study.
   At this stage, it is too early to tell if any of the supplemented feeds used in this project will be adopted by feed mill nutritionists as mitigation strategies of nutritional stress.

Standard growth performance parameters (specific growth rate (SGR), weight gain, feed conversion ratio (FCR), and condition factor) were evaluated for significance using ANOVA with Tukey's post-hoc test (significance level set at $p < 0.05$). Based on performance metrics the natural reference diet outperformed all experimental feeds, as expected; the fishmeal control diet was the second best-performing diet. No significant differences in performance were detected among the soy-based diets regardless of supplementation (see Table 2). Tissue samples are being analyzed using NMR spectroscopy.

Expected Outputs/Deliverables - List each deliverable identified in the project, indicate whether or not it was supplied and if not supplied, please provide an explanation as to why.

1) The required USB progress reports (submitted quarterly): supplied.
2) Final report to be submitted within 30 days of project completion: supplied (addendum final report to be submitted upon completion).
3) A technical bulletin for distribution to be submitted within 120 days of project completion: we

anticipate at least one manuscript to be developed from this project for submission to a peer-reviewed journal.

4) Results will be presented at Aquaculture America or the International Fish Biology or other scientific meeting: we will be presenting results from this project at the 2019 World Aquaculture Triennial meeting in New Orleans (oral presentation accepted).

Describe any unforeseen events or circumstances that may have affected project timeline, costs, or deliverables (if applicable.)

The major unforeseen event that has delayed the NMR-based metabolomics analysis of tissue extracts has been an instrument malfunctioning (NMR 700 MHz spectrometer hosted at the Hollings Marine Laboratory) in connection with hurricane Florence. Unfortunately, this event has delayed our analyses, but they are scheduled to be resumed soon. This delay will not cause any increases in cost of the project to SAA/USB. An addendum to this final report will be submitted upon completion of all planned analyses for this project.

What, if any, follow-up steps are required to capture benefits for all US soybean farmers? Describe in a few sentences how the results of this project will be or should be used.

If our proposed supplementation protocols prove to be able to improve the overall metabolic fingerprint of juvenile red drum as judged based on the comparison with the two best performing reference diets, new supplementation protocols can be developed that can be adopted by fish nutritionists to produce new alternative feeds containing at least 60% soybean meal-derived protein for red drum and possibly other fish species.US soybean farmers will benefit from increased inclusion levels of US soy-derived protein in fish feeds for aquaculture. The next major follow-up step would be an economic analysis of the high soy protein supplemented feeds evaluated in this study to determine the long-term overall cost of the proposed feeds and their potential viability at the commercial production scale.

List any relevant performance metrics not captured in KPI's.

Table 1. Feed Formulations for experimental feeds evaluated in this project.

Ingredient (g/100 g dry weight basis)							
Diet	**Soybean meal 60% (Unsupplemented)**	**Fishmeal Control (and conditioning diet)**	**Folate (5X)**	**B12 (5X)**	**Met (2X)**	**2XMet/ 5XFolate/ 5XB12**	**Soybean meal 60% (Supplemented)**
Soybean Meal 48%CP	59.00	0.00	59.00	59.00	59.00	59.00	59.00
Fish Meal SeaPro-75	0.00	33.40	0.00	0.00	0.00	0.00	0.00
Squid - CSF (squid meal)	4.00	4.00	4.00	4.00	4.00	4.00	4.00
SC Blood 13 (blood meal)	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Menhaden fish oil	13.25	10.46	13.25	13.25	13.25	13.25	13.25
Wheat flour	12.02	41.93	12.02	12.02	11.92	11.92	6.43
Lecithin - Yelkinol AC dry lecithin	0.00	0.00	0.00	0.00	0.00	0.00	1.00
Stay-C 35 (Vitamin C)	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin premix ARS 702	1.00	1.00	1.00	1.00	1.00	1.00	1.00
NaCl	0.00	0.00	0.00	0.00	0.00	0.00	0.28
Magnesium Oxide	0.00	0.00	0.00	0.00	0.00	0.00	0.06
Potassium chloride	0.00	0.00	0.00	0.00	0.00	0.00	0.56
Monocalcium Phosphate	2.40	2.60	2.40	2.40	2.40	2.40	4.20
Choline Cl 50%	0.60	0.60	0.60	0.60	0.60	0.60	0.60
DL-Methionine	0.75	0.21	0.75	0.75	0.85	0.85	0.85
Lysine HCl	1.30	0.13	1.30	1.30	1.30	1.30	2.32
Threonine	0.38	0.37	0.38	0.38	0.38	0.38	0.65
Taurine	0.00	0.00	0.00	0.00	0.00	0.00	0.50
TM ARS 1440 (Trace mineral premix)	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin B12	0.00	0.00	0.00	0.0000012	0.0000000	0.0000012	0.0000000
Folate			0.001	0.000	0.000	0.001	0.000

Table 2. Performance characteristics from this project.

Diet	Specific Growth Rate (SGR)	Weight Gain (% initial)	Feed Consumed (g/fish)	Feed Conversion Ratio (FCR)
Diet #1	1.3 ± 0.08	201.04 ± 20.01	183.51± 23.82	0.94 ± 0.02
Diet #2	1.7 ± 0.07	327.71 ± 26.57	249.20 ± 1.92	0.77 ± 0.04
Diet #3	1.3 ± 0.12	203.37 ± 29.50	205.27 ± 20.14	1.00 ± 0.08
Diet #4	1.3 ± 0.16	197.85 ± 41.55	181.88 ± 25.59	0.93 ± 0.10
Diet #5	1.3 ± 0.04	207.91 ± 10.00	194.18 ± 6.31	0.96 ± 0.05
Diet #6	1.3 ± 0.15	205.73 ± 38.29	187.81 ± 11.73	0.94 ± 0.12
Diet #7	1.3 ± 0.07	204.92 ± 17.01	199.86 ± 14.41	0.99 ± 0.02
Natural Diet	2.2 ± 0.08	565.67 ± 33.80	1364.01 ± 44.77	2.43 ± 0.06
P (significant if <0.05)	0.0023	0.0006	0.0038	0.0243