Gene editing for growth enhancement in channel catfish, *Ictalurus punctatus*, and xenogenesis in common carp, *Cyprinus carpio* to produce blue catfish, *I. furcatus,* sperm

by

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Abstract

Xenogenesis is a method of reproduction where successive generations differ from each other and no genetic material is transmitted from the parent to the offspring. Xenogenesis can be accomplished by transplanting spermatogonial stem cells (SSCs), oogonial stem cells (OSCs) or primordial germ cells (PGCs) from the desired diploid donor species into a sterilized host. Many beneficial ecological and aquaculture applications are possible from this process. Specifically, the production of embryos from hybridizing channel catfish (*Ictalurus punctatus*) females with blue catfish (*I. furcatus*) males could be refined to a simpler and more cost-effective practice of xenogenesis. Xenogenesis could alleviate the reliance on growing blue catfish males to maturity and sacrificing them for gonad extraction and subsequent artificial spawning. Male blue catfish cannot be stripped of milt for artificial fertilization and must be sacrificed for sperm collection. Thus, one objective was to produce xenogenic common carp, *Cyprinus carpio,* to become biological blue catfish milt factories.

Common carp embryos, and larvae were injected with blue catfish oogonial stem cells and spermatagonial stem cells at various points in relation to their development. Fish were injected beginning at 0-degree days, every 23-degree days until 621-degree days. In total, 152 potential common carp xenogens were sampled and 57 of them were confirmed to be hosting blue catfish stem cells through polymerase chain reaction (PCR), giving a total 37.5% success rate of common carp hosting blue catfish cells in the gonads. The highest percentage of positive samples were observed at 0 to 46-degree days and 483-575 degree days, averaging 62.3% and 56.7% success respectively. Rate of xenogenesis was similar for male and female common carp

hosts. The mean survival among all the treated groups through the first 34 days was 47%, however, among groups injected during embryonic development, mean survival was 8.6%. During the grow out stage (34-545 dpf), mean survival for all treated groups was 70%. At one and half years of age, xenogenic common carp males produced blue catfish sperm based upon DNA analysis of the expressed sperm.

In addition to stronger reproductive control, some targeted genetic alterations were performed to aid in higher growth rates and increase yield within catfish aquaculture. Two induced mutations were investigated, *melanocortin-4 receptor* (*mc4r*) and *myostatin* (*mstn*) gene in channel catfish. In a earthen pond environment stocked at 18,508 fish per hectare, the mean weight for channel catfish *mc4r* P1, *mc4r* F1 X control, control X *mc4r* F1, and *mc4r* F1 X *mc4r* F1 mutants was 361, 266, 521, and 426 grams, respectively, was larger ($P>0.05$,$P>0.05$, $P<0.01$, $P<0.01$) than control (C) channel catfish, 299 grams. In a recirculating system environment, during the first year of growth (354 days post fertilization), channel catfish *mc4r* F1 mutants had a mean weight of 22.6 grams and channel catfish controls were 10.5 grams, a 73.3% increase in body weight in the mutant fish ($P<0.0001$). During the next 161 days, *mc4r* mutants grew 29.2% faster ($P<0.0001$) than controls, reaching mean body weights of 66.6 grams and 49.7 grams respectively. Additionally, the effects of a CRISPR/Cas9 mediated *mc4r* and *myostatin* (*mstn*) gene knockouts on disease resistance to *Flavobacterium covae*, in channel catfish and blue catfish (*I.furcatus*) were examined. Both *mc4r* and *mstn* channel catfish mutants had better survival ($P=0.0061$, $P=0.0150$) when challenged with *F. covae* compared to the control channel catfish. Observed survival of blue catfish *mc4r* mutants was higher than blue catfish controls but was not significantly different ($P=0.21$).

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List of Abbreviations

2N	Diploid
3N	Triploid
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AUMPC	Area Under the Disease Progress Curve
CASA	Computer assisted sperm analysis
CB hybrid	Channel catfish (*Ictalurus punctatus*) ♀ x blue catfish (*I. furcatus*) ♂ hybrid catfish
CPE	Carp pituitary extract
DMEM	Dulbecco's Modified Eagle's Medium
DO	Dissolved oxygen
DPF	Days post fertilization
EDTA	Ethylenediamine tetraacetic acid
ESC	Enteric septicemia of catfish
FBS	Fetal bovine serum
HBSS	Hanks' Balanced Salt Solution
HCG	Human Chorionic Gonadotropin

IACUC	Institutional Animal Care and Use Committee
ICo	Intracoelomic
LHRHa	Luteinizing hormone-releasing hormone analogue
MAS	Motile Aeromonas septicemia
mc4r	melanocortin-4 receptor
MS-222	Tricaine methanesulfonate
mstn	myostatin
OSC	Oogonial stem cell
PAM	Protospacer adjacent motif, NGG
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGC	Primordial germ cell
PGD	Proliferative gill disease
RAS	Recirculating aquaculture system
RGNNV	Red spotted grouper nervous necrosis virus
SCPP-1	secretory calcium-binding phosphoprotein
SNPs	single nucleotide polymorphisms
SSC	Spermatagonial stem cell

TAE	Tris-acetate-EDTA
TAN	Total ammonia-nitrogen

Gene editing of melanocortin-4 receptor (*mc4r*) and myostatin (*mstn*) for growth and disease resistance in channel catfish, *Ictalurus punctatus*, and blue catfish, *I. furcatus*

Abstract

Growth of F_1 and F_2 CRISPR/Cas9 mediated *melanocortin-4 receptor* (*mc4r*) gene knockout in channel catfish (*Ictalurus punctatus*) was investigated. In an earthen pond environment stocked at 18,508 fish per hectare, the mean weight for channel catfish *mc4r* P1, *mc4r* F1 X control, control X *mc4r* F1, and *mc4r* F1 X *mc4r* F1 mutants was 361, 266, 521, and 426 grams, respectively, was larger ($P>0.05$,$P>0.05$, $P<0.01$, $P<0.01$) than control (C) channel catfish, 299 grams. In a recirculating aquaculture system environment, during the first year of growth (354 days post fertilization), channel catfish *mc4r* F1 mutants had a mean weight of 22.6 grams and channel catfish controls were 10.5 grams, a 73.3% increase in body weight in the mutant fish ($P<0.0001$). During the next 161 days, *mc4r* mutants grew 29.2% faster ($P<0.0001$) than controls, reaching mean body weights of 66.6 grams and 49.7 grams respectively. Additionally, the effects of a CRISPR/Cas9 mediated *mc4r* and *myostatin* (*mstn*) gene knockouts on disease resistance to *Flavobacterium covae,* in channel catfish and blue catfish (*I.furcatus*) were examined. Both *mc4r* and *mstn* channel catfish mutants had better survival ($P=0.0061$, $P=0.0150$) when challenged with *F. covae* compared to the control channel catfish. Observed survival blue catfish *mc4r* mutants was higher than blue catfish controls but was not significantly different ($P=0.21$).

1. Introduction

Freshwater aquaculture is constantly evolving to become more profitable while keeping operational costs stable. The United States produced 1.5 billion dollars of aquaculture produced seafood in 2018, and profit margins are vital for encouraging this continued growth of the industry (NOAA, 2022). Feed is the largest variable and overall expense for raising fish to harvest (Lipton and Harrell, 1990), and current catfish feed prices and global fish feed prices are high (Chen 2023, Gregory 2023) damaging profitability and sustainability. Losses to diseases are also substantial in the catfish industry and for aquaculture, in general (Owens 2012, Wright 2018; Zhou et al. 2018; Peterman & Posadas 2019). The overall production of catfish on US farms has declined from a peak in 2003 due to a variety of external factors such as recession and the importation of Vietnamese *Pangasianodon* (Engal et al., 2021), which have a cheaper production cost. However, the adaptation of intensive aeration and intensive split pond culturing, has enabled slight economic recovery.

One solution to this challenge is using genetic modifications to enhance growth rate, feed conversion efficiency, and increase disease resistance, reducing feed cost and loss from mortalities. New options have been added to complement traditional selective breeding programs, including targeted modifications to the genome, resulting in altered performance in multiple traits for the modified organism (Dunham, 2023). Genetic manipulations, specifically mutation of melanocortin-4 receptor *(mc4r)* and myostatin (*mstn),* growth regulating genes, may further close the gap between production potential of US based catfish farms and their overseas competitors in the future.

The application of clustered regularly interspaced short palindromic repeat/CRISPR-associated 9 (CRISPR/Cas9) gene editing was first introduced in 2013 (Ahmad and Amiji, 2018). This genetic modification method has proven to be an exciting genome editing tool for many reasons. When comparing CRISPR/Cas9 to other genomic tools such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), CRISPR/Cas9 provides high predictability, straightforward design and construction, and easier multiplex targeting (Ahmad and Amiji, 2018). Since its introduction, CRISPR/Cas9 work has been applied in a multitude of species. *Mst*n and *mc4r* gene knock-out have been successful in channel catfish (*Ictalurus punctatus*) among other species (McPherron et al., 1997; Coogan, 2021; Khalil et al., 2017; Coogan et al., 2022a).

The melanocortin-4 receptor inactivation in mice resulted in hyperphagia, hyperinsulinemia, and hyperglycemia associated with maturity and onset obesity syndrome (Husazar et al., 1997). In mammals, the *mc4r* gene is expressed in the central nervous system and aides in the regulation of energy homeostasis (Tao, 2010). Humans with heterozygous *mc4r* mutations account for 1 to 6% of early onset or severe adult obesity cases. However, no clinical data is available for cases of homozygous mutations, but this genotype has been identified in humans (Lubrano-Berthleier et al., 2006). *Mc4r* has also been shown to affect various physiological functions such as pigmentation, steroid production, feed intake and sexual function in teleost species (Janprai et al., 2011). In aquaculture, these phenotypic changes can be strategically used to maximize growth and feed conversion rate efficiency (FCR). For example, channel catfish *mc4r* mutants had an observed mean body weight between 13-48% larger than that of controls (Coogan et al., 2022a). In goldfish (*Carassius auratus*), intracerebroventricular

(icv) injection of the goldfish *mc4r* antagonist increased food intake, thus increasing growth (Cerdá-Reverter et al., 2003).

A study on the cyprinid fish, *Spinibarbus hollandi*, demonstrated relationships between *mc4r* promoters and five growth parameters: body weight, body length, total length, body depth, and body width. In fish with the genotype "GG", expression level of *mc4r* in the brain was lower and this genotype had higher growth rates for all five parameters than the other two genotypes, "AA and AG" (Yang et al., 2018). The five growth traits of the AG genotype fish were also observed to be greater than those of the AA genotype fish, although the differences were not significant (Yang et al., 2018). Due to the causations provided from altered *mc4r* gene expression and complete modification, this gene has potentially profound impacts on the ability to change the animal production sector, specifically in that of the aquaculture industry.

Myostatin (*mstn*, growth and differentiation factor 8, GDF8) belongs to the transforming growth factor-b (TGF-b) superfamily (Thomas et al., 2000; Zhang et al., 2020). It serves as a negative regulator of skeletal muscle growth in vertebrates, which if knocked out or down regulated can result in hyperplasia and hypertrophy, of skeletal muscle (Thomas et al., 2000). This mutation causes these changes by inhibiting myogenesis and hypertrophy as increased muscle fibers can result from increased myoblast proliferation and delayed differentiation (Thomas et al., 2000; Coogan et al., 2022a). The outcome of these mutations is sometimes referred to as "double muscling" and one of the most notable natural forms of this mutation was seen in Belgian Blue cattle (*Bos taurus*), thus also being known as the Belgian Blue mutation (Zou et al., 2019). When this mutation when induced in sheep (*Ovis aries*), significant increases were observed for mean leg yield, loin yield, proportion of loin yield and total yield (Hickford et al., 2010).

Positive effects on growth parameters were also seen in *mstn* mutant common carp (*Cyprinus carpio*). Body weight, total body length, body width and body depth in gene edited common carp were significantly higher than the control genotype (Shahi et al., 2022). In stocker stage channel catfish, P1 *mstn* mutants were 88% larger than controls as well as 27% larger than controls at market size (Coogan et al., 2022b). Heterozygous F1 mutants were 218% larger than controls when grown in aquaria (Coogan et al., 2022b). This double muscling phenomenon has been shown to increase carcass yield in red sea bream (*Pagrus major*) by 7-8% compared to the wild type of the same length, making it an obvious advantageous modification in an aquaculture setting (Washio et al., 2021).

However, while *mstn* knock-out provides growth benefits, it has also been shown to have integral effects on the organism's disease resistance and immune response capabilities (Chiang et al., 2016; Coogan et al., 2022b). Disease resistance is the most important aquaculture trait as disease causes 40% of all catfish production mortality (Tucker et al., 2012). In 2016, the most predominantly diagnosed diseases in catfish aquaculture from east Mississippi catfish production facilities were columnaris disease, caused by *Flavobacterium covae*, enteric septicemia of catfish (ESC) caused by *Edwardsiella ictaluri*, and motile Aeromonas septicemia (MAS) caused by *Aeromonas hydrophila* and related motile aeromonads, as well as *Henneguya ictaluri* (proliferative gill disease, PGD) (Peterman et al., 2019). Thus, improvements are needed in the disease resistance of catfish in aquaculture. In 2016, in East Mississippi alone, an estimated 10 million dollars in loss was caused from disease, with columnaris disease, responsible for 1.4 million dollars in loss (Peterman et al., 2019). When challenged with *Edwardsiella ictaluri*, channel catfish *mstn* mutants performed equally or better than controls (Coogan et al., 2022b). However, the immune system response of *mstn* mutant medaka (*Oryzias latipes*) challenged with

red spotted grouper nervous necrosis virus (RGNNV), was less than that of the control wildtype as quantified by reduced expression levels of interferon-stimulated genes, appearing to possess partially suppressed or undeveloped immune systems, while demonstrating the enhanced growth characteristics of the *mstn* mutation (Chiang et al., 2016).

The objectives of this study were to evaluate growth of *mc4r* and *mstn* knock-out fish of varying families and zygosity cultured at varying stocking densities in pond and recirculating aquaculture systems (RAS) environments. A second major objective was to determine potential pleiotropic effects related to diseases resistance of these growth-regulated, gene edited channel catfish. If *mc4r* or *mstn* mutants do have better growth but less disease resistance, the gain in production from growth could be negated by the higher mortality.

2. Methodology

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) guidelines.

2.1 *Mc4r* and *mstn* mutant production.

The channel catfish used in this growth study were progeny of a P1 and F1 generations of *mc4r* mutant channel catfish described in (Coogan et al., 2022a; Coogan et al., 2022b). For the pond study, 7 channel catfish families were stocked, female x male crosses: 07453304 cntrl x 51283993 cntrl, 51587305 cntrl x 10701567 cntrl, B20 adipose clipped cntrl, F1 MC4R x cntrl 46B, 40a mc4r x mc4r, P1 50A mc4r and cntrl x B20 F1. Four channel catfish families were followed through time in tanks, female x male crosses: cntrl (0018 x 6953), *mc4r* (P1 7006 x P1

7014) F1, *mc4r* (P1 7230 x P1 7235) F1 and *mc4r* (F1 R27 x F1 4877) x (R30 x 4593) F2. The control and *mc4r* mutant channel catfish families produced for the disease challenge consisted of a 2019 control x 2019 control cross as well as a 2019 F1 *mc4r* x 2019 F1 *mc4r* mutant cross. The *mstn* mutant families produced for the challenge were comprised of a 2018 P1 *mstn* x 2018 P1 *mstn* cross, a 2019 F1 *mstn* x 2019 F1 *mstn* cross, and a 2019 F1 *mstn x 2018* Control cross. The blue catfish families used in the disease challenge consisted of 2018 Control x 2018 F1 *mc4r* cross and a 2017 Control x 2017.

The fish used in the study were spawned in the summer of 2021 through standard catfish pen spawning. The selected pairs of parental catfish were removed from the ponds and stocked into flow through fiberglass spawning tanks divided into 0.3 x 0.6 x 0.3-meter sections. The tank used was 6.1 meters in length, 0.9 meters in depth, and 0.61 meters in width for a total volume of 1,700 L. Water flowing into the tank was approximately 26 to 27 ℃ flow rate was set at 24 L/min to ensure dissolved oxygen was above 5 mg/L and ammonia was as near 0 ppm as possible. Channel catfish females were then administered a priming intracoelomic (Ico) injection of luteinizing hormone-releasing hormone analogue (LHRHa) at 20 μg/kg and human chorionic gonadotropin (HCG) at 1,600 IU / kg bodyweight, followed by a resolving dose of 100 μg LHRHa / kg bodyweight 12 hours later and males were administered a single dose of 20 μg/kg of LHRHa and HCG at 1600 IU/ kg bodyweight at the time of the female's' initial dose. When an egg mass was observed, it was collected, weighed, and an estimated number of eggs was calculated by counting the number of eggs per gram. The embryos were moved to a trough filled with a solution of water supplemented with CaCl2 at 50 ppm for water hardening before incubating in a flow-through hatching trough. Embryos were then moved to plastic baskets in

hatching troughs with paddlewheel style incubators (7.5 m × 0.5 m × 0.45 m) with water ranging from 27-30 °C at a 15 L/min flow rate (Dunham et al., 2000).

The *mc4r* mutants used in the *F. covae* challenge were produced through standard pen spawning as described above. The *mstn* mutants used in the challenge were produced through induced spawning and artificial fertilization. Myostatin gene edited females were given the same dose of LHRHa as described above and placed into mesh spawning bags secured to the side of the spawning tanks. Once eggs were observed on the bag, the females were anesthetized in an 18 L bucket with 100 ppm tricaine methanesulfonate (MS-222, Syndel USA, Ferndale, WA) and buffered with sodium bicarbonate to a pH of 7.0. Upon sedation, the female catfish was then removed, dried, and eggs were stripped into metal spawning pans (~25 g of eggs/pan) coated in Crisco All-Vegetable shortening (Crisco, USA, Parsippany, NJ) to reduce egg adhesion. After egg collection, males were selected and anesthetized, dried, testes were surgically removed, and males were sutured to close the incision. All tools used in the procedure were sterilized with 70% ethanol prior to the initial incision. Once the testes were removed from the coelomic cavity, they were cleaned to avoid contamination from blood vessels, connective tissues, and the peritoneum. After cleaning, macerating, and rinsing with 9-ppt saline, testes were weighed, and the milt was placed in into plastic 50 milliliter tubes, at a dilution rate of 10 mL of saline per 1 gram of testes. A *mstn* mutant female channel catfish produced approximately 25 g of eggs/pan, which were fertilized by 6 different males (control channel catfish, control blue catfish, two *mstn* blue catfish, and two *mstn* channel catfish). Egg masses were then moved to vexar baskets suspended in hatching troughs with active paddlewheels (Dunham et al., 2011).

2.2 Fish culture in ponds

Mixed families of *mc4r* mutants of varying zygosity levels, and wildtype channel catfish were reared together in a 0.04-hectare earthen pond located at Auburn Universities Fish Genetics Research Unit at E.W. Shell Fisheries Research Center in Auburn Alabama. The fish in this pond (G27) were fed Purina Catfish 32 floating feed; (crude protein: ≥32.00%, crude fat: ≥4.00%, crude fiber: 7.00%, and phosphorus: 0.80%). Feeding was ad libitum seven days a week from March through November, and five days a week in the winter months. The pond was stocked at 18,508 fish per hectare and was seined using a 3.8 cm mesh seine over a two-year period to measure growth.

2.3 Fish culture in tanks

Four families of channel catfish were spawned in the summer of 2021. They consisted of Family 1: control x control (3 tanks), Family 2: *mc4r* x control (F1, 2 tanks), Family 3: control x *mc4r* (F1, 1 tank), Family 4: *mc4r* x *mc4r* (F2, 3 tanks). Before stocking fish into the experimental tanks, fish were briefly reared in 60 L glass aquaria in an RAS system at densities calculated to be 11 fish per L then reduced to 1 fish / 1 L. Fish were stocked into 106 L experimental tanks, densities were 1 fish / 1 L in the glass aquaria. Fish were stocked in the 106 L tanks, May 18th, 2022, and individual weights were taken of all fish and pit tagged for individual identification (Biomark AP12). Stocking densities in the 106 L rectangular tanks (RAS) was 0.442 fish/L. Fish were fed ad libitum daily with Triton premium aquaculture feed 4010 (crude protein: ≥40.00%, crude fat: ≥10.00%, crude fiber: ≤4.00%, and phosphorus: 1.00%).

2.4 *F. covae* challenge design

For the *F. covae* challenge, 11 total replicates of varying species (*I. punctatus* and *I. furcatus*), families, and generations were infected with *Flavobacterium covae* (strain ALG-00-530)) by exposing the fish to the bacteria culture in static water for 45 minutes. The final inoculum concentration was 4.75 x 10^8 CFU/mL, and the dosing rate was 100mL per 10L. To achieve the target of 4.75 x 10^6 CFU mL final exposure in the water. A total of 1800mL of this inoculum was added into two 275 L tanks. Sterile, modified Shieh Broth (1800mL) was used in an identical singular mock-challenged tank. Mortalities were monitored for 167 hours, and mortalities were removed every three hours. Body weight and total length were recorded, and samples were placed into 1.5 mL Eppendorf tubes, and stored in a liquid nitrogen Dewar until being transferred into a −80 °C freezer for future analysis.

2.5 Mutation analysis

Samples of *mc4r* and *mstn* mutant fish were taken by clipping a pelvic fin. The fin samples were placed into 1.5 mL Eppendorf tubes on ice and then held at −80 °C until DNA extraction. DNA was extracted using proteinase K digestion followed by protein and ethanol precipitation (Waldbieser and Bosworth, 2008). DNA concentrations were brought to 500 ng/µL using Mili-Q water as the diluent and concentrations were measured with a NanoDrop® 2000 spectrophotometer (Thermo Fisher Scientific). Next, using the TOPO™ TA cloning™ kit without competent cells from Thermo Fischer Scientific, *E. coli* cells were cultured and stored at -80 °C, and then thawed immediately before use. Eight clones per sample (40 fish: 320 clones total) were sent to Sequetech Corporation in Mountain View, California for DNA sequencing. Sequences of potential mutants were compared to that of a wildtype sequence using MAFFT version 7 software. Nucleotides were only considered if within 715 bases' before or after the

sgRNA integration site. Mutations were then analyzed to determine if the predicted amino acid profile was altered by the mutation using the National Center for Biotechnology Informations' online open reading frame finder. If a mutation caused the amino acid signature to differ from the control wildtype sequence, it was considered a mutated genotype for the desired gene manipulation.

2.6 Statistical analysis

All data was analyzed using the GraphPad Prism 10 statistical analysis software (v.10.0.3; GraphPad Software Inc., Boston, MA, USA). An ANOVA with Dunnett's multiple comparisons test was used to calculate differences in body weight between *mc4r* mutants and controls in both the pond and RAS environment. Mean final body weight in ponds was adjusted with the regression technique of to account for initial weight differences. Fisher's exact test was used to calculate significance regarding the results of the disease trial for channel catfish *mc4r* mutants and channel catfish controls, channel catfish *mstn* mutants and channel catfish controls, as well as blue catfish *mc4r* mutants and blue catfish controls. For the disease challenge, Area Under the Mortality Progress Curve (AUMPC) was calculated with the formula:

AUMPC $=\sum[(yi+yi+1)/2]n-1i=1(ti+1-ti)$, where yi is the number of mortalities within the replicate at time ti and n is the total number of time points. This formula is used to quantitatively describe a diseases' intensity or organisms' susceptibility to the pathogen through time.

3.0 Results

3.1 Fish culture in earthen ponds.

After 469-days of culture at a stocking density of at 18,508 fish per hectare. P1 *mc4r* , F1 *mc4r* X control, control X F1 *mc4r* and F1 *mc4r* X F1 *mc4r* genetic types were 20.7, -11.0, 74.2, and 42.5% larger than the control (Table 1). Mean adjusted body weights attained were between 261 and 521 g, and all genetic types were larger than the control except for control X F1 *mc4r*.

3.2 Culture in tanks

After the first 12 months of growth, mean body weight of control, F1 *mc4r* X control, control X F1 *mc4r* and F1 *mc4r* X *mc4r* channel catfish was 10.7, 12.1, 10.3 and 28.1 g, respectively, with the F_2 being the largest (Table 2, $P<0.0001$). At 41 months, mean body weight of control, F1 *mc4r* X control, control X F1 *mc4r* and F1 *mc4r* X F1 *mc4r* channel catfish was 46.4, 58.8, 44.7, and 81. 2 g, respectively. F1 *mc4r* X F1 *mc4r* was the largest genetic type followed by control X F1 *mc4r* ($P< 0.0001$,$P<0.05$). Relative growth rate of and F1 *mc4r* X F1 *mc4r* had slowed but final body weight was still the largest.

Table 1. Initial, final and adjusted final body weights of P1 melanocortin-4 receptor *(mc4r* gene edited), control X control, F1 *mc4r* X control, control X F1 *mc4r*, F1 *mc4r* X F1 *mc4r* and control channel catfish, *Ictalurus punctatus*, grown in an earthen pond at 18,508 fish per hectare for 469 days.

Treatment	N	Mean Body Weight (g) Initial +/- SD	Mean Body Weight final (g) +/- SD	Mean Adjusted Body Weight final (g) +/- SD
Control	54	61 +/- 13.5	299 +/- 101	299 +/- 100
P1	33	29 +/- 3.1**	265 +/-74*	361 +/- 101
F1 X control	45	90 +/-3.4**	353 +/- 152	266 +/- 115
Control X F1	93	23 +/-9.2**	407 +/-148**	521 +/- 190**
F1 X F1	37	42 +/-2.8**	369 +/- 132*	426 +/- 153**

*Mean adjusted body weight was different from the control ($P<0.05$; Dunnett's multiple comparisons test)

** Mean adjusted body weight was different from the control ($P<0.01$; Dunnett's multiple comparisons test)

Table 2. Body weights of F1 *melanocortin-4 receptor (mc4r* gene edited), control channel catfish, F1 *mc4r* X control, control X F1 *mc4r*, and F1 *mc4r* X F1 *mc4r, Ictalurus punctatus*, grown in 60 L aquaria in a recirculating aquaculture system RAS at 0.45 fish/L for 12 months and then 1 fish /L in 106 L tanks in an RAS until 16.5 months of age.

Treatment	N (tanks)	Total Body Weight (g) at 12 Months +/- SD	Total Body Weight (g) at 16.5 Months +/- SD	Mean survival (%)
control (0018 x 6953)	4	10.66 +/- 3.81	46.43 +/- 15.30	72.64
mc4r het. (7006 *mc4r* x 7014 control) F1	2	12.05 +/- 6.07	58.85 +/- 10.55*	88.67
mc4r het. (7230 control x 7235 *mc4r*) F1	1	10.32 +/- 3.43	44.74 +/- 12.42	95.00
mc4r homo. (R27 x 4877) x (R30 x 4593) F2	3	28.18 +/- 5.15**	81.23 +/- 16.9**	86.67

*Mean adjusted body weight was different from the control (P<0.05; Dunnett's multiple comparisons test)

** Mean adjusted body weight was different from the control (P<0.01; Dunnett's multiple comparisons test)

3.3 *F. covae* challenge

Survival rates of the various catfish genetic types ranged from 0 to 100% during the *F. covae* challenge (Table 3). observed survival rates ranged from 0 to 100%. Both *mc4r* and *mstn* channel catfish mutants had better survival , P= 0.006 and 0.015, respectively, compared to the control channel catfish (Fig. 1 and 2). Survival of *mstn* and *mc4r* channel catfish mutants averaged 80 % and 83.3% survival, respectively. The observed survival of blue catfish *mc4r* mutants, 30%, was higher, although not significant (P=0.21) compared to blue catfish controls, which had no survival (Fig. 3). Individual families varied in total mortality, and rates of mortality varied during the challenge (Table 3, Figs. 4-6).

3.4 Mutation analysis

Genome sequencing of the *mc4r* families revealed a variety of mutations within exon 1 of the melanocortin-4 receptor (*mc4r*) gene coding sequence. The types of mutations observed ranged from single nucleotide deletions, insertions, and mostly substitutions. These mutations were considered impactful if the expected amino acid expression based on the wildtype genome of channel catfish differs from the observed amino acid profile and overall charge within the mutant sequence (Fig. 7). The mean number of mutations that caused a change in the amino acid sequence per fish was 5.0 and mean number of overall mutations was 12.7 per individual. Showing that some changes to the genome from the induced *mc4r* mutation potentially did not cause a difference in expression, however all fish with altered expected charges within the amino acid sequence were classified as positive mutants.

Table 3. Survival of channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*) wild types, F1 melanocortin-4 receptor (*mc4r*) mutant blue catfish, F2 *mc4r* channel catfish, challenged with *Flavobacterium covae*.

Family information	Generation of the mutants	Species	Number survived	mortalities	survival %
8944 (neg *mc4r*) x 4293 (pos *mc4r*)	F1	Blue catfish	3	7	30
Blue catfish control	n/a	Blue catfish	0	10	0
7142 (pos *mstn*) x 3995 (pos *mstn*)	F1	Channel catfish	10	0	100
7412 (pos *mstn*) x 9942 (pos *mstn*)	F2	Channel catfish	7	3	70
7412 (pos *mstn*) x R20 (wildtype)	F1	Channel catfish	7	3	70
7119 (pos *mc4r*) x 7013 (pos *mc4r*)	F2	Channel catfish	8	2	80
7119 (pos *mc4r*) x 7013 (pos *mc4r*)	F2	Channel catfish	9	1	90
7119 (pos *mc4r*) x 7013 (pos *mc4r*)	F2	Channel catfish	8	2	80
6417 (neg mc4r) x 3687 (neg mc4r)	n/a	Channel catfish	3	7	30
Channel catfish control	n/a	Channel catfish	2	8	20
Channel catfish control	n/a	Channel catfish	9	1	90

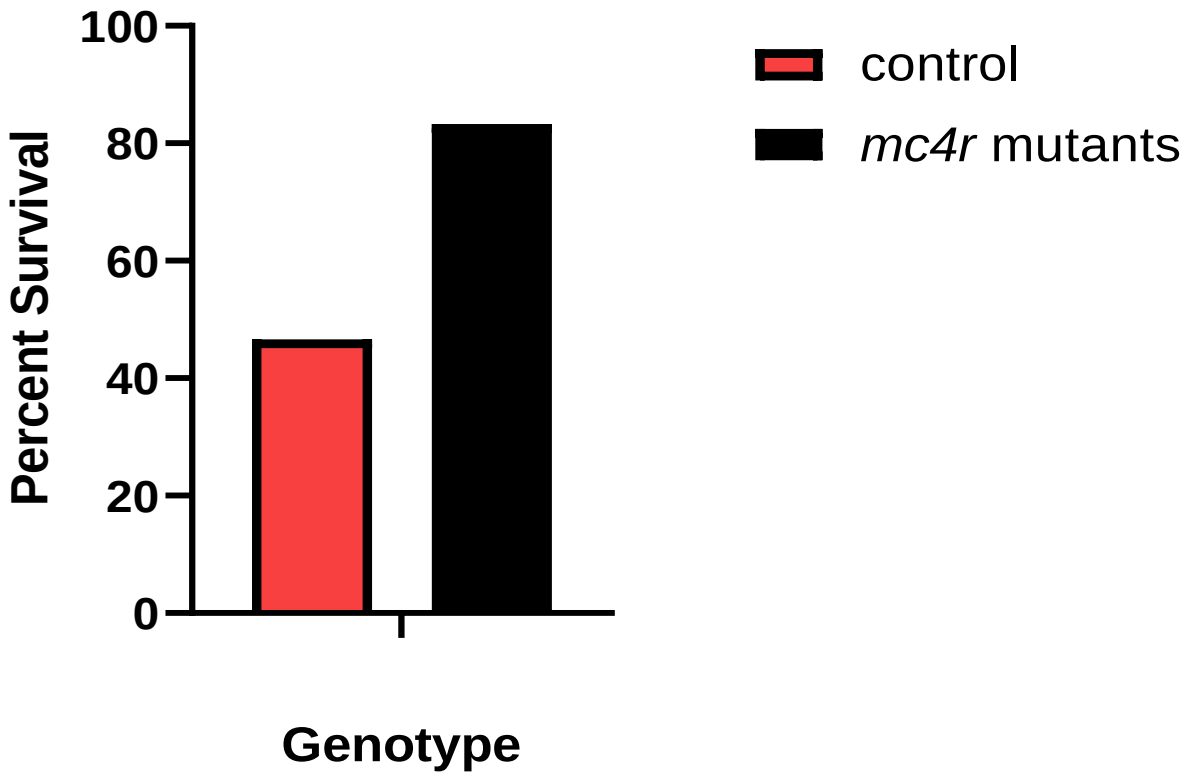

Figure 1. Survival of channel catfish (*Ictalurus punctatus*) mutated for melanocortin-4 Receptor (*mc4r)* (N=30) and control channel catfish (N=30) when challenged with *Flavobacterium covae*. (Fisher's exact test, P=0.0061)

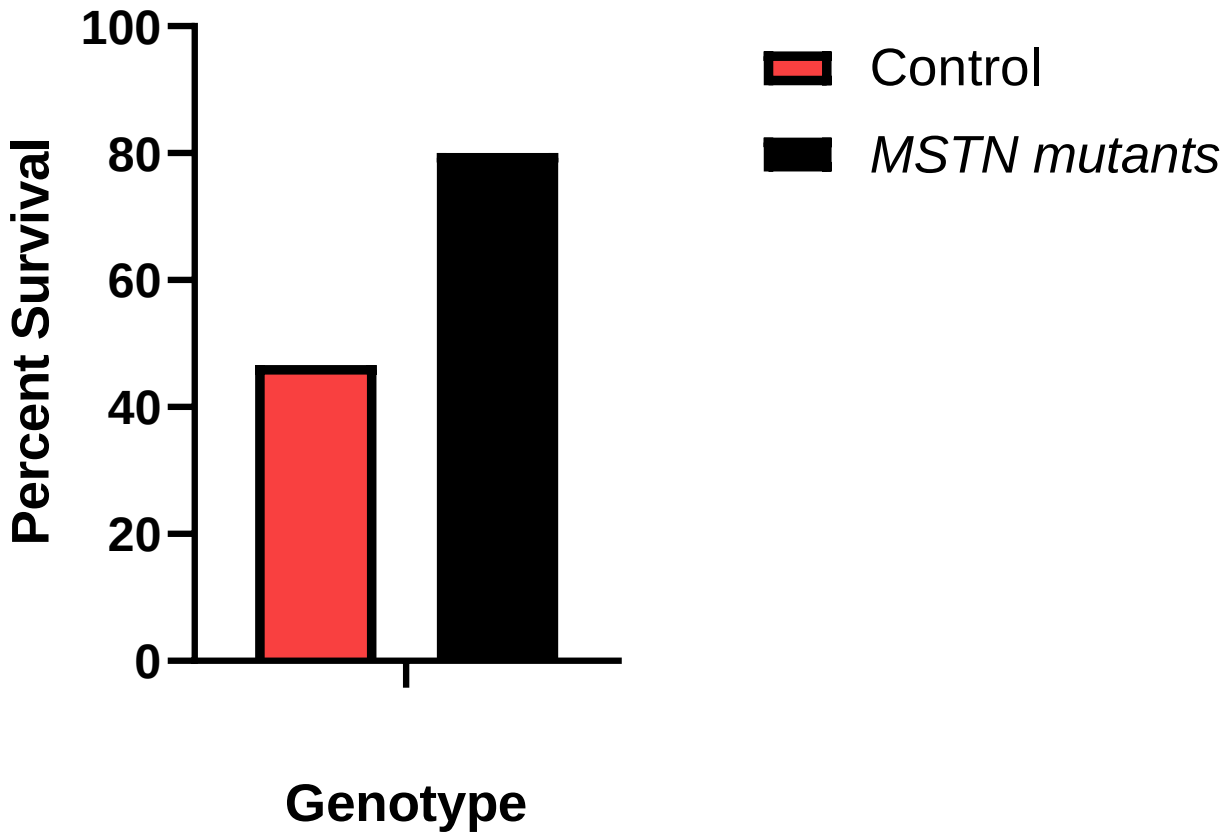

Figure 2. Survival of channel catfish (*Ictalurus punctatus*) mutated for myostatin (*mstn*) (N=30) and control channel catfish (N=30) when challenged with *Flavobacterium covae*. (Fisher's exact test, P=0.015).

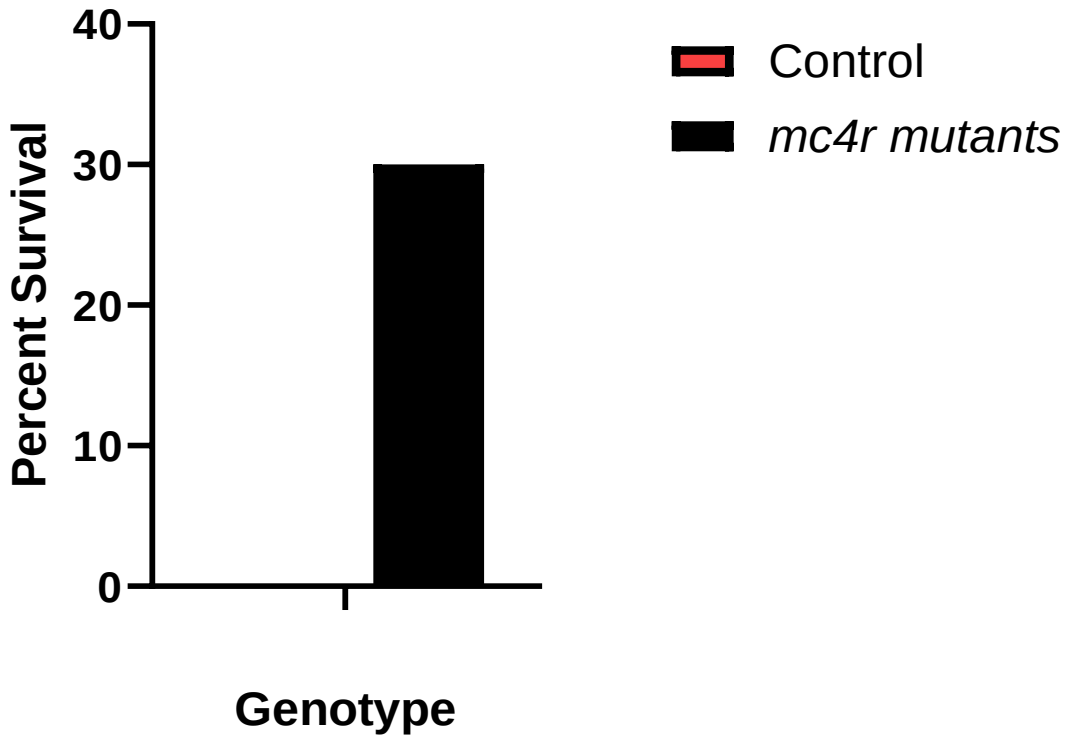

Figure 3. Survival of blue catfish (*Ictalurus furcatus*) mutated for melanocortin-4 receptor (*mc4r*) (N=10) and control blue catfish (N=10) when challenged with *Flavobacterium covae*. (Fisher's exact test, P=0.21).

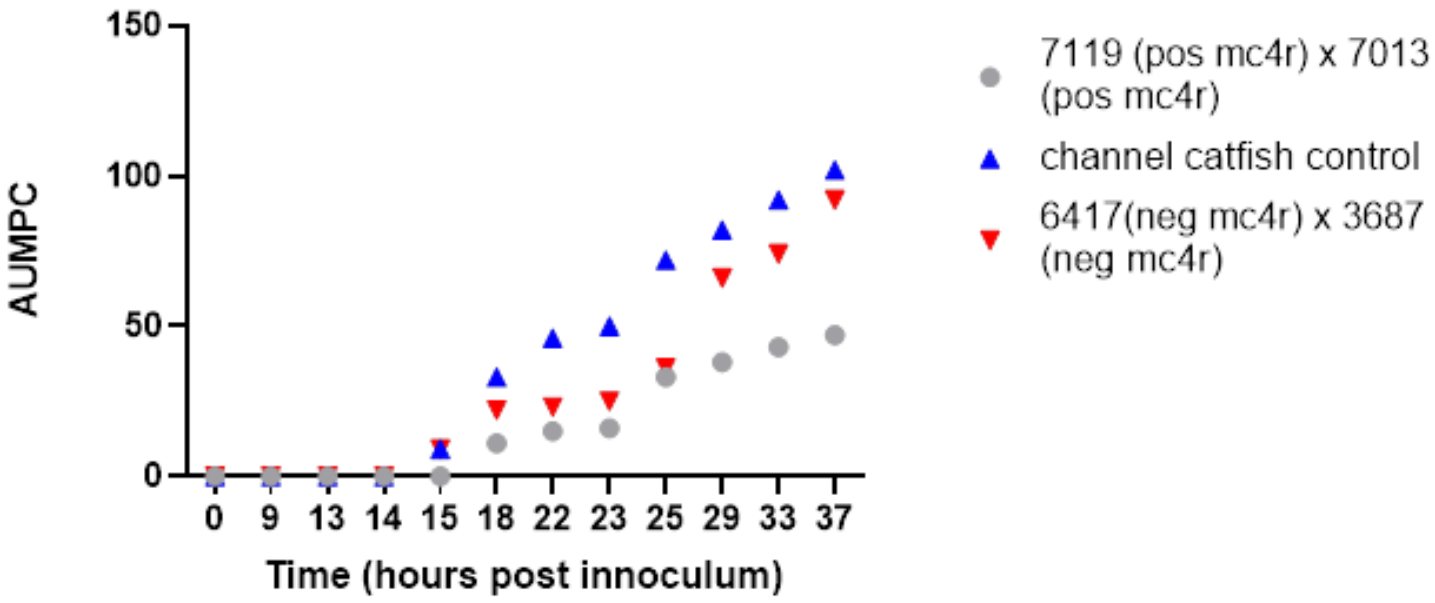

Figure 4. Area under mortality progress curve through time, comparing a homozygous F_2 *mc4r* mutant channel catfish family, *Ictalurus punctatus*, and two different control families (injected "neg" and non-injected controls) during a challenge with *Flavobacterium covae*.

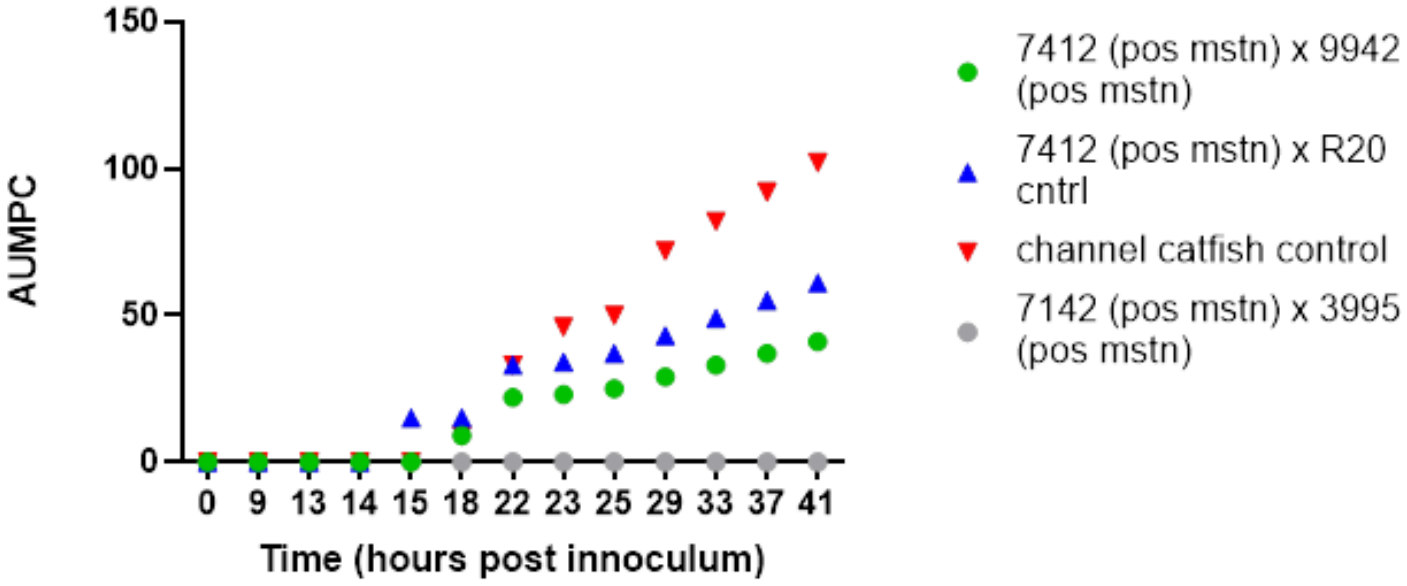

Figure 5. Area under mortality progress curve through time, comparing F2 *mstn* mutant channel catfish, *Ictalurus punctatus*, family, F1 *mstn* mutant channel catfish family, a F2 *mstn* mutant backcross to wild type channel catfish family, and a wild type control family during a disease challenge with *Flavobacterium covae.*

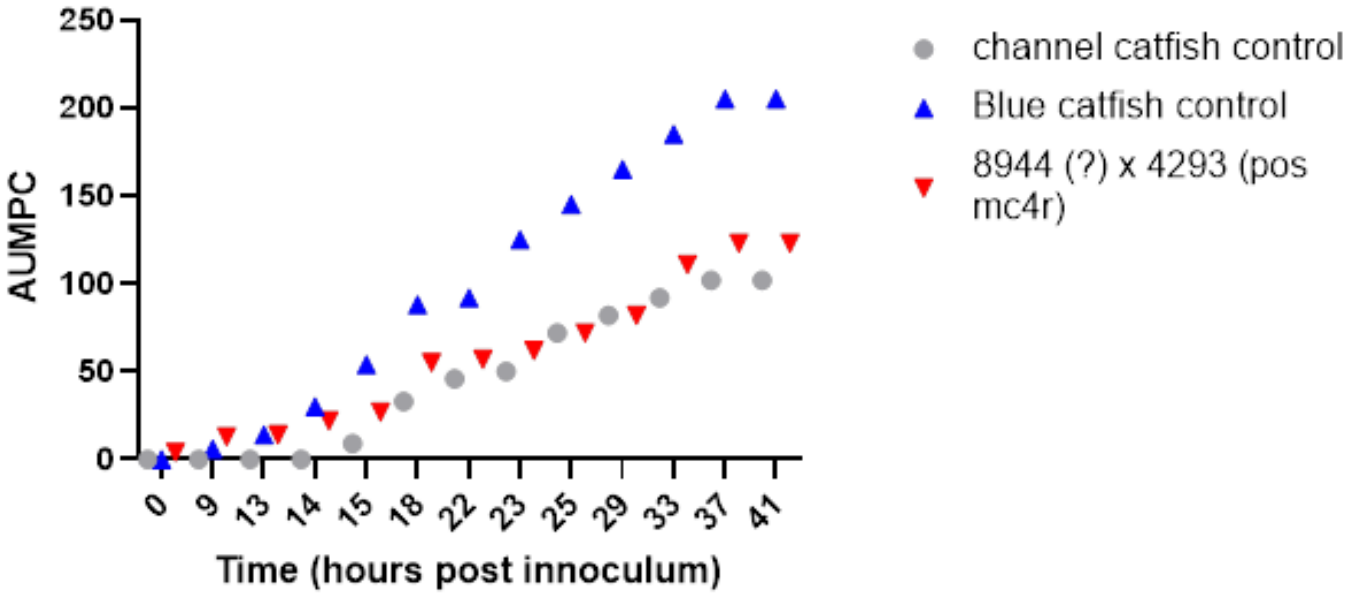

Figure 6. Area under mortality progress curve through time, comparing a F1 backcross *mc4r* mutant blue catfish, *Ictalurus furcatus*, family with a wild type channel catfish, *I. punctatus*, control family and with a wild type blue catfish family challenged with *Flavobacterium covae*.

Wild-type

ggagatggaggacacggaagagactcgcagattagaataaacgcag**ATG**AAGACGGAAAGCGGAGGACTGTG
GTGAGGAGGTCTTGCGGATATGAACGTGTCGGAGCACCACGGGATGCAGCATGCAC
ACCGGAACCACAGCCTGGGCGTGCAGATTGGAAACAAAGCCGGCTCGGGGGAAAG
GAACTCGGAGTCGGGCTGCTACGAGCAGCTGTTGATCTCCACCGAGGTCTTCATCAC
GCTAGGGTTGGTCAGCCTTCTGGAGAACATCCTGGTAATCGCGGCCATCGTCAAGAA
CAAGAACTTCCACTCGCCCATGTACTTCTTCATCTGCAGCCTGGCGGTGGCCGACCT
GCTGGTGAGCGTATCGAACGCGACAGAAACGGCTGTGATGGCGCTGATCACCAGCG
GCAACCTGACCATCTCTGGAGACGTCGTGAAAAGCATGGACAATGTGTTCGACTCCA
TGATCTGCAGCTCACTCCTGGCCTCCATTTGGAGTCTCCTGGCCATCGCCGTGGACC
GCTACGTCACCATCTTCTACGCCCTGCGCTACCACAACATCATGACCCAACGCCGCG
CGGCGCTCATCATCGTATGCATATGGAGCTTCTGCACGGCGTCCGGTGTGCTCTTCA
TCATCTACTCGGAGAGCGCTACAGTCCTCATCTGCCTTATCAGCATGTTCTTCACCAT
GCTGGCCCTCATGGCCTCGCTTTACGTGCACATGTTCCTCTTGGCGCGGCTTCACATG
AAACGCATCGCCGCCTTACCGGGGAACGGCCCCGTGTGGCAGGCGGCCAACATGAA
GGGCGCCGTGACGCTCACCATCCTGCTCGGAGTGTTTGTCGTGTGCTGGGCGCCGTT
TTTTCTCCACCTCATTCTCATGAtctcttgtccgaggaacccgtattgcgtctgcttcatgtctc

Wild sequence

5’aag // CACACCG // GTCAAGA // CTCACTC // ATGACCC // GTGACGC // GTTTGTC // ATTCTCA // tat3’

AA: MNV // MQHAHRNH // GLVSLLE // AIVKNKN // SLLAIAVD // RYHNIMTQRR // GAVTLTI // HLILMIS // 3’

5’ (000 // 00+0++0+ // 0+00++- // 0000000 // 0++0000- // ++000000++ // 0000+00 // ++0+000) // 3’

***mc4r* x Control F1**

Mutated sequence

5’aag // CACGCCG / GTCGAGA // CTCGCTC // ATGGCCC // GTGGCGC // GTTCGTC // ATTTTCA // tat3’

AA: MNV // MQHARRNH // GLVRLLE // AIVENKN // SLLVIAVD // RYHSIMAQRR // GAVALTI // HLIFMIS // 3’

5’ (000 // 00+0++0+ // 0+0+++- // 000-000 // 0++000- // ++000000++ // 0000+00 // ++0000) // 3’

Figure 7. CRISPR/Cas9 induced mutations within exon 1 of melanocortin-4 receptor (*mc4r*) gene coding sequence of channel catfish (*Ictalurus punctatus*) F1 *mc4r* mutant compared to

wild-type a channel catfish. The exons and introns are indicated by upper and lower case and the underlined bold uppercase is the start codon. The primers used in PCR are indicated in red. The guide RNA target sites are indicated in green as well as the PAM (Protospacer adjacent motif, NGG) in blue. Double slashes indicate continuance of the wild type sequence for ease of reading. Individual red letters are representative of single nucleotide polymorphisms (SNPs). "AA" represents the corresponding expected amino acid sequence from Genbank (Accession No. LBML01001141.1). Under the predicted amnio acid profile are the corresponding charges associated with the amino acids being expressed, "+" being positive, "-" being negative, and "0" being neutral. Red symbols or numbers within this line express charge alterations caused by the change of the expected amino acid.

4.0 Discussion

When grown in aquaria during the early fry/fingerling phase, F_2 *mc4r* mutants grew 2.8X faster than wild type and reciprocal F1 *mc4r* families. Once reaching stocker size the F_2 *mc4r* mutants were 75.0 % larger than the controls. Additionally, *mc4r* X control backcross was 26.7% larger than the control. Similar results were observed in the earthen pond as control X F1 *mc4r* and F1 *mc4r* X F1 *mc4r* genetic types were 74.2, and 42.5% larger, respectively, than the control. *Mc4r* genetic types exhibited higher resistance to columnaris for both natural and artificial challenge infections. Channel catfish *mstn* mutants also had higher survival than wild type controls when challenged with columnaris. The growth rate obtained for the P_1 *mc4r* in ponds, 20.7% faster than the control, was similar to that observed by Khalil et al. (2023) for P_1 *mc4r* in tanks which was 20% to 38% compared to full-sibling controls at the juvenile stage.

In the current study and that of Coogan et al. (2022), P_1 or F_1 mated with the wild type results in wild type and heterozygous knockout progeny. Mating of P_1 individuals or mating of F_1 individuals results in progeny that are a combination of wild type, heterozygous knockout and homozygous knockout progeny. The data of Coogan et al. (2022) indicated that in regards to body weight, the mutation acts as a recessive and the increased growth is only displayed by homozygous individuals. F_1 homozygous *mc4r* mutant channel catfish were reaching market size 30% faster than F1 heterozygous *mc4r* mutants in earthen ponds, and at the stocker stage (~ 50 g), homozygous channel catfish *mc4r* mutants grew to be 40% larger than the mean of combined wildtype families and 54% larger than F1 *mc4r* heterozygous mutants (Coogan et al., 2022). The results from the ponds was similar in the current study, control X F1 *mc4r* and F1 *mc4r* X F1 *mc4r* genetic types were 74.2, and 42.5% but the fish were around a third of the size of the 1.3 kg fish of Coogan et al. (2022). There was the exception of the one heterozygous backcross family actually exhibiting the best growth (the other heterozygous backcross family did not have increased growth) which contradicts the results of (Coogan et al. 2022) regarding recessive gene action of the mutation. Possible explanations of the unexpected heterozygote growth improvement include family effects, heterobeltiosis or not all of the mutations exhibit the same type of gene action. A similar result was obtained in the tanks, although the fish were only grown to about 60g. One of two heterozygous families showed improved growth compared to the control, but the family with the most homozygotes had the best growth, a 75% improvement. Verifying the conclusion of Coogan et al. (2022), there were no apparent genotype-environment interactions when comparing growth of *mc4r* F1 and F2 channel catfish mutants to wildtype controls between tank traditional catfish pond farming environments.

When infected with *Flavobacterium covae*, percent survival of channel catfish *mc4r* and *mstn* mutant families had higher survival rates compared to controls. Blue catfish *mc4r* mutants had a survival rate of 30% while blue catfish controls had a 0% survival rate, not yielding significant results but a promising avenue for future research. Different genotypes experienced different timelines for their mortality events.

In the channel catfish *mstn* mutants, both heterozygous and homozygous *mstn* mutant channel catfish performed better than control wild type channel catfish. Coogan et al. (2022) observed that when F_1 *mstn* mutant channel catfish were challenged with *Edwardsiella ictalurid*, causative agent of enteric septicemia (ESC) of catfish challenge, performed equally or better than controls, however, the infection was severe resulting in total mortality with the mutants having longer survival time. In the current study, the improved disease resistance of both F_1 and F_2 *mstn* mutant channel catfish was dramatically better than the wildtype control when challenged naturally or artificially with *F. covae*. Previous genetic comparisons (Dunham et al. 2002, Wang et al. 2024) have shown that ESC challenges and mortalities tend to be more severe and the genetic differences less in these challenges compared to columnaris challenges. In regard to enhanced growth and disease resistance, the *mstn* knockout/mutation appears to act in a dominant fashion.

Our results contradict what has been observed in other species, as previous studies in fish with *mstn* gene knockout indicated some impairment of the immune system, increasing the inherent susceptibility to different diseases (Sahoo and Paul, 2023). In medaka the *mstn* mutation resulted in decreased immune performance and a failure to initiate an ISG (interferon-stimulated gene) response, suggesting decreased innate immune response ability (Chiang et al., 2016). However, F2 mutant medaka has significantly increased body weight and body length when

compared to controls (Chiang et al., 2016) similar to what was seen in the current study. Two loci exist for *mstn* complicating evaluations with one locus perhaps more focused on growth and the other on immune responses. Perhaps, different species are at different points in the evolution of the role of these two loci and interactions of the two have not been studied.

Disease resistance of the mutant *mc4r* channel catfish was almost identical to that of the *mstn* mutants. However, no completely heterozygous families were evaluated. Blue catfish tend to be less resistant to *F. columnare* when compared to channel catfish (Dunham et al. 1993). In the current study, heterozygous blue catfish *mc4r* mutants had higher observed survival than wild type blue catfish, which suggests that not only do these *mc4r* mutants receive growth benefits from their genetic alteration, but they may also gain heightened immune response against *F. covae*.

Both *mc4r* and *mstn* mutation enhance growth and disease resistance of channel catfish and increased disease resistance in blue catfish. *Mstn* mutation appears to act in dominant fashion for both growth and disease resistance enhancement, whereas *mc4r* mutation appears to act in a dominant fashion for disease resistance based on the initial observation in blue catfish and in a recessive manner for growth enhancement. These gene edited catfish would be beneficial for enhancing catfish farming.

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Xenogenesis for reproductive enhancement of channel catfish (*Ictalurus punctatus*) x blue catfish (*I. furcatus*) hybrid embryo production using common carp (*Cyprinus carpio*) as a host for blue catfish sperm

Abstract

Xenogenesis is a method of reproduction where successive generations differ from each other and no genetic material is transmitted from the parent to the offspring. Xenogenesis can be accomplished by transplanting spermatogonial stem cells (SSCs), oogonial stem cells (OSCs) or primordial germ cells (PGCs) from the desired diploid donor species into a sterilized host. Specifically, the production of embryos from hybridizing channel catfish (*Ictalurus punctatus*) females with blue catfish (*I. furcatus*) males could be refined to a simpler and more cost-effective practice of xenogenesis. Xenogenesis could alleviate the reliance on growing blue catfish males to maturity and sacrificing them for gonad extraction and subsequent artificial spawning. Common Carp (*Cyprinus carpio*) males can be hand stripped of milt, which is a much-desired quality in the aquaculture setting due to the relative ease of gamete collection for artificial spawning. Thus, common carp embryos, and larvae were injected with blue catfish oogonial stem cells and spermatagonial stem cells at various points in relation to their development. In total, 152 potential xenogens were sampled and 57 of them were confirmed positive through PCR, giving a total 37.5% success rate of common carp hosting blue catfish. The highest percentage of positive samples were observed at 0 to 46-degree days and 483-575 degree days, averaging 62.3% and 56.7% success respectively. Rate of xenogenesis was similar for male and female common carp hosts. Confirmed by our statistical analysis, sex played no

significant part in the ability of common carp hosts to be colonized by blue catfish stem cells ($P>0.05$). Considerably more variable, weight by sex ranged from 4 grams to 160 grams. Both male and female show no statistical evidence that weight was affected by the phenotypic sex. However, when examined weight by treatment, we see statistical evidence that the timing of injection may affect the growth rate of the individual, regardless of sex or the presence of PKH26 dye, which was used to label oogonial stem cells and spermatagonial stem cells. Growth of common carp xenogens shows a bimodal curve with the highest growth rates centered around 23- degree days and 483-degree days at implantation. The mean survival among all the treated groups through the first 34 days was 47%, however, among groups injected during embryonic development, mean survival was 8.6%. During the grow out stage (34-545 dpf), mean survival for all treated groups was 70%. No statistical means were produced to verify this, but visually, a trend appears of the common carp injected at earlier stages having higher rates of mortality than those injected at later dates. During the grow out stage, this trend is no longer present, with mean mortality in all treatments never reaching above 49%, potentially suggesting that once past nearly 30 days in development, common carp xenogens have a mean chance of survival of 70% and the impact of early trauma is not as prevalent. At one and half years of age, xenogenic common carp males produced blue catfish sperm based upon DNA analysis of the expressed sperm.

1. Introduction

Freshwater aquaculture in the United States is primarily dominated by the production of *Ictalurid* species such as the channel catfish (*Ictalurus punctatu*s) ♀ x blue catfish (*I. furcatus*) ♂ hybrid catfish (CB) and channel catfish (*Ictalurus punctatus)*. Overall yields in 2018 were: 164.1 million kilograms of catfish, 101.4 million kilograms of crawfish, and 30.1 million kilograms of trout (NOAA, 2022). The US aquaculture sector has faced a variety of struggles since its introduction such as water consumption, strict regulation, disease control, feed expense, and animal husbandry. However, the expansion of the channel catfish industry since 1960s is an instance of one of the success stories of the aquaculture industry (Stickney 2009). Today, still facing the same challenges that all aquaculture commodities face, the catfish aquaculture industry provided 47.5 percent of U.S. freshwater fishery products by value in 2018 (NOAA, 2022).

A major change for the catfish industry was the introduction of the hybrid catfish during the late 1990s, but especially in 2005. The channel catfish female and blue catfish male (CB) hybridization has shown to provide increased growth, bacterial disease resistance, seinability, angling vulnerability, carcass yield, and low oxygen tolerance, when compared to the pure strain of their parent species (Dunham et al., 2018). Currently, interspecific hybrid catfish channel catfish ♀ × blue catfish ♂ (CB hybrid), represents approximately 70% of US catfish production (Dunham and Elaswad, 2018). However, generating this cross comes with its own unique set of challenges. Natural hybridization of the two species is rare. To overcome these reproductive obstacles, the use of carp pituitary extract (CPE) or luteinizing hormone-releasing hormone analogue (LHRHa) in addition to in vitro fertilization became the standard procedure for

inducing channel catfish females for CB hybrid embryo production (Dunham and Masser., 2012; Su et al. 2013), but now LHRHa and other GnRHs are almost exclusively utilized. This process still requires the sacrifice of the mature blue catfish male to artificially fertilize the channel catfish eggs that can be stripped from the female. This process highlights a major issue with CB hybrid production, growing blue catfish males to maturity for a one-time use during spawning.

Xenogenesis is a method of reproduction where successive generations differ from each other and no genetic material is transmitted from the parent to the offspring. Xenogenesis can be accomplished by transplanting spermatogonial stem cells (SSC), oogonial stem cells (OSC) or primordial germ cells (PGCs) from the desired diploid donor species, into the sterilized triploid host (Hettiarachchi et al., 2022). One way to potentially avoid the reproductive barrier in CB hybrid production is through xenogenesis.

Xenogenesis has been shown to be successful in a variety of species, environments, and phylogenetic distances. For instance, operational Japanese yellowtail (*S. quinqueradiata*) sperm was produced in jack mackerel (*T. japonicus*) as a result from intraperitoneal transplanted spermatogonia (Morita et al., 2015). Channel catfish have also successfully been used to host and ultimately produce blue catfish fry as a result of blue catfish stem cell transplantation (Hettiarachchi et al., 2022,). Primordial germ cells (PGCs) from zebrafish (*Danio rerio*), pearl danio (*Danio albolineatus*), goldfish (*Carassius auratus*), and loach (*Cobitidae Spp.*) have been transplanted into blastula-stage zebrafish embryos. The PCG migration toward the gonadal region of the host embryo was shown to be at comparatively high rate in all cyprinid species listed above (Saito et al., 2010). This pioneering technology would allow for the production of CB hybrids in a more simplistic fashion by mating the two xenogens together as if they were wild type diploids or stripping the male for its milt (when using a strippable male host species),

which in this case would contain haploid blue catfish gametes to be used to fertilize channel catfish eggs.

In this study, common carp (*Cyprinus carpio*) were evaluated as the host species to receive the blue catfish germ cells to produce blue catfish sperm. They may make a good host as common carp males can be readily stripped of milt. This surrogacy would allow for the common carp to serve as biological sperm factories and obviate the need to raise and ultimately cull the male blue catfish brood stock. The overall goal in this study was to evaluate the optimal time for stem cell implantation, using common carp as host for blue catfish gametes for peak proliferation and colonization.

2. Methodology

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) guidelines.

2.1. Common carp selection and triploid production

Mature common carp of both sexes were selected from various 0.04-ha earthen ponds located at Auburn University Fish Genetics Research Unit at E.W. Shell Fisheries Center in Auburn Alabama. Female common carp were chosen that had strong secondary sexual characteristics (softening of the abdomen as well as reddening and protrusion of the genital papilla), and males based on overall size (body weight and body length) as well as ease of milt secretion and obvious tubercle development on the pectoral fins and operculum. The fish in these

ponds were fed (Purina Catfish 32, floating feed; crude protein: ≥32.00%, crude fat: ≥4.00%, crude fiber: 7.00%, and phosphorus: 0.80%) 7 days per week during summer and 3 days per week during winter. The common carp were then switched from a grow out feed to a customized brood stock feed containing 36% protein, two months before brood stock collection to enhance spawning capabilities.

The selected common carp were then removed from the ponds and stocked into 1,688-liter fiberglass spawning tanks, using flow through water. The females were administered an intracoelomic injection of Ovaprim at 0.5 ml/kg (Ovaprim™, Ferndale, WA). The females were then placed into spawning bags and secured to the tank and were checked every three hours between 12 and 24 hours after injection. The males were kept in the same tank as the females but were separated by a partition downstream from the females to avoid early fertilization. Once eggs were noticed on the bag, the females were anesthetized in an 18 L bucket with 100 ppm tricaine methane sulfonate (MS-222, Ferndale, WA) buffered with sodium bicarbonate to a neutral pH. Once sedated, the common carp was then removed, dried, and eggs were stripped onto metal spawning pans coated in vegetable oil (~25 g of eggs/pan). After egg collection, males were selected and identically anesthetized, dried, and milt was stripped into plastic 50 milliliter tubes. Three pairs were made and induced with Ovaprim, however, only one female and male were ultimately used.

After allowing for 5 minutes gamete contact time, Fullers' earth solution was added to the sperm-egg mixture to start the fertilization process and remove any potential egg adhesion. The Fullers' earth solution was prepared by mixing 6 g of Fullers' earth powder (Starwest Botanicals, Sacramento, CA) into 1 L of reservoir water. During the gamete contact time, the fertilizing eggs were then moved into a cylindrical pressure chamber (340 mm height, 70 mm diameter), which

was then placed onto a Carver press (Carver, Inc., Wabash, IN). At 5 minutes post-fertilization, the embryos were subjected to a pressure shock of 8,000 psi for 5 minutes to induce triploidy. After the pressure shock, eggs were moved to a 450 L hatching trough of flowthrough water supplemented with 50 ppm of CaCl2 for water hardening (1 hour). After hardening, embryos were then moved 5 L tubs of Holtfreters solution (NaCl 3.46g, KCl 0.05g, CaCl2 0.1g, NaHCO3 0.2g per liter) in water baths ranging from 22-24 °C (due to daily fluctuation of flowthrough temperatures). Embryos and larvae remained in Holtfreters solution for 28 days and were then moved to 10L glass aquaria on an RAS system. Survival is potentially one of the most crucial metrics of success and was measured in two major life stages of this project.

2.2. Gonad preparation and stem cell isolation

Sexually immature blue catfish were selected based on size, described by Hettiarachchi et al. 2020. The donor blue catfish were then euthanized and sterilized with 70% ethanol. All tools used in the procedure were also sterilized with 70% ethanol prior to the initial incision. Following the methods of Shang et al. (2015), the ovaries and testes were removed from the coelomic cavity, avoiding contamination from blood vessels, connective tissues, and the peritoneum. Gonadal weights were recorded and the gonads were placed in 100 mm × 15 mm sterile petri dishes containing 5 mL of Hanks' Balanced Salt Solution [(HBSS, SH30031.03, GE Healthcare Life Sciences) supplemented with 1.0 μg/mL NaHCO3 (Church & Dwight Co., NG) and 100 unit/mL penicillin - streptomycin (I15140–122, Life Technologies)] which were ultimately transferred to a biosafety cabinet for cleaning and sterilization. Using sterile scalpel blades, any connective tissue and blood cells were removed. The gonads were then rinsed three times with 1 mL of anti-agent medium and soaked in a 5 mL 0.5% bleach solution prepared with

double-distilled H_2O for two minutes. After the soak was completed, three rinses of HBSS and phosphate-buffered saline (PBS; J62692, Alfa Aesar) were completed respectively. Gonads were then macerated with a sterile scalpel blade and moved to a 50 mL autoclaved glass flask with stir bar and 0.25% trypsin - ethylenediamine tetraacetic acid (EDTA; 25200–072, Life Technologies) was added at 50 times of the weight of the gonads. Samples were then incubated on ice for 30 minutes, and afterwards samples were brought to 22 °C on a hotplate with a magnetic stirrer to attain higher digestion effectiveness. The cell suspension was then filtered through a 40 μm cell strainer with nylon mesh (352340, VWR International) and centrifuged at 500g (Eppendorf Centrifuge 5418 R) for 20 min to separate cells from trypsin, and supernatant was discarded. The pellet was then resuspended in 2 mL of Dulbecco's Modified Eagle's Medium/DMEM [DMEM (10–090-CV, Corning cellgro,) supplemented with 10% fetal bovine serum (FBS; 10438018, Life Technologies), 100 unit/mL penicillin - streptomycin (15140-122, Life Technologies), and 200 mM l-glutamine (A2916801, Life Technologies) to offer a complimentary environment for the cells. Next, 5 μL of cell suspension was gradually hybridized with 45 μL of 0.4% trypan blue (15250061, Life Technologies). Then 10 uL of the cells were examined under an Olympus objective microscope (BH2), equipped with a 20× objective, to determine the total number of both live and dead cells, total number of live and dead SSCs, and total number of live and dead OSCs using of a Neubauer hemocytometer. Cell count was performed in four corners (1 mm^2 each area) of the hemocytometer, and number of cells in 1mL was computed according to Louis and Siegel (2011) with the dilution factor of 2 (cell suspension: trypan blue with 1:1 ratio). This process was repeated two additional times to ensure cell concentration where the total number of cells per mL = average number of cells in the four squares (each 1 mm2) × dilution factor ×

10,000. This process was repeated from day 0 (fertilization) through day 27 post fertilization, allowing for fresh stem cells to be used for each replicate.

2.3. Stem cell labelling and implantation into the triploid common carp host

Half of the freshly isolated stem cells were then labeled with PKH26 red fluorescence cell linker (CGLDIL, Sigma-Aldrich, St. Louis, MO) following manufacturer's instructions and the other half were unlabeled. Common carp embryos and fry were injected (PKH26 and no dye) every other day for 27 days (fry around 50 per replicate, embryos roughly 500 per replicate). Glass capillary tubes were heated and pulled into a needle with a Flaming / Brown micropipette puller (model P-97). Then 10 μL was 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 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. Individual 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. Each embryo and fry were injected with roughly l μL of cell suspension. The embryos were injected within the cell body to avoid disrupting the yolk and for better embryo uptake, and larvae were injected near the genital papilla anticipating cells to potentially migrate and colonize the gonadal ridge. After injection, the embryos and fry were placed back into Holtfreters solution. The application of PKH26 dye and no dye to the undifferentiated stem cells to be injected was also to be examined

2.4. Primer testing, detection limits, and PCR analysis

Fish were sampled randomly from each treatment for PCR analysis. The 18-month-old common carp were sacrificed by pithing and gonads surgically removed. The separated gonad samples were placed into 1.5 mL Eppendorf tubes on ice and then held at −80 °C until DNA extraction. DNA was extracted using proteinase K digestion followed by protein and ethanol precipitation (Waldbieser and Bosworth, 2008). DNA concentrations were brought to 500 ng/µL using ultra purified water and measured with a NanoDrop® 2000 spectrophotometer (Thermo Fisher Scientific). Thermocycling was done in a Bio-Rad T100-thermalcycler, and electrophoresis was performed in a Labnet ENDURO™ Gel XL E0160 Electrophoresis System for 30 minutes in a 1% TAE buffer. Gels were read using a Biorad GelDoc™ XR+ molecular imager, and the ImageLab software.

Five different primers, four being designed around mitochondrial DNA and one based on a secretory calcium-binding phosphoprotein (SCPP) gene family gene found in some ray-finned fishes (SCPP1, Lemopoulos and Montoya-Burgos, 2021), were tested through PCR to identify the most reliable sequence for detecting blue catfish DNA within common carp samples. After testing all primers together on pure blue catfish and pure common carp DNA, SCPP1 was chosen for the lack of banding in common carp DNA. Cycle number, annealing temperature, and detection limits were all tested to identify any limitations in recognition of blue catfish DNA signals. The annealing temperature was 60°C and 30 cycles were needed to adequately provide banding. We were able to detect blue catfish DNA at a concentration of 3.75 ng/µL(lowest tested), diluted with common carp DNA. Success of blue catfish stem cell colonization in the common carp hosts was determined by the confirmation of the presence of blue catfish DNA

fragments using polymerase chain reactions. Samples tested consisted of surgically removed gonadal material and fin clips.

2.5. Sperm collection

Extraction of sperm from the common carp xenogens and control common carp were initiated by an injection of human chorionic gonadotropin (HCG) marketed as Chorulon from Intervet, Inc. HCG was administered at 400 international units per kilogram body weight 24 hours before sampling. The following day, sampling was done one of two ways. Initially, a hand strip was attempted on the males and was collected by a micropipette and stored in an Eppendorf tube. If no milt was given by the fish, testes were surgically removed from the coelomic cavity upon sacrificing the male.

2.6. PKH26 analysis

Fingerling common carp suspected to be colonized by the injected, PKH26 labeled blue catfish stem cells were randomly chosen from various replicates for PKH26 analysis. Fish were euthanized through MS-222 overdose and gonads were subsequently collected using aseptic techniques with sterilized instruments. Gonadal tissue was macerated and placed on a sterile glass microscope slide. After slide preparation, images were taken using a Zeiss Imager A2 microscope equipped with a camera (Axio-cam 202) and Zen Pro v.6.1 software (Zeiss, Oberkochen, Germany).

2.7. Statistical analysis

All data were analyzed using GraphPad Prism 10 statistical analysis software (v.10.0.3; GraphPad Software Inc., Boston, MA, USA). After the PCR analysis, logistical calculations were performed to elucidate success rate based on the number of fish sampled and number of positives. Un-paired t-tests were used to compare total body weight (grams) of triploid common carp xenogens which were injected with blue catfish stem cells, comparing positive and negative xenogens. A Chi-square assessment of the percent of positive common carp hosts based on the presence of blue catfish DNA separated by the timing of injection was performed. Fisher's exact test was used to describe the relationship between the sex of common carp hosts and their readiness to be colonized by blue catfish stem cells, compared between all fish tested regardless of the time of injection. A T-test with Welch's correction was used to demonstrate the relationship between the sex of the common carp xenogen hosts and their total body weight. Survival during two time periods were analyzed using Chi-square analyses, the injection and incubation period while in the 5 L tubs of Holtfreters solution (4/12/21 – 5/16/21) and the grow out period while stocked in 10 L RAS glass aquaria (5/16/21-11/14/22). Survival was compared among no dye and PKH for both time periods (ND: no fluorescent dye added to stem cells and PKH: blue catfish stem cells labeled with PKH26 dye). Alpha was set to 0.05.

3.0 Results

3.1 Rate of xenogenesis

Overall, positive xenogen samples containing blue catfish DNA were seen at each time point with the highest percent of positive samples around 0 to 46-degree days and 483-575

degree days, averaging 62.3% and 56.7% success respectively (Table 1). Although no statistically significant results were seen, visual trends can be seen, and a bi-modal curve is present **(**Fig. 1**)**. The distribution is observed around early and later injections, with the highest percent achieved being 70% in the 483-degree days class and lowest percent in 391-degree days with 7% positive (Fig. 1). This trend may represent that both early and late-stage implantations may have various advantages and disadvantages, with respect to the xenogen's ability to be colonized. Results from the PCR tests indicated positives in gonad samples, fin samples, and both or neither.

Table 1. Percentage of putative triploid common carp (*Cyprinus carpio)* containing blue catfish (*Ictalurus furcatus*) DNA as confirmed by PCR of SCPP1 marker in blue catfish, in gonad, fin, or both at 581 days post fertilization after being injected with a blue catfish spermatagonial stem cells and oogonial stem cells 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 host common carp.

Degree days of development when injected	N	Positive in Gonad Only	Positive in Fin Only	Positive in Both	Total % Positive in Gonad
0	15	7 (46%)	1 (7%)	2 (13%)	60
23	10	2 (20%)	1 (10%)	3 (30%)	50
46	5	2 (40%)	0 (0%)	1 (20%)	60
161	10	0 (0%)	0 (0%)	1 (10%)	10
207	4	1 (25%)	0 (0%)	1 (25%)	50
253	10	1 (10%)	6 (6%)	0 (0%)	10
299	10	3 (30%)	1 (10%)	0 (0%)	30
322	20	2 (10%)	2 (10%)	2 (10%)	20
345	12	3 (25%)	0 (0%)	0 (0%)	25
391	15	1 (7%)	2 (13%)	0 (0%)	7
483	10	6 (60%)	0 (0%)	1 (10%)	70
529	10	5 (50%)	0 (0%)	0 (0%)	50
575	10	5 (50%)	0 (0%)	0 (0%)	50
621	11	4 (36%)	0 (0%)	0 (0%)	36

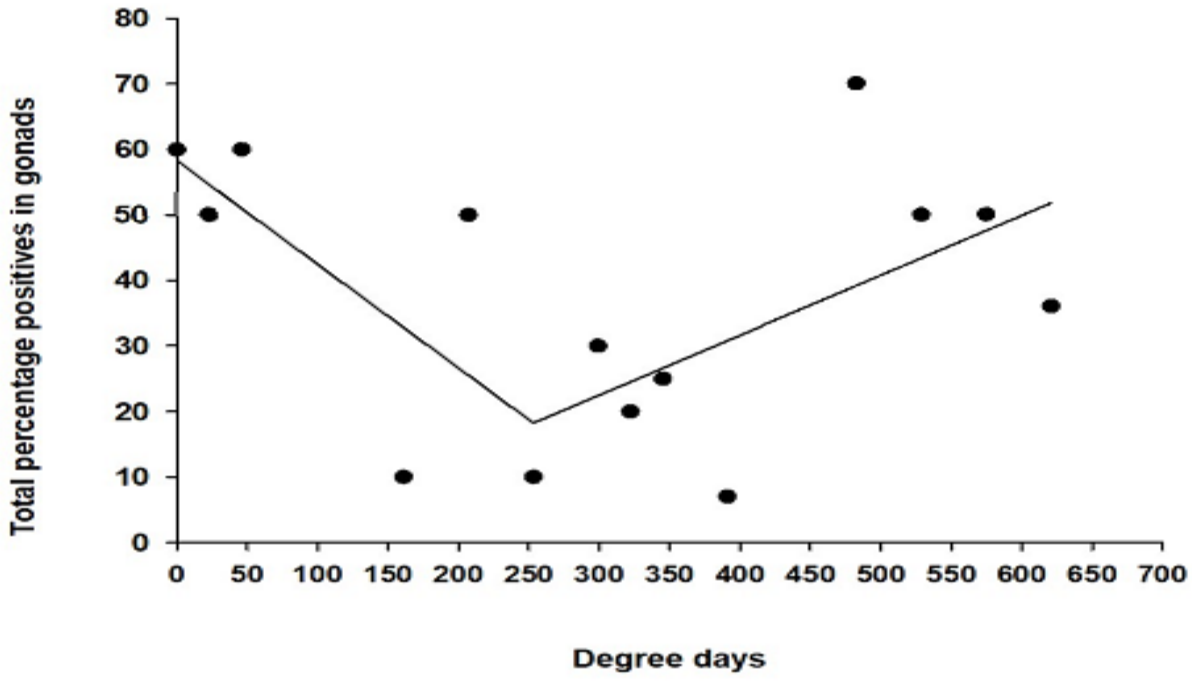

Figure 1. Percentage of xenogeneic common carp (*Cyprinus carpio*) hosting blue catfish DNA in gonad, or both gonad and fin at final sampling (581 days post fertilization), when injected with a mixture of SCCs and OSGs extracted from blue catfish (*Ictalurus furcatus*). Embryos and fry were injected from 0-degree days to 621-degree days after fertilization. A sample was considered positive when an at least an ovary or testes from a common carp germline had identical banding to a gonad sample from a blue catfish based on PCR of the SCPP1 gene in blue catfish. Highest percent number of positive samples came from injection blue catfish stem cells at 483-degree days (70%), and the lowest percentage of positive samples was seen at 391-degree days (7%). (Breakpoint analysis, $R^2 = 0.437$)

3.2 Sex effect on colonization

Genetic or phenotypic display sex had no effect on the individual's ability to host blue catfish gametes. Phenotypic variation was large as the largest common carp weighed 150 grams while the smallest was less than 7.5 grams (Fig. 2). Percentage of male and female xenogens did not differ (P>0.05, Fig. 2).

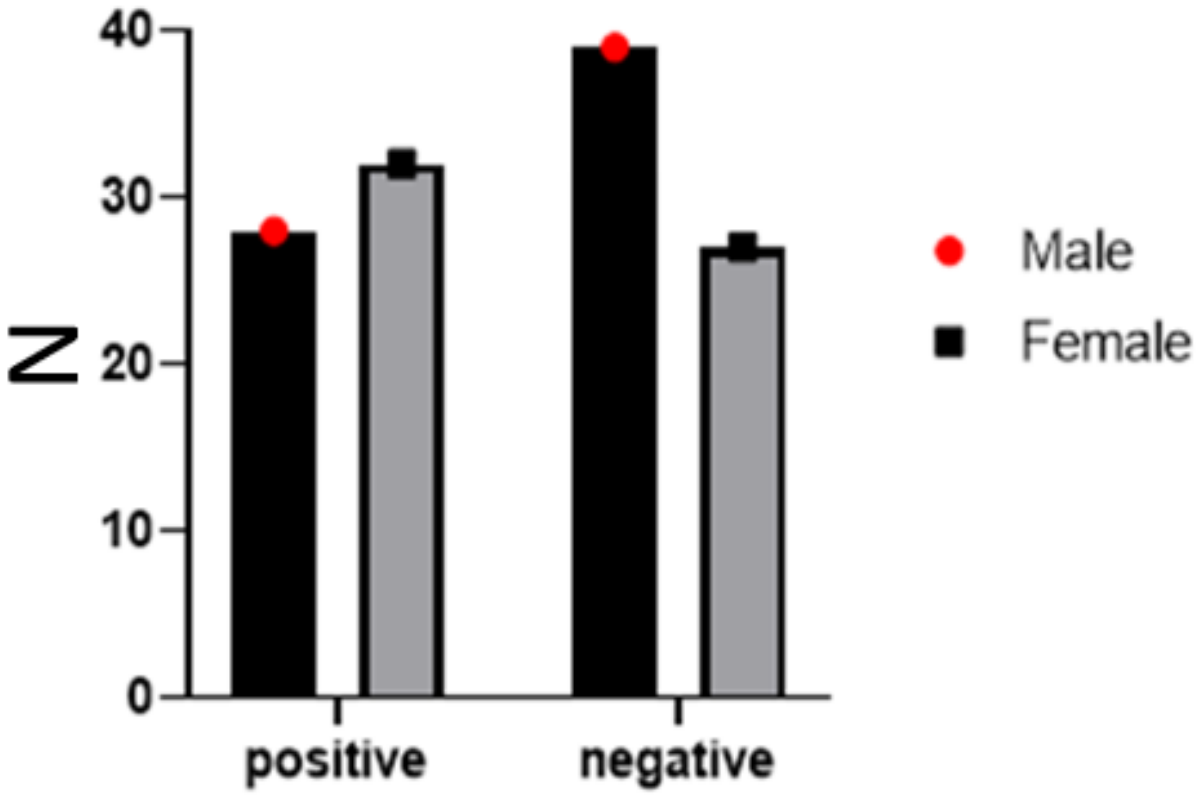

Figure 2. Relationship between the sex of common carp (*Cyprinus carpio*) xenogen hosts and their colonization by blue catfish (*Ictalurus furcatus*) stem cells. (Fisher's exact test: P>0.05).

3.3 Growth

Regarding overall weight of common carp xenogens and controls, we found no significant relationship between weights of fish that were colonized by blue catfish stem cells (positive) and fish that were not (negative and 2N/3N controls). However, when split among the various timepoints of stem cell injection, some significant relationships were identified. Top performers in terms of weight at the final sampling at 581 days post fertilization being 23 and 575-degree days (61 g, 49 g). Notably, the triploid and diploid controls total weight were 41.53 grams and 19.06 grams, reinforcing the ability of triploid common carp to out preform diploid common carp in terms of physical growth. Sex was also examined to see if it was a factor in the individual's total weight. Mean body weight of positive common carp xenogens was 35.43 g and that of negative xenogens was 33.38 grams, which were not different ($P > 0.05$).

3.4 Survival

For no dye injection and incubation (34 dpf) survival, the highest percent survival was at 391-degree days and 2N (diploid) control with 100% survival and lowest percent survival was at 253 and 437-degree days at 0% survival (Fig. 3). For survival through injection and incubation of common carp injected with PKH, highest percent survival was at 345-degree days and with 93 % survival and lowest percent survival at 69 and 437-degree days with 0% (Fig. 4). When these two treatments (PKH and no dye) are combined, percent survival was highest in 345-degree days and 2N controls, 95 and 100 percent, respectively, and the lowest observed in 69 and 207-degree days, 0% survival (Fig. 5). However, no statistically significant differences ($P> 0.05$) were observed but, a trend develops in mid to late injection timing in development with higher relative

survival around 345 and 529-degree days (Fig. 5). The next stage of development that was examined was grow out (5/16/21-11/14/22), and it consisted of the time spent in RAS 10L glass aquaria. When examining survival for common carp injected with un-dyed stem cells through the grow out stage, 46, 207, and 621-degree days had the highest percent survival at 100 percent and the lowest were at 299 and 322-degree days with 46 and 39 percent survival respectively (Fig. 6). For common carp injected with dyed stem cells, the 253 and 391-degree day injections both had a percent survival of 100 while 483 and 565-degree days had the lowest percent survival of 37 and 50 percent, respectively (Fig. 7). When both dyed and un-dyed cells are combined for total survival through the grow out stage, 621 and 161-degree days demonstrated the highest percent survival at 95 and 88 percent, respectively, while 2N control and 483-degree days had the lowest percent survival of 51% (Fig. 8).

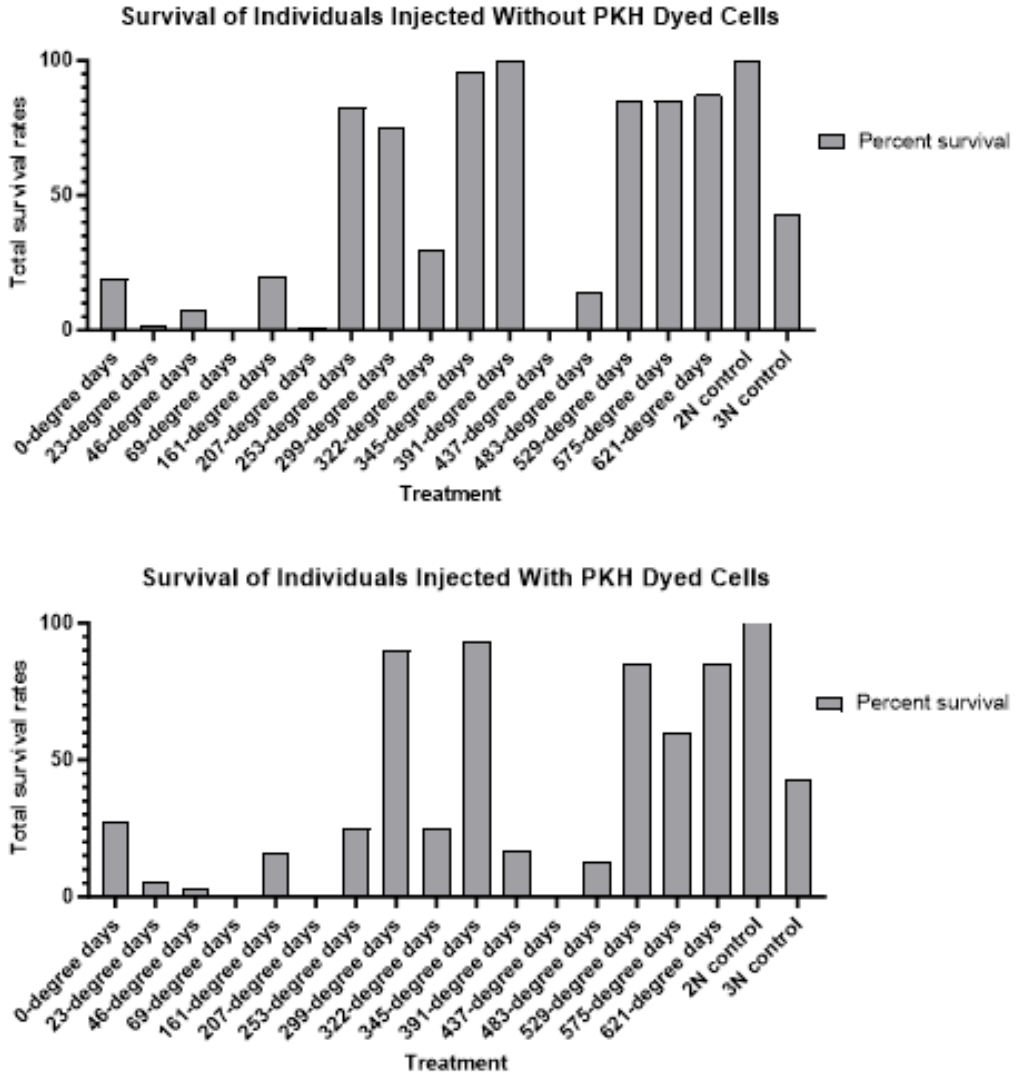

Figure 3. Survival of common carp (*Cyprinus carpio*) receiving blue catfish (*Ictalurus furcatus*) stem cells with and without PKH26 dye at 34 days post-fertilization that had been injected at different time points. Highest percent survival among the no dye group was at 345-degree days and 2N control with 93 and 100% and lowest percent survival at 69 and 437-degree days with 0%. Highest percent survival among the PKH26 group was at 345-degree days, 93%, and lowest percent survival at 69 and 437-degree days with 0%.

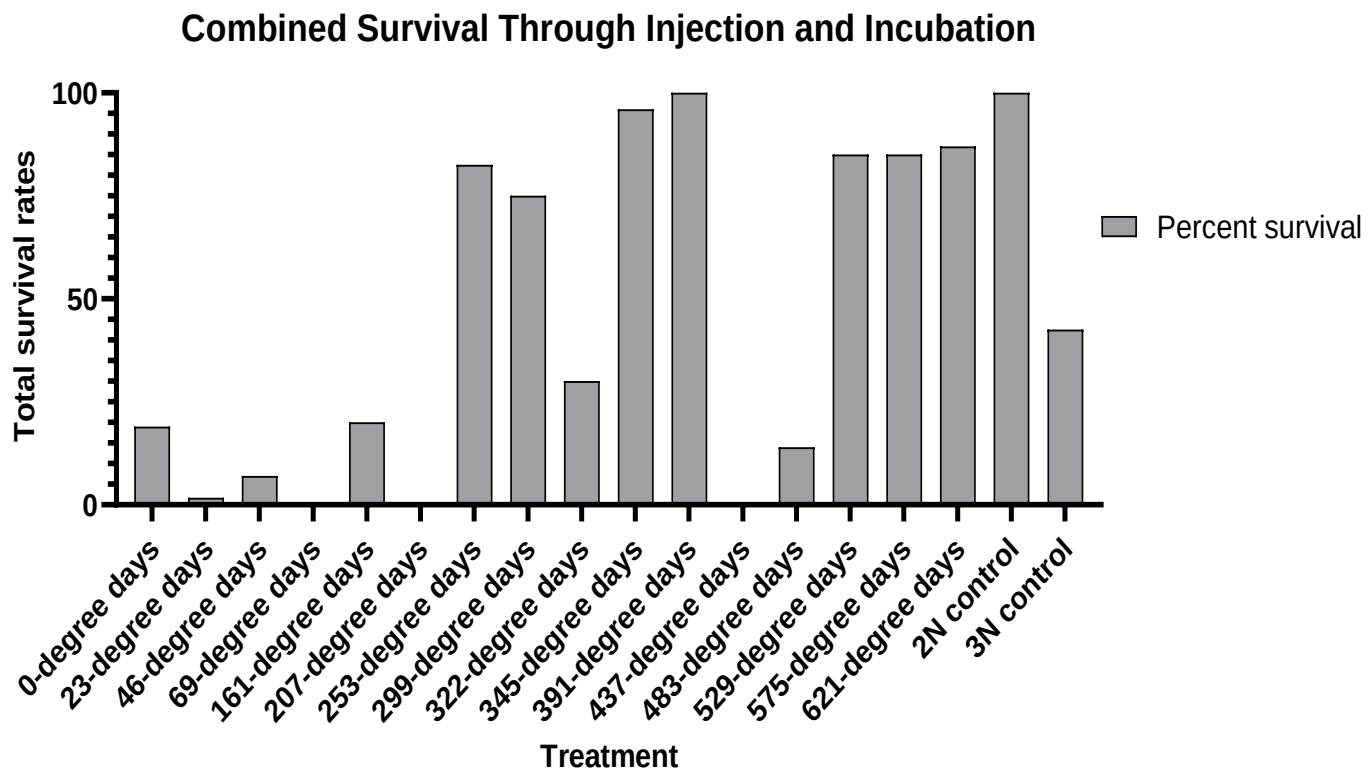

Figure 4. Survival of common carp (*Cyprinus carpio*) receiving blue catfish (*Ictalurus furcatus*) stem cells with and without PKH26 dye at 34 days post-fertilization that had been injected at different time points through the injection and incubation period (4/12/21 – 5/16/21). Overall, percent survival was highest in 345-degree days and 2N controls at 95 and 100 percent survival.

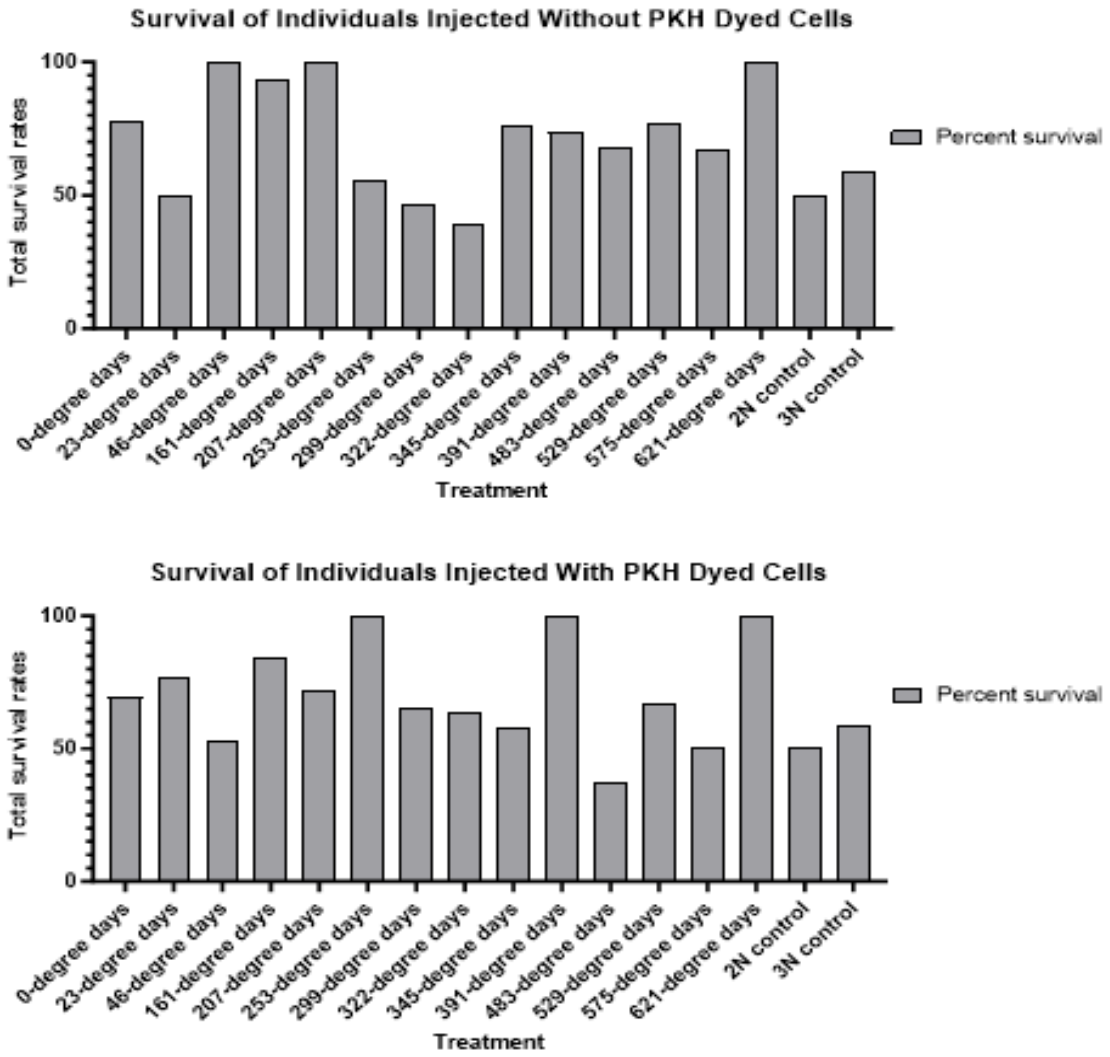

Figure 5. Survival of common carp (*Cyprinus carpio*) receiving blue catfish (*Ictalurus furcatus*) stem cells with and without PKH26 dye from 34 days to 454 days post-fertilization that had been injected at different time points. During the grow out period of no dye stem cell injected fish at 207 and 621-degree days had the highest percent survival. The lowest no dye stem cell injected common carp were at 299 and 322-degree days with 46 and 39 percent survival, respectively. The highest percent survival of the PKH26 injected common carp was the 253 and 391-degree day injections, both having 100 percent survival, while 483 and 575-degree days had the lowest percent survival of 37 and 50% respectively.

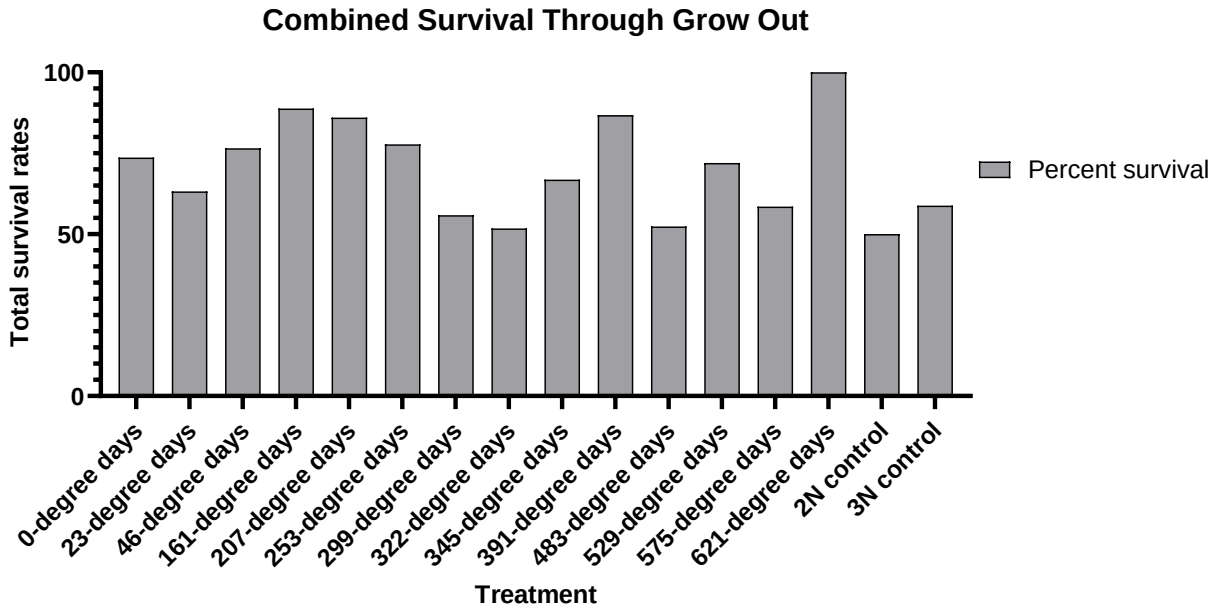

Figure 6. Survival of common carp (*Cyprinus carpio*) receiving blue catfish (*Ictalurus furcatus*) stem cells with and without PKH dye from 34 days to 454 days post-fertilization that had been injected at different time points. Fish injected at 621 and 161-degree days demonstrated the highest percent survival at 95 and 88%, respectively. While 2N control and 483-degree days had the lowest percent survival of 51%.

3.5 Xenogenesis confirmation

Samples were considered positive if gonad material tested positive for containing blue catfish DNA. In total, 152 potential xenogens were sampled and 57 of them were confirmed positive through PCR, giving a total 37.5% success rate of common carp hosting blue catfish (Table 1). Table 2 describes the forward and reverse SCPP1primer used for pcr confirmation and the respective amplicon size. The results of the PCR analysis are shown in figure 6. PKH analysis demonstrated that the blue catfish stem cells had colonized the common carp gonad and were proliferating (Fig. 7)

Table 2. The primer [SCPP1 (secretory calcium-binding phosphoproteins)] 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

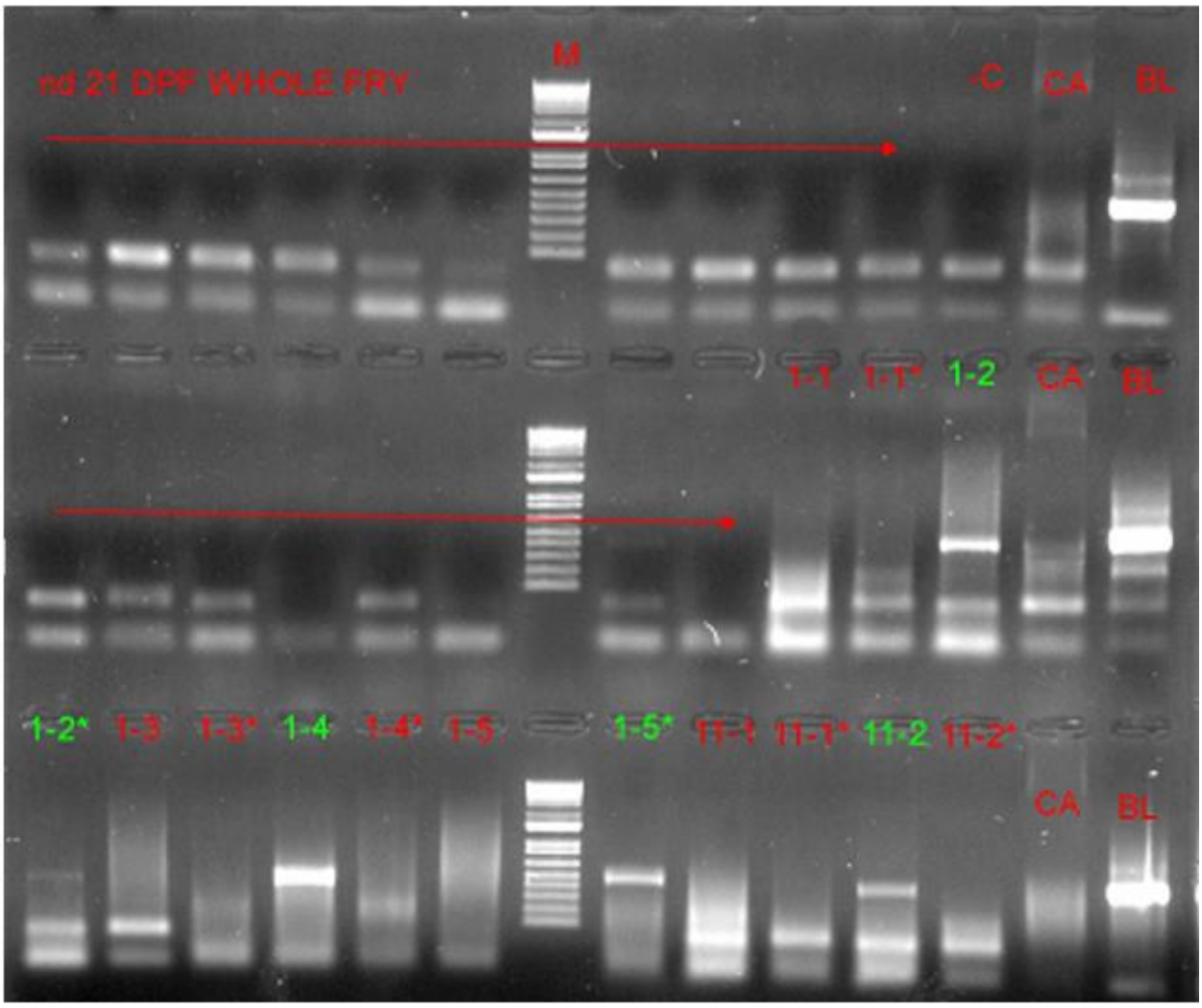

Figure 6. Example of PCR results when testing gonad and fin samples from suspected common carp (*Cyprinus carpio*) xenogens harboring blue catfish (*Ictalurus furcatus*) DNA. Labels in green are samples with banding patterns identical to that of the blue catfish control (BL), and labels in red are samples that had no identical banding and 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.

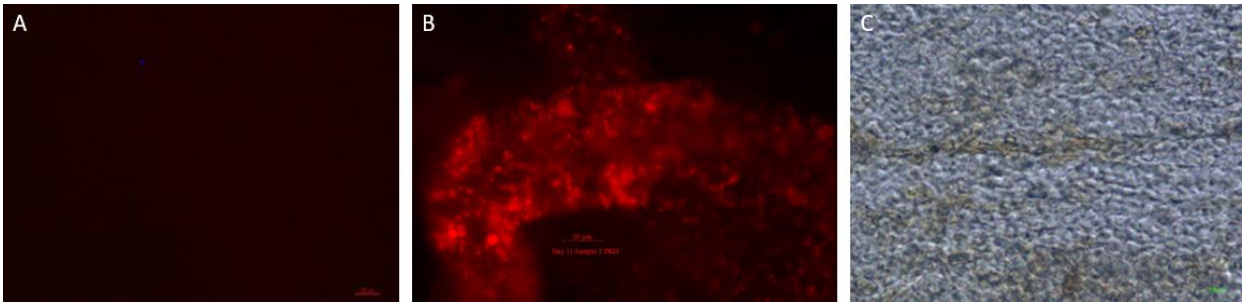

Figure 7. Common carp (*Cyprinus carpio*) control and common carp xenogenic reproductive tissues evaluated for fluorescence from PKH26 dyed blue catfish (*Ictaluras furcatus*) injected stem cells. Control triploid common carp at 45 days post fertilization showing no fluorescence (A). Common carp injected at 253-degree days, exhibiting fluorescence (B) and the corresponding brightfield view of the same section of tissue (C). The injected fish were 45 days post fertilization at the time of this image. Scale bars are set at 20 μm.

3.6 Gonad morphology and sperm production

Testes and ovaries of the putative xenogenic common carp had the appearance of normal common carp gonads when the fish were 454 days old. Some were well developed and others were atrophied (Figs. 8,9). Ova had the appearance of common carp ova or possibly that of immature catfish ova.

Of 76 males tested, 64 had sperm and 24 (42.5%) tested positive for blue catfish DNA. Testes of the other 12 males were tested for blue catfish DNA, and 41.7% were positive. A total of 76 total females were evaluated, 60 had eggs, 20 (33.3%) tested positive for blue catfish DNA. The ovarian tissue from 16 females that had no discernable eggs were analyzed, 5 tested positive for blue catfish DNA.

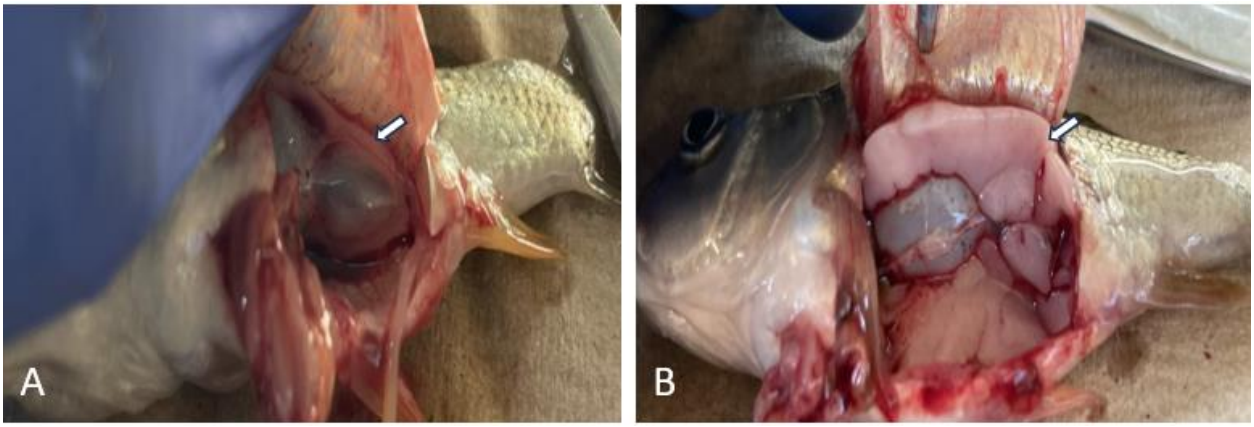

Figure 8. Morphology of testes of putative male xenogen common carp (*Cyprinus carpio*) producing blue catfish (*Ictalurus furcatus*) sperm. (A) underdeveloped testes, (B) further developed and nearly mature testes filled with sperm. Both fish were 454 days post fertilization.

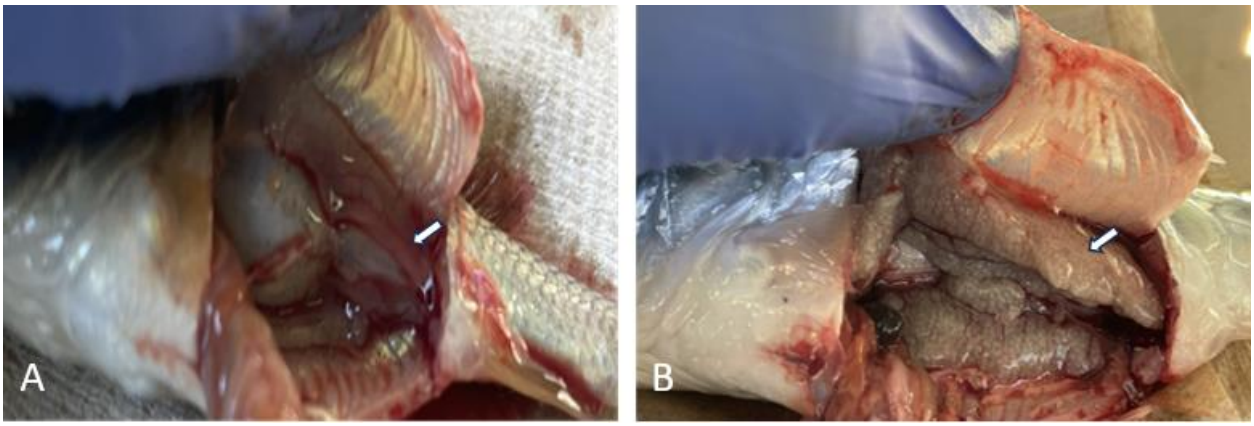

Figure 9. Morphology of ovaries of putative female xenogen common carp (*Cyprinus carpio*) producing blue catfish (*Ictalurus furcatus*) ova. (A) underdeveloped ovaries, (B) further developed and nearly mature ovaries filled with underripe eggs. Both fish were 454 days post fertilization.

4.0 Discussion

Xenogenic common carp were produced that contained blue catfish cells in their gonads based on the presence of DNA markers and the presence of blood catfish cells that had been fluorescently labeled. Based on DNA analysis of resulting sperm and eggs, the xenogenic common carp were capable of making blue catfish gametes. Some of the injected xenogenic stem cells migrated to the fin rather than the genital ridge. This type of mosaicism has never been reported in xenogenic fish. Injection at different points in development influenced rate of xenogenesis and survival. Rate of xenogenesis was the same in male and female embryos. The developmental times best for colonization of donor stem cells was not correlated between male and female common carp embryos and fry.

The success of a host species carrying gametic cells of a separate species have been documented in a wide variety of species and in an assortment of environments (Hettiarachchi et al., 2022; Morita et al., 2015; Saito et al, 2008; Pšenička et al., 2015). This process of xenogenesis has the potential to circumvent the need for the sacrifice of male blue catfish in the production of CB hybrid catfish. In fact, two generations of xenogenesis can eliminate the need for blue catfish brood stock as the gametes, particularly sperm, can be perpetuated in the common carp xenogenic host. CB hybrid catfish have been successfully produced with the use of control channel catfish females and xenogenic male channel catfish hosting blue stem cells (Hettiarachchi et al., 2023). The advantage to this system is that hand stripping can be avoided. The advantage of using common carp as a blue catfish sperm factory, is the copious amounts of strippable sperm produced, making the artificial fertilization technology more efficient and reducing the space needed for brood stock development as catfish males would no longer be

needed. Theoretically, common carp can produce much more sperm on a smaller footprint than catfish males.

The best treatments in the current study had a 50-100% xenogenesis rate. This is a better success rate than that of Franěk et al., (2019) when transplanting spermatogonia of common carp into goldfish, which achieve 40% success. For salmonid xenogenesis, a success rate of 30 to 50% was achieved among injected host embryos (Okutsu et al., 2007). However, success rates as high as 80% have been reported in channel catfish hosting blue catfish gametes (Hettiarachchi et al., 2022).

Injecting at the critical developmental stage has a large effect on colonization and proliferation of the stem cells. Two ranges of peak stem cell colonization were found between 0 to 46-degree days and 483 to 575-degree days, where successful detection of blue catfish DNA is 62.3% and 56.67% success respectively. These results indicate that there exist two critical injection ranges, early development (23 to 69-degree days) and later development (483 to 621-degree days). Common carp were unable to reject allografts before 2 weeks post fertilization (Zapata et al., 2006). Thus, the early spike in acceptance is potentially caused by the diminished immune response from embryonic or freshly hatched larval common carp. The later increase in success is potentially related to the relative ease and precision of injecting larger fry directly unto the gonadal ridge, and the innate ability to be injected without being subjected to mechanical damage from the injection process. However, immunity may not be involved at all as juvenile and adult diploid and triploid pejerrey, tilapia and channel catfish have been made xenogenic with donor stem cells colonizing the gonad (Majhi et al. 2009, Lacerda et al. 2012, Perera et al. 2017.

Growth and sex of common carp xenogens was also examined. Sex of the host did not affect the colonization of blue catfish stem cells in common carp hosts. However, there was no significant correlation between the two sexes in regard to the overall peaks of colonization. Timing for optimum colonization may be affected by sex. This is logical as timing for sex determination varies between sexes in catfish (Wang et al. 2022) and common carp (Komen et al. 1992.

Body weight ranged from 4 grams to 160 grams and at this stage sexual dimorphism was yet to emerge. Timing of injection affected growth rate. There was a bimodal curve with the highest growth rates centered around individuals injected with stem cells 23- degree days and 483-degree days after fertilization. Additionally, triploid common carp usually grow slower than diploids through our experimental timeline (Basavaraju et al., 2002). Mean body weight of 1-year-old triploid common carp was about 85% of that of control diploids (Cherfas et al., 1994). However, triploid common carp grow faster to food size when compared to the diploid common carp (Cai et al., 2021). The triploid common carp controls out preformed diploid common carp controls to approximately 30g in the current study. Both diploid and triploid non-injected controls grew slower than the highest preforming injected fish.

For the efficient production of commercial sized xenogenic brood stock populations, both rate of xenogenesis and survival are key. Hatch and early survival are potentially impacted by the process of hydrostatic shock for triploidy, anesthetization prior to injection, and the injection of the stem cells. Initial 34-day hatch/survival of the 2N and 3N common carp controls was 100 % and 60%, respectively. Large mortality occurred during the first 34-day period. As expected, survival after this period was much higher.

The primary goal of this study was to create biological sperm factories for blue catfish sperm. Triploid common carp hosting blue catfish gonads produced blue catfish sperm. The xenogenic common carp were one and a half years of age, 6 months shy of the typically accepted standard of two years to reach sexual maturity (Wang et. al., 2023).

In conclusion, the timeline for optimal colonization of blue catfish stem cells within common carp lies in early embryonic development and later stages of fry development. Injection of embryos is relatively easier than that of a newly hatched fry, but mortality is very high at both these stages. Handling and injection precision were likely highest at those later stages of development and some of these time points appear optimum for mass production of xenogenic common carp. This technology has potential to greatly improve the catfish aquaculture industry by reducing the cost of producing CB hybrids. The next critical step is to confirm that the blue catfish sperm produced by xenogenic common carp can fertilize catfish eggs, and fully grown and mature xenogenic males can produce large numbers of viable sperm. This technology has also been shown to be successful in other species, and could have implications in other industries as well, such as conservation and the aquarium trade.

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