Effects of Donor Size, Host and Technician Skill for Success of Xenogenesis for Sturgeon or Catfish Gametogonium Transplantation into Triploid Catfish

by

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Abstract

Xenogenesis is an innovative reproductive technology for the gamete production of biologically and commercially important species. As the primary source of caviar sold commercially, wild sturgeon populations have been experiencing declines linked to over exploitation and habitat alterations. However, cultural and demographic shifts along with increased incomes and the improved availability of premium products is leading to increased customer demand for such luxury products. An increased demand for caviar as well as pressure being placed on natural sturgeon populations provide opportunities to explore xenogenic solutions, specifically the use of ictalurid hosts as surrogates for producing sturgeon gametes. Oogonial and spermatagonial stem cells from two sturgeon species, Siberian sturgeon (*Acipenser baerii*) and lake sturgeon (*A. fulvescens*) were isolated and transplanted into white catfish, *Ameiurus catus* ♀ x blue catfish, *Ictalurus furcatus* ♂ hybrid catfish and channel catfish (*I. punctatus*) hosts. Results from this study indicate that there were significant increases in cell area ($P = 0.002$) and cluster area ($P = 0.006$) over the sampling points for white x blue hybrid catfish injected with lake sturgeon gametogonium. Moreover, a total of 88.9% of sampled fish displayed fluorescence from dyed donor cells during the post injection sampling period, indicating that the donor stem cells from this biologically important species can successfully proliferate in triploid white x blue hybrid catfish recipients. Although there were no statistically significant increases in cell area ($P = 0.702$) or cluster area ($P = 0.150$) in triploid channel catfish transplanted with Siberian sturgeon OSCs, there were still observed increases for both metrics. Furthermore, 76.7% of the triploid channel catfish transplanted with donor Siberian sturgeon OSCs sampled displayed fluorescence during the sampling period, indicating that these cells can survive in

triploid channel catfish hosts. The results from the current study suggest that white catfish may be better xenogenic hosts for increasing cell and cluster proliferation compared to channel catfish hosts.

A second study investigated the relationship between body size characteristics and live gametogonium quantity for channel catfish (*I. punctatus*). Xenogenesis is becoming a well-documented technology for overcoming the reproductive barriers to produce the valuable hybrid catfish (channel catfish ♀ with blue catfish ♂). Currently, there are limited resources available for selecting the optimal size donor fish to maximize cell quantity and quality, which is critical to increase the efficiency of xenogenesis procedures. Therefore, a study was conducted to determine the relationships between total length (T_L), total weight (T_W), and the quantity and quality of extracted gametogonium in channel catfish donors. Relationships were observed between T_W and the number of extracted live OSCs ($r^2 = 0.460$; $P = < 0.001$) as well as the T_L and number of live OSCs ($r^2 = 0.449$; $P = < 0.001$), indicating that an approximate T_W of 150 to 350 g and T_L of 28 to 38 cm yield the largest quantity of stem cells. Similarly, in males T_L ($r^2 = 0.149$; $P = < 0.001$) and T_W ($r^2 = 0.233$; $P = < 0.001$) revealed a relationship to the number of extracted live SSCs, where 20 to 40 cm and 350 to 600 g males yielded the highest number of extracted live stem cells. These observed relationships are intended to be used as biomarkers to aid in the donor selection process.

Lastly, a third study assessed the impact of technical aspects in the xenogenesis procedure on xenogen production by examining the impact of microinjection techniques as well as technician skill level. Although no significant relationships were found between low and high experience classes using the manual or automatic injectors ($P = 0.387$ and $P = 0.369$ respectively) and no significant improvements for experience classes as they became more

familiar with injection methods after subsequent repetitions (low experience class, manual and automatic, P = 0.362 and P = 0.875 respectively; high experience class, manual and automatic, P = 0.193 and P = 0.086 respectively). This is likely due to highly variable data points, but some trends were observed. Slight improvements were made in both experience classes between their first and final repetitions using the manual injector indicating technicians were becoming more proficient with more experience. Furthermore, slight decreases in injection success were noted for both experience classes using the automatic injector between their first and final repetitions. This potentially stems from blockage issues with the glass needles used in the automatic injecting apparatus. Observations were also made comparing the success of individual technicians. Technicians had higher success rates while using the manual injector compared to the automatic injector (57.5% compared to 49.4%, respectively). The two lowest experience technicians also made notable improvements while using the manual injector and automatic injector, respectively. Technicians' preference to either injection method was also examined. In 4 of 5 cases, the percentage xenogens produced was very similar between the preferred and non-preferred methods. In one case, the technician preferred the automatic injector but produced almost double the xenogens using the manual injector.

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List of Abbreviations

IUCN	International Union for Conservation of Nature
USD	United States dollar
CB hybrid	Channel catfish (*Ictalurus punctatus*) ♀ x blue catfish (*I. furcatus*) ♂ hybrid catfish
WB hybrid	White catfish (*Ameiurus catus*) ♀ x blue catfish (*I. furcatus*) ♂ hybrid catfish
PGC	Primordial germ cell
OSC	Oogonial stem cell
SSC	Spermatagonial stem cell
IACUC	Institutional Animal Care and Use Committee
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
LHRHa	Luteinizing hormone-releasing hormone analogue
MS-222	Tricaine methanesulfonate
DPH	Days post-hatch
DPI	Days post-injection
RAS	Recirculating aquaculture system
T_L	Total length
T_W	Total weight
HBSS	Hanks' Balanced Salt Solution
PBS	Phosphate-buffered saline
EDTA	Ethylenediamine tetraacetic acid
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal bovine serum

Chapter 1: Isolation and Transplantation of Sturgeon Gametogonium into the Genital Ridge of Triploid Ictalurid Catfish

Abstract

Xenogenesis is emerging as an innovative reproduction technology with the potential to be utilized as a tool for producing the gametes of biologically and economically important species. As the primary source of caviar sold commercially, sturgeon populations have been experiencing severe declines which can be linked to over exploitation and habitat alterations. However, customer demand for luxury products is increasing in relation to cultural and demographic shifts along with increased incomes and the improved availability of premium products. An increased demand for caviar coupled with the concern for natural sturgeon populations provide opportunities to utilize ictalurid hosts as xenogenic surrogates for producing sturgeon gametes. Therefore, the current study was conducted to demonstrate the potential of interspecific transplantation of oogonial stem cells (OSCs) and spermatagonial stem cells (SSCs) from two sturgeon species, Siberian sturgeon (*Acipenser baerii*) and lake sturgeon (*A. fulvescens*) into white catfish, *Ameiurus catus* ♀ x blue catfish, *Ictalurus furcatus* ♂ hybrid catfish and channel catfish (*I. punctatus*) hosts. Results indicate that there were significant increases in cell area ($P = 0.002$) and cluster area ($P = 0.006$) over the sampling points for white x blue hybrid catfish injected with lake sturgeon gametogonium. Moreover, a total of 88.9% of fish displayed fluorescence from dyed donor cells at 82-100 days post injection. This indicates that the donor stem cells from this biologically important species can successfully proliferate in triploid white x blue hybrid catfish recipients. Although there were no statistically significant increases in cell area ($P = 0.702$) or cluster area ($P = 0.150$) in triploid channel catfish

transplanted with Siberian sturgeon OSCs, there were still increases observed for both metrics and controls exhibited no fluorescence. Furthermore, 76.7% of the triploid channel catfish transplanted with donor Siberian sturgeon OSCs sampled displayed fluorescence during the sampling period, indicating that these cells can survive in triploid channel catfish hosts. Xenogenic host systems were also compared between the triploid hosts used in this study. The results from the current study suggest that white x blue hybrid catfish may be better xenogenic hosts for increasing cell and cluster proliferation compared to channel catfish hosts, however, the slower growth and lower survival of the hybrid may negate this potential advantage.

1. Introduction

Sturgeons are species of fish belonging to the Acipenseridae family originally distributed throughout the Northern hemisphere. As the primary source of commercial caviar, sturgeon populations have undergone severe declines linked to over exploitation along with habitat alteration (Zaidi and Ireland, 2008). Due to these drastic population declines, the International Union for Conservation of Nature (IUCN) listed all sturgeon species used commercially in Annex II of the CITES regulations in 1997, which required trade quotas and protection for these species (Bronzi and Rosenthal 2014). These regulations resulted in the current form of production, where caviar is almost completely produced through aquacultural operations. Being the most expensive aquaculture product with prices ranging up to 5,000 USD/kg, caviar production continues to increase with projections that indicate a maximum production of 3,000 t/year which is mostly attributed to the shift from wild capture to farmed sturgeon (Sicuro, 2019). However, customer demand for luxury products is increasing in relation to cultural and demographic shifts, along with increased household incomes and the improved availability of premium products. Specifically, the worldwide luxury food market value as of 2016 was

estimated to range between 46 and 76 billion € (roughly between 49 billion and 81 billion USD) with a projected increase of 5% (Hartmann et al., 2017). Despite the movement of production to farmed caviar, the 2009 reassessment of the Acipenseriformes status determined that the population condition of nearly all species were still declining with > 44% of species classified as more threatened compared to the 1996 assessment (IUCN Red List). This increased demand for caviar coupled with the concern for natural sturgeon populations provide opportunities to utilize technologies emerging in other sectors of aquaculture production, specifically using ictalurid hosts as xenogenic surrogates.

The catfish industry is the largest segment of aquaculture in the United States. Originally, the channel catfish (*Ictalurus punctatus*) was the primary species used for food fish production. Now, 70% of catfish production in the United States is from the interspecific hybrid channel catfish x blue catfish (CB hybrid, channel catfish ♀ x blue catfish, *I. furcatus* ♂) (Dunham and Elaswad, 2018). The CB hybrid was adopted by the industry due to the hybrid's rapid growth rate, feed conversion efficiency, tolerance concerning low levels of dissolved oxygen, improved resistance to disease, higher survival rates, improved carcass yield, as well as their ease of capture (Guidice, 1966; Yant and Green, 1975; Chappell, 1979; Tave et al., 1981; Dunham et al., 1983; Dunham et al., 1986; Dunham et al., 1987; Dunham et al., 1990; Huang et al., 1994; Wolters et al., 1996; Argue et al., 2003; Bosworth et al., 2004; Li et al., 2004; Dunham et al., 2008; Arias et al., 2012; Bosworth, 2012). Although utilization of the CB hybrid provides many advantages compared to the production of channel catfish, difficulties still exist. The most direct limitation when producing the CB hybrid is the reproductive isolation between the two parent species (Dong et al., 2017). Assisted reproduction and artificial spawning techniques have helped to mitigate this challenge, but still require the sacrifice of the blue catfish male to obtain the

sperm (Dunham and Argue, 2000). Xenogenesis is one method which can overcome the need to dispatch the valuable blue catfish broodstock males (Perera et al., 2017).

Xenogenic organisms are comprised of elements typically foreign to its species (Takeuchi et al., 2004; Takeuchi et al. 2009). Xenogenesis is a reproductive method in which successive generations differ from each other with no genetic information being transferred from parent to offspring (Dunham, 2023). The process of xenogenesis involves isolating primordial germ cells (PGCs), spermatogonial stem cells (SSCs), or oogonial stem cells (OSCs) from a diploid donor species and transplanting them into a host typically sterilized via triploidy (Perera et al., 2017; Hettiarachchi et al., 2020; Hettiarachchi et al., 2022). The majority of research concerning xenogenesis has centered on the introduction of cells from the donor to a host in the same genera. However, totipotent cells can successfully be transplanted between different families and orders of fish (Saito et al., 2010; Silva et al., 2006). PGC transplantation into developing embryos during the blastula-stage is one method to produce xenogenic fish and there has been well-documented success using zebrafish (*Danio rerio*) (Giraldez et al., 2005; Ciruna et al., 2002; Saito et al., 2008 and 2010; Lacerda et al., 2013). Another approach is to introduce isolated OSCs or SSCs into the host fish by transplantation into recently hatched triploid fry (Hettiarachchi, 2022; Perera et al., 2017). Donor cells transplanted into these young fish with undeveloped immune systems have the ability to survive and differentiate into mature reproductive organs (Okutsu et al., 2006).

Several factors determine the success and efficiency of cell transplantation. The size and life stage of the injected host appears to play a significant role in determining the colonization success of injected cells (Perera et al., 2017). Furthermore, recent studies indicate that the size

and maturity of the cell donor also significantly affects the quantity and quality of cells (Hettiarachchi et al., 2022).

The rate of gonadal development has been observed to be largely dependent on the rate of sexual development of the recipient host spp. (Rex Dunham, personal communication). Therefore, by utilizing xenogenesis, the production of sturgeon gametes using host species of Ictaluridae may prove to be a faster and more economically feasible option opposed to traditional caviar production methods while also providing opportunities for species conservation if gametes demonstrate full viability due to the earlier sexual maturity of catfish (Bates et al., 2001; Delwiche et al., 2016).

The aim of this current study was to demonstrate the potential of interspecific transplantation of OSCs and SSCs from two sturgeon species, Siberian sturgeon (*Acipenser baerii*) and lake sturgeon (*A. fulvescens*) into white catfish, *Ameiurus catus* ♀ x blue catfish ♂ hybrid catfish (WB hybrid catfish) and channel catfish hosts.

2. Materials and Methods

All experimental studies and investigations performed using animals were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.

2.1. Triploid host production

During the spring spawning season of 2022, sexually mature and visibly gravid channel catfish and white catfish females were collected from earthen ponds (0.04 ha) located at Auburn University's Fish Genetics Research Unit at E.W. Shell Fisheries Center (Auburn, AL). Gravid channel catfish and white catfish females had ovulation induced through the use of luteinizing

hormone-releasing hormone (LHRHa) implants which were intraperitoneally injected. A primary dose of 20 μg/kg body weight was administered followed by the resolving dose of 100 μg/kg body weight 12 hours later. Females were then placed into soft mesh bags and submerged in tanks (7.5 m × 0.5 m × 0.45 m) containing flow-through pond water at 26 to 28 °C with a flow rate of 24 L/min. Thirty-six hours after the primary dose, bags were then checked every 4-6 hours until eggs were observed on the bags. Once eggs were observed, the ovulated females were removed from the bags and anesthetized using a 100 ppm buffered tricaine methanesulfonate (MS-222, Ferndale, WA) solution. The female was then removed from the solution and dried thoroughly. Eggs were obtained by hand stripping into metal spawning pans containing a thin layer of vegetable shortening (Crisco, B&G Foods, Parsippany, NJ, USA).

Mature channel catfish and blue catfish males were euthanized for sperm collection using a 250 ppm MS-222 solution. The male's gonads were surgically removed, and subsequent sperm solution prepared via methods described by Dunham and Masser (2012). In brief, testes were surgically removed and cleaned using 0.9% saline solution, then dried with paper towel to remove excess solution and any remaining blood. Scalpel blades were then used to mince the testes to a fine consistency. Next, 100 μm mesh was used to filter the resulting teste/sperm mixture. The sperm solution was then diluted using 10 mL of 0.9% saline solution for every 1 g of testes used. Sperm solution was then applied to the eggs at a ratio of 2 mL of solution for every 25 g of eggs. Channel catfish sperm was used to fertilize channel catfish eggs (pure channel catfish, channel catfish ♀ x channel catfish ♂) and blue catfish sperm was used to fertilize white catfish eggs (WB hybrid catfish, white catfish ♀ x blue catfish ♂). Sperm and eggs were then gently mixed for 2 minutes to allow for adequate gamete contact time and fertilization.

A solution of Fullers' earth powder (Starwest Botanicals, Sacramento, CA, USA) was then made by adding 6 g of powder to approximately 1 L of pond water and thorough mixing. The Fullers' earth solution was added to the egg/sperm mixture and swirled continuously for 3 minutes. At 5 minutes post-fertilization the eggs were pressure shocked using a Carver press and cylindrical pressure chamber (340 mm height, 70 mm diameter). A hydrostatic pressure of 7000 psi was applied to the eggs for 5 minutes to induce triploidy.

Following the pressure treatment, eggs were removed from the pressure chamber and placed back into metal pans which were submerged in pond water containing 50 ppm $CaCl_2$ for 1 hour to facilitate water hardening. After 1 hour, eggs were moved to flow-through troughs which contained a paddle wheel and aeration. A reservoir was the source of water. Eggs were incubated in hanging mesh baskets between the paddle wheel blades within the trough until hatching.

2.2. Gonad collection

Two species of sturgeon, Siberian sturgeon (*Acipenser baerii*) and lake sturgeon (*Acipenser fulvescens*), were used during this study. Siberian sturgeon were acquired from the University of Georgia's Cohutta Fisheries Center (5350 Red Clay Rd, Cohutta, GA 30710). The Siberian sturgeon were hatched in 2006 and gonads were removed surgically on site using aseptic techniques. Seven Siberian sturgeon females with weights ranging from 1.81 kg to 9.53 kg were selected and euthanized via blunt force prior to gonad removal. Gonads were assessed based on the level of gamete maturity and gonads with minimal gamete development prioritized to increase the probability of isolating OSCs. After removal, gonads were cut into smaller pieces (~ 2.5 cm x 2.5 cm) in order to fit into 50 mL tubes. Short-term storage of the tissue was accomplished using methods described by Franěk et al. (2019). Briefly, Dulbecco's Modified Eagle's Medium/DMEM [DMEM (Corning Cellgro)] was added until completely covering the

tissue and tubes were kept on ice during transportation to Auburn, AL where they were kept under refrigeration at 4 °C.

Lake sturgeon were contributed by the Warm Springs National Fish Hatchery (5308 Spring Street Warm Springs, GA 31830). The lake sturgeon were hatched in 2021 and were delivered live to Auburn University's Fish Genetic Research Unit. Nine fish were received with weights ranging from 49.76 g to 114.13 g and total lengths ranging from 20.32 cm to 28.57 cm. Lake sturgeon were euthanized via blunt force, and gonads were surgically removed using aseptic techniques. All tools used during these procedures were cleaned with 70% ethanol prior to surgery and between samples.

2.3. Stem Cell Isolation

Two extractions were performed with the gonads collected from the two Siberian sturgeon females with the least mature gonads. The first fish selected was 5.90 kg and 55.88 cm in total length, the second fish was 1.81 kg and 45.72 cm. Gonads were removed from the cell culture media and 56.2 g of gonads were used for the first extraction followed by 112.69 g for the second. Regarding the lake sturgeon gonads, gonads from the 9 fish were pooled together due to their level of gonad immaturity to facilitate ease of processing. The gonads had a combined weight of 1.35 g. Stem cells for both species were extracted using techniques described by Hettiarachchi et al. (2020), Abualreesh et al. (2020, 2021a, 2021b), and Shang et al. (2015). After extraction, stem cells were quantified according to standard protocols (Hettiarachchi et al., 2020; Abualreesh et al., 2020, 2021a, 2021b; Shang et al., 2015).

2.4. Stem cell labelling and implantation into triploid hosts

Half of the isolated stem cells from both species were labelled using PKH26 red fluorescent cell linker (CGLDIL, Sigma-Aldrich, St. Louis, MO) which was prepared via the manufacturer's instructions. Labelled and unlabeled cells were then transplanted into 5-6 days post-hatch (DPH) channel catfish and WB hybrid catfish fry that had been incubated at 28 °C. Triploid channel catfish fry were injected with either Siberian sturgeon OSCs or Siberian sturgeon OSCs stained with PKH26 (approximately 39.3% of the injected cell suspension). Triploid WB hybrid catfish fry were injected with either lake sturgeon gametogonium or lake sturgeon gametogonium stained with PKH26 (approximately 44.7% of the injected cell suspension). Non-injected controls were generated for both triploid channel catfish and triploid WB hybrid catfish. Each triploid channel catfish treatment had six replicates containing 30 fry each, 3 non-injected control replicates of 30 fish were also kept (15 replicates x 30 fry, 450 total fry). Each triploid WB hybrid catfish treatment had three replicates containing 60 fish each, three control replicates containing 30 fish each were also kept ((6 treatment replicates x 60 fry) + (3 control replicates x 30 fry), 450 total fry). All channel catfish fry and half of the WB hybrid catfish fry for each treatment were injected individually using a 33-guage needle (outer diameter: 0.209 mm; inner diameter: 0.108 mm) with a gas tight syringe attached to a repeater (Hamilton, Reno, NV). Injection for the remaining WB hybrid catfish fry was executed using compressed nitrogen pressure injection. Glass needles used for the injections were produced using a Flaming/Brown micropipette puller (model P-97).

A Fischer Scientific 10 μL micropipette was used to load 10 μL of stem cell solution into the needles and needles were secured into a manual micromanipulator (MM 33, Märzhäuser Wetzlar). The manual micromanipulator was then attached to an MPPI-3 pressure injector

(Applied Scientific Instruments Inc.) set to 15 psi / pulse duration 3-4. The apparatus was controlled via 99% pure pressurized nitrogen gas. All injected fish received 1 μg containing ~80,000 unsorted cells of the respective cell suspensions.

2.5. Growth and survival

Following gametogonium transplantation, fry were moved to an aerated recovery container containing pond water then moved to aerated 5 L containers housed in a pond water flow-through trough with the water level kept at approximately 60% of the height of the containers to minimize temperature fluctuations. Fry were cultured at a density of 30 fish per container. Water was exchanged once per day, removing ~80% of the water during each exchange. After treating with 4 ppt salinity for 1 week, fry were moved to 60 L tanks in a recirculating aquaculture system (RAS), with both treatment and control fish having a density of 30 fish per aquaria. Total ammonia-nitrogen, nitrate, nitrite, pH, temperature, dissolved oxygen, alkalinity, and hardness were maintained within acceptable ranges of <0.5 mg/L, <15 mg/L, <0.5 mg/L, 7.0 ± 0.1, 25.4 ± 1.5 ◦C, 6.9 ± 1.0 mg/L, and 35.8 ± 6 ppm, respectively. Standard commercial catfish feeds were used (Purina Catfish fry feed; crude protein: 40%, crude fat: 4%, crude fiber: 7%, and phosphorus: 0.8%) to feed fry until satiated (3 feedings per day). Survival data was collected for 87 DPH for both treatments and their corresponding triploid controls. Evaluation of weight and length were determined at 87, 107, and 511 DPH for the triploid channel catfish and 87, 95, 105, and 492 DPH for the triploid WB hybrid catfish.

2.6. PKH Analysis

Triploid channel catfish with labelled cells injected were sampled at 82- and 100- days post injection (DPI) with 3 fish selected at random from 5 of the PKH replicate treatments.

Triploid WB hybrid catfish fingerlings were sampled at 82, 90, and 100 DPI with 3 fish randomly selected from each of the 3 replicate labelled treatments. Three control fish from both the channel catfish and WB hybrid catfish were also sampled. The fingerlings were euthanized and gonads were removed using aseptic techniques. Gonads were then prepared on sterile microscope slides for digital imaging. All tools were cleaned with 70% ethanol between samples to prevent cross contamination. Digital images were acquired using an Axio-cam 202 camera attached on a Zeiss Imager A2 microscope and analyzed using Zen Pro v.6.1 software (Zeiss, Oberkochen, Germany). Gonadal tissue samples with observed red fluorescence indicated the presence of donor derived cells. Fluorescent image analysis took place using ImageJ software where total cell area and cell cluster areas were determined. Cell areas were defined as observed fluorescence with areas < 150 μm^2 while cell cluster areas were defined as observed fluorescence with areas > 150 μm^2.

2.8. Statistical analysis

All data were analyzed using GraphPad Prism statistical analysis software (v.10.0.3; GraphPad Software Inc., Boston, MA, USA). Data for injected sturgeon cells and their respective hosts were analyzed separately. One-way ANOVA models were used to compare percent cell area as well as percent cluster area over time. Post-hoc testing was done using Tukey's test. Alpha was set at 0.05.

2.8.1. Lake sturgeon gametogonium and triploid white x blue hybrid catfish

Growth measurements including total length (cm) and weight (g) were collected at 87, 95, 105, and 492 DPH for both treatment and control fish. Unpaired t-tests were used at each time point to compare both growth metrics between transplanted hosts and non-injected triploid

catfish. Survival data comparing treatment mortality and triploid control catfish was collected at 29, 58, and 87 DPH and analyzed using unpaired t-tests at each time point. Stem cell colonization and proliferation data was collected at 82, 90, and 100 DPI. Paired t-tests were used to compare percent cell area as well as percent cluster area between the two sampling points. Alpha was set at 0.05.

2.8.2. Siberian sturgeon OSCs and triploid channel catfish

Growth measurements including total length (cm) and weight (g) were collected at 87, 107, and 511 DPH for both treatment and control fish. Unpaired t-tests were used at each time point to compare both growth metrics between transplanted hosts and triploid control catfish. Survival data comparing treatment mortality and non-injected triploid catfish was collected at 29, 58, 87, and 492 DPH for fish injected with lake sturgeon cells and their corresponding controls and 29, 58, 87, and 492 DPH for fish injected with Siberian sturgeon cells and their corresponding controls. Survival data was analyzed using unpaired t-tests at each time point. Stem cell colonization and proliferation data was collected at 82 and 100 DPI.

3. Results

3.1. Lake sturgeon gametogonium transplanted to triploid white x blue hybrid catfish

Of the fish sampled to detect fluorescently dyed donor cells, 88.9% displayed fluorescence. The sturgeon cells were proliferating rapidly, as percent cell area was doubling every 8-10 days inside of the catfish gonad from 82, 90, and 100 DPI increasing between 82-90 DPI, 82-100 DPI, and 90-100 DPI ($P = 0.005, 0.002, 0.021$ respectively, Fig. 1.2A). Similarly, the percent cluster area quadrupled between 82 and 100 DPI increasing between 82-90 DPI and

82-100 DPI (P = 0.026 and 0.006 respectively, Fig. 1.2B) with no significant increase in cluster area between 90-100 DPI (P = 0.186, Fig. 1.2B).

Triploid white x blue catfish injected with lake sturgeon gametogonium grew at half the rate of control triploid white x blue catfish up to 87 DPH (P = 0.019, Fig. 1.3A/B). Then the growth of the control slowed, and the difference was less at 95 DPH (P = 0.005, Fig. 1.3A/B), and by 105 through 492 DPH there were no significant differences observed (P = 0.709 and P = 0.660 respectively, Fig. 1.3C/D). Total length between treatment fish and non-injected controls was not different at 87, 95, 105, and 492 DPH (P = 0.095, 0.070, 0.937, 0.943 respectively, Fig. 1.3E-H). Observed survival of treatment fish was about 30% lower than non-injected triploid control catfish at 29, 58, and 87 DPH but only showed significance at 87 DPH (P = 0.113, 0.137, 0.051 respectively, Fig. 1.3I-K). At 492 DPH injected fish had higher survival compared to controls but not significantly different (P = 0.817, Fig. 1.3L). At approximately 6 to 29 DPH however, the aquaria in which these fish were housed experienced an outbreak of *Aeromonas hydrophila* which resulted in heightened levels of mortality during this period. Channel catfish hosts experienced no significant diseases throughout the study. For channel catfish hosts, the mean survival at 29 DPH was 73.06% while white x blue catfish hosts displayed a mean survival of 65.56% (Fig. 1.5G and Fig. 1.3I, respectively).

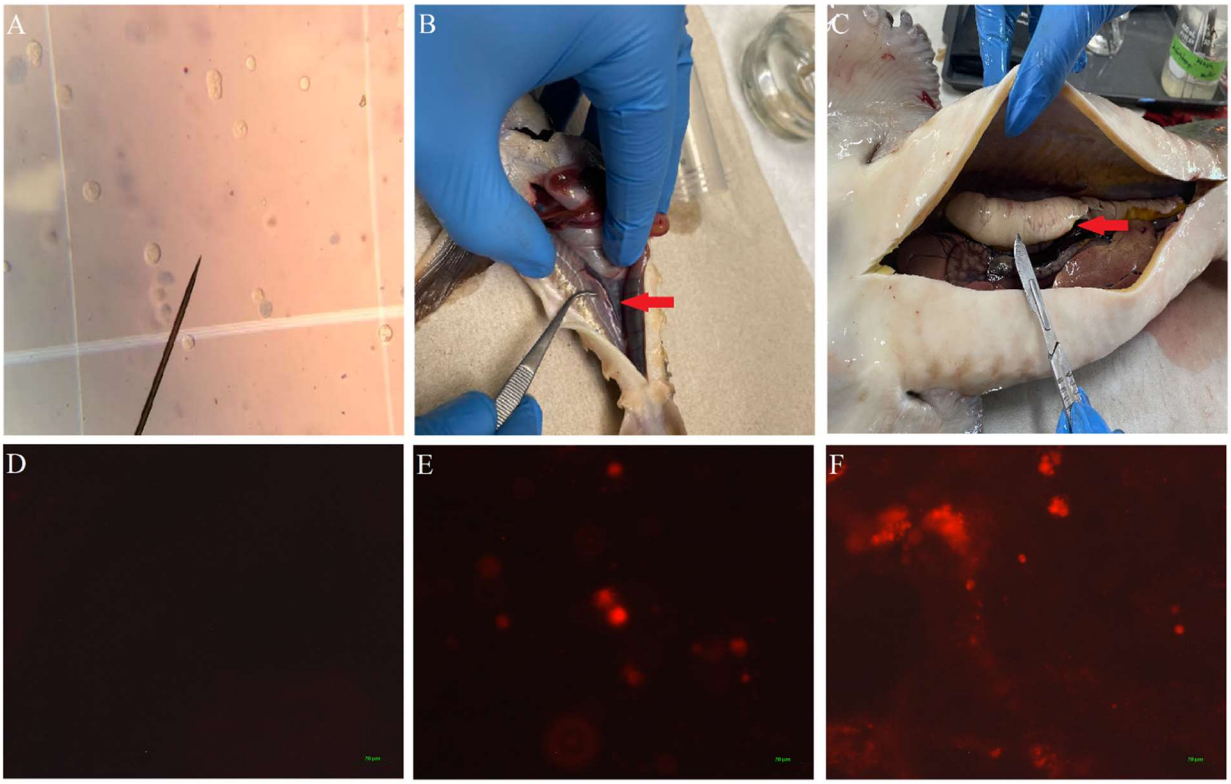

Fig. 1.1. Isolated lake sturgeon (*Acipenser fulvescens*) stem cells with PKH26 fluorescent labelling (A). Lake sturgeon gonad development from donor fish selected for cell extractions (B). Siberian sturgeon (*Acipenser baerii*) gonad development from donor fish selected for cell extractions (C). Gonadal tissues showing fluorescence from PKH26 dyed lake sturgeon donor stem cells. The non-injected control triploid hosts showed no fluorescence (D), while the cell injected treatments showed proliferation and increased fluorescence from 82 DPI to 100 DPI (F).

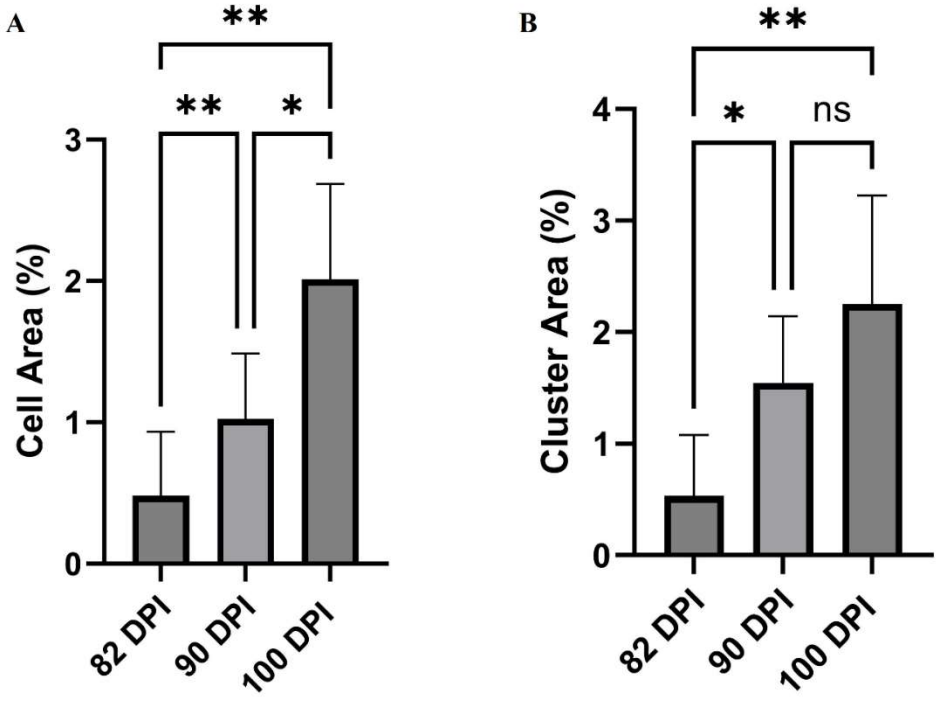

Fig 1.2. Cell area (A) and cluster area (B) for triploid white catfish, *Ameiurus catus* ♀ x blue catfish, *Ictalurus furcatus* ♂ hybrid catfish injected with lake sturgeon (*Acipenser fulvescens*) gametogonium. Sampling took place at 82, 90, and 100-days post-injection, each time point was compared (82 - 90 DPI, 82 - 100 DPI, and 90 - 100 DPI) to determine significance for both cell area (P = 0.005, 0.002, 0.021 respectively; A) and cluster area (P = 0.026, 0.186, 0.006 respectively; B). Comparisons were made using one-way ANOVA and Tukey's honestly significant difference.

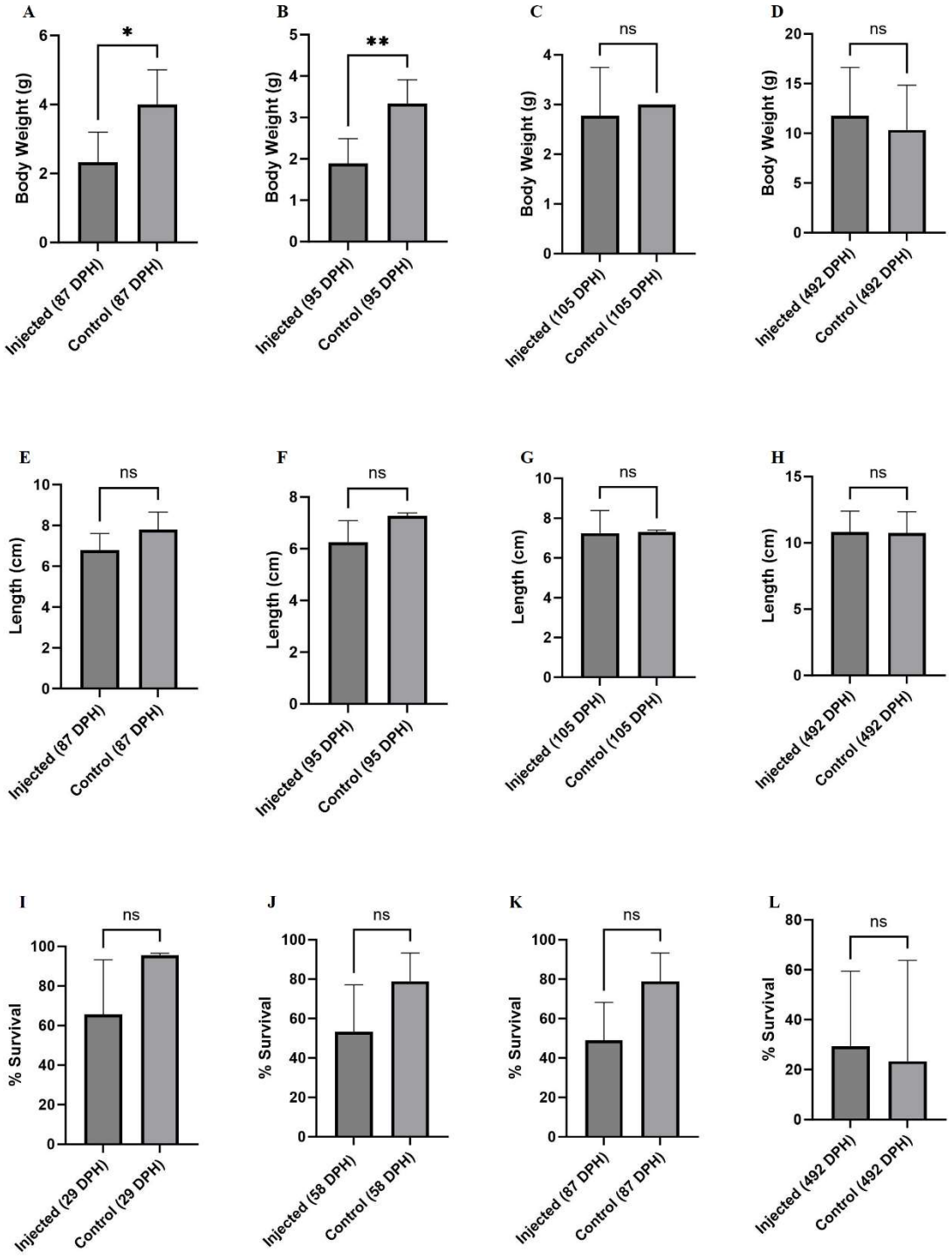

Fig. 1.3. Growth rate and survival between triploid white catfish, *Ameiurus catus* ♀ x blue catfish, *Ictalurus furcatus* ♂ hybrid catfish injected with lake sturgeon (*Acipenser fulvescens*) gametogonium and their corresponding non-injected triploid controls. Body weight differences were evaluated at 87, 95, 105, and 492-days post-hatch (P = 0.019, 0.005, 0.710, 0.660, respectively; A-D). Total length differences were determined at 87, 95, 105, and 492-days post-

hatch (P = 0.095, 0.070, 0.937, 0.943 respectively; E-H). Survival differences were determined for the treatment and control groups at 29, 58, 87, and 492-days post-hatch (P = 0.113, 0.137, 0.051, 0.817 respectively; I-L). Unpaired t-tests were used for statistical analysis.

3.2. Siberian sturgeon OSCs transplanted to triploid channel catfish

Of the fish sampled to detect fluorescently dyed donor cells, 76.7% displayed fluorescence over the sampling periods. Percent cell area observed at 82 and 100 DPI showed no significant increase (P = 0.702, Fig. 1.4A). The observed percent cluster area doubled from 82 and 100 DPI but showed no significant increase (P = 0.150, Fig. 1.4B).

Triploid channel catfish injected with Siberian sturgeon OSCs had observed body weights approximately 50% larger than controls compared at 87 and 105 DPH, however this was not significantly different (P = 0.107 and 0.172, respectively, Fig. 1.5A-C). Neither group grew between 87 and 105 DPH. By 511 DPH triploid channel catfish controls surpassed the injected fish in body weight (by 10%) but not significantly (P = 0.582 respectively, Fig. 1.5A-C), despite heavier mortality of the injected fish. Total length of treatment fish and non-injected controls at 87, 105, and 511 DPH was different, with injected fish longer at 87 and controls longer at 511 DPH (P = 0.031 and 0.022 respectively, Fig. 1.5D/F) while no significant difference was observed at 105 DPH (P = 0.094, Fig. 1.5E).

Survival of the treatment fish was 22% lower than non-injected controls but not significantly different at 29 DPH (P = 0.226, Fig. 1.5G-I). However, treated fish had lower survival than controls at 58 and 87 DPH (P = 0.086 and 0.086 respectively, Fig. 1.5G-I). At 511

DPH injected fish has a higher observed survival compared to controls but not significantly different (P = 0.583, Fig. 1.5J).

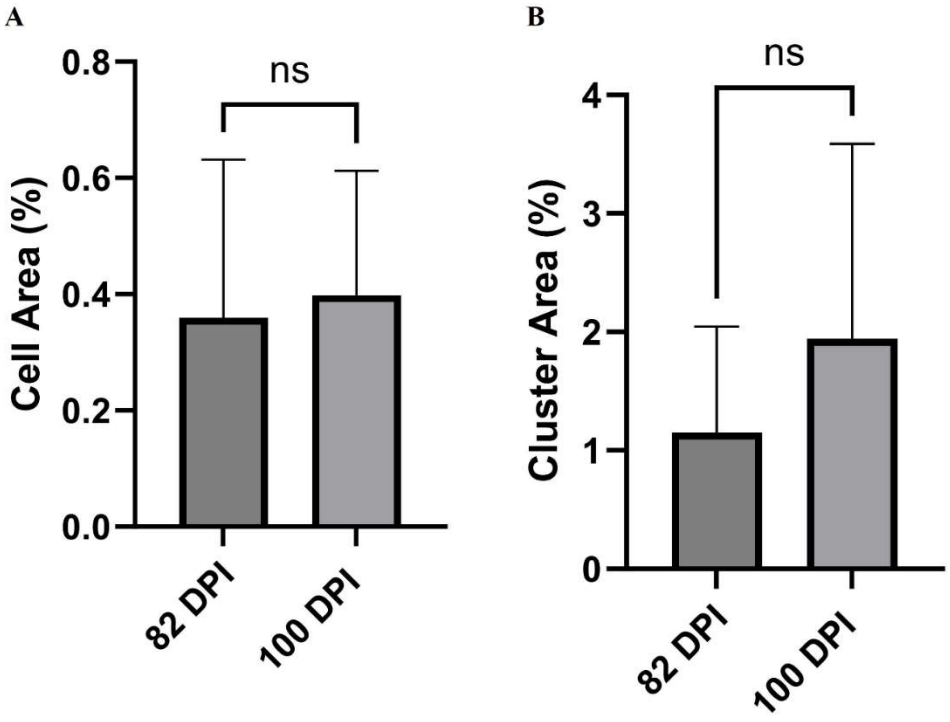

Fig 1.4. Cell area (A) and cluster area (B) for triploid channel catfish (*Ictalurus punctatus*) injected with Siberian sturgeon (*Acipenser baerii*) oogonial stem cells. Sampling took place at 82 and 100-days post-injection to determine significance for both cell area (P = 0.702; A) and cluster area (P = 0.150; B). Paired t-tests were used for statistical analysis.

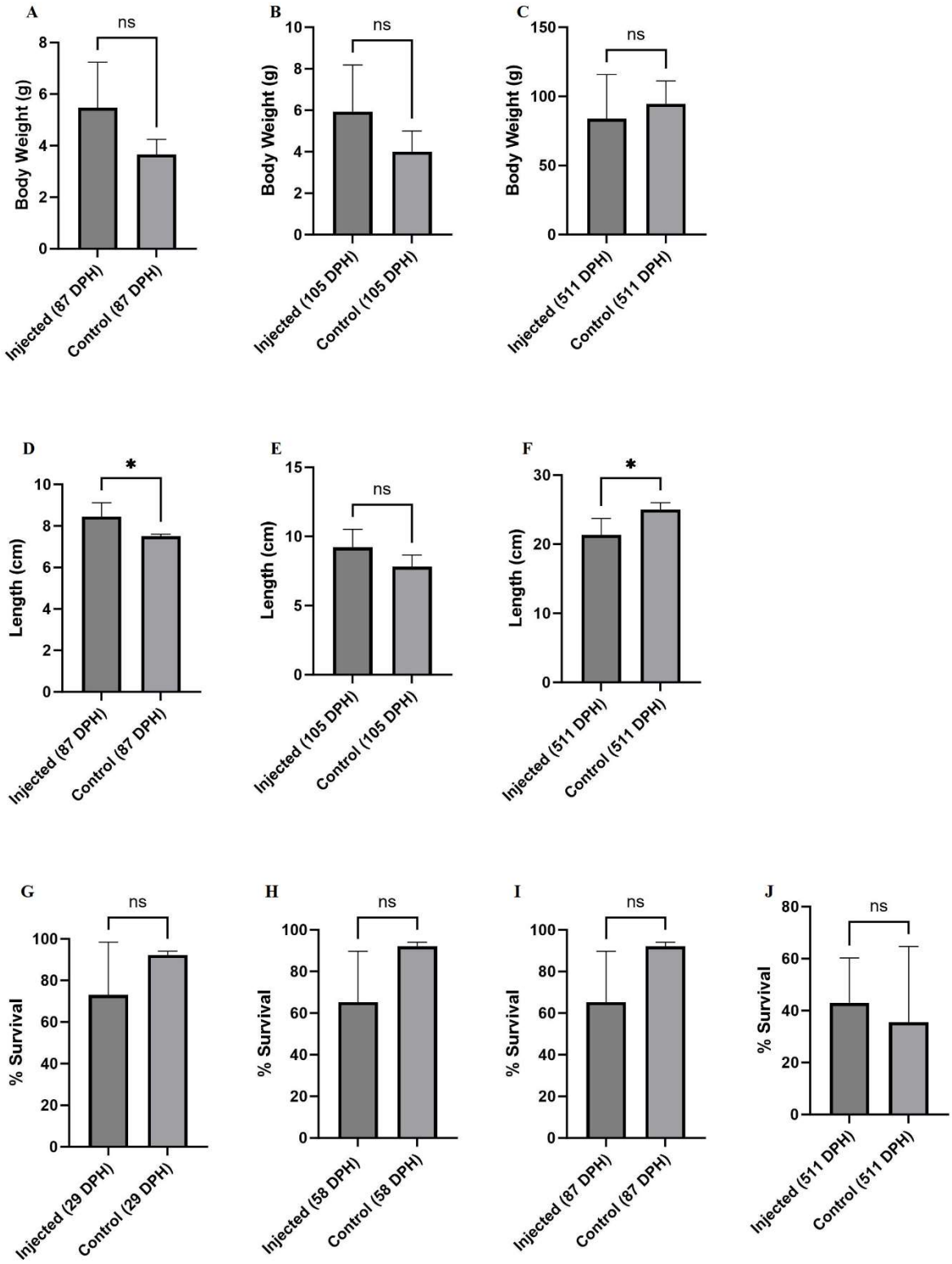

Fig. 1.5. Growth rate and survival between triploid channel catfish (*Ictalurus punctatus*) injected with Siberian sturgeon (*Acipenser baerii)* oogonial stem cells and their corresponding non-

injected triploid controls. Body weight differences were evaluated at 87, 105, and 511-days post-hatch (P = 0.107, 0.172, 0.582 respectively; A-C). Total length differences were determined at 87, 105, and 511-days post-hatch (P = 0.031, 0.094, 0.022 respectively; D-F). Survival differences were determined for the treatment and control groups at 29, 58, 87, and 511-days post-hatch (P = 0.226, 0.086, 0.086, 0.583 respectively; G-J). Unpaired t-tests were used for statistical analysis.

3.3. Host system comparisons-current study

As xenogenic hosts, channel catfish grew faster and had potentially better survival compared to the white x blue hybrid hosts. The mean body weight and length for channel catfish hosts increased from 5.47 g to 83.8 g and 8.45 cm to 21.33 cm over 424 days (approximately 0.19 g/day and 0.03 cm/day), while white x blue hybrid hosts increased from 2.33 g to 11.78 g and 6.79 cm to 10.81 cm over 405 days (approximately 0.02 g/day and 0.01 cm/day). At the final sampling point, the mean survival of channel catfish hosts was 43.00% while white x blue hybrid hosts had a mean survival of 29.33%. The number of total cells within these two hosts were similar. Cell proliferation, however, was lower in the channel catfish hosts with the mean area of cell area showing no increase over 18 days (0.33% at 82 and 100 DPI) compared to white x blue hosts which experienced an increase from 0.48% to 2.01% over the same period (Table 1.1). Both hosts showed increased mean cell cluster proliferation over the 18-day period with 1.01% to 1.84% and 0.53% to 2.25% for channel catfish and white x blue hybrid catfish hosts, respectively.

3.4. Host system comparisons-multiple catfish studies

In related catfish studies, Hettiarachchi et al. (2022a; 2022b) and Gurbatow (2020) observed comparable trends for channel catfish and white catfish hosts injected with blue catfish

and channel catfish donor cells (Table 1.1). Channel catfish hosts transplanted with blue catfish stem cells exhibited mean cell area increases of 0.30% to 0.43% and 2.80% to 2.88% at 50 to 90 DPI (Hettiarachchi et al., 2022a and 2022b respectively) and 0.25% at 90 DPI (Gurbatow, 2020). Mean cell cluster area for channel catfish hosts in these studies increased from 2.52% to 7.10% and 3.86% to 8.45% at 50 to 90 DPI and 0.96% at 90 DPI, respective to the studies. Research from Hettiarachchi et al. (2022b) also indicated white catfish xenogenic hosts had mean cell proliferation from 0.50% to 5.87% and 0.36% to 6.45% and mean cell cluster proliferation from 5.93% to 12.01% and 4.84% to 14.63% at 50 and 90 DPI for hosts injected with blue catfish stem cells and channel catfish stem cells, respectively.

Table 1.1. Comparisons of percent cell area and percent cluster area in channel catfish (CC, *Ictalurus punctatus*), blue catfish (BC, *Ictalurus furcatus*), white catfish (WC, *Ameiurus catus*), and white catfish ♀ x blue catfish ♂ hybrid catfish hosts. Donor cells transplanted into the hosts were isolated from blue catfish, channel catfish, lake sturgeon (LS, *Acipenser fulvescens*), and Siberian sturgeon (SS, *Acipenser baerii*). Sampling points include 45-50, 82, 90, and 100-days post-injection (DPI).

		% Cell Area (DPI)				% Cluster Area (DPI)			
Host	**Donor**	**45-50**	**82**	**90**	**100**	**45-50**	**82**	**90**	**100**
[1]CC	BC			0.25				0.96	
[2]CC	BC	2.80		2.88		3.86		8.45	
[2]CC	BC	0.30		0.43		2.52		7.10	
[2]WC	BC	0.50		5.87		5.93		12.07	
[2]WC	CC	0.36		6.45		4.84		14.63	
[3]WB	LS		0.48	1.02	2.01		0.53	1.55	2.25
[3]CC	SS		0.33		0.33		1.01		1.84

[1]Gurbatow [2] Hettiarachchi [3]Current study

4. Discussion

In the present study, donor stem cells from Siberian sturgeon and lake sturgeon survived and proliferated in triploid catfish hosts. These results reinforce the potential of xenogenesis as a reproductive tool for producing the gametes of biologically and economically important species.

Numerous examples exist for successful SSC and OSC transplantation involving a variety of cell donors and hosts (Ciruna et al., 2002; Giraldez et al., 2005; Saito et al., 2008 and 2010; Lacerda et al., 2012; Silva et al., 2006). Successful cell transplantation has even been demonstrated between fish of different families and orders (Saito et al., 2010; Silva et al., 2006). This promising and innovative reproductive technology opens potential avenues for shortening generation intervals, cloning the gonads of valuable individuals, and expanding populations of threatened species.

Prior to sexual maturation, one of the most powerful tools available for determining the effectiveness of germ cell colonization and proliferation is the labelling of donor derived cells using fluorescence (Parish, 1999; Lee and Yoshizaki, 2016; Ye et al., 2021). In this study, fluorescently labeled donor cells in triploid catfish hosts were observed at 82 and 100 DPI and 82, 90, and 100 DPI for Siberian sturgeon and lake sturgeon, respectively. In addition, there were significant increases in cell area and cluster area during subsequent sampling points for white x blue hybrid catfish injected with lake sturgeon gametogonium. This indicates that the donor stem cells from this biologically important species can successfully proliferate in triploid white x blue hybrid catfish recipients. Although there were no statistically significant increases in cell or cluster area in triploid channel catfish transplanted with Siberian sturgeon OSCs, there were still overall increases observed for both metrics over the two sampling points. Theoretically, PKH26 should disappear at around 100 days. Thus, the proliferation may be grossly underestimated if

PKH26 was in the process of fading at this time. Furthermore, 76.67% of the triploid channel catfish transplanted with donor OSCs sampled displayed fluorescence. This indicates that these cells have the ability to survive in triploid channel catfish hosts.

The channel catfish grew faster and had better survival compared to the white x blue catfish as xenogenic hosts. Contributing to the lower survival of white x blue hybrid catfish was their susceptibility to a bacterial outbreak (*A. hydrophila*). Channel catfish hosts were not notably affected by disease during this study. The total number of cells present in each host were similar between hosts and the rate of cluster proliferation was slightly higher in channel catfish (channel catfish, 0.53% to 2.25%; WB hybrid, 1.01% to 1.84%). However, the rate of cell proliferation was lower in channel catfish compared to white x blue hybrid catfish (channel catfish, unchanged at 0.33%; WB hybrid, 0.48% to 2.01%). The results from the current and previous studies suggest that white catfish may be better xenogenic hosts for increasing cell and cluster proliferation compared to channel catfish hosts. This might be partially offset by lower survival of white catfish xenogens (Hettiarachchi et al., unpublished). We hypothesized that white x blue hybrid catfish might be a good host as they have the white catfish maternal genome and we predicted that they would have higher survival, however, they were more susceptible to disease.

Xenogenic techniques have already had documented success in the conservation of endemic/endangered species as well as the improvement in reproduction for commercially important species including rainbow trout (*Oncorhynchus mykiss*) (Kobayashi et al., 2003; Yoshizaki and Lee, 2018), cherry salmon (*Oncorhynchus masou*) (Lee et al., 2016), and blue catfish (Abualreesh et al., 2020, 2021 a,b), among others. More specifically, Pšenička et al. (2015) demonstrated a successful xenogenic transplantation of germ line cells from Siberian

sturgeon (*Acispenser baerii*), while Ye et al. (2017) demonstrated successful cell transplantation from the critically endangered Chinese sturgeon (*Acispenser sinesis*). In the present study, Siberian sturgeon and lake sturgeon gametogonium were successfully transplanted into triploid catfish recipients. Together, this suggests the utility of xenogenic reproductive technologies for future production of valuable species for population restoration or commercialization.

In conclusion, the current study demonstrated that triploid channel catfish and white x blue hybrid catfish are potentially viable hosts for Siberian sturgeon and lake sturgeon germ line cells, allowing for colonization and proliferation after receiving intraperitoneal injections of cell suspensions at the genital ridge. It is notable that one donor sturgeon can provide thousands of viable gametogonium which can be used to transplant many recipient hosts. Therefore, this technology provides excellent opportunities for future xenogenesis applications to substantially impact declining populations of wild sturgeons along with new possibilities for producing economically important aquaculture products for expanding global markets. Colonization and proliferation of the sturgeon stem cells are evident, and next their ability to morph into viable gametes needs to be demonstrated and further examined.

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Chapter 2: Optimizing Donor Size Selection for Channel Catfish (*Ictalurus punctatus*) Gametogonium Isolation

Abstract

Xenogenesis requires large quantities of stem cells from the donor, thus identifying the size of donor to maximize yield of stem cells is important for efficient application of this technology. Previously, Hettiarachchi et al. (2020) determined that the optimum size to collect spermatogia A and oogonial stem cells in blue catfish, *Ictalurus furcatus*, was 25 to 39.9 cm and 100 to 499.9 g and 25 to 39.9 cm and 200 to 600 g, respectively. It is not known if the same size would be the best when the closely related channel catfish, *I. punctatus*, which matures at younger ages and smaller sizes than blue catfish, is utilized as a donor species. Therefore, the current study was conducted to determine the relationships between total length (T_L), total weight (T_W), and the quantity and quality of extracted gametogonium in channel catfish donors. Relationships were observed between T_W and the number of extracted live OSCs ($r^2 = 0.460$; $P = < 0.001$) as well as the T_L and number of live OSCs ($r^2 = 0.449$; $P = < 0.001$), indicating that an approximate T_W of 150 to 350 g and T_L of 28 to 38 cm yield the largest quantity of stem cells. Similarly, in males T_L ($r^2 = 0.149$; $P = < 0.001$) and T_W ($r^2 = 0.233$; $P = < 0.001$) revealed a relationship to the number of extracted live SSCs, where 20 to 40 cm and 350 to 600 g males yielded the highest number of extracted live stem cells. These results were similar to results found in blue catfish, the slightly smaller optimal size observed for channel catfish may be a result of the earlier age at which channel catfish reach maturity compared to blue catfish.

1. Introduction

Freshwater aquaculture production in the United States consists primarily of catfish species with the hybrid catfish (channel catfish*, Ictalurus punctatus* ♀ with blue catfish, *I. furcatus* ♂) serving as a critical component of the industry (Dunham and Masser, 2012; Perera et al., 2017). The industry's adoption of the hybrid catfish is a result of the genotype's consistently superior production traits including, growth rate, feed conversion, survivability, fillet yield, dress out percentage, and harvestability (Yant et al., 1976; Dunham et al., 1983; Dunham et al., 1987; Dunham and Argue, 1998; Bosworth et al., 2004; Dunham and Masser, 2012; Arias et al., 2012; Dunham et al., 2014). Despite the advantages of producing hybrid catfish, challenges are still present, and include the reproductive barriers which inhibit natural production and require the sacrifice of blue catfish males for sperm (Argue et al., 2003; Hu et al., 2011). The lack of natural hybridization between channel and blue catfish has necessitated improvement of artificial spawning and assisted reproduction techniques to allow for industry expansion (Dunham and Masser, 2012; Bosworth, 2012; Boxrucker and Kuklinski, 2006). Although these technologies have aided the establishment of hybrid catfish production, the techniques are labor intensive and need new or improved hatchery technologies to facilitate the continued growth of the industry (Dunham and Argue, 2000). Xenogenesis is an innovative technology which may provide solutions to overcoming reproductive barriers such as these.

Xenogenesis is a reproductive method where successive generations differ from each other (Dunham, 2011). The procedure results in xenogenic organisms which are comprised of elements typically foreign to its species (Cavaco et al., 1999; Takeuchi et al., 2004; Takeuchi et al., 2009; Dunham, 2011; Higuchi et al., 2011). The process of xenogenesis involves isolating and transplanting primordial germ cells (PGCs), spermatogonial stem cells (SSCs), or oogonial

stem cells (OSCs) from a diploid donor species into a sterile host (Hettiarachchi et al., 2022). This procedure is accomplished by injecting one or more of the cell types from the donor fish (or developing embryo in the case of PGCs) into a host which is typically sterilized via triploidy with the intention of producing gametes of the donor species within the transplanted host (Perera et al., 2017; De Siqueira-Silva et al., 2018). This concept was demonstrated in mice via the successful isolation and transplantation of donor SSCs to sterile recipients (Brinster and Avarbock, 1994).

In fish, a similar technique was developed using PGCs carrying green fluorescent protein (GFP), where cells were transplanted into the body cavity of *Onchorhynchus mykiss* larvae (Kobayashi et al., 2003). Following transplantation, the PGCs exhibited an ability to migrate and colonize at the genital ridge. This led to the formation of germ line cells corresponding to the development ovaries or testis based on the transplanted individual's genetic sex. Examples of this technique are diverse and becoming well-documented, and applications include the conservation of biologically important species, as well as the improving the production of commercially-valuable species. Previous research includes PGC transplantations from donor pearl danio (*Danio albolineatus*), zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), and loach (*Misgurnus anguillicaudatus*) into host zebrafish embryos (Ciruna et al., 2002; Satio et al., 2008; Satio et al., 2010; Lacerda et al., 2013).

Successful OSC and SSC transplantations have also been documented. Specific OSC transplantation examples include pejerrey (*Odontesthes bonariensis*), where ovarian germ cells were transplanted into *Odontesthes hatcheri* hosts, and in Chinese sturgeon where *Acipenser sinensis* ovarian germ cells were transplanted into *Acipenser dabryanus* hosts (Majhi et al., 2009; Ye et al., 2017). Rainbow trout OSCs were also successfully used by researchers to produce

donor derived phenotypes with intraspecific transplantation (Yoshizaki et al., 2010). SSC transplantation in fish has also been demonstrated, including studies where donor cells from jundia catfish (*Rhamdia quelen*) transplanted into Nile tilapia (*Oreochromis niloticus*), rainbow trout transplanted into cherry salmon (*Oncorhynchus masou*), Siberian sturgeon (*Acispenser baerii*) transplanted into sterlet sturgeon (*Acispenser ruthenus*), and Chinese sturgeon (*Acispenser sinesis*) transplanted into Darby's sturgeon (*Acispenser dabryanus*) (Silva et al., 2006; Yoshizaki et al., 2010; Pšenička et al., 2015; Ye et al., 2017).

Breeding experiments have also been conducted with ictalurid catfish, where xenogenic male channel catfish containing cells transplanted from blue catfish displayed normal courtship behaviors when paired with diploid channel catfish females which were induced to ovulate via hormone injection (Perera et al., 2017). Although the eggs did not hatch after natural spawning, a small number of hybrid catfish were produced after removing the xenogenic gonads to manually fertilize hand-stripped eggs from a channel catfish female. Even though only a small number of fry were produced, the study illustrated the potential for xenogenic technology to be used in the industry as an alternative method to generate channel x blue hybrid catfish embryos.

To achieve the adoption of this fry production method by the aquaculture industry, xenogenesis still requires optimization to increase viability and efficiency. An important aspect is the ability to reliably identify donor individuals which yield large amounts of germ line cells for transplantation. Previous work has already been concluded regarding this subject where blue catfish stem cell yield and viability has been studied in relation to donor body size (total length (T_L) and total weight (T_W)) (Hettiarachchi et al., 2022). There are certain applications that channel catfish could be the appropriate donor, thus it is beneficial to determine the optimum size to harvest stem cells from this species.

The aim of the study conducted here is to investigate the relationship between donor body size (total length (T_L) and total weight (T_W)) and germ line cell production and viability for channel catfish. Ultimately, the goal of this research is to illustrate reliable biomarkers which may be used for selecting appropriate stem cell donor fish.

2. Materials and Methods

During this research, experiments and investigations performed using animals were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.

2.1. Catfish husbandry:

The fish used for this experiment were reared in 0.04-ha aquaculture ponds with an average depth of 1.2 m. Ponds were located at the Fish Genetics Research Unit at Auburn University, AL, USA (32.6622° N, 85.4960°W). All fish were fed a commercial floating feed (Purina Catfish 32) containing 32% crude protein, 4% crude fat, 7% crude fiber, and 0.8% phosphorus. Fish were fed once daily until satiated. The fish chosen for this study were selected to include a diverse size range (14.0-67.3 cm) which ensured that a variety of developmental stages were sampled. Twenty-seven males and 23 females were harvested from the ponds. After capture, fish were euthanized using a 300 mg/L solution of buffered tricaine methanesulfonate (MS-222) and transferred on ice to Auburn University's Fish Genetics Laboratory to measure T_L (± 0.1 cm) and T_W (± 0.05 g) along with stem cell isolation and quantification.

2.2. Stem cell isolation and quantification

Fish were sterilized using a 70% ethanol solution and stem cells were isolated following procedures described by Shang et al. (2015). Briefly, the ventral side of the fish was opened to gain access to the peritoneal cavity where the gonads were then removed while avoiding contamination from blood vessels or connective tissues. Gonad weight was then recorded before transferring samples to individual sterile petri dishes containing 5 mL of Hanks' Balanced Salt Solution ((HBSS, GE Healthcare Life Sciences) supplemented with 1.0 µg/mL NaHCO3 (Church & Dwight Co., NG) and 100 µ/mL penicillin - streptomycin (Life Technologies)). Samples were then directly transported to a biosafety cabinet for further processing. Cleaning was accomplished by using sterile scalpel blades to remove and discard any remaining blood and connective tissues. Sterilization of the gonads took place via rinsing three times with ~ 1 mL of anti-agent medium followed by rinsing in 5 mL of 0.5% bleach solution for 2 minutes. The next phase of cleaning and sterilization included three separate rinses in HBSS then three separate rinses with phosphate-buffered saline (PBS, Alfa Aesar). Following the final rinsing phase, samples were homogenized via mincing with a sterile scalpel until a fine consistency was achieved. Samples were then transferred to individual autoclaved glass flasks (50 mL) containing magnetic stir bars. After transferring samples, 0.25% trypsin - ethylenediamine tetraacetic acid (EDTA, Life Technologies) was added to each flask at a 50:1 ratio of trypsin to gonad weight. Flasks were capped with aluminum foil to prevent contaminants from entering the samples.

Ice was then used to incubate the samples for 45 minutes prior to transferring the samples to a magnetic stir plate for 1 hour at 22 °C to facilitate digestion. Two phases of filtration followed the digestion using 70 µm then 40 µm nylon mesh cell strainers (VWR International, Radnor, PA, USA). The filtered solution was captured in sterile 50 mL tubes, which were then

centrifuged at 500 x g (Eppendorf Centrifuge 5418 R) for 10 minutes to form the cells into a pellet to facilitate removal of the trypsin. After discarding the supernatant, the pellets were resuspended using 2 mL of Dulbecco's Modified Eagle's Medium/DMEM [DMEM (Corning Cellgro) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 unit/mL penicillin - streptomycin (15140-122, Life Technologies), and 200 mM L-glutamine (Life Technologies) to promote cell survivability.

Subsamples of the cell solutions (10 μL) were then removed and gently mixed with 10 μL of 0.4% trypan blue (Life Technologies) and were observed under an Olympus objective microscope (BH2), equipped with a 20× objective. Samples (10 μL) were loaded onto a hemocytometer and cell counts were obtained to determine the total number of live and dead germ line cells. In each of the four corner quadrants of the hemocytometer (1 mm^2 area) the cells were counted and the total number of cells for a 1 mL sample was calculated using methods described by Louis and Siegel (2011) with a dilution factor of two (trypan blue and cell suspension in a 1:1 ratio). Counts were repeated for each sample where cells/mL = number of cells in the four quadrants x dilution factor x 10,000.

2.3. Statistical analysis

All data were analyzed using SigmaPlot statistical analysis software (v15.0; SPSS Inc., San Jose, CA, USA). Nonlinear regression analyses were used to relate T_W and T_L to the number of live SSCs for males, and to relate the T_W and T_L to the number of live OSCs for females. Alpha was set to 0.05.

3. Results

Channel catfish females ranged from 14.0 to 52.8 cm and weighed from 17.5 to 1113.5 g (Fig. 1). Upwards to 1.33×10^7 live OSCs (per fish) were extracted from the females. relationship between the T_L and the number of extracted live OSCs was ($y = 9.6386*\exp(-.5*((x-33.6766)/4.7416)^2)$; $r^2 = 0.449$; $P = < 0.001$; Fig. 2.1A). The relationship between T_W and the number of extracted live OSCs was ($y = - 10.2026*\exp(-.5*((x-239.3632)/115.4560)^2)$; $r^2 = 0.460$; $P = < 0.001$; Fig. 2.1B).

Channel catfish males ranged from 15.25 to 67.25 cm and weighed from 18.9 to 1924.5 g (Fig. 2). Upwards to 2.06×10^7 live SSCs (per fish) were extracted from the males. The relationship between the T_L and the number of extracted live SSCs was ($y = 6.4240*\exp(-.5*((x-31.0075)/9.1666)^2)$; $r^2 = 0.149$; $P = < 0.001$; Fig. 2.2A). The relationship between T_W and the number of extracted live SSCs was ($y = 7.8750*\exp(-.5*((x- 463.9128)/163.3854)^2)$; $r^2 = 0.233$; $P = < 0.001$; Fig. 2.2B).

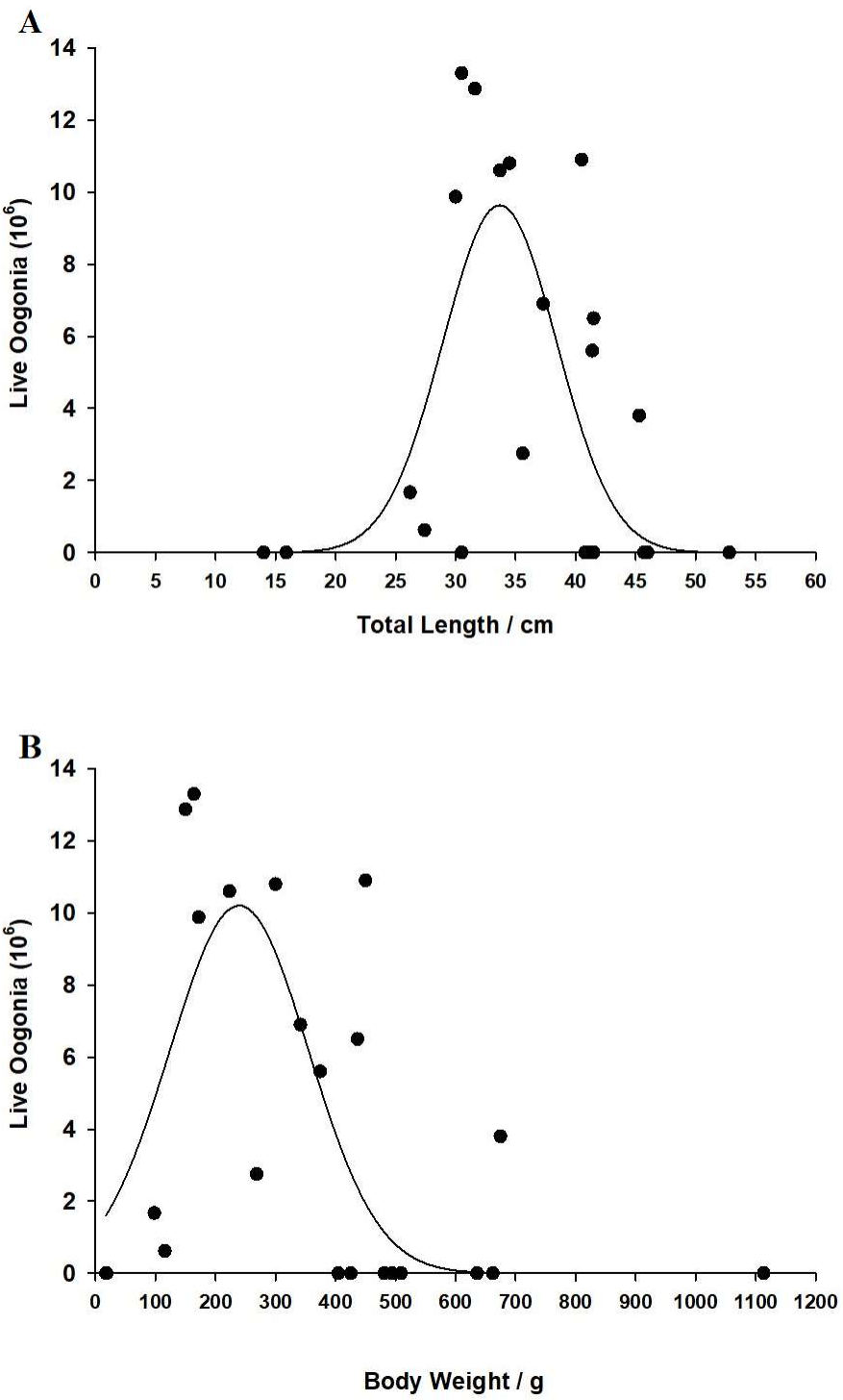

Fig 2.1. Relationships between total length and live OSC production ($r^2 = 0.449$; P = < 0.001; A) as well as total weight and live oogonial stem cell (OSC) production ($r^2 = 0.460$; P = < 0.001; B) for female channel catfish (*Ictalurus punctatus*). Nonlinear regression models were used for statistical analysis.

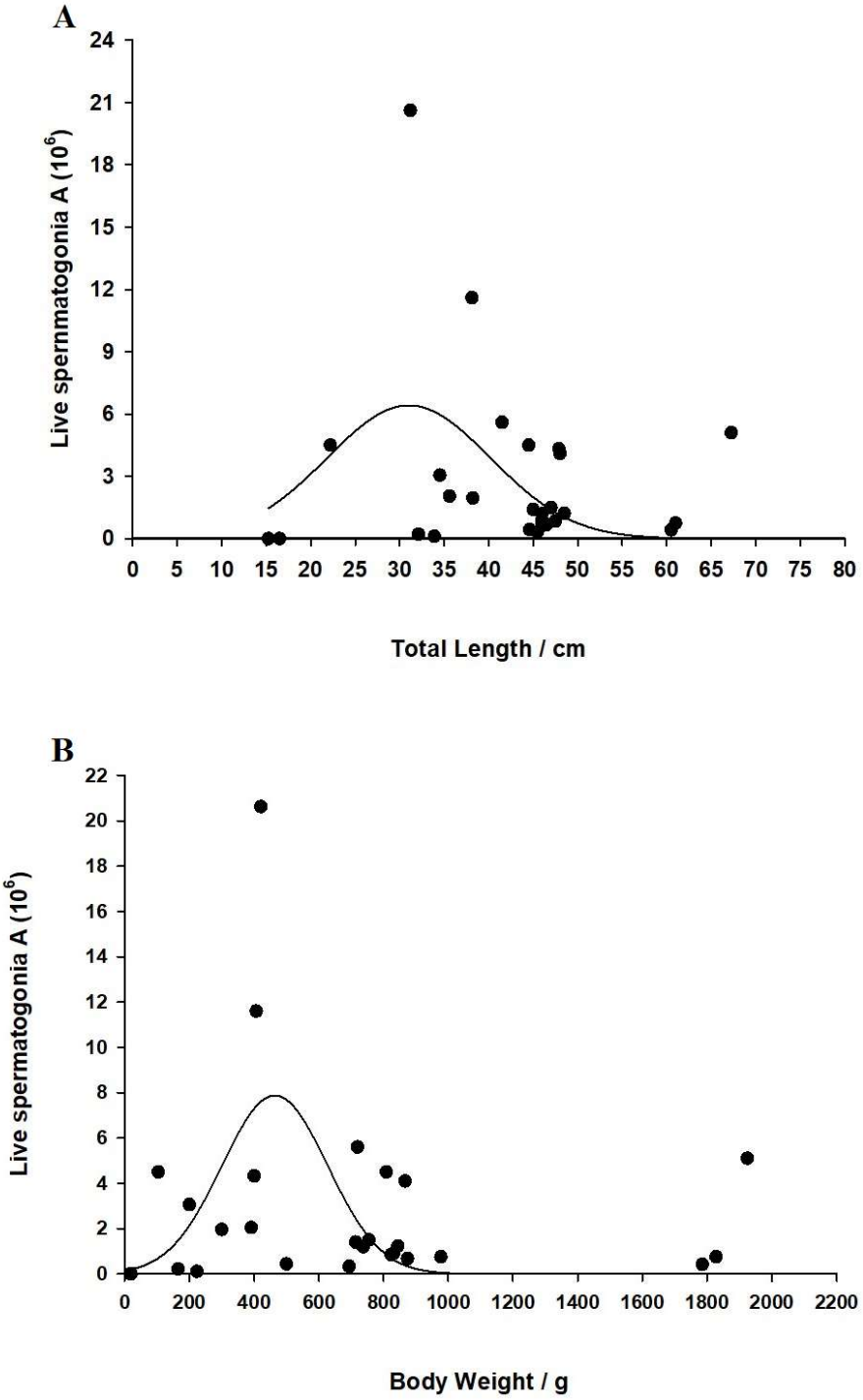

Fig 2.2. Relationships between total length and live spermatagonial stem cell (SSC) production ($r^2 = 0.149$; P = < 0.001; A) as well as total weight and live SSC production ($r^2 = 0.233$; P = <

0.001; B) for male channel catfish (*Ictalurus punctatus*). Nonlinear regression models were used for statistical analysis.

4. Discussion

The relationship between live OSCs and T_L of channel catfish females was a nonlinear, dome-shaped function showing an optimal size for collecting OSCs at approximately 28 to 38 cm. Similarly, the optimal weight for maximizing cell extraction was approximately 150 to 350 g. These results were similar to those reported by Hettiarachchi et al. (2020) for the closely related blue catfish female where the optimal female size for donor cell collection was 25 to 39.9 cm and 200 to 600 g. In this case, the optimal size for collection of OSCs was slightly smaller for channel catfish and may be a reflection of the earlier sexual maturity and smaller size at sexual maturity compared to blue catfish. In that case, it would be expected that OSCs would start transitioning to other cell types at a smaller size compared to blue catfish.

In the case of catfish males, Hettiarachchi et al. (2020) described the relationship between T_W and T_L and SSC quantity in blue catfish males for selecting the best donor fish to maximize stem cell harvest. The relationship observed between male T_W and T_L regarding SSC production indicated that an optimal length and body weight of blue catfish males for relative SSC number was 25 to 39.9 cm and 100 to 499.9 g. In the current study optimal size for relative SSC harvest in channel catfish was similar to blue catfish at approximately 20 to 40 cm and 350 to 600 g.

Many examples of successful OSC transplantations exist in the literature which involve various donor species including pejerry (Majhi et al., 2014) where mean T_W and mean T_L of donor fish were recorded, rainbow trout (Yoshizaki et al., 2010) where age and mean T_L were recorded, and zebrafish (Wong et al., 2011) where age was recorded. In these studies, however,

no selection techniques were implemented during the donor selection process other than focusing on immature females.

The success of SSC transplantation through xenogenesis has also been well documented in the literature using a variety of donor species including jundia catfish (Silva et al., 2006), rainbow trout (Yoshizaki et al., 2010), Siberian sturgeon (Pšenička et al., 2015), and Chinese sturgeon (Ye et al., 2017) among others. However, in these experiments, randomly selected immature individuals were used to isolate donor SSCs.

In conclusion, the relationship between body size and quantity of OSCs or SSCs reveals results which are promising for use as biomarkers for determining the optimal size of donor fish during selection processes. However, when considering the coefficient of determination (r^2) values from each model, the accuracy of predicting the expected amount of stem cells using T_W and T_L appears to be high for females ($r^2 = 0.460$ and $r^2 = 0.449$ respectively) compared to males which had significantly weaker relationships ($r^2 = 0.264$ and $r^2 = 0.149$ respectively). The ability to determine the relationship between size characteristics of the donor fish and the quantity and quality of extracted donor cells is important for optimizing the efficiency of germline transplantation for xenogenesis applications. Finally, the data presented in this study will be useful to predict the number of stem cells at specific body sizes without the need for sacrificing fish to strengthen the efficiency of germ cell collection.

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Chapter 3: Impact of Cell Microinjection Techniques (Manual vs Automatic Injectors) and Technician Skill Level on Xenogen Production

Abstract

Xenogenesis is becoming a viable reproductive method for producing the valuable hybrid catfish (channel catfish*, Ictalurus punctatus* ♀ with blue catfish, *I. furcatus* ♂). Although initial research appears promising, several factors play roles in the efficiency and success of xenogenesis which are important when considering technology transfer and commercialization. In addition to certain biological variables in need of optimization, there are technical aspects of the procedure which require examination for increasing efficiency. Two major technical variables in present xenogenesis procedures include the skill level and experience of technicians who are injecting cells into the host as well as the method for delivery of these cells (manual or automatic injectors). Therefore, the current study examined the variability between injection technicians with different experience levels using both manual and automatic injectors to determine the impact on successful xenogen production. Although no significant relationships were found between low- and high-experience classes using the manual or automatic injectors ($P = 0.387$ and $P = 0.369$, respectively) and no significant improvements were seen for experience classes as they became more familiar with injection methods after subsequent repetitions (low experience class, manual and automatic, $P = 0.362$ and $P = 0.875$ respectively; high experience class, manual and automatic, $P = 0.193$ and $P = 0.086$ respectively). This is likely due to highly variable data points, but some trends were observed. Slight improvements were made in both experience classes between their first and final repetitions using the manual injector indicating technicians are becoming more proficient with more experience. Furthermore, slight decreases in injection

success were noted for both experience classes using the automatic injector between their first and final repetitions potentially stemming from blockage issues with the glass needles used in the automatic injecting apparatus. Technicians had higher success rates while using the manual injector compared to the automatic injector, 57.5% and 49.4%, respectively. The two lowest experience technicians also made notable improvements while using the manual injector and automatic injector, respectively. Technicians' preference to either injection method was also examined. In 4 of 5 cases, the percentage xenogens produced was very similar between the preferred and non-preferred methods. In one case, the technician preferred the automatic injector but produced almost double the xenogens using the manual injector. Not surprisingly, individuals with almost no experience were not as proficient as technicians with more extensive experience.

1. Introduction

In the United States, hybrid catfish produced by crossing channel catfish (*Ictalurus punctatus*) ♀ with blue catfish (*Ictalurus furcatus*) ♂ are the dominant fish produced by the aquaculture industry. The widespread adoption of the hybrid catfish is due to the increased feed conversion, growth rate, harvestability, survivability, dress out percentage, and fillet yield displayed by the interspecific hybrid compared to channel catfish (Yant et al., 1976; Dunham et al., 1983; Dunham et al., 1987; Dunham and Argue, 1998; Bosworth et al., 2004; Dunham and Masser, 2012; Arias et al., 2012; Dunham et al., 2014). Although the value of the hybrid was quickly recognized by the industry, challenges in production still exist, namely the lack of natural hybridization between the two species which requires the sacrifice of valuable blue catfish males for sperm collection (Hu et al., 2011). Improved hatchery techniques and technologies, such as xenogenesis, may provide an avenue for producing this hybrid on a commercial scale.

Xenogenesis is a method of reproduction where no genetic information is transferred to the offspring from the parent and successive generations differ from each other (Dunham, 2023). The process of creating xenogenic fish involves transplanting primordial germ cells (PGCs), oogonial stem cells (OSCs), or spermatagonial stem cells (SSCs) isolated from a diploid donor into a sterile host which results in the production of donor-derived gametes (Takeuchi et al., 2004; Takeuchi et al., 2009; Dunham, 2023; Perera et al., 2017, De Siqueira-Silva et al., 2018). The self-renewal characteristics displayed by PGCs, OSCs, and SSCs throughout their lives make them valuable as donor cells for xenogenesis (Yoshizaki et al., 2010; Yoshizaki and Lee, 2018). After transplanting in the host, the injected cells migrate to the genital ridge and proliferate resulting in male or female germ cells corresponding to the genetic sex of the host after gonad differentiation.

The successful application of this technique to improve the production of commercially valuable species and for conservation purposes has been demonstrated using these three cell types. Specific examples of PGC transplantation include work done with rainbow trout (*Onchorhynchus mykiss*) (Kobayashi et al., 2003), along with transplantations from donor pearl danio (*Danio albolineatus*), zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), and loach (*Misgurnus anguillicaudatus*) into host zebrafish embryos (Ciruna et al., 2002; Satio et al., 2008; Satio et al., 2010; Lacerda et al., 2013). Successful OSC and SSC transplantation research has been conducted with pejerrey where *Odontesthes bonariensis* OSCs were transplanted into *Odontesthes hatcheri* hosts and with Chinese sturgeon where *Acipenser sinensis* OSCs were transplanted into *Acipenser dabryanus* hosts (Majhi et al., 2009; Ye et al., 2017). Concerning SSCs, examples include jundia catfish SSCs (*Rhamdia quelen*) transplanted into Nile tilapia (*Oreochromis niloticus*), rainbow trout SSCs transplanted into cherry salmon (*Oncorhynchus*

masou), Siberian sturgeon SSCs (*Acispenser baerii*) transplanted into sterlet sturgeon (*Acispenser ruthenus*), and Chinese sturgeon SSCs (*Acispenser sinesis*) transplanted into Darby's sturgeon (*Acispenser dabryanus*) (Silva et al., 2006; Yoshizaki et al., 2010; Pšenička et al., 2015; Ye et al., 2017).

Although initial research appears promising, several factors play roles in the efficiency and success of xenogenesis. For example, the life stage and body size of the injected host has a significant role in relation to the colonization and proliferation of injected cells (Perera et al., 2017; Hettiarachchi et al., 2022, Hettiarachchi et al., 2023). Body size and life stage also appear to play important roles in the quantity and quality of the cells obtained from the donor (Hettiarachchi et al., 2022). In addition to biological variables in need of optimization, there are likely technical aspects of the procedure which require examination for increasing the efficiency of production. Two major technical variables in the present xenogenesis procedure include the skill level and experience of technicians who are injecting cells into the host as well as the method by which they deliver these cells (manual or automatic injectors). These variables are especially pertinent to technology transfer and commercialization.

To facilitate the adoption of xenogenesis by the aquaculture industry as a viable method of producing the valuable channel x blue hybrid, it is important to define the best procedure to be used as well as demonstrating that large investments in highly trained technicians are not paramount for the successful application of these techniques. Thus, the focus of the current study was to examine the variability between injection technicians at different experience levels using both manual and automatic injectors to determine the impact on successful xenogen production.

2. Materials and Methods

All experimental studies and investigations performed using animals were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.

2.1. Catfish husbandry

Fish used in this experiment were grown in 0.04-ha aquaculture ponds with a 1.2 m average depth. The ponds were located in Auburn University, AL, USA (32.6622° N, 85.4960°W) at the Fish Genetics Research Unit. A commercial floating feed (Purina Catfish 32) containing 32% crude protein, 4% crude fat, 7% crude fiber, and 0.8% phosphorus was used to feed the fish. Two months prior to spawning, brood fish were fed a floating feed containing 36% crude protein, 10% crude fat, 3% crude fiber, and 0.8% phosphorus. All fish were fed one time daily until satiation was achieved, this was changed to 5 days per week for 2 months prior to spawning. Fish were harvested from the ponds as needed via pond seining with a ~4cm mesh seine net. Following capture, channel catfish were used for spawning procedures while blue catfish were anesthetized solution of tricaine methanesulfonate (MS-222) with a concentration of 300 mg/L and transferred on ice to Auburn University's Fish Genetics Laboratory for stem cell isolation, as described below.

2.2. Triploid host production

During the spring spawning season of 2022, visibly gravid and sexually mature channel catfish females were selected and were intraperitoneally injected with luteinizing hormone-releasing hormone luteinizing hormone-releasing hormone (LHRHa) to induce ovulation. When applying the hormone, a primary dose of 20 μg/kg body weight was administered followed by a

resolving dose 12 hours later of 100 μg/kg body weight. The females were then moved to soft mesh bags submerged in flow-through pond water tanks (7.5 m × 0.5 m × 0.45 m) with a flow rate of 24 L/min. Females were then checked every 4-6 hours until a small number of eggs could be observed on the bags. When eggs were observed, females were removed from the bags and anesthetized using a 100-ppm solution of MS-222. Once fully anesthetized, the females were removed from the solution and thoroughly dried. Eggs were then acquired via hand stripping the female into metal spawning pans coated with a thin layer of vegetable shortening (Crisco, B&G Foods, Parsippany, NJ, USA) to prevent eggs adhering to each other and the pan.

Sexually mature channel catfish males were selected and euthanized for sperm collection. The male's testes were surgically removed, and a sperm solution prepared via methods described by Dunham and Masser (2012). Briefly, after testes were surgically removed, they were cleaned using a 0.9% saline solution and dried using paper towel to remove any remaining blood or excess solution. Testes were processed to a fine consistency by using sterilized scalpel blades to mince them. Following mincing, a 100 μm mesh was used to filter the sperm mixture. The resulting sperm solution was captured in a 50 mL tube and diluted using 10 mL of 0.9% saline solution for every 1 g of testes used. For every 25 g of eggs, 2 mL of sperm solution was applied followed by 2 minutes of gentle mixing to facilitate adequate gamete contact time and fertilization.

A Fullers' earth solution was then made by adding 6 g of Fullers' earth power (Starwest Botanicals, Sacramento, CA, USA) to approximately 1 L of pond water and mixing thoroughly by hand. The resulting solution was then added to the gamete mixture and continually mixed for 3 minutes. When 5 minutes post fertilization was reached, the eggs were transferred to a cylindrical pressure chamber (340 mm height, 70 mm diameter) to be used in conjunction with a

Carver press to pressure shock the eggs to induce triploidly. A hydrostatic pressure of 7000 psi was applied for 5 minutes to accomplish this.

Following the pressure shock, eggs were transferred from the pressure chamber back to metal pans which were submerged in pond water containing 50 ppm $CaCl_2$ for one hour to achieve water hardening of the eggs. After the 1 hour hardening phase, eggs were moved to flow-through pond water troughs containing aeration and a paddle wheel. A reservoir was the source of water. Until hatching, eggs were stored in hanging mesh baskets between the paddle wheel blades within the trough.

2.3. Stem cell isolation

Blue catfish were sterilized by wiping the body down with a 70% ethanol solution and stem cells were isolated following techniques and procedures described by Shang et al. (2015). In brief, an incision was made on the ventral side of the fish to gain access to the peritoneal cavity. The gonads were then removed carefully to ensure no contamination by blood or connective tissues. The weight of the gonads was first recorded before transferring them to sterile petri dishes containing 5 mL of Hanks' Balanced Salt Solution ((HBSS, GE Healthcare Life Sciences) supplemented with 1.0 µg/mL NaHCO3 (Church & Dwight Co., NG) and 100 unit/mL Penicillin - Streptomycin (Life Technologies)). Samples were then brought to a biosafety cabinet for further cleaning and processing. Cleaning the samples took place by using sterile scalpel blades to remove any remaining connective tissue or blood. The cleaned samples were then sterilized via rinsing three times with ~ 1 mL of anti-agent medium followed by rinsing in 5 mL of 0.5% bleach solution for 2 minutes. Another phase of cleaning took place via 3 rinses in HBSS followed by 3 rinses in phosphate-buffered saline (PBS, Alfa Aesar). After the six rinses were complete, samples were processed to a fine consistency by mincing with a sterile scalpel

blade. The samples were then transferred to autoclaved glass flasks (50 mL) which contained magnetic stir bars. Upon transferring the samples, 0.25% trypsin - ethylenediamine tetraacetic acid (EDTA, Life Technologies) was added to each flask at a 50:1 ratio of trypsin to gonad weight.

Samples were then incubated on ice for approximately 45 minutes before moving them to a magnetic stir plate for 1 hour at room temperature (22 °C) for digestion. After digesting for 1 hour, samples were filtered in two phases using a 70 μm followed by a 40 μm nylon mesh cell strainer (VWR International, Radnor, PA, USA). The filtrate was captured in 50 mL tubes then centrifuged for 10 minutes at 500g (Eppendorf Centrifuge 5418 R) to form a cell pellet. Trypsin was removed via gentle pipetting and the cells were resuspended using 2 mL of Dulbecco's Modified Eagle's Medium/DMEM [DMEM (Corning Cellgro) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 unit/mL penicillin - streptomycin (Life Technologies), and 200 mM L-glutamine (Life Technologies) to promote cell health. After extracting, stem cell quantification took place according to standard protocols (Hettiarachchi et al., 2020; Abualreesh et al., 2020, 2021a, 2021b; Shang et al., 2015).

2.4. Technician selection and host injection

Six technicians with variable experience with microinjection techniques were selected. Skill level was assumed based on the number of years that each individual had conducted microinjections. Skill levels ranged from no prior experience microinjecting to highly skilled with 14 years of microinjecting experience. Of the technicians assigned to the low experience class for manual injections (technicians 1-3), technician one had no prior experience, technician two had one-month of experience, and technician three had almost one-year of experience. For technicians with high experience for manual injections (technicians 4-6), technician four had

three years of experience, technician five had four years of experience, and technician six had five years of experience. Regarding the automatic injections, of the low experience technicians (1, 2, and 4), technician one had no prior experience, technician two had one-month experience, and technician four had almost one-year of experience. For technicians in the high experience class for the automatic injector (6, 3, and 5), technician six had one year of experience, technician three had two years of experience, and technician five had approximately fourteen years of experience. Upon completion of their repetitions using both injection methods, the technician was asked to indicate which injection method they preferred.

When the triploid channel catfish fry reached 5 days post-hatch (DPH) they were transported to the lab for transplantation. Individual fry were anesthetized using 10 mg/L MS-222 solution and observed microscopically at 1.5× (Amscope, Irvine, CA) to facilitate injecting the cells near the genital ridge. Fry were positioned on their side to allow for needle insertion between the yolk sac and anal fin at the expected location of the genital ridge. Injections consisted of 1 uL unsorted cell suspension containing roughly 80,000 cells. Technicians first used a hand-held injector having a 33-gauge needle (inner diameter: 0.108 mm; outer diameter: 0.209 mm; Hamilton, Reno, NV) with a manual microinjector (Hamilton, Reno, NV) to inject 10 fish for 3 repetitions (30 fish total per technician) while time was recorded for each repetition.

Next, technicians were asked to repeat the injections using the automatic injector. For the automatic injector, a Flaming / Brown (model P-97) micropipette puller was used to heat and pull glass capillary tubes into needles. Next, a Fisher Scientific 10 µL micropipette was used to load 10 µL of cell suspension into the glass needle which was subsequently loaded into the injection apparatus. The apparatus consisted of MPPI-3 pressure injector (Applied Scientific Instruments), set at a pulse duration of 4 and 15 psi, connected to a 99% pure pressurized

nitrogen canister. To allow for needle manipulation and injection, the pressure injector was connected to a manual micromanipulator (MM 33, Märzhäuser Wetzlar) and secured to a magnetic base (MHC Industrial Supply Company). Again, technicians completed 3 repetitions of injections consisting of 10 fish each (30 fish total per technician) while time for each repetition was being recorded. Injected fry were then recovered in ~ 1 L of pond water with aeration before being moved to flow through pond water troughs with individual baskets for each repetition of 10 fish.

2.5. DNA analysis

After 5 weeks of grow out, fry were euthanized and had gonads surgically removed. Gonad samples were then placed in individual 1.5 mL tubes for DNA extraction. Prior to extraction, samples were held at -80 ºC. DNA extractions took place using proteinase K digestion which was followed by protein and ethanol precipitation as described by Waldbieser and Bosworth (2008). DNA concentrations were measured using a NanoDrop® 2000 spectrophotometer (Thermo Fisher Scientific) and adjusted to ~ 500 ng/µL via dilutions with RNase/Dnase free water. PCR was performed using 10 µL microcentrifuge tubes prepared with 1 µL of the DNA sample, 2.2 µL of RNase/Dnase free water, 0.6 µL of each primer follistatin (Fst) and hepcidin (Hamp), and 5 µL of 2x Eco. Thermocycling was accomplished using a Bio-Rad T100-thermalcycler. The Hamp and Fst amplification products were resolved on an ethidium-bromide-stained agarose gel (2%), and gels were analyzed using a Biorad GelDoc™ XR+ molecular imager, and the ImageLab software.

2.6. Statistical analysis

All data were analyzed using GraphPad Prism statistical analysis software (v.10.0.3; GraphPad Software Inc., Boston, MA, USA). Firstly, technicians were divided into two groups determined by prior experience using manual or automatic injection techniques. With respect to the injection method, technicians with less than one year experience were assigned to the low experience class, while technicians with over one year experience were assigned to the high experience class. Unpaired t-test models were then used to compare the injection success rates with either injection method between the two classes, the injection success rates between the two classes regardless of injection method, and the injection success rates between the two methods regardless of experience class. Paired t-test models were used to determine the improvement of each class by comparing the injection success rates achieved during their first and last injection repetitions for each injection method as well as comparing the improvement for each injection method independently regardless of experience class. Alpha was set to 0.05.

3. Results

In this study, injection success was defined as the number of xenogens produced per repetition. Xenogens were confirmed via DNA analysis. Technician proficiency was examined by comparing individual injection success rates and the success rate of xenogen production adjusted to a standard amount of time (one hour).

Low experience technicians had success ranging from 11.11% to 87.5% using the manual injector and 20% to 80% using the automatic injector. High experience technicians had success ranging from 25% to 80% using the manual injector and 33.33% to 57.14% using the automatic injector. There was found to be no significant relationship between low and high experience

groups for the manual injector (P = 0.386, Fig. 3.1A) or for the automatic injector (P = 0.369, Fig. 3.1B). No significant relationships were found in the comparisons between pooled observations for low experience and high experience classes regardless of injection method (P = 0.215, Fig. 3.1C) or for the comparison between injection methods regardless of technician experience class (P = 0.214, Fig. 3.1D). No significant improvement was found for the low experience class using either manual or automatic injection techniques (P = 0.362 and P = 0.875 respectively, Fig. 3.2A/B). No significant improvement was found for the high experience class using either manual or automatic injection techniques as well (P = 0.193 and P = 0.086 respectively, Fig. 3.2C/D). When comparing improvements made between the first and last repetitions for manual and automatic injections regardless of technician experience level no significant relationships were found (P = 0.065 and P = 0.528 respectively, Fig. 3.2E/F).

The time taken for each technician to complete a full repetition of ten injections was also recorded for each of the injection methods so that the number of injections per hour and the number of successful injections per hour could be determined (Table 3.1A/B). Across all technicians, time to complete one repetition ranged from 3.95 minutes to 14.93 minutes for the manual injector and 3.72 minutes to 14.93 minutes for the automatic injector. For technicians using the manual injector the mean success rates over the 3 repetitions were 49.7, 58.1, 79.6, 54.2, 39.9, and 63.7% for technicians one through six respectively. The mean xenogens produced per hour for the manual injector were 23, 29.8, 66.8, 48.2, 51.8, and 58.4, respectively. Of all technicians, regardless of experience, the mean success rates using the manual injector was 57.5%, the mean number of xenogens produced was 46.3. Using the automatic injector, the mean values for success rate were 48.3, 51.6, 57.6, 42.1, 44.4 and 52.3%, respectively for technicians 1, 2, 4, 6, 3, and 5. Mean values for xenogens produced per hour for technicians using the

automatic injector were 24.3, 32.1, 30.2, 23.4, 38.8 and 73.9, respectively. Of all technicians, regardless of experience, the mean success rate using the automatic injector was 49.4%, the mean number of xenogens produced per hour was 37.1.

The improvement made by individual technicians was determined by comparing the injection success rates (number of xenogens produced) between their first and subsequent repetitions (Table 3.1A/B). Regarding the manual injector, technicians one and four showed improvement over both subsequent repetitions. Technicians three and five showed improvement during their second repetition but their success rates were not notably different in their third repetitions. Technicians two and six had decreased success rates in their second repetitions but showed improvement in their final repetitions. While using the automatic injector, technician two had notable improvement over both subsequent repetitions. Technicians one and six had decreased success rates in their second repetitions but showed improvement in their final repetitions. Technician four showed no improvement in their second repetition and decreased success in their final repetition. Technician three showed constant injection success over all repetitions, and technician five showed decreased success in their second repetition and no notable change in their final repetition.

Upon completion of their injections for all repetitions using both injection methods technicians were asked to choose which injection method they preferred. Technicians 1, 2, and 3 preferred using the automatic injector over the manual injector. Technicians 4 and 6 preferred the manual injector while technician 5 had no preference between methods.

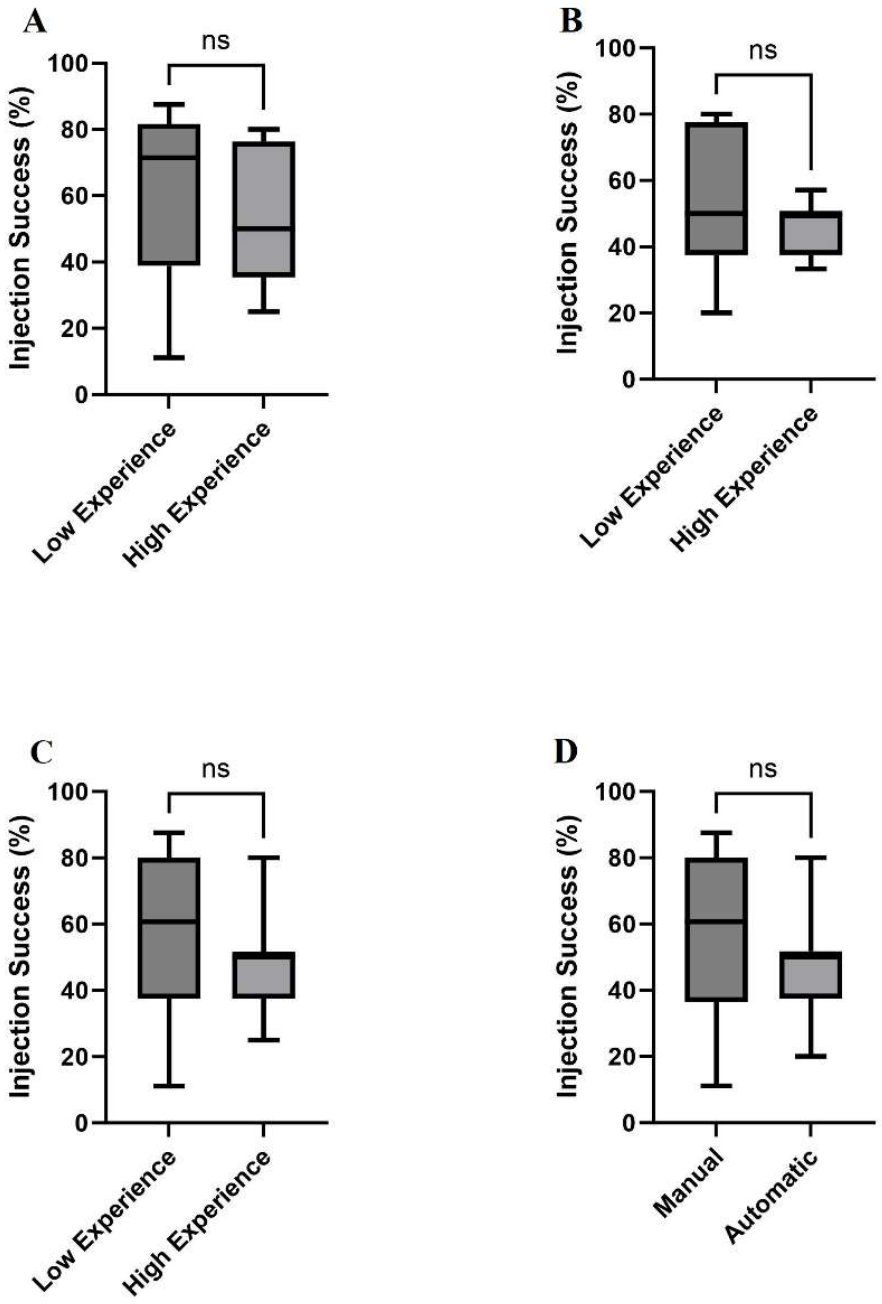

Fig. 3.1. Injection success rates with manual and automatic injection methods between high and low experience technicians (P = 0.386 and P = 0.369, A-B respectively), the injection success rates between the two classes regardless of injection method (P = 0.215, C), and the injection success rates between the two methods regardless of experience class (P = 0.214, D). The box represents values from quartile 1 to quartile 3 with the bold line indicating the median. Unpaired t-tests were used for statistical analysis.

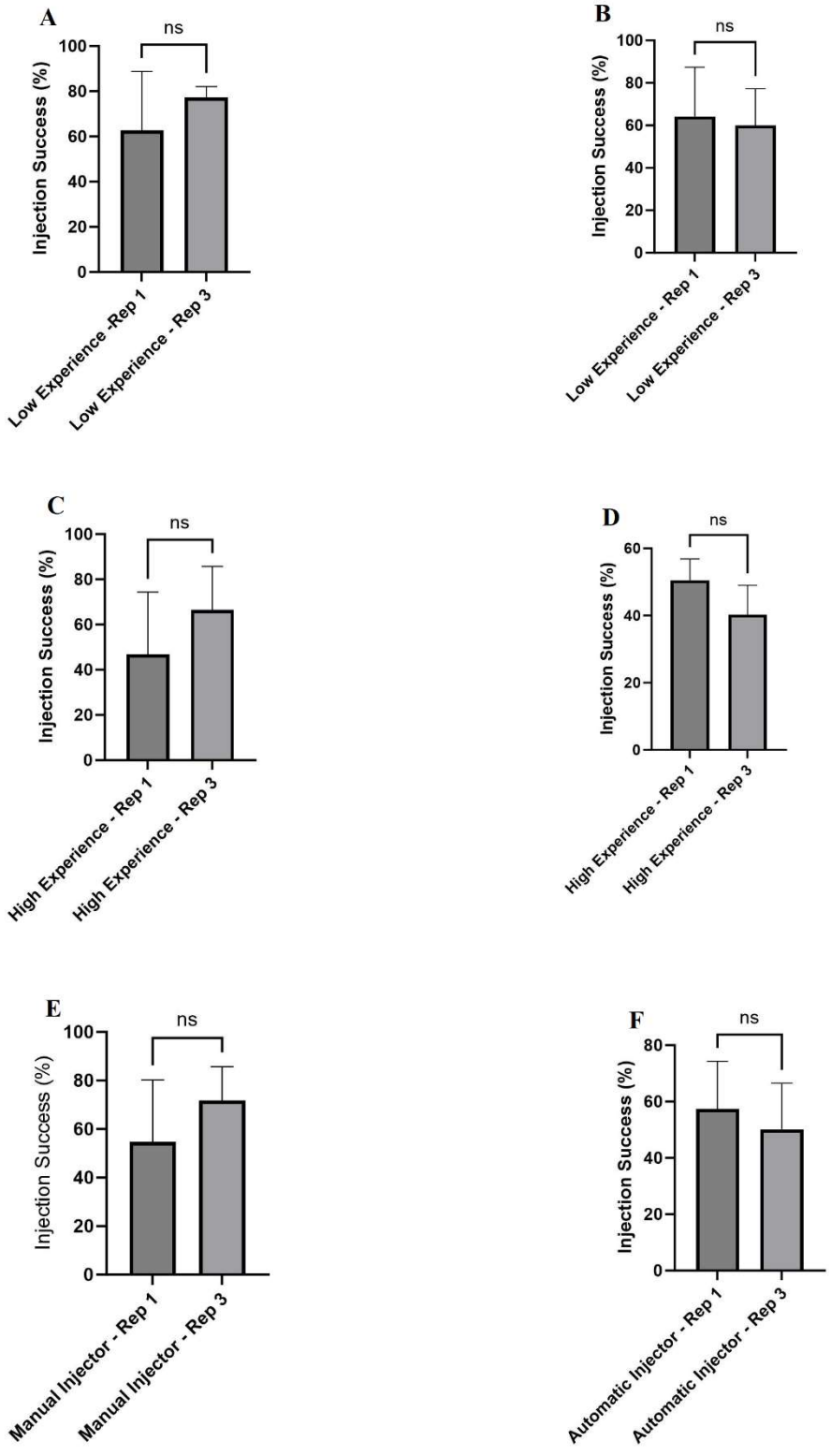

Fig. 3.2. Improvement of low experience and high experience technicians by comparing the injection success rates achieved during their first and last injection replicates for manual (P = 0.362 and P = 0.193 respectively, A/C) and automatic (P = 0.875 and P = 0.086 respectively, B/D) injection methods as well as comparing the improvement for manual and automatic

injection methods regardless of experience class. (P = 0.065 and P = 0.528 respectively, E-F). Paired t-test models were used for statistical analysis.

Table 3.1. Comparisons for technician mean success rates (% of injections resulting in xenogen production) for technicians one through six, their improvement over subsequent repetitions compared to the success rate of their initial repetition, and the mean number of xenogens produced per hour while using the manual injector. Underlined values indicate technician preference for injection method (A). Comparisons for technician mean success rates (the % of injections resulting in xenogen production) for technicians one through six, their improvement over subsequent repetitions compared to the success rate of their initial repetition, and the mean number of xenogens produced per hour while using the automatic injector. Underlined values indicate technician preference for injection method (B). Technicians are listed in order from least to most experience according to the injection method.

Technician	Experience	Injection Success (%, Mean/SD) Manual	Improvement Rep 2	Improvement Rep 3	Xenogens Produced / hour (Mean/SD)
1	0	49.7/19.5	+	+	23/9.6
2	1 month	58.1/33.3	-	+	29.8/21.8
3	11 months	79.6/8.1	+	0	66.8/8.2
4	3 years	<u>54.2/19.1</u>	+	+	48.2/29.0
5	4 years	39.9/13.1	+	0	51.8/23.2
6	5 years	<u>63.7/26.4</u>	-	+	58.4/21.4
Total (Mean/SD)		57.5/13.5			46.3/16.8

Technician	Experience	Injection Success (%, Mean/SD) Automatic	Improvement Rep 2	Improvement Rep 3	Xenogens Produced / hour (Mean/SD)
1	0	<u>48.3/27.5</u>	-	+	24.3/9.9
2	1 month	<u>51.6/24.5</u>	+	+	32.1/7.6
4	11 months	57.6/19.7	-	+	30.2/5.9
6	1 year	42.1/4.0	-	0	23.4/4.7
3	2 years	<u>44.4/9.6</u>	0	-	38.8/8.2
5	14 years	52.3/4.4	0	0	73.9/8.2
Total (Mean/SD)		49.4/5.6			37.1/18.9

SD = standard deviation; + = notable improvement; - = decreased success; 0 = no notable change

4. Discussion

Two major technical variables in xenogenesis procedures include the experience or skill level of technicians performing cell injections as well as the method by which the cells are delivered (manual or automatic injectors). In the present study however, no significant differences were found between low experience or high experience classes for technicians using either injection method. This is likely due to highly variable data points, but some trends were observed. Although there were no significant relationships observed between the experience classes, the high experience class had lower variation regarding injection success compared to the low experience class for both the manual and automatic injection techniques. The lower level of variance in relation to injection success might indicate that technicians with more than one year experience are more consistent with their injections compared to technicians with less than one year experience.

Another important aspect to consider is the relative improvement made by technicians as they gain more experience with injection methods and procedures. In this study, improvement was determined by comparing the success rate of the first and final replicates that each experience class acquired for the two injection methods. Although no significant improvements were determined, a consistent slight improvement in success rate was observed for technicians using the manual injector across both experience classes. Only the least experienced technicians showed improvement over replications using the automatic injector. One potential reason for the observed decrease in injection success rates observed in subsequent repetitions using the automatic injector may be linked to the glass needles used in the injection apparatus. As technicians performed their injections using the automatic injectors, blockages would sometimes occur, and if these blockages went unnoticed by the operating technician, it could result in no

cells being transplanted until the blockages were detected and cleared. As technicians used the same needle for each succeeding repetition, the relative frequency of blockages may have increased leading to slightly lower success rates over time.

Technicians had more success while using the manual injector compared to the automatic injector, 57.5% and 49.4%, respectively. It is also notable that the two least experienced technicians (technicians one and two) improved their injection success rates over three repetitions while using the manual and automatic injectors, respectively. Technician preference for injection method did not predict success. In 4 of 5 cases, the percentage xenogens produced was very similar between the preferred and non-preferred methods. In one case, the technician preferred the automatic injector but produced almost double the xenogens using the manual injector. Those who preferred using the automatic injector attained equal, or in some cases, much better success by using the manual injector. The one technician with no preferred method however achieved higher injection success while using the automatic injector.

The 2 individuals with a month or less of experience produced much fewer xenogens per hour with the manual injector compared to the other 4 individuals. The automatic injector seemed to level the field, and xenogens per hour per individual were similar accept one technician who was able to produce 2-3X as many xenogens per hour compared to the other participants. This individual had 14 years of experience of microinjecting one-cell embryos with an automatic system. That skill appeared to translate to automatic injection of fry.

The percentage of xenogens produced was much less than the 60-80% produced by some of the same participants in recent studies (Hettiarachchi et al., 2022, Hettiarachchi et al., 2023). Perhaps performance was hindered by the pressure of knowing that the injections were timed.

Future experiments to study the effects of expertise may want to consider this factor in the design.

In conclusion, although few differences were identified due to high variability, valuable trends were observed. Technique has an effect on the stem cell transplantation efficiency, and comfort for one procedure compared to another does not translate to better results. In general, experienced operators are able to more successfully produce xenogens than non-trained and newly trained technicians. Although experience is important, a business focused on xenogenesis could have some initial success with untrained operators.

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