**Project Title:** Development of an All-Female Yellow Perch Population: A Strategic Approach Using Thermal Manipulation, Sperm Selection, and Genomic Data Analysis [Termination Report]

**Total Funds Committed:** $162,261

**Initial Project Schedule:** July 1, 2017 to June 30, 2019

**Current Project Year:** July 1, 2-2017 to June 30, 2018

**Participants:** Sepulveda Villet, O.J., University of Wisconsin-Milwaukee; Dabrowski, K.E., The Ohio State University

**Extension Liaison**: Jim Held*, replaced by J. E. Morris, Iowa State University*

**Industry Liaison**: Stinton, A., RDM Shrimp, Fowler, Indiana

**Reason for Termination:** Completion of project objectives.

## Project Objectives

1. To determine the influence of temperature on gonadal differentiation in Yellow Perch of Ohio origin raised at low 14°C (57°F) or high 24°C (75°F) water temperature from fertilization until completion of sex differentiation.
2. Examine the sex ratio and growth rate of progenies sired by potentially sex reversed males (obtained from objective 1) reared in parallel groups (OSU) and separately in a "common garden" design by factorial crossing (UW-Milwaukee). Additionally, outcross performance (fertilization, survival, growth rates at 30 and 90 days, feed efficiency) will be evaluated among crosses of Ohio strain and hybrids between UW- MILWAUKEE genetically improved yellow perch x Ohio perch sperm.
3. To determine if the use of a flow-cytometry-based cell sorting method will correctly identify and segregate "Y"- and "X"-sperm, using a fluorescent nuclear tag and differential fluorescence as separation criteria (UW-Milwaukee).
4. To characterize DNA from "X"-sperm and utilize a novel yellow perch genome to identify putative sex-linked markers that can be used to increase efficiency of cell- sorting or other molecular-based sperm selection methods (UW-Milwaukee).
5. To optimize high-throughput cryopreservation methods for yellow perch sperm and develop a pilot cryo-bank of sex-reversed ("XX") male yellow perch sperm, which will be immediately available for use by fish farmers in the North-Central region to produce all-female progenies for grow-out (OSU).

## Deliverables

1. The development of standardized methods for collection, extension, cryopreservation and distribution of yellow perch semen.
2. A technique of thermal manipulation that will result in sex-reversed male yellow perch, which produce all-female progenies when crossed to female yellow perch.
3. The identification of putative sex-determining gene(s) for yellow perch.
4. A method to screen and select sperm, as a strategy to produce monosex lines.
5. Primary, peer-reviewed literature highlighting our research products.
6. Technical white paper(s) on collection techniques and use of cryopreserved semen in commercial fishfarms.
7. A web-based outreach and training program for the use of cryopreserved semen in commercial farms.

## Project Summary

The problem addressed by this research project is the lack of analytical and research tools needed for development of a sustainable method to produce all-female fingerlings, and to reliably preserve semen from genetically improved and sex- reversed yellow perch. The technologies and resources gained from these efforts will benefit the aquaculture industry by increasing hatchery efficiency and enabling the production of all-female populations for grow out. This research proposal directly addresses USDA/ NCRAC targeted research area (TRA) A-1: "Reproduction/ Early life history", with activities for "Broodstock quality/ management" and "Monosex production", as well as TRA A-5: "Enhanced Growth Technology" through activities for "Improved strains". This project will increase the number of aquaculture facilities dedicated to yellow perch aquaculture by increasing the year-round availability of yellow perch gametes, and will reduce the scope and size of broodstock operations by reducing the number of male breeder fish required to supplement out of cycle-spawning in commercial facilities. The increased availability of yellow perch gametes will also develop a commodity product, similar to that found in porcine, cattle and poultry industries, with the sale of high quality, pedigreed and validated gametes for commercial hatchery use. The development of molecular sex markers will allow further development of mono-sex yellow perch strains, which will reduce operational variation in fish size ranges, and reduce operation costs through the abatement of labor-intensive processes, such as size grading and sorting.

Finally, this project will secure the availability of yellow perch fingerlings by facilitating the storage and availability of locally-adapted genetic resources found in the Great Lakes region and beyond.

## Technical Summary and Analysis

*Objective 1.* – To determine the effect of temperature on gonad differentiation in Yellow Perch, 11 full-sibling progenies were produced at OSU in April 2018. Each sibling group was divided to two individual tanks in two separate recirculation systems and reared at low (14°C; 57°F) and high (27°C; 81°F) water temperature. Fish were exposed to these two different temperatures from the start of exogenous feeding until presumed sex differentiation was completed (mean total length of individuals reached approximately 30mm; 1.2in). Growth and survival were monitored throughout (56 days at high temperature, 144 days at low temperature) and samples were taken once fish reached target body size for histological analysis of gonad formation. Temperatures were then adjusted to follow seasonal variation for continued grow-out.

We attempted to determine sex ratio externally in May 2019. However, due to average small size of fish from high ([22.9±10g; 13.4±1.7cm] [0.8±0.4oz; 5.2±0.7in]) and low ([6±2g; 8.3±1.1cm] [0.2±0.1oz; 3.3±0.4in]) temperature groups, not all fish had reached externally identifiable sexual maturity, such as release of sperm. We observed male-biased sex ratios in several high temperature groups (Table 1). Spermiating males from low and high temperature groups were chosen randomly to fertilize eggs and identify possible neomales (Objective 2.) Due to the potential presence of neomales without open sperm ducts, these fish continued to be grown-out for final determination of sex ratio externally, January 2020.

*Objective 2.*– Extended samples of OSU Yellow Perch semen were received by UW-Milwaukee in 2018, to establish a common protocol of cross-strain fertilization. Semen was collected from OSU normal males. OSU staff macerated testes from putative neomales, and UW-Milwaukee Choptank strain males were also used. Semen samples were distributed to small sections of freshly spawned eggs (UW-Milwaukee Choptank strain) to allow for fertilization. Data on the number of non-viable eggs, eggs per 2.5cm (1in) grid, and fertilized eggs after 2 hours were collected. Fertilization was low for both the OSU normal males, and OSU putative neomale semen samples, ranging from 0-10.35% fertilization. Use of fresh semen, stripped from control males, resulted in high fertilization (81.86-93.08%, n = 25), underscoring a need to improve methods and protocols for the transportation of cryopreserved and extended semen samples.

In spring 2019, spermiating males from high and low temperature groups at OSU were randomly chosen for fertilization trials in order to produce progenies and at later staged to identify potential neomales produced by methods previously described in Objective 1. The number of spermiating males across high and low temperature groups at OSU in April 2019 was low, and males only gave small volumes of sperm. In addition, egg quality of OSU perch females was low due to unsuitable spring water temperatures (accelerated temperature increased from 10 to 18°C [50 to 64°F]), resulting in low hatching success across all progenies produced. Fifteen progenies from thirteen different 2018 males (from five high temperature and one low temperature 2018 groups) were divided and stocked to individual aquaria in three recirculation systems. Progenies are currently being reared in high (27°C;

81°F), mid-range (23°C, 73°F), and low (14°C; 57°F) temperatures.

In June 2019, average weight of fish in high temperature (19.3±14.2mg; 0.3±0.2gr) was significantly ($p<0.001$) greater than mid-range (14.7±11.3mg; 0.2±0.2gr) and low (11.7±3.7mg; 0.2±0.05gr) temperature groups after 14 days of rearing at target temperatures. Average length of fish in high (13.2±2.3mm; 0.5±0.1in) and low (12.9±2.9mm; 0.5±0.1in) temperature groups were significantly ($p<0.0001$) greater than in low temperature groups (12±1.1mm; 0.5±0.04in) after 14 days of rearing at target temperatures. After 42 days of rearing at target temperatures, in July 2019, average weight of fish in high (69.4±34mg; 1.1±0.5gr) and mid-range (66.5±30.9mg; 1±0.5gr) temperature groups were significantly ($p=0.0057$) greater than in low (54.8±21.3mg; 0.8±0.3gr) temperature groups, but not significantly different from each other. Average length of the high temperature group (19.7±2.5mm; 0.8±0.1in) was significantly ($p=0.0448$) greater than the low temperature group (18.8±2.1mm; 0.7±0.08in), but not the mid-range group (19.5±2.4mm; 0.8±0.09in) at 42 days. Average survival of the low temperature group (67.6%) to 14 days of rearing at target temperature was higher than average survival in the mid-range (62.3%) and high (56.2%) temperature groups. Average survival at 42 days of rearing was highest in the high temperature group (63.8%), followed by the mid-range (57.3%) and then low (46.1%) temperature groups. This suggests that rearing at the high and mid-range temperatures accelerated growth of fish and did not have a significant impact on survival. These temperatures may not only produce XX-neomales, but ensure that neomales reach sexual maturity before fish reared in lower temperatures. This may lead to participation in spawning as 1- and 2-year olds, respectively.

Progenies will continue to be reared in target temperatures until the end of August 2019. Fish will then be reared in seasonal temperatures for grow-out until reaching sexual maturity in Spring 2020. Fish will be dissected at this time to determine sex ratio of each group. It will allow us to conclude which sires, produced in 2018 (Objective 1), are XX- neomales.

*Objective 3.* — Two fluorescent nuclear dyes were identified as compatible with freshly collected yellow sperm cells, and compatible with two flow-cytometry platforms, Thermo Fisher Scientific's Attune NxT flow cytometer, and BD Facsaria III Fusion flow cytometer with cell sorter. While initial trials were unable to distinguish distinct populations of preserved sperm cells, we will continue trials to determine appropriate methods to sort sperm cell populations based on nuclear density and volume, following methods used in cattle to separate male- from female-bearing sperm cells.

*Objective 4.* — A high resolution nuclear genome of the yellow perch has been completed, using a number of resources, including existing transcriptome data from USDA-ARS, as well as new data generated in Illumina Hi-Seq and Pacific Biosciences RSII analyzers. Total read coverage of the new genome exceeds 87x coverage, with a putative size of 1.1Gbp. Additionally, a histone-based spatial scaffold has been constructed using Dovetail Genomics' HiC method. This resulted in an annotated scaffold containing 24 likely chromosomes, matching already known karyotypes for yellow perch. Predicted protein transcripts identified at least 7 likely sex-determination genes also observed in other teleost fishes. Further analyses will determine if these genes are concentrated in a specific putative chromosome. To fulfill this effort, a research consortium was developed among researchers from UW-M, USDA- ARS, Mississippi State University, and with contract work from Dovetail Genomics. This high-resolution genome comprises annotated gene sequences, predicted protein transcripts, as well as spatial and putative chromosome-level information (derived from Dovetail Genomics' HiC method).

*Objective 5.* — UW-Milwaukee staff have collected sperm from March-spawning male broodstock, and have processed it as described in Miller et al. (2018). A portion of these samples for long term storage (beginning the cryobank at UWM, 25 individuals collected from two strains, (50 individuals total), and another portion will be used for the fertilization trials in March. OSU staff are currently conducting a literature review on cryopreservation of percid sperm. This literature review is part of the funded PhD student's dissertation and is expected to be completed by December 2019. During 2018 spawning season the amount of sperm from "XX-neomales" was not sufficient due to their body size and volume of sperm produced, to establish the depository. Potential neomales are expected to be identified once progenies have been grown- out to 5g individual mass and can be dissected to determine sex ratio. Sperm from identified neomales will then be collected in early Spring 2020 and cryopreserved following the methods of Miller et al. (2018) in order to establish the depository. These resources will be made available to the North Central Region aquaculture community. Additionally, UWM will transfer 30 fish (15 female, 15 male, F4 Choptank strain) adult brooders, as an initial broodstock exchange

between the two institutions. This will allow the development of outcrosses, additional collection of germplasm, and initiate a redundant or contingency stock, to reduce risk of accidental loss of improved strains at UWM.

## Principal Accomplishments

*Objective 1.* – Temperature manipulation was used as a possible technique to produce sex-reversed Yellow perch. External sex determination of high temperature reared experimental groups showed presence of male-biased sex ratios. Low temperature reared groups did not reach externally identifiable sexual maturation in the first year. Fish are continuing grow-out for evaluation of sex ratio when fish reach age-2 in 2020.

*Objective 2.* – Males from low and high temperature reared groups were used to sire progenies in Spring 2019 to evaluate progeny sex ratios and identify XX-neomales produced in Objective 1. Progenies are being reared in high, mid-range, and low temperature. Growth and survival monitoring of progenies showed that high temperature fish grew largest after 14 days of rearing at high temperature, compared to mid-range and low temperature groups. However, there was no significant difference in average fish weight of high and mid-range temperature from 14 to 42 days of rearing. Average survival in the high temperature groups was lowest at 14 days of rearing (56.2%), but highest from 14 to 42 days of rearing (63.8%) compared to the other groups. Results suggest warmer rearing temperatures benefit fish growth without significantly decreasing survival. Progenies will be grown-out and dissected to determine sex ratios in Spring 2020.

*Objective 3.* − The initial identification of compatible dyes and flow cytometry platforms will allow to develop common, standardized, and replicable methods to separate male- from female- bearing populations of yellow perch sperm cells, thus providing an additional method to obtain all-female cohorts of yellow perch.

*Objective 4.* − The completion of a high resolution, annotated genome marks a watershed point for development of improved strains of yellow perch, as marker assisted selection can now take place at an accelerated rate. By having this genomic resource, gene families associated with desirable traits, such as growth rate, disease resistance, sex- determination, etc., can now be more easily identified. This type of effort and product has resulted in improved strain development in other species, such as catfish, tilapia, and rainbow trout.

*Objective 5.* − The start of a yellow perch sperm cryobank at UWM and OSU establishes the beginning of a framework that will eventually allow fish farmers to reduce their broodstock sizes by obtaining cryopreserved semen from these institutions, and any future partners. This will allow more farmers to produce their own stocks, as vertically integrated broodstock production is currently not common, due to the high costs of feeding large numbers of adult breeders. By reducing those broodstocks to only females, this expense can be reduced.

## Impacts

- First data investigating the effect of thermal manipulation on Yellow perch sexual determination
- XX-neomale sperm will be cryopreserved and made available to the North Central Region farmers and researchers through a cryobank located at OSU once fish reach sexual maturation in Spring 2020
- Completed fully annotated and chromosome-mapped nuclear genome of yellow perch, with corresponding RAD panels develop to screen for sex-differential gene activity.
- Compatible dyes identified for flow-cytometry based sorting of yellow perch sperm cells in two flow-cytometry platforms.
- Development of a practical method for collection and processing of yellow perch semen, including the design and testing of a collection funnel to minimize cross contamination during specimen retrieval.
- Three graduate students supported by this grant (1 PhD student at OSU, 2 MS students at UWM), with a fourth UWM MS student participating as a collaborator in objectives 2 and 4.

## Publications, Manuscripts, Workshops, and Conferences

See the Appendix for a cumulative output for all NCRAC-funded Yellow Perch activities.