Project Title: Formulation and Assessment of a New Generation of Starter Diets for Largemouth Bass (*Micropterus salmoide*s) and Yellow Perch (*Perca flavescens*) larvae [Termination Report]

Project Period: March 1, 2017-February 28, 2019; extension to August 30, 2020.

NCRAC Funding Level: $35,000

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Extension Liaison: Alex Primus, University of Minnesota

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Reason for Termination: Completion of project objectives.

Project Objectives:

1. To raise larval LMB and YP on live rotifer/brine shrimp nauplii diets as a control and transition to commercial formulated feed (Otohime) or laboratory prepared microparticulate diets.
2. To prepare a *Pichia pastoris* (yeast) culture in order to obtain sufficient biomass of the product proven to be successful in diets for marine and freshwater fish larvae. In addition, we will express salmon trypsinogen in *Pichia* to increase the protein digestion capacity of larval fish fed with this ingredient.
3. To compare growth rate, survival, and swim bladder inflation of LMB and YP in side-by-side laboratory (OSU and UW-Madison) and practical, on-farm (Coral Reef, New Albany, OH) experiments during the larval-juvenile transition period.

Project Summary

Previous experiments provided evidence that rearing largemouth bass and yellow perch larvae on formulated diets from beginning of food intake to replace live feeds results in high mortality despite food acceptance. Therefore, we analyzed formulated diets that included a yeast species successfully used in initial feeding of other fish species, and modified this yeast to express proteolytic enzyme and facilitate digestion process. Enrichment of live food with Pichia had a positive effect on performance of juvenile Zebrafish, compared to algae enrichment alone. We found Zebrafish fed formulated dry diet at first-feeding had inferior performance to those fed live feed. Transformation of fish trypsinogen
linear DNA into Pichia was expected to increase larval fish protein digestion capacity. Salmon trypsinogen gene was cloned and transformed into Pichia, but trypsinogen protein was not expressed. In 2020, Pichia was transformed to express cod trypsinogen with fusion protein GFP, but only GFP expressed alone provided fluorescent signal. Results of this work linked failure of expression of trypsinogen to the specific Salmon transgene, and concluded lack of expression is not an issue in the yeasts themselves or the expression system used.

Technical Summary and Analysis

Objective 1.— Experiments carried out in OSU lab in 2019 concentrated on zebrafish as the surrogate, model Experiments carried out in OSU lab in 2019 concentrated on zebrafish as the surrogate, model species only due to the delay of the award in the first year of the project. In 2019 spawning season of both yellow perch (May) and largemouth bass (June) were already completed before the first culture batch of Pichia was produced. In 2020, because of the COVID-19 pandemic, travel and laboratory work was not allowed from March to August. The first feeding experiment addressed the effect of live food (*Artemia* naupli) enrichment with Pichia yeast along other commercial enrichments on zebrafish performance during juvenile stage and follow-up to the phase of maturation. Zebrafish larvae wer estocked at the density of 110-114 larvae in 6 cylindrical containers (6L) and conditions followed the procedure described earlier, 3 ppt saline, constant light and 28–29 ºC (Dabrowski and Miller (2018). During the first 7 days (5-11 days post fertilization; dpf), larvae in all containers were fed marine rotifers *Brachionus plicatilis*. At 12 dpf, larvae were divided into 3 groups (2 replicates) and transitioned to feeding with enriched Artemia, with one of 3 prepared diets; Nannochloropsis algae (NA) (Nanno 3600 Instant Algae®), baker's yeast (*Saccharomyces cerivisiae*) (SC), or live, concentrated Pichia yeast (PY). This phase lasted 3 weeks. At 34 dpf, fish from each group was divided into 3 replicates and were transferred to a recirculating Zebrafish rack system (2 L containers) and fish were transitioned to feeding dry food (Otohime B2® diet). Fish were fed 4-5 times/day based on fish biomass at 26.5-27 °C and photoperiod at 12:12 (L:D). The mean body weight for fish was 60.7, 79.7 and78.7, and survival 87, 82.7 and 85.9%, for

NA, SC and PY, respectively. Differences between treatments were not significant at this point. Following transition to dry feed differences in performance widened, and zebrafish at 91 dpf showed differences in the mean weight, 296.6,316.3 and 307.7 mg. We concluded that enrichment of live food with Pichia during larval-juvenile transition have positive effect on fish performance in comparison to algae enrichment alone. The second experiment in 2019 addressed the estimation of live feed (enriched rotifers and/or Artemia nauplii) (labelled with Fluorescent GFP-Pichia; Green Fluorescent Protein expressed) in comparison to dry formulated feed acceptance, intestinal transition time and performance (growth and survival in the similar rearing unite set-up). Larvae used in this experiment (Casper (transparent) strain, 7 dpf and wild type pigmented fish, 5 dpf, were divided randomly into 6 groups with 2 replicates (40 larvae/replicate). Larvae were subjected to six feeding treatments (T) as follows: 1. Treatment 1(Rotifer): larvae were fed on rotifers during the 2phases of experiment (Rotifers were fasted for 16 h). 2. Treatment 2 (Rotifer-P): larvae were fed on Pichia enriched rotifers during the 2 phases of experiment (Rotifers were enriched with Pichia for 16 h). 3. Treatment 3 (Dry diet-P): larvae were fed on Pichia containing dry diet (size 106-212 μm) during the 2 phases of experiment. 4. Treatment 4 (Rotifer-Artemia): larvae were fed on rotifers during the first phase, 7 days (rotifers were fasted for 16 h) then shifted to Artemia during the second phase, 7 days (artemia were starved for 17-18 h). 5. Treatment 5 (Rotifer-Artemia-P): larvae were fed on rotifers during the first phase (rotifers were starved for 16 h) then shifted to Pichia enriched Artemia during the second phase (Artemia were enriched for 17-18 h). 6. Treatment 6 (Rotifer-Dry diet-P): larvae were fed on rotifers (rotifers were starved for 16h) during the first phase then shifted to Pichia containing dry diet (size 106-212 μm). Fish were fed manually 4-5 times /day. Fish for observations under the fluorescent microscope (Nikon 80i Epifluorescent microscope) collected after 1 h of feeding (Figure 1). Green light images demonstrate intake of fluorescent marker in rotifers (A and B) and Artemia nauplii (Fig. 1C and D). The anesthetized fish from the first 3groups were taken at 8dpf and at 13th dpf (1st day of phase 1 and 2, respectively). In the second phase, samples were taken from the other 3groups (4-6) at 15 dpf (1st day of phase 2) and at 20 dpf (5 days after feeding of the designed food in this phase). After 21 dpf, larvae were counted in all the tanks for survival and samples (n = 12) were taken for measuring weight (g) and length (mm) of juveniles (Table 1). These samples were kept in -80oC to be used for the gene expression (trypsin enzyme) analysis. At the completion of this experiment significant differences were recorded in mean weight of zebrafish that indicate highly inferior performance of fish fed formulated dry feed (Table1).

Dr.Rappley's laboratory carried out studies aimed at transforming linear DNA sequence of Atlantic salmon and cod (in 2020) trypsinogens into Pichia cells. To address this objective, previously characterized trypsin-II (ST-II) mRNA sequence of salmon trypsin (CAA49678.1 trypsin II) was used to extract the full sequence of salmon trypsinogen. >XM_014155449.1, predicated Salmo salar trypsinogen II, mRNA sequence,was then used. A synthetic gene was made that lacked the secretion signal so that the trypsin would be maintained within the yeast cells rather than secreted into the medium. This gene was then inserted into a cloning vector (pCR600), and transformed into E.coli cells following the direction of (Invitrogen kit manufacturer). After that, the plasmid DNA of the recombinant clones was purified, digested SalI to produce linear DNA, and transformed into Pichia GS115. Four transformants of each construct were tested for salmon trypsinogen expression by immunoblotting using the FLAG or 6xHIS epitope tags. For constitutive expression vectors (i.e. glyceraldehyde-3-phosphate dehydrogenase(GAPDH) promoter, and translation elongation factor (TEF1), the cells were grown in 2 ml of minimum glucose medium overnight at 30oC. For constructs that use the methanol-regulated AOX1 promoter, cells were first gown up overnight in 2 ml of minimum glycerol (1%) medium at30oC. After that, cell were harvested by centrifugation and cultured in minimum Pichia base with added methanol to final concentration of 0.5, 1and 2 % and incubated at 30oC with shaking. Cloning of Salmon trypsinogen II (ST-II) gene and its transformation into Pichia pastoris were successfully achieved and confirmed by sequencing. For constructs using the TEF1 promoter, none of the transformants showed expression of the trypsinogen protein. However, expression of a GFP transgene under control of the same promoter was successfully detected. The same results were obtained when trypsinogen genes were expressed from the methanol-induced promoter AOX1. These results directly linked the failure in expression of trypsinogen to the Salmon trypsinogen transgene and not a problem in the yeasts themselves or the expression system employed. Consequently, future work will attempt to use alternate fish trypsinogens such as cod trypsinogen and cunner fish (*Tautogolabrus adsperus*) (Macouzet et al.2005). We have requested synthesis of primers. Targeted Audiences Yellow Perch operations in the

North Central Region (NCR) have all experienced difficulties in out of season reproduction and raising perch larvae in captivity, in recirculated systems. The industry has long recognized that expanding Yellow Perch culture from ponds to indoor systems would require replacement of live feeds with nutrient complete diets from larval stage to broodstock. Cost-effective starter diets or minimizing duration of live feed (rotifer and brine shrimp) use is a prerequisite of the economic viability of perch culture within NCR.

Principal Accomplishments

Through the first experiment, we concluded that enrichment of live food with Pichia during larval-juvenile transition have positive effect on fish performance in comparison to algae enrichment alone. We developed a protocol for enriching live food with Pichia yeast, and utilized an optimal rearing system. In experiment two, we developed a protocol for producing Pichia containing dry diet for first feeding and compared that to live food. At the completion of this experiment, we recorded significant differences in mean weight of zebrafish, indicating highly inferior performance of fish fed formulated dry feed (Table1). These results during the feeding trials are directly linked to the failure in the yeast's expression of trypsinogen to the Salmon trypsinogen transgene and not a problem in the yeasts themselves or the expression system employed. We anticipate that use of an alternate fish trypsinogen will result in expression of this protein, increasing performance of fish fed Pichia containing dry diet. We have thus developed a protocol for preparing this yeast with a trypsinogen transgene, though alternate sources need to be investigated, and subsequently preparing diets containing this transgenic yeast for first feeding. In 2020, we extended studies on Zebrafish and included effect of nutritional history of juveniles on the reproductive capacity of Zebrafish. Namely, Artemia were enriched with 3different products: Nannochloropsis algae, Saccharomyces cerevisiae and Pichia pastoris yeast for 24 h enrichment period. Growth performance was not significantly different among the different groups at the end of both phases (enriched Artemia phase and dry feed phase) ($P<0.050.05$). Female weight and length as well as male length were not affected by the Artemia enrichment ($P<0.05$). Larvae fed Saccharomyces cerevisiae enriched artemia showed the highest male weight compared with the other 2 groups. Pichia enriched Artemia fed as juveniles showed improved average fecundity ($P < 0.05$), fertilization rate and average spawning efficiency ($P<0.05$).

Impacts

This work has made a significant contribution to the development of formulated diets for first feeding in commercial species in order to replace live feeds. The results produced in this project provide valuable and necessary preliminary knowledge and methods for formulating dry diets containing transgenic yeast to enhance fish performance. Availability of these diets in the future, after further research and development, will make culture of Largemouth bass and yellow perch feasible year-round. Industry would be able to avoid the limitations of unpredictable weather effects on pond zooplankton and the short growing season in the NCR.

Recommended follow-up activities

We anticipate publishing these studies in peer-reviewed scientific journals in 2021. These experiments have developed a protocol that can be applied to commercial species in the future. Additionally, future work will attempt to use fish trypsinogens other than Salmon, such as cod and cunner fish, in order to achieve expression of the trypsinogen proteins in Pichia. We anticipate that successful expression of these proteins will result in greater performance of fish fed these Pichia containing diets as first food.

Table 1. Growth and survival of Zebrafish larvae at 21 dpf (initial mean weight of zebrafish larva is 0.22 mg).

Treatments	Body weight (mg)	Body length (mm)	Survival (%)
Rotifer	61.2± 5.0 [a]	19.7 ± 0.5 [a]	80.6 ± 9.1
Rotifer-P	69.1± 2.1[a]	20.4 ± 0.2 [a]	78.4 ± 3.1
Dry diet- P	8.1 ±2.6 [b]	6.8 ± 1.0 [d]	62.4 ± 2.2
Rotifer-Artemia	60.5 ±3.7[a]	17.3 ± 0.1 [b]	85.9 ± 6.6

Rotifer- Artemia-P	61.0 ±3.7 [a]	17.2 ±0.5 [b]	82.8 ± 6.6
Rotifer-Dry diet-P	16.3 ±1.7 [b]	11.6 ± 0.2[c]	89.0 ± 2.2
P-value	< 0.0001	< 0.0001	0.0334

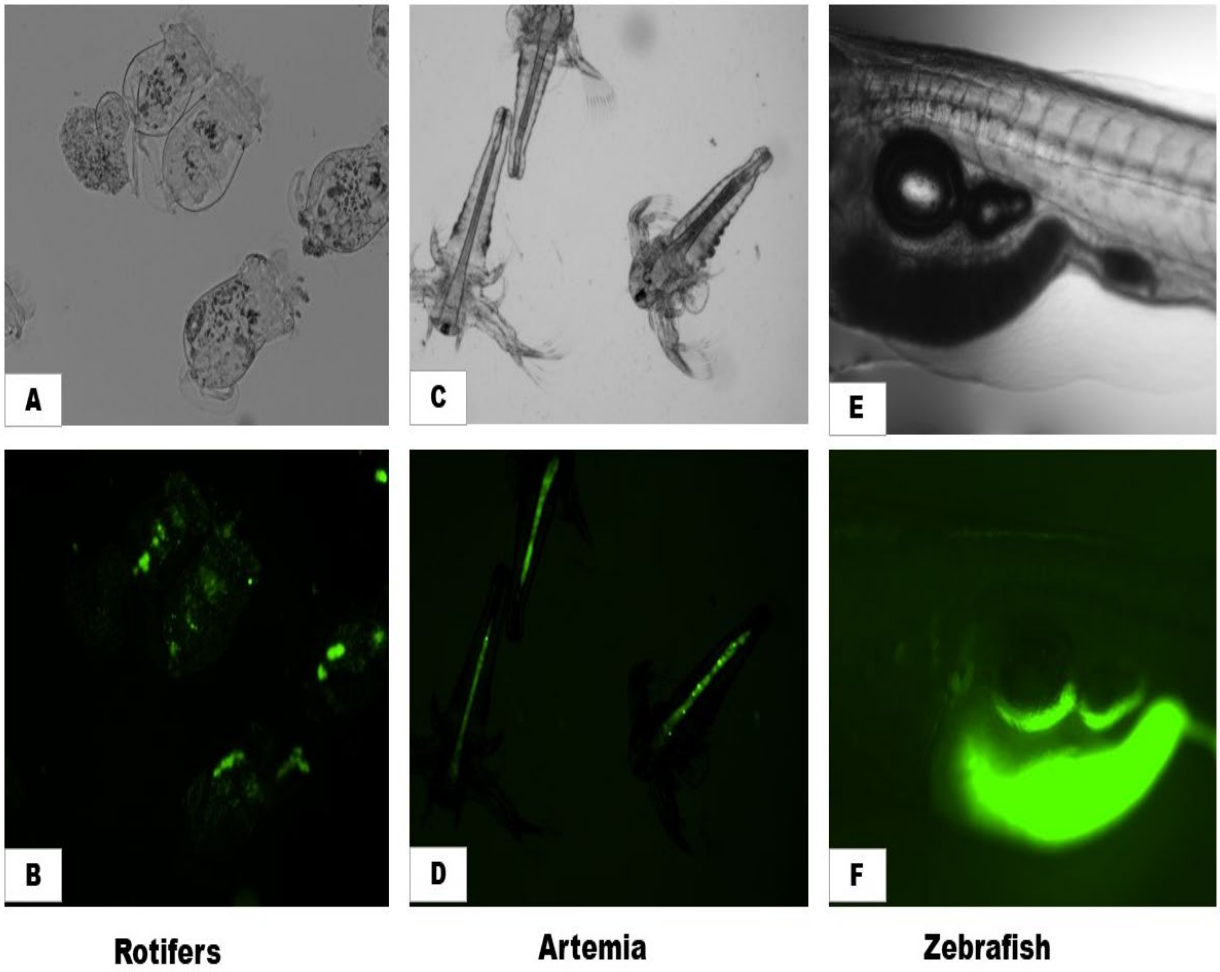

Figure 1. Enriched rotifers (A and B) which were enriched with Pichia for 16 h; Enriched *Artemia* (C and D) which were enriched with *Pichia* for 17-18 h; zebrafish larvae (E and F) as larvae was fed on rotifers during the first phase then shifted to Pichia containing dry diet during the second phase. First row of pictures is under natural light while the second row is using fluorescence. All pictures were captured using the Nikon 80i fluorescent microscope, 4x.