Project Title: Genetically Improved All-Female Walleye for Intensive Aquaculture Production in the Great Lakes Region [Termination Report]

Total Funds Committed: $225,421
Initial Project Schedule: July 1, 2019-June 30, 2021 [Extended to June 30, 2023]
Current Project Year: September 1, 2022-August 30, 2023
Participants: K. Dabrowski (Ohio State University), Osvaldo Johnathan Sepulveda-Villet (University of Wisconsin-Milwaukee)
Extension Liaison: Alexander 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

The first experiments established the basis for using heterologous sperm from yellow perch in order to secure homozygous walleye (XX) progeny for further steps of the work, namely mono-sex population and sex -reversed (XX) neomales following androgen treatment. Fig. 1 illustrates normal control cross, and normal hybrid between non-irradiated sperm of yellow perch and walleye eggs. Further, negative control constitutes insemination of walleye eggs with UV-irradiated yellow perch spermatozoa (fragmented, nonfunctional) resulting in production of haploid gynogens, and the final variant identical to haploid gynogen supplemented with meiotic shock to activated walleye egg preventing the second polar body (pronucleus) expulsion.

Optimization of the use of pressure shocks to produce triploid walleyes was carried out in 2019 and resulted in production of triploids in April 2019 (Fig. 2). We evaluated the effectiveness of 3 different pressure shock protocols applied 4 minutes post fertilization: 7,000psi for 40 minute duration, 8,000psi for 30 minutes, and 9,000psi for 12 minutes. The 8,000psi shocked group had a triploidy induction rate of 68.8%, while the 9,000psi shocked group was induced at a rate of 95% and had the highest embryonic survival of the shocked groups at 135 hours post fertilization (hpf, eyed-stage, 17.5%). Control, diploid walleye was 100% diploid, with survival of 11.4% at 135 hpf. The 7,000psi shocked group did not survive to hatching, and survival of the 8,000psi shocked group at the eyed-stage (135 hpf) was very low (1.5%). The 9,000psi shocked group and control, diploid fish were stocked to 37L aquaria (260 fish/aquarium) in a closed-recirculation system for first-feeding. Aquaria were maintained at 20°C, with salinity of 3-6ppt, and algal paste

(*Nannochloropsis*) provided turbidity (8-15NTU). Walleye were fed live *Artemia* nauplii as first food and remained in the aquaria system for the first 17 days of feeding. At the end the aquarium culture phase, survival of control, diploid groups (44.9±8.8%) was higher than that of the triploid groups (30.0±2.7%). There was no significant difference in mean weight between control, diploid (53.4±12.7mg) and triploid walleye (50.0±11.4mg) at the end of the aquarium culture phase (35 days post fertilization, dpf). Fish were transferred to 60L fiberglass tanks in a flow-through system for grow-out and transitioned solely to dry diet (Otohime B2) after 40 days of exogenous feeding (58dpf) on *Artemia.* No significant differences in weight between control, diploid and triploid groups were observed during this phase of rearing (Table 1). At 250dpf (December 2020), fish from each experimental group were PIT tagged and then combined and transferred to 400L tanks for common garden grow-out. Control, diploid group mean weight (23.8±7.2g) was significantly greater than that of triploid group (19.6±10.2g). Fish were measured again on July 2020. There was no significant difference in mean weight between control, diploid (51.7±9.4g) and triploid groups (48.6±14.1g). Weight data over the course of grow-out, from 28-470dpf is shown in Table 1. Fish continue to be grown-out and growth, survival, and gonad development were being monitored. We have not observed a growth advantage of triploid walleye compared to diploids from hatching through15 months of age. However, it is likely that a growth advantage will not be seen until control, diploid fish begin to sexually mature and grow the gonads, while it is likely that triploid individuals will not mature sexually, instead, continuing to invest energy in body growth.

Gynogenetic progenies were then 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.

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 is 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.

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. UWM has 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.

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 year 2 of the project, we began working on objective 1 and produced gynogenetic walleye which then underwent MT treatment to induce sex reversal. We are uncertain if these neomales will be functional, i.e. possess functional sperm ducts capable of releasing sperm. These fish are currently being grown out at OSU until we are able to determine sex ratios. We also produced mixed-sex and potential all-female, diploid and triploid progenies in order to compare growth and survival (objective 3) and are currently monitoring and growing-out these fish at OSU. Objective 2 of the project was completed in year 1. 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.

Recommended Follow Up Activities

There is a high potential for walleye to become a major contributing species to private aquaculture in the North Central Region and beyond. New genetic lines of all-female walleye and depository of neomales (XX males) with validated inheritance of monosex progeny must be performed regularly. Large scale production will necessitate creation of cryopreserved sperm (X) bank where farmers can obtain high quality (fertilizability) sperm year round. However, the gap in knowledge on the walleye production potential and value have delayed the development of this species (domestication) for aquaculture. Therefore, advances in research that provide solutions to the challenges associated with intensive culture, high density, enhanced tolerance to intermittent hypoxia, formulated feeds specifically targeting broodstock nutritional requirements such as high level of polyunsaturated fatty acids, vitamin concentration that were linked to gametes quality (C, E, A and B1) of walleye could result in a more profitable aquaculture industry. In addition, walleye was recently listed as an invasive species in several western states, thus the method of producing sterile fish (all female triploids) is appealing.

Publications, Manuscripts, Workshops, and Conferences

Papers Presented:

Miller, M., K. Dabrowski. 2023, Developing technology to induce tetraploidy in saugeye (*Sander vitreus* x *Sander canadensis*) as a means to establish saugeye aquaculture in the U.S. Aquaculture America 2023, February 23-26, New Orleans, Louisianna. (NCRAC acknowledged as funding source)

Miller, M., J. Grayson, K. Dabrowski. 2020. Induction of triploidy in walleye *Sander vitreus* and evaluation of growth differences between triploid, control, and hybrid walleye. Aquaculture America 2020, February 9-12, Honolulu, Hawaii. (NCRAC acknowledged as funding source)

Dabrowski, K., M. Miller, A. Kramer, J. Grayson. 2020. Production of the gynogenetic female walleye *Sander vitreus* and induction of sex reversal with methyltestosterone. Aquaculture America 2020, , February 9-12, Honolulu, Hawaii. (NCRAC acknowledged as funding source)

Manuscripts:

Schmitz, A.M., Sepulveda-Villet, O.J. Short term temperature fluctuations affect embryonic and larval development of yellow perch (Perca flavescens). J Aquac Mar Biol . 2021;10(4):168-176. DOI: 10.15406/jamb.2021.10.00318

Publications:

Dabrowski, K. Production of the gynogenetic female walleye and induction of sex reversal with methyltestosterone. 2020. *In:* Sterility in Aquaculture – Advances, Performance, Impacts. World Aquaculture Magazine 51(2): 51.

Sepulveda Villet, O.J. Warming climate affecting Wisconsin's fish population. Spectrum News 1. October 11 2021. https://spectrumnews1.com/wi/milwaukee/news/2021/10/11/warming-climate-trend-is-affecting-wisconsin-fish

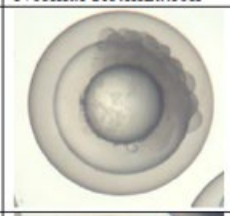
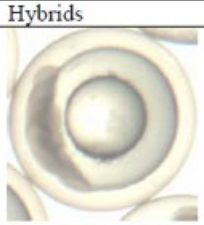
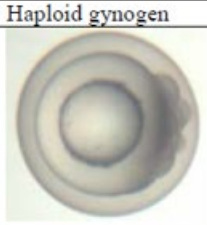
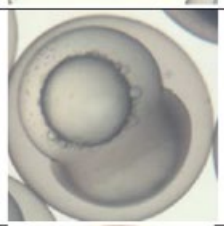
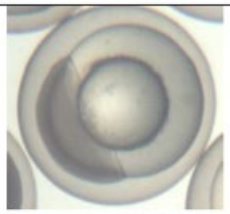
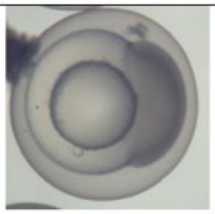
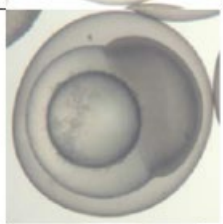
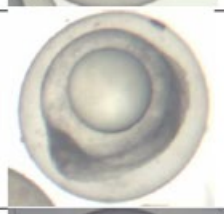
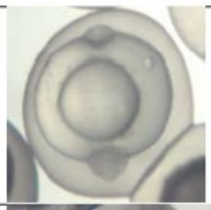
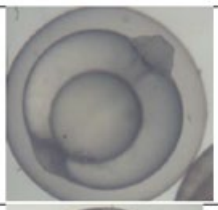
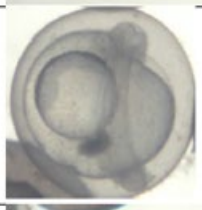
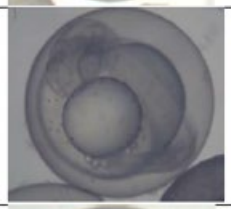
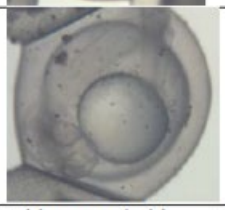
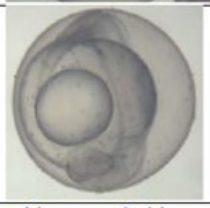
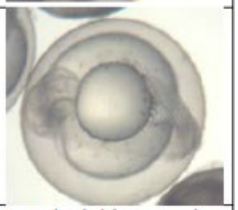
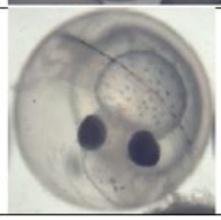
Miller, M. 2020. Induction of female monosex polyploid Yellow perch (*Perca flavescens*) and production of monosex stocks in order to increase efficiency of Yellow perch aquaculture. Dissertation. *Not yet available online.* Ohio State University, Columbus, Ohio. (December 13, 2020). (NCRAC acknowledged as funding source)

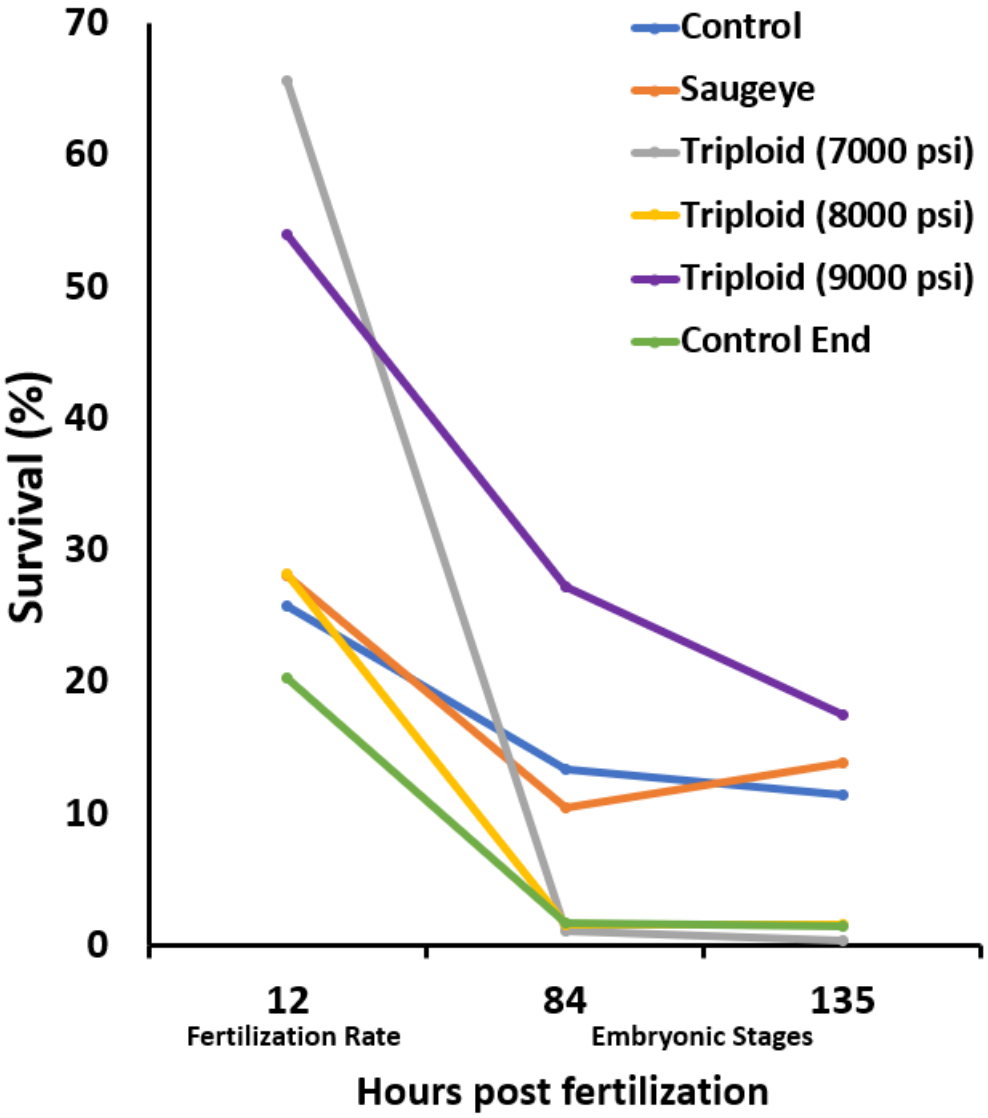
Haley M. Lucas, 2020. Analysis of plant biomass production comparing decoupled aquaponics against equivalent single-loop aquaponic and hydroponic systems growing *Lactuca sativa.* University of Wisconsin-Milwaukee, Milwaukee, Wisconsin. *Not yet available online* (August 19, 2020).

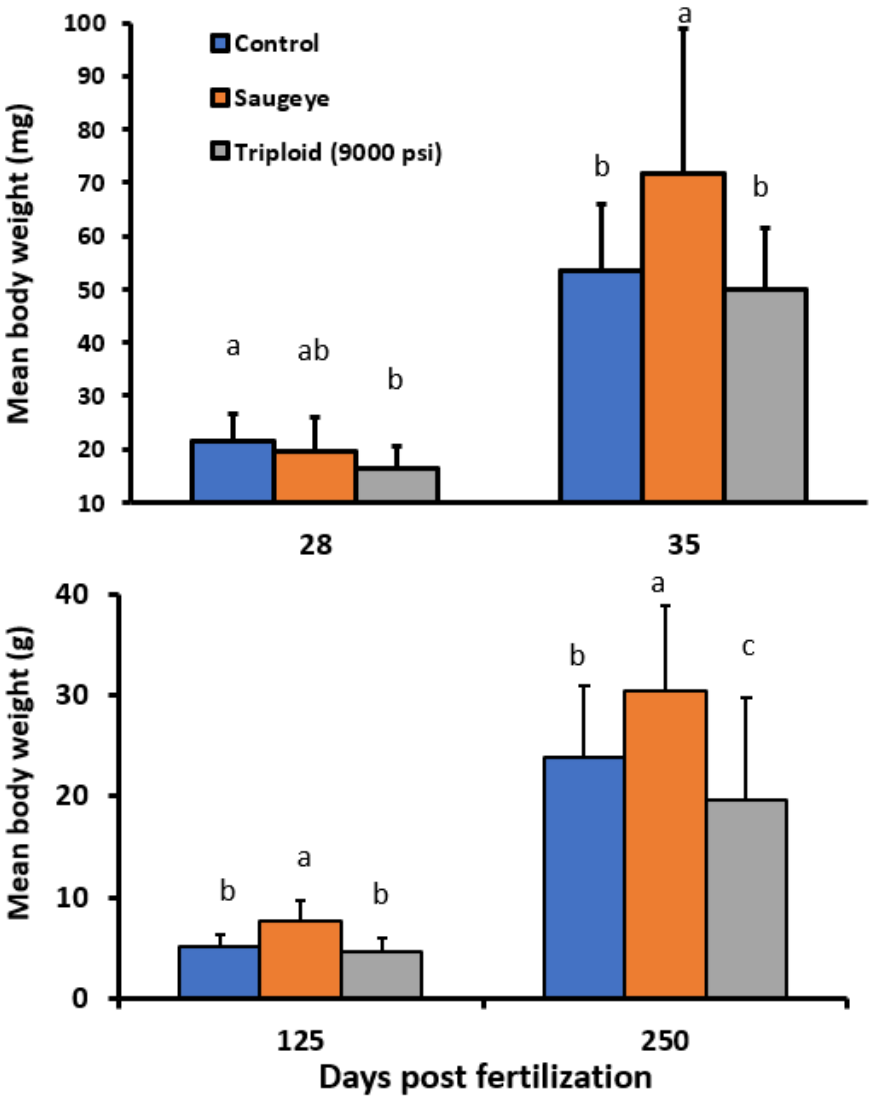
Sonya C. Ponzi, 2020. Evaluation of sperm cryopreservation for yellow perch (*Perca flavescens*) broodstock management. University of Wisconsin-Milwaukee, Milwaukee, Wisconsin. *Not yet available online* (December 19, 2020).

Table 1: Weight data of triploid and control, diploid walleye from 28 through 470 days post fertilization.

Treatment Group	Days post fertilization (dpf)				
	28	35	125	250	470
Control, diploid	21.5±5.0mg	53.4±12.7mg	5.1±1.2g	23.8±7.2g	51.7±9.4g
Triploid	16.3±4.4mg	50.0±11.4mg	4.7±1.2g	19.6±10.2g	48.6±14.1g

	Normal fertilization	Hybrids	Haploid gynogen	Diploid gynogen
Blastodisc cleavage				
Epiboly				
Head and trunk formation				
Formation of eye lenses				
Pigmentation of eye lenses		Did not reach this stage before end of experiment	Did not reach this stage before end of experiment	Reached this stage, but no pictures taken


70
60
50
40
30
20
10
0
Survival (%)
Control
Saugeye
Triploid (7000 psi)
Triploid (8000 psi)
Triploid (9000 psi)
Control End
12
Fertilization Rate
84
135
Embryonic Stages
Hours post fertilization


Control
Saugeye
Triploid (9000 psi)
Mean body weight (mg)
100
90
80
70
60
50
40
30
20
10
a
ab
b
b
a
b
28
35
Mean body weight (g)
40
30
20
10
0
b
a
b
b
a
c
125
250
Days post fertilization