

Final Report for Project# 14332

“Determination and validation of transcriptomic and metabolite biomarkers for dietary soybean meal utilization”

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Period covered: August 1, 2014 to December 31, 2015

EXECUTIVE SUMMARY

Inclusion of soybean meal (SBM) in diets had varying negative effects on rainbow trout, red drum and cobia with rainbow trout appearing to be more sensitive to SBM with the experimental diet formulations used. Rainbow trout showed decreased feed efficiency and increased relative liver weight (hepatosomatic index). Cobia and red drum showed no clinical effects in growth performance although growth may have not been optimal. Histological examination showed an effect of SBM on intestinal tissue and liver cells in all three species which was correlated with SBM inclusion level. The results of the first objective suggest antinutritional factors present in SBM may have dissimilar effects on different species. The variation observed in the histological parameters among species warrants further investigation. In Objective 2, gene expression in intestine and/or liver tissue was correlated in all species with SBM inclusion. Potential genetic biomarkers observed among the genes that were screened across species included transforming growth factor β , Interleukin-2 and “calprotectin”. Calprotectin merits further examination since it may be detected in sloughed cells as with other vertebrates and could lead to a non-invasive detection method. In Objective 3, Plasma metabolomic profiles for each species were examined and resulting changes could be explained to varying levels using PCA models. All objectives of this study were met with the three putative biomarkers listed above warranting additional investigation for their utility in early and non-invasive detection of SBM-caused gastric enteritis.

PERFORMANCE TIMELINE

The University of Idaho was notified of this award on September 15, 2014. Post-award negotiations between the Office of Sponsored Programs (OSP), University of Idaho, and SAA began on that date. An internal budget was established for investigators on September 26, 2015. A no-cost extension was applied for and granted extending the performance period for the task order from July 31, 2015 until December 31, 2015.

GOALS AND OBJECTIVES

The long-term goal of the research is to develop a suite of biomarkers or biomarker signatures that will quantitatively correlate to performance indices of growth and efficiency when soybean meal (SBM) is utilized in diets of rainbow trout, red drum and cobia. To accomplish this goal, three primary objectives were addressed:

- 1) Define correlations between growth rates and dietary SBM concentrations (anti- nutrient levels)
- 2) Validate molecular markers in the liver and intestine across the three species that correlate with growth performance as well as intestinal histological evaluation, and
- 3) Define metabolite changes in plasma that correlate with growth performance.

RESEARCH ACTIVITIES AND RESULTS

Objective 1: Define correlations between growth rates and dietary SBM concentrations (anti- nutrient levels)

Rainbow trout, red drum and cobia were fed for 12 weeks using 3 experimental diet formulations containing (10, 20 and 40%) soybean meal and a control diet containing 0% soybean meal. After 12 weeks, weight gain, feed efficiency and hepatosomatic index (the ratio of liver weight to body weight) were measured in all groups along with several other growth parameters (Table 1). Feed efficiency and hepatosomatic index (HSI) were affected by the diets in rainbow trout with efficiency declining and relative liver size increasing with increasing SBM in the diet. Red drum and cobia weight gain, feed efficiency and HSI were not significantly affected over 12 weeks of feeding.

Table 1. Percent weight gain, feed efficiency and hepatosomatic index for rainbow trout, red drum and cobia fed diets containing 0, 10, 20, and 40% SBM over 12 weeks.

Treatment	Rainbow Trout			Red Drum			Cobia		
	%weight gain	feed efficiency	Hepatosomatic Index	%weight gain	feed efficiency	Hepatosomatic Index	%weight gain	feed efficiency	Hepatosomatic Index
Control	318	0.89	1.19	660	0.75	2.81	298.2	1.01	2.40
10%SBM	301	0.90	1.21	684	0.84	2.98	287.5	1.10	2.39
20%SBM	315	0.89	1.28	667	0.85	2.94	282.3	1.08	2.43
40%SBM	294	1.02	1.12	726	0.82	2.77	282.8	1.12	2.37
SEM	5.700	0.032	0.033	73.680	0.050	0.973	5.198	0.020	0.058
p value	0.361	<0.001	0.088	0.698	0.180	0.962	0.737	0.175	0.989

Histological examination of liver and intestine samples revealed significant changes to multiple histological parameters suggesting an inflammatory response to SBM in the diets in all three species (Table 2). Histological data also indicate differences in inflammation in each species. For example, abnormal vacuolization (Abn Vacs) was significant rainbow trout and red drum but not in cobia. Conversely, glycogen vacuoles (Glyc Vac) and changes in nuclear size (Nuc Change) were significant in rainbow trout and cobia liver tissue but not in red drum.

Table 2. Histological changes observed in rainbow trout, red drum and cobia fed diets containing 0, 10, 20, and 40% SBM over 12 weeks. (Intestine: submucosal width, submucosal cellularity, lamina propria width, lamina propria cellularity, normal vacuolation and abnormal vacuolation; Liver: glycogen vacuolation, overall inflammation, and nuclear change).

Organ	Parameter	Rainbow Trout		Red Drum		Cobia	
		H	P value	H	P value	H	P value
Intestine	Sub Width	5.677	0.128	1.460	0.692	3.283	0.350
	Sub Cell	3.382	0.336	2.939	0.401	0.979	0.806
	LP Width	7.376	0.061	0.082	0.994	0.227	0.973
	LP Cell	8.177	0.042	16.194	0.001	1.093	0.779
	Norm Vacs	10.903	0.012	4.533	0.209	17.841	<0.001
	Abn Vacs	15.729	0.001	12.643	0.005	7.309	0.063
Liver	Glyc Vac	17.935	<0.001	6.802	0.078	8.556	0.036
	Liver Infl	2.145	0.543	1.400	0.706	1.977	0.577
	Nuc Change	11.785	0.008	3.000	0.392	11.500	0.009

Pairwise correlations and canonical correlations of co-variates indicate strong relationships between the percentage of SBM in the diet and changes in growth performance and/or histological parameters in all species. For example, decreases feed efficiency in rainbow trout were highly correlated with SBM percentage in the diet ($r=0.832$; $y=0.867+0.003x$; $P<0.001$).

Objective 2: Validate molecular markers in the liver and intestine across the three species that correlate with growth performance as well as intestinal histological evaluation

Previous microarray analyses indicated significant dysregulation in 226 and 264 candidate biomarkers in the intestine and liver of rainbow trout, respectively. Further analysis defined statistical correlations between these putative markers in rainbow trout and soy-induced enteritis. We prioritized the most promising of these genes for validation across species: glutathione peroxidase, thioredoxin reductase, catalase, superoxide dismutase 1, hypoxia inducing factor, tumor necrosis factor α , Interleukin-1 β , Interleukin-2, Interleukin-6, complement factor 4, C-reactive protein, nuclear factor- κ B, nuclear factor erythroid 2-related factor 2, Heat shock protein 70, Heat shock protein 29, Heat shock protein 90, protein kinase C and “calprotectin”.

Results indicate that several of the putative biomarkers examined (Interleukin -2, transforming growth factor β and “calprotectin”) show significant differences in expression with increasing levels of SBM in the diet. Rainbow trout showed the greatest differences in expression likely due to the acknowledged greater sensitivity of rainbow trout to soy-induced enteritis. Red drum and cobia both showed a less dramatic and most times insignificant increase in the same genes. Figure 1 shows expression of a portion of “calprotectin” a putative heterodimer consisting of two SA100 calcium-binding proteins. One of two portions of this heterodimer was highly correlated to SBM inclusion in the diet.

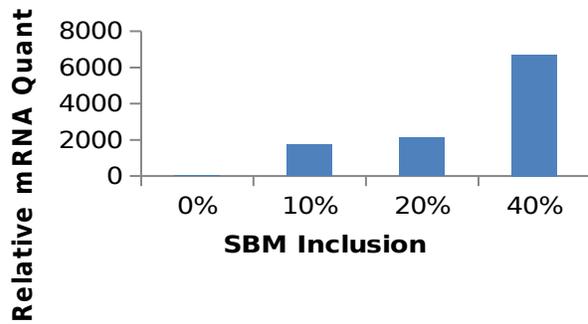


Figure 1. Expression of one calcium-binding dimer from putative “calprotectin” in rainbow trout distal intestine with increasing levels of dietary SBM.

However, Figure 2 shows the expression of what is believed to be the constitutively expressed portion of the heterodimer. In this instance the SA100A1 protein is expressed in the proximal intestine in an inverse relationship with SBM inclusion in the diet. Expression of SA100A1 in the distal intestine was not responsive to dietary SBM.

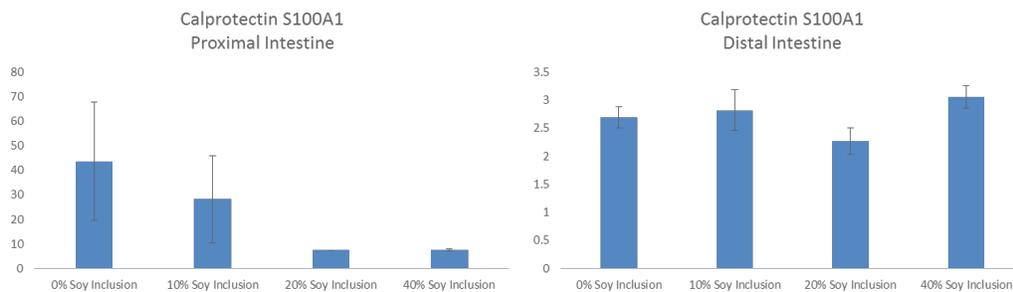


Figure 2. Expression of the constitutive portion of “calprotectin” in rainbow trout proximal and distal intestine with increasing levels of dietary SBM.

Expression of transforming growth factor β showed differences between proximal and distal intestine with proximal intestine expression unresponsive to SBM inclusion in the diet. In the distal intestine, expression of transforming growth factor β correlates with the increase of SBM % in the diet (Figure 3).

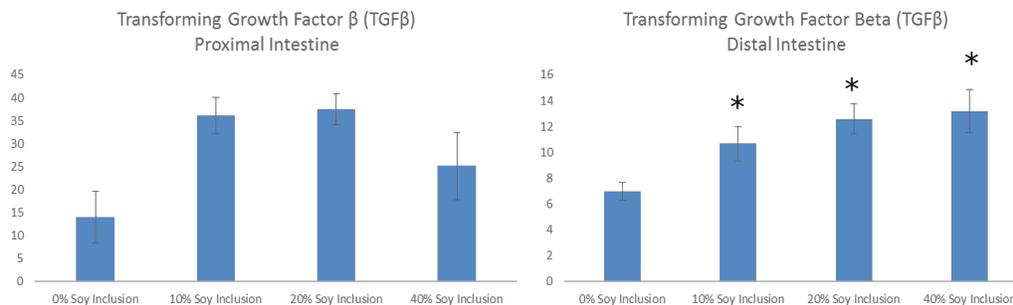


Figure 3. Expression of transforming growth factor β in rainbow trout proximal and distal intestine with increasing levels of dietary SBM.

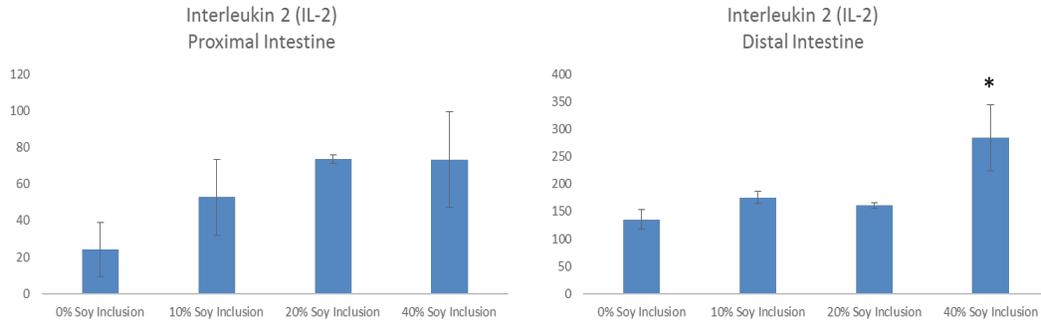


Figure 4. Expression of interleukin-2 in rainbow trout proximal and distal intestine with increasing levels of dietary SBM.

Expression of interleukin-2 was significantly higher in the distal intestine only in the diet containing 40% SBM. Expression in the proximal intestine indicates a trend toward increased expression with the inclusion of SBM. Many aquaculture species tolerate levels of dietary SBM below 40%. Interleukin-2 is a signaling molecule for the immune system or a “cytokine”. Its increased expression at 40% SBM should be further explored as a potential threshold biomarker - a gene whose expression is induced only after a threshold has been reached. In this case, SBM inclusion in the diet greater than 20% may induce expression of this cytokine and trigger an immune response.

Objective 3: Define metabolite changes in plasma that correlate with growth performance

Blood plasma from all three species were examined at the end of the growth period using nuclear magnetic resonance (NMR) and multivariate analysis to examine difference in the metabolome with diet as a factor. PCA analyses were able to explain 58.2, 64.8 and 75.6% of the variance associated with the diets in rainbow trout, red drum and cobia respectively. Initially, PCA analyses included the T=0 plasma samples as a phenotypic anchor point but this was later considered to be a confounding effect.

The plasma PCA model for the rainbow trout study at the terminal point explains 58.2 % of the variance in the data set using PC1 and PC2. In Error: Reference source not found, the sequential diets with increasing SBM are

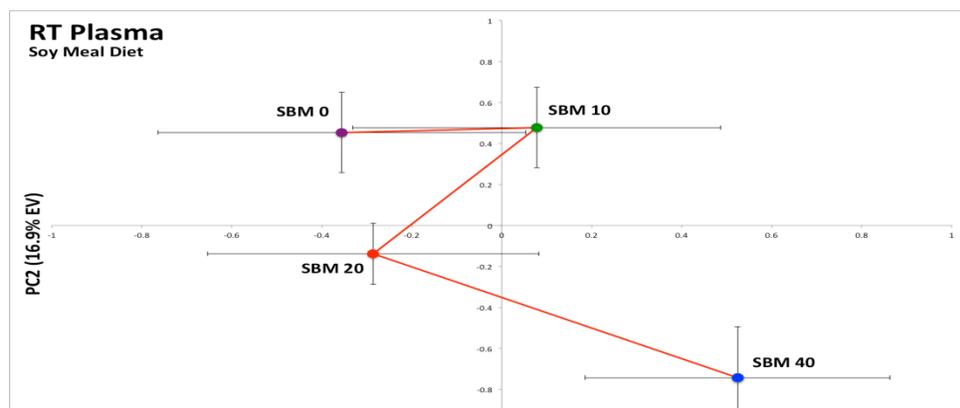


Figure 1 - PCA model of the Rainbow Trout data. Error bars are +/- 1 SEM.

connected with a solid line so that any diet-dependent pattern would be distinguishable.

The analysis shows that at T=84 days, there was no significant difference among the experimental diets in the PC1 axis; however a soy-content trend is apparent in PC2 (16.9 % explained variance) which should be further explored.

The plasma PCA model for the red drum study at the terminal point explains 64.8 % of the variance in the data set using PC1 and PC2, down slightly from the T0-included model (67.6 %) possibly linked to the reduced number of samples in the model. In this figure (Figure 6), the sequential diets with increasing SBM are connected with a solid line so that any diet-dependent pattern would be distinguishable. The analysis shows that at T=84 days, the two lower soybean meal diets are distinct from the two higher soybean meal diets.

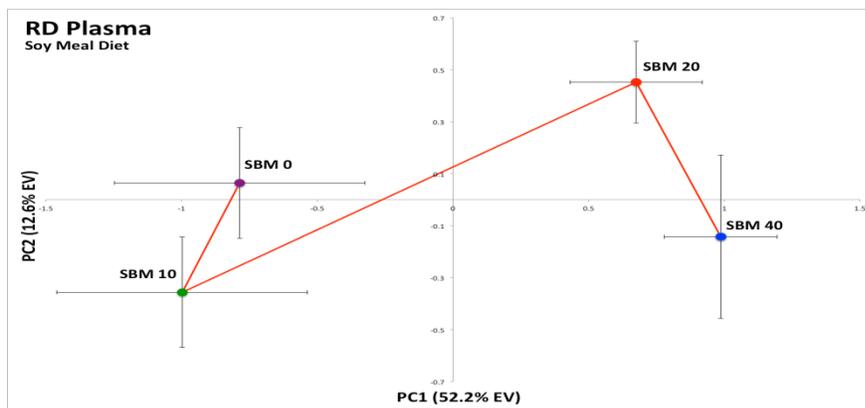
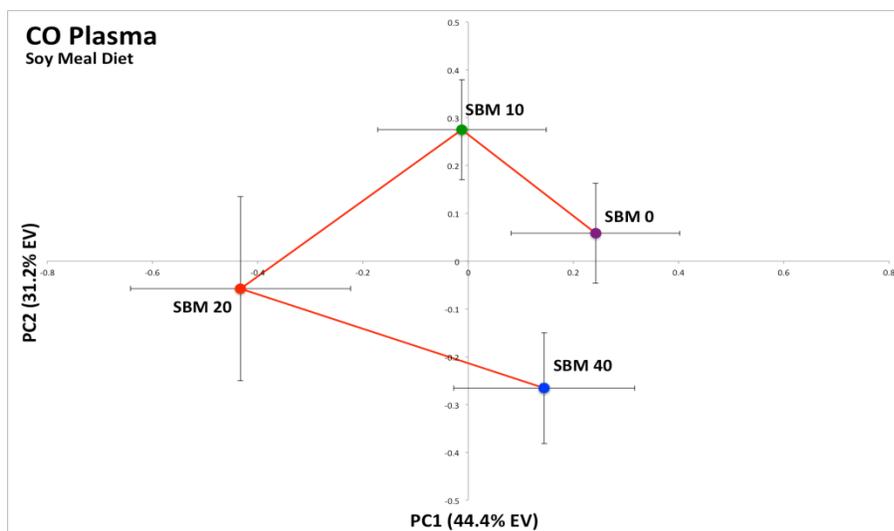


Figure 6 - PCA model of the Red Drum data. Error bars are +/- 1 SEM.

The plasma PCA model for the cobia study explains 75.6 % of the variance in the data set using PC1 and PC2. In Figure 7, the sequential diets with



increasing SBM are connected with a solid line so that any diet-dependent pattern would be distinguishable. The analysis shows that at T=84 days, there was little consistent significant difference among the experimental diets.

Figure 7 - PCA model of the Cobia data. Error bars represent +/- 1 SEM.

CONCLUSIONS

Data generated from this study highlight seven important points:

1. With respect to changes in growth and histological changes, a clinical effect (inflammatory effect) was achieved in all three species with the experimental diets used.
2. Growth and/or histological changes were correlated with increasing SBM in the diet.
3. Rainbow trout are generally considered to be more sensitive to SBM than red drum and cobia and this was generally borne out by lower feed efficiency and a larger HSI in rainbow trout compared to what was observed in the the other two species who were fed the same diets.
4. Histological examination revealed putative inflammatory effects in all three species even though growth was not apparently affected in red drum and cobia. Moreover, significant changes were observed in different histological parameters in each species suggesting mechanisms to compensate for antinutritional factors (ANFs) found in SBM may be different in each species or alternatively, different ANFs have dissimilar effects in these species.
5. Gene expression data revealed three genes which may be suitable as biomarkers for SBM-induced gastric enteritis (transforming growth factor β , interleukin 2 and calprotectin) in all three species.
6. The use of "calprotectin" as a potential biomarker warrants further investigation. In other vertebrates genes encoding calcium-binding proteins are highly upregulated with inflammation and translated into protein sequences that form a heterodimer referred to as "calprotectin". This heterodimer can represent a substantial portion of the cytoplasmic protein found in neutrophils and squamous epithelial tissue (tissue in the intestine). As such, sloughing of intestinal cells and neutrophils into the intestinal lumen carry a lot of this protein. The protein can be detected non-invasively using antibodies. This is a promising idea for non-invasive sampling in cultured fish.
7. Metabolomic analysis revealed a trend toward increasing dietary SBM inclusion in rainbow trout and differences between diets containing 0 - 10% SBM and 20 - 40% SBM in red drum. Metabolomic analysis of

cobia plasma showed no overall differences between dietary treatments. These data underscore the point that different species have different metabolic responses to dietary SBM.

Products and Events

Presentations of these data include:

The 2016 U.S. Soy QSSB Aquaculture Educational Opportunity in Villahermosa, Mexico on January 12, 2016.

The triennial meeting of the World Aquaculture Society in Las Vegas, NV on February 22-26, 2016. Three separate abstracts were presented (abstracts are attached in Appendix).

The International Symposium on the Feeding and Nutrition of Fish to be held in Sun Valley, Id on June 5-10, 2016. (abstract is attached in Appendix)

APPENDIX

NMR-BASED METABOLOMICS FOR FINFISH FEED STUDIES

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The biochemical effects on aquaculture species fed various ratios of ingredients such as protein or lipid sources or alternative novel ingredients that are meant to improve growth or health status can be assessed in numerous ways. The metabolome is the suite of endogenous, small-molecule metabolites related to biochemical pathways as the products and agents in the fully expressed gene and protein biochemistry of living organisms. It has been postulated that this particular 'omic' measure (metabolomics) is strongly related to the expressed phenotype and could hold the keys to 'discovery' of novel interactions and responses to varying feed formulations. The operating hypothesis of this approach is that the instantaneous metabolite profile reflects the physiological status of the organism and a thorough understanding of the metabolome and its dynamics can lead to diagnostic insight for individuals and populations that could result in optimal feed development, faster growth, and reduced disease. Metabolomics can help investigate broad-based, unbiased biochemical responses to all types of stimuli.

This presentation will give some background details of the nuclear magnetic resonance (NMR) based metabolomics measurement approach for the assessment of small-molecule metabolites in tissues and biofluids. Practical examples from studies that we have undertaken across multiple species using tissue-specific analyses with several alternative diet formulations will illustrate the utility of this novel approach.

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180

NUTRIGENOMIC BIOMARKERS F



EFFECTS OF GRADED LEVELS OF DIETARY SOYBEAN MEAL INCLUSION ON GROWTH OF COBIA *Rachycentron canadum*

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The demand for quality seafood continues to increase while production from natural fisheries have stabilized over the last few decades requiring aquaculture to increase in production to meet this increased demand. Aquaculture raised fish are typically fed diets high in fishmeal as its primary protein sources which are harvested from these wild fisheries. Research utilizing alternative protein sources are being implemented for many aquaculture species both to ensure sustainability of these wild fisheries as well as to allow producers efficient and cost-effective choices for fish feeds. Cobia is considered a great candidate for aquaculture by scientific and industry professionals due to its high growth rates and excellent flesh. Cobia are an upper trophic level carnivore requiring moderate levels of crude protein typically obtained from fish meal. This research uses graded levels of soybean meal (SBM) to determine inclusion levels which optimize growth and feed conversion in cobia. A natural diet was used as a positive control of optimum growth to compare to pelleted diets. A 12-week feeding study was conducted on juvenile (initial mean weight, 93.3 g) cobia utilizing four experimental pelleted diets: a 0% SBM control diet, a 10% SBM, a 20% SBM, a 40% SBM,. Fish (n=20) were held in fifteen 1,500 L recirculating tanks, with three randomly assigned replicate tanks per diet, and fed twice daily to satiation. Weight gain, feed conversion ratios (FCR), standard growth rate (SGR) and intestinal morphology were compared between treatments at the conclusion of the trial to determine effects from increased SBM. Growth rates were not statistically different between treatments suggesting diets including up to 40% protein replaced by soybeans are viable options for cobia diets. Growth rates of cobia fed pelleted diets were comparable to natural diets.

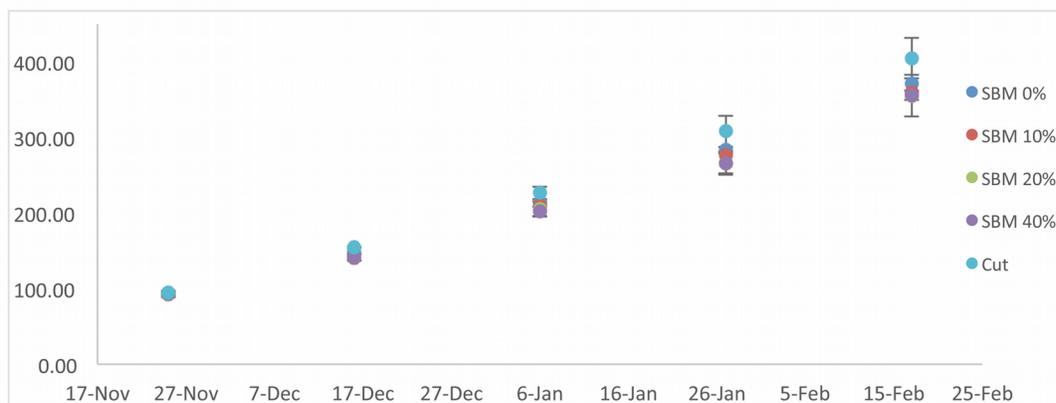


Fig 1 Red drum average weight per fish in grams for each treatment throughout the 12 week study.

COMPARATIVE DIETARY SOYBEAN MEAL UTILIZATION IN RAINBOW TROUT, RED DRUM AND COBIA

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Intolerance to dietary soybean meal (SBM) has been assessed in several aquaculture species. In this study we assessed physiological and metabolic responses of rainbow trout (*Oncorhynchus mykiss*), red drum (*Sciaenops ocellatus*) and cobia (*Rachycentron canadum*) to graded dietary SBM inclusion (0, 10, 20 and 40%) and correlated conventional growth performance response variables with gene expression and metabolomics. Results provide comparative data for three species that vary in SBM intolerance when fed the same diet. Both FCR and feed intake were affected in rainbow trout and cobia along with energy retention depending on dietary SBM level. In red drum, feed efficiency decreased with increasing SBM level in the diet. Histologically rainbow trout showed the greatest intestinal changes as SBM inclusion in diet increased. Red drum and cobia had inflammatory changes more confined to focal areas of the gut with reduced vacuolization and changes to submucosal width and cellularity. Expression of C-reactive protein, heat shock 70 protein or superoxide dismutase in the liver did not correlate with SBM inclusion in any species. However, expression of interleukin-2, tumor necrosis factor α and transforming growth factor β did correlate with increasing SBM in the intestine.

Metabolomic patterns generated using NMR methods varied across species with no significant difference observed in rainbow trout (Figure 1a) or cobia (not shown) but significant variation observed in red drum (Figure 1b). These comparative data indicate physiological and metabolic interactions do not necessarily follow the same pattern for each species even though diet formulations were the same.

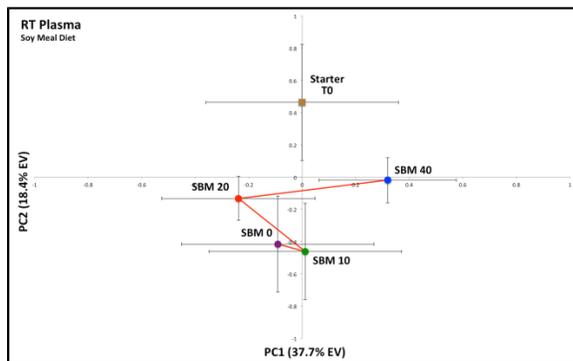


Figure 1a. Metabolomic profile of rainbow trout (RT) fed diets containing graded levels of SBM for 12 weeks.

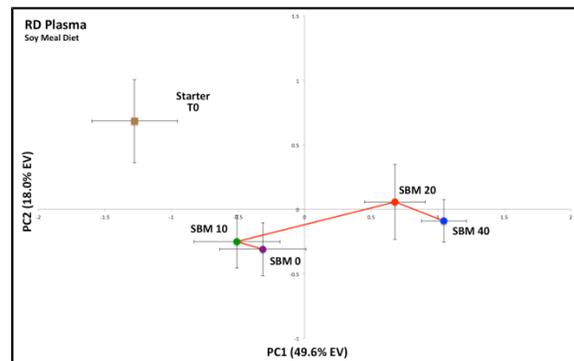


Figure 1b. Metabolomic profile of red drum (RD) fed diets containing graded levels of SBM for 12 weeks.