Effects of Cell Number, Host, and Timing of Injection on Efficiency of Xenogen Production in Ictalurid Catfish

by

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Keywords: Xenogenesis, surrogate, cell colonization,
cell proliferation, triploids, reproductive technology

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Abstract

Xenogenesis is a reproduction technology that has been identified for hybrid catfish (♀ channel catfish, *Ictalurus punctatus* × ♂ blue catfish, *I. furcatus*) embryo production. The xenogeneic process can be accomplished by transplanting primordial germ cells (PGCs), spermatogonial stem cells (SSCs), or oogonial stem cells (OSCs), derived from a donor diploid fish into sterile recipients, which then enables the recipient fish to produce donor-derived gametes. The most widely researched form of xenogenesis for the hybrid catfish industry involves transplanting undifferentiated blue catfish cells into triploid channel catfish surrogates. However, there is potential for further technological advancement, particularly as efforts to increase sustainability and efficiency within the U.S. catfish industry continue. Therefore, several experiments were conducted to enhance the efficiency of hybrid catfish embryo production through xenogenesis.

The primary objectives of this thesis were to assess donor cell quantities and explore surrogate species options, The first study aimed to determine whether 80,000 or 100,000 blue catfish cells per fry impacted rates of xenogenesis in surrogate channel catfish. It was identified that 100,000 cells per fry did increase both proliferation and colonization of donor cells in surrogates. The second study also aimed to determine whether 80,000 or 100,000 cells per fry impacted xenogen output, but white catfish (*Ameiurus catus*) surrogates were used along with both blue catfish and channel catfish donor cells, respectfully. It was identified that the white catfish is a suitable surrogate for hybrid catfish xenogen production, 100,000 cells per fry does lead to increased cell colonization, and no donor species was superior to the other. The third study assessed the feasibility of utilizing the common carp (*Cyprinus carpio*) as a blue catfish

sperm surrogate for hybrid catfish production implications. Common carp accepted undifferentiated blue catfish cells, which migrated to and colonized the anal fin, muscle, pectoral fin, and gonad. Donor cells did not migrate to the eye, liver, intestine, or heart. Overall, these findings will enhance the efficiency of germ cell transplantation for commercial-scale hybrid catfish production.

Keywords: Xenogenesis, Cell colonization, Cell proliferation, Stem cells, Reproduction Technology

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List of Abbreviations

2N	Diploid
3N	Triploid
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
BGC	Blue catfish gonadal cell
BW	Body weight
CAFO	Concentrated animal feeding operations
CGC	Channel catfish gonadal cell
DMEM	Dulbecco's Modified Eagle's Medium
DPH	Days post-hatch
EDTA	Ethylenediamine tetraacetic acid
FBS	Fetal bovine serum
FCR	Feed conversion ratio
fst	Follistatin
hamp	Hepcidin
HBSS	Hanks' Balanced Salt Solution
IACUC	Institutional Animal Care and Use Committee
LHRHa	Luteinizing hormone-releasing hormone analogue
MS-222	Tricaine methanesulfonate
MtDNA	(Blue catfish specific) mitochondrial DNA
OSC	Oogonial stem cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGC	Primordial germ cell
RAS	Recirculating aquaculture system
scpp-1	secretory calcium-binding phosphoprotein
SSC	Spermatagonial stem cell
TAE	Tris-acetate-EDTA
TL	Total length
U.S.	United States

Chapter 1: General Introduction

1.1 United States Aquaculture Industry

Aquaculture production is crucial for expanding the United States (US) food industry in a sustainable way while accounting for the growing human population. As climate change looms, it is important to rely less on importation and heavy carbon-emitter products such as beef. Due to the importation of ~80% of seafood products today, the US is in a ~$17 billion trade deficit (Andersen et al., 2021). For food security purposes alone, improving the aquaculture sector is exceedingly important. Over the last 10 years, other countries such as China, Vietnam, Norway, and Indonesia have rapidly increased production of domesticated aquatic organisms for food purposes, but the US has yet to fully follow suit (FAO, 2022). By expanding each aquatic species industry individually, change can be made in conjunction with an increase in consumer education and a cultural shift to consume more farmed fish.

1.2 United States Catfish Industry

For decades, the catfish industry in the US has greatly contributed to the expansion of the national aquaculture industry. Today, catfish is the market leader for the industry, making up ~75% of the entire finfish market (USDA, 2022). Despite intensive competition from imports, the catfish industry still provides livelihoods for farmers across the Southern region of the US. Alabama, Mississippi, and Arkansas are the top producers of catfish, which generated $447 million pond bank in 2022 (USDA, 2023). The hybrid catfish (♀channel catfish, *Ictalurus punctatus*, × ♂blue catfish, *I. furcatus*) is the market leader due to its favorable growth rate, high dress out percentage, increased fillet yield, efficient feed conversion ratio (FCR), improved tolerance to low dissolved

oxygen, increased survival rates, and high seinability (Yant et al., 1976; Dunham and Smitherman, 1984; 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 positive impact the hybrid catfish has had on the industry, a shortcoming is the intense labor necessary to produce hybrid embryos. The female channel catfish must be hormone injected, bag-spawned, and stripped of her eggs upon ovulation (Hu et al., 2011). The male blue catfish must be sacrificed, and his testes must be surgically removed and processed into a sperm solution to use in artificial fertilization (Dunham and Masser, 2012). This process is relatively labor intensive, time consuming, and requires extra labor costs, space, and resources (Argue et al., 2003).

1.3 Xenogenesis

As the US works to not repeat past mistakes of unsustainable growth and operation among concentrated animal feeding operations (CAFOs), it is important aquaculture grows in an environmentally conscious way in which the surrounding air, water, and land is not polluted (Donham et al., 2007). When looking at ways to continue to grow the hybrid catfish industry in the most efficient and sustainable way, new and emerging technologies such as xenogenesis can contribute towards this goal. Xenogenesis is a method of reproduction in which successive generations differ from each other and no genetic material is transmitted from the parent to the offspring (Dunham, 2023). Though this technology is still being refined, overarching goals entail finding a more efficient and sustainable way to create the hybrid catfish for the aquaculture industry.

Using xenogenesis is one way the hybrid catfish industry can become more sustainable. In this case, the channel catfish is the surrogate species and a mature female channel catfish is bag spawned. When she begins to ovulate, her eggs are stripped and artificially fertilized by a channel catfish sperm solution (Perera et al., 2017). Shortly following fertilization, eggs undergo triploidization, typically by using hydrostatic pressure. Eggs are left to incubate, and 4-6 days after hatching, fry are injected with donor blue catfish stem cells to create a xenogenic fish (Hettiarachchi et al., 2023). Primordial germ cells (PGCs) from developing embryos, spermatogonial stem cells (SSCs), and oogonial stem cells (OSCs) are the cell types that can be isolated and introduced into surrogate species (Yoshizaki et al., 2003; Perera et al., 2017). Cells must be harvested from an immature donor fish as their gonadal cells have yet to fully differentiate. The optimal size for donor ictalurid catfish is roughly 300-500 g (Hettiarachchi et al., 2023b). Undifferentiated cell injection into the triploid fry surrogates leads to the development of the donor species' gonads and gametes in the surrogate fish (Perera et al., 2017; De Siqueira-Silva et al., 2018).

Once a xenogenic individual grows and matures, they will be able to spawn naturally, and if paired correctly, a hybrid can be created without artificial fertilization or sacrifice of the male (Perera et al., 2017). This process has been proven to be successful in channel catfish surrogates injected with blue catfish stem cells (Perera et al., 2017) along with utilizing a white catfish (*Ameiurus catus*) surrogate with both channel catfish and blue catfish donor cells (Hettiarachchi et al., 2024).

Since xenogenesis is a new and emerging technology, additional research is needed to optimize the techniques for commercialization. Recent studies have analyzed optimal days post-hatch for stem cell injection (Hettiarachchi et al., 2023a), used different surrogate species to create

varying xenogen types (Hettiarachchi et al., 2024), analyzed effects of seasonality for optimizing germ cell extraction (Hettiarachchi et al., 2023b), and developed and utilized cryopreservation techniques (Hettiarachchi et al., 2022) for both oogonia (Abualreesh et al., 2021a) and spermatogonia (Abualreesh et al., 2020, 2021b). In an effort to expand on these past efforts, more research to continue to sustainably develop xenogenesis needs to be done.

1.4 Objectives

Successful unsorted gonadal cell transplantation is dependent upon surrogate's acceptance and incorporation of the foreign cells. Cell quantity is an aspect of transplantation that has not thoroughly been analyzed for the creation of xenogenic catfish. Different surrogate species', aside from the channel catfish, acceptance of foreign cells also is an area not thoroughly explored for the hybrid catfish industry. This thesis aims to analyze the impact of differing cell quantities for the creation of both channel catfish and white catfish xenogens. This thesis also aims to assess the capabilities of the white catfish to accept and colonize both blue catfish and channel catfish donor cells. Lastly, this thesis aims to assess the incorporation of the common carp (*Cyprinus carpio*) as a surrogate species injected with blue catfish cells, to further benefit the hybrid catfish industry through xenogenesis.

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Chapter II: Impacts of donor cell density and day of injection for channel catfish, *Ictalurus punctatus,* xenogen production

Abstract

Hybrid catfish (♀ channel catfish, *Ictalurus punctatus* × ♂ blue catfish, *I. furcatus*) account for ~70% of the catfish market due to superior performance over parent species. Xenogenesis has successfully enabled the production of hybrid catfish embryos by transplanting unsorted gonadal cells from donor diploid blue catfish into triploid channel catfish surrogates. The objective of this study was to assess how the density of unsorted gonadal cells impacts xenogenesis success when injected into surrogates at different days post-hatch (DPH). Triploid channel catfish fry were injected with either 80,000 or 100,000 unsorted gonadal cells, labeled with PKH26 fluorescence dye, at 4, 5, and 6 DPH. At 45 and 90 DPH, total length (TL), body weight (BW), and survival were evaluated. Colonization of donor cells was assessed by calculating percent cell area (<150 μm²) and cluster area (>150 μm²). PCR determined the percentage of xenogens from gonadal tissues. Results indicated that density of injected cells did not impact survival (P = 0.212) of surrogate fish. Cell density impacted percent cluster area, where fry injected with 100,000 cells/fry had increased rates of proliferation and colonization compared to fry injected with 80,000 cells/fry (P = 0.004). Injection day had no impact on percentage of xenogens produced. Percent cell area increased from 45 to 90 DPH (P = 0.007). PCR analyses showed that 78% and 67% of surrogates were xenogens for 80,000 and 100,000 cells, respectively (P = 0.260). These findings enhance the efficiency of germ cell transplantation for commercial hybrid catfish production.

Key words: Xenogenesis, Blue catfish, Stem cell, Reproductive technology, Surrogate

1.0 Introduction

The catfish industry continues to be the market leader in the US aquaculture sector, with Mississippi, Alabama, Arkansas, and Texas producing 96% of total domestic sales in the US, which generated $437 million in pond bank sales in 2023 (NASS, 2024). Today, hybrid catfish (♀ channel catfish, *Ictalurus punctatus* × ♂ blue catfish, *I. furcatus*) is the most commonly cultured genotype in the catfish industry (NASS, 2024) due to its high fillet yield, seinability, high dress out percentage, tolerance to low dissolved oxygen, increased disease resistance, efficient feed conversion ratio (FCR), and rapid, yet uniform, growth rate (Yant et al., 1976; Dunham et al., 1983; Dunham et al., 1987; Dunham and Argue, 1998; Bosworth et al., 2004; Arias et al., 2012; Dunham et al., 2014). An impediment to the industry is the labor-intensive *in vitro* fertilization (IVF) process that is used to create hybrid catfish. For instance, IVF entails hormonal stimulation, bag spawning, manual egg stripping for the females, and sacrifice of males for sperm removal (Hu et al., 2011), which can take 4 to 6 years of resources to raise the males to maturity (Argue et al., 2003; Hu et al., 2011).

To improve hybrid catfish embryo production and the catfish industry, a new technology, xenogenesis, is emerging (Dunham, 2023). Xenogenesis is a method of reproduction in which successive generations differ from each other and no genetic material is transmitted from the parent to the offspring (Dunham, 2023). A xenogen is an organism comprised of elements typically foreign to its species. In this case, xenogenesis would be utilized by naturally or semi-naturally (hormone-induced tank spawning in pairs) mating a channel catfish female with a xenogenic triploid channel catfish producing sperm of a blue catfish, resulting in 100% hybrid catfish embryos. This technology has been successfully utilized to produce channel catfish × blue catfish

hybrids (Perera et al., 2017) as well as a white catfish (*Ameiurus catus*) surrogate with either a channel catfish or blue catfish gonad (Hettiarachchi et al., 2024).

To continue to improve xenogenesis technology for efficiency and economic viability, a variety of studies have been conducted. Recent studies have assessed how day of injection impacts triploid fry success (Hettiarachchi et al., 2023a), how donor body size and seasonality impacts the quantity of extracted gonadal stem cells (Hettiarachchi et al., 2022; Hettiarachchi et al., 2023b), and how *in vitro* culturing impacts the health of blue catfish stem cells (Hettiarachchi et al., 2022). Cryopreservation techniques for oogonia (Abualreesh et al., 2021a) and spermatogonia have been developed to facilitate xenogen production and create gene banks (Abualreesh et al., 2020, 2021b). Most recent hybrid catfish xenogenic transplantation studies have been conducted with limited knowledge on how the density of unsorted blue catfish gonadal cells impacts the colonization and proliferation of donor cells when injected into surrogates (Shang et al., 2015; Çek et al., 2016; Perera et al., 2017; Shang et al., 2018; Hettiarachchi et al., 2020, 2022, 2023a, 2023b). Up to 80,000 cells/fry have been injected in both channel catfish (Hettiarachchi et al., 2020, 2022, 2023a, 2023b) and white catfish surrogates (Hettiarachchi et al., 2024), but exceeding 80,000 cells/fry has yet to be investigated.

The current study aims to fill this knowledge gap to further improve xenogenic technology for future hybrid catfish applications. Thus, the objective of this study was to assess how the density of unsorted gonadal cells (80,000, 100,000, or 120,000 cells/fry) from blue catfish impacts the success of xenogenesis when injected into surrogate channel catfish at 4-, 5-, or 6- days post-hatch (DPH).

2.0 Materials and methods

All investigations and experimental studies on animals were conducted according to the Institutional Animal Care and Use Committee (IACUC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) protocols and guidelines for Auburn University Institutional Animal Care and Use Committee (AU-IACUC # 2021:3893).

2.1 Broodstock management

Broodstock were housed at the Auburn University E.W. Shell Fisheries Center in Auburn, Alabama in 2022 and 2023. Channel catfish and blue catfish were cultured in 0.04- ha earthen ponds (~1 meter in depth) and fed five days a week with 32% protein pellet feed during the summer and three days per week during the winter. Leading up to the spawning season, feed was shifted to a 36% protein broodstock feed provided five days per week. In June, during peak spawning season, mature (3- to 4-year-old) channel catfish females (N = 5, mean body weight ± SEM = 0.8 ± 0.2 kg) and males (N = 5, mean body weight ± SEM = 0.7 ± 0.3 kg) were harvested from the earthen ponds by seining, using a 3.8 cm mesh net.

2.2 *In vitro* fertilization procedures

Upon harvest, gravid channel catfish females were administered luteinizing hormone releasing hormone analogue (LHRHa) at 90 μg/kg body weight via intraperitoneal implantation. Following implantation, the females were placed into individual mesh bags (38 cm × 56 cm) and held ~30 cm apart in 670 to 750 L flow through (30 L/min) pond water holding tanks. Temperature

in the tanks ranged from 26 to 28ºC. At 36 h post-implantation, bags were checked every 4 h for eggs attached to the spawning bag, which indicates that the female is ovulating. Once eggs were detected, the females were anesthetized with 100 mg/L tricaine methanesulfonate (MS-222, Ferndale, WA) and 100 ppm $NaHCO_3$ solution.

Once anesthetized, the female fish were rinsed with pond water and dried thoroughly. Crisco® vegetable shortening was carefully rubbed on the underside of the females and eggs were hand-stripped into Crisco® coated metal pans (~25 g of eggs/pan). Males were euthanized and their sperm were collected for IVF following protocols by Dunham and Masser (2012) and Hettiarachchi et al. (2022). After euthanasia, testes were removed from the body cavity with a sterile scalpel and forceps. Testes were rinsed with 0.9% saline solution to remove any blood. After rinsing, the testes were gently dried and then minced with a scalpel blade. Following mincing, the testes were filtered with a 100 μm mesh. Thereafter, 10 mL of 0.9% saline was added for each 1 g of testes (w/v). Following filtration, the sperm solution was ready for fertilization.

2.3 Triploid induction

All IVF was conducted at 27 to 28ºC. To accomplish triploid induction for channel catfish embryos, the hand-stripped eggs were first mixed in a metal pan with freshly collected mature channel catfish sperm at a rate of 2 mL of sperm solution (10 mL of 0.9% saline per 1 g of testes) per 25 g of eggs for 2 min (Hettiarachchi et al., 2023a). Next, a fertilizing solution was prepared by adding 6 g of powdered Fullers' Earth (MP Biomedicals, Santa Ana, CA) to 1 L of water. The fertilizing solution was mixed with the sperm and egg solution to prevent egg adhesion. After 3 min of gentle mixing with the fertilizing solution, eggs were transferred into a hydrostatic press,

and at 5 min post-fertilization 7,500 psi of hydrostatic pressure was applied for 5 min (Perera et al., 2017; Hettiarachchi et al., 2022, 2023a). Following pressure shock, eggs were moved into a flow-through pond water hatching trough (supplemented with $CaCl_2$ at 50 ppm) and left undisturbed for 1 h for hardening. Temperature in the hatching trough ranged from 26 to 28ºC and the flow rate was held at 3.79 L/min. After 1 h, eggs were moved into hanging mesh baskets (7.0 m × 0.4 m × 0.2 m), which were suspended in a flow-through pond water hatching trough with paddlewheel agitation and compressed aeration. Temperature remained between 26 to 28ºC and the flow rate was 15 L/min in the hatching trough.

2.4 Isolation of donor gonadal cells from immature blue catfish

In preparation for cell injections, sexually immature 1- to 2-year-old blue catfish (mean total length ± SEM = 34.76 ± 6.35 cm and mean body weight ± SEM = 349.69 ± 123.14 g) were harvested from a 0.04-ha earthen pond with a 3.8 cm mesh seine net and kept in a flow-through pond tank (7.5 m × 0.4 m × 0.25 m). Blue catfish were selected each day from the holding tank and euthanized (N = 4 fish per day × 7 days = 28 total). Following euthanasia, gonad extraction and isolation were performed to retrieve unsorted gonadal cells, including oogonia and spermatogonia cells, using protocols described by Hettiarachchi et al. (2020, 2022, 2023a, 2023b). Oogonia and spermatogonia A are equally effective in producing xenogens (Hettiarachchi et al. 2022). In brief, extracted gonads from male or female blue catfish were separately placed on a sterile petri dish (100 mm × 15 mm) which contained 5 mL of Hanks' Balanced Salt Solution [(HBSS, GE Healthcare Life Sciences, Marlborough, MA) supplemented with 1.0 µg/mL $NaHCO_3$ (Church & Dwight Co., NG) and 100 U/mL Penicillin - Streptomycin (Life Technologies,

Carlsbad, CA)]. Samples were individually placed in freshly prepared 0.5% bleach solution for 2 min to remove blood and then rinsed 3 times in both HBSS and phosphate-buffered saline (PBS; Alfa Aesar, Haverhill, MA). Samples were then diced with a sterile scalpel blade and 0.25% trypsin - ethylenediamine tetra acetic acid (EDTA; Life Technologies) was added at 50 times the weight of each sample. Following trypsinization, filtration was done using a 70 µm and 40 µm cell strainer (VWR International) and the remaining solution was centrifuged at 500 g (Eppendorf Centrifuge 5418 R) for 10 min. The resulting pellet was resuspended in 2 mL Dulbecco's Modified Eagle's Medium/DMEM (DMEM; Corning Life Sciences, Corning, NY) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 unit/mL penicillin-streptomycin (Life Technologies), and 200 mM L-glutamine (Life Technologies). The resulting suspension was comprised of unsorted gonadal cells including spermatogonial stem cells (SSC) or oogonial stem cells (OSC) (Fig. 1.1A).

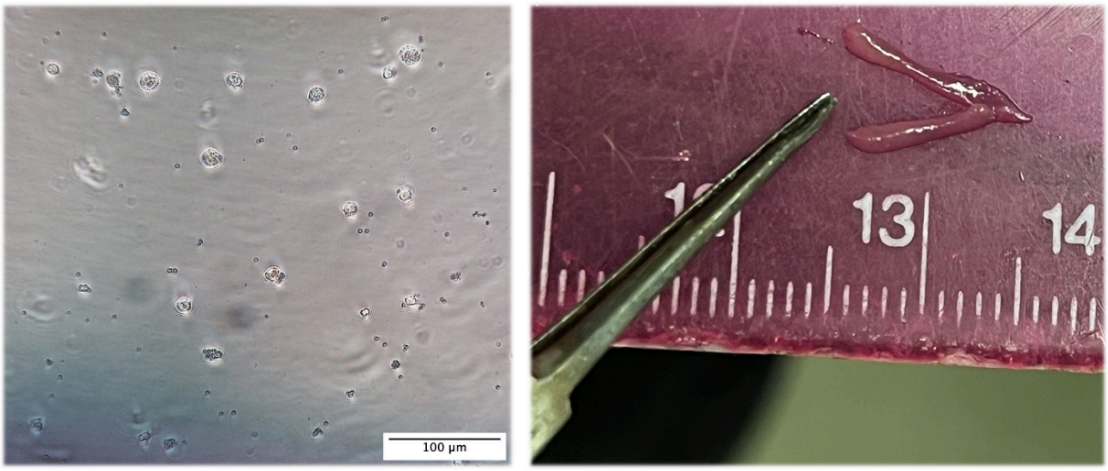

Figure 1.1. (A) Unsorted gonadal cells extracted from immature blue catfish (*Ictalurus furcatus*) gonads. (B) Surgically removed gonad from male triploid channel catfish (*I. punctatus*) surrogate fry at 90 days post-hatch.

2.5 Gonadal cell labeling and transplantation

Unsorted gonadal cells including SSCs and OSCs, from immature blue catfish were labeled with PKH26 (CGLDIL, Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. PKH26 is a red fluorescence cell linker used to determine colonization and proliferation rates of injected cells within 100 days of injection (Lee and Yoshizaki, 2016; Hettiarachchi et al., 2020, 2022, 2023a, 2023b). Unsorted cells were counted using a hemocytometer and divided into three separate tubes: 80,000 cells/µL, 100,000 cells/µL, and 120,000 cells/µL. Concentrations of cells were factorially transplanted into triploid channel catfish fry at 4, 5, or 6 DPH following standard protocols described by Hettiarachchi et al. (2020, 2022, 2023a, 2023b). The original design included 120,000 cells/fry, but due to viscosity these injections were not possible, and the treatment was eliminated.

Triploid channel catfish fry (2 cell density concentrations × 3 injection days × 3 replicates × 20 fry = 360) were anesthetized using 100 ppm MS-222 buffered with 10 ppm $NaHCO_3$ solution. The anesthetized fry were placed in a Petri dish (100 mm × 15 mm) and observed microscopically at 1.5× (Amscope, Irvine, CA) while being manually injected with a 33-gauge needle (outer diameter: 0.209 mm; inner diameter: 0.108 mm; Hamilton, Reno, NV) containing a 1 µL cell suspension containing either 80,000 cells/µL or 100,000 cells/µL PKH labelled unsorted gonadal cells. The needle was carefully inserted into the body cavity between the anal fin and yolk sac, which is the area where the genital ridge is expected to form. After injection, the fry were placed in a 5.7 L recovery container with proper aeration before being moved back to mesh fry cages (0.2 m × 0.2 m × 0.25 m) in flow-through troughs (7.5 m × 0.4 m × 0.15 m) at a density of 20 fry/cage.

2.6 Data collection

2.6.1 Growth and survival

Following cell transplantation, fry were fed a standard commercial catfish fry feed (crude protein: 50.00%, crude fat: ≥4.00%, crude fiber: 7.00%, and phosphorus: 0.80%) three to five times per day to satiation. The feed pellet size was increased gradually as their mouth size increased. To determine growth, nine fingerlings were randomly selected from each treatment at 45 and 90 DPH, and total length (TL) and body weight (BW) were determined. Survival rates were determined between day 0 (day of injection) through each sampling point (45- and 90-days post-injection).

2.6.2 PKH26 analysis

From each treatment, three fingerlings were randomly selected to analyze the colonization and proliferation of the unsorted gonadal cells inside triploid channel catfish surrogates. Fingerlings were sacrificed and gonads were surgically removed (Fig. 1.1B). Upon removal, gonads were carefully placed on a sterile microscope slide (1600221, Life Technologies). Slides were analyzed and digital images were taken using a Zeiss Imager A2 microscope equipped with a digital camera (Axio-cam 202) and Zen Pro v.6.1 software (Zeiss, Oberkochen, Germany). Gonad regions that fluoresced red were viewed as "positive" cells for successful cell colonization, and images that failed to fluoresce were viewed as "negative" cells. Three fluorescent images were taken for each gonad region and were further analyzed using ImageJ software (Fig. 1.2). Percent cell area and cluster area were determined based on ImageJ analysis. Cell areas were defined as

having a fluorescence area <150 μm^2 and cluster areas were defined as having a fluorescence area >150 μm^2.

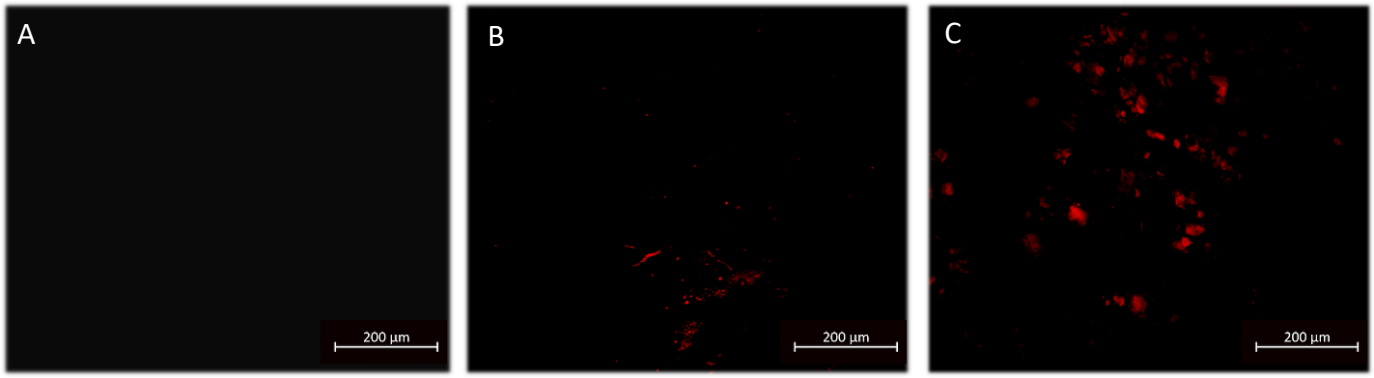

Figure 1.2. (A) Gonadal tissues of a triploid channel catfish (*Ictalurus punctatus*, surrogate) expressing fluorescence from PKH26 dyed donor-derived unsorted gonadal cells. (A) A non-injected control treatment sampled at 45 days post-hatch (DPH), showing no fluorescence. (B) Cell areas/clusters in a triploid channel catfish (surrogate) sampled at 45 DPH injected with 80,000 blue catfish (*I. furcatus*) unsorted gonadal cells/fry at 5 DPH. (C) Cell areas/clusters in a channel catfish (surrogate) sampled at 45 DPH injected with 100,000 blue catfish unsorted gonadal cells/fry at 5 DPH.

2.6.3 DNA extraction and PCR analysis

Three additional fingerlings were sacrificed per treatment at both sampling intervals (45 and 90 DPH) to determine the presence of channel catfish and blue catfish DNA. Gonads were extracted from the sacrificed fingerlings (Fig. 1.1B) and placed into 1.5 mL Eppendorf tubes for DNA extraction. Samples were kept on ice and then held at -80ºC until DNA extraction was conducted. Proteinase K digestion was first used for extraction and then protein/ethanol

precipitated. Following DNA extraction, PCR analysis was conducted using 0.6 µL of each primer follistatin (*fst*) and 0.3 of each µL hepcidin (*hamp*), 5 µL of 2x Eco, 1.7 µL of RNase/DNase free water, and 1.5 µL of the DNA sample. Following thermal cycling, a 2.0% agarose gel was used for ethidium-bromide for staining (Waldbieser and Bosworth, 2008, Table 1.1).

Table 1.1. The primers [*fst* (follistatin) and *hamp* (hepcidin antimicrobial protein)] that were used in PCR analysis to differentiate channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*). Primers were previously described by Waldbieser an Bosworth (2008).

Gene	Forward primer	Reverse primer	Amplicon (bp)	
			Channel catfish	Blue catfish
fst	ATAGATGTAGAGGAGCATTTGAG	GTAACACTGCTGTACGGTTGAG	348	399
hamp	ATACACCGAGGTGGAAAAGG	AAACAGAAATGGAGGCTGGAC	222	262

2.6.4 Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro-Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). Survival, growth (BW, TL), and fluorescent image analyses (cell area and cluster area) were analyzed using three-way ANOVA models which contained the cell density [80,000, 100,000 cells/fry (120,000 cells could not be injected because of viscosity)], sampling interval (45 and 90 DPH), and fry injection day (4, 5, 6 DPH) main effects,

as well as associated interactions. If higher-order interactions were detected the saturated models were decomposed into lower-order ANOVA models. If no significant interactions were detected, the cell density, DPH, and sampling interval main effects were interpreted. Data were arcsin square root or $\log_{10}$ transformed to meet ANOVA assumptions when necessary. Post-hoc testing was done using Tukey's HSD test with alpha set at 0.05 for interactions and main effects. One-way ANOVAs were run to compare non-injected triploid channel catfish fry to treatment fry. Paired t-tests were used to compare the percentage of xenogens produced with 80,000 or 100,000 cells/fry at both 45 and 90 DPH. Fisher's Exact Test was used to compare the percentage of xenogens detected using PCR compared to those detected using PKH26.

3.0 Results

3.1 Survival and growth

No significant interactions were detected for fry survival ($P \geq 0.212$; Fig. 1.3A). Cell density ($P = 0.390$; Fig. 1.3B), sampling interval ($P = 0.999$; Fig. 1.3C), and injection day ($P = 0.982$; Fig. 1.3D) also had no impact on fry survival. Survival of non-injected controls was not different from that of the fry injected with cells after 45 ($P = 0.924$) and 90 ($P = 0.882$) days post-injection.

For fry BW, the injection day × sampling interval interaction was significant ($P = 0.006$); therefore, the model was revised to examine the effect of injection day at each sampling interval (Fig. 1.3F, G). At 45 DPH, BW did not differ between the injection days ($P = 0.482$; Fig. 1.3F), while at 90 DPH, fry injected on 4 DPH were significantly heavier than those injected on 5 or 6 DPH ($P = 0.001$; Fig. 1.3G). Cell density had no impact on fry BW ($P = 0.185$, Fig. 1.3H), and

BW of non-injected controls did not differ from that of the fry injected with cells after 45 (P = 0.063) and 90 (P = 0.084) days of growth.

Fry TL had a significant injection day × cell density interaction (P = 0.019). Therefore, the effect of injection day in relation to cell density was examined to understand the interaction (Fig. 1.3). Cell density had no impact on fry TL at 4 (P = 0.910; Fig. 1.3J), 5 (P = 0.751; Fig. 1.3K), and 6 DPH (P = 0.504; Fig. 1.3L). At 90 DPH, fry were significantly longer than those sampled at 45 DPH (P = 0.001; Fig. 1.3M), and TL of non-injected controls did not differ from that of the fry injected with cells after 45 (P = 0.071) and 90 (P = 0.233) days of growth.

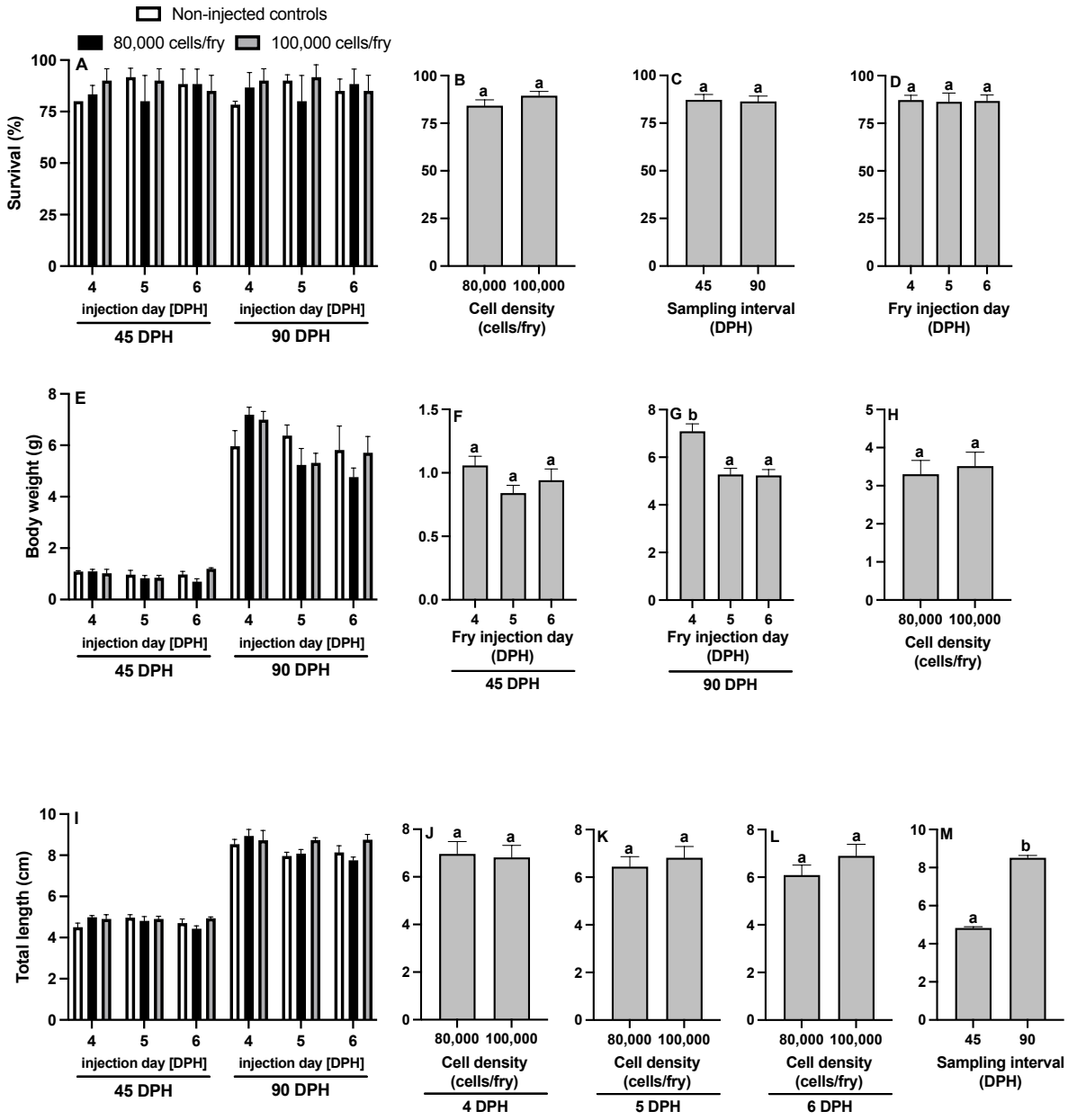

Figure 1.3. Percentage survival of triploid xenogenic channel catfish (*Ictalurus punctatus*) injected with blue catfish (*I. furcatus*) unsorted gonadal cells and non-injected controls at (A) 45 days post-hatch (DPH) and 90 DPH, comparing injection days (4, 5, & 6 DPH) and injection density (80,000 and 100,000 cells/fry). (B) Survival based on cell density quantities injected: 80,000 or 100,000 cells/fry. (C) Survival based on sampling interval: 45 or 90 DPH. (D) Survival based on injection day: 4, 5, 06 DPH. (E) Body weight (BW) at 45 DPH and 90 DPH, for triploid

xenogenic channel catfish injected with blue catfish unsorted gonadal cells from 4 to 6 DPH with either 80,000 or 100,000 cells/fry (N=63). (F) BW at 45 DPH, at each injection day (4, 5, and 6 DPH). (G) BW at 90 DPH, at each injection day (4, 5, 6 DPH). (H) BW for fry injected with either cell density: 80,000 or 100,000 cells/fry. (I) Total length (TL) at 45 DPH and 90 DPH for triploid xenogenic channel catfish injected with blue catfish unsorted gonadal cells from 4 to 6 DPH, with either 80,000 or 100,000 cells/fry (N=63). (J) TL cell density comparison for fry injected at 4 DPH. (K) TL cell density comparison for fry injected at 5 DPH. (L) TL cell density comparison for fry injected at 6 DPH. (M) sampling interval comparison for TL. [ab]Means followed by the same letter are not different (P > 0.05, three-way ANOVA model).

3.2 Quantifying PKH26 florescent labeling

Unsorted gonadal cell colonization and proliferation was quantified using PKH26 dye (Fig. 1.2). For percent cell area, there were no significant interactions detected (P = 0.943; Fig. 1.4A). Cell density did not have an impact on colonization or proliferation rates in fry for percent cell area (P = 0.445; Fig. 1.4B). Percent cell area increased significantly from 45 to 90 DPH (P = 0.007; Fig. 1.4C), whereas injection day had no impact on percent cell area (P = 0.813; Fig. 1.4D).

Percent cluster area (>150 μm^2, %) also had no significant interactions (P = 0.777; Fig. 1.4E). Cell density had a significant impact on colonization and proliferation rates in surrogate fry for percent cluster area (P = 0.004; Fig. 1.4F), such that 100,000 cells/fry led to increased fluorescing regions in surrogates. Sampling interval (P = 0.097; Fig. 1.4G) and injection day (P = 0.422; Fig. 1.4H) had no impact on percent cluster area.

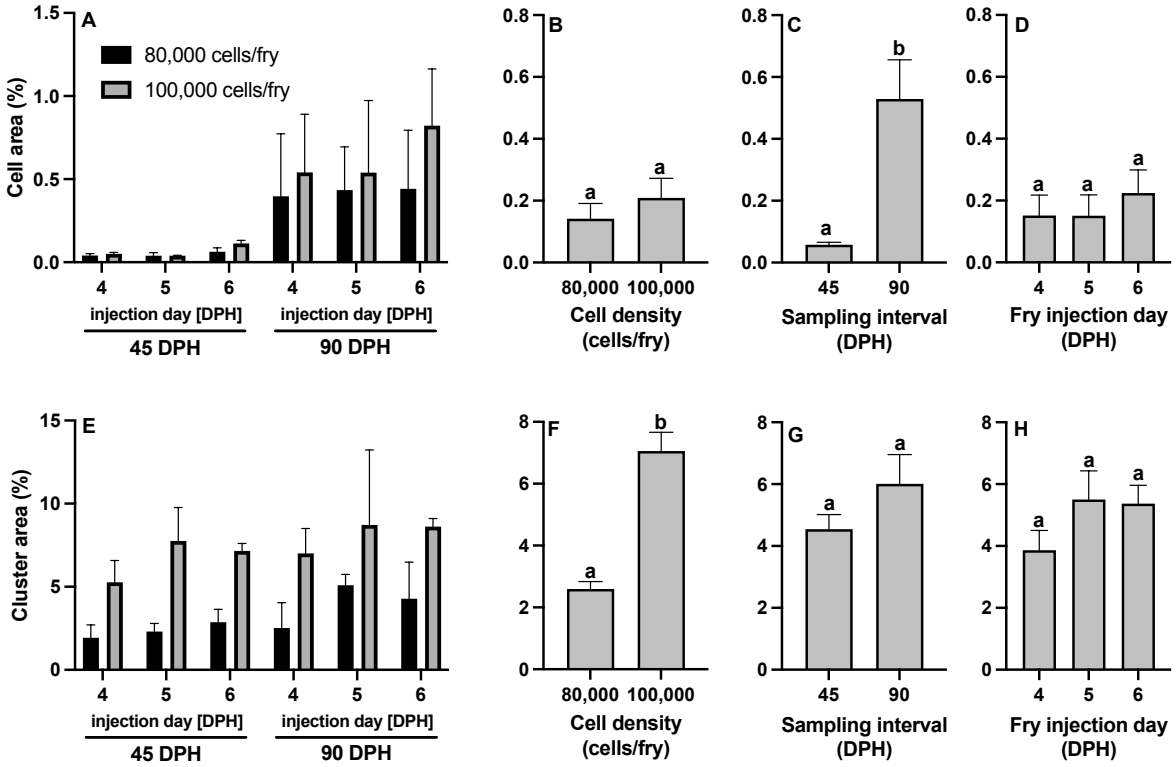

Figure 1.4. (A) Cell area (%, <150 µm2) and (E) cluster area (%, >150 µm2) at 45 days post-hatch (DPH) and 90 DPH of triploid channel catfish (*Ictalurus punctatus*) injected with blue catfish (*I. furcatus*) unsorted gonadal cells from 4 to 6 DPH with either 80,000 or 100,000 cells/fry (N = 18). (B) Cell area based on cell density quantities injected (80,000 or100,000 cells/fry). (C) Cell area based on sampling interval (45 and 90 DPH). (D) Cell area based on fry injection day (4, 5 or 6 DPH). (F) Cluster area based on cell density quantities injected (80,000 or100,000 cells/fry). (G) Cluster area based on sampling interval (45 and 90 DPH). (H) Cell area based on fry injection day (4, 5 or 6 DPH). [ab]Means followed by the same letter are not different ($P < 0.05$, three-way ANOVA model).

3.3 Rate of xenogenesis

PCR analysis detected blue catfish donor-derived cells in the gonads of triploid channel catfish fry when injected with 80,000 and 100,000 cells/fry (Fig. 1.5). When surrogate fish were observed at 45 DPH, the percentage of xenogens detected was 83.7% and 79.3% for surrogates injected with 80,000 and 100,000 cells/fry, respectively. At 90 DPH, the percentage of xenogens detected was 77.78% and 66.67% for surrogates injected with 80,000 and 100,000 cells/fry, respectively. No significant differences were found when comparing percent xenogens detected by DNA analysis for surrogates injected with 80,000 cells/fry vs. 100,000 cells/fry ($P = 0.260$).

Percent xenogens also were not different ($P < 0.999$) for surrogates injected with 80,000 cells/fry (100.0%) vs. 100,000 cells/fry (94.4%) as detected by PKH26 analysis. Significantly more ($P=0.01$) xenogens were detected using PKH26 (97.2%) than with PCR (77.8%).

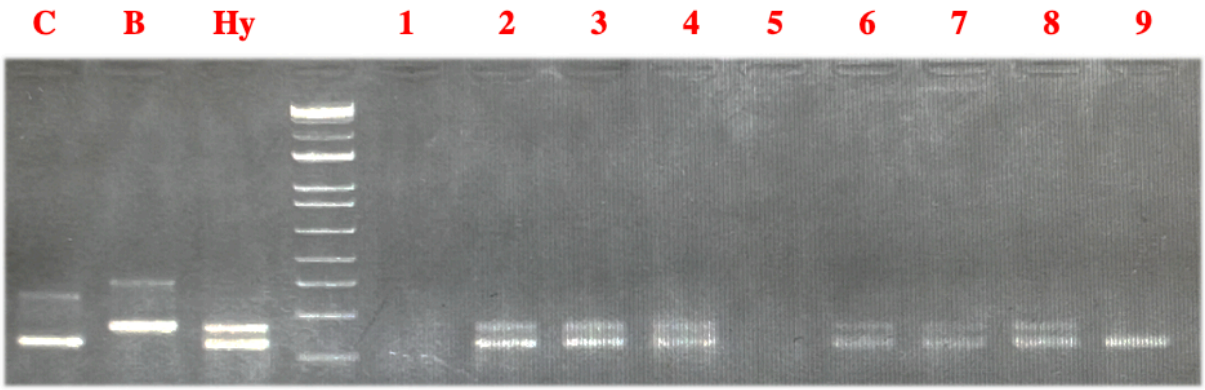

Figure 1.5. Sample results from PCR for detecting blue catfish (*Ictalurus furcatus*) donor cells in the testes of triploid channel catfish (*I. punctatus*). Blue catfish and channel catfish cells were differentiated with PCR using follistatin (*fst*) and hepcidin antimicrobial protein (*hamp*) genes as markers. C = channel catfish control, B = blue catfish control, Hy = female channel catfish × male blue catfish hybrid controls.

4.0 Discussion

The present study demonstrates that (i) survival and growth of xenogens were not impacted by injecting unsorted gonadal cells; (ii) 100,000 cells/fry leads to increased percent cluster area colonization and proliferation in xenogenic catfish surrogates; and (iii) both percentage of cell and cluster area increased from 45 to 90 DPH, demonstrating increased rates of proliferation. These results add to the current body of knowledge on xenogenesis for catfish (Perera et al., 2017; Hettiarachchi et al., 2020, 2022, 2023a, 2023b) along with past studies using other surrogate species (Morita et al., 2015; Lujić et al., 2018; Franěk et al., 2021, 2022).

Increased utilization of xenogenic catfish could improve hybrid catfish hatchery efficiency. However, increasing the number of injected cells inside the surrogate could lead to increased mortality, as transplantation of cells into sterile fry can often result in injury and stress (Hettiarachchi et al., 2023a). As such, mortalities are likely to occur as the number of cells injected into the surrogate increases, especially due to the sensitivity of fry during these "critical" early life stages. However, we found no differences in survival between fry injected with 80,000 or 100,000 cells, suggesting that 100,000 cells/fry can be utilized for increasing xenogen output.

In this study, we used percent cell area and cluster area to quantify colonization and proliferation rates of injected cells in surrogate gonads. Past studies using various forms of surrogacy applications have used 5,000 spermatogonia cells/fry in germ cell-depleted zebrafish (*Danio rerio*) surrogates (Franěk et al., 2022), ~15,000 germline stem cells/fry in rainbow trout (*Oncorhynchus mykiss*) surrogates (Marinović et al., 2022), and 30,000 to 50,000 germ stem cells/fry in goldfish (*Carassius auratus*) surrogates (Franěk et al., 2021). Thus, the number of cells/fry varies based on species. In our experiments only ~50 to 60% of injected unsorted gonadal cells are SSCs and OSCs (Perera et al., 2017; Shang et al., 2018). Despite this, our injection density

is still at the higher end of the spectrum, as compared to most aquatic species. Injecting germ stem cells in surrogates is the most common method for creating xenogens, but cell extraction protocols vary by species (Lujić, et al. 2018; Hettiarachchi et al., 2020, 2022, 2023a, 2023b; Franěk et al., 2021, 2022; Marinović et al., 2022). A universal protocol is not feasible as species morphology and physiology differs.

In preliminary studies, we attempted to inject 120,000 cells/fry. However, needle clogs (outer diameter: 0.209 mm; inner diameter: 0.108 mm) occurred frequently and successful injection was not possible (unpublished data). This prevented us from testing cell injection quantities >100,000 cells/fry. A larger needle gauge (outer diameter: 0.261 mm; inner diameter: 0.133 mm) yielded fewer clogs but resulted in large puncture wounds in the fry, often piercing through the body cavity. Thus, at present, 100,000 cells/fry appears to be the upper injection threshold, until more suitable injection techniques become available.

Both the percent colonization as well as the proliferation are important for determining success of xenogenesis. In the current study, two techniques, PCR and PKH26 fluorescence were used to determine the success of the stem cell injections. The PCR is a good and rapid technique to identify xenogens. However, it does not distinguish between low and high colonization (Lee and Yoshizaki, 2016; Hettiarachchi et al., 2020) without elaborate DNA measurements. The PKH26 technique requires some extra effort but the quantification of the colonization and proliferation is likely a better indicator of the future progeny production for a particular treatment.

One would expect that PCR would be more sensitive for detecting potential xenogens than PKH26 analysis. However, 35 of 36 (97.2%) individuals evaluated using PHK26 were deemed xenogenic while 28 individuals (77.8%) from another random sample of 36 at the same time points were identified as xenogenic, which is the opposite expectation. We do not have a good explanation

for these results unless there is some type of competitive effect during PCR amplification since DNA from both the donor and surrogate are present.

Advances in PKH26 technology have improved our understanding of colonization and proliferation of donor cells in xenogens (Hettiarachchi et al., 2023a). Results have varied from one study to another (Hettiarachchi et al. 2022; 2023a), highlighting the need for improved injection techniques to more consistently increase colonization and proliferation of the donor stem cells. Tracking the present surrogates overtime to assess maturity and spawning rates should be the next phase of this study to further understand how higher cell quantities impact maturity and the output of the desired donor progeny.

In the current study, cell area percentage increased as surrogate fry aged. This aligns with past studies by Hettiarachchi et al. (2023a), demonstrating successful colonization and continued proliferation of cells in surrogates. In other species, colonization and migratory potential are strongly influenced by injection age, often decreasing as the age at injection increases (Franěk et al., 2022). In channel catfish surrogates, injecting cells after 7 DPH decreased colonization success (Hettiarachchi et al., 2023a). Hettiarachchi et al. (2023a) found the optimal injection days for channel catfish fry to be 4 to 6 DPH. Percent cell area in the surrogates decreased slightly (~5 to 4 %) from the first to the second sampling interval, but percent cluster area increased in size at the second sampling interval (~4 to 9%), likely due to cell areas combining to create cluster areas (Hettiarachchi et al., 2023a). A similar study by Hettiarachchi et al. (2022) using xenogenic channel catfish, also found both percent cell area and cluster area to increase from the first to the second sampling interval when using both fresh and cryopreserved SSCs and OSCs (Hettiarachchi et al., 2022). Across other species, there are inconsistencies whether or not injected cell colonies tend to increase or decrease as surrogate fry age, as the biggest factor tends to be related to

surrogate acceptance of the foreign cells (Franěk et al., 2022). Of course, sampling time and the eventual fading of the fluorescent dye are variables that can have a strong impact on the variation among these studies.

Future research for producing xenogenic catfish should focus on utilizing pure populations of stem cells to reduce the number of cells injected. This would require some type of purification procedure. As stated previously, for the production of xenogenic catfish, ~50 to 60% of injected cells are stem cells (Perera et al., 2017; Shang et al., 2018). Thus, the number of somatic cells injected could be reduced by improving *in vitro* pure stem cell culture. This would likely improve colonization and proliferation rates in the gonad region and reduce the need for higher quantities of unwanted cells being injected into surrogates. Current cell purification techniques for catfish stem cells are inefficient and lead to excessive cell death (Hettiarachchi et al., 2022). Thus, improving cell culture to generate sustained cell growth, development, and regeneration could also reduce the number of immature donor fish sacrificed. Bhattarai et al. (2023) have successfully cultured (*in vitro*) black crappie (*Pomoxis nigromaculatus)* and white crappie (*P. annularis)* ovarian tissue primary cells. Similar cell culture efforts with the blue catfish would be beneficial for the creation of the xenogenic catfish.

5.0 Conclusion

In conclusion, increasing gonadal cell quantities did not impact fry survival and growth but did enhance colonization and proliferation rates. Over time, cell proliferation was higher when 100,000 cells were injected compared to 80,000 cells. These findings are important for advancing the hybrid catfish industry along with development of xenogenesis that is technically and economically feasible for commercial use.

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Chapter III: Impacts of donor blue catfish (*Ictalurus furcatus*) and channel catfish (*I. punctatus*) cell density for creating surrogate xenogenic white catfish (*Ameiurus catus*)

Abstract

As xenogenesis technology continues to develop for hybrid channel catfish, *Ictalurus punctatus* ♀ × blue catfish, *I. furcatus* ♂ embryo production, improving surrogacy systems is desirable. The white catfish (*Ameiurus catus*) has a short maturation time making it an ideal candidate for commercial application of xenogenesis. In this study, white catfish was assessed as a surrogate species while also analyzing gonadal cell density quantities for proliferation and colonization. Triploid white catfish fry were injected with 80,000 or 100,000 blue catfish (BGCs) or channel catfish gonadal cells (CGCs)/fry labeled with PKH26 dye at 4-, 5-, and 6-days post-hatch (DPH). At 45 and 90 DPH, survival of recipients, growth performance (body weight [BW] and total length [TL]), and colonization/proliferation of donor cells were evaluated (cell area < 150 μm^2 and cluster area > 150 μm^2). PCR was used to determine percentage of xenogens from gonad samples. Growth and survival were not impacted by cell density or donor. CGCs surrogates injected with 100,000 cells/fry had a larger cell area than those injected with 80,000 cells/fry ($P < 0.05$). BGCs and CGCs surrogates injected with 100,000 cells/fry had larger cluster areas than those injected with 80,000 cells/fry ($P < 0.05$). Both cell area and cluster area increased in size by 90 DPH for BGCs ($P < 0.05$) and CGCs surrogates ($P < 0.05$). PCR analysis confirmed that >78% at 45 and >83% at 90 DPH of sampled surrogates were positive xenogens. To conclude, white catfish are a viable surrogate for producing xenogenic fry.

Keywords: Xenogenesis, Blue catfish, Channel catfish, White catfish, Reproductive technology

1.0 Introduction

The catfish industry remains a top producer for the United States (US) aquaculture industry, accounting for ~75% of total finfish production (USDA, 2022). Farmers often grow the channel catfish, *Ictalurus punctatus* ♀ × blue catfish, *I. furcatus* ♂ hybrid due to increased growth rates, harvestability, increased disease resistance, and high dress out percentage (Dunham and Brummett, 1999; Brown et al., 2011; Arias et al., 2012; Dunham et al., 2014; Dunham and Elaswad, 2018). Creating hybrid catfish involves an in vitro fertilization process (Dunham, 2023), which is labor intensive and time consuming (Argue et al., 2003; Hu et al., 2011). To aid in the efficiency and sustainability of creating hybrid catfish, an innovative technology known as xenogenesis is being developed (Dunham, 2023).

Xenogenesis is a method of reproduction in which successive generations differ from each other and no genetic material is transmitted from the parent to the offspring (Dunham, 2023). Primordial germ cells (PGCs), spermatogonial stem cells (SSCs), or oogonial stem cells (OSCs) are derived from gonads of immature donor diploid fish and then transplanted into sterile triploid recipients (Perera et al., 2017; Shang et al., 2018). This transfer leads to the development of donor-derived gametes in the surrogate (Amer et al., 2001; Yoshizaki et al., 2010; Wong et al., 2011; Perera et al., 2017; De Siqueira-Silva et al., 2018, Hettiarachchi et al., 2022, 2023a, 2023b, 2024). Since PGCs, SSCs, and OSCs, are able to migrate and colonize after transplantation, they are able to produce either an ova or sperm in the surrogate depending on the individual's predetermined sex (Yoshizaki and Lee, 2018).

Past studies have refined the xenogenesis technology (Shang et al., 2015; Perera et al., 2017; Hettiarachchi et al., 2020; Abualreesh et al., 2020, 2021a, 2021b; Hettiarachchi et al., 2022, 2023a, 2023b, 2024) and efforts continue to advance the sustainable advancement of the hybrid

catfish industry. Past studies have improved stem cell culturing techniques and enabled SSC specific marker identification (Shang et al., 2015); used xenogenesis to successfully produce channel × blue hybrid catfish (Perera et al., 2017); found the optimal donor size for stem cell extraction (Hettiarachchi et al., 2020); created cryopreservation techniques for oogonia (Abualreesh et al., 2021a) and spermatogonia (Abualreesh et al., 2020, 2021b); produced xenogenic catfish with cryopreserved testes and ovarian tissues (Hettiarachchi et al., 2022); assessed the ideal age to inject triploid fry (Hettiarachchi et al., 2023a); assessed the effects of seasonality on germ cell extraction (Hettiarachchi et al., 2023b); and determined impacts of stem cell quantity for proliferation and colonization (Pottle et al., unpublished data). These recent studies have utilized the channel catfish as the triploid surrogate species. Using other surrogate species is an area of research that needs further attention as the technology continues to develop.

White catfish (*Ameiurus catus*) are a potential surrogate species to aid in xenogenic technology. The white catfish reaches sexual maturity in only 1 to 2 years, compared to the 2 to 4 years for channel catfish, or 5 to 6 years for blue catfish (Dunham and Smitherman, 1981; Goudie et al., 1983). Due to the shortened time to reach sexual maturity, using xenogenic white catfish to produce hybrid catfish could result in reduced feed costs, decreased holding space, and reduced labor costs (Hettiarachchi et al., 2024). Luteinizing hormone releasing hormone analogue (LHRHa) hormone implants at 90 µg/kg can also be used to induce spawning in white catfish (Fobes, 2013), making this species a prime candidate for culture (Hettiarachchi et al., 2024).

A recent study by Hettiarachchi et al. (2024) assessed the feasibility of using white catfish as a surrogate species to produce xenogenic catfish to further aid in the hybrid catfish industry. Blue catfish (BGCs) and channel catfish (CGSs) donor gonadal cells were transplanted into surrogate triploid white catfish fry from 0 to 12 days post-hatch (DPH). A survival rate of >81.2%

was found after transplantation for fry injected between 4 to 5.5 DPH, demonstrating acceptance of the foreign cells (Hettiarachchi et al., 2024). Once these white catfish surrogates mature, assessing whether they can successfully produce channel × blue hybrid catfish when pair mated will be next landmark for understanding feasibility.

The current study aims to further assess the feasibility of using white catfish as a surrogate species for xenogenesis to create hybrid catfish. Specifically, we (i) investigate if either BGCs or CGCs are more suitable for cell transplantation and (ii) identify what cell density (80,000, 100,000, or 120,000 cells/fry) provides the highest degree of colonization and proliferation after transplantation into triploid recipients at 4-, 5-, and 6-DPH.

2.0 Materials and Methods

All investigations and experimental studies on animals were conducted according to the Institutional Animal Care and Use Committee (IACUC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) protocols and guidelines.

2.1 White catfish husbandry

White catfish broodstock (1.5 – 3 years old) were reared at the Fish Genetics Research Unit at the Auburn University E.W. Shell Fisheries Center in Auburn, Alabama, USA. Broodstock were cultured in 400 m^2 earthen ponds with an average depth of ~1 m. White catfish were fed to satiation with a 32% floating feed protein pellet (crude protein: ≥32.00%, crude fat: ≥4.00%, crude fiber:

7.00%, and phosphorus: 0.80%) 5 days per week during summer and 3 days per week during winter. Starting in March 2023, white catfish were fed a 36% protein broodstock feed to enhance spawning capabilities. Using a 3.8 cm mesh seine net, sexually mature (2- to 3-year-old) male (N = 4, mean body weight ± SEM = 0.75 ± 0.1 kg) and female white catfish (N = 4, mean body weight ± SEM = 0.7 ± 0.15 kg) were harvested from earthen ponds and stocked into 670 to 750 L flow through holding tanks using pond water at a rate of 30 L/min.

2.2 White catfish spawning

Shortly after harvest, gravid white catfish females and mature white catfish males were administered an intraperitoneal implant of LHRHa at 90 µg/kg body weight and pairs were placed in 60 L clear glass aquaria. The pairs were left undisturbed for 36 h before each tank was observed every 4 h for signs of ovulation as indicated by a few eggs observed on the bottom of the tanks. Upon signs of ovulation, females were removed from tanks and anesthetized in an 18 L bucket with 100 ppm tricaine methanesulfonate (MS-222, Syndel USA, Ferndale, WA) buffered with $NaHCO_3$ to a pH of 7.0.

Following anesthesia, females were carefully rinsed with pond water and thoroughly dried. Crisco® vegetable shortening was rubbed on the female's underside, and eggs were manually stripped into metal pans coated with Crisco® (~25 g of eggs/pan). Male white catfish were euthanized and gonads removed to create a sperm solution. In brief, testes were surgically removed, cleaned in a 0.9% saline solution, dried, minced, and filtered with a 100 µm mesh (Dunham and Masser, 2012; Hettiarachchi et al., 2022). After filtration, the sperm were combined with saline at a rate of 10 mL of 0.9% saline/1 g of testes to produce the sperm solution.

2.3 Fertilization and triploid induction

Water temperature during fertilization ranged from 20 to 22ºC. In brief, ~25 g of eggs were combined with 2 mL of white catfish sperm solution and gently mixed for 2 min in a metal pan. Fullers' Earth (MP Biomedicals, Santa Ana, CA) solution was created by mixing 6 g of powder per 1 L of pond water and 250 mL of the solution was added into the metal pans to prevent adhesiveness of the eggs. The Fuller's Earth solution was gently mixed with the egg and sperm solution for 3 min to initiate fertilization. At 5 min post-fertilization, embryos were induced to be triploids (Perera at al., 2017; Hettiarachchi et al., 2022, 2023a, 2024). Eggs were gently poured into a cylindrical pressure chamber (340 mm height, 70 mm diameter), which was then placed onto a Carver press (Carver, Inc., Wabash, IN) for triploid induction. Hydrostatic pressure was applied at 7,500 psi for 5 min. Following pressure shock, eggs were moved into a 450 L hatching trough supplemented with $CaCl_2$ at 50 ppm for 1 h to harden. Water temperature was 20 to 24ºC, and the hatching trough had a flow rate of 3.8 L/min. After hardening, eggs were moved into hanging mesh baskets (7 m × 0.4 m × 0.2 m) in a flow-through hatching trough (flow rate: 15 L/min) with a paddle wheel and compressed aeration. The hatching trough temperature was 20 to 24ºC.

2.4 Isolation of donor stem cells

Prior to hatching of triploid embryos, sexually immature (1- to 2-year-old) channel catfish (N = 6 [3 male and 3 female], mean total body length ± SEM = 30.925 ± 2.175 cm, mean total body weight ± SEM = 299.575 ± 69.925 g) and blue catfish (N = 6 [3 male and 3 female], mean

total body length ± SEM = 33.552 ± 1.99 cm, mean total body weight ± SEM = 357.4 ± 79.88 g) were harvested from earthen ponds with a 3.8 cm seine net and kept in a 670 to 750 L flow-through pond water holding tank. Once white catfish eggs had hatched and fry reached 4-, 5-, and 6-DPH, channel catfish and blue catfish were selected and euthanized (2 fish per day × 6 days = 12 fish total). Following euthanasia, gonad removal and gonadal cell extraction was performed for both BGCs and CGCs following the methods of Shang et al. (2015), Abualreesh et al. (2020, 2021a, 2021b), and Hettiarachchi et al. (2020, 2022, 2023a, 2023b, 2024). The protocol resulted in a cell suspension comprised of unsorted gonadal cells including SSCs, OSCs, and PGCs.

2.5 Labeling stem cells and injection

Following gonadal cell extraction and isolation, BGCs and CGCs were labeled with PKH26 red fluorescence cell linker (CGLDIL, Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions. Unsorted cells for both BGCs and CGCs were counted using a hemocytometer and divided into six separate tubes: 80,000, 100,000, and 120,000 stem cells/µL for BGCs and 80,000, 100,000, and 120,000 stem cells/µL for CGCs. Attempts were made to transplant each stem cell quantity from BGCs and CGCs donors into the triploid channel catfish fry at 4-, 5-, and 6-DPH following the standard protocols (Hettiarachchi et al. 2023a, 2023b, 2024). The 120,000 cells/fry treatment group ultimately failed due to viscosity and was removed from further analyses. Similar issues occurred when injecting channel catfish surrogates (Pottle et al., unpublished data).

Cell transplantation began by anesthetizing white catfish triploid fry (2 donor species × 2 cell density concentrations × 3 injection days × 3 replicates × 20 fry = 720) with 10 mg/L tricaine

methanesulfonate (MS-222) buffered with 10 mg/L sodium bicarbonate solution. Fry were placed in a petri dish (100 mm × 15 mm), observed under a microscope at 1.5× (Amscope, Irvine, CA), and manually microinjected (Hamilton, Reno, NV). For injections, 1 uL of unsorted cell suspension (BGCs and CGCs) containing either 80,000 or 100,000 cells were injected with a 33-gauge needle (outer diameter: 0.209 mm; inner diameter: 0.108 mm; Hamilton, Reno, NV). The needle was inserted in the region where the genital ridge was expected to form; the cavity between the anal fin and yolk sac (Hettiarachchi et al., 2023a, 2024) (Fig. 2.1A). Following injection, fry were moved to mesh cages (0.2 m × 0.2 m × 0.25 m) in aerated pond water flow-through hatching troughs (flow rate: 15 L/1 min) at a density of 20 fry/cage.

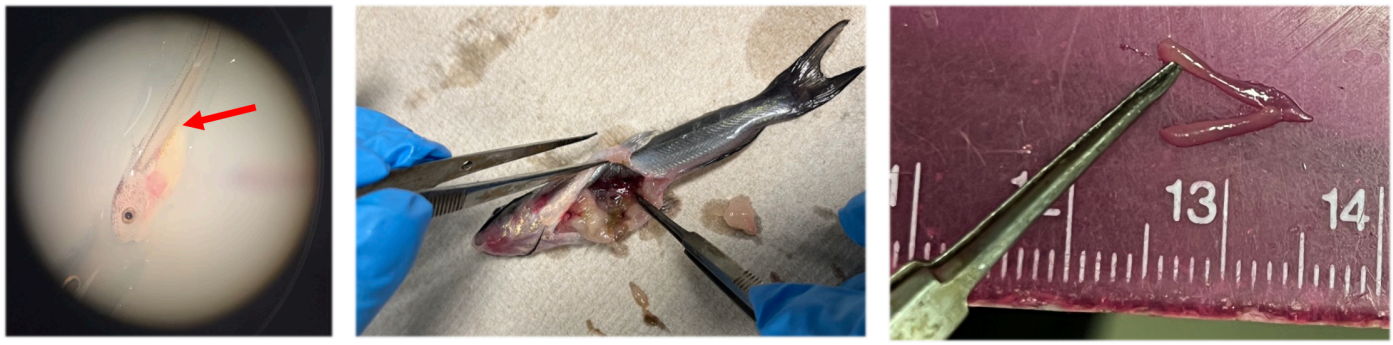

Figure 2.1. (A) Injection site for transplantation (intraperitoneally) of donor derived gonadal cells into triploid white catfish (*Ameiurus catus*). Injection site of transplanted cells, which then migrate to the genital ridge of the recipient and initiate oogenesis or spermatogenesis. (B) Gonad removal from surrogate fry. (C) Gonad from putative xenogenic white catfish female fry prior to PKH26 analysis, DNA extraction, and PCR analysis.

2.6 Survival and growth

Following a 12-h recovery period, fry were fed a standard commercial catfish fry feed (crude protein: 50.00%, crude fat: ≥4.00%, crude fiber: 7.00%, and phosphorus: 0.80%) 4 to 6 times per day to satiation. Feed pellet size was gradually increased to accommodate fry growth (Hettiarachchi et al., 2020, 2023a, 2024). Two sampling intervals, 45 and 90 DPH, were used to record fry survival, growth, PKH26 fluorescence, and genomic DNA/PCR data. At both sampling intervals fry survival data were collected by counting remaining fry, and 9 fingerlings were randomly sampled from each treatment to collect total length (TL) and body weight (BW) data.

2.7 PKH26 observations

To evaluate colonization and proliferation of BGCs and CGCs in triploid white catfish surrogates, 3 fingerlings were randomly selected from each treatment, sacrificed, and their gonads were surgically removed (Fig. 2.1BC). Each gonad was placed on a sterile microscope slide (1600221, Life Technologies) and observed microscopically. Digital images were taken using a Zeiss Imager A2 microscope equipped with a digital camera (Axio-cam 202) and Zen Pro v.6.1 software (Zeiss, Oberkochen, Germany). If a sample had a fluorescing region, it was deemed positive with transferred cells, while if no fluorescing region was detected, it was deemed negative. Three fluorescent images were taken of each positive gonad sample and further analyzed using Image J software. In Image J, fluorescing regions were measured, where cell areas had a fluorescence area <150 μm^2 and cell cluster areas had a fluorescence area >150 μm^2 (Fig. 2. 2).

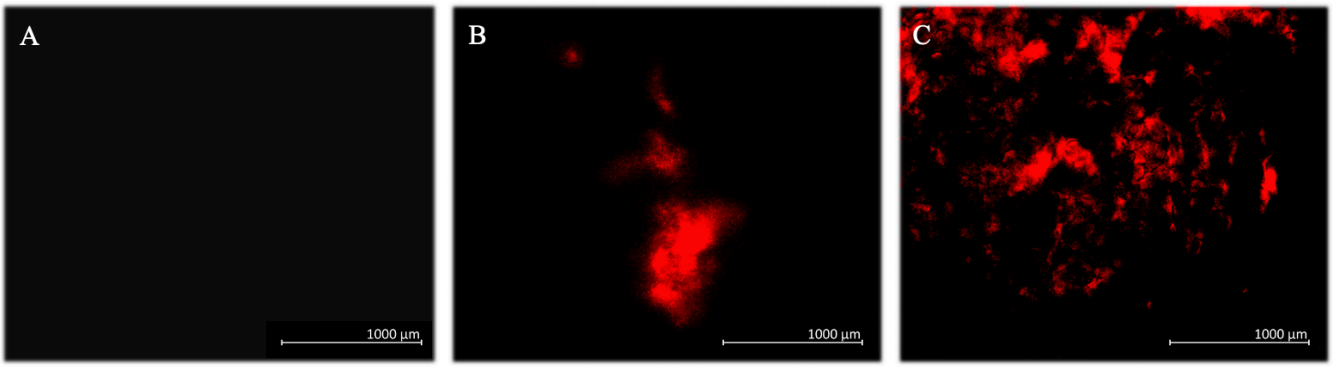

Figure 2.2. Gonadal tissues of a triploid white catfish (*Ameiurus catus*, surrogate) expressing fluorescence from PKH26 dyed donor-derived unsorted gonadal cells. (A) A non-injected control treatment sampled at 45 days post-hatch (DPH), showing no fluorescence. (B) Cell areas/clusters in a triploid white catfish (surrogate) sampled at 45 DPH injected with 80,000 blue catfish (*Ictalurus furcatus*) unsorted gonadal cells/fry at 6 DPH. (C) Cell areas/clusters in a white catfish (surrogate) sampled at 45 DPH injected with 100,000 blue catfish unsorted gonadal cells/fry at 6 DPH.

2.8 Genomic DNA/PCR analysis

Following PKH26 analysis, diagnostic assays were run to determine if sampled gonads had blue catfish or channel catfish DNA. Sampled gonads were placed into 1.5 mL Eppendorf tubes and held on ice and at -80 ºC until DNA extraction. Here, samples were digested using proteinase K, followed by protein and ethanol precipitation for DNA extraction. PCR analysis was performed using a 10 µL reaction volume. Microcentrifuge tubes contained 0.6 µL of primer follistatin (*fst*) and 0.3 µL hepcidin (*hamp*), 5 µL of 2x Eco, 1.7 µL of RNase/DNase free water, and 1.5 µL of the DNA sample before being run in a thermal cycler. Samples were run on a 2.0% agarose gel, using

ethidium-bromide for staining, for the *fst* and *hamp* amplification products to be resolved (Waldbieser and Bosworth, 2008; Hettiarachchi et al., 2020, 2023a, 2024; Table 2.1).

Table 2.1. The primers [*fst* (follistatin) and *hamp* (hepcidin antimicrobial protein)] that were used in PCR analysis to differentiate channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*) and white catfish (*Ameiurus catus*). Primers were previously described by Waldbieser and Bosworth (2008).

Gene	Forward primer	Reverse primer	Amplicon (bp)	
			Channel catfish	Blue catfish
fst	ATAGATGTAGAGGAGCATTTGAG	GTAACACTGCTGTACGGTTGAG	348	399
hamp	ATACACCGAGGTGGAAAAGG	AAACAGAAATGGAGGCTGGAC	222	262

2.9 Statistical analyses

SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA) was used for data analyses. To ensure assumptions were met, residuals were tested for normality (Shapiro-Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). Three-way ANOVA models were run to analyze survival, growth (BW and TL), and fluorescent imaging analyses (cell area and cluster area). ANOVA models contained cell density (80,000 and 100,000 cells/fry),

sampling interval (45 and 90 DPH), and fry injection day (4-, 5-, and 6-DPH) main effects, and the associated interactions. If higher-order interactions were detected, the saturated models were decomposed into lower-order ANOVA models. If no significant interactions were detected, cell density, sampling interval, and fry injection day main effects were interpreted. When necessary, data was arcsin square root or $\log_{10}$ transformed to meet ANOVA assumptions. Alpha was set at 0.05 for main effects and interactions. Post-hoc testing was done using Tukey's HSD test.

Paired t-tests were used to compare growth and performance of non-injected 4 DPH control fry to treatment surrogates. Paired t-tests were used to compare survival, growth, and cell density of surrogate white catfish fry injected with BGCs to those injected with CGCs. Paired t-tests were also used to compare percentage of xenogens produced with 80,000 or 100,000 cells/fry at both 45 and 90 DPH. Fisher's Exact Test was used to compare the percentage of xenogens detected using PCR compared to those detected using PKH26.

3.0 Results

3.1 BGC surrogates: survival, growth, and PKH26 fluorescent labeling

No significant interactions were detected for survival of surrogates injected with BGCs (Fig. 2.3A). Cell density (Fig. 2.3B), sampling interval (Fig. 2.3C), and injection day (Fig. 2.3D) had no impact on fry survival (Table 2.2). For both TL and BW, no significant interactions were detected (Fig. 2.3E and Fig. 2.3I). Cell density (Fig. 2.3FJ) and injection day (Fig. 2.3HL) had no impact on fry growth. Surrogates were significantly longer and heavier at the 90 DPH sampling interval compared to the 45 DPH interval when injected with BGCs (Fig. 2.3GK, Table 2.2). Non-

injected control fry at 4 DPH also had no performance differences from surrogates at 45 and 90 DPH ($P > 0.05$).

There were no significant interactions for BGC surrogate fry when analyzing both cell area (<150 μm^2, %, Fig. 2.4A) and cluster area (>150 μm^2, %, Fig. 2.4E). Cell density (Fig. 2.4B) and injection day (Fig. 2.4D) also had no impact. At 90 DPH, cell area increased in size (Fig. 2.4C), showing increased proliferation over time in BGCs surrogates (Table 2.2).

For cluster area, cell density impacts rates of colonization for fry injected with 100,000 cells/fry having a larger cluster area than those injected with 80,000 cells/fry. Injection day had no impact on rates of colonization or proliferation (Fig. 2.4H). From the 45 to the 90 DPH sampling interval, cluster area significantly increased in size for BGC surrogates (Fig. 2.4C; Table 2.2). Non-injected controls had no cell area or cluster area at 45 and 90 DPH.

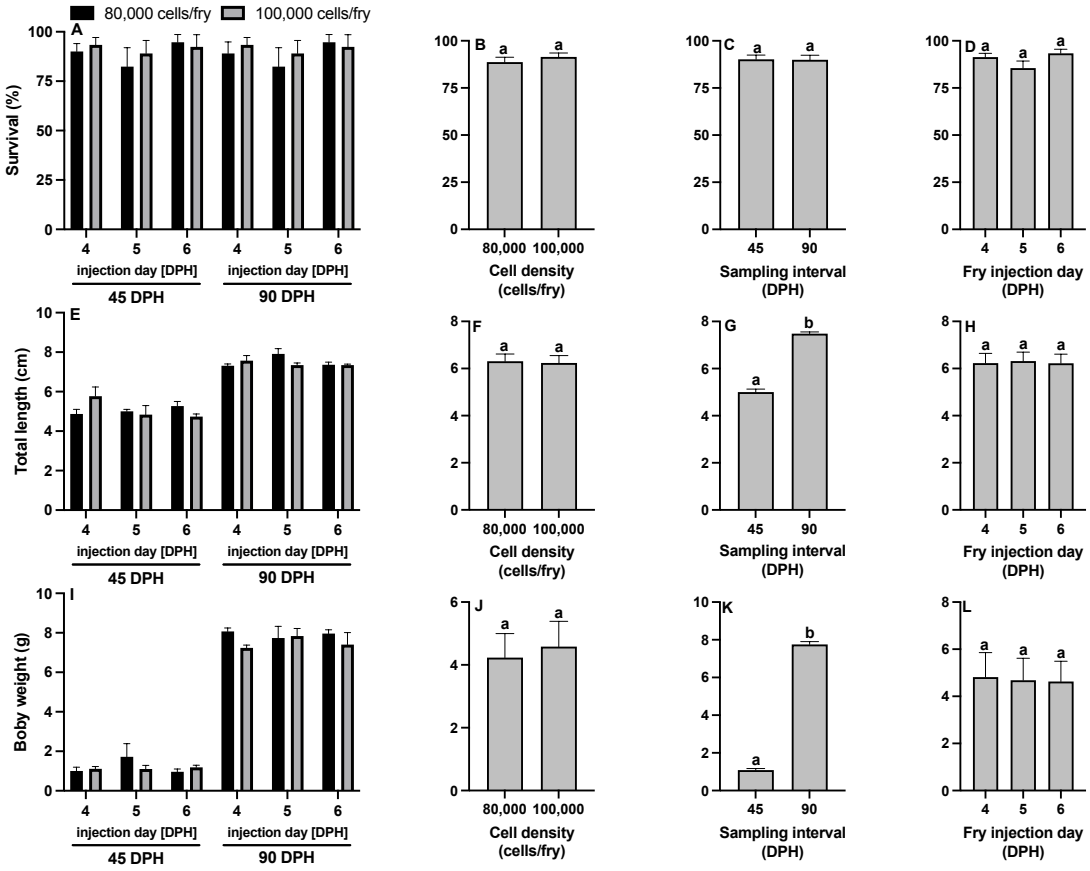

Figure 2.3. Survival (%), total length (TL), and body weight (BW) of triploid white catfish (*Ameiurus catus*) injected with either 80,000 or 100,000 blue catfish (*Ictalurus furcatus*) gonadal cells (BGCs)/fry at 4-, 5-, and 6-days post-hatch (DPH). Fry were sampled at both 45 and 90 DPH. (A) Survival for injection day at both 45 and 90 DPH, (B) survival for fry injected with 80,000 and 100,000 cells/fry, (C) survival at 45 and 90 DPH sampling intervals, and (D) survival at 4-, 5-, and 6-DPH injection day. (E) TL for injection day at both 45 and 90 DPH, (F) TL for fry injected with 80,000 and 100,000 cells/fry, (G) TL at 45 and 90 DPH sampling intervals, and (H) TL at 4-, 5-, and 6-DPH injection day. (I) BW for injection day at both 45 and 90 DPH, (J) BW for fry injected with 80,000 and 100,000 cells/fry, (K) BW at 45 and 90 DPH sampling intervals, and (L) at 4-, 5-, and 6-DPH injection day. [ab]Means followed by the same letter are not different ($P > 0.05$, three-way ANOVA model).

Table 2.2. Results of the analysis of variance (three-way ANOVA with interaction) for triploid white catfish (*Ameiurus catus*) injected with blue catfish stem cells (*Ictalurus furcatus*). Main effects include injection day (ID), cell density (CD), and sample day (SD). (DFN = numerator degrees of freedom, DFD= denominator degrees of freedom, *f* = *f* value, p = p value, BW = body weight (g), TL = total length (cm), cell area <150 μm^2, cluster area >150 μm^2). Alpha was set at 0.05 for main effects and interactions.

			Survival		*BW*		*TL*		*Cell area*		*Cluster area*	
Effect	DFN	DFD	*(f)*	*(P)*	*(f)*	*(P)*	*(f)*	*(P)*	*(f)*	*(P)*	*(f)*	*(P)*
Injection day (ID)	2	12	0.42	0.666	2.95	0.09	3.46	0.07	0.51	0.615	0.37	0.696
Cell density (CD)	1	12	0.17	0.684	0.43	0.53	0.1	0.76	3.39	0.091	11.87	**0.005**
ID × CD	2	12	0.13	0.882	0.49	0.62	0.05	0.95	0.04	0.959	0.43	0.663
Sample day (SD)	1	12	0.03	0.871	5.05	**0.04**	370	**0.01**	8.34	**0.014**	26.34	**0.001**
ID × SD	2	12	0.03	0.973	0.15	0.87	2.81	0.1	0.09	0.91	0.5	0.618
CD × SD	1	12	0.03	0.871	0.05	0.82	0.1	0.75	0.7	0.418	0.7	0.42
ID × CD × SD	2	12	0.03	0.973	0.58	0.58	0.08	0.93	0.08	0.923	0.44	0.654

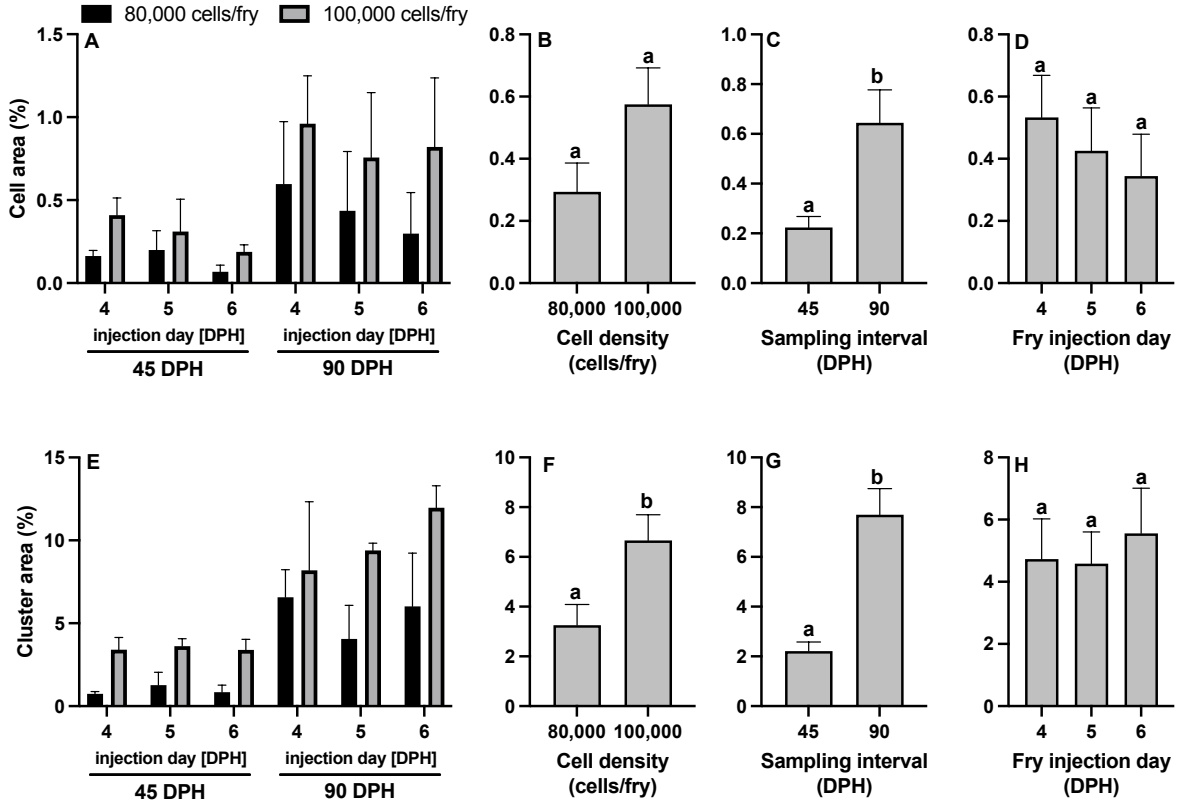

Figure 2.4. (A) Cell area for Cell area (%, >150 μm^2) and cluster area (%, <150 μm^2) for triploid white catfish (*Ameiurus catus*) injected with either 80,000 or 100,000 blue catfish (*Ictalurus furcatus*) gonadal cells (BGCs)/fry at 4-, 5-, and 6-days post-hatch (DPH). (A) Cell area for injection day at both 45 and 90 DPH, (B) cell area for fry injected with 80,000 and 100,000 cells/fry, (C) cell area at 45 and 90 DPH sampling intervals, and (D) cell area at 4-, 5-, and 6-DPH injection day. (E) Cluster area for injection day at both 45 and 90 DPH, (F) cluster area for fry injected with 80,000 and 100,000 cells/fry, (G) cluster area at 45 and 90 DPH sampling intervals, and (H) cluster area at 44-, 5-, and 6-DPH injection day. [ab]Means followed by the same letter are not different (P > 0.05, three-way ANOVA model). Non-injected controls have zero variance, violating ANOVA assumption of variance, and were not included in the model.

3.2 CGC surrogates: survival, growth, and PKH26 fluorescent

No significant interactions were detected for survival (Fig. 2.5A), TL (Fig. 2.5E), BW (Fig. 2.5I), cell area (Fig. 2.6A), or cluster area (Fig. 2.6E) for CGC injected surrogates (Table 2.3). Cell density (Fig. 2.4BFJ) and injection day (Fig. 2.5DHL) also had no impact on fry survival or growth (Table 2.3). Fry were significantly longer (Fig. 2.5G) and heavier (Fig. 2.5K) by the 90 DPH sampling interval, yet no significant survival (Fig. 2.5C) differences were found over time (Table 2.3). Non-injected control fry at 4 DPH also had no performance differences from surrogates at 45 and 90 DPH ($P > 0.05$).

For both cell area and cluster area, cell density was impacted rates of colonization for CGC surrogates as fry injected with 100,000 cells/fry had a larger cell area and cluster area compared to fry injected with 80,000 cells/fry (Fig. 2.6BF; Table 2.3). Both fluorescing regions increased in size from the 45 to 90 DPH sampling interval (Fig. 2.6CG), demonstrating increased proliferation over time in CGCs surrogates (Table 2.3). Injection day had no impact on cell area or cluster area (Fig. 2.6DH). Non-injected controls had no cell area or cluster area at 45 and 90 DPH.

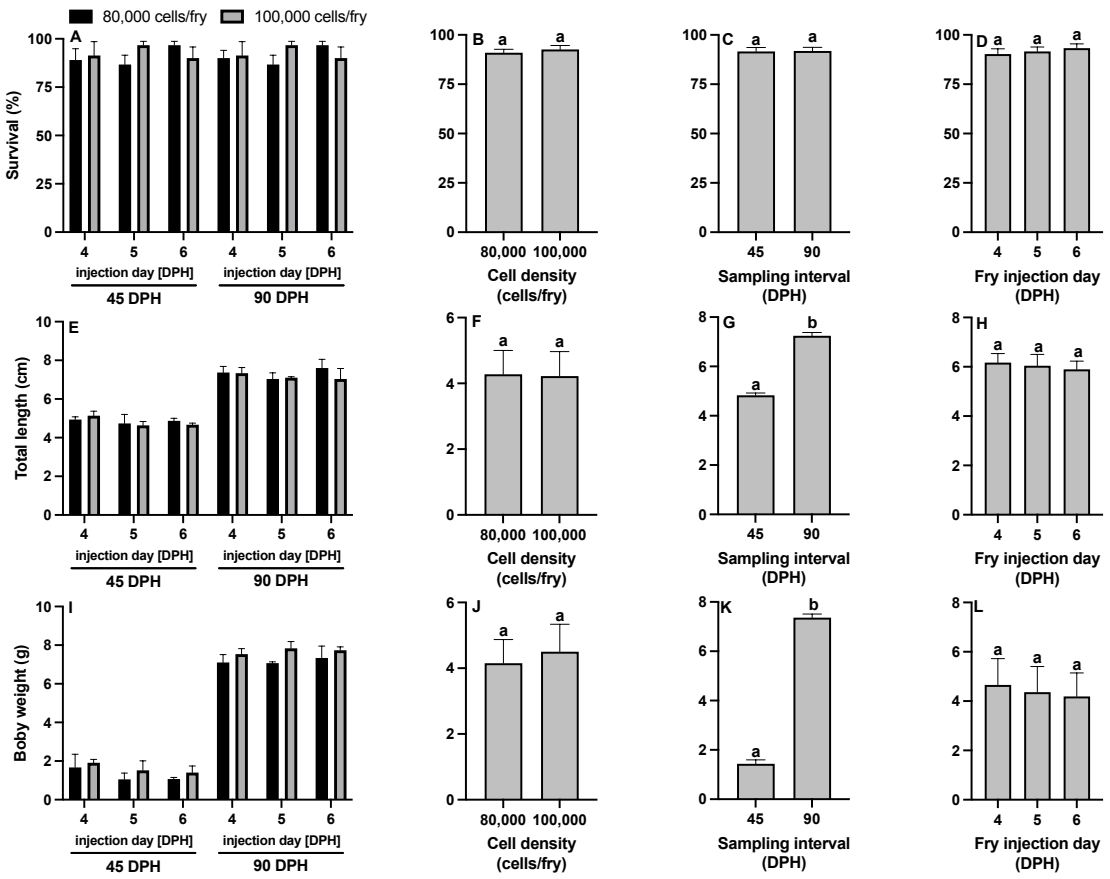

Figure 2.5. Survival (%), total length (TL), and body weight (BW) of triploid white catfish (*Ameiurus catus*) injected with either 80,000 or 100,000 channel catfish (*Ictalurus punctatus*) gonadal cells (BGCs)/fry at 4-, 5-, and 6-days post-hatch (DPH). Fry were sampled at both 45 and 90 DPH. (A) Survival for injection day at both 45 and 90 DPH, (B) survival for fry injected with 80,000 and 100,000 cells/fry, (C) survival at 45 and 90 DPH sampling intervals, and (D) survival at 4-, 5-, and 6-DPH injection day. (E) TL for injection day at both 45 and 90 DPH, (F) TL for fry injected with 80,000 and 100,000 cells/fry, (G) TL at 45 and 90 DPH sampling intervals, and (H) TL at 4-, 5-, and 6-DPH injection day. (I) BW for injection day at both 45 and 90 DPH, (J) BW for fry injected with 80,000 and 100,000 cells/fry, (K) BW at 45 and 90 DPH sampling intervals, and (L) at 4-, 5-, and 6-DPH injection day. [ab]Means followed by the same letter are not different ($P > 0.05$, three-way ANOVA model).

Table 2.3. Results of the analysis of variance (three-way ANOVA with interaction) for triploid white catfish (*Ameiurus catus*) injected with channel catfish stem cells (*Ictalurus punctatus*). Main effects include injection day (ID), cell density (CD), andand sample day (SD). (DFN = numerator degrees of freedom, DFD = denominator degrees of freedom, *f* = *f* value, p = p value, BW = body weight (g), TL = total length (cm), cell area <150 μm^2, cluster area >150 μm^2). Alpha was set at 0.05 for main effects and interactions.

			Survival		*BW*		*TL*		*Cell area*		*Cluster area*	
Effect	DFN	DFD	*(f)*	*(P)*	*(f)*	*(P)*	*(f)*	*(P)*	*(f)*	*(P)*	*(f)*	*(P)*
Injection day (ID)	2	12	0.18	0.841	0.26	0.774	0.83	0.46	1.71	0.221	0.55	0.589
Cell density (CD)	1	12	0.46	0.518	0.11	0.749	0.62	0.446	5	**0.045**	12.23	**0.004**
ID × CD	2	12	1.32	0.304	0.51	0.614	0.25	0.786	0.18	0.835	2.33	0.14
Sample day (SD)	1	12	0.03	0.871	14.21	**0.003**	757.15	**0.001**	19.07	**0.001**	19.17	**0.001**
ID × SD	2	12	0.03	0.973	2.31	0.142	1.34	0.299	0.42	0.666	0.93	0.421
CD × SD	1	12	0.03	0.871	0.27	0.611	1.04	0.329	0.5	0.495	0.96	0.347
ID × CD × SD	2	12	0.03	0.973	1.78	0.211	0.24	0.792	0.32	0.734	0.57	0.581

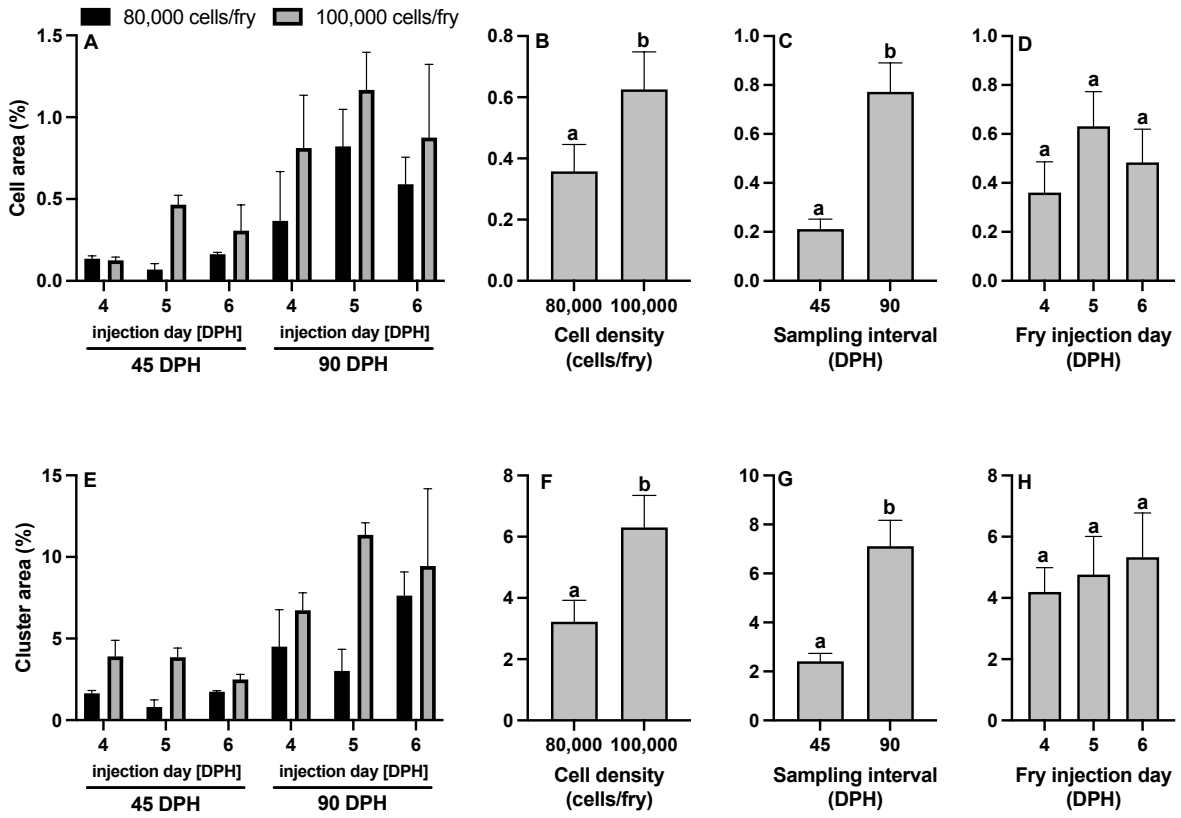

Figure 2.6. Cell area (%, <150 μm^2) and cluster area (%, <150 μm^2) at for triploid white catfish (*Ameiurus catus*) injected with either 80,000 or 100,000 channel catfish (*I. punctatus*) gonadal cells (CGCs)/fry at 4-, 5- and 6-days post-hatch (DPH). (A) Cell area for injection day at both 45 and 90 DPH, (B) cell area for fry injected with 80,000 and 100,000 cells/fry, (C) cell area at 45 and 90 DPH sampling intervals, and (D) cell area at 4-, 5-, and 6-DPH injection day. (E) Cluster area for injection day at both 45 and 90 DPH, (F) cluster area for fry injected with 80,000 and 100,000 cells/fry, (G) cluster area at 45 and 90 DPH sampling intervals, and (H) cluster area at 4-, 5-, and 6-DPH injection day. [ab]Means followed by the same letter are not different (P > 0.05, three-way ANOVA model). Non-injected controls have zero variance, violating ANOVA assumption of variance, and were not included in the model.

3.3 Donor species comparison

Survival, growth performance, and fluorescent labeling data for surrogate white catfish fry injected with BGCs were compared to surrogate fry injected with CGCs to assess the viability of each donor species. At the first and second sampling intervals, no differences were observed among surrogates injected with BGCs or CGCs and between 80,000 or 100,000 cells/fry for survival, TL, BW, cell area, and cluster area (Table 2.4).

Table 2.4. Statistical results summary for comparison between triploid white catfish (*Ameiurus catus*) injected with channel catfish (*Ictalurus punctatus*) and blue catfish gonadal cells (*I. furcatus*) injected with 80,000 or 100,000 cells/fry. (DF = degree of freedom, DPH = days post-hatch, TL = total length, BW = body weight, cell area <150 μm^2, cluster area <150 μm^2). Paired T-tests were used, and alpha was set at 0.05.

Trait	Density (cells/fry)	DF	*P*-value (45 DPH)	*P*-value (90 DPH)
Survival	80,000	8	0.73	0.628
TL	80,000	8	0.776	0.132
BW	80,000	8	0.073	0.359
Cell area	80,000	8	0.658	0.567
Cluster area	80,000	8	0.171	0.779
Survival	100,000	8	0.812	0.812
TL	100,000	8	0.841	0.175
BW	100,000	8	0.937	0.952
Cell area	100,000	8	0.969	0.739
Cluster area	100,000	8	0.929	0.718

3.4 Xenogenic detection

PCR analysis indicated transplanted BGCs and CGCs were present in the gonads of surrogate white catfish fry during the 45 and 90 DPH sampling intervals (Fig. 2.7). At 45 DPH, the percentage of xenogens detected in surrogates injected with BGCs was 77.78% and 88.89%

when recipients were injected 80,000 and 100,000 cells/fry, respectively (P = 0.543). The percentage of xenogens detected in recipient fish injected with CGCs at 45 DPH was 77.78% for both 80,000 and 100,000 cells/fry (P = 0.999).

At 90 DPH, the percentage of xenogens detected in surrogate fish injected with BGCs was 83.33% and 91.67% when recipients were injected with 80,000 and 100,000 cells/fry, respectively (P = 0.792). The percentage of xenogens detected in recipient fish injected with CGCs at 90 DPH was 75% and 83.33% for 80,000 and 100,000 cells/fry, respectfully (P = 0.455) (Fig. 2.7).

Percent xenogens were not different (P = 0.104) for BGCs surrogates injected with 80,000 cells/fry (77.8%) vs. 100,000 cells/fry (100.0%) as detected by PKH26 analysis. Similarly, percent xenogens were not different (P < 0.999) for CGCs surrogates injected with 80,000 cells/fry (88.8%) vs. 100,000 cells/fry (88.8%). No significant (P = 0.5030) differences were found among xenogens detected using PKH26 (86.11%) vs. PCR (81.9%).

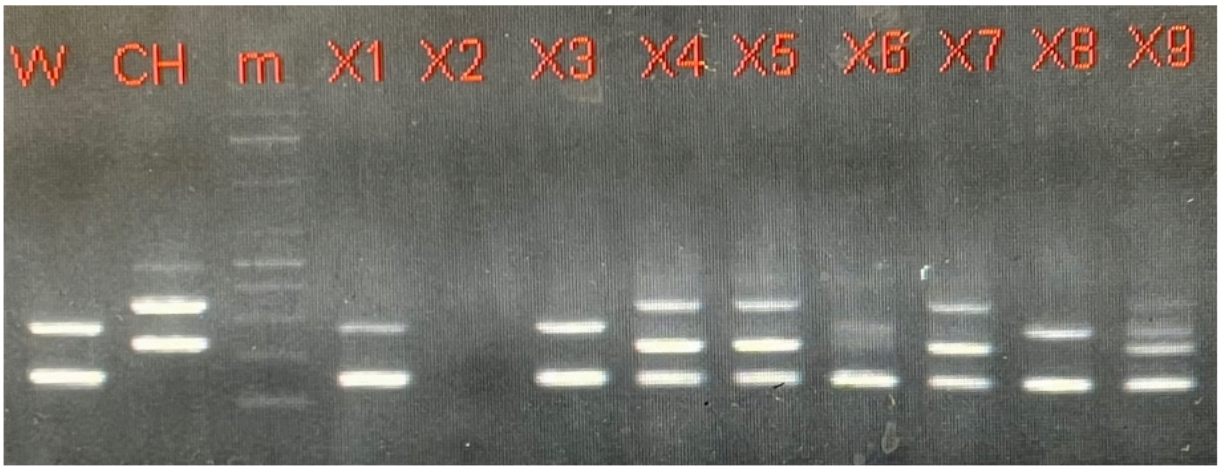

Figure 2.7. Sample results from PCR for detecting channel catfish (*Ictalurus punctatus*) donor cells in the testes of triploid white catfish (*Ameiurus catus*). Follistatin (*fst*) and hepcidin antimicrobial protein (*hamp*) genes were used as markers to differentiate channel and white catfish cells. W = white catfish control, CH = channel catfish control, m = marker.

4.0 Discussion

The present study is in accordance with recent findings by Hettiarachchi et al. (2024), showing white catfish as a feasible surrogate for BGCs and CGCs donor cells. Similarly, Pottle et al. (unpublished data) injected 100,000 cells/fry into triploid channel catfish which showed increased proliferation and colonization in surrogates. The present study also demonstrates that rates of proliferation increased over time for both BGCs and CGCs injected fry. Additionally, we showed that neither donor species were superior to the other. This is an important result, especially if the goal is to mate xenogenic white catfish females producing channel catfish eggs with xenogenic white catfish males producing blue catfish sperm to produce hybrid progeny. Due to favorable characteristics of white catfish, such as early sexual maturity and good spawning rates, incorporation of this species into xenogenic technology could be a pivotal change for the hybrid catfish industry. The demonstration that the white catfish surrogate readily accepts BGCs and CGCs donor cells adds further validation of using white catfish to produce channel-blue hybrid catfish embryos (Hettiarachchi et al., 2024).

Utilizing surrogate species with shortened generation times for increased efficiency has been explored within other aquaculture systems (Ryu et al., 2022). For example, chinook salmon (*Oncorhynchus tshawytscha*) females take 3 years and males take 5 years to reach sexual maturity. Rainbow trout (*Oncorhynchus mykiss*) females can reach sexual maturity in 2-3 years and males in 1-2 years. Using xenogenesis to produce chinook salmon germ cells in surrogate rainbow trout larvae can shorten generation time and enhance production (Ryu et al., 2022).

The surrogate's acceptance of foreign donor cells could be an obstacle for using some species for xenogenic technology development. Some surrogate species may reject foreign cells (Lee and Yoshizaki, 2016). If surrogate acceptance is achieved and colonization is confirmed,

genetic tools such as selective breeding can be used to further advance a surrogate's performance (Yoshizaki and Yazawa, 2019). Selection for the best phenotypes and genotypes could take place for both immature donor and juvenile surrogate fish, though surrogate selection may be more difficult depending on the optimal DPH for injection (Yoshizaki and Yazawa, 2019). Using specific DNA markers can enable simpler and quicker identification of ideal phenotypes in larvae fry and donor juveniles (Abdelrahman et al., 2017). Incorporating both host specification and selective breeding into xenogenesis can not only enhance efficiency but also sustainability within the technology.

The finding that 100,000 cells injected/fry compared to 80,000 cells injected/fry leads to increased colonization is another way to increase successful xenogen injections and production. Additionally, reducing the number of unidentified cells and increasing the number of pure stem cells injected for xenogenic catfish production, may be the next important step for increasing the efficiency of xenogenesis in ictalurid catfish. Currently, ~50-60% of injected cells are stem cells (Perera et al., 2017; Shang et al., 2018) capable of colonization, and 100,000 cells injected per fry is the maximum density that allows passage through the injection needle.

A review by Ryu et al. (2022) suggests several options to increase transplantation success, and one way discussed is to increase donor cell quantities. An example of this can be seen in medaka (*Oryzias latipes*) surrogates (Calvi et al., 1998). When receiving over 3,000 unsorted testicular cells, medaka larvae had significantly higher colonization rates (63.3%) compared to surrogates who received <3,000 unsorted cells (1.1-29.0%) (Calvi et al., 1998). As we have found similar results when increasing gonadal cell quantities, increasing stem cell purity is the next step for enhancement of xenogenic catfish production.

The white catfish presents a unique opportunity for potential adoption of xenogenesis in the catfish industry with its shortened time to maturation and smaller body size, yet there are some drawbacks to utilizing the white catfish (Hettiarachchi et al., 2024). In pond culture, white catfish have lower survival, seinability rates, and fighting issues (especially among males), which can lead to injuries and secondary infections (Fobes, 2013). One way to avoid these challenges with the white catfish is to use recirculating aquaculture systems (RAS) for culture of the broodstock instead of pond culture. RAS allows for a more controlled setting such as stocking density control, incorporation of shelters to alleviate fighting, and eliminates harvesting issues and is relatively sustainable compared to other concentrated animal feeding operations (Hettiarachchi et al., 2024).

5.0 Conclusion

The white catfish is a suitable candidate for xenogenesis application. An increase in donor cell enhanced proliferation and colonization in surrogates. Both BGCs and CGCs were accepted by the surrogate species and neither were superior to the other, demonstrating the feasibility of using xenogenic white catfish to produce channel catfish female × blue catfish male hybrid embryos. Continued efforts to further establish the technology should be made, especially as the aquaculture industry continues to grow in the US. Enhancing purity of donor cells will be a crucial next step in xenogenesis technology for ictalurid catfish to further improve efficiency, sustainability, and feasibility of this system.

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Chapter IV: Tissue mosaicism in xenogenic common carp (*Cyprinus carpio*) injected with gonadal stem cells from blue catfish (*Ictalurus furcatus*)

Abstract

Xenogenesis is a method of reproductive surrogacy in which successive generations differ from each other and no genetic material is transmitted from the parent to the offspring. Theoretically, spermatogonial stem cells (SSCs) or oogonial stem cells (OSCs) from a donor species injected near the genital ridge of a sterile surrogate species should colonize that region and eventually become donor gametes, resulting in the surrogate only being able to produce donor progeny. A myriad of studies on xenogenesis focus upon this expected fate of the donor stem cells, and there are no reports of these introduced cells migrating outside the genital region. We found that when SSCs or OSCs from blue catfish (*Ictalurus furcatus)* were injected into triploid common carp (*Cyprinus carpio*) embryos and fry, some cells not only colonized the gonads but also migrated and colonized the pectoral fin, anal fin, or muscle in some individuals based upon DNA analysis, resulting in mosaicism in these tissues, although they retained the normal phenotypic appearance of common carp. This migration also occurred without colonization of the gonads. However, no catfish-carp mosaicism was observed in heart, liver, intestine, and eye, indicating some migration pathways are not possible or these tissues were less receptive to colonization. The genital ridge was colonized as late as 27 days post fertilization but the pectoral fin was not colonized later than 19 days post fertilization. In general, individuals injected at later time points in development had reduced or no mosaicism. Triploid induction was not 100% effective and blue catfish cells were also detected in the gonad, muscle, anal fin, and pectoral fin of diploid common carp surrogates. Blue catfish cells colonized (87.5%) the gonads of triploid common carp at a higher rate (42.9%; $P<0.05$) than in diploid common carp. The

incidence of non-target tissues containing blue catfish DNA was 50% higher in triploids than diploids. This is the first report of mosaic tissues being generated outside of the gonadal region in fish during xenogen creation.

Keywords: Reproductive technology, xenogenesis, mosaicism, common carp, blue catfish

1.0 Introduction

Xenogenesis is a method of surrogate reproduction in which successive generations differ from each other and no genetic material is transmitted from the parent to the offspring (Dunham, 2023). Xenogenesis can be accomplished by transplanting undifferentiated germ cells, such as primordial germ cells (PGCs), spermatogonial stem cells (SSCs), or oogonial stem cells (OSCs) derived from donor diploid fish into surrogate triploid fish (Hettiarachchi et al., 2022) or fish sterilized by other methods. Applications of xenogenesis for the aquaculture industry include shortening generation times, achieving repeated gamete production of semelparous fish, minimizing broodstock maintenance, and preserving superior strains paired with cryopreservation (Ryu et al., 2022).

Xenogenesis has been explored for a variety of fish species. One application has been for the salmonid industry. Rainbow trout (*Oncorhynchus mykiss*) surrogates were used to shorten generation time by hosting Chinook salmon (*Oncorhynchus tshawytscha*)'s germ cells to produce donor-derived sperm and eggs in only 2 years (Takeuchi et al., 2001). Goldfish (*Carassius auratus*) surrogates have been used to maintain the carp gene bank by producing common carp (*Cyprinus carpio*) cells (Franěk et al., 2021). Sterlet sturgeon (*Acipense ruthenus)* germ cells were transferred to beluga (*Huso huso)* surrogates to increase fecundity (Franěk et al., 2022) and *dead end*-knockout was used to create germ cell-depleted zebrafish (*Danio rerio*) surrogates (Li et al., 2017). Spermatogonia was transplanted into triploid Nibe croaker (*Nibea mitsukurii*) surrogates to evaluate the suitability of hemizygous pHSC-GFP transgenic (gfp/−) donor cells in surrogates (Yoshikawa et al., 2017).

For the hybrid catfish (♀ channel catfish, *Ictalurus punctatus* × ♂ blue catfish, *I. furcatus*) industry in the US, xenogenesis has been accomplished by transplanting

undifferentiated blue catfish gonadal cells intro triploid channel catfish surrogates (Perera et al., 2017). White catfish (*Ameiurus catus*) surrogates have also been used for producing hybrid embryos, accepting both channel catfish and blue catfish donor cells (Hettiarachchi et al., 2024). Due to reproduction challenges of the hybrid catfish, finding more ways to incorporate xenogenesis into the industry remains a goal for sustainable aquaculture development (Dunham and Masser, 2012).

The common carp (*Cyprinus carpio*) is a potential candidate to be incorporated into the hybrid catfish xenogenesis technology development as it reaches sexual maturity in ~2 years (Sivakumaran et al., 2003; Smith and Walker, 2004) (sometimes as early as 1 year), exhibits high fecundity (Swee and McCrimmon, 1966), tolerates a wide range of environmental conditions (Edwards and Twomey, 1982; Weber et al., 2010), can be spawned year-round under controlled conditions (Swee and McCrimmon, 1966; Smith and Walker, 2004), and helps control the overgrowth of plants/algae in commercial ponds with its omnivorous diet (Weber and Brown, 2012). Sperm can also be hand stripped from live common carp, offering an advantage over catfish. In catfish, sperm must be surgically retrieved either by sacrificing the fish (Billard et al., 1995) or by surgically removing testes to extract sperm and then repairing the wound with sutures (Bart et al. 1998). The common carp is an economical species to rear in captivity as feed costs are low and maintenance is minimal (Weber and Brown, 2012).

Theoretically, common carp could be a surrogate to produce blue catfish sperm. Due to the high fecundity of common carp, large quantities of blue catfish sperm could potentially be collected through hand stripping. Since blue catfish take 5 – 6 years to reach sexual maturity (Hettiarachchi et al., 2020), but the common carp can reach sexual maturity in ~1 – 2 years

(Sivakumaran et al., 2003; Smith and Walker, 2004), successful xenogenesis would save time, resources, and reduce costs for hybrid catfish embryo production.

The motivation for this research was to develop a system using xenogenic common carp to produce blue catfish sperm. During this process, we discovered xenogenic common carp with blue catfish DNA outside (the pectoral fin) of the colonization target, gonad. All previous research on xenogenesis in fish examined colonization in the target gonads, and this is the first report of mosaicism or colonization outside of the target tissue in xenogenic fish, Thus, the objective of this study was to determine the extent of blue catfish-common carp mosaicism in tissues (gonad, muscle, eye, pectoral fin, pelvic fin, heart, liver and intestine) of putative xenogenic common carp produced by injecting blue catfish gonadal cells near the genital ridge of different ages of common carp fry.

2.0 Materials and methods

All investigations and experimental studies on animals were conducted according to the Institutional Animal Care and Use Committee (IACUC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) protocols and guidelines.

2.1 Broodstock selection

Common carp broodstock were reared in 0.04- ha earthen ponds averaging ~1 m in depth at the E.W. Shell Fisheries Center, Fish Genetics Research Unit, at Auburn University in Auburn, Alabama. Males and females were fed a 32% protein pellet feed (Purina Catfish) five days per week during summer and three days per week during winter. Prior to spawning,

broodstock were transitioned to a customized feed containing 36% protein to enhance spawning capabilities.

A 3.8cm seine was used to harvest common carp from the ponds. Broodstock were examined based on secondary sexual characteristics before being chosen for spawning. Females were chosen based on softening of the abdomen, reddening, and protrusion of the genital papilla. Males were chosen based on overall size (body weight [~1.6kg] and body length [~50 cm]), ease of milt expression, and clear tubercle development on the pectoral fins and operculum.

2.2 Broodstock spawning

Upon selection, common carp were removed from the earthen ponds and stocked into a flow-through, pond water, holding tank (3.0 m × 0.4 m × 0.25 m). Females were administered an intracelomic injection of Ovaprim at 0.5 ml/kg (Ovaprim™, Ferndale, WA) and placed upstream in the holding tank, in a green mosquito net mesh enclosure. Males received Ovaprim at a dosage of 0.1 ml/kg and were subsequently placed downstream of the females, separated by a partition. Genders were separated to avoid early fertilization. 10-16 h after hormone injection, females were checked every 30 min for signs of ovulation (few eggs seen on the mesh enclosure).

Once ovulation had begun, the female was anesthetized with 100 ppm tricaine methanesulfonate (MS-222, Ferndale, WA) and 100 ppm sodium bicarbonate to achieve a neutral pH. Once anesthetized, the female was removed from the water, thoroughly dried, and the eggs were manually stripped from the body into a metal spawning pan coated in Crisco® vegetable shortening (~25 g of eggs/pan). Simultaneously, males were anesthetized, thoroughly dried, and milt was hand stripped from their body into plastic 50 mL tubes. Milt was added to eggs dry to prevent immediate fertilization.

2.3 Triploid induction

Fullers' earth powder (Starwest Botanicals, Sacramento, CA) was combined with pond water at a rate of 6g/1L and was mixed with the sperm and eggs to accomplish fertilization and prevent adhesion. Eggs were then transferred into a cylindrical pressure chamber (340 mm height, 70 mm diameter) which was then placed in a Carver press (Carver, Inc., Wabash, IN). At 5 min post fertilization, the eggs underwent a 5 min pressure shock treatment at 8,000 psi to induce triploidy.

Following pressure shock, eggs were placed into 5L tubs filled with Holtfreter's solution (comprising 3.46 g NaCl, 0.05 g KCl, 0.1 g $CaCl_2$, 0.2 g $NaHCO_3$ per liter) in water baths. Eggs remained in Holtfreter's solution for three days until hatch at 22 – 24 °C. After hatch, eggs were placed in a diluted Holtfreter's solution for 25 days before being transferred to 60-L glass aquaria in a recirculating aquaculture system (RAS).

Hatched fry were fed *Artemia nauplii* 5 times per day for the initial 25 days. Once carp were moved to RAS they were transferred to a Purina AquaMax powdered feed five times per day for two months. Carp fingerlings were then fed Aquaxcel WW Fish Starter 4512 three times per day for two months. Juvenile fish were fed WW 4010 Transition feed three times per day for the remaining duration. All fish were fed to satiation.

2.4 Gonadal cell extraction from immature blue catfish

Sexually immature blue catfish (1 – 2 years old) were harvested from earthen ponds with a 3.8 cm seine and euthanized. Blue catfish were then cleaned with 70% ethanol, thoroughly dried, and their immature gonads were harvested from the coelomic cavity following protocols by Shang et al. (2018) and Hettiarachchi et al. (2020). Upon removal, the gonads were placed in

100 mm × 15 mm sterile petri dishes containing 5 mL of Hanks' Balanced Salt [(HBSS, GE Healthcare Life Sciences, Marlborough, MA) supplemented with 1.0 μg/mL $NaHCO_3$ (Church & Dwight Co., NG) and 100 U/mL Penicillin - Streptomycin (Life Technologies, Carlsbad, CA)] and were transferred to a biosafety cabinet for cleaning and sterilization. Unsorted gonadal cell extraction was then conducted following protocols described by Shang et al. (2018). The extraction resulted in a cell suspension which comprised of unsorted gonadal cells, including spermatogonia stem cells (SSCs) and/or oogonia stem cells (OSCs) (Fig. 1).

2.5 Unsorted gonadal cell transplantation

Both embryos (~500 per replicate) and eggs (~50 per replicate) were injected with unsorted cells every two days from day 0 (fertilization) through day 27 post fertilization for the first spawning set. For the second spawning, fry were injected with unsorted cells at 2-, 12-, 13-, 14-, 15-, 16, and 17-days post-hatch. Fresh cells were extracted from blue catfish donors (pooled male and female cells) and used each day. Prior to injections, fry were anesthetized with 100 mM buffered MS-222 solution and placed on a petri dish filled with a 1% TAE gel to allow for easier handling. For injections, a standard protocol by Perera et al. (2017) was followed. In brief, glass capillary tubes were heated and pulled into a needle with a Flaming / Brown micropipette puller (model P-97). Ten μL of stem cells were loaded into the needles with a Fischer Scientific 10 μL micropipette. Next, a 99% pure pressurized nitrogen gas canister connected, to a MPPI-3 pressure injector (15 psi, pulse duration 3-4) from Applied Scientific Instruments, was incorporated. The MPPI-3 was then connected to a manual micromanipulator MM 33 produced

by Märzhäuser Wetzlar and secured to a magnetic base designed by MHC Industrial Supply Company.

Each fry and embryo were injected with ~1 μL of cell suspension. Fry were injected in the cavity between the anal fin and yolk sac where the genital ridge is expected to form. Embryos were injected in the cell body to avoid disrupting the yolk and for better embryo uptake. Following injections, fry and embryos were placed back into Holtfreter's solution for recovery.

2.6 Culture

After recovering from stem cell transplantation, both groups of common carp surrogates were transferred into 5 L tubs of Holtfreters solution for a 5-week (4/12/21 – 5/16/21 and 4/13/23 – 5/18/23, respectfully) incubation period. Next both fry groups were stocked in 10 L RAS glass aquaria (5/16/21-11/14/22 and 5/18/203 – 10/23/23, respectfully) for growth.

2.7 Triploid analysis

To analyze triploid success and how this correlates with mosaicism, the second group of common carp surrogates were sampled 6 months after initial injections. Five μm of blood was drawn from 36 suspected positive common carp using a 33-gauge needle (outer diameter: 0.209 mm; inner diameter: 0.108 mm; Hamilton, Reno, NV). Blood was stored on ice and transported to Warm Springs National Fish Hatchery in Warm Springs, GA for ploidy testing. In brief, 1.0 mL of the sampled blood was mixed with 10 mL of isotonic saline and placed in a coulter counter machine. The Coulter counter measured both cell counts and volume of the sampled blood. Samples with a nucleus size >2.75 microns were considered triploids, and <2.75 microns

were considered diploids. Due to difficulty drawing blood in the immature common carp, only 24 of the 36 sacrificed carp had blood tested.

2.8 Mosaicism analysis

From the first group of injected carp, 152 had their gonads and pectoral fins sampled. Fish that were microinjected with blue catfish gonadal cells from 0 - 27 days post-fertilization (DPF) were sampled. From the second group of injected carp, 36 had blood drawn for triploid analysis and were sacrificed using methanesulfonate (250mg/L; MS-222, Ferndale, WA). These common carp were dissected and the gonad, muscle (around dorsal fin), pectoral fin, anal fin, eye, liver, intestine, and heart were removed and stored in labeled, separate, 1.5 mL Eppendorf tubes on ice. Samples were held at -80 ^{o}C until DNA extraction could occur. For DNA extraction, protocols by Waldbieser and Bosworth (2008) were followed. In brief, proteinase K was used for digestion which was followed by protein and ethanol precipitation (Waldbieser and Bosworth, 2008) (Table 3.1). In a few cases for the second group, some tissues could not be sampled due to the small size of the immature common carp.

2.9 Primer development, PCR analysis

To further confirm the presence or absence of blue catfish DNA within common carp samples, five different primers were tested to determine the most accurate sequence. Three nuclear markers, *fst*, *hamp,* (Table 3.1), and SCAL, were tested along with a blue catfish specific mitochondrial DNA (mtDNA) marker. A final primer, *scpp-1*, was tested which is based on secretory calcium-binding phosphoprotein family gene found in some ray-finned fishes (Table 3.2, Lemopoulos and Montoya-Burgos, 2021, Bern, 2024).

Pure blue catfish and common carp DNA samples were used for initial primer testing. After PCR analysis, *scpp-1* was chosen as the most accurate sequence primarily due to the lack of banding for common carp DNA. Thus, for PCR analysis, cycle number, annealing temperature, and detection limits were all tested to identify any limitations to recognizing blue catfish DNA (Bern, 2024).

Testing concluded the optimal annealing temperature should be 60°C and 30 cycles were needed to adequately provide banding. Blue catfish DNA was detected at DNA concentrations as low as 3.75 ng/µL, diluted with common carp DNA, yet ~50 ng/µL was the optimal concentration to see banding (Bern, 2024).

To determine success of blue catfish cell colonization in common carp surrogates, PCR was used to confirm the presence of blue catfish DNA in common carp samples. PCR was conducted in a 10 µL reaction volume in microcentrifuge tubes containing 0.5 µL of the *scpp-1* forward, 0.5 µL of the *scpp-1* reverse, 5 µL of 2x Eco, 3.5 µL of RNase/Dnase free water, and 0.5 µL of the DNA sample. DNA concentrations remained at 0.05 µm (Bern, 2024).

Table 3.1: Primers used for genes [*fst* (follistatin) and *hamp* (hepcidin antimicrobial protein)] to differentiate channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*). Primers were previously described by Waldbieser and Bosworth (2008).

Gene	Forward primer	Reverse primer	Amplicon (bp)	
			Channel catfish	Blue catfish
fst	ATAGATGTAGAGGAGCATTTGAG	GTAACACTGCTGTACGGTTGAG	348	399
hamp	ATACACCGAGGTGGAAAAGG	AAACAGAAATGGAGGCTGGAC	222	262

Table 3.2. The primer set [*scpp-1* (secretory calcium-binding phosphoproteins) gene] that was used in PCR analysis to detect the presence of and blue catfish (*Ictalurus furcatus*) DNA within the samples obtained from potential xenogens.

Gene	Forward primer	Reverse primer	Amplicon (bp)	
			Blue catfish	Channel catfish
scpp-1	TGGAGAGCCCAGAGAAAAAC	GGTGGTCTCAGTGGACTCGT	437	407

2.10 Statistical analysis

SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA) and GraphPad Prism statistical analysis software (v.10.0.3; GraphPad Software Inc., Boston, MA, USA) were both used for all data analyses. After the PCR analysis, logistical calculations were performed to elucidate success rate based on the number of fish sampled and number of fish positive for blue catfish DNA. Fisher's exact test was used to compare the frequency of blue catfish stem cells colonizing tissues other than gonad.

3.0 Results

3.1 Initial rates of xenogenesis

Sampled common carp were regarded xenogenic (positive) if PCR analysis confirmed that blue catfish DNA was present (Fig. 3.1; Fig. 3.2). For the first set of common carp, 152 potential xenogens were sampled, and 57 of those sampled were confirmed positive (37.5%).

Positive xenogen common carp containing blue catfish DNA were seen at each time point (0-, 23-, 45-, 161-, 207-, 253-, 299-, 322-, 345-, 391-, 483-, 529-, 575- and 621-degree days). The highest percentage of positive samples were injected between 0 - 46-degree days (62.3%) and 483 – 575-degree days (56.7%), with 483 degree days yielding the highest percent positive achieved (70%). Lowest percent xenogens was at 391-degree days (7%). Individuals with no blue catfish DNA, in the pectoral fin only, in the gonad only and in both tissues simultaneously were observed (Table 3.3).

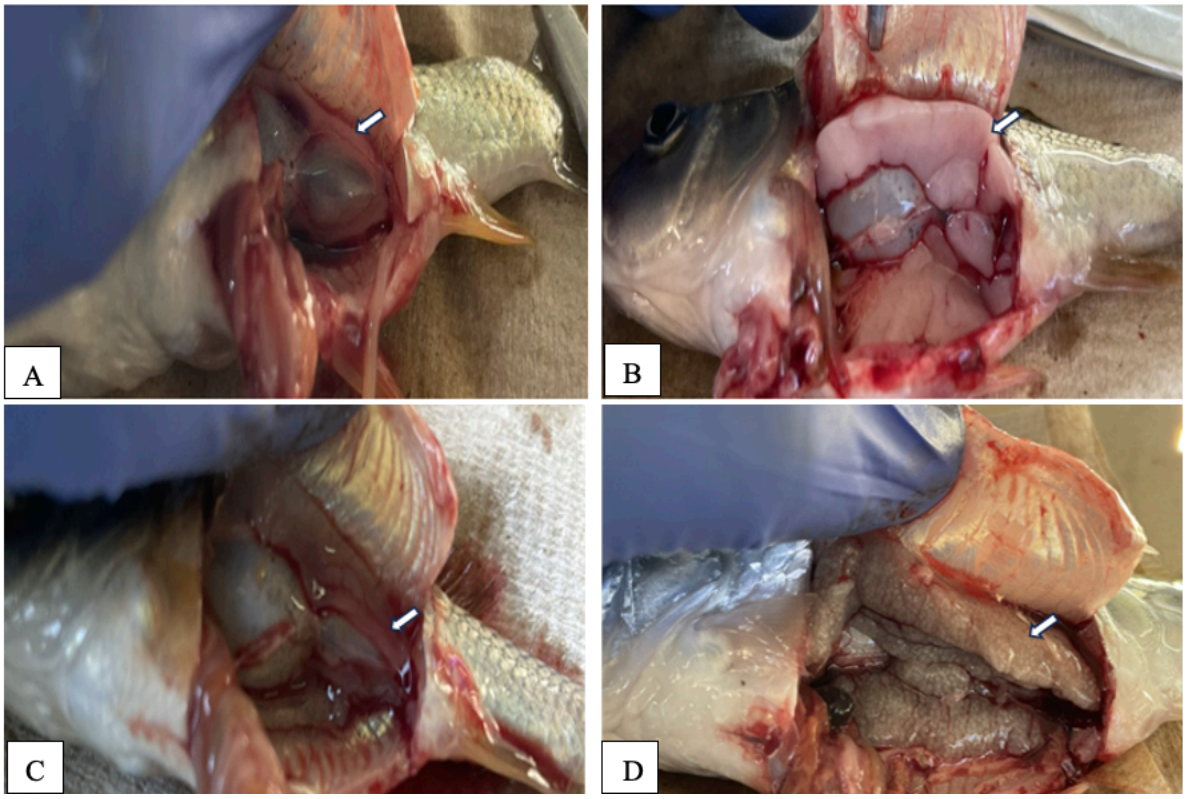

Figure 3.1. A) Underdeveloped male testes of xenogen common carp (*Cyprinus carpio*) suspected of hosting blue catfish (*Ictalurus furcatus*) sperm. B) Further developed and nearly mature carp testes filled with sperm. C) Underdeveloped female ovaries of xenogen common carp suspected of hosting blue catfish eggs. D) Furrther developed and nearly mature ovaries filled with underripe eggs. Both fish were 454 days post fertilization.

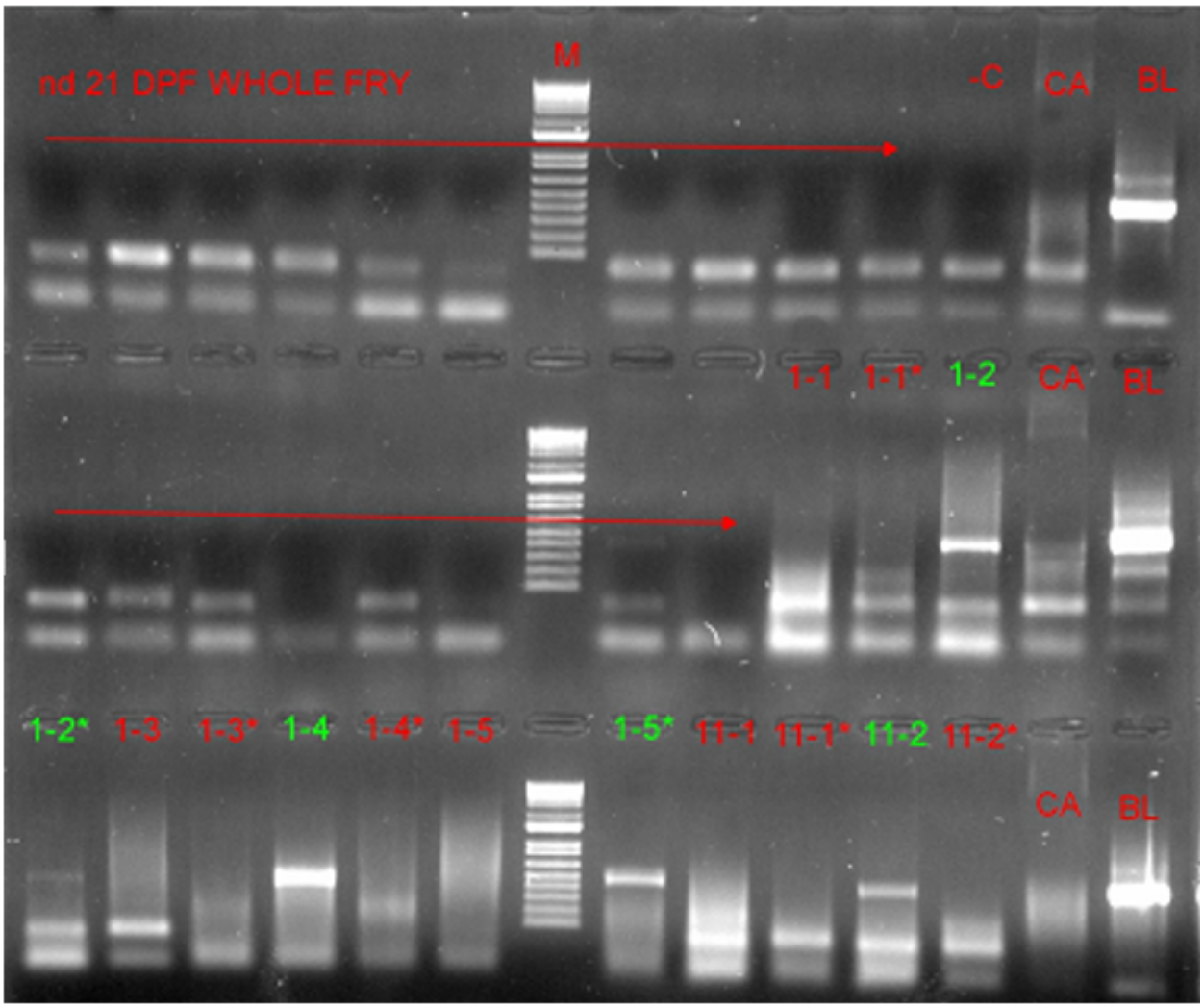

Figure 3.2. Example of PCR results when testing gonad and fin samples from suspected common carp (*Cyprinus carpio*) xenogens harboring blue catfish (*Ictalurus furcatus*) DNA. Labels in green are samples with banding patterns identical to that of the blue catfish control (BL), and labels in red are samples that had no identical banding. M is the marker to identify the band size. "CA" represents a diploid common carp control and "-C" represents a negative control to ensure no contamination occurred during PCR analysis. The asterisk at the end of the sample identification correspond to the sample type being gonad derived and no asterisk is fin derived (Bern, 2024).

Table 3.3. Percentage of putative triploid common carp (*Cyprinus carpio)* containing blue catfish (*Ictalurus furcatus*) DNA as confirmed by PCR of the *scpp-1* marker in blue catfish in gonad, fin, or both at 581 days post fertilization after being injected with a blue catfish primordial germ cell (PCGs) at various stages of development. Embryos and fry were injected periodically from 0-degree days to 621-degree days after fertilization. Treatment refers to the point in development when stem cells were injected into the sterile surrogate common carp (Bern, 2024).

Degree days of development when injected	N	Positive in Gonad Only (%)	Positive in Fin Only (%)	Positive in Both	Total % Positive in Gonad
0	15	46.0	**7.0**	**13.0**	60.0
23	10	20.0	**10.0**	**30.0**	50.0
46	5	40.0	0.0	**20.0**	60.0
161	10	0.0	0.0	**10.0**	10.0
207	4	25.0	0.0	**25.0**	50.0
253	10	10.0	**6.0**	0.0	10.0
299	10	30.0	**10.0**	0.0	30.0
322	20	10.0	**10.0**	**10.0**	20.0
345	12	25.0	0.0	0.0	25.0
391	15	7.0	**13.0**	0.0	7.0
483	10	60.0	0.0	**10.0**	70.0
529	10	50.0	0.0	0.0	50.0
575	10	50.0	0.0	0.0	50.0
621	11	36.0	0.0	0.0	36.0

3.2 Triploidy

Of the 24 carp whose blood was sampled to check ploidy levels in 2023, 66.67% were triploids, and 33.33% were diploids (Table 3.4; Table 3.5; Fig. 3.3). Mosaicism was seen in both triploids and diploids along with unknown individuals. Highest rates of mosaicism was seen in

triploid surrogates and the lowest in diploid surrogates (P<0.05). Both triploid and diploid individuals only expressed carp bands in the eye, liver, intestine, and hear (Table 3.4; Table 3.5; Fig. 3.3, Fig. 3.4).

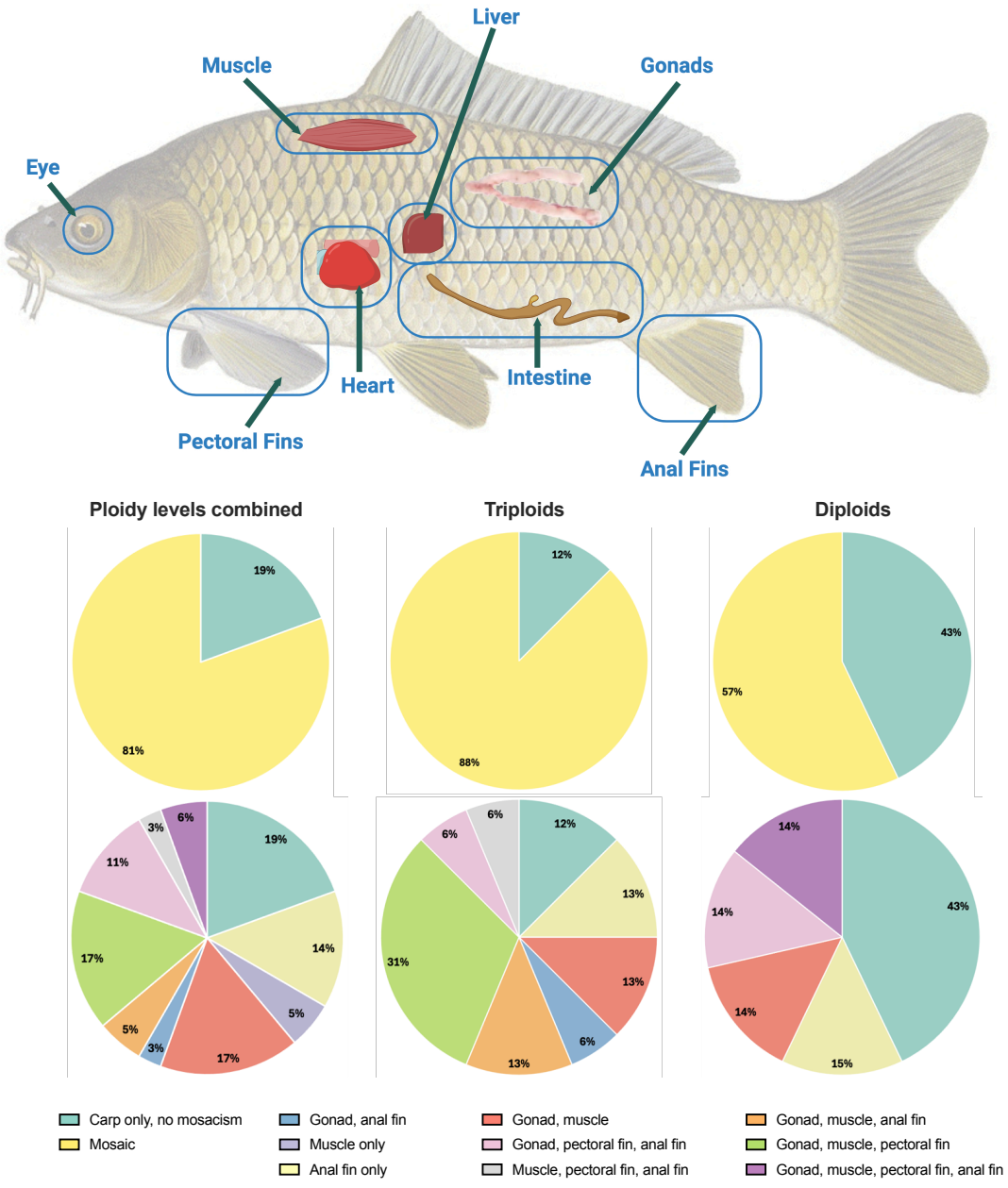

Figure 3.3. (A) Sampled areas of suspected common carp (*Cyprinus carpio*) xenogens harboring blue catfish (*Ictalurus furcatus*) DNA. The eye, muscle, liver, gonads, intestine, anal fins, heart,

and pectoral fins were removed and tested for mosaicism using PCR. (B) Percent mosaicism from suspected common carp xenogens harboring blue catfish DNA (N = 36). Surrogates had carp only DNA (no mosaicism), anal fin only - muscle only - gonad and anal fin - gonad and muscle – muscle, pectoral fin anal fin – gonad, pectoral fin, anal fin – gonad, muscle, anal fin – gonad, muscle, pectoral fin - gonad, muscle, pectoral fin and anal fin. Both triploid (N = 16) and diploid (N = 8) surrogates were sampled along with unknown ploidy levels (N = 12).

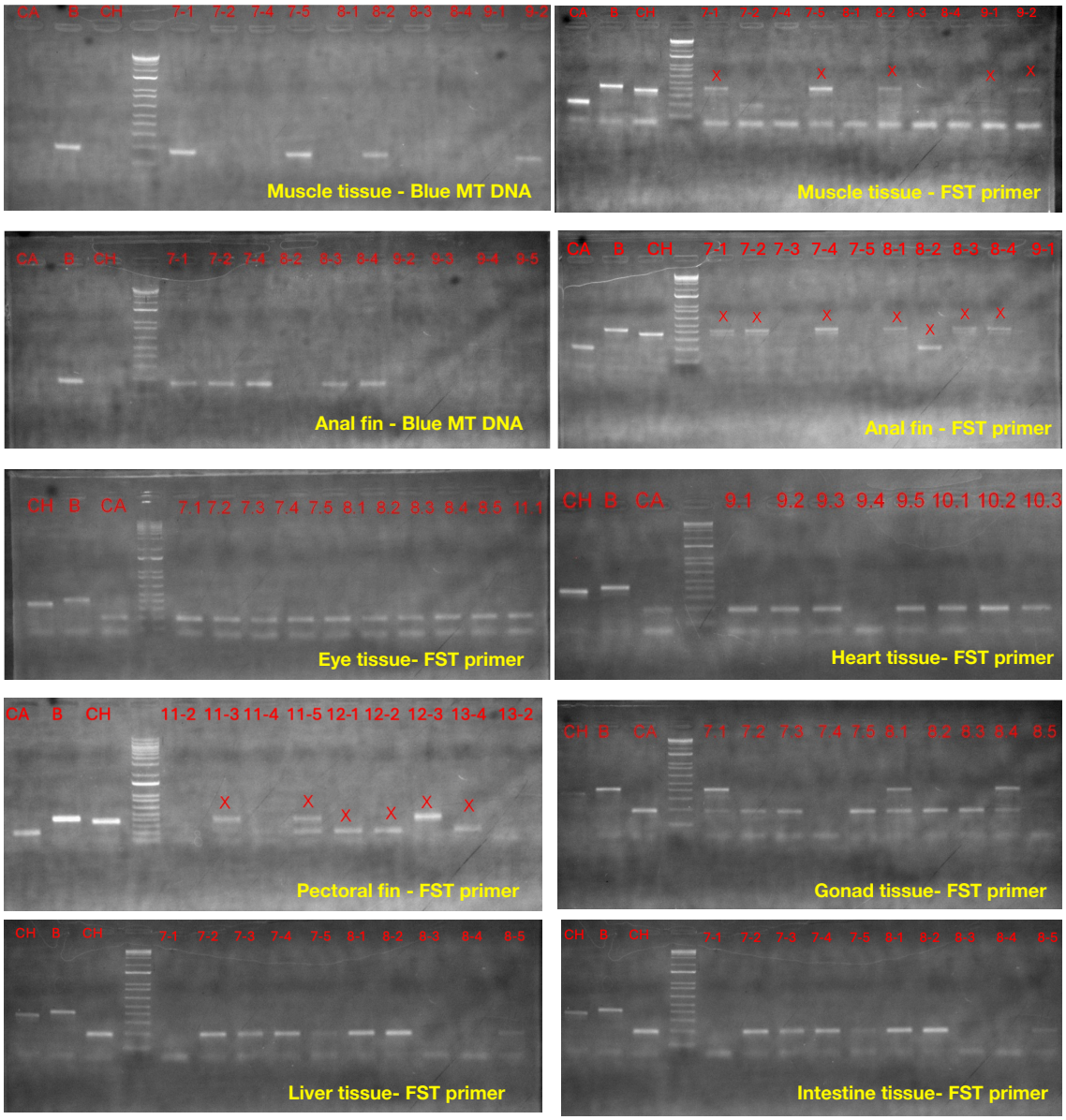

Figure 3.4. Example of PCR results when testing gonad, muscle, anal fin, pectoral fin, heart, eye, liver, and intestine samples from suspected common carp (*Cyprinus carpio*) xenogens harboring blue catfish (*Ictalurus furcatus*) DNA. CA = control common carp, B = control blue catfish, and CH = control channel catfish. Several primers were used for amplification including a blue catfish specific mitochondrial DNA (mtDNA), *fst* (follistatin), blue catfish MT DNA, and a primer based on the secretory calcium-binding phosphoprotein family gene (*scpp-1*).

Table 3.4. Frequency of common carp (*Cyprinus carpio)* DNA only and a combination of common carp and blue catfish (*Ictalurus furcatus*) DNA in gonad, muscle, pectoral fin, anal fin, eye, liver, intestine, and heart of common carp microinjected with blue catfish gonadal cells as confirmed by PCR of the Follistatin (*fst*), a blue catfish specific mitochondrial DNA (mtDNA), and a primer based on the secretory calcium-binding phosphoprotein family gene (*scpp-1*). N = number of samples analyzed for that specific tissue.

Sample type	N	Common carp DNA only (%)	Common carp and blue catfish DNA (Total % mosaic)
Gonad	30	33.3	66.7
Muscle	28	32.1	67.9
Pectoral fin	23	47.8	52.2
Anal fin	21	28.6	71.4
Eye	28	100.0	0.0
Liver	18	100.0	0.0
Intestine	24	100.0	0.0
Heart	13	100.0	0.0

Table 3.5. Percentage of putative triploid common carp (*Cyprinus carpio)* containing blue catfish (*Ictalurus furcatus*) DNA as confirmed by PCR of the Follistatin (*fst*), a blue catfish specific mitochondrial DNA (mtDNA), and a primer based on the secretory calcium-binding phosphoprotein family gene (*scpp-1*) amplifying blue catfish DNA in gonad, muscle, pectoral fin, anal fin, eye, liver, intestine, and heart tissues. Sampled individuals were unknown ploidy level, diploid (2N), or triploid (3N). N = number of samples analyzed for that specific tissue. The total percentage of positive mosaicism for each tissue is displayed. N = number of samples analyzed for that specific tissue. + = positive samples.

Sample type	N	Diploid (N)	Triploid (N)	Unknown ploidy (N)	+ unknown mosaic (%)	+ diploid mosaic (%)	+ triploid mosaic (%)	+ total mosaic (%)
Gonad	30	10	6	14	70.0	50.0	68.8	66.7
Muscle	28	11	4	13	63.6	50.0	76.9	67.9
Pectoral fin	23	6	4	13	50.0	33.3	53.8	52.2
Anal fin	21	8	4	9	62.5	75.0	77.8	71.4
Eye	28	10	4	14	0.0	0.0	0.0	0.0
Liver	18	6	4	8	0.0	0.0	0.0	0.0
Intestine	24	6	6	12	0.0	0.0	0.0	0.0
Heart	13	2	1	10	0.0	0.0	0.0	0.0

3.3 Mosaicism

Blue catfish DNA was detected in the gonads, muscle (sampled from around the dorsal fin), pectoral fin, and anal fin along with common carp DNA (Table 3.4; Table 3.5). Only common carp DNA was detected in the eyes, livers, intestines, and hearts. For combined diploid and triploids, in the gonad, 66.7% of samples possessed blue catfish DNA. The anal fin yielded the highest rates of migrated cells, 71.4%, followed by the muscle, 67.9%. The pectoral rate had the lowest rate of blue catfish cell colonization at 52.1% (Table 3.4, Fig. 3.3, Fig. 3.4).

Among confirmed triploid individuals, the anal fin also yielded the highest rate of migrated cells at 77.8%, followed by the muscle at 76.9%. The gonad had the third highest rate of migrated cells at 68.8%, lastly followed by the pectoral fin at 53.8% (Table 3.5, Fig. 3.3, Fig. 3.4).

For known diploids, 75% of sampled fry had migrated cells in the anal fin. 50% of surrogates had blue catfish DNA in gonad and muscle samples. The pectoral fin yielded lowest rate of migrated cells at 33.3% in diploids (Table 3.5, Fig. 3.3, Fig. 3.4).

4.0 Discussion

Blue catfish gonadal stem cells colonized the target tissue, which were the gonads of triploid common carp surrogates. Mosaicism, defined here as colonization of tissues other than the target genital ridge, was detected. This is the first report of mosaicism in studies of xenogenesis. Mosaicism was revealed by PCR, which revealed the presence of blue catfish DNA in pectoral fin, anal fin, and dorsal fin muscle in some individual common carp surrogates. Interestingly, there was no colonization and proliferation in eye, liver, heart, and intestine.

Mosaic individuals displayed the normal common carp body phenotype. The genital ridge could be colonized as late as 27 days post-fertilization, but the pectoral fin could not be colonized later than 19 days post fertilization. Triploid induction was not 100% effective. Blue catfish cells were also detected in the gonad, muscle, anal fin, and pectoral fin of diploid common carp surrogates but again not in eye, heart, liver and intestine. Blue catfish cells colonized the gonads of triploid common carp at a higher rate (87.5%) than in diploid common

carp (42.9%). The incidence of non-target tissue mosaicism containing blue catfish DNA was 50% higher in triploids than diploids.

Injecting stem cells during critical developmental stages significantly impacted colonization and proliferation. Peak stem cell colonization occurred over two distinct time ranges: 0 to 46-degree days and 483 to 575-degree days. Blue catfish DNA was detected in the carp gonads at 62.3% and 56.67%, respectively, for these two critical injection periods. This is consistent with the results of Zapata et al. (2006) with regard to immune response to foreign cells/tissues. Juvenile common carp did not reject allografts until two weeks post-fertilization (Zapata et al., 2006). In our study of stem cell recruitment, this lack of rejection is even longer, as there was no evidence of rejection of the injected blue catfish cells up to 27-days post fertilization. However, immunity might not be involved, as juvenile and adult diploid and triploid pejerrey, tilapia, and channel catfish have successfully colonized donor stem cells in their gonad regions (Majhi et al., 2009; Lacerda et al., 2012; Perera et al., 2017). The critical time of transplantation for maximum colonization and proliferation varies among species (Franěk et al., 2022; Hettiarachchi et al., 2023; Hettiarachchi et al., 2024) but has rarely been studied.

There are many possibilities for the existence and pattern of mosaicism that developed from the stem cell introduction. The anal fin is near the microinjection site so it is not surprising that transplanted stem cells might stray to that structure and its muscle, as long as there is a tissue layering division that could provide the appropriate ‘cellular highway,’ especially if that region was receptive to colonization. We sampled muscle just below ventral to the dorsal fin. Both the muscle and the pectoral fin are a large distance from the injection site, requiring a long migration to colonize those tissues. Injected stem cells did not colonize the eye, heart, liver, and intestine.

There was either no natural path for the stem cells to follow or these tissues were at a developmental stage that was unreceptive to colonization.

During normal development, stem cells routinely migrate significant differences away from their point of origin, following existing paths through and between tissue layers. Stem cells 'home' into the target tissues via a combination of cytoskeletal rearrangement, chemokine signaling, attaching to cell adhesion molecules, and moving along extracellular pathways that guide the cells to their ultimate locations. Sometimes cells use proteolytic or other activities to open up the pathway; matrix metalloproteinases are key to these functions (Lau and Li, 2013). Aberrant migration can be responsible for initiation of some cancers (López-Lázaro, 2015).

There could be several reasons why there was a complete lack of blue catfish stem cell recruitment in the eye, liver, intestine, and heart. During development in vertebrates, the central nervous system develops before more distally positioned regions of the anatomy (for example, axial limbs, and the posterior components of the vertebrate column) (Katz et al., 2013). Thus, the anterior regions, including the eye might prevent invasion of migrating stem cells, while gonads, gametes, muscles, and fins were further behind in development and may be less restrictive about accepting injected stem cells (Ozair et al., 2013). The liver and intestine arise from the vegetal portion of the early embryo, and actually provide stem cells to the genital ridge; they actively migrate away from the vegetal pole. Therefore, we would not expect stem cells to populate the intestines. Distal organs, including the gonads, gametes, muscles, and fins are more likely to accept migratory stem cells (McKay, 1997).

The "accepting" tissues might be more receptive or less selective towards the integration of a heterogeneous population of cells. This receptivity could be due to the inherent biological properties of these tissues, such as higher regenerative capacity, more highly aligned with paths

into the tissue, or a generally more permissive microenvironment that can accommodate a variety of cell types, including those that are not fully characterized or purified stem cells. The lack of acceptance of unpurified stem cells in the liver, intestine, eye, and heart, might indicate that these tissues have stricter requirements for cell migration and integration. This could be due to tighter tissue homeostasis, a less permissive microenvironment, or active mechanisms that prevent the colonization of foreign or unrecognized cells, particularly when the cell population is heterogeneous and unpurified (Gross-Thebing et al., 2020).

Another potential explanation as to why we are finding positive blue catfish DNA in the muscle and fin tissues is that they are of the same originating germ cell layer (mesoderm). The eye is of the nervous system/skin cell layer of the germ cell layers; the heart, liver and intestine are of the endodermal germ cell origin. The liver and intestine also have a very high endogenous stem cell activity and cell turnover. Liver is the least differentiated of the major organs of a vertebrate, and the heart, from very early embryogenesis, very actively pumps the blood. The pumping action could be so hard that cells that get to it via the circulation (which would be most effective) might not be able to easily recruit to the interior of the blood vessels, including the heart. The eye, liver, intestine, and heart are moreover in an entirely different germ cell layer, so they may never even see the injected cells (Doitsidou et al., 2002).

The mesoderm forms into two types that form the muscles, and then also the lateral plate mesoderm, which forms bones and the paraxial mesoderm that forms somites and muscle. The eye and its orbit have their origins in the neural crest, which is already well developed as the axial skeleton forms (Duke University, 2012)

The gonadal ridge is the source of the mesodermal cells that populate the muscles and form somites. Also, the fins arise from the bony development of the somites, which means they

are segmented and immediately become associated with the muscle (Yan et al., 2022). In fish, the genital ridge originates from the coelomic epithelium, a cell layer that thickens on the ventral surface of the mesonephros. The genital ridge is made up of a cluster of somatic cells. These somatic cells form by coelomic epithelium proliferation, epithelial-to-mesenchymal transition, and migration. Since the genital ridge is bipotential (i.e., it can develop into either an ovary or testis), the genetic sex that is established at fertilization determines which type of gonads develop. The somatic cells of the genital ridge become the gonad wall/structure; the primordial germ cells become the gametes (Knaut et al., 2003).

Germ cells develop at some distance from the gonads, only to later migrate to them, where they differentiate into eggs or sperm. The separation of site of origin from the final destination seems to be a mechanism to exclude germ cells from the general developmental processes (Wolpert, 2015).

In zebrafish, laminin is a key protein in the basement membrane of tissues, playing a crucial role in cell adhesion, migration, and differentiation during development. The laminin is the “highway” and seems to be a major component of the basement membrane which is responsible for preventing passage of cells through a particular zone. The laminin is also thought to limit cell migration along the surface. In zebrafish, PGC migration is directed by the chemokine Cxcl12a, which binds with its receptor, Cxcr4b, on the cell surface (Doitsidou et al., 2002; Knaut et al., 2003; Molyneaux and Wylie, 2003). However, polarized bleb formation and cell motility can occur independently of chemokine signaling, making them fundamental behaviors of PGCs (Doitsidou et al., 2002; Gross-Thebing et al., 2020). Despite this, migrating PGCs still rely on Cxcl12a from surrounding cells along their migration route to reach their intermediate and final destinations (Doitsidou et al., 2002; Boldajipour et al., 2008).

Future efforts should determine whether cell migration patterns seen in zebrafish and common carp are seen in other species. Morita et al. (2015), Okutsu et al. (2008), Franěk et al. (2019), and Hettiarachchi et al. (2022; 2024) did not detect mosaicism in jack mackerel, yellow tail, goldfish, channel catfish or white catfish surrogates, however, they did not examine this possibility. Few studies assess the true function of cell migration in teleost species. Therefore, future efforts should examine tissue selectivity and combine this understanding with developments in xenogenesis technology.

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