Project Title: Genetically Improved All-Female Walleye for Intensive Aquaculture Production in the Great Lakes Region [Progress Report]
Total Funds Committed: $225,421
Initial Project Schedule: July 1, 2019-June 30, 2021 [Extended to June 30, 2022]
Current Project Year: November 1, 2020-August 30, 2021
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Extension Liaison: Alex Primus (University of Minnesota)
Industry Liaison: Adam Hater, Jones Fish, Cincinnati, Ohio

Project Objectives

The overall goal of the proposed project is to use genetic methods to produce triploid walleye *Sander vitreus* that will result in superior growth of the female genotype (30% growth advantage over males) (Malison et al. 1990) and avoidance of nutrients expenditure associated with sexual maturation by polyploidy (sterilization). Specific objectives are to:

1. Produce meiotic gynogenetic (XX) walleye and to compare two methods (immersion and feeding) to sex reverse gynogenetic fish into neomales (sperm producing XX fish) using 17α-methyltestosterone(MT).
2. Optimize the use of pressure shocks to produce triploid walleyes.
3. Compare growth, survival, and gonad development of the following four experimental progeny groups: (a) diploid walleyes (sex genotypes: XX & XY), (b) triploid walleyes (XXX & XXY), (c) all-female diploid walleyes (XX), and (d) all-female triploid walleyes (XXX). These experiments will be conducted in raceway tanks (OSU, UW-Madison, and Reef Systems Coral FarmInc, New Albany, OH) and micro-ponds (Northey Farms LLC, Deerfield, WI).
4. Refine walleye sperm cryopreservation methods and develop a pilot cryobank for walleye neomale sperm to allow for immediate availability to research laboratories and fish farms in the North Central Region
5. Record short videos over the span of 2 years of research and extension (work on the farms) that will include all the phases of life cycle of walleye and the methods conducted in the laboratory, including production of gynogens and triploids (pressure shock), sperm cryopreservation and use in practical field conditions, and results of the project.

Project Summary

This project aims to optimize and combine feminization and triploidy to produce walleye *Sander vitreus* with superior production traits. We are working toproduce gynogenetic masculinized walleye (XX-genotype) and cross these individuals with walleye females. Resulting progenies will be pressure shocked to produce triploid (sterile) all-female offspring. Our proposed methods eliminate possibility of escaped domesticated fish interbreeding with wild stocks, thus addressing major public concerns about impact of aquaculture on conservation of aquatic resources. We will compare growth, survival, and gonad development of: triploids of female walleye x male walleye cross, control diploids of female walleye x male walleye cross, all-female triploids offemale walleye x walleye neomale cross, and control diploids of female walleye x walleye neomale cross in tanks. These techniques are likely to accelerate growth, enhance production efficiency, and improve flesh quality. The technologies developed will be immediately delivered to industry. Neomale sperm will be cryopreserved and stored in a pilot cryo-bank and will be made available to research laboratories and fish farms.

Anticipated Benefits
The technologies and resources gained from this research will directly benefit the aquaculture industry by increasing production efficiency and providing means for production of improved triploid all-female stocks for grow-out. The economic analysis included in this proposal will substantiate our predictions on the improvements gained by production of triploid all-female walleye stocks. This project will expand the production of walleye in the North Central Region by increasing the profitability of walleye aquaculture through these improved strains. This technology has proven successful in other commercial species, such as the production of all-female triploid rainbow trout, produced and sold by Troutlodge, Washington, U.S. By providing year-round availability of walleye neomale sperm, there will be a reduction in the size of broodstock operations needed by reducing the number of breeding males required to conduct out-of-season spawning in commercial facilities. There is also an opportunity for the future development of a commodity market for high-quality, validated walleye gametes for commercial hatchery use (again, the triploid rainbow trout currently produced by Troutlodge is a convincing example). There is also a major economic incentive for the production and sale of triploid all-female walleye eggs to states, agencies, and programs that don't have their own broodstocks, similar to what is currently done with rainbow trout. Troutlodge Inc, the largest trout egg producer in the word, charges$34/1000 triploid all-female rainbow trout eggs, compared to $15/1000 diploid mixed-sex eggs.

Project Progress
Objective 1. — Gynogenetic progenies were produced in spring of 2021 at OSU using gametes collected from wild, Mosquito Lake walleye and UV irradiated sperm of OSU broodstock Yellow perch. A pressure shock of 9,000 PSI applied at 4 minutes post fertilization for a duration of 12 minutes was applied to induce chromosome duplication in gynogen groups and flow cytometry analysis confirmed successful gynogen production. Gynogenetic and control sibling embryos were incubated in McDonald jars until hatching (13 days post insemination). Newly hatched larvae were then kept in flow-through troughs until free-swimming stage (4-9 days post hatching) and then stocked to nine, 50L conical tanks housed in a recirculating system (4 tanks gynogens, 5 tanks control). Conditions within the larvae culture system included elevated salinity (4-5ppt), algal turbidity, continuous availability of live food, Artemia nauplii, and use of specialized spray heads for incoming water.

After 10 days of feeding on live food, fish were sampled and split for transfer - half of the fish to UWM for grow-out and MT treatment via immersion and half to remain at OSU for MT treatment via feeding. On April 28, 2021, UWM received 864 gynogenotes, and 1,291 control walleye larvae, through a transfer from OSU. Although the fish were initially received with some mortalities due to road transport (7-hour transport time), losses to cannibalism and maladaptation to culture conditions led to not having sufficient individuals to complete the MT immersion trials as initially planned. At OSU, fish were transferred to 10gal aquaria (24 tanks, n=100fish/tank) for transitioning to dry-diet and MT treatment via feeding (Otohime B1). MT diets (30mg/kg dose) were prepared by diluting MT into EtOH and then spraying this solution on 1kg dry feed. A control diet, sprayed with EtOH, was prepared alongside. MT diets were fed ad libitum to fish for 43 days, until fish reached a mean total length of 40.5mm. Treatment groups are currently being grown-out for later determination of sex and evaluation of gonads. Survival and growth is

monitored throughout. UWM attempted to secure genetically defined strains of walleye from colleagues at UW- Stevens Point, but the enforced shutdowns due to COVID-19 emergency impeded this activity. We continued to refine our processes to cryopreserve percid semen using a controlled rate freezer.

Objective 2. — This objective was completed in the first year of the project. Further detail is given in the Year 1 progress report.

Objective 3. — Researchers from OSU used MT treated males produced in 2018 to produce potential all-female diploid and triploid progenies, as well as diploid and triploid mixed-sex progenies from non-treated males in spring 2021. Flow cytometry confirmed induction of triploidy in shocked groups. These fish are currently being grown out at OSU so that we can analyze sex ratios. Survival and growth are being monitored.

Due to COVID travel restrictions and restrictions placed on research activities, all grow-out is currently being conducted at OSU rather than on-farm. UWM will use an internally developed genome for walleye to seek gene candidates for sex-determination to assist in evaluating these objectives following the modified timeline. UWM will also perform pedigree analyses for OSU progeny and parental crosses to determine whether success in triploidy and gynogenesis is linked to maternal lineages.

Objective 4. — Researchers from UWM continued to refine our processes to cryopreserve percid semen using a controlled rate freezer and using yellow perch semen from our laboratory stocks as a proxy to walleye semen. At UWM researchers have been able to store percid semen in a -150°C freezer with post-thaw sperm cell viability of 10-15% beyond the 120-day evaluation period, with viability unaffected through 7 months in 2021.

Objective 5. — Postponed and possibly will not be done due to loss of the Extension Liaison (Primus) associated with this project.

Outreach Overview
Results of triploidy induction (objective 2), gynogenesis (objective 1), and hormonal sex reversal (objective 1) experiments conducted at OSU were presented at the 2020 Aquaculture America conference in Honolulu, Hawaii in February 2020. Results of the gynogenesis experiments were also published in the World Aquaculture Magazine within an article entitled: Sterility in Aquaculture – Advances, Performance, Impacts.

Due to COVID, there were no research presentations given at professional conferences from March 2020-November 2021. However, results of the project were shared with OSU students enrolled in the SENR 5355 Aquaculture course. With the no-cost extension granted to this project, we anticipate further dissemination of results in 2022.

Target Audiences
Fish farmers in the North Central Region, fish farmers across the U.S., aquaculture industry professionals, fisheries managers, scientists and researchers, graduate and undergraduate students.

Deliverables (Outputs)
The research conducted during 2020 and 2021 directly contributed to the education of undergraduate students enrolled in the OSU Aquaculture course during both spring semesters, as students were trained in fish reproduction, embryology, and larviculture through hands-on learning. In addition, this project provided four undergraduate interns an opportunity to gain experience in hatchery methods, fish husbandry, and research throughout 2021. The first year of this project also directly contributed to the training of three graduate students, one of which completed her doctorate degree December 2020.

We have also determined optimal pressure shock conditions for walleye meiotic gynogenesis and induction of triploidy, as well as MT treatment methods. UWM's share of this effort resulted in two graduating MS thesis students (Haley Lucas, and Sonya Ponzi), with two additional students involved in the research project as undergraduate (Emma Li Gilbertson) and graduate internship (Adam Jeschke) experiences. Additionally, resources developed through this and a previous NCRAC-funded project allowed the enrichment of four courses offered at UWM (Principles of

Aquaculture systems, Sustainable Finfish Aquaculture and Nutrition Principles, Fish Health, and Wisconsin Aquaponics: Hemp and Hops). Two MS theses were produced, and two journal manuscripts are in development for publication.

Outcomes/Impacts
Short term outcomes:
- Increased knowledge of optimized methods to obtain triploid walleye through pressure shocks
- Increased knowledge of performance (growth, survival) of mixed sex triploid walleyes in comparison to mixed sex diploid walleyes in indoor culture
- Increased knowledge of methods to obtain gynogenetic walleye through use of irradiated yellow perch or walleye sperm
- Increased knowledge and optimization of the production and performance from early life stage through adulthood of sex reversed gynogenetic walleye
- Increased knowledge of the transition from live to formulated diets and the treatment of walleye with MT via feeding method

Medium term outcomes:
- Delivery of technology developed thus far to the scientific community and industry professionals (WAS, Hawaii 2020)
- Undergraduate and graduate students gaining knowledge and understanding of this technology through participation in OSU and UWM courses and internships

Impacts Summary

Relevance. — There is a high potential for walleye to become a major contributing species to private aquaculture in the North Central Region and beyond. However, the gap in knowledge on their production potential and value have delayed the development of this species for aquaculture. Therefore, advances in research that provide solutions to the challenges associated with intensive culture, high density, formulated feeds, of walleye could result in a more profitable aquaculture industry. In addition, walleye was recently named an invasive species in several western states, thus the method of producing sterile fish is appealing.

*Respons*e. — The proposed project will specifically address the questions of sex ratio and superior growth of triploid sterile all-female walleye. During the first year of the project, we completed objective 2, optimization of pressure shocks to produce triploid walleye, and began work on objective 1, production of meioticgyno genetic walleye and subsequent hormonal sex reversal. For the first time, we are collecting data on the growth and survival of 100% female,gynogenetic, sex reversed and triploid walleye stocks and comparing them to traditional diploid mixed sex stocks, in order to quantify the value of culturing female monosex triploid walleye.

Results. — The proposed project directly addresses a major constraint to the aquaculture industry in the North Central Region and has begun providing critical knowledge, essential to the development of this new alternative fish species for U.S. aquaculture, walleye, to the professional and scientific communities. We anticipate that as this project progresses, we will gather additional knowledge, which will lead to changes in industry priorities as walleye aquaculture expands in the NCR and beyond. We have also provided graduate and undergraduate students with valuable, hands-on training in these technologies, which will aid in the project's long-term goals as these individuals enter the workforce.

Recap. — We have developed technology to produce walleye triploids through pressure shock, as well as all-female walleye gynogens and potential hormonally sex-reversed gynogens. These technologies are being further developed and refined and will be disseminated to industry after completion of the project.

Publications, Manuscripts, Workshops, and Conferences

See the Appendix for a cumulative output for all NCRAC-Funded Walleye activities.