| | |
|---|---|
| **Project Number:** | USB #1830-352-0501-J |
| **Project Title:** | Effects of dietary soy replacement on burbot (freshwater cod) growth and immune function |
| **Organization:** | University of Idaho; Department of Fish and Wildlife Sciences |
| **Principal Investigator Name:** | Dr. Ken Cain |

**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.

Project design summary: This SAA project focused on fishmeal replacement with soy protein products for a novel and upcoming aquaculture species, burbot (freshwater cod). The project aimed to evaluate fish growth performance with 25 and 50% fishmeal protein replacements with soybean meal (SBM), a commercial soy protein concentrate (SPC), and a bioprocessed soybean meal (BSBM), both at the juvenile and subadult life stages. Following the juvenile growth trial, burbot were challenged with a common aquaculture pathogen of interest, *Aeromonas* spp, to evaluate and diet-related benefits to burbot health. Following the completion of the juvenile trial, soy protein digestibility metrics were evaluated and the two top performing diets from the juvenile trials (25% SBM and 25% SPC) were provided to subadult (grow-out stage) burbot to compare growth performance.

Specific project accomplishments:
*Objective 1. Determine palatability and digestibility of soy protein ingredients in burbot*

A standardized digestibility trial was performed using adult burbot and a 70/30 diet formulation that allowed for ingredient digestibility to be computed (Hardy and Barrows, 2012). Data were analyzed for the apparent digestibility coefficients (ADCs) of dry matter (DM), protein (P), lipid (L), and energy (E) for the trial diets. The calculated results are:

| Metric (%) | Reference | SBM | SPC | BSBM | *P*-value |
|---|---|---|---|---|---|
| **ADC-DM** | 66.6±0.8$^{a}$ | 62.8±0.010$^{b}$ | 65.8±0.5$^{ab}$ | 65.0±0.5$^{ab}$ | ***0.028*** |
| **ADC-P** | 81.1±0.4$^{c}$ | 84.4±0.4$^{ab}$ | 84.7±0.3$^{a}$ | 83.1±0.3$^{b}$ | ***<0.001*** |
| **ADC-L** | 88.8±0.9 | 83.9±1.5 | 88.1±0.3 | 87.2±1.3 | ***0.053*** |
| **ADC-E** | 77.6±0.7$^{a}$ | 73.5±1.0$^{b}$ | 77.5±0.4$^{a}$ | 75.9±0.5$^{ab}$ | ***0.009*** |

SPC ingredient protein ADC (93.5 %) in fish was the highest level compared to other treatments and is followed by SBM (92.3%) and MESBM (88.1 %) diets. The SPC ingredient protein ADC was significantly higher than MESBM digestibility, though there were no differences in ingredient digestibility between SPC and SBM diets. Trial results demonstrated the greatest protein digestibility in the soy protein concentration (SPC) group, and clearly demonstrate separation from the reference diet for the soybean meal (SBM) and bioprocessed soybean meal (BSBM). Lipid and dry matter digestibility were found to be overall decreased for the plant-based products, although not significantly for the lipids. Energy digestibility was lowest for the SBM treatment group.

*Objective 2. Investigate growth performance in juvenile (age 1) and subadult (age 1) burbot fed soy protein sources at varied fishmeal protein replacement rates*

Juvenile growth performance trial: The juvenile feeding trial ran for 91d, and took place from Dec 2018 through March 2019. Diet-related differences were detected in relative growth ($P$=0.012) and specific

growth rate (SGR; *P*=0.017). For relative growth, the fishmeal replacement with 25% soybean meal (SBM; 176.6%) was found to have outperformed the bioprocessed soybean meal (BSBM) diets, as well as the high-level FM replacements with SBM and soy protein concentrate (SPC). Similar findings were found for SGR, with the SBM-25% and reference diets outperforming the other FM replacement groups. No differences were detected in the average weight per fish across the treatments (*P*=0.055), but the SBM-25% group did demonstrate the highest individual weights (90.4g). Analysis of FCR did not yield significant differences (*P*=0.055), but the 25%-SBM replacement showed the best conversion (1.17), while the 50%-BSBM FCR was not as favorable (1.89). Survival was not significantly different throughout the trial period (*P*=0.533) and final average tank survival across all diets was 92%. There appeared to be no dietary impacts on juvenile burbot anatomy, as discerned through an evaluation of organosomatic indices post-feeding trial. There were no differences in visceral weight (*P*=0.171), visceral fat (*P*=0.393), liver size (*P*=0.642), fillet yield (*P*=0.595) or spleen size (*P*=0.645). Thus, it can be inferred that the soy protein replacement did not change the overall fish morphology and provided fillet yields that were comparable to the Reference diet. Burbot whole-body proximate analysis (feeding trial endpoint) results were completed and did not yield any diet-related compositional differences in protein (*P*=0.410), lipid (*P*=0.226), energy (*P*=0.141). Thus, study results demonstrate that a 25% fishmeal replacement level with traditional soybean meal is appropriate for juvenile burbot.

Subadult grow-out trial: Based on these final growth study results, the reference diet, and 25% FM replacements with SBM, and 25% FM replacement with SPC were used as dietary treatment groups for the subadult grow-out trial. The team began the final grow-out, subadult feeding trial, on July 16$^{th}$. Nine flow-through tanks were stocked with fifty 200g burbot, temperatures are 14°C, and photoperiod is 12 hours dark 12 hours light. The tanks are divided into three treatment groups, 3 tanks receiving a base reference diet, 3 tanks receiving a diet with 25% of the fishmeal replaced with soy protein concentrate, and 3 tanks receiving a diet with 25% of the fishmeal replaced with soybean meal. Initially burbot were fed based on bodyweight, however due to wasted feed and poor feed conversion ratios the tanks are now currently handfed to satiation twice a day, and the study period was increased in duration. Sixty days into the handfeeding portion of this study feed conversion ratios are as follows, 1.73 for the SPC diet, 1.92 for the SBM diet, and 1.81 for the Reference diet. Also, relative growth at sixty days is 10.2% for soy protein concentrate, 8.09% for soybean meal, and 9.4% for the reference group. SGR values at sixty days were 0.17g/day for the SPC diet, 0.13g/day for SBM, and 0.16g/day for the reference diet. This trial will be completed on November 26$^{th}$ 2019 (final weigh-day), and full growth results will be reported in a separate manuscript from the juvenile/disease work, along with the digestibility findings.

*Objective 3. Characterize gastrointestinal health, disease susceptibility and immune function in burbot fed soy protein sources*

The juvenile burbot were challenged at 24h following the completion of the growth performance trial. For challenge, 15 fish from each growth trial tank were transferred to 19L tanks and challenged with a virulent dose of *Aeromonas* sp. (Strain 141; $OD_{600}$=0.164 and 1.3x10$^8$ CFU/fish) for 28d at 15°C. Mock fish for each dietary treatment groups were also sham-challenged with sterile PBS as served as a control. Final cumulative mortality was analyzed at the end of the trial and no diet-related differences were found (*P*=0.170), although the SPC-25% and BSBM-50% FM replacements yielded the highest mortality of the dietary groups. Analysis of distal intestine samples using a previously established scoring system for Atlantic cod (Colburn et al. 2012) revealed no differences in lamina propria thickness (*P*=0.489) or cellularity (*P*=0.979), and the amount of connective tissue of the submucosa was found to be similar (*P*=0.972), indicative of no diet-related complications. Sera lysozyme levels were not detectable pre-challenge, but showed some small lysozyme levels post-challenge, although not across dietary treatment groups (*P*=0.975). These low basal levels of circulating lysozyme have been previously reported in Atlantic cod (Caipang et al. 2009), so burbot appear to have a similarly low innate lysozyme response. No treatment differences were detected in burbot hematocrit (*P*=0.972), but hematocrit levels post-challenge were found to be elevated (*P*=0.010). Head kidney-derived macrophages were cultured prior to and

following challenge trial completion to observe any changes in respiratory burst potential (RBA; leukocyte killing ability). No dietary differences in RBA were observed pre-challenge (*P*=0.887) or post-challenge (*P*=0.307). In summary, the soy protein sources did not appear to have any influence on survival during pathogen challenge, when compared to the reference diet group. Overall, there appeared to be no morphological changes to the distal intestine following 91d on the soy-based proteins.

**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.

The long-term goals (KPIs for this project included the:
1.*Determination of optimal soy protein inclusion rates for both juvenile and grow-out phases of burbot culture via growth performance trials.*
Results from the juvenile feeding trial discerned that a 25% FM-protein replacement with SBM or a SPC is optimal for juvenile burbot, and allows for comparable growth to a reference/traditional formulation. This information is now available for diet formulations for burbot producers and allows for a reduction in animal protein for these diets.

2.*Comparison of upgraded and enhanced soy products in burbot formulations (i.e., soy protein concentrates and fermented soy products).*
The juvenile feeding trial results, along with the digestibility data demonstrate that the traditional, unprocessed soybean meal and the soy protein concentrate were the top candidates for use in burbot formulations. These results may be explained by a more primitive gastrointestinal tract (in comparison to salmonids) and in Atlantic cod, there is a region in the intestine where fiber can be stored and fermented for enhanced nutrient uptake. Thus, they may have an affinity for more unprocessed feedstuffs due to their natural gastric function. As such, this project has allowed for a Ph.D. student in our lab to run a small-scale experiment to evaluate gastrointestinal ingredient (including soy protein) transit times (Winter 2020) to further discern these project findings.

*3.Characterization of overall burbot health and physiological/immunological response in burbot fed experimental diets containing soy products.*
Undoubtedly, support from this project has further advanced the knowledge of burbot health and immunology. The team has successfully cultured burbot head kidney leukocytes and used them in culture as a health index (i.e., respiratory burst function). Additionally, the role of burbot lysozyme (sera) and gene expression (TLRs) have been investigated, as they relate to dietary treatments. Lastly, this project has allowed for our team to successfully isolate IgM from burbot sera and we are currently working with Washington State University to develop a monoclonal antibody for use in ELISA development and this tool will be made available to burbot researchers in the near future.

**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.

**Publications:**

Bruce, T.J., J. Ma, L. P. Oliver, B.R. Lafrentz, and K.D. Cain. Disease diagnostics in cultured burbot (*Lota lota*) and experimental challenge with *Flavobacterium columnare* and *Aeromonas* sp. from a commercial production facility. *In preparation for submittal to the Journal of Fish Diseases in November 2019.*

Bruce, T. J., Oliver, L.P., Small, B.C., Hardy, R.W., Brown, M.L., Craig, S.R., and Cain, K.D. Evaluation of fishmeal replacement with soy protein sources on the growth response and health of cultured burbot (*Lota lota*). *In preparation for submittal to Aquaculture in Dec 2019.*

***Another publication presenting the digestibility results and the sub-adult growth findings will be prepared in Early 2020, targeting Aquaculture Research or the North American Journal of Aquaculture.

**Presentations:**

Bruce, T. J., Oliver, L.P., Ma, J., Small, B.C., Hardy, R.W., Brown, M.L., Craig, S.R., and Cain, K.D. Growth and immune responses of cultured burbot *Lota lota maculosa* fed varied inclusions of soy protein sources. Accepted abstract for a platform poster presentation at Aquaculture America 2020 in Honolulu, HI.

Ma, J., Bruce, T. J., Oliver, L.P., Lafrentz, B.R., and Cain, K.D. Disease diagnostics in cultured burbot (*Lota lota)* and experimental challenge with novel *Flavobacterium columnare* and *Aeromonas* spp. isolates. 2019. Platform presentation. Western Fish Disease Workshop/AFS FHS Annual Meeting. Ogden, UT.

Bruce, T. J., Ma, J., Oliver, L.P., and Cain, K.D. Immunological parameters in cultured burbot (*Lota lota*) following long-term feeding of soy protein variants and exposed to pathogenic *Aeromonas* spp. 2019. Platform presentation. Western Fish Disease Workshop/AFS FHS Annual Meeting. Ogden, UT.

Bruce, T. J., Oliver, L.P., Small, B.C., Hardy, R.W., Brown, M.L., Craig, S.R., and Cain, K.D. Evaluation of fishmeal replacement with soy protein sources on the growth response and health of cultured burbot (*Lota lota*). 2019. Poster presentation. Aquaculture America 2019. New Orleans, LA.

Bruce, T. J., Ma, J., Oliver, L.P., Gulen, S., Lafrentz, B.R., and Cain, K.D. Disease diagnostics and evaluation of immunological parameters in cultured burbot (*Lota lota*). 2019. Platform presentation. Aquaculture America 2019. New Orleans, LA.

**Students and postdoctoral researchers:**

This study supported a thesis project for Sinem Gulen, a M.S. student in Dr. Cain's (PI) laboratory. Sinem completed the digestibility work as a chapter in her thesis. Luke Oliver, a Ph.D. student in Dr. Cain's has also assisted with the subadult feeding trial and will use these data in a chapter of his dissertation. This project also supported Dr. Tim Bruce (Co-investigator), a postdoctoral fellow in Dr. Cain's lab, and Dr. Bruce assisted with project management, trial execution, immune assay development, and report preparation.

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

*Juvenile burbot feeding trials and digestibility*: No unforeseen events or circumstances impacted the project outcomes for these SAA project components. All trials were conducted as planned and yielded beneficial results for producers and indicated optimized soy protein replacement rates for burbot diet formulations.

*Sub-adult feeding trial*: The team encountered some feeding issues with the larger-sized burbot. This sub-adult life stage in the burbot lifecycle often has been characterized by a decrease in feed consumed. As such, the burbot did not appear to respond to an autofeeder providing the diets. As such, the team switched to hand-feeding and this has appeared to mitigate the issue of having excessive feed wastes. This information will also be valuable to report to producers in a manuscript form. As such, this issue caused a small delay in completing the sub-adult trial, which is now expected to end in November.

*Burbot immunology*: Overall, this project has provided a great deal of knowledge in the area of burbot immunology, which is virtually unexplored. The team did have to work to overcome some research obstacles at the bench, as this is a new aquaculture species and limited published work on their physiology. Our team experienced some difficulties in choosing the appropriate immune gene primers,

which were based on Atlantic cod. Several primer sets were evaluated and the team was able to find genes of interest to evaluate post-diet, but this took a bit longer than expected and delayed the gene expression component by a few months. Although difficult, this project component was still included and will provide valuable information to be included in the upcoming manuscript for other researchers looking to investigate burbot gene function. Another obstacle for the burbot immunology work was the selection of a monoclonal antibody to measure *Aeromonas* spp.-specific titers post-challenge. Although we had previously assessed the compatibility of a commercially-available Atlantic cod IgM monoclonal antibody, it appears that this product no longer reacts in the expected way. Since we did not see great reactivity, we took a different route and purified IgM from burbot sera and are now working with Washington State University to develop a monoclonal antibody. This will provide a new and unique tool to determine the magnitude of an adaptive immune response for burbot. As such, we were not able to complete the specific antibody work at this time but will report on agglutinating antibody titer development.

**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.

As a follow-up to our manuscripts in preparation and professional presentations, the team will also plan to share data with producers in the Pacific Northwest via some extension articles. The team is working with Gary Fornshell, the University of Idaho Aquaculture Extension Educator, to provide updates to regional producers and promote burbot aquaculture as part of a Western Regional Aquaculture Center (WRAC) project.

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

Although not originally planned in the long-term goals, this project has also allowed for:

1. The development a monoclonal antibody for burbot IgM that will be used in upcoming burbot health research.
2. A small-scale offshoot project evaluating the transit time of soy protein ingredients in the burbot gastrointestinal tract
3. An additional publication evaluating the virulence/administration route of the *Aeromonas* spp. from the pilot challenge trials