Genetic Biotechnology to Improve Reproduction of North American Catfish for Aquaculture, Genetic Enhancement and Genetic Conservation

By

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the requirements for the
Degree of
Doctor of Philosophy

Auburn, Alabama
December 14, 2024

Keywords: Xenogenesis, Spermatogonial Stem Cell Type A, Hybrid Catfish, Common Carp, CRISPR/Cas9, Transgenesis

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Abstract

Xenogenesis is an emerging technology for hybrid catfish production using primordial germ cells (PGCs), spermatogonial stem cells (SSCs), or oogonia stem cells (OSCs) transplanted to a sterile host species. The present study investigated the recovery of spermatogonial stem cells through short-term culture before transplantation using various incubation conditions. Stem cell research is a rapidly growing area that has the potential to generate therapeutic drugs to treat diseases as well as study disease progression from the beginning for humans. Many of the same techniques apply to human pluripotent stem cell culture as they do to normal mammalian cell culture. However, maintaining the undifferentiated state of human pluripotent stem cells (hPSCs) requires extra considerations to ensure that the cells keep their key traits of self-renewal and pluripotency. Such information is not available for blue catfish, *Ictalurus furcatus*, to enable catfish xenogenesis research. SSCs were extracted and isolated from the immature gonads of blue catfish. The maintenance of spermatogonial stem cells was investigated in this work used short-term culture prior to transplantation. The SSCs were incubated with and without 5% CO_2 at 24-30°C temperature range and with 0-75mM or ROCK I. Spermatogonia produced in vitro discovered to be the best treatment at 30°C, with 50-75 mM ROCK I with 5% CO_2 ($p=0.001$) for 72 hours.

The timing of implantation and the number of transplanted cells were varied in order to optimize the selection of channel catfish (*Ictalurus punctatus*) fry and to evaluate the proliferation of donor cells into functional gonadal tissues. Xenogenic progeny were successfully produced in channel catfish when donor cells were implanted between 3 and 5 days post-hatch. Further experiments aimed to assess the efficiency of germ cell transplantation by introducing

spermatogonial germ cells from blue catfish and channel catfish into sterile common carp fry, resulting in xenogenic common carp capable of producing channel or blue catfish gametes. The most effective injection window was determined to be between 15 and 25 days post-hatch.

The effects of hormonal treatments on spawning success and reproductive performance in CRISPR/Cas9-generated melanocortin-4 receptor (*mc4r*) knockout channel catfish (*Ictalurus punctatus*) were investigated. Varying hormonal regimens, including luteinizing hormone-releasing hormone analog (LHRHa) and human chorionic gonadotropin (HCG), were evaluated for their impact on spawning rates, relative fecundity, hatch rates, and fry yield per kilogram of female body weight. Results demonstrated that HCG was crucial for successful spawning in *mc4r* mutants, with spawning failure observed in its absence, despite the presence of pronounced secondary sexual traits. The combination of HCG and LHRHa significantly enhanced reproductive outcomes, with *mc4r* x *mc4r*, pairings exhibiting fecundity and hatch rates comparable to wild-type controls under optimized hormonal protocols.

Dedication

This dissertation work is dedicated to, in honor of and in memory of my mother, Karen D. Alston, who is my motivation. She taught me that "I can do all things through Christ who strengthens me." Philippians 4:13. I am truly thankful for having her in my life. This work is also dedicated to my sister, Taylor P. Alston, who have always loved me unconditionally, and whose good example taught me to work hard for the things that I aspire to achieve. Lastly to my husband, Tobias T. Donnell, who was up with me with the sunrise and out late in the evening supporting me throughout this entire process.

Acknowledgments

First and foremost, I want to express my gratitude to Dr. Rex Dunham, my adviser, for his guidance, advice, and support throughout my PhD program. Dr. Moss, Dr. Butts, and Dr. Chen, members of my committee, deserve special gratitude for their advice and comments on my work. Again, a special thanks to Dr. Ian Butts for his guidance on statistical analysis. I would like to thank all my colleagues in Dr. Dunham's lab, both past and current. To all of those who helped me achieve this goal I am deeply thankful to have you in my life.

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Overview

Catfish farming has become a cornerstone of U.S. aquaculture, thriving in the southeastern United States due to its favorable environmental conditions and strategic advancements. Beginning with the historical and ecological importance of channel catfish (*Ictalurus punctatus*), this section highlights technological innovations such as floating feed pellets and water quality management practices that have enhanced the industry's efficiency and sustainability. Genetic breakthroughs, including hybrid catfish production through artificial fertilization techniques, have played a key role in significantly boosting productivity. These advancements, alongside the influence of government programs, economic pressures, and international competition, reflect the intricate connections between science, policy, and economics that support the industry.

From channel catfish, the focus shifts to common carp (*Cyprinus carpio*), a species that has become both a valuable resource in aquaculture and a significant ecological challenge as an invasive species. Its adaptability and reproductive capacity have driven advancements in production methods, including controlled spawning and hatchery practices. At the same time, the need to mitigate its ecological impact highlights the ongoing balance between maximizing aquaculture yields and protecting native ecosystems.

Stem cell research introduces another dimension to this narrative, offering groundbreaking possibilities in biotechnology, regenerative medicine, and aquaculture. With their unique ability to self-renew and specialize, stem cells have driven advancements in genetic research and cellular reprogramming, particularly through the development of induced pluripotent stem cells (iPSCs). Progress in culturing techniques and the study of stem cell

microenvironments further demonstrates the potential of this field to transform both science and medicine. Together, these interconnected topics illustrate the dynamic evolution of aquaculture practices, ecological management, and cutting-edge biotechnological innovation.

Introduction

Catfish farming remains the most significant section of American aquaculture due to its well-established infrastructure in the southeastern United States, driven by optimal environmental conditions; strong market demand for its versatile and affordable protein source; advancements in production efficiency through selective breeding and optimized feeding; significant contributions to rural economies; continuous support from government programs, research institutions, and extension services; and its resilience in adapting to market challenges, such as competition from imported fish, by emphasizing the quality and safety of domestically produced catfish (Engle, et al., 2022). The farmers in the United States have demonstrated the flexibility and resourcefulness to adapt to the changing economic conditions. The catfish has a mild flavor, is low in calories and high in protein. The channel catfish, *Ictalurus punctatus*, are the most abundant catfish species in North America and a famous sport fish (Wilson, 2017). North American catfishes consist of seven genera and have at least 45 species (Haubrock et al., 2021). The channel catfish (*Ictalurus punctatus*) belongs to the family Ictaluridae and is native to a range extending from the St. Johns River in Florida to Delaware along the eastern coast of the United States, which lies east of the Appalachian Mountains. Its native range also spans from the Gulf of Mexico northward to the Hudson Bay drainage (Dunham and Smitherman, 1984). This species is widely distributed across the southern United States, reaching as far as the northern regions, including areas along the eastern seaboard.

The first catfish farm was established in Kansas in 1930, but there was little catfish farming in the US until the late 1960s (Dunham and Smitherman 1984). In the 1970's, catfish farming grew in popularity in the southeastern United States, with Mississippi becoming the leading catfish-producing state (Hanson et al.2020). The reason for this exponential growth was due to several factors such as the environmental conditions, economic opportunity, innovation in technology, and the higher demand for domestic seafood. The warmer climate and abundance of freshwater resources in the southeastern United States provide ideal conditions for catfish farming. Mississippi, in particular, has a number of large rivers and a high-water table, making it well-suited for catfish production (Hanson et al.2017). The availability of cheap land and labor, as well as government support in the form of loans and technical assistance made catfish farming an attractive economic opportunity for farmers in the region. During the late 1960s and early 1970s, the price of soybean, cotton and other crops was low, providing incentive for large farms in Mississippi to convert this land to catfish ponds as catfish was providing a better profit margin. Many small farmers in the southeastern United States saw catfish farming as a way to diversify their operations and generate additional income (Wellborn 1983).

Researchers and farmers in the southeastern United States developed innovative techniques for catfish production, including improved feeds and water quality management practices (Hegde et al.2022), which led to increased production and efficiency in the industry. The growth of the catfish industry in the United States also reflects a growing demand for domestic seafood. Catfish is a popular food fish, particularly in the southern United States, and many consumers prefer to purchase locally produced seafood (Freed et al., 2020). These factors, among others, contributed to the growth of the catfish industry in the southeastern United States, with Mississippi becoming the leading catfish-producing state 40-50 years ago followed by

Alabama, Arkansas and Louisiana (now replaced by Texas). Today, catfish farming remains an important industry in the region, providing jobs and economic opportunities for many communities (Jones, 2022).

The introduction of floating pellets and improved water quality management practices had a significant impact on catfish farming in North America in the 1980s (Boyd et al., 2020). The floating feed improved nutrition, increased efficiency, provided better water quality, reduced disease and more importantly greater feeding management. Nutrition improvement provided balanced nutrition formulation, enhanced digestibility and absorption, optimized growth and development, reduction of nutrient imbalances, and sustainable feeding practices. Floating pellets are formulated to provide a balanced diet for catfish, containing essential nutrients such as proteins, lipids, carbohydrates, vitamins, and minerals in optimal proportions. Precise control over nutrient composition ensures that catfish receive all necessary nutrients for growth and development, promoting better overall health and performance (Gatlin et al. 2007). Floating pellets are designed to be highly digestible, allowing catfish to efficiently absorb nutrients from the feed (Kemp & Britz 2008). By meeting the nutritional requirements of catfish, floating pellets promote faster growth rates and higher yields, resulting in increased productivity and profitability for farmers (Boyd 2012). Nutrient imbalances can lead to stunting of growth, metabolic disorders, and increased susceptibility to diseases. Through precise formulation and feeding practices, floating pellets minimize the risk of nutrient deficiencies or excesses (Salhi et al. 2004). Floating pellets contribute to sustainable feeding practices in catfish farming by reducing feed wastage and environmental impacts. The buoyant nature of the pellets prevents them from sinking to the bottom of the pond, where they can contribute to nutrient runoff and water pollution. By optimizing feeding efficiency and minimizing feed losses, floating pellets

help conserve resources and promote the long-term sustainability of catfish aquaculture operations (Gatlin et al. 2007). The adoption of floating pellets represents a significant advancement in catfish nutrition management, offering a scientifically formulated and efficient feeding solution.

Ecologically, catfish inhabit diverse freshwater systems, including rivers, lakes, ponds, and swamps, where DO levels can fluctuate significantly due to factors such as high temperatures, organic matter decomposition, and limited water flow (Boyd, 2012). These habitats often experience hypoxic conditions, particularly in warm climates or during periods of eutrophication, where nutrient overload leads to increased biological oxygen demand (Diaz & Rosenberg, 2008). The improvement of water quality management practices overall improved the health and growth of catfish, minimized disease risks, and promoted overall farm productivity. The use of aerators and water circulation systems helped maintain optimal levels of dissolved oxygen and prevented the buildup of harmful bacteria and toxins (Cole & Boyd, 1986). Dissolved oxygen is one of the most critical water quality parameters in aquaculture. Adequate oxygen levels are essential for the aerobic respiration of fish and beneficial microorganisms, while low oxygen concentrations can lead to stress, reduced growth, and even mortality (Colt et al., 2006). The water quality improvements were only maintained when stocking density did not increase, thus with an increase in stocking density, water quality remains an issue correlated with increased rates of diseases (Danaher et al., 2007). Overall, the introduction of floating feed pellets and improved water quality management practices led to significant improvements in the efficiency and profitability of catfish farming in North America. These non-genetic advancements have helped the catfish industry grow and remain an important source of high-quality protein and economic opportunity in the region.

In the 1990s, the United States Department of Agriculture (USDA) implemented several programs to help expand the catfish industry and increase exports. The four main pillars to support the expansion of the catfish industry were research and development, marketing and promotions, technical assistance and lastly, loans and grants. The USDA Agricultural Research Service (ARS) conducted research on catfish nutrition, genetics, and disease management to help improve the efficiency and profitability of catfish farming (U.S. Department of Agriculture, Agricultural Research Service, n.d.). The USDA also provided funding for research projects conducted by universities and private companies. The USDA Foreign Agricultural Service (FAS) worked to promote U.S. catfish in international markets, organizing trade missions and participating in international trade shows (U.S. Department of Agriculture, Farm Service Agency, n.d.). The FAS also provided information and assistance to U.S. catfish exporters on issues such as import regulations and labeling requirements. The USDA Natural Resources Conservation Service (NRCS) provided technical assistance to catfish farmers on issues such as soil and water management, nutrient management, and erosion control (U.S. Department of Agriculture, Natural Resources Conservation Service, n.d.). The NRCS also provided funding for conservation practices that helped improve water quality and reduce environmental impacts. The USDA Farm Service Agency (FSA) also provided loans and grants to catfish farmers to help them establish or expand their operations. The FSA also helped farmers who suffered losses due to natural disasters or other causes. These programs helped support the growth of the catfish industry in the United States, both domestically and internationally. Today, the catfish industry is an important source of high-quality protein and economic opportunity for many communities in the southern part of the United States.

Catfish farming has been the largest aquaculture industry in the U.S., producing around 350 million kilograms (about 771,617,000 lb.) annually and accounting for approximately 70% of all U.S. aquaculture production in 2000 (Wise et al. 2021; Fantini-Hoag et al. 2022). However, due to high production costs, the U.S. recession, and competition with low-priced imported fish, such as Asian catfish, production declined to 138 million kilograms (about 304,237,560 lb.) by 2011 (NASS, 2012). Presently, Vietnam supplies 70% of the catfish fillets consumed in the U.S. (Shang, 2013). Despite a slight increase to approximately 150 million kg (about 330,693,000 lb.) in 2015-2017 (Hanson and Sites, 2015; Wise et al., 2017), catfish production remains relatively slow growing, with 158 million kg (about 348,329,960 lb.) produced in 2019 (NASS, 2020). Although profitable, the surviving catfish farms need to improve efficiency, productivity, and sustainability to avoid susceptibility during the next economic downturn or a potential rise in fuel and feed costs, which is currently happening. Genetic research is one potential avenue for enhancing sustainability and profitability for catfish production in the United States.

Without realizing it, the first ictalurid genetics and breeding program was initiated by Dr. Homer Swingle and Ellis Prather of Auburn University during the 1950s and 1960s. Their research compared different species (species are genetically different) of catfish such as *Ameiurus catus* (white catfish), *Ictalurus furcatus* (blue catfish), Pylodictis olivaris (flathead catfish), and channel catfish to determine their suitability as aquaculture species (Dunham and Smitherman 1984; Robinson & Li, 2020). The channel catfish was the most suitable for aquafarming. The spawning season for channel catfish in the United States spans from April to August, coinciding with water temperatures ranging from 21 to 30 °C (Lenz, 1947; Wolters, 1996). To facilitate natural spawning, artificial spawning nests, such as containers, are

strategically placed in ponds and monitored every other day for fertilized egg masses (Busch, 1983; Steeby, 1987; Tucker & Robinson, 1990). Employing the open pond method, channel catfish brooders exhibit a spawning success rate of 30% to 50% (Brauhn, 1971; Bondari, 1984; Wolters, 1993). The incubation period for channel catfish eggs varies between 5 to 10 days post-spawning, depending on the ambient water temperature (Wolters, 1993). In comparison to open-pond spawning, pen spawning is a semi-natural method that offers enhanced management and monitoring capabilities. Selected male and female catfish are placed in large net cages or enclosures submerged in a pond or other natural water body, providing a controlled environment that increases the likelihood of successful spawning interactions. Pen spawning enables precise control over environmental factors that trigger spawning behavior, such as temperature adjustments to mimic seasonal fluctuations, thereby activating the reproductive response. Moreover, the enclosed environment ensures that eggs remain within the pen, protecting them from external predators and simplifying the collection and handling of fertilized eggs. This method is often preferred by commercial fisheries due to its improved control and monitoring capabilities compared to open-pond spawning, potentially leading to higher success rates. However, pen spawning requires more labor and infrastructure to set up and maintain the enclosures. Despite its advantages, pen spawning, along with open-pond spawning, is less favored for hybrid embryo production due to these higher operational demands and the need for precise environmental control, which is more effectively achieved through artificial fertilization methods

In the early 1960s, the United States Fish and Wildlife Service (USFWS) conducted natural breeding and genetic enhancement programs. The program was led by O.L. Green, Harry Dupree, and John Giudice. The research focused upon interspecific hybridization. The seven

main ictalurid species were hybridized in almost all possible combinations (Dunham, 2006). The female channel catfish hybridized with male blue catfish exhibited heterotic growth at low densities in ponds (Giudice et al. 1966). However, no other hybrid combinations exhibited enhanced aquaculture potential except the channel catfish female X white catfish male had heterotic growth at early life stages only (Dunham, 2006).

The channel catfish female-blue catfish male hybrid was then evaluated at commercial densities at Auburn University, and still exhibited heterobeltiotic growth as well as superior feed conversion efficiency and dressout percentage compared to channel catfish (Smitherman et al., 1983). For the next 40 years the majority of the research on hybrids was conducted at Auburn University with hybrids being advantageous, exhibiting faster growth, better feed conversion, higher carcass yield, higher tolerance to crowded conditions, and low oxygen levels among other desirable traits (Abass et al., 2022).

Moreover, hybrid catfish are known for their superior meat quality, characterized by firmer texture and lower fat content, which aligns with consumer preferences and enhances marketability (Morris et al., 1999). They also exhibit greater tolerance to a wider range of environmental stressors, including fluctuations in temperature and water quality, which improves their adaptability and survivability in diverse aquaculture settings (Tidwell et al., 1999). These combined advantages—faster growth, better feed efficiency, increased disease resistance, higher survival rates, improved meat quality, and greater environmental tolerance—make hybrid catfish a preferred choice for commercial aquaculture, offering significant economic benefits and improved product quality over traditional channel catfish farming (Boyd, 2016).

The rapid growth of the catfish industry in the 1980s and 1990s led it to become one of the most important agricultural activities in states such as Mississippi, Arkansas, and Alabama. From

the late 1990s until 2004, there was a consistent but low commercial application of hybrid catfish producing 1-5 million fry/year utilizing artificial fertilization via hormone induced ovulation (Su et al. 2013) with carp pituitary extract (CPE) (Fig 1). In 2005, hybrid catfish fry production had a dramatic increase as Auburn University and Eagle Aquaculture introduced a new artificial fertilization technology centered around ovulation with luteinizing hormone releasing hormone analogue (LHRHa). Hybrid catfish production now accounts for more than 50% and upwards to 70% of US catfish production (Wang et al., 2024).

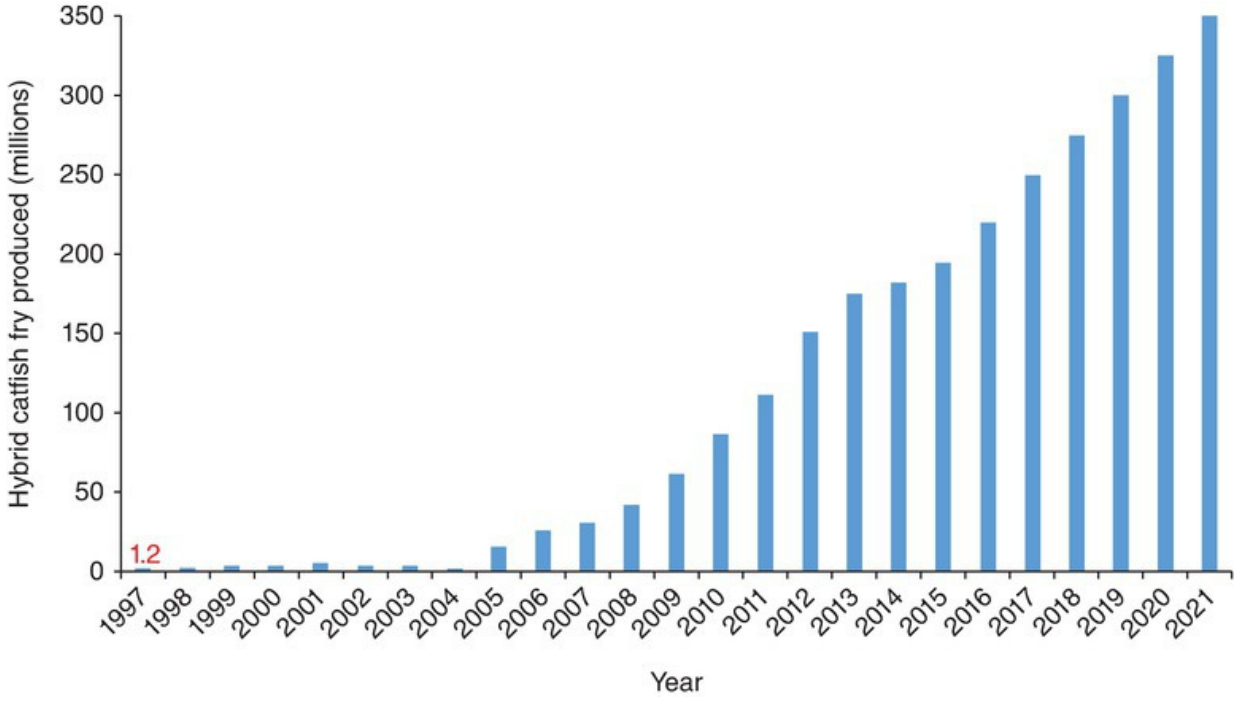

Figure 1 Production of channel catfish (*Ictalurus punctatus*) female × blue catfish (*Ictalurus furcatus*) male hybrid catfish fry production over time, illustrating the adoption of this hybrid by the U.S. catfish industry (Dunham, Aquaculture and Fisheries Biotechnology: Genetic Approaches, pp. 349–355, 2023).

Common Carp

Common carp exhibit intricate spawning behaviors both in the wild and in captivity, reflecting their adaptability and reproductive efficiency. In their natural habitat, common carp prefer shallow, vegetated waters for spawning (Kucharczyk et al., 2008). These environments provide protection for their adhesive eggs, which are scattered randomly and adhere to underwater vegetation, grasses, and other substrates. A typical adult female carp can lay approximately 300,000 eggs per spawning period, and over a million eggs annually (Fishing, 2005). During the spawning process, carp cluster in shallow waters rich in aquatic plants. Females release their eggs in close proximity to these plants, where males then fertilize them. The fertilization process is time-sensitive; sperm must enter the egg through a micropyle, which remains open for about 30–60 seconds (Kucharczyk et al., 2008). The micropyle closes regardless of successful fertilization (Horváth & FAO, 2015). The adhesive nature of the eggs ensures they remain attached to whatever surface they encounter, initiating development soon after. The development time for carp eggs is temperature-dependent, with fry typically emerging within 2-8 days post-fertilization (Sapkale et al., 2011). Although carp predominantly spawn in the spring, they are capable of spawning multiple times throughout the year, especially in response to favorable environmental conditions such as increased water temperatures (17-26°C) and rainfall. In tropical regions, carp can spawn year-round, demonstrating their ecological versatility. In captivity, common carp exhibit similar spawning behaviors but under more controlled conditions. Aquaculture facilities often simulate the natural environmental triggers—such as water temperature and photoperiod—to induce spawning. The presence of aquatic vegetation or artificial spawning substrates is crucial to mimic the natural conditions required for egg adhesion and subsequent development. The fertilized eggs are then treated to reduce

stickiness, a crucial step to prevent clumping and ensure better aeration and development during incubation (Site, 2009). These treated eggs are subsequently placed in hatchery jars, where they are incubated until hatching. Artificial spawning in captivity not only ensures a high yield of fry but also allows for the precise control of genetic lines and the timing of production cycles (Rinchard & Kestemont, 1996).

Interestingly, recent research has revealed that a small percentage of fertilized common carp eggs can survive passage through the digestive tracts of waterfowl and hatch successfully after being excreted. This remarkable finding, reported by Lovas-Kiss et al. (2020), suggests an additional natural dispersal mechanism for carp eggs, potentially aiding in their widespread distribution. The efficient reproductive strategies of common carp, both in the wild and in captivity, underscore their resilience and adaptability. These behaviors not only ensure high reproductive output but also facilitate the species' proliferation across diverse environments.

The incubation environment is meticulously controlled to maintain optimal conditions for egg development. Once the eggs hatch, the larvae are transferred to large rearing jars where they continue to develop. At the onset of exogenous feeding, the larvae are moved to nursery ponds. These ponds are designed to provide ample food and suitable environmental conditions to support the rapid growth and development of the juvenile fish (László Horváth & Seagrave, 1992; Horváth & FAO, 2015). This method is vital for meeting the high demand for common carp, both as a food source and for ornamental purposes. By refining techniques such as hypophysation and controlled incubation, aquaculture operations can optimize the reproductive output and overall health of their carp populations, contributing to the sustainability and efficiency of fish farming practices.

Invasive Carp

The introduction and proliferation of invasive carp species, notably the common carp, bighead carp (*Hypophthalmichthys nobilis*), silver carp (*Hypophthalmichthys molitrix*), and grass carp (*Ctenopharyngodon idella*), have significantly disrupted ecosystems across the globe. Common carp have been introduced to 59 nations on six continents, largely through human activities such as aquaculture, ornamental fish trade, and accidental releases (Chick & Pegg, 2001; Lake, 2013; FAO, 2020). In the United States, common carp are present in 48 states, heavily populating the southern two-thirds of the country (FAO, 2020). These species exhibit high reproductive rates and feeding habits that allow them to dominate their environments. Common carp, for instance, are known for their bottom-feeding behavior, which involves uprooting and disturbing submerged vegetation, leading to habitat degradation and a reduction in water quality. This activity releases phosphorus into the water, promoting algal blooms that can further deteriorate the ecosystem (Chick & Pegg, 2001; Sampson et al., 2009; Hayer et al., 2014). Such changes adversely affect native fish and waterfowl populations, which rely on aquatic vegetation for food and habitat.

The economic impact of invasive carp is substantial. Local governments and agencies spend millions annually on management and control efforts. For example, in Victoria, Australia, common carp are classified as a noxious species, allowing for unlimited fishing to reduce their numbers (Sanders & Peterson, 2011). Australians have come up with an innovative use for captured carp include converting them into plant fertilizer, providing an environmentally friendly method of population control and resource utilization (Sanders & Peterson, 2011). The success of these interventions varies, and ongoing research is essential to develop more effective and

sustainable management strategies. In Utah, significant efforts have led to an estimated 75% reduction in the carp population in Utah Lake through netting operations (FAO, 2020)

Overall, the invasive nature of various carp species has led to significant ecological and economic challenges. Their ability to alter habitats and outcompete native species necessitates continued efforts in management and control to mitigate their impact. Intervention methods to control carp populations include mechanical removal, chemical treatments, and biological controls. Mechanical removal involves the use of seine nets to capture large numbers of carp, especially during their breeding season when they congregate in shallow waters. Chemical treatments, such as the application of rotenone, are used in targeted areas to eliminate carp by poisoning them without affecting other species (Sanders & Peterson, 2011). Additionally, some regions have explored biological controls, including the development of genetically modified carp that are sterile, thus preventing reproduction (Lake, 2013).

The history of biological control in managing invasive species dates back several decades and has evolved significantly with advancements in genetic technology. One promising avenue is the development of genetically modified carp that are sterile, thereby preventing their reproduction and curtailing population growth (Xu et al., 2022). This approach, known as genetic biocontrol, involves altering the genetic makeup of carp eggs so that the resulting offspring are incapable of reproduction. Genetic biocontrol strategies, while potent, require careful consideration to minimize potential negative impacts on the environment (Burt, 2003; Teem et al., 2020). Implementing triploidy in fish populations has the potential to mitigate the environmental impacts of invasive carp, although this approach has not been tested. Male triploids exhibit sexual behavior and can induce diploid females to ovulate (Dunham 2023). .

However, the triploid male cannot fertilize the eggs, and, theoretically the overall carp population decreases, leading to the restoration of native flora and fauna.

Stem Cells and their History

Stem cells can develop into at least one type of mature, differentiated cell as well as endless or protracted self-renewal (Weissman, 2000). According to Till and McCulloch (1980), stem cells are precursor cells with the capacity to self-renew and give rise to a variety of mature cell types. Understanding what defines a stem cell as a stem cell—that is, the cell's "stemness"—is prerequisite knowledge for understanding stem cell cultivation. According to Aponte and Caicedo (2017), stemness is the combination of a cell's capacity to reproduce itself, produce differentiated cells, and engage with its surroundings to strike a balance between quiescence, proliferation, and regeneration (Takahashi & Yamanaka, 2006). There are several types of stem cells that have potential use in aquaculture and fisheries as well as other medicinal fields. According to Aponte and Caicedo (2017), there are three main categories of stem cells: induced pluripotent stem cells (iPSCs), non-embryonic stem cells (sometimes called adult stem cells), and embryonic stem cells.

History of Stem Cells

Since the 1950s, cells have been cultivated in vitro to create enzymes, vaccines, growth factors, monoclonal antibodies and hormones, and have served as key models for biological and physiological study. Henrietta Lacks', a 31-year-old African American mother who died of cervical cancer on October 4, 1951, cells (HeLa cells) are the oldest human cell line (Skloot, 2010). The original HeLa cells were harvested from her cervix without her permission on February 8, 1951, consequently resulting in the most used cell line and many medical innovations. HeLa cell genome and Henrietta Lacks' original genome show very little similarity

when compared to each other today. Stem cells survival rates exceed that of ordinary cells (Borowski et al., 2008); therefore, increasing the chance for these cells to accumulate genetic mutations. Several mutations are required for an individual cell to lose control over its self-renewal, growth, and become the source of cancer. Lacks' genome originally contained 46 normal chromosomes while most HeLa cells have 70-90 chromosomes with over 20 translocations, some of which are highly complex, involving multiple chromosomal rearrangements (Heng et al., 2004; Borowski et al., 2008; Mummery, 2014). The HeLa cells from Henrietta Lacks are naturally mutated immortal cells, while stem cells are natural nonmutated immortal cells. Artificially created immortal cells use Simian virus 40 (SV40) T, an antigen that can induce telomerase activity in the infected cells (Foddis et al., 2002). Scientists have utilized HeLa cells to find a cure for polio, create the human papilloma virus (HPV) vaccines, improve cell culturing practices, discover how to count chromosomes within the somatic cells and develop genome mapping protocols for the human genome project (MacDonald, 2018; Tjio & Levan, 2010).

Stem cells can be used for both therapeutic and reproductive cloning. In 2012, John Gurdon and Shinya Yamanaka were awarded the Nobel Prize for Physiology or Medicine for their groundbreaking discoveries related to cellular reprogramming. Their work demonstrated that mature, specialized cells can be reprogrammed to become pluripotent, meaning they can develop into any type of cell in the body (Hummel, 2013). In January of 2018, Chinese scientists in Shanghai announced the successful use of fetal fibroblasts to clone two female macaque monkeys by somatic cell nuclear transfer (Tien Yin Wong et al., 2013; Liu et al., 2020). Gene therapies also look to stem cell therapy to help find methods to cure and reduce the amount of

cancer cells and tumors within patients by curating a health program that is made of the individual (de Pagter et al., 2015; Dvorak et al., 2014; Ramirez, 2017).

Stem cell manipulation has created a new branch of study for scientists to utilize to enhance the world we live in. In the early 2000s, researchers discovered that the following growth factors have a roll in differentiating the embryotic stem cells into specific types of cells: basic fibroblast growth factor (bFGF), transforming growth factor beta1 (TGF-beta1), activin-A, bone morphogenic protein 4 (BMP-4), hepatocyte growth factor (HGF), epidermal growth factor (EGF), beta nerve growth factor (betaNGF) and retinoic acid (Bagutti et al., 2001; Hwang et al., 2005; Schuldiner et al., 2000). The effects of these growth factors were proven by the differentiation of the embryotic stem cells morphing into cells with different epithelial or mesenchymal morphologies (Schuldiner et al., 2000). Mouse embryotic stem cells can be maintained in their pluripotent state either by culturing them on mouse embryonic fibroblast feeder layers or in medium containing leukemia inhibitory factor. Leukemia inhibitory factor is a cytokine released from the mouse embryonic fibroblast feeder layers (Thomson, 1998; Fecek et al., 2008; Lutolf et al., 2009). Current practices to maintain human embryonic stem cells in an undifferentiated state typically depend on the support of feeder cells such as mouse embryonic fibroblasts or an extracellular matrix such as Matrigel (Villa-Diaz et al., 2012). Embryotic stem cells have strong plasticity and potentially unlimited capacity for self-renewal.

Human embryotic stem cell (ESCs) have been extensively studied for their potential in regenerative medicine, given their ability to differentiate into a wide range of specialized cell types (Precious Earldom Mulaudzi et al., 2023). However, one of the primary challenges in using ESCs in clinical applications is immune rejection. When ESC-derived cells or tissues are introduced into a patient, the recipient's immune system recognizes them as foreign, leading to

graft rejection (Gale et al., 1987; Ho & Soiffer, 2001). Overcoming this barrier is critical for the success of stem cell therapies.

Embryonic stem cells are derived from the inner cell mass of the blastocyst during early embryonic development. These cells are characterized by their pluripotency, meaning they have the potential to give rise to all three germ layers—endoderm, mesoderm, and ectoderm—which form the tissues and organs of both aquatic and terrestrial species (Thomson, 1998). Early research demonstrated that embryonic stem cells can proliferate in vitro, maintaining their undifferentiated state while retaining the ability to differentiate into various specialized cell types under appropriate conditions (Thomson, 1998).

The pluripotent nature of these cells extends beyond human applications. In species such as catfish, pluripotent cells can differentiate into specific cell types, highlighting their potential not only in regenerative medicine but also in aquaculture biotechnology. Advances in stem cell technology, particularly in directing the differentiation of ESCs into specialized cell types, hold promise for a variety of applications, ranging from treating human diseases to improving aquaculture practices (Bongso et al., 1994; Thomson, 1998; Trounson, 2006).

Technology and Stem Cells

Technologies has been developed to derive embryotic stem cell-like pluripotent stem cells from differentiated somatic cells. The process of the conversion of these cells is referred to as “pluripotential reprogramming” (Kimura et al., 2002; Kim et al., 2011). Reprogrammed cells demonstrate pluripotent characteristics such as reactivation of pluripotency-related genes, inactivation of tissue-specific genes, differentiation potential to all three germ layers, and a specific epigenomic state corresponding to the pluripotent cells (Thomson, 1998; Ho and Soiffer, 2001; Fecek et al., 2008; Kim et al., 2011; McKee and Chaudhry, 2017). Somatic cells can

acquire a pluripotent state following fusion with pluripotent stem cells such as embryotic stem cells, embryonic germ cells, and embryonal carcinoma cells (M. et al., 1997, 2001; Surani, 1999; Kimura et al., 2002; Han et al., 2008; J.T. et al., 2008). The methylation patterns of imprinted genes in somatic cells differ between embryonic germ cells and somatic hybrid cells derived from embryonic stem cells (M. et al., 1997). The methylation pattern of imprinted genes in somatic cells remains unchanged after fusion with embryonic stem cells, with methylation occurring on the maternal allele, similar to somatic cells. In contrast, fusion with embryonic germ cells results in the absence of methylation on both alleles. These findings suggest that embryonic germ cells possess an additional capacity to induce methylation changes in somatic cells (Cowan, 2005; Surani, 2005; Han et al., 2008; Kim et al., 2011).

Non-embryonic stem cells, also known as adult or somatic stem cells, are limited in their self-renewal capabilities and differentiation capacity. All tissues have their own chamber of stem cells, and adult-somatic stem cells are present in all types of organs and tissues in the organism (Till & McCulloch, 1980; Chagastelles & Nardi, 2011). There are five types of adult stem cells: hematopoietic stem cells, mesenchymal stem cells, neural stem cells, epithelial stem cells, and skin stem cells. These stem cells are responsible for replenishing cells that die within a given organ, either due to damage or disease (Laertis Ikonomou et al., 2022). Adult stem cells are produced during a process known as ontogenesis (Rodgers &d Jadhav, 2018; Rodeo, 2019; Liu et al., 2020; Posa et al., 2021). Ontogenesis is the development of an individual organism or anatomical or behavioral feature from the earliest stage to maturity (Bändel, 1988). Adult tissue-specific stem cells are rare and generally do not have surface markers that would readily distinguish them from mature cells of the same tissue (Wanjare and Huang, 2019). Therefore,

they cannot be readily ‘isolated' like embryotic stem cells, but there are many different protocols that have succeeded in enriching stem/progenitor cells to different degrees of purity.

The survival, dormancy, and activation of cells are contingent upon specific signals within their microenvironment (Rezza, Sennett, and Rendl, 2014). The expression and/or secretion of these factors by surrounding components establish a localized niche, thereby modulating stem cell activity. Stem cells can also receive signals from more distant cells within the tissue or even from external tissues. These signals are categorized into survival signals, which promote cell survival and prevent apoptosis, and activating signals, which stimulate cellular processes or activate specific cellular pathways (Rezza, Sennett, and Rendl, 2014). Numerous transcription factors have been investigated in the context of stem cell-autonomous survival. For instance, transforming growth factor myostatin is expressed by satellite stem cells to maintain their dormancy in muscle tissue (McCroskery et al., 2003). A genetic mutation resulting in the deletion of the Notch signaling effector RBPJ (recombination signal binding protein for immunoglobulin kappa J region) leads to the loss of dormancy in satellite stem cells in resting muscle (Bjornson et al., 2012). The Notch pathway is crucial for cell fate decisions, including quiescence and self-renewal, and serves as a key regulator in stem cell biology. Adult stem cells are capable of long-term self-renewal and producing mature cells with specialized functions (Weissman, 2000; Liu et al., 2019; Charitos et al., 2021). When an adult stem cell goes through the self-renewing process, one cell will remain as a self-renewing stem cell, and the second daughter cell will be replicated and differentiated into a mature cell type (Trounson, 2006). These generated cells are called precursor or progenitor cells, which after several rounds of mitosis, give rise to differentiated cells (Chagastelles & Nardi, 2011).

In the tissues, stem cells and their progeny are closely associated with mesenchymal/ stromal cells which have been shown to play a central role in stem cell regulation (Rezza, Sennett and Rendl, 2014). This process is demonstrated by increasing the number of sertoli cells in the seminiferous epithelium. Creating more niches for spermatogonia stem cells to develop and inhibiting the production of these supportive signals from the sertoli cells will decrease the number of spermatogonia stem cells numbers (Oatley and Brinster, 2012). The growth factor glial cell line-derived neurotrophic factor (GDNF) plays an essential role in stem cell quiescence in mice. GDNF deficient mice have seminiferous tubules that lack germ cells due to the inability of sertoli cells to sustain undifferentiated stem cells (H. et al., 2004; McCroskery et al., 2003; Oatley & Brinster, 2012; Parker et al., 2012; Meng et al., 2000). The effect of GDNF signaling on spermatogonia stem cells maintenance is promoted in vitro by fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF), and insulin-like growth factor (IGF) (Heng et al., 2004; Kubota et al., 2004; Hwang et al., 2005). Several additional secretory factors are important for maintaining stem cells within the body. These factors are still being studied in detail. The extracellular matrix basement membrane and other adhesion molecules are also key players in many adult stem cell niches. Mature stem cells can be manipulated to become pluripotent (Colman, 2013; Wong et al., 2013). Pluripotent cells can give rise to all the cell types that make up the body. These cells will be later defined as induced pluripotent stem cells. Induced pluripotent stem cells are used to produce embryonic stem cells and anytime of cell, tissue, or organ. Almost any mature cell type in the body can be reprogrammed into induced pluripotent stem cells and then be differentiated into tissue-specific cells of desired lineages (Singh et al., 2015; Zahumenska et al., 2020). A non-evasive and easily accessible method to obtain mature somatic cells through the urine in humans for the development and induction of pluripotent stem

cells (Schosserer et al., 2015). The total procedure to convert the mesenchymal stem cells from the urine involves two weeks of cell culturing and three to four weeks of reprogramming. Consequently, a high number of induced pluripotent stem cells are produced with excellent differentiation potential (Zhou et al., 2012). Zhou in 2012 established a protocol that required only a 30-ml sample of urine, which is simple, relatively fast, cost-effective, and universal (Zhou et al., 2012; Schosserer et al., 2015). Urine samples have been shown to be a good alternative option for harvesting induced pluripotent stem cells to be differentiated into different cell subtypes across the body and its system.

The urine derived stem cells studies have shown that urine cells might demonstrate two types of cloning (Zhou et al., 2012). In direct in vivo cellular reprogramming, lineage-restricted transcription factors and microRNAs have the potential to reprogram local somatic cells to differentiate into specific types of cells without an intermediary stem/progenitor cell stage (Surani, 1999; Kim et al., 2011). Induced pluripotent stem cells were found to be less efficient and to exhibit greater variability deficiencies that could be improved with culturing technique alterations when compared to embryonic stem cells (Hu et al., 2010). In 2012, Yamanaka discovered that four growth factors; Oct4, Sox2, Klf4, and c-Myc, with the capacity to induce pluripotency and enable cells to develop into any of 220 cell types (Evans and Kaufman, 1981; Takahashi et al., 2007; Lowry et al., 2008; Colman, 2013). Naïve human induced pluripotent stem cells can be generated directly from somatic cells with Oct4, Sox2, Klf4, and c-Myc when overexpressed (Tripathi et al., 2021). These cells are culture nutrient rich media with a shorter tissue culture time and more extended passages (Evans & Kaufman 1981; Nichols, 2009; Weinberger, 2016; Kilens et al., 2018). There may be genetic and epigenetic variations among different induced pluripotent stem cells lines (Liang & Zhang, 2013). Differences may be

inherited from donor somatic cells produced during reprogramming or culturing the cells. To combat the chance of genomic instability in the cells, Doss and Sachinidis (2019) proposed ten minimum quality criteria required for clinical grade induced pluripotent stem cells and their differentiated products to protect the host once the cells are introduced to the body (Liziane Raquel Beckenkamp et al., 2024). These criteria include maintaining genomic stability to prevent mutations during culture, verifying pluripotency to ensure differentiation into all three germ layers, and ensuring sterility to avoid microbial contamination. Additionally, they emphasize the necessity of eliminating undifferentiated iPSCs in differentiated products to prevent tumor formation. Immunogenicity testing is crucial to assess the cells' potential to provoke an immune response, while functional assays are needed to demonstrate the capabilities of the differentiated cells. Regular karyotype analysis is required to check for chromosomal integrity, and molecular markers should be used for identity confirmation of the cell type. Furthermore, ensuring high cell viability post-thaw or post-culture and monitoring apoptosis and necrosis to maintain low levels of cell death during production are also critical. These rigorous standards aim to uphold the quality and reliability of iPSCs for clinical use.

Culturing induced pluripotent stem cells in feeder-free conditions is extremely important (Jung et al., 2012; M.X and A., 2019). Self-renewal culture technique calls for placing induced pluripotent stem cells, mice or human, on a monolayer of feeder-cells such as a primary mitotically inactivated mouse embryonic fibroblast cell culture (Thomson et al., 1998; Qiu et al., 2016). For human induced pluripotent stem cells, culture media may improve stem cell differentiation capacity if it is nutrient-rich, xeno-free, and serum-free as these conditions provide a defined and controlled environment that minimizes variability, reduces the risk of contamination, and supports the maintenance of pluripotency while facilitating efficient and

reproducible differentiation into specific cell lineages. (Gong et al., 2009; S. et al., 2012). There is significant concern over the risk of xenopathogen contamination, which would make human embryonic stem cells unsafe for regenerative medicine (Ilic, 2006; F. and G.A, 2007). There are many ways that pluripotent stem cells can be induced, such as using genetic factors, signaling molecules, small molecules, microRNAs, chemicals and biomaterials. The modifying of induced pluripotent stem cells via biomaterials offers an approach to increasing the reforming efficiency and scalability (Teng Songsong et al., 2013; Song et al., 2021). Upon reprogramming, induced pluripotent stem cell growth and differentiation can be improved by using a stem cell niche. These niches are an environment that mimics the natural microenvironment of stem cells, and thereby modulates stem cell phenotype development, proliferation, and differentiation (Mohamed and van der Walle, 2008; Danhier et al., 2012; Ankrum et al., 2014). Genetic factors may change the induced pluripotent stem cells (iPSc). These factors include whether a desired normal cell phenotype or undesired cell phenotype, for example, a nonspecific or cancerous type of cell, is formed. Biomaterials can govern the kinetics of reprogramming factors via nanoparticle and microparticle based systems. In addition, they can regulate stem cell fate and function as well as be employed to facilitate induced pluripotent stem cell transplantation (Dellatore et al., 2008; Lutolf et al., 2009; Higuchi et al., 2011; Peppo et al., 2013). Induced pluripotent stem cells lower risks of immune rejection, contamination, infection, and the ability to create large quantities for personalized medicine. When selecting an induced pluripotent stem cell reprogramming method, it is important to minimize risk, maintain pluripotency, and enhance the ability to direct a specific cell to a location for its development.

Fish have two major types of cell lineages that originate from a fertilized egg, the germline cells and the soma cells. Germline cells are the cells that give rise to eggs or sperm and

are responsible for passing genetic information from one generation to the next (Liu et al., 2018). Male germ cells are continuously formed throughout adult life by the stem cells in the testes (Mummery et al., 2014). The spermatogonial stem cells (SSCs) are responsible for the formation of the sperm. These cells are unique in that they undergo meiosis to produce haploid cells with half the number of chromosomes as the parent cell. Somatic cells, on the other hand, are all the other cells in the body that are not involved in reproduction (Tat et al., 2011). These cells make up the tissues and organs of the body and do not pass on genetic information to offspring.

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Chapter One

Survival Rates of Unsorted Spermatogonial Type A Stem Cells of Blue Catfish *(Ictalurus furcatus)* Incubated with Rho Protein Kinase Inhibitor (ROCK I) At 0, 25, 50, and 75mM at a Temperature Gradient With and Without 5% CO_2

Abstract

Spermatogonial stem cells (SSCs), which are capable of both self-renewal and creation of multiple differentiated germ cells, sustain continuous sperm production in the testes. Maintaining the undifferentiated state requires extra considerations to ensure that the cells keep their key traits of self-renewal and pluripotency while waiting for manipulation, but such information is not available for blue catfish, *Ictalurus furcatus*. SSCs were extracted and isolated from the immature gonads of blue catfish. The maintenance of spermatogonial stem cells was investigated in this work using short-term culture prior to transplantation. The SSCs were incubated with and without 5% CO_2 at 24-30°C temperature range and with 0-75uM of Y-27632, a specific inhibitor of Rho kinase (ROCK I). Spermatogonia incubated at 72 hour *in vitro* survived best at 28°C, utilizing 50-75 uM ROCK I with 5% CO_2 ($p=0.001$) for 72 hours.

1.1 Introduction

The hybrid, channel catfish, *Ictalurus punctatus*, female X blue catfish*, I. furcatus*, male, has superior characteristics such as faster growth rate, higher survival rate, better disease resistance, low oxygen tolerance, higher carcass and fillet yield, (Dunham & Elaswad, 2018) and accounts for approximately 50-70% of all catfish cultured in the United States (USDA, nd) Moreover, hybrid catfish are known for their superior meat quality, characterized by firmer texture and lower fat content, which aligns with consumer preferences and enhances marketability (Morris et al., 1999). They also exhibit greater tolerance to a wider range of environmental stressors, including fluctuations in temperature and water quality, which improves their adaptability and survivability in diverse aquaculture settings (Tidwell et al., 1999). These combined advantages—faster growth, better feed efficiency, increased disease resistance, higher survival rates, improved meat quality, and greater environmental tolerance—make hybrid catfish a preferred choice for commercial aquaculture, offering significant economic benefits and improved product quality over traditional channel catfish farming (Boyd, 2016).

Artificial fertilization is the most common way for commercially producing hybrid catfish embryos; however, various drawbacks include intense effort, time, and the sacrifice of valuable blue catfish males. It has been demonstrated that mating a normal channel catfish female with a xenogeneic channel catfish male will generate blue catfish sperm, as an appropriate alternative for making hybrids (Perera et al., 2016), although this method also needs increased efficiency. 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) producing organisms comprised of elements typically foreign to its species known as xenogens.

This process could increase efficiency of reproduction for hybridization and reproduction efficiency in difficult to spawn species. Primordial germ cell transplantation to obtain donor-derived offspring, within and between species, has been demonstrated in various animal species, including teleost fish (Yamaha et al., 2007; Saito et al., 2008). In 2010, Saito's transplantation research demonstrated that the mechanism of primordial germ cell migration is highly conserved beyond the family barrier in fish and transplantation of a single primordial germ cell is an efficient method for producing inter-species germ-line chimeras (Saito et al., 2010).

Germ cells can be specifically labeled and isolated for culture and transplantation, providing tools for reproduction of endangered species in close relatives (Selokar et al., 2018). Xenogenesis of aquatic organism has established surrogate brood stock to produce high values and/or difficult to spawn fish (Lin et al., 1992; Ciruna et al., 2002; Takeuchi, Yoshizaki and Takeuchi, 2003, 2004; Yamaha et al., 2007; Saito et al., 2008).

To improve the application of xenogenesis in catfish, both short term and long term culture of stem cells would be beneficial. In mice, stem cells can be cultured for long time intervals in vitro without losing their functional characteristics (Mummery et al., 2014). For catfish stem cell culture protocols, similar to those for human and mouse cell culture, it is crucial to determine the optimal temperature and carbon dioxide levels that support the survival and proliferation of gonadal stem cells. This optimization would allow breeders to select superior genetic groups and clone this information into multiple triploid fish.

Other factors such as Rho-associated protein kinase 1 (ROCK I) added to cell culture media could contribute to successful culture. ROCK I is a member of the protein kinase family, sharing 45% to 50% homology with other actin cytoskeletal kinases, including myotonic dystrophy kinase, myotonic dystrophy-related Cdc42-binding kinase, and citron kinase

(Munugalavadla et al., 2007). The ROCK signaling pathway is involved in the regulation of cell division and proliferation. ROCK can phosphorylate and inactivate the myosin phosphatase, leading to increased phosphorylation of myosin light chain (MLC) and enhanced cell contractility. This influences the progression of the cell cycle and cell proliferation.

ROCK I plays a pivotal role in regulating cellular growth, adhesion, migration, metabolism, and apoptosis by controlling the assembly of the actin cytoskeleton and cell contractility (Riento and Ridley, 2003; Munugalavadla et al., 2007). Inhibition of ROCK can induce apoptotic cell death through membrane blebbing, which increases actin-myosin contraction and activates caspase signaling pathways, leading to apoptosis (Kurosawa, 2012; Cheng et al., 2015). The activation of Rho GTPase; the ROCK pathway is initiated by the activation of Rho GTPases, primarily RhoA, which can be activated by various extracellular signals, such as growth factors or mechanical stimuli (Figure 2). When activated, RhoA exchanges GDP for GTP, becoming active. The active RhoA-GTP binds to the coiled-coil region of ROCK, resulting in a conformational change that exposes the kinase domain of ROCK, allowing it to become active. Following the phosphorylation of myosin light chain (MLC) and activation of myosin by ROCK leads to the contraction of actin filaments and the reorganization of the actin cytoskeleton, promoting cell contractility and cell shape changes. This is important for processes such as cell migration and cell adhesion. ROCK signaling pathway also influences gene expression through the regulation of transcription factors and cofactors. ROCK can phosphorylate and activate several transcription factors, such as serum response factor (SRF) and myocardin-related transcription factors (MRTFs), which control the expression of genes involved in cell proliferation and differentiation.

Y-27632 is a pyridine derivative of ROCK I that has been widely used in various biological systems, including cultured cells, isolated tissues, and animal models. This chemical is soluble in distilled water and stable for at least four weeks at room temperature. Y-27632 absorption is time, temperature and saturation dependent when administered to the culture media (Köksel et al., 2005; Fernandes et al., 2006). ROCK I optimized the in vitro survival of neural progenitors produced from mice embryonic stem cells, mouse intestinal stem cells, and human keratinocytes (Fernandes et al., 2006; Koyanagi et al., 2008). After fluorescence-activated cell sorting, ROCK I was utilized to increase cell recovery of human embryonic stem cells. Cells cluster must be separated to reveal induced cells before using a fluorescence-activated cell sorting method. Due to dissociation-induced apoptosis, cell recovery efficacy after sorting will be limited. The survival rate of post-sorted cells was improved after the addition of Y-27632 was added to the plating medium. During apoptosis, ROCK-mediated force generation causes morphological alterations such as contraction, dynamic membrane blebbing, and nuclear disintegration (Coleman and Olson, 2002). In Mozambique tilapia, *Oreochromis mossambicus*, ROCK inhibitor/feeder layer supplementation has been reported to be advantageous for selecting epithelial-like cells and shortening time to immortalization (Gardell et al., 2014).

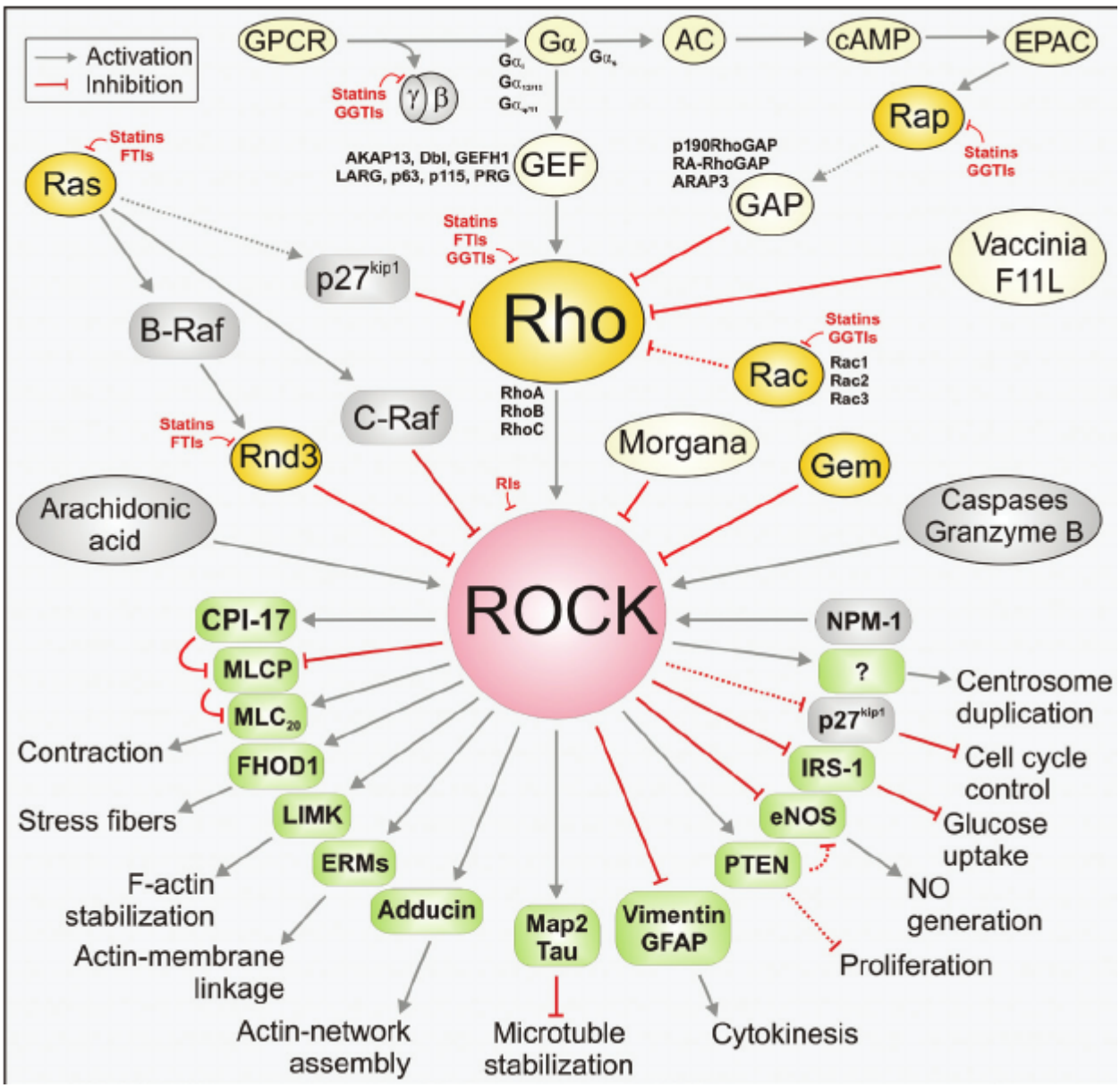


Figure 2. ROCK signaling pathway from Amin, et al. (2013).

The objectives of this study were to examine the effects of ROCK I, temperature and CO_2 on the short-term survival of catfish spermatogonia stem cells. This has implications for the preparation of these stem cells and the timing of transplantation of these stem cells into host catfish fry This study also provides an indicator of the possible importance of these factors in long-term culture of spermatogonia A.

1.2 Materials and Methods

Cell culture medium, ROCK I preparation, and incubator preparation:

Twelve 2-year-old blue catfish males (mean body weight: 478.9±114.7 g, mean testis weight: 0.08±0.03 g) were utilized to obtain stem cells. After euthanization, the external body area of fish was sterilized with 70% ethanol and then stem cells were isolated according to methods described by Shang et al. (2015). After initial data collection total length (TL), body weight (BW), and gonadal weight of fish, testes were carefully removed from the peritoneal cavity to avoid contamination with connective tissues, peritoneum, and blood vessels. Gonads were placed on a sterile petri dish (100 mm > 15 mm) containing 5 mL of anti-agent medium [Hanks' Balanced Salt solution (HBSS, SH30031.03, GE Healthcare Life Sciences, MA) supplemented with 1.0 µg/mL $NaHCO_3$ (Church & Dwight Co., NG) and 100 unit/mL Penicillin - Streptomycin (I15140-122, Life Technologies,CA)], which were then transferred to a biosafety cabinet for cleaning and sterilization. Within the biosafety hood, connective tissue and coagulated blood cells were removed from samples using sterile scalpel blades and tweezers. Gonads were rinsed three times with 1 mL of anti-agent medium and soaked in 5 mL 0.5% bleach solution prepared with double-distilled H_2O for 2 min. This was followed by three rinses with HBSS and three rinses with phosphate-buffered saline (PBS; J62692, Alfa Aesar, MA). Each gonad was then minced with a sterilized scalpel blade and transferred to a 50 mL autoclaved glass flask with a

stir bar. For each sample, 0.25% trypsin ethylenediamine tetraacetic acid (EDTA; 25200-072, Life Technologies) was added at 50 times the weight of the gonads. Samples were then incubated on crushed ice for 30 min followed by 1 h incubation at 22 °C with a magnetic stirrer to achieve higher digestion efficiency. Cell suspension from each replicate was then filtered using a 70 μm (352350, VWR International) cell strainer with mesh, 40 μm (352340, VWR International) cell strainer with nylon mesh, and centrifuged at 2900 RPM (Eppendorf Centrifuge 5418 R) for 20 min to separate cells from trypsin. The resulting supernatant was discarded and harvested pellet was resuspended in 2 mL of media Dulbecco's Modified Eagle's Medium (DMEM) (10-090-CV, Corning Cellgro) supplement 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 provide a favorable environment for the cells. Thereafter, 5 μL of the cell suspension was gently mixed with 45 μL of 0.4% Trypan Blue (15250061, Life Technologies). Cells (10 μL) were then observed under a BH2 Olympus objective microscope, supplemented with a 20x objective, to determine the total number of cells, total number of live and dead cells, total number of live and dead spermatogonial stem cells, SSCs, and total number of live and dead OSCs with the aid of a hemocytometer. A hemocytometer was utilized for cell count and cell density (cell/mL) in four corner quadrants (each 1 mm2 area) in triplicate and cell density (cells/mL) was calculated according to Louis and Siegel (2011) with the dilution factor of 10 (cell suspension: trypan blue with 1:9 ratio).

Total cell number equation (TCN) per mL:

$$(\text{total number of counted cells} \times \text{dilution factor} \times 10^4)/(\text{number of squares})$$

ROCK I was prepared following the protocol from the producer. A volume of 1.5 mL of sterile water was added into the 5 mg vial of ROCK I and mixed thoroughly to produce a 10 mM stock solution. The aliquot were frozen to be stored at -20°C or -80 °C into the working volume to avoid repeated freeze-thaw cycles. The stock solution was diluted into cell culture medium immediately before use. The concentration of the final solution (0, 25, 50, 75 μM) was prepared from the stock solution, followed the formula below.

Final Solution Concentration (FSC):

concentration of stock solution x volume of stock / volume of final solution.

Incubators were set either with 5% CO_2 or without CO_2 with various temperatures set to 24, 26, 28, and 30 degrees Celsius during the treatment trials.

Treatments of stem cells in incubator and ROCK I concentrations

Thirty-two total treatments were examined utilizing a 4 X 4 X 2 factorial design. Cells cultured with four Rock I concentrations (0,25,50,75 μM) with and without 5% medical grade carbon dioxide (CO2) at four temperatures; 24, 26, 28, and 30 ^{o}C was evaluated .

The cells in the suspension (9.8-10 x 10^5 SSCs) were divided into 6-well plates that contained cell culture medium supplemented with ROCK I at the four concentrations in cell culture media 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 provide a favorable environment for the cells. 4 wells contained 5mL of cell culture media and seeded with 6.8-7 x 10^5 SSCs. Two control wells were included within each

treatment group, a single well contained 5mL of cell culture media and one cell contained 5mL of deionized sterilized water. Three replicates of each SSCs treatment were utilized per ROCK I concentration.

All treatments plates were incubated in an incubator (VWR International Catalog Number: (10810-888)) at the previously referenced experimental temperature and CO_2 levels. During daily culture harvest, cells were sampled, stained with trypan blue, and observed under the microscope to determine stem cell viability and cell count.

During an 86-hour period, cells, including stem cells and other gonadal cells, were subjected to culture and quantified at 24-hour intervals. The cell culture media remained unchanged throughout the entire 0 to 86-hour period. Subsequently, a solitary well containing the cells was collected every 24 hours and centrifuged to generate a compact cell pellet. This pellet was subsequently resuspended in 1 mL of cell culture media to be counted with the hemocytometer.

Statistical analysis

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; PROC UNIVARIATE; SAS Institute, 2003) and homogeneity of variance (plot of residuals vs. predicted values; PROC GPLOT; SAS Institute, 2003). Stem cell data was log transformed and alpha was set at 0.05 for main effects and interactions (Zar, 1996). Temporal changes in stem cell production and Gonadosomatic Index (GSI) were analyzed using repeated measures mixed model ANOVA (PROC MIXED).

1.3 Results

At 24 hours, the viability of type A spermatogonial stem cells (SSCs) not treated with ROCK I exhibited a greater survival rate (Table 7 P= 0.0004) at 28°C with carbon dioxide (CO_2) when compared to 24°C with and without CO_2 (Table 1). When incubated at 28°C, 25 μM ROCK I with CO_2 and 50 μM ROCK I without CO_2 had exhibited a slower decline at 0.50×10^4 cells/mL and 0.42×10^4 cells/mL per hour between 24 and 48 hours respectively (Figure 8 and Figure 5) when compared to 24°C incubation without medical grade carbon dioxide 25 μM (1.04×10^4), and 50 μM 0.83×10^4 (Figure 3). Over a 48-hour timeframe, the viability of type A spermatogonial stem cells (SSCs) exhibited a longer survival rate in the presence of CO_2 compared to treatments without CO_2. The influence of CO_2 exposure demonstrated a significant impact across all interactions, including temperature, ROCK I concentration, and time (Figures 3-10). Notably, treatments conducted at warmer temperatures (26 and 28 ℃, as indicated in Tables 3-4; Figures 5-6 and Tables 7-8; Figures 9-10) exhibited prolonged survival rates in contrast to lowest and highest temperatures ($P < 0.001$). When comparing treatments utilizing 75 mM and 50 mM of ROCK I, no statically significant differences ($P>0.05$) in survivability were observed. However, distinctions became evident when comparing 0 mM and 25 mM of ROCK I treatments with those involving 50 mM and 75 mM of ROCK I ($P < 0.001$). Biological factors, such as temperature and CO_2, played significant roles in cell survivability ($P < 0.001$), while the combined effects of ROCK I with CO_2 treatments, durations exceeding 48 hours, and cooler temperatures did not exhibit similar outcomes.

As time progressed to 48 and 72 hours, significant differences were still evident between the biological factors ($P < 0.05$), however, the cells began to go through apoptosis Table 1-8 and Figures 3-11.. At 26°C cells were still surviving when exposed to CO_2 at 86 hours (Table 6)

when compared to those without (Table 2) from 0 to 50mM of ROCK I.. At 28 and 30°C cells some cells were surviving without the use of medical grade CO_2 at 50 and 75mM of ROCKI .

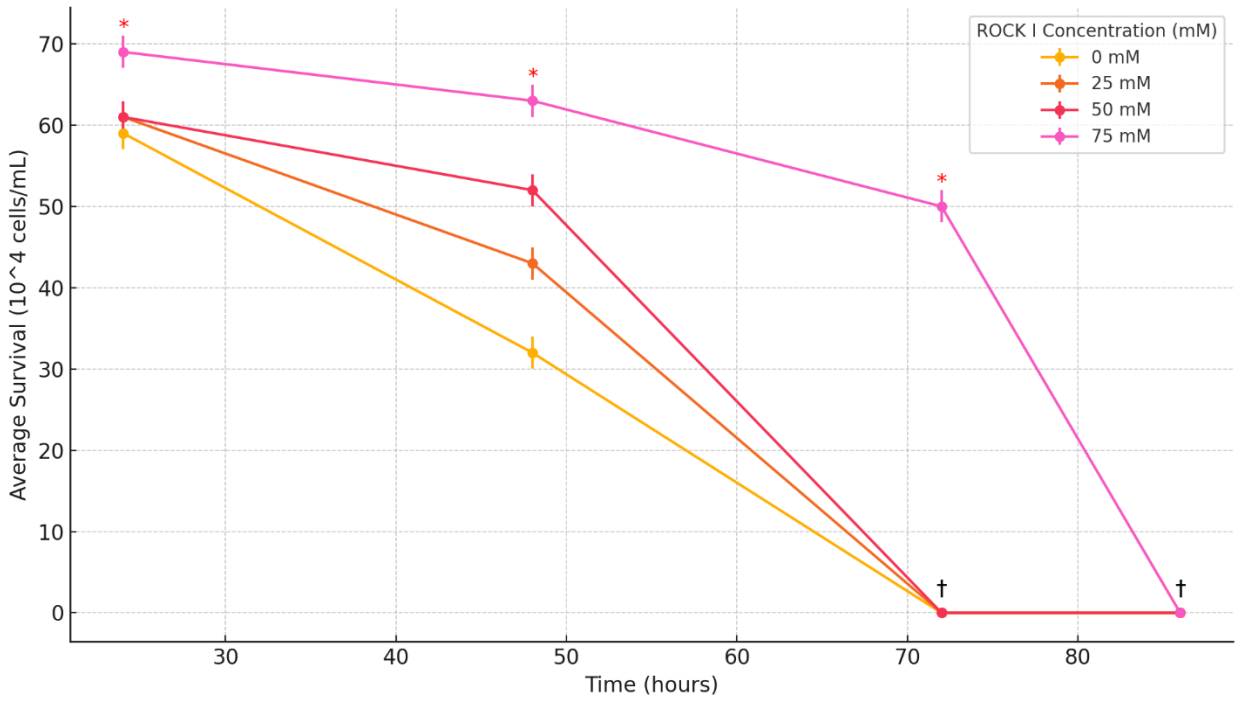
Table 1 Channel catfish, *Ictalurus punctatus*, type A spermatogonial stem cells (SSCs) mean survival ± standard deviation when incubated at 24 degrees Celsius without medical grade carbon dioxide at a ROCK I concentration gradient. All treatments were seeded with 100,000 cells (9.8-10 x 10^5 SSCs), and the results are the average percentage of three trials.

ROCK I Concentrations (mM)	Survival at 24 hours	Survival at 48 hours	Survival at 72 hours	Survival at 86 hours
0 mM	59.46 ± 1.58^{bx}	32.13 ± 1.11^{by}	0.0^{bz}	0.0^{z}
25 mM	61.32 ± 1.61^{bx}	43.09 ± 1.15^{by}	0.0^{z}	0.0^{z}
50 mM	61.24 ± 1.13^{bx}	52.25 ± 1.05^{by}	0.0^{z}	0.0^{z}
75 mM	69.31 ± 1.93^{ax}	63.05 ± 1.21^{ay}	$50.43 \pm 1.22^{a?}$	0.0^{z}

[ab]Means followed by different letters in a column are different ($p \leq 0.05$; Tukey post-hoc comparisons).

[xyz]Means followed by different letters in a row are different ($p \leq 0.05$; Tukey post-hoc comparisons).

Figure 3 Channel Catfish, *Ictalurus punctatus* Type A spermatogonial stem cells (SSCs) 86-hour survival curve when incubated at 24 degrees Celsius without medical grade carbon dioxide at a ROCK I concentration gradient



Between 24 and 48 hours, the rates of decline for the 0 mM, 25 mM, 50 mM, and 75 mM groups were approximately 0.83 x 10^4, 1.04 x 10^4, 0.83 x 10^4, and 0.21 x 10^4 cells/mL per hour, respectively. From 48 to 72 hours, the rates increased to 1.67 x 10^4, 1.25 x 10^4, 1.25 x 10^4, and 1.25 x 10^4 cells/mL per hour, respectively. This suggests a dose-dependent effect of ROCK I, with higher concentrations (75 mM) initially slowing the rate of cell death but ultimately leading to complete mortality by 86 hours. † Denotes treatments where 100% mortality was observed. * Cell survivability in the 75 mM ROCK I treatment was significantly higher at each time point compared to the 0 mM and 25 mM groups ($p \leq 0.05$), based on ANOVA with Tukey post-hoc comparisons.

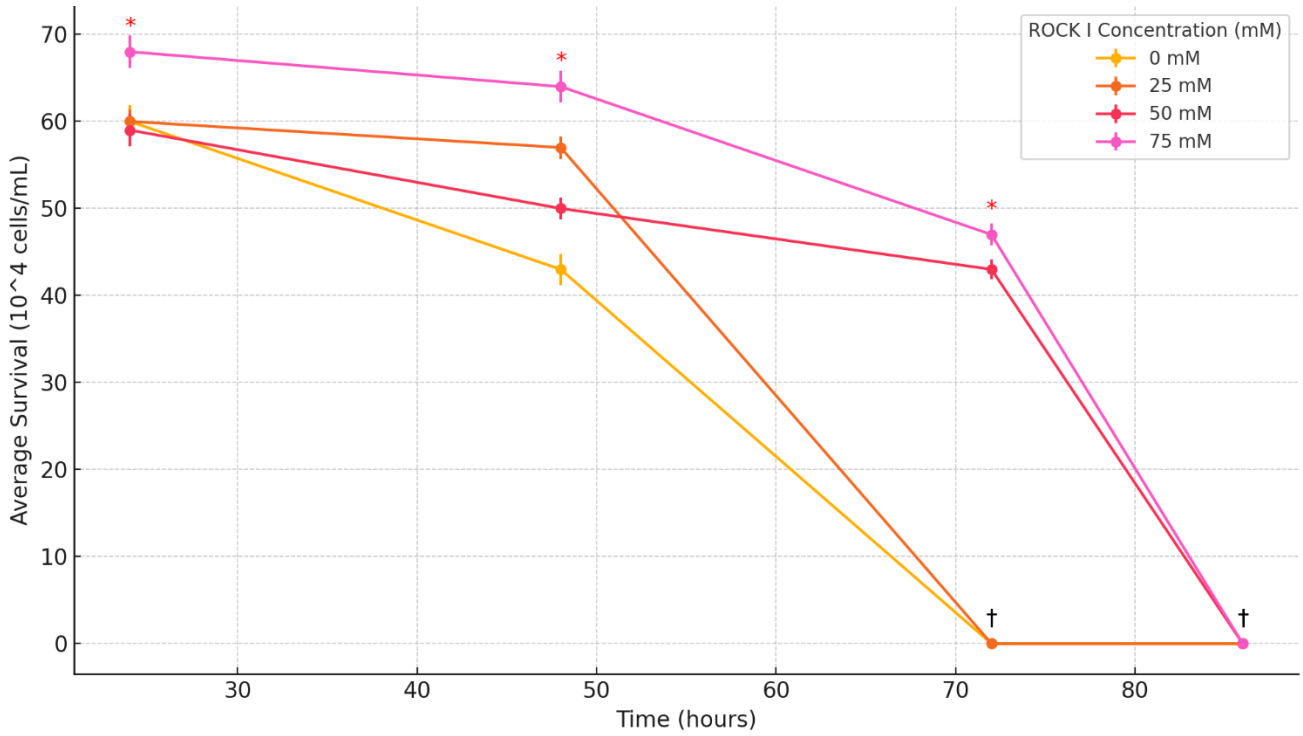
Table 2 Channel catfish, Ictalurus punctatus, type A spermatogonial stem cells (SSCs) mean survival +/- standard deviation when incubated at 26 degrees Celsius without medical grade carbon dioxide at a ROCK I concentration gradient.
All treatments were seeded with 100,000 cells (9.8-10 x 10^5 SSCs), and the results are the average percentage of three trials.

ROCK I Concentrations (mM)	Survival at 24 hours	Survival at 48 hours	Survival at 72 hours	Survival at 86 hours
0 Mm	60.45 ± 1.89^{bx}	43.08 ± 1.79^{by}	0^{y}	0
25 Mm	60.38 ± 1.43^{bx}	57..15 $\pm 1.29^{by}$	0^{y}	0
50 mM	59.13 ± 1.88^{bx}	50.27 ± 1.28^{by}	43.09 ± 1.12^{by}	0
75 Mm	68.22 ± 1.85^{ax}	64.31 ± 1.83^{ay}	47.36 ± 1.24^{ay}	0

^{ab}Means followed by different letters in a column are different ($p \leq 0.05$; Tukey post-hoc comparisons).

^{xy}Means followed by different letters in a row are different ($p \leq 0.05$; Tukey post-hoc comparisons).

Figure 4 Channel Catfish, *Ictalurus punctatus* Type A spermatogonial stem cells (SSCs) 86-hour survival curve when incubated at 26 degrees Celsius without medical grade carbon dioxide at a ROCK I concentration gradient



The rate of cell survival decline varies across treatments. For the 0 mM and 50 mM groups, the rate of decline remained consistent at 1.25×10^4 cells/mL per hour between 24 and 72 hours. In the 25 mM group, the rate of decline was highest between 24 and 48 hours at 1.46×10^4 cells/mL per hour, slowing to 0.83×10^4 cells/mL per hour between 48 and 72 hours. In contrast, the 75 mM group showed a much slower decline of 0.21×10^4 cells/mL per hour between 24 and 48 hours, but accelerated to 1.25×10^4 cells/mL per hour from 48 to 72 hours. † Denotes treatments where 100% mortality was observed. † Denotes treatments where 100% mortality was observed. * Cell survivability in the 75 mM ROCK I treatment was significantly higher at each time point compared to the 0 mM and 25 mM groups ($p \leq 0.05$), based on ANOVA with Tukey post-hoc comparisons.

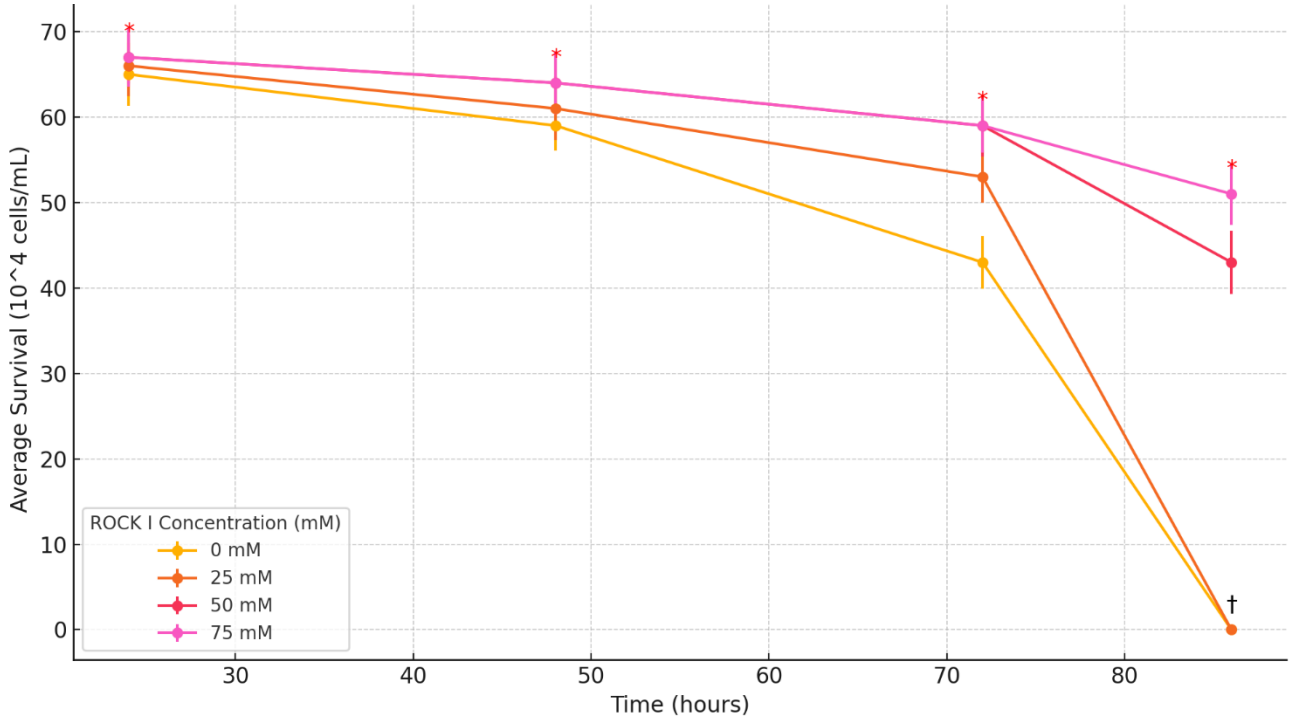
Table 3 Channel catfish, *Ictalurus punctatus*, type A spermatogonial stem cells (SSCs) mean survival +/- standard deviation when incubated at 28 degrees Celsius without medical grade carbon dioxide at a ROCK I concentration gradient.
All treatments were seeded with 100,000 cells (9.8-10 x 10^5 SSCs), and the results are the average percentage of three trials.

ROCK I Concentrations (mM)	Survival at 24 hours	Survival at 48 hours	Survival at 72 hours	Survival at 86 hours
0 mM	65.41 ± 3.71^{bx}	59.46 ± 2.95^{by}	43.11 ± 3.06^{by}	0
25 mM	66.13 ± 3.58^{bx}	61.32 ± 3.73^{by}	53.42 ± 3.03^{by}	0
50 mM	67.29 ± 3.11^{abx}	64.49 ± 3.59^{aby}	59.43 ± 3.63^{abyz}	43.44 ± 3.71^{abz}
75 mM	67.32 ± 3.43^{ax}	64.09 ± 3.66^{ay}	59.27 ± 3.16^{ayz}	51 ± 3.64^{az}

[ab]Means followed by different letters in a column are different ($p \leq 0.05$; Tukey post-hoc comparisons).

[xyz]Means followed by different letters in a row are different ($p \leq 0.05$; Tukey post-hoc comparisons).

Figure 5 Channel Catfish, *Ictalurus punctatus* Type A spermatogonial stem cells (SSCs) 86-hour survival curve when incubated at 28 degrees Celsius without medical grade carbon dioxide at a ROCK I concentration gradient



The rate of cell survival decline differs across the treatment groups. In the 0 mM group, the rate of decline between 24 and 48 hours was 0.625×10^4 cells/mL per hour, increasing to 1.46×10^4 cells/mL per hour between 48 and 72 hours. The 25 mM group showed a consistent rate of decline at 1.04×10^4 cells/mL per hour for both intervals. The 50 mM group exhibited a slower decline between 24 and 48 hours at 0.42×10^4 cells/mL per hour, accelerating to 1.04×10^4 cells/mL per hour between 48 and 72 hours. In the 75 mM group, the decline was the slowest, with 0.21×10^4 cells/mL per hour from 24 to 48 hours and 0.83×10^4 cells/mL per hour from 48 to 72 hours. † Denotes treatments where 100% mortality was observed. † Denotes treatments where 100% mortality was observed. * Cell survivability in the 75 mM ROCK I treatment was significantly higher at each time point compared to the 0 mM and 25 mM groups ($p \leq 0.05$), based on ANOVA with Tukey post-hoc comparisons.

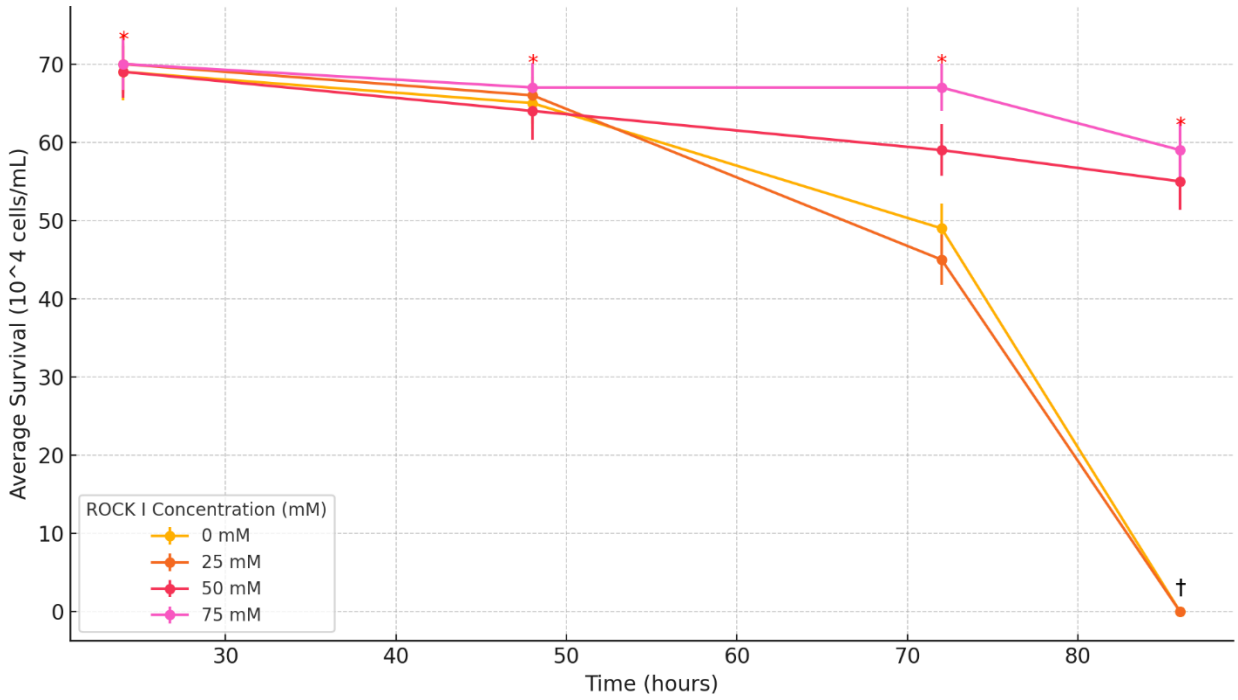
Table 4 Channel catfish, *Ictalurus punctatus*, type A spermatogonial stem cells (SSCs) mean survival +/- standard deviation when incubated at 30 degrees Celsius without medical grade carbon dioxide at a ROCK I concentration gradient.
All treatments were seeded with 100,000 cells (9.8-10 x 10^5 SSCs), and the results are the average percentage of three trials.

ROCK I Concentrations (mM)	Survival at 24 hours	Survival at 48 hours	Survival at 72 hours	Survival at 86 hours
0 mM	69 ± 3.65^{bx}	65 ± 3.34^{by}	49 ± 3.19^{bz}	0
25 mM	70 ± 3.72^{bx}	66 ± 3.59^{by}	45 ± 3.23^{bz}	0
50 mM	69 ± 3.34^{bx}	64 ± 3.67^{by}	59 ± 3.32^{bz}	55 ± 3.67^{bz}
75 mM	70 ± 3.31^{ax}	67 ± 3.14^{ay}	67 ± 3.03^{az}	59 ± 3.62^{az}

[ab]Means followed by different letters in a column are different ($p \leq 0.05$; Tukey post-hoc comparisons).

[xyz]Means followed by different letters in a row are different ($p \leq 0.05$; Tukey post-hoc comparisons).

Figure 6 Channel Catfish, *Ictalurus punctatus* Type A spermatogonial stem cells (SSCs) 86-hour survival curve when incubated at 30 degrees Celsius without medical grade carbon dioxide at a ROCK I concentration gradient



0 mM and 25 mM groups, the rate of decline between 24 and 48 hours was 00.42×10^4 cells/mL per hour, increasing to 1.67×10^4 cells/mL per hour between 48 and 72 hours. The 50 mM group exhibited a slower decline, with a rate of 0.21×10^4 cells/mL per hour between 24 and 48 hours, accelerating to 1.04×10^4 cells/mL per hour between 48 and 72 hours. The 75 mM group had the slowest rate of decline, showing 0.083×10^4 cells/mL per hour from 24 to 48 hours and 0.54×10^4 cells/mL per hour from 48 to 72 hours. † Denotes treatments where 100% mortality was observed. * Cell survivability in the 75 mM ROCK I treatment was significantly higher at each time point compared to the 0 mM and 25 mM groups ($p \leq 0.05$), based on ANOVA with Tukey post-hoc comparisons.

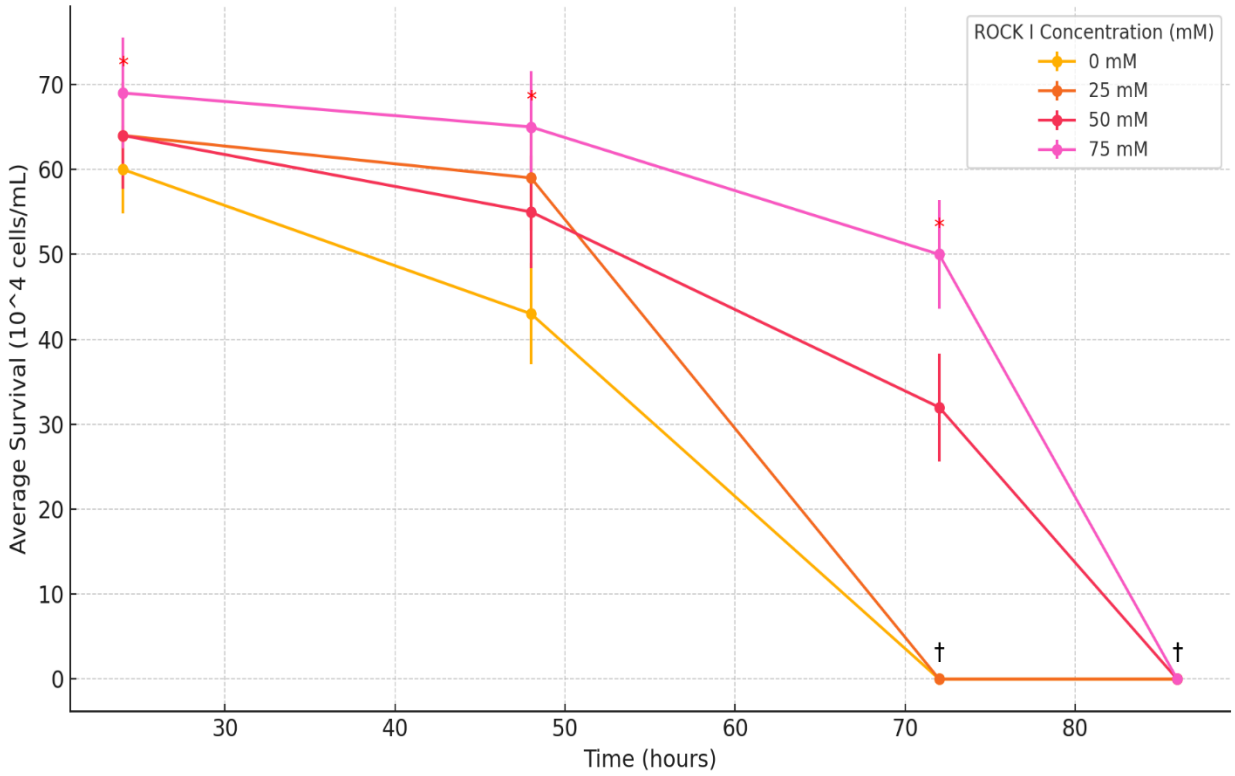
Table 5 *Ictalurus punctatus* (Channel Catfish) Type A somatic stem cells (SSCs) mean survival +/- standard deviation when incubated at 24 degrees Celsius with 5% medical grade carbon dioxide at a ROCK I concentration gradient
All treatments were seeded with 100,000 cells (9.8-10 x 105 SSCs), and the results are the average percentage of three trials.

ROCK I Concentrations (mM)	Survival at 24 hours	Survival at 48 hours	Survival at 72 hours	Survival at 86 hours
0 mM	60.37 ± 5.14^{bx}	43.05 ± 5.94^{by}	0	0
25 mM	64.06 ± 6.17^{bx}	59.47 ± 6.13^{by}	0	0
50 mM	64.18 ± 6.25^{bx}	55.26 ± 6.58^{by}	32.47 ± 6.36^{bz}	0
75 mM	69.25 ± 6.50^{ax}	65.16 ± 6.61^{ay}	50.36 ± 6.37^{az}	0

^{ab}Means followed by different letters in a column are different ($p \leq 0.05$; Tukey post-hoc comparisons).

^{xyz}Means followed by different letters in a row are different ($p \leq 0.05$; Tukey post-hoc comparisons).

Figure 7 Channel Catfish, *Ictalurus punctatus* Type A spermatogonial stem cells (SSCs) 86-hour survival curve when incubated at 24 degrees Celsius with 5% medical grade carbon dioxide at a ROCK I concentration gradient



0 mM group, the rate of decline was 0.83×10^4 cells/mL per hour between 24 and 48 hours, increasing to 1.67×10^4 cells/mL per hour between 48 and 72 hours. Similarly, in the 25 mM group, the rate of decline was 0.92×10^4 cells/mL per hour from 24 to 48 hours and 1.58×10^4 cells/mL per hour from 48 to 72 hours. The 50 mM group exhibited a slower initial decline at 0.42×10^4 cells/mL per hour from 24 to 48 hours, accelerating to 1.25×10^4 cells/mL per hour from 48 to 72 hours. The 75 mM group showed the slowest decline, with 0.21×10^4 cells/mL per hour from 24 to 48 hours and 0.83×10^4 cells/mL per hour from 48 to 72 hours. † Denotes treatments where 100% mortality was observed. * Cell survivability in the 75 mM ROCK I treatment was significantly higher at each time point compared to the 0 mM and 25 mM groups ($p \leq 0.05$), based on ANOVA with Tukey post-hoc comparisons.

Table 6 *Ictalurus punctatus* (Channel Catfish) Type A somatic stem cells (SSCs) mean survival +/- standard deviation when incubated at 26 degrees Celsius with 5% medical grade carbon dioxide at a ROCK I concentration gradient.
All treatments were seeded with 100,000 cells (9.8-10 x 105 SSCs), and the results are the average percentage of three trials.

ROCK I Concentrations (mM)	Survival at 24 hours	Survival at 48 hours	Survival at 72 hours	Survival at 86 hours
0 mM	61.31 ± 3.87^{bx}	40.14 ± 3.04^{bx}	0	0
25 mM	63.10 ± 3.69^{bx}	55.29 ± 3.87^{bx}	0	0
50 mM	65.36 ± 3.52^{bx}	62.45 ± 3.69^{bx}	54.23 ± 3.87^{bx}	32.41 ± 3.04^{bx}
75 mM	69.04 ± 3.35^{ax}	64.37 ± 3.52^{ax}	57.01 ± 3.69^{ax}	43.26 ± 3.87^{ax}

[ab]Means followed by different letters in a column are different ($p \leq 0.05$; Tukey post-hoc comparisons).

[x]Means followed by different letters in a row are different ($p \leq 0.05$; Tukey post-hoc comparisons).

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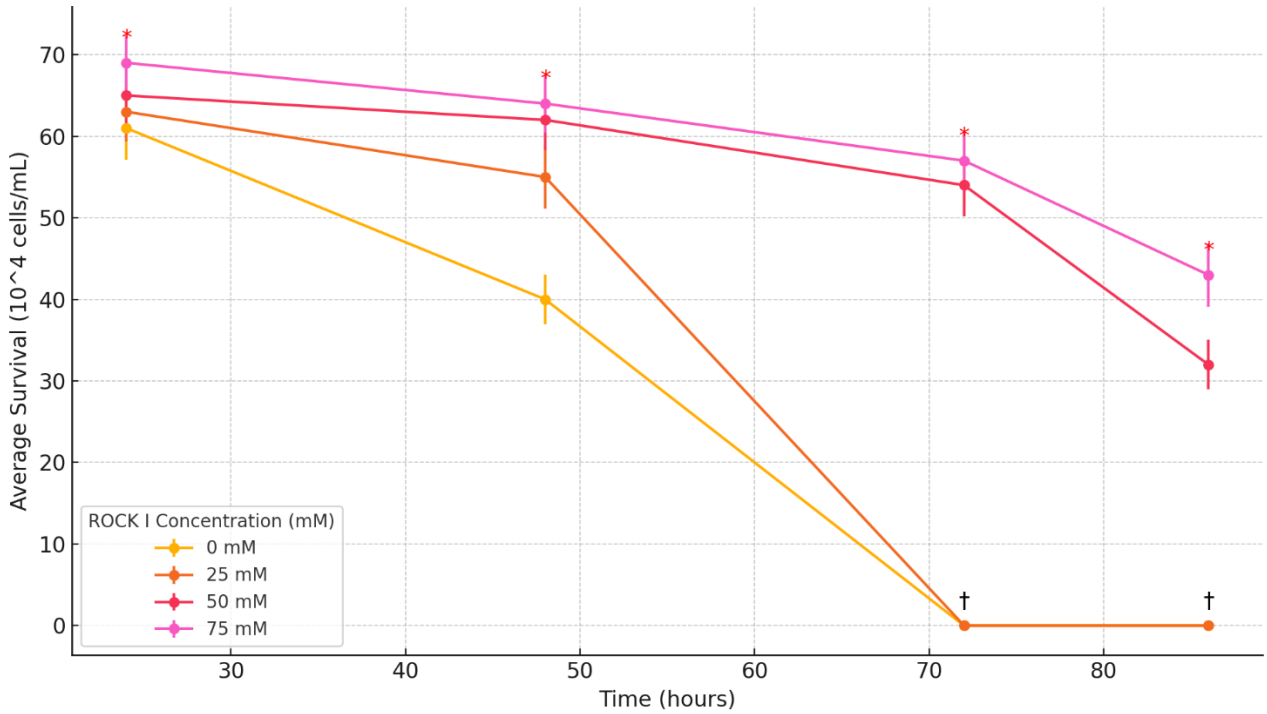
Figure 8 Channel Catfish, *Ictalurus punctatus* Type A spermatogonial stem cells (SSCs) 86-hour survival curve when incubated at 26 degrees Celsius with 5% medical grade carbon dioxide at a ROCK I concentration gradient



0 mM group, the rate of decline was 0.83×10^4cells/mL per hour between 24 and 48 hours, increasing to 1.67×10^4 cells/mL per hour between 48 and 72 hours. Similarly, in the 25 mM group, the rate of decline was 0.75×10^4 cells/mL per hour from 24 to 48 hours and 1.75×10^4 cells/mL per hour from 48 to 72 hours. The 50 mM group exhibited a slower decline at 0.21×10^4 cells/mL per hour between 24 and 48 hours, increasing to 1.07×10^4 cells/mL per hour from 72 to 86 hours. The 75 mM group had the slowest rate of decline, with 0.21×10^4 cells/mL per hour from 24 to 48 hours and 0.71×10^4 cells/mL per hour from 72 to 86 hours. † Denotes treatments where 100% mortality was observed. * Cell survivability in the 75 mM ROCK I treatment was significantly higher at each time point compared to the 0 mM and 25 mM groups ($p \leq 0.05$), based on ANOVA with Tukey post-hoc comparisons.

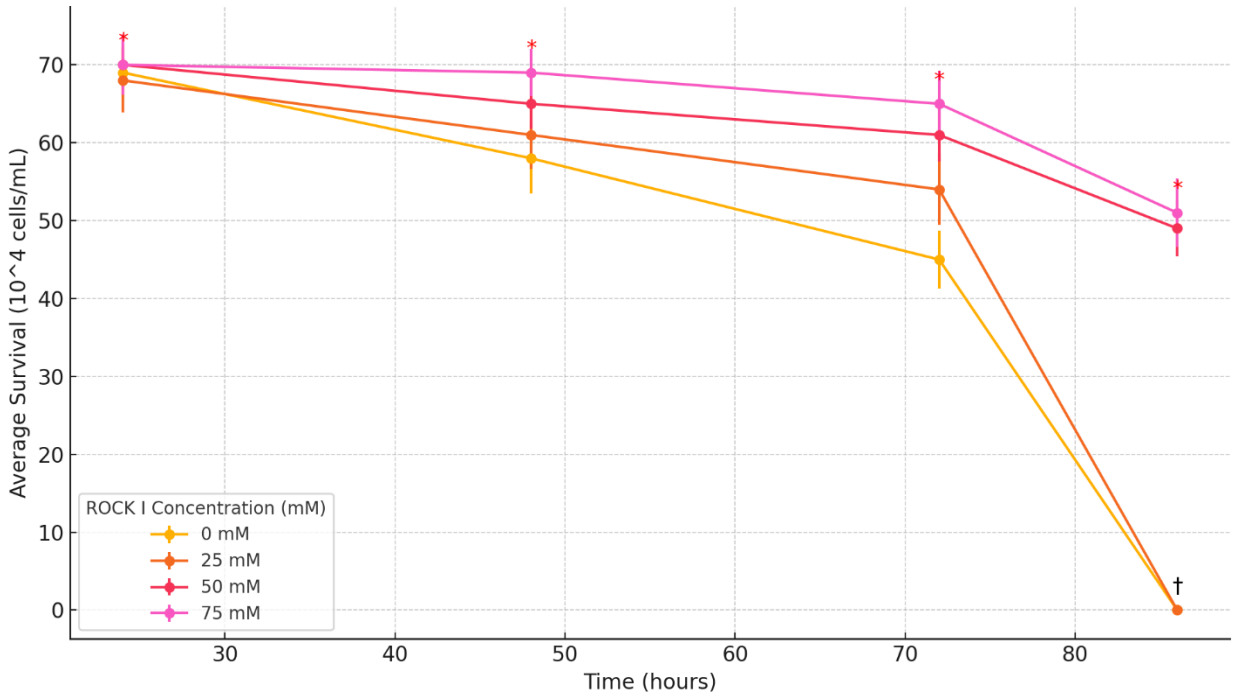
Table 7 *Ictalurus punctatus* (Channel Catfish) Type A somatic stem cells (SSCs) mean survival +/- standard deviation when incubated at 28 degrees Celsius with 5% medical grade carbon dioxide at a ROCK I concentration gradient.
All treatments were seeded with 100,000 cells (9.8-10 x 105 SSCs), and the results are the average percentage of three trials

ROCK I Concentrations (mM)	Survival at 24 hours	Survival at 48 hours	Survival at 72 hours	Survival at 86 hours
0 mM	69.14 ± 3.39^{bx}	58.15 ± 4.56^{by}	45.41 ± 3.73^{by}	0
25 mM	68.25 ± 4.12^{bx}	61.46 ± 4.39^{by}	54.10 ± 4.56^{by}	0
50 mM	70.43 ± 3.04^{bx}	65.39 ± 3.21^{by}	61.19 ± 3.39^{by}	49.16 ± 3.56^{by}
75 mM	70.38 ± 3.87^{ax}	69.15 ± 3.04^{ay}	65.39 ± 4.21^{ay}	51.33 ± 4.39^{ay}

^{ab}Means followed by different letters in a column are different ($p \leq 0.05$; Tukey post-hoc comparisons).

^{xy}Means followed by different letters in a row are different ($p \leq 0.05$; Tukey post-hoc comparisons).

Figure 9 Channel Catfish, *Ictalurus punctatus* Type A spermatogonial stem cells (SSCs) 86-hour survival curve when incubated at 28 degrees Celsius with 5% medical grade carbon dioxide at a ROCK I concentration gradient



0 mM group, the rate of decline was 0.42×10^4 cells/mL per hour between 24 and 48 hours, increasing to 1.67×10^4 cells/mL per hour between 48 and 72 hours. Similarly, in the 25 mM group, the rate of decline was 0.50×10^4 cells/mL per hour from 24 to 48 hours and 1.58×10^4 cells/mL per hour from 48 to 72 hours. The 50 mM group showed a slower decline at 0.29×10^4 cells/mL per hour between 24 and 48 hours, increasing to 1.07×10^4 cells/mL per hour from 72 to 86 hours. The 75 mM group exhibited the slowest decline, with rates of 0.21×10^4 cells/mL per hour from 24 to 48 hours and 0.57×10^4 cells/mL per hour from 72 to 86 hours. † Denotes treatments where 100% mortality was observed. * Cell survivability in the 75 mM ROCK I treatment was significantly higher at each time point compared to the 0 mM and 25 mM groups ($p \leq 0.05$), based on ANOVA with Tukey post-hoc comparisons.

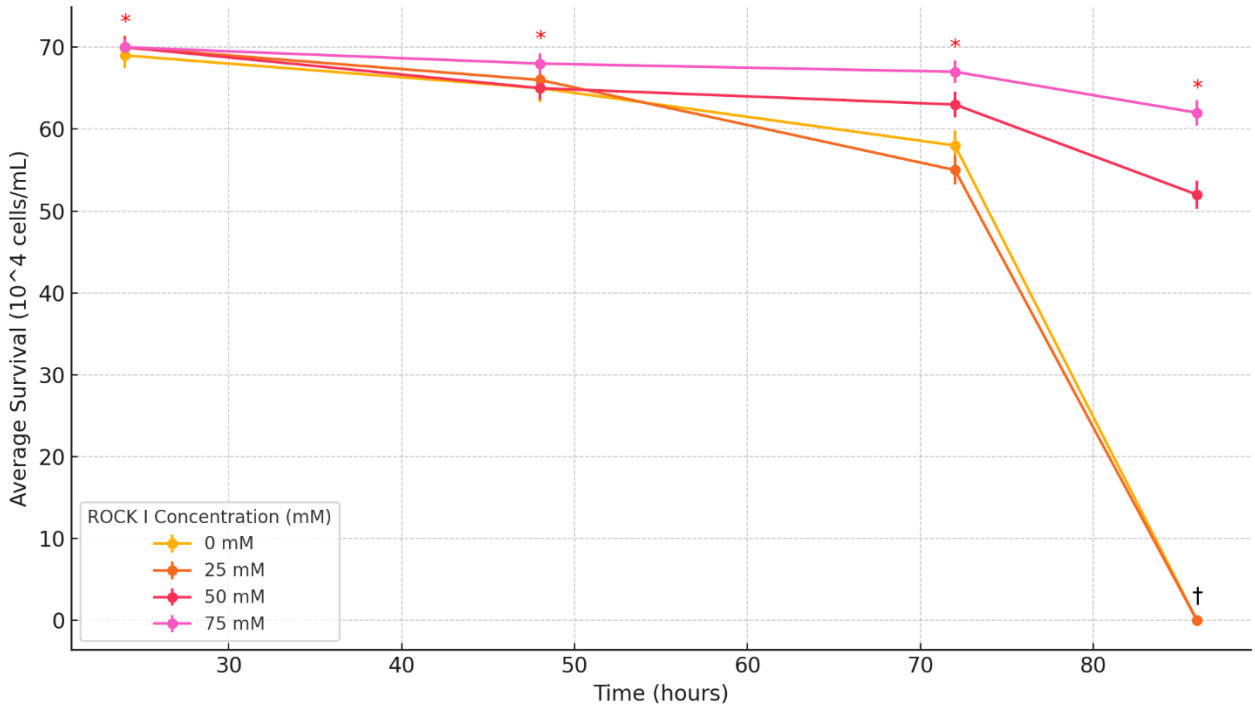
Table 8 *Ictalurus punctatus* (Channel Catfish) Type A somatic stem cells (SSCs) mean survival +/- standard deviation when incubated at 30 degrees Celsius with 5% medical grade carbon dioxide at a ROCK I concentration gradient.
All treatments were seeded with 100,000 cells (9.8-10 x 105 SSCs), and the results are the average percentage of three trials

ROCK I Concentrations (mM)	Survival at 24 hours	Survival at 48 hours	Survival at 72 hours	Survival at 86 hours
0 mM	69.09 ± 1.56^{bx}	65.13 ± 1.73^{by}	58.29 ± 1.91^{by}	0
25 mM	70.30 ± 1.39^{bx}	66.25 ± 1.56^{by}	55.04 ± 1.73^{by}	0
50 mM	70.42 ± 1.21^{bx}	65.13 ± 1.39^{by}	63.29 ± 1.56^{by}	52.30 ± 1.73^{by}
75 mM	70.15 ± 1.04^{ax}	68.14 ± 1.21^{ay}	67.13 ± 1.39^{ay}	62.35 ± 1.56^{ay}

[ab]Means followed by different letters in a column are different ($p \leq 0.05$; Tukey post-hoc comparisons).

[xy]Means followed by different letters in a row are different ($p \leq 0.05$; Tukey post-hoc comparisons).

Figure 10 Channel Catfish, *Ictalurus punctatus* Type A spermatogonial stem cells (SSCs) 86-hour survival curve when incubated at 30 degrees Celsius with 5% medical grade carbon dioxide at a ROCK I concentration gradient



0 mM and 25 mM groups, the rate of decline was 0.21×10^4 cells/mL per hour between 24 and 48 hours, increasing to 1.88×10^4 cells/mL per hour between 48 and 72 hours, followed by 0.71×10^4 cells/mL per hour from 72 to 86 hours. The 50 mM group exhibited a slower decline, with 0.13×10^4 cells/mL per hour from 24 to 48 hours, accelerating to 0.71×10^4 cells/mL per hour between 48 and 86 hours. The 75 mM group showed the slowest rate of decline, with 0.08×10^4 cells/mL per hour from 24 to 48 hours, 0.54×10^4 cells/mL per hour from 48 to 72 hours, and 0.36×10^4 cells/mL per hour from 72 to 86 hours. † Denotes treatments where 100% mortality was observed. * Cell survivability in the 75 mM ROCK I treatment was significantly higher at each time point compared to the 0 mM and 25 mM groups ($p \leq 0.05$), based on ANOVA with Tukey post-hoc comparisons.

Cell survivability was lower (P</= 0.05) at 24°C and 26 °C incubation temperatures when compared to 28°- and 30°C. The 75 mM of ROCK I had greater survival (p <=0.05) when compared with lower concentrations of ROCK I at 0- and 25-mM of ROCK I at every temperature and time point. The addition of CO_2 at 30 °C had increased (p <=0.05) cell survivability at every time interval when compared to all treatments without CO_2. Higher temperatures (28- and 30°C) and higher ROCK I concentrations, (50- and 75-mM) of ROCK I, produced a significant impact on cell survivability at 72- and 86 hours when compared to lower temperatures and lower concentrations of ROCK I. No significant difference when 50mM of ROCK I is used compared to 75 mM at all interactions.

1.4 Discussion

Stem cell research is a rapidly growing discipline with the ability to enhance therapeutic drugs for disease treatment as well as reproductive enhancement and treatment. Many of the same techniques apply to human pluripotent stem cell culture can be applied to mammalian cell to mammalian and fish germ cell cultures, supporting advancements in fields like xenogenesis (Chase and Firpo, 2007). For instance, fish germ cells, including spermatogonial stem cells, have been shown to maintain their ability to differentiate when cultured under appropriate conditions, making them viable for use in reproductive enhancement techniques (Yoshizaki et al., 2011). However, maintaining the undifferentiated state of pluripotent stem cells requires extra factors to ensure that the cells keep their essential qualities of self-renewal and pluripotency (Yao et al., 2006; McDevitt and Palecek, 2008; Borowski et al., 2012). Survival of the stem cells is obviously critical to make reproductive enhancement such as xenogenesis feasible.

In the current study, our goal was to determine what the best culture conditions were for blue catfish SSCs. Results from the current studies revealed the importance of the biological

effects, temperature and CO_2. Undifferentiated cells had better survival in a controlled environment with 5% CO_2 and a range of 28-30 ℃, consistent with previous studies that highlight the importance of CO_2 and temperature control in maintaining cell viability (Thomson, 1998; Eidet et al., 2015). The Rho protein kinase inhibitor increased the survival rate of the cells and prevented them from undergoing apoptosis, aligning with earlier research on the protective role of ROCK inhibitors in preventing cell death under stress conditions (Codogno & Meijer, 2005; Kurosawa, 2012). ROCK I enhance the survivability for cells exposed to low temperature environments without CO_2. ROCK I may have had the same effects on cells in an ideal environment, but effects were overshadowed by the warmer temperature when CO_2 is utilized. The data across all concentrations reveals a clear dose-dependent response, where higher concentrations of ROCK I result in slower rates of cell death. This dose-dependent effect is consistent with other studies showing ROCK inhibition's capacity to prolong cell survival (Riento & Ridley, 2003). The rapid decline in the 0 mM and 25 mM groups, particularly between 48 and 72 hours, contrasts with the slower and more gradual decline observed in the 50 mM and 75 mM groups, supporting the hypothesis that ROCK I inhibits apoptosis-related pathways. This aligns with findings that suggest ROCK inhibition blocks apoptotic signaling, particularly in the early stages of cell stress (Watanabe et al., 2007) . Given that the 75 mM group maintained the highest cell survival rates across all time points, it is likely that this concentration optimally supports cell membrane integrity and mitigates stress-induced apoptosis. The differences in the rates of change further suggest that ROCK I may influence specific cellular mechanisms at different stages of incubation. For example, the pronounced protection in the early time points (24 to 48 hours) at higher concentrations may involve the suppression of early apoptotic signaling, while the gradual decline after 48 hours suggests that ROCK I delays rather than fully

inhibits cell death pathways over longer durations. This is consistent Rungsiwiwut et al. (2013) findings in other stem cell models.

Stem cell culture has been established for other aquatic organisms. For culturing various cell types, several processes are required, such as thawing frozen stocks, plating cells in culture vessels, changing media, passaging, and cryopreservation (Yao et al., 2006; Borowski et al., 2012). Primary cell cultures of aquatic invertebrates have been established from a variety of tissues, including cnidarian regenerating and differentiated tissues (Ambrosone and Tortiglione, 2013; Barnay-Verdier et al., 2013), sponge tissue explants or dissociated cells (Batel et al., 1993; Akpiri, Konya and Hodges, 2017), cultures from embryonic/larval stages and different organs from marine and freshwater bivalves and gastropods (Bändel, 1988; Shin et al., 2011), various shrimp (Decapoda, Arthropoda) cell types (Bloem et al., 2007), and regenerating organs of echinoderms (Barker et al., 2009; Bely, 2010; Barnay-Verdier et al., 2013; Ballarin et al., 2018).

Innovations by researchers in Japan have resulted in mass-production of rainbow trout germline stem cells for the first time (Iwasaki-Takahashi et al., 2020). Sertoli cells were extracted from juvenile rainbow trout with a single type-A spermatogonium stage and grown in vitro to create a feeder layer (Shikina and Yoshizaki, 2010; Iwasaki-Takahashi et al., 2020). Second, they used derived feeder layer and a newly designed culture medium including rainbow trout blood plasma to establish a method for in vitro development of rainbow trout type A spermatogonium stage. Combining stem cell cryopreservation with subsequent transplantation into recipient fish is potentially a potent strategy for long-term preservation of endangered fish genetic resources. Future research with catfish might include examination of some of these approaches.

These results have significant implications for the use of ROCK inhibitors in cell culture and preservation, particularly in spermatogonial stem cells where maintaining cell viability over extended periods is critical. The ability of higher concentrations of ROCK I to slow down apoptosis suggests potential applications in a long-term culturing system to produce stem cells and preserve the spawning ability of optimum catfish males and females can be created with the use of culturing the extracted stem cells. This production system can prolong the time period in which juvenile fish can be implanted. This would allow for extraction to take place 1-2 day prior to the day of fry implantation. Short-term in vitro culturing could allow spermatogonia to recover surface proteins required for successful incorporation into the recipient genital ridge. This would further the creation of xenogeneic channel catfish to be used to produce channel catfish female X blue catfish male hybrid embryos.

Future studies should investigate the specific intracellular pathways modulated by ROCK I, as well as the potential for even higher concentrations to further extend cell viability and include stem cell – based genetic manipulation. Exploring these intracellular pathways of stem cells for genetic modification in catfish, such as gene editing using CRISPR technology. Investigating the potential of stem cells to deliver and integrate modified genetic material into catfish germline cells, enabling targeted genetic modifications for traits of interest in catfish aquaculture. In conclusion, ROCK I exhibits a clear dose-dependent effect on the survival of *Ictalurus punctatus* SSCs, with the 75 mM concentration providing the most significant protection against cell death. Due to the high cost of ROCK I this study also showed that 50mM and 75mM concentration did not have a significant difference. Additionally, optimum temperature and CO_2 levels are important. These findings highlight the potential for ROCK I to enhance the longevity of stem cells in vitro, with promising applications in cell-based therapies.

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Chapter Two

Utilization of Xenogenic Channel Catfish, *Ictalurus punctatus*, and White Catfish, *Ameiurus catus*, Hosts to Produce Blue Catfish, *Ictalurus furcatus*, and Channel Catfish Progeny

Abstract

The potential of xenogenesis utilizing *Ameiurus catus* (white catfish) and *Ictalurus punctatus* (channel catfish) to produce other types of ictalurid progeny such as channel catfish and blue catfish (*Ictalurus furcatus*) was evaluated. Several treatments, mostly those involved with utilizing primordial germ cells or low numbers of spermatogonial stem cells to produce the original xenogens had low or no success in producing donor progeny. However, implantation of 80,000 donor gonadal cells on days 3- 5 post-hatch to create xenogenic brood stock resulted in 80.0%% of these spawns producing donor progeny . Putative xenogenic brood stock produced by injecting stem cells 0-2 days post hatch did not become gravid. The fecundity, and hatch percentages and fry/kg of spawns producing donor progeny were not different (P>0.05) than spawns producing host progeny. Xenogenic white catfish produced both channel catfish and blue catfish progeny, and xenogenic channel catfish produced blue catfish progeny. The critical window for successful xenogenesis appears to be 3 to 5 post-hatch or beyond, highlighting the importance of timing in cell implantation. This is the first report of successful production of xenogenic derived donor progeny in ictalurid catfish. This study demonstrates the feasibility of cloning gonads from superior male catfish into triploid hosts, ensuring the propagation of high-quality genetic material and potentially enhancing hybrid catfish production. Future research could address long-term breeding programs and conservation efforts, utilizing xenogenic techniques to rejuvenate declining fish populations.

2.1 Introduction

As the global population increases, the demand for affordable and sustainable protein sources continues to rise (Worm et al., 2009). Overfishing of wild stocks has created a reduction in aquatic biodiversity and shifted the overall ecosystem. In response, the aquaculture industry is expanding to meet these demands (Tacon, 2019; van Doan et al., 2020) with improved techniques and technologies (Dunham, 2023) for farmers, including catfish farmers, to increase production (El Bilali, Strassner, and Ben Hassen 2021) without a rise in production footprint. Catfish farming in Alabama, Arkansas, Mississippi, and Texas accounts for nearly 70% of total United States freshwater aquaculture production (Torrans and Ott, 2019). Initially, channel catfish were almost exclusively grown in the catfish industry, but now more than 60% of production is in hybrid catfish.

Channel catfish (*Ictalurus punctatus*) female mated with blue catfish (*Ictalurus furcatus*) male hybrid catfish offspring possess favorable production traits compared to channel catfish, and the culture of hybrid catfish is more economical than that of channel catfish (Towers 2014; Perera et al., 2017a). Hybrid catfish have better performance than channel catfish and/or blue catfish for several traits such as enhanced growth rate, efficient food conversion, tolerance to low dissolved oxygen, improved disease resistance, higher survival rate, dress out percentage, fillet yield, and seinability (Dunham et al., 1983, Dunham & Masser, 1998, Bosworth et al., 2004, Phelps et al., 2011, Arias et al., 2012; Bosworth, 2012; Chatakondi et al., 2016, Perera et al., 2017b;).

Regarding growth, hybrid catfish exhibited superior growth compared to channel catfish with hybrid catfish demonstrating 41% higher final body weight than channel catfish (Giudice, 1966) at low densities in ponds. The hybrids have more favorable growth rates than channel

catfish, growing 35% more rapidly when raised under communal pond conditions and higher densities (Masser and Dunham, 1998; Argue, Liu and Dunham, 2003; Dunham, 2011). When fed at full ration, the channel x blue hybrid catfish grew faster from May to September than the purebred channel catfish due to hybrid catfish consuming a greater percentage of body weight per feeding (Green and Rawles 2010). The average cost of hybrid catfish fingerling production is 15-22.5% lower than the cost of channel catfish production (Ligeon et al. 2004; Kumar & Engle, 2011; Gosh et al., 2021).

Additional studies by Dunham et al. (1986), Masser and Dunham (1998), and Torrans and Ott (2018) further contribute to our understanding of the growth patterns and characteristics of hybrid catfish. These studies, together with Dunham et al. (1982), provide valuable insights into the uniform growth rates exhibited by hybrids compared to channel catfish. Specifically, three distinct size classes for catfish were identified, measuring 35.6 cm, 38.1 cm, and 40.6 cm. Notably, a higher percentage of hybrids (87%) fell within these size classes compared to channel catfish (76%). This consistency in growth patterns facilitates effective marketing strategies for farmers, while the uniform size of the hybrids offers advantages in output production for processing plants. However, an issue arises with "oversized" fish among hybrid and channel catfish, which can disrupt standard processing and market expectations (Gosh et al., 2021). Hybrid catfish, due to their faster growth and sometimes unpredictable size, may exceed the target size, leading to logistical challenges for processing plants that are optimized for consistent sizes (Gosh et al., 2021; Fantini-Hoag et al., 2022). Addressing this requires additional strategies for managing growth rates in hybrid catfish to ensure that their size aligns with production standards, reducing the risk of oversized fish impacting efficiency. Hybrids also have higher survival and are more tolerant to diseases as compared to channel catfish. Hybrids had greater

survival rate (93.8%) than channel catfish (85.4%) when cultured in ponds (Li et al., 2008). Hybrid fry survival or disease resistance was also greater than that of channel catfish in pond culture with channel catfish fry survival of 29.5% and hybrid catfish fry survival of 100% with the mortality primarily caused by columnaris disease (Dunham et al., 1990). When subjected to an immersion bath with *Edwardsiella ictaluri*, hybrid catfish and channel catfish had survival rates of 73.8% and 62.0%, respectively (Wolters et al., 1996). The hybrid catfish are also less susceptible to the parasitic infection from *Ichthyophthirius multifiliis* (ich or white spot disease), Flavobacterium *columnare* (columnaris disease), *Aeromonas hydrophila* and *Edwardsiella ictaluri* of catfish (ESC), compared to channel catfish (Truong and Bullard, 2021).

Hybrids exhibit increased tolerance to low dissolved oxygen (DO) (Dunham et al., 1983;). In oxygen-deprived ponds, survival rates were approximately 49.5% for the channel catfish and 92.5% for the hybrid catfish. In cages, mortality rates were about 87.5% of channel catfish and 51.0% of hybrids. Mortality rate in concrete tanks due to lethal levels of low DO was 100% for the channel catfish and 33% for the hybrids. Their resilience adds a level of insurance against mortality in the event that an aeration system is not automatic or in the event of aerator failure (Tucker & Hargreaves, 2004). The capability to withstand hypoxic conditions positions hybrid catfish as resilient species amid climate change, which is expected to exacerbate the frequency and intensity of low oxygen events in aquatic systems (Diaz & Breitburg, 2009).

Spawning technique may influence the opportunities to benefit from xenogenesis to produce hybrid catfish. To facilitate the spawning of channel catfish (*Ictalurus punctatus*) in aquaria, fish are meticulously selected and administered hormones to enhance reproductive outcomes (Graham, 1999). In controlled environments, such as 120 L rectangular fiberglass tanks, channel catfish exhibit a spawning success rate ranging from 22% to 58% when paired or

grouped (Bates & Tiersch, 1998). Furthermore, Dunham et al. (unpublished) have consistently achieved higher spawning success rates of 75% to 90% in aquaria settings. Unlike their female counterparts, male channel catfish have the capability to spawn multiple times annually under hatchery conditions, thereby increasing reproductive efficiency (Legendre et al., 1996).

Reproduction is fundamental for the production of hybrid catfish between channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*). Channel catfish typically achieve sexual maturity significantly earlier than blue catfish, with females reaching maturity at approximately three years of age, whereas blue catfish males generally do not mature until five to six years old (Tucker & Robinson, 1990).

The reproductive techniques for hybridizing channel catfish (*Ictalurus punctatus*) with blue catfish (*Ictalurus furcatus*) potentially encompass three primary methodologies: open-pond spawning, pen spawning, and artificial fertilization involving induced ovulation followed by hand stripping. Pen spawning, a more controlled variant of natural spawning, confines broodstock within smaller enclosures or pens within a larger pond, facilitating closer monitoring and potentially higher success rates. These pens, typically constructed with netting, create a confined space that allows for more precise observation and collection of fertilized eggs (Busch, 1983; Steeby, 1987; Tucker & Robinson, 1990). However, pen spawning to produce hybrids has erratic results (Dunham et al., 2000).

Recent advances in artificial spawning and assisted reproduction programs have facilitated the commercial-scale production of interspecific hybrid embryos, which exhibit superior production characteristics (Boxrucker & Kuklinski, 2006; Dunham & Masser, 2012; Bosworth, 2012). Artificial fertilization begins with the administration of hormone treatments, such as synthetic analogs of gonadotropin-releasing hormone (GnRH), to female catfish. These

hormones stimulate the maturation and release of eggs from the ovaries. Once ovulation occurs, the eggs are manually extracted through hand stripping

These gametes are then combined in a controlled environment to ensure high fertilization rates, with subsequent incubation in hatching troughs or jars to ensure optimal development (Legendre et al., 1996; Graham, 1999; Bates & Tiersch, 1998; Su et al. 2013). This method, though labor-intensive and requiring specialized expertise, allows for precise control over the breeding process, resulting in higher quality and more consistent offspring production. Su et al. (2013) have reported spawning success rates of 75% to 90% using this technique. Artificial fertilization offers significant advantages in terms of precise timing, synchronization, and control over the reproductive process. It allows for the collection of large quantities of eggs, which can be reared under optimal conditions to produce substantial numbers of fingerlings for stocking or research purposes. The artificial spawning technique has led to the establishment of the hybrid catfish industry that now produces 350 million hybrid fry per year. However, this technology is labor intensive and the male blue catfish are utilized only once after culturing them for approximately years.

Xenogenesis

Xenogenesis represents an advanced biotechnological strategy aimed at enhancing hybrid catfish embryo production through the fertilization or development of one species facilitated by the gametes or embryos of another. This approach seeks to elevate the genetic quality of cultivated fish populations, promoting improvements in growth rate, disease resistance, and other advantageous traits. By integrating desirable genetic characteristics across species, xenogenesis holds potential to streamline the production of high-performance fish stocks, contributing to sustainable aquaculture practices and improved industry outcomes. Xenogenesis involves the use

of a surrogate species to carry and produce offspring that are genetically distinct from the surrogate parents (Dunham, 2023). This process utilizes key reproductive cells such as primordial germ cells (PGCs), type A spermatogonial stem cells (SSCs), and oogonial stem cells (OSCs) (Perera et al., 2017). These cells are harvested from donor species, which possess desirable genetic traits, and transplanted into sterilized host or surrogate species (Silva et al., 2016) as depicted in Figure 2. The production of xenogeneic catfish (Figure 2) illustrates the transplantation of blue catfish germ cells into the genital ridge of the triploid channel catfish. A xenogen (an organism comprised of elements typically foreign to its species) acts as a host or surrogate to produce gametes of the donor species (Tomoyuki et al., 2006; Yoshizaki & Yazawa, 2019). After implantation the cells will proliferate and differentiate into a functional reproductive system.

Primordial germ cells (PGCs) are critical for the initial development of an organism's reproductive system. PGCs migrate during embryogenesis to the developing gonads, where they differentiate into gametes (sperm or eggs) (Ball et al., 2019). In xenogenesis, PGCs from a donor species can be transplanted into the embryos of a sterile host species, leading the host to produce sperm or eggs of the donor species. This technique is potentially and highly beneficial for aquaculture application. Type A spermatogonial stem cells (SSCs) are another option for producing xenogens. SSCs are responsible for the continuous production of sperm in male organisms. Similarly, oogonial stem cells (OSCs) are essential for the generation of oocytes or eggs in female organisms. Transplanting OSCs from a donor species into a host organism allows the host to produce eggs of the donor species, which can then be fertilized by sperm from SSC-transplanted males, ensuring the production of hybrid offspring with desired traits (Austin, 2012).

Xenogenesis has been successfully used to conserve endangered and endemic species and enhance reproductive models in various fish species. Notable examples include the zebrafish (*Danio rerio*) (Lacerda et al., 2013), rainbow trout (*Oncorhynchus mykiss*) (Bouma & Nagler, 2001; Tomoyuki et al., 2006), pejerrey (*Odontesthes bonariensis*) (Majhi et al., 2009), and nibe croaker (*Nibea mitsukurii*) (Cabrita et al., 2023). SSC transplantation has shown effectiveness across species with significant genetic divergence and closely related species. Examples include SSCs from rainbow trout injected into cherry salmon (*Oncorhynchus masou)* (Nagler et al., 2001), jundia catfish (*Rhamdia quelen*) SSCs transplanted to Nile tilapia (*Oreochromis niloticus*) (Silva et al., 2016), and *Solea senegalensis* SSCs to *Scophthalmus maximus* (Pacchiarini et al., 2014). Further instances encompass Siberian sturgeon (*Acipenser baerii*) SSCs to starlet sturgeon (*Acipenser ruthenus*) (Pšenička et al., 2015), tiger puffer (*Takifugu rubripes*) SSCs to grass puffer (*Takifugu niphobles*) (Hamasaki et al., 2017), Chinese sturgeon (*Acipenser sinensis*) SSCs to Dabry's sturgeon (*Acipenser dabryanus*) (Cao et al., 2017), and blue catfish (*Ictalurus furcatus*) SSCs to channel catfish (*Ictalurus punctatus*) (Perera et al., 2017) Procedures for oogonial stem cell (OSC) transplantation are similar to those for SSCs, and their transfer has been successfully applied in species such as rainbow trout (Yoshizaki et al., 2010), zebrafish (Rehn et al., 2011), and Siberian sturgeon (Pšenička et al., 2015). Successful transplantation of spermatogonial stem cells from blue catfish (*Ictalurus furcatus*) and channel catfish (*I. punctatus*) into triploid white catfish (*Ameiurus catus*) fry has been demonstrated (Hettiarachchi et al., 2024). Additionally, blue catfish stem cells have been effectively transplanted into triploid channel catfish, creating xenogenic fry (Hettiarachchi et al., 2023). Xenogenesis offers numerous advantages for producing hybrid catfish, including the ability to bypass reproductive barriers that complicate traditional hybridization techniques, thereby ensuring higher genetic

diversity and scalability for large-scale production. The transplantation of primordial germ cells (PGCs), SSCs, and OSCs into surrogate hosts could significantly enhance the efficiency of hybrid production and ensure the consistent propagation of desirable genetic traits, improving the overall yield and quality of hybrid catfish.

Channel catfish could function as hosts to produce blue catfish sperm (Perera et al. 2017). However, white catfish has advantageous traits, such as earlier sexual maturity compared to blue catfish and channel catfish as a xenogenic host to produce channel catfish, blue catfish and hybrids.

Figure 11 Production of xenogeneic catfish (adapted from Perera et al., 2017)

The primary objective of this research is to enhance the hybrid embryo production between channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatu*s) through the augmentation of gonadal stem cell proliferation within channel catfish, thereby enabling the production of blue catfish gametes. Alternatively, white catfish (*Ameiurus catus*) could act as a potential host for both blue and channel catfish gonadal stem cells providing an alternative system to produce hybrid. This approach also has potential advantages for generating channel catfish and blue catfish. Xenogens produced with a variety of injection parameters will be

spawned. Reproductive traits, including the percentage of spawning, relative fecundity (number of eggs per kilogram of fish), hatch rate percentage, and the number of fry per kilogram of female body weight, between the xenogeneic progeny and control fish will be evaluated. This investigation will provide valuable insights into optimizing xenogeneic progeny production in catfish and contribute to the advancement of reproductive techniques in aquaculture.

2.2 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.

Preparation of Brood Stock

Channel catfish and white catfish brood stock were maintained in 0.04-hectare earthen ponds, with an average depth of 1 meter. These ponds were equipped with a ½ horsepower surface aerator (Air-O-Lator) to ensure that the dissolved oxygen levels remained above 3 mg/L. The brood stock were fed a diet consisting of a 32% protein catfish pellet, administered at a rate of 1-2% of their body weight, five days per week. Selection of individuals for breeding was based on both health status and secondary sexual characteristics indicative of reproductive readiness. Males were identified by their well-developed papillae, large, muscular heads, and bodies that exhibited a pronounced width at the head region. Additionally, males displaying dark coloration and signs of scarring from territorial fighting were considered ideal candidates, as these features are often associated with robust reproductive health. For females, selection criteria included a soft, well-rounded abdomen, which was noticeably wider than the head, along with a swollen urogenital opening, indicating imminent spawning readiness.

To minimize stress and avoid compromising the health of the broodstock, handling was kept to a minimum, and fish were held in tanks only for the shortest duration necessary before spawning. This approach was designed to ensure the maintenance of optimal physiological conditions for successful breeding and high-quality gamete production.

Production of Triploid Channel Catfish Fry

Mature channel catfish females and males were carefully selected based on their reproductive readiness. To induce ovulation, gravid females received an intramuscular implantation of luteinizing hormone-releasing hormone analogue (LHRHa) at a dosage of 90 µg per kg body weight, following established protocols (Lambert et al., 1999; Dunham et al., 2000; Hutson, 2006; Kristanto et al., 2009). These females were then placed in spawning bags within flow-through spawning tanks. Selected males, displaying pronounced secondary sexual characteristics, were sacrificed for sperm collection.

Eggs visible outside the spawning bags signals ovulation. The ovulating females were anesthetized using buffered 100 mg/L tricaine methanesulfonate (MS-222) until opercular movement ceases. Hand stripping of the females was conducted with approximately 25 grams of eggs collected into spawning pans followed by the addition of sperm from the channel catfish males. To initiate the fertilization process, Fullers' earth solution (MP Biomedicals, Santa Ana, CA) was prepared by dissolving 6 grams of Fullers' earth powder in 1 liter of pond water at 27°C, was added to the pans to separate the eggs from the egg mass.

At three minutes post-fertilization, the fertilized eggs are transferred to a round chamber (34 cm height, 7 cm diameter) (Figure 12a). Five minutes after fertilization, the eggs were pressurized for an additional five minutes at either 7,000 or 7,500 psi to induce triploidy. Following the hydrostatic pressure shock, the eggs were transferred to tanks containing calcium

chloride ($CaCl_2$) 100–150 mg/L for a one-hour treatment before being incubated in flow-through hatching troughs equipped with paddle wheel aeration.

The triploid eggs typically hatch within 5-7 days at temperatures ranging from 25.5 to 27.4°C. Six months post-hatching, the triploidy of the channel catfish was verified using a Coulter counter to measure erythrocyte nucleic volume, a method validated by Beck and Biggers (1983).

Isolation of Donor Stem Cells from Blue Catfish

Type A spermatogonial stem cells (SSCs) and primordial germ cells (PGCs) were isolated from blue catfish. To obtain SSCs, sexually immature male blue catfish with an average body weight of 678.5±291.1g and mean testis weight of 0.43±0.22g were selected and euthanized using 300 mg/L MS-222 until opercular movement ceased. Post-euthanasia, the fish were washed with tap water, placed on ice, and dissected. The external surface was sterilized with 70% ethyl alcohol. Each fish was weighed, the abdomen was opened, and the testes were collected. The testes were weighed, washed in a 0.5% bleach solution for 1-2 minutes, and placed in a petri dish containing 5 mL of an anti-agent medium composed of Hank's Balanced Salt Solution (HBSS) with $NaHCO_3$, penicillin, and streptomycin.

Connective tissues and blood vessels were removed, and the testes were washed thrice with phosphate-buffered saline (PBS) and the anti-agent medium within a biosafety cabinet. The testes were minced with sterilized blades and transferred to autoclaved flasks containing 0.25% trypsin-EDTA. The flasks were incubated on ice for 30 minutes and then at room temperature for 60 minutes on a stirrer. The suspension was filtered using a 40-μm and then followed by a 60 - μm strainer and centrifuged at 500 g for 10 minutes. The supernatant was discarded, and the cell

pellets were re-suspended in a cell culture medium containing L-15 Leibovitz, HEPES, penicillin, streptomycin, NaHCO3, L-glutamine, ES Cell Fetal Bovine Serum, and bFGF.

The number of SSCs was determined using a microscope and hemocytometer. In addition to SSCs, PGCs from blue catfish embryos were also isolated. The procedure for collecting PGCs mirrored that of the SSCs. Midsections, including genital ridges from the embryos, were dissected before hatching and processed similarly to the SSC collection method. The number of cells in 1 mL was calculated according to Louis and Siegel (2011) with the dilution factor of 2 (cell suspension: trypan blue with 1:1 ratio). Three counts were done for each sample and the mean used for further analysis.

Total cell number equation (TCN) per mL:

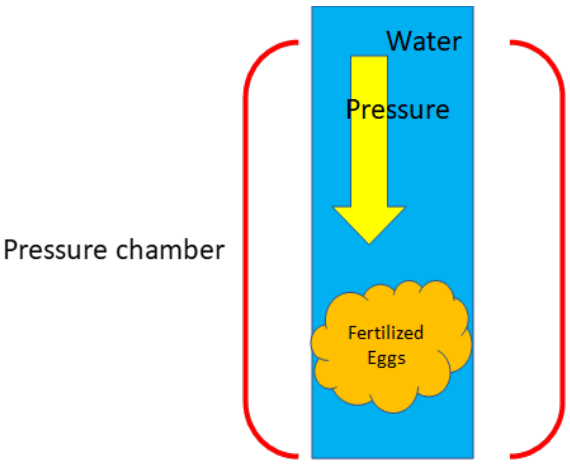
(total number of counted cells × dilution factor × 10^4) / (number of squares)

Transplantation of Sorted SSCs or PGCs into Triploid Recipients

Several treatments were evaluated including brood stock developed from blastula and day 0 DPH fry that were injected by Vo (2019). Those included blastula stage transplanted with 4,000 SSCs or 8-14 PGCs and newly hatched triploid fry injected with 7,200 cells (4,000 SSCs) or 8-14 PGCs. In the current effort triploid fry were each microinjected with 80,000 unsorted gonadal derived cells for which about 50% are expected to be SSCs (Shang et al. 2018). In the current study, the treatments injected with 80,000 unsorted cells 0-5 DPH were added. Some brood stock lost their identifying brands or pit tags but were used to increase the use of known treatments and to increase probability producing fry from putative xenogenic brood stock. Channel catfish fry, pressurized at 7,000 - 7,500 psi, were prepared for transplantation with primordial germ cells (PGCs) or spermatogonial stem cells (SSCs). Once the triploid channel fry hatched (8.9±0.5 mm in length), they were anesthetized in a solution of 10 mg/L MS-222

buffered with 10 mg/L sodium bicarbonate. The anesthetized fry were then placed in a petri dish and examined microscopically at 1.5X magnification (Amscope, Irvine, CA). Each fry was injected with 1 µL of cell suspension containing a total 7,200, 40,000, or 80,000 cells (unsorted SSCs or PGCs) using a gastight syringe attached to a repeater (Hamilton, Reno, NV) (Figure 12C). Site of injection is at the genital ridge of the triploid fry (Figure 12D). Following the injection, the fry were incubated in baskets within flow-through hatching troughs with aeration and maintained for six months before being tagged and stocked into ponds.

A: 

B: 

C: 

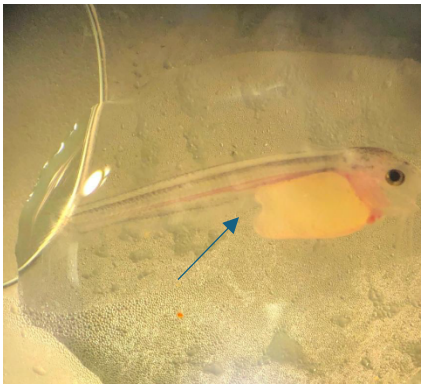
D: 

Figure 12 A Microinjection procedure for triploid channel catfish, *Ictalurus punctatus* fry used for transplantation of blue catfish, *Ictalurus furcatus*, type A spermatogonial stem cells (SSCs) or primordial germ cells (PGCs)
A: Carver press with hydrostatic pressure chamber to make triploid eggs. Five min post-fertilization, eggs were loaded into the steel chamber and then pressurized at 7,000-7,500 psi for 5 min. B: Carver pressure chamber internal mechanistic illustration. C: A gastight syringe attached to a repeater was used to transplant the unsorted SSCs into the peritoneal cavity of triploid fry. D: Channel catfish fry at 12-36 h post-hatch transplanted with germline stem cells (unsorted) with a gastight syringe attached to a repeater. The genital ridge is located on the wall of the peritoneal cavity. The total length of newly hatched fry was approximately 9 mm.

Spawning of Transplanted Fish

From 2019-2021, two to three -year-old transplanted fish were seined from ponds and selected for spawning based on their pronounced secondary sexual characteristics. These selected fish were then transferred to indoor facilities. Concurrently, normal channel catfish males and females were also chosen to mate with the transplanted individuals. The transplanted males and females were segregated and induced to spawn using luteinizing hormone-releasing hormone analogue (LHRHa) implants at a dosage of 100 μg/kg body weight, a protocol also applied to the normal channel catfish used in mating.

Individual males were paired with either normal or transplanted females in spawning aquaria measuring 91 cm x 32 cm x 61 cm, filled with approximately filled with 155-165 1liters of water ensuring controlled conditions. The water flow rate was maintained at 4 L/min with a temperature range of 26.4-27.8°C and dissolved oxygen levels of 4.8-5.7 mg/L. Post-spawning, the egg masses were collected and sampled for deoxyribonucleic acid (DNA) analysis. Subsequently, these egg masses were placed in baskets suspended in hatching troughs equipped with air diffusers and water flow systems. Formalin or chelated copper was applied at 100 mg/L

every 8 hours to prevent fungal and bacterial growth with each treatment lasting 15-30 minutes. The newly hatched fry were then sampled and transferred to rearing tanks where they were fed a diet containing 50% protein.

DNA Extraction from Eggs and Newly Hatched Fry Samples

Eggs and newly hatched fry samples were collected and immediately placed into 1.5 mL microfuge tubes on ice before being stored at -80°C. DNA extraction was performed using a proteinase K digestion method, adhering to the protocols established by Liu et al. (1998) and modified by Waldbieser and Bosworth (2008). Additionally, DNA from diploid channel catfish, blue catfish, and hybrid catfish was extracted to serve as controls.

To begin, 300 µL of cell lysis buffer—comprised of 100 mM NaCl, 10 mM Tris-HCl (pH 8), 25 mM EDTA, and 0.5% sodium dodecyl sulfate—was added to each tube containing 20-30 mg of tissue. Following this, 1.5 µL of Proteinase K (Sigma) was introduced, and the samples were incubated at 55°C for 2-4 hours. Throughout the incubation period, the tubes were periodically checked and vortexed to ensure complete dissolution of the tissue. Once the tissue was fully dissolved, the samples were vortexed for 15 seconds before adding 170 µL of protein precipitation solution. The tubes were then vortexed for 20 seconds and placed in a -20°C freezer for 10 minutes. After freezing, the samples were centrifuged at 13,200 RPM for 7 minutes, and the supernatant was transferred to new tubes containing 600 µL of 100% ethyl ethanol. These tubes were centrifuged again at 13,200 RPM, and the liquid was removed by pipetting, leaving behind a white pellet. Next, 600 µL of 75% ethyl ethanol was added to the tubes, which were then vortexed briefly and centrifuged at 13,200 RPM for 3 minutes to precipitate the DNA. After removing the ethyl ethanol by pipetting, the white pellet was resuspended in 10-100 µL of RNA/DNA-free water. The DNA concentration was measured using an ND-1000

spectrophotometer (NanoDrop Technologies). The same DNA extraction procedure was applied to the control samples from diploid channel catfish, blue catfish, and hybrid catfish.

PCR Detection of Genetic Markers

To distinguish between channel catfish, blue catfish, and their hybrids, polymerase chain reaction (PCR) was employed based on the methodology detailed by Waldbieser and Bosworth (2008). The specific primers used for PCR amplification targeting genes in channel catfish and blue catfish are outlined in Table 17. The marker genes selected for differentiation were follistatin (Fst) and hepcidin antimicrobial protein (Hamp).

PCR reactions were assembled in a 10.0 µL volume, which included 20-250 ng of genomic DNA, and the reaction mix comprised 1.0 µL of 10 mM Tris-HCl (pH 8.0), 0.4 µL of 50 mM MgCl2, 0.8 µL of 2.5 mM of each dNTP, 0.6 µL of 10 µM Fst primers, 0.3 µL of 10 µM Hamp primers, 0.1 µL of 5U/µL Platinum Taq polymerase, and 3.9 µL of water.The thermocycling protocol began with an initial denaturation step at 95°C for 3 minutes. The first amplification cycle consisted of denaturation at 95°C for 1 minute, annealing at 65°C for 1 minute, and extension at 70°C for 1 minute, repeated for 35 cycles. This was followed by a second amplification cycle with denaturation at 95°C for 30 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 1 minute, also for 35 cycles. The process concluded with a final extension at 72°C for 10 minutes.The PCR products of Fst and Hamp were visualized on a 2.0% agarose gel stained with ethidium bromide. Amplicon sizes were determined using the TrackIt™ 100 bp DNA Ladder (Invitrogen, Carlsbad, CA).

Table 9 Primers used for Fst (follistatin) and Hamp (hepcidin antimicrobial protein) genes to differentiate channel catfish, *Ictalurus punctatus*, and blue catfish, *I. furcatus*.

Primers were previously described by Waldbieser and Bosworth (2008).

			Amplicon(bp)	
			Channel Catfish	Blue Catfish
Gene	Forward Primer	Reverse Primer		
Fst	ATAGATGTAGAGGAGCATTTGAG	GTAACACTGCTGTACGGTTGAG	348	399
Hamp	ATACACCGAGGTGGAAAAGG	AAACAGAAATGGAGGCTGGAC	222	262

Statistical analysis

The dataset underwent comprehensive analysis utilizing R and RStudio with binary logistic regression employed to investigate the relationships within the data. Initially, univariable binary logistic regression models were fitted to each predictor individually. To meet the assumptions of normality and stabilize variance, the data were subjected to logarithmic transformation. The significance threshold was set at an alpha level of 0.05 for both main effects and interaction terms. Mann-Whitney U tests were used to compare the percent spawn, relative fecundity, hatch rate, and fry/kg female BW between xenogenic pairs and control pairs.

2.3 Results

Eighty percent of *Ameiurus catus* (white catfish) implanted with 40,000-80,000 donor gonadal cells on day 3 post-hatch and days 4-5 post hatch produced donor derived progeny

(Table 10-12). In the case of xenogenic channel catfish injected with 80,000 unsorted gonadal cells, 40% injected 3-4 DPH and 67% injected 4-5 DPH produced blue catfish fry (Table 15). Putative xenogenic brood stock produced by injecting stem cells after 7 days post hatch did not become gravid. None of 25 xenogenic channel catfish produced by injecting primordial germ cells produced blue catfish progeny and only 5.0% of putative xenogenic brood stock that had received 7,200 unsorted gonadally cells produced blue catfish fry. The fecundity, hatch percentages and fry/kg female BW of xenogenic spawns were comparable to those of non-xenogenic and control groups (P=0.997). Relative fecundity across treatment groups reveals that xenogenic and non-xenogenic broodstock maintain egg production comparable to controls. Xenogenic *Ameiurus catus* implanted with *Ictalurus furcatus* cells showed fecundity rates comparable to non-xenogenic groups (Table 10-12). Xenogenic *Ictalurus punctatus* treated with 80,000 cells at 4–5 DPH demonstrated fecundity levels of 1471.4 ± 532.7 eggs per female, aligning closely with the non-xenogenic groups (Table 15). Hatch rates across xenogenic and non-xenogenic groups generally fell within productive ranges, with xenogenic *Ameiurus catus* (Table 18-20) and *Ictalurus punctatus* (Table 13-15) groups achieving rates between 70% and 85%, similar to control levels at 79.5%. Fry yield per kilogram of female BW, a critical metric for reproductive productivity, remained high in both xenogenic and non-xenogenic groups, with xenogenic *Ictalurus punctatus* achieving 922.9 ± 652.6 fry/kg in 2021 (Table 15). Xenogenic *Ameiurus catus* demonstrated a similarly productive yield at 719.84 ± 254.32 fry/kg in 2021 (Table 12). Comparative statistical analyses showed no significant differences in spawning rate, relative fecundity, hatch rate, and fry yield per kilogram of female BW between xenogenic and control groups, with p-values > 0.05 across metrics

Table 10 Percentage spawning, relative fecundity, fry/kg female body weight (BW) of putative xenogenic, *Ameiurus catus* spawning pairs mated in aquaria with luteinizing hormone releasing hormone analogue (LHRHa) hormone injection in 2019.

Treatments							
Xenogenic		N	% Spawn	Relative Fecundity	Hatch %	Fry/Kg Female BW	Progeny
Female	Male						
3 DPH 80,000 unsorted cells: *Ictalurus punctatus*	3 DPH 80,000 unsorted cells: *Ictalurus punctatus*	1	100	4878.3	80.7	3936.8	*Ictalurus punctatus*
Non - Xenogenic							
Implanted 80,000 unsorted cells: *Ictalurus punctatus*	Implanted 80,000 unsorted cells: *Ictalurus punctatus*	10	61.6	4919.9 ± 2011	59.40 ± 29.2	3410.7 ± 2255	*Ameiurus catus*
Control							
Control	Control	3	100	1415.6 ± 911	82.5 ± 11.3	1167.1 ± 841	*Ameiurus catus*

Table 11 Percentage spawning, relative fecundity, fry/kg female body weight (BW) of putative xenogenic, *Ameiurus catus* spawning pairs mated in aquaria with luteinizing hormone releasing hormone analogue (LHRHa) hormone injection in 2020.

Treatments							
Xenogenic		N	% Spawn	Relative Fecundity	Hatch %	Fry/Kg Female BW	Progeny
Female	Male						
3 DPH 80,000 unsorted cells: *Ictalurus punctatus*	3 DPH 80,000 unsorted cells: *Ictalurus punctatus*	1	100	2281.3	84.9	1161.1	*Ictalurus punctatus*
Non - Xenogenic							
Implanted 80,000 unsorted cells: *Ictalurus punctatus*	Implanted 80,000 unsorted cells: *Ictalurus punctatus*	5	100	2032.2 ± 556.5	59.1 ± 7.4	1204.7 ± 453.0	*Ameiurus catus*
Implanted 80,000 unsorted cells: *Ictalurus punctatus*	No Record	2	100	19250	75.7	14578.2	*Ameiurus catus*
No Record	Implanted 80,000 unsorted cells: *Ictalurus punctatus*	3	66.6	4100.5 ± 12841.4	75.6 ± 44.4	8392 ± 8550.8	*Ameiurus catus*
Control							
Control	Control	4	100	1328.6 ± 756.5	74.3 ± 24.4	987.4 ± 344.7	*Ameiurus catus*

Table 12 Percentage spawning, relative fecundity, fry/kg female body weight (BW) of putative xenogenic, *Ameiurus catus* spawning pairs mated in aquaria with luteinizing hormone releasing hormone analogue (LHRHa) hormone injection in 2021.

Treatments							
Xenogenic		N	% Spawn	Relative Fecundity	Hatch %	Fry/Kg Female BW	Progeny
Female	Male						
4-5 DPH 40,000 unsorted cells: *Ictalurus furcatus*	4-5 DPH 40,000 unsorted cells: *Ictalurus furcatus*	4	100	904.42 ± 230.40	78.35 ± 9.01	719.84 ± 254.32	*Ictalurus furcatus* DNA
Non-Xenogenic							
3 DPH 40,000 unsorted cells: *Ictalurus punctatus*	No Record	1	100	965.3	66	637.1	*Ameiurus catus*
4 DPH 40,000 unsorted cells: *Ictalurus furcatus*	5 DPH 40,000 unsorted cells: *Ictalurus furcatus*	1	100	1090.7	74.3	810.4	*Ameiurus catus*
No Record	Implanted 80,000 unsorted cells: *Ictalurus furcatus*	1	100	770	81.8	629.9	*Ameiurus catus*
Control							
Control	Control	4	100	1044.0 ± 330.4	86.2 ± 12.1	900.3 ± 294.7	*Ameiurus catus*

Table 13 Percentage spawning, relative fecundity, fry/kg female body weight (BW) of putative xenogenic, *Ictalurus punctatus* spawning pairs mated in aquaria with luteinizing hormone releasing hormone analogue (LHRHa) hormone injection in 2019

Treatments							
Non - Xenogenic		N	% Spawn	Relative Fecundity	Hatch %	Fry/Kg Female BW	Progeny
Female	Male						
Implanted with primordial germ cells: Ictalurus furcatus	Implanted with primordial germ cells: Ictalurus furcatus	11	80.4	1990.2 ± 334.7	80.7 ± 98.4	1591.7 ± 98.4	*Ictalurus punctatus*
Control							
Control	Control	5	100	1297.4 ± 444.6	71.2	902.8 ± 105.2	*Ictalurus punctatus*

Table 14 Percentage spawning, relative fecundity, fry/kg female body weight (BW) of putative xenogenic, *Ictalurus punctatus* spawning pairs mated in aquaria with luteinizing hormone releasing hormone analogue (LHRHa) hormone injection in 2020

Treatments							
Xenogenic		N	% Spawn	Relative Fecundity	Hatch %	Fry/Kg Female BW	Progeny
Female	Male						
No Record	7000psi 7,200 unsorted cells: *Ictalurus furcatus*	1	100	2397.7	74.6	1939.2	*Ictalurus furcatus*
Non- Xenogenic							
Implanted 7,200 unsorted cells: Ictalurus furcatus	Implanted 7,200 unsorted cells: Ictalurus furcatus	9	71.4	2812.3 ± 856.7	73.8 ± 6.18	2049.24 ± 458.53	*Ictalurus punctatus*
Implanted 7,200 unsorted cells: Ictalurus furcatus	No Record	1	100	3418.0	69.4	2373.5	*Ictalurus punctatus*
No Record	Implanted 7,200 unsorted cells: Ictalurus furcatus	1	100	1560	74.2	1157.5	*Ictalurus punctatus*
Control							
Control	Control	7	100	1397.4 ± 524.21	79.5	860.3 ± 172.2	*Ictalurus punctatus*

Table 15 Percentage spawning, relative fecundity, fry/kg female body weight (BW) of putative xenogenic, *Ictalurus punctatus* spawning pairs mated in aquaria with luteinizing hormone releasing hormone analogue (LHRHa) hormone injection in 2021

Treatments							
Xenogenic		N	% Spawn	Relative Fecundity	Hatch %	Fry/Kg Female BW	Progeny
Female	Male						
4-5 DPH 80,000 unsorted cells: *Ictalurus furcatus*	4-5 DPH 80,000 unsorted cells: *Ictalurus furcatus*	2	100	1471.4 ± 532.7	84.3 ± 59.6	922.9 ± 652.6	*Ictalurus furcatus*
No Record	3 DPH 80,000 unsorted cells: *Ictalurus furcatus*	2	100	1044.73 ± 49.12	71.15 ± 5.73	744.74 ± 94.79	*Ictalurus furcatus* DNA
Non-Xenogenic							
3 DPH 80,000 unsorted cells: *Ictalurus furcatus*	4 DPH 80,000 unsorted cells: *Ictalurus furcatus*	1	100	1240	75.5	936.2	*Ictalurus punctatus*
3-4 DPH 80,000 unsorted cells: *Ictalurus furcatus*	implanted with primordial germ cells: *Ictalurus furcatus*	2	100	982.4 ± 97.6	74.87 ± 16.1	727.7 ± 84.9	*Ictalurus punctatus*

7500 psi implanted with primordial germ cells: Ictalurus furcatus	4 DPH 80,000 unsorted cells: *Ictalurus furcatus*	1	100	1467.0	69.5	1019.6	*Ictalurus punctatus*

2.4 Discussion

This study evaluated the reproductive performance of *Ameiurus catus* (white catfish) and *Ictalurus punctatus* (channel catfish) broodstock implanted with *Ictalurus furcatus* (blue catfish) cells. The reproductive performance of *Ameiurus catus* (white catfish)) broodstock implanted with *Ictalurus punctatus* (channel catfish) cells. By assessing reproductive metrics—spawning success, relative fecundity, hatch rates, and fry yield per kilogram of female body weight (BW)—across various implantation timings and cell amount this study demonstrates that xenogenic approaches can reliably produce viable donor-derived progeny, thus advancing aquaculture productivity through xenogenesis, as supported by prior research (Yoshizaki et al., 2010; Hettiarachchi et al., 2023; Hettiarachchi et al., 2024). White catfish spawns (80%) resulted in donor progeny, blue catfish or channel catfish fry, when donor cells, 40,000-80,000, were implanted 3- to 5 -days post-hatch . This was the most successful treatment, 80,000 blue catfish gonadal cells transplanted at 3-5 days post hatch, for xenogenic channel catfish , although the observed success rate, 40-67% of brood stock producing donor fry, was lower than that for white catfish. This could have implications for which xenogenesis system is best for deployment. None of the xenogens microinjected with 7,200, 40,000, or 80,000 gonadal cells after 7 DPH became gravid indicative of inadequate colonization and/or proliferation when stem cells are introduced at that developmental stage. This aligns with Yoshizaki et al.'s (2010) work, which highlighted

improved germ cell integration with fry and similar findings in medaka (Yi et al., 2010) Transplantation of primordial germ cells into triploid channel catfish failed to produce xenogens that produced donor fry, and only 5.0% of those injected with 7,200 unsorted gonadal cells produced blue catfish fry. Relative fecundity, hatch rate, and fry/kg female BW are important factors for determining the most effective xenogenesis system for ictalurids and other families of fish. Xenogenic *Ameiurus catus* exhibited fecundity levels comparable to non-xenogenic groups. Likewise, xenogenic Ictalurus punctatus implanted with 80,000 cells at 4–5 DPH showed fecundity of 1471.4 ± 532.7 eggs per female, consistent with non-xenogenic levels. This outcome suggests that xenogenic transplantation can maintain fecundity under optimized conditions (Silva et al., 2016; Lacerda et al., 2018). Hatch rates across xenogenic and non-xenogenic groups generally fell within productive ranges, with *Ameiurus catus* and *Ictalurus punctatus* achieving hatch rates between 70% and 85%, which was close to the control level of 79.5%. Hatch rates in xenogenic broodstock injected at 3-5 DPH underscore the efficacy of xenogenic methods in producing viable progeny. This is supported by Saito et al. (2008), who demonstrated that timing in germ cell transplantation can influence the viability of resulting progeny. Vo (2019) produced donor progeny via xenogenesis in Ictalurus spp., but the current study achieved improved success rates for the best treatments, highlighting the refined methodologies and effectiveness of timing in the current research Hettiarachchi et al. (2023 & 2024). Fry yield per kilogram of female body weight (BW), a key productivity metric, remained high across xenogenic, non-xenogenic, and control groups. Xenogenic *Ictalurus punctatus* achieved a yield of 922.9 ± 652.6 fry/kg, while xenogenic *Ameiurus catus* showed a similar yield of 719.84 ± 254.32 fry/kg. These values affirm that xenogenic techniques can sustain reproductive output levels similar to traditional broodstock, aligning with the results of Bartley et

al. (2000) who noted the potential of xenogenic interventions to maintain fry productivity without compromising host reproductive efficiency.

The results align with findings from xenogenic research in other species, including zebrafish, medaka, and salmonids (Takeuchi et al., 2004; Yoshizaki et al., 2011; Lacerda et al., 2018; Hettiarachchi et al., 2024), demonstrating the feasibility of germ cell transplantation across fish species. Previous work in salmonids and medaka by Takeuchi et al. (2004) and Yi et al. (2010) also supports the current study's outcomes, as xenogenesis proved effective in enhancing production of donor-derived progeny. The success rates for Ictalurus punctatus were slightly lower than for Ameiurus catus, highlighting the potential for refined system selection depending on species-specific applications.

DNA analysis confirmed that progeny from xenogenic Ameiurus catus were Ictalurus furcatus, validating the efficacy of donor-derived progeny production. In contrast, control groups produced progeny consistent with their host species, verifying the genetic specificity of xenogenic progeny. Such genetic verification aligns with prior research (Saito et al., 2008) and supports the reliability of xenogenesis for generating species-specific offspring within a surrogate system.

These results demonstrate the potential of xenogenesis to produce donor progeny from later-maturing fish species through surrogacy in faster-maturing species. For example, xenogenic *Ameiurus catus* achieved sexual maturity within three years and produced *Ictalurus furcatus* fry, compared to the 4–6 years required for *Ictalurus furcatus* to mature. This finding suggests significant potential for genetic improvement programs in aquaculture, where delayed maturity in donor species presents limitations. Moving forward, xenogenesis could support innovative breeding programs by preserving elite germ lines and promoting genetic diversity in aquaculture

The timing of cell implantation is critical with day three to day five post-hatch resulting in the greatest spawning percentage of the resulting xenogenic brood stock. Based on the measurement of stem cell colonization and proliferation in xenogenic channel catfish and white catfish, Hettiarachchi et al. (2023;2024) generated a similar outcome, 4-6 DPH.

Moving forward, these advancements could be applied in innovative ways in short- and long-term breeding program. One option is the preservation and cloning of the spawning capabilities of the best males and females from diverse family lines. Applications also lie in conservation efforts. For instance, xenogenic fish could be utilized to rejuvenate declining populations. The current study serves as proof of principle as one species functioned as a surrogate to produce progeny of another species. This xenogenic technique can also be used to produce hybrid embryos. The precision and success of our methodologies demonstrate the significant potential for these techniques to enhance the efficiency and sustainability of aquaculture practices. Several key factors for further enhancing xenogenesis in catfish include further refinement of identifying optimal implantation days, improved techniques for implanting cells into the host, improving culture of stem cells and perfecting the process for extracting cells from the donor parent.

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Chapter Three

Production of Triploid Common Carp, *Cyprinus carpio*, using Hydrostatic Pressure and Xenogenic Common Carp by Injection of Catfish Stem Cells

Abstract

The production of xenogenic common carp (*Cyprinus carpio*) capable of producing catfish gametes marks a significant advancement in aquaculture. This study explores the potentional of utilizing xenogenic common carp (*Cyprinus carpio*) to produce gametes from channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and hybrid catfish (*I. punctatus* female x *Ictalurus furcatus* male) , thus transforming common carp into ictalurid sperm factories, thereby eliminating the need to sacrifice male blue catfish to make hybrid embryos. By employing hydrostatic pressure to produce 99% triploid common carp, we have simplified gamete extraction. Of 60 individuals sampled, 17, 12 and 10 had gonads containing DNA from hybrid catfish blue catfish and channel catfish, respectively. stem cells (10). The mean days post hatch (DPH) for xenogenic carp was 22.5 days. A chi-square test ($\chi^2 = 8.24$, $p = 0.016$) and ANOVA ($F = 4.76$, $p = 0.012$) confirmed a significant association between cell type and xenogenesis. Logistic regression further demonstrated that lower DPH and Hybrid stem cells significantly increased xenogenic likelihood ($p < 0.05$). No significant sex bias was observed, with a nearly equal distribution of male and female xenogenic carp. These findings highlight the potential of using xenogenic carp in aquaculture, offering a sustainable and efficient method for hybrid catfish production. The study provides critical insights into optimizing xenogenesis, paving the way for future research and commercial application in aquaculture.

3.1 Introduction

Aquaculture boasts a rich history, spanning many millennias with practices deeply embedded in human civilization. Wild common carp (*Cyprinus carpio*) inhabit various aquatic environments, including river middle and lower reaches, floodplains, lakes, oxbow lakes, and reservoirs. Although primarily bottom feeders, carp will forage throughout the water column. In Europe, carp ponds are typically shallow and eutrophic, with muddy bottoms and abundant aquatic vegetation (Arlinghaus & Mehner, 2003). The adaptability of carp to diverse ecological conditions has facilitated their widespread cultivation. For over 8,000 years, carp have served as a cultivated food source (Harland 2019), ornamental garden element, and symbol of strength in Asia (Harland, 2019).

The mid-1800s saw waves of immigrants introducing common carp to the United States, where a fish once prized, was now despised. By the early 20th century, both public agencies and sportsmen increasingly viewed common carp (*Cyprinus carpio*) as an invasive species detrimental to aquatic ecosystems. Despite large-scale efforts to harvest carp from local waters, their taste was considered inferior to that of selectively bred European carp or native game fish, reducing their appeal as a food source (Chick, 2001; Kopf et al., 2017). Moreover, rapid carp population growth contributed to declining water quality and threatened biodiversity, as their presence was associated with the deterioration of clear, nutrient-rich lakes and waterways (Doherty et al., 2016)

With annual production exceeding 3 million metric tons (Blvd et al., n.d.; FAO, 2020), various strains of common carp, including Koi, Oujiang color carp, and Hebao red carp, have gained popularity as ornamental fish due to their diverse skin colors and scale patterns. The economic importance of common carp has driven extensive research into their physiology,

development, immunology, disease control, genetic breeding, and transgenic manipulation (Rinchard & Kestemont, 1996; Bianka Tóth et al., 2020). Additionally, common carp are frequently utilized as model organisms in ecological, environmental toxicological, evolutionary, and breeding/spawning studies.

Artificial Spawning

Artificial spawning of common carp in captivity is a well-established practice aimed at maximizing reproductive efficiency and ensuring mass production. In commercial aquaculture, this process is often initiated through hypophysation, where lyophilized pituitary extract is injected into the fish (Sigal Drori et al., 1994; Lin et al., 1995). This method induces spawning by promoting gonad maturation and the synthesis of sex steroids through the gonadotropic hormones present in the pituitary extract (Salar Dorafshan et al., 2003). During this procedure, both male and female common carp are prepared for stripping, a technique where sperm and eggs are manually extracted. The fertilized eggs are then treated to reduce stickiness, a crucial step to prevent clumping and ensure better aeration and development during incubation (Site, 2009). Artificial spawning in captivity not only ensures a high yield of fry but also allows for the precise control of genetic lines and the timing of production cycles (Rinchard & Kestemont, 1996).

Triploidy is a technique successfully applied to species such as catfish, zebrafish, trout, salmon, and loach (Phelps et al., 2011; Perera et al., 2017; Hettiarachchi et al., 2024), resulting in sterilization . Triploid fish, possessing three sets of chromosomes instead of the usual two found in diploid fish, are rendered sterile, preventing their reproduction and subsequent population growth. Triploidy is achieved through several techniques, each with specific methodologies and outcomes.

One common method involves the application of physical shocks, such as temperature or pressure, to newly fertilized eggs, which disrupts normal cell division and results in the retention of an extra set of chromosomes (Benfey, 1999). Chemical treatments using agents like colchicine can also induce triploidy by interfering with the spindle apparatus during cell division (Chourrout et al., 1986). These procedures need to be precisely timed and carefully managed to ensure the successful creation of triploid individuals. The first goal of this study is to create triploidy common carp through the use of physical shocks by applying pressure to the fertilized eggs. This is a critical first step, generating a sterile host, for xenogenesis. Triploid induction can be difficult for common carp due to the narrow window of time required for effective physical or chemical treatment during early embryonic development, the adhesive nature of the eggs that complicates uniform exposure to treatment, and the sensitivity of embryos to induced stress, which can significantly reduce survival rates.

Hybrid Catfish

The hybrid catfish, channel catfish (*Ictalurus punctatus*) female crossed with the blue catfish (*I. furcatus*) male, is of significant commercial importance in aquaculture. One of the primary reasons for the commercial success of hybrid catfish is their enhanced performance traits compared to their parent species. Hybrid catfish exhibit superior growth rates, higher feed conversion efficiency, and greater resistance to diseases. These traits make them highly desirable for commercial production, as they lead to increased yield and profitability. Moreover, hybrid catfish have improved tolerance to environmental stressors, which is crucial for maintaining high survival rates in varying aquaculture conditions (Dunham et al., 1990; Bosworth, 2012).

However, creating hybrid catfish presents several challenges. The primary difficulty lies in the reproductive incompatibility between the parent species. Channel catfish and blue catfish

have different breeding behaviors and environmental requirements for spawning, making natural hybridization rare (Dunham et al., 1990) . To overcome this, artificial spawning techniques must be employed. These techniques involve hormone-induced spawning, manual stripping of eggs and sperm, and careful fertilization processes to ensure successful hybridization (Su et al. 2013). The current protocol is labor intensive and requires the sacrifice of blue catfish males to obtain sperm for a one-time use after growing them for 4-7 years.

An alternative approach is xenogenesis, 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). Offspring develop from transplanted germline stem cells from donor fish. This process can utilize various types of germline stem cells, such as primordial germ cells (PGCs), spermatogonial stem cells (SSCs), and oogonial stem cells (OSCs), extracted from donor diploid fish and transplanted into sterile hosts. These transplanted cells enable the host to produce donor-derived gametes, bypassing the host's sterility. The second goal of this research was to produce xenogenic common carp capable of producing blue catfish or channel catfish milt. This could lead to a new, innovative and more efficient technology for making hybrid catfish embryos and possibly eliminate the need of blue catfish on the farm.

Objective of Research

The objective of this research is to engineer xenogenic common carp (*Cyprinus carpio*) that are capable of developing functional gonads of channel (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*) through the transplantation of catfish spermatogonial stem cells (SSCs). By establishing surrogate carp that can produce viable catfish gametes, we aim to evaluate the feasibility of interspecies xenogenesis within aquaculture, potentially crossing *Cyprinidae* (carp) and *Ictaluridae* (catfish) to expand breeding versatility.

This innovative approach could greatly enhance aquaculture by overcoming the reproductive challenges posed by species-specific environmental and behavioral requirements, enabling surrogate-based propagation of catfish without the need for direct breeding of the species. Success in this endeavor would provide valuable insights into the biological compatibility of species across taxonomic families, paving the way for conservation efforts, enhanced genetic diversity, and selective breeding.

3.2 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.

Spawning common carp

Twenty healthy, disease-free, and fully mature common carp, 10 females and 10 males, between 2 to 4 years of age were selected for spawning. These spawners were seined and transferred to greenhouse tanks for acclimatization. After a 24-hour acclimation period, the female common carp showing signs of readiness for spawning, such as a swollen abdomen, were randomly divided into two groups and placed in upstream sections of two separate tanks. Similarly, the male common carp were divided into groups and placed downstream from the females to facilitate controlled exposure..

To induce spawning, common carp were subjected to hypophysation, a technique involving the injection of lyophilized carp pituitary extract. The injection is typically administered intramuscularly or intraperitoneally, often in the evening to align with the natural spawning rhythms of the fish. Hormone injections were 2 to 4 mg/kg body weight for females and 1 to 2

mg/kg for males. Following hormone injection, priming dose of about 0.5 to 1 mg/kg, followed by a larger resolving dose of the remaining 1.5 to 3 mg/kg approximately 24 hours later, males and females were monitored closely. Males exhibited increased activity, while females laid a few adhesive eggs on the side of the tanks. The females were anesthetized using buffered 100 mg/L tricaine methanesulfonate (MS-222) until opercular movement slowed. Hand stripping of the females was conducted, with approximately 25 grams of eggs collected into spawning pans. The eggs were then fertilized with sperm from the common carp males. To initiate the fertilization process, Fullers' earth solution (MP Biomedicals, Santa Ana, CA), prepared by dissolving 6 grams of Fullers' earth powder in 1 liter of pond water at 27°C, was added to the pans.

Production of Triploid Common Carp Fry

Three minutes post-fertilization, the fertilized eggs were transferred to a round chamber (34 cm height, 7 cm diameter) and placed on a Carver press to generate hydrostatic pressure. Five minutes after fertilization, eggs were pressure shocked for 5 min at 8,500 psi to induce triploidy.

Incubation

After completing pressure shock, eggs were removed from the chamber, placed in tanks supplemented with calcium chloride ($CaCl_2$) for 1 hour. Embryos were placed in four-liter tubs of Holtfreter’s solution with 10 mg/L doxycycline kept at 27°C with continuous aeration. The solution was changed, and dead embryos were removed daily. After about 5 days, or when the embryos were moving rapidly within the egg membrane and close to hatch, doxycycline treatment was discontinued. At 20 dph fry were moved to aquaria in recirculating systems until

large enough to be PIT (Passive Integrated Transponders) tagged and then moved to earthen ponds.

Isolation of Donor Stem Cells from Blue Catfish, Channel Catfish, and Hybrid Catfish

Type A spermatogonial stem cells (SSCs) were isolated from blue catfish ,channel catfish and hybrid catfish. To obtain SSCs, sexually immature male blue catfish and hybrid catfish with an average body weight of 678.5±291.1g and mean testis weight of 0.43±0.22g were selected and euthanized using 300 mg/L MS-222 until opercular movement ceased. To obtain SSCs, sexually immature male channel catfish with an average body weight of 691.3±182.6g and mean testis weight of 0.56±0.69g were selected and euthanized using 300 mg/L MS-222 until opercular movement ceased Post-euthanasia, the fish were washed with tap water, placed on ice, and dissected. The external surface was sterilized with 70% ethyl alcohol. Each fish was weighed, the abdomen was opened, and the testes were collected. The testes were weighed, washed in a 0.5% bleach solution for 1-2 minutes, and placed in a petri dish containing 5 mL of an anti-agent medium composed of Hank's Balanced Salt Solution (HBSS) with $NaHCO_3$, penicillin, and streptomycin.

Within a biosafety cabinet, connective tissues and blood vessels were removed, and the testes were washed thrice with phosphate-buffered saline (PBS) and the anti-agent medium. The testes were minced with sterilized blades and transferred to autoclaved flasks containing 0.25% trypsin-EDTA. The flasks were incubated on ice for 30 minutes and then at room temperature for 60 minutes on a stirrer. The suspension was filtered using a 40-µm and then 60 -µm strainer and centrifuged at 500 g for 10 minutes. The supernatant was discarded, and the cell pellets were re-suspended in a cell culture medium containing L-15 Leibovitz, HEPES, penicillin, streptomycin, NaHCO3, L-glutamine, ES Cell Fetal Bovine Serum, and bFGF.

The number of SSCs was determined using a microscope and hemocytometer. The number of cells in 1 mL was calculated according to Louis and Siegel (2011) with the dilution factor of 2 (cell suspension: trypan blue with 1:1 ratio). Three counts were done for each sample and the mean used for further analysis.

Transplantation of Sorted SSCs or PGCs into Triploid Recipients

Putative triploid common carp fry were injected 0 days post hatch to 60 days post hatch with catfish stem cells. They were anesthetized in a solution of 10 mg/L MS-222 buffered with 10 mg/L sodium bicarbonate. The anesthetized fry were then placed in a petri dish and examined microscopically at 1.5X magnification (Amscope, Irvine, CA). Each fry received an injection of 1 μL of unsorted stem cell solution containing total 80,000 cells via a 33-gauge needle and a gastight syringe. The injection site was the cavity between the anal fin and yolk sac, where the genital ridge was anticipated to form (Figure 13). Thirty common carp fry were individually injected once, this was done daily with fresh blue catfish or hybrid catfish SSCs, while 30 common carp fry were injected with channel catfish SSCs. The injections period was from 0 day post hatch to 60 days pot hatch. The treatment groups were kept separately after the injections. The fry were transferred to recovery troughs with aeration and maintained for six months before being tagged, half of each treatment group were stocked into ponds.

Figure 13 Juvenile common carp (*Cyprinus carpio*) that was implanted with catfish spermatogonia A stem cells 19 days post hatch at approximately 17mm in total length. The injection consisted of 1 μL of unsorted catfish stem cell solution containing a total of 80,000 cells via a 33-gauge needle and a gastight syringe.

Sperm and Egg Collection

One year after SSC implantation sperm and egg samples were collected from randomly selected treated common carp. Female common carp selected for egg collection were first acclimated to the hatchery environment. During the spawning season, when water temperatures range between 22-25°C, females displaying signs of ripeness, such as a swollen abdomen. To induce ovulation, females were administered hormonal injections, carp pituitary extract (CPE). Approximately, 12-24 hours post hormone -injection, the females were ready for egg collection. The abdomen was

carefully massaged in a downward motion towards the genital opening, allowing the eggs to be expelled into a clean, dry container (figure 13A & B).

A:  B: 

Figure 14.

A: Hand stripping 1 year old potential xenogenic female common carp, *Cyprinus carpio*. Eggs were visibly larger than urogenital, making it difficult for them to be expelled.

B: Hand stripping of 1-yea- old potential xenogenic male common carp, *Cyprinus carpio*.

The injections were administered intramuscularly. Careful monitoring follows to observe behavioral changes and physical signs of spawning readiness, such as increased activity. To collect the milt, the abdomen was pressed gently towards the genital opening, and the milt was collected directly into a clean, dry container (Figure 14B). Eggs were collected from the females in a similar way (Figure 14A).

DNA Extraction from Eggs and Milt from Common Carp

Milt and egg samples were collected and immediately placed into 1.5 mL microfuge tubes on ice before being stored at -80°C. DNA extraction was performed using a proteinase K digestion method, adhering to the protocols established by Liu et al. (1998) and modified by Waldbieser and Bosworth (2008). Additionally, DNA from diploid channel catfish, blue catfish, and hybrid catfish was extracted to serve as controls.

To begin, 300 µL of cell lysis buffer comprised of 100 mM NaCl, 10 mM Tris-HCl (pH 8), 25 mM EDTA, and 0.5% sodium dodecyl sulfatewas added to each tube containing 20-30 mg of tissue. Following this, 1.5 µL of Proteinase K (Sigma) was introduced, and the samples were incubated at 55°C for 2-4 hours. Throughout the incubation period, the tubes were periodically checked and vortexed to ensure complete dissolution of the tissue.

Once the tissue was fully dissolved, the samples were vortexed for 15 seconds before adding 170 µL of protein precipitation solution. The tubes were then vortexed for 20 seconds and placed in a -20°C freezer for 10 minutes. After freezing, the samples were centrifuged at 13,200 RPM for 7 minutes, and the supernatant was transferred to new tubes containing 600 µL of 100% ethyl ethanol. These tubes were centrifuged again at 13,200 RPM, and the liquid was removed by pipetting, leaving behind a white pellet. Next, 600 µL of 75% ethyl ethanol was added to the tubes, which were then vortexed briefly and centrifuged at 13,200 RPM for 3 minutes to precipitate the DNA. After removing the ethyl ethanol by pipetting, the white pellet was resuspended in 10-100 µL of RNA/DNA-free water. The DNA concentration was measured using an ND-1000 spectrophotometer (NanoDrop Technologies). The same DNA extraction procedure was applied to the control samples from diploid channel catfish, blue catfish, and common carp.

PCR Detection of Genetic Markers

To distinguish between channel catfish, blue catfish, hybrid catfish, and common carp, polymerase chain reaction (PCR) was employed based on the methodology detailed by Waldbieser and Bosworth (2008). The specific primers used for PCR amplification targeting genes in channel catfish and blue catfish are outlined in table 17. The marker genes selected for differentiation were follistatin (Fst) and hepcidin antimicrobial protein (Hamp).

PCR reactions were assembled in a 10.0 µL volume, which included 20-250 ng of genomic DNA, and the reaction mix comprised 1.0 µL of 10 mM Tris-HCl (pH 8.0), 0.4 µL of 50 mM MgCl2, 0.8 µL of 2.5 mM of each dNTP, 0.6 µL of 10 µM Fst primers, 0.3 µL of 10 µM Hamp primers, 0.1 µL of 5U/µL Platinum Taq polymerase, and 3.9 µL of water.

The thermocycling protocol began with an initial denaturation step at 95°C for 3 minutes. The first amplification cycle consisted of denaturation at 95°C for 1 minute, annealing at 65°C for 1 minute, and extension at 70°C for 1 minute, repeated for 35 cycles. This was followed by a second amplification cycle with denaturation at 95°C for 30 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 1 minute, also for 35 cycles. The process concluded with a final extension at 72°C for 10 minutes.The PCR products of Fst and Hamp were visualized on a 2.0% agarose gel stained with ethidium bromide. Amplicon sizes were assessed using the TrackIt™ 100 bp DNA Ladder (Invitrogen, Carlsbad, CA).

Ploidy Analysis

Common carp samples were sent to Warm Springs Fish Technology Center in Warm Springs, Georgia 31830-2140 for ploidy analysis. Ploidy was determined with a Coulter Counter.

Statistical Analysis

To determine optimal days post-hatch for injection to maximize catfish gamete production in common carp fry, we conducted comprehensive statistical analyses focusing on gamete production patterns in both males and females. A time-series analysis to uncover production trends across specific time phases. Gamete production data were divided into three naturally distributed phases: Early (1-10 Days Post Hatch, DPH), Middle (11-30 DPH), and Late (31-49 DPH). This segmentation allowed us to observe and quantify gamete production changes over time. For each phase, we calculated the mean and standard deviation of gamete production for males and females individually, as well as the combined totals. To compare gamete production between males and females, we applied a two-sample t-test to the mean number of individuals producing catfish gametes in each group.

3.3 Results

Observations

The xenogenic eggs from the common carp that were positive for catfish DNA appeared like those in in Figure 14A. The eggs appear to exhibit qualities indicative of healthy oocyte development, such as uniform size, shape, and color. The eggs had a difficult time exiting the urogenital opening, also known as the vent because the eggs appeared to be larger than control carp eggs. The golden-yellow hue and consistent granularity are characteristic of mature catfish eggs, which suggests a well-defined vitellogenesis stage—a critical phase in oocyte maturation where yolk proteins accumulate in the oocytes to support embryo development. This consistency in color and texture is typically a sign of adequate nutrient deposition, which is necessary for successful fertilization and embryogenesis.

Rate of Xenogenesis

A total 37 males and 23 females common carp produced strippable gametes. 35 out of the 37 males and 21 out of 23 females are putative xenogenic individuals. Of 25 out of 35 (71.4%) common carp male producing strippable gametes were xenogenic (Table 16), and 14 out of 21 (66.6%) common carp female were xenogenic individuals produced common carp gametes. Xenogenic common carp producing catfish gametes were found for those being injected on days 1-49 post hatch. There appeared to be peaks for those injected at about 1, 2, 4, 9-, 16-, 22-, and 49-days post hatch. These days showed high responsiveness, with the highest number of fish giving gamete days post hatch.

Table 16 The number of putative xenogenic common carp (*Cyprinus carpio*) injected with type A somatic stem cells (SSCs) of channel catfish (*Ictalurus punctatus*), blue catfish (*Ictaluru furcatus*), and hybrid (*Ictalurus punctatus* female x *Ictaluru furcatus* male) catfish from 1-49 days post hatch that produced strippable gametes and the percentage of these gamete collections that contained ictalurid DNA.

Days Post Hatch	Male		Female	
	Giving gametes	% giving catfish gametes	Giving gametes	% giving catfish gametes
1	1	100%	1	100%
2	4	100%	1	100%
3	-	-	1	100%
4	2	100%	1	100%
5	2	50%	-	-
6	-	-	1	100%
8	1	0%	-	-
9	1	100%	1	100%
10	3	33%	2	50%
12	1	0%	2	50%
16	2	100%	1	100%
17	4	75%	-	-
19	-	-	1	0%
20	3	66%	-	-
21	1	100%	-	-
22	1	100%	1	100%
23	-	-	1	0%
25	-	-	2	50%
26	1	100%	2	0%

27	1	0%	-	-
28	1	0%	-	-
31	1	100%	-	-
35	-	-	1	100%
37	-	-	1	100%
39	2	100%	-	-
45	1	100%	-	-
46	1	0%	-	-
48	-	-	1	100%
49	1	100%	-	-
Control	2	0%	2	0%

The optimal day post-hatch for injecting the common carp fry to maximize catfish gamete production is day 2. Male common carp produced 4 fish producing gametes with catfish DNA, while females only produce 1 when implantation day is two days after hatch. Males also showed successful implantation day on days 17 with 3 and the 20^{th} and 39^{th} with 2 males on each day producing gametes with catfish DNA. Female common carp that produce gametes of catfish were injected in the middle phase (11-30 DPH). While males tend to produce more gametes, the difference is not statistically significant ($p \approx 0.059$), suggesting a trend where males may have higher gamete production success than females at one year of age.

3.4 Discussion

This study successfully produced xenogenic common carp that were capable of generating eggs and sperm from ictalurid catfish, highlighting the potential of xenogenesis as a transformative approach in aquaculture. The colonization and proliferation of ictalurid spermatogonial A stem cells in common carp were observed at multiple points from 1 to 49 days post-hatch (DPH). . There were peaks of successful xenogenesis at, 2, 4, 9, 16, 22, and 49 days post hatch. The lower DPH were correlated with a higher probability of xenogenesis. The results align with existing literature, which emphasizes the significance of early transplantation for successful stem cell integration and differentiation (Yoshizaki et al., 2010; Jin et al., 2021). Early-stage hosts, particularly those in larval or juvenile phases, are known to exhibit enhanced stem cell acceptance due to an immunologically permissive environment, a factor that has been similarly noted in salmonid and cyprinid xenogenic models (Goto et al., 2015).

While the study did not find a statistically significant deviation from a 1:1 sex ratio, the skew towards male xenogenic individuals suggests a potential biological bias. One plausible explanation for this skew is that stem cells may preferentially colonize the developing male gonad due to differential rates of gonadal maturation between sexes. Males in common carp generally reach sexual maturity earlier than females, and since DNA analyses were performed only on individuals with strippable gametes, young xenogenic males would be more likely to reach maturity sooner than females (MartÃnez et al., 2014; Begum et al., 2022). Similar sex-based biases have been reported in previous xenogenic studies, where host species with faster male maturation rates yielded higher male-biased xenogenic outcomes (Hutchings & Gerber, 2002). However, this hypothesis requires further study, as understanding sex ratios in xenogenic individuals is essential for refining applications where balanced sex representation is critical.

Interestingly, 35% of the putative xenogenic individuals producing strippable gametes were found to lack detectable ictalurid DNA. This discrepancy suggests the possibility of misidentification or incomplete xenogenesis, where diploid common carp may have been mistaken for triploid individuals, a scenario previously observed in grass carp, *Ctenopharyngodon idella* (Dunham, 2023). Although triploid stem cells are generally incapable of such replication, rare occurrences in similar cyprinid species suggest a potential anomaly that warrants further investigation, especially in xenogenic and triploid carp lines. A targeted study examining DNA integrity and stem cell propagation in these cases would clarify whether the observed instances are genuine anomalies or attributable to procedural inconsistencies.

The next step in developing xenogenic common carp as viable gamete donors lies in evaluating the fertilizing ability of ictalurid sperm produced through xenogenesis. If the catfish sperm generated in xenogenic carp proves to be functional, this method could obviate the need to sacrifice valuable male blue catfish. By utilizing xenogenic common carp males as continuous sperm donors, aquaculture operations could establish efficient “sperm factories” for blue catfish. This approach has broader implications for resource allocation; it would enable farms to reduce broodstock populations and dedicate more space to marketable fish. Furthermore, successive generations of xenogenic common carp could propagate the desired genetic material, potentially eliminating the need to maintain live blue catfish on-site. Similar applications have shown promise in salmonid production, where surrogate broodstock have been used effectively to propagate rare or endangered genetic lines (Yoshizaki & Yazawa, 2019).

This study successfully identified key time points, particularly 2, 4, 9, 16, 22, and 49 DPH, as optimal for producing xenogenic common carp capable of generating ictalurid gametes.

These findings lay essential groundwork for refining xenogenic techniques across various fish species, especially within the context of conservation and resource-efficient aquaculture. The use of host species as “sperm factories” has promising applications beyond cyprinids and ictalurids, potentially extending to other commercially significant species like salmon and trout. Future studies could explore optimizing host and donor compatibility, as well as refining stem cell transplantation techniques to improve integration rates and functional gamete yield. Such advancements could enable aquaculture to achieve unprecedented levels of efficiency, sustainability, and genetic management, helping to meet the global demand for seafood in an environmentally sustainable manner. Xenogenesis holds transformative potential for enhancing fish breeding programs, reducing broodstock requirements, and ensuring genetic diversity and sustainability in farmed fish populations.

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Chapter Four

Reproduction of Melanocortin-4 Receptor Gene Edited Channel Catfish, *Ictalurus punctatus*

Abstract

The spawning success and the influence of hormonal treatments on melanocortin-4 receptor (*mc4r*) gene-edited channel catfish (*Ictalurus punctatus*) was examined.. Fish were hormone induced with luteinizing hormone-releasing hormone analog (LHRHa) and human chorionic gonadotropin (HCG), and spawning rates, relative fecundity, hatch rates, and fry yield per kilogram of female body weight were determined. Administration of HCG was crucial for successful spawning in *mc4r* mutants. Without HCG, spawning was unsuccessful despite robust secondary sexual characteristics in *mc4r* fish. The addition of HCG to LHRHa treatments significantly enhanced reproductive outcomes, with *mc4r* x *mc4r* pairings exhibiting comparable spawning rates (P=0.133), fecundity and hatch rates to controls under optimal hormonal conditions. In contrast, *mc4r* mutants lacking HCG failed to spawn, reinforcing its necessity in overcoming the impaired reproductive capabilities associated with *mc4r* mutations. Control fish consistently achieved high spawning success with standard LHRHa treatment alone, outperforming the genetically modified counterparts in terms of fecundity and hatch rates.

4.1 Introduction

The melanocortin-4 receptor (*mc4r*) is a critical component of the G protein-coupled receptor (GPCR) family, specifically within the melanocortin receptor subgroup, which also includes MC1R through MC5R (Liu et al., 2019). This receptor is instrumental in regulating energy homeostasis, sexual function and reproduction, glucose homeostasis, bone metabolism and many other physiological functions. Located predominantly in the hypothalamus, the *mc4r* is activated by melanocyte-stimulating hormone (MSH), a neuropeptide derived from proopiomelanocortin (POMC) (Liu et al., 2019). The activation of *mc4r* by MSH plays a vital role in suppressing appetite and stimulating energy expenditure. Conversely, the activity of *mc4r* is antagonized by agouti-related peptide (AGRP), which acts to increase appetite and reduce metabolism and energy expenditure, highlighting its role in energy balance (Liu et al., 2019). This receptor is intricately connected within the neuroendocrine network, acting downstream of leptin and ghrelin, two hormones integral to hunger and satiety signaling, and upstream of kisspeptin, which is essential for reproductive hormone release (Liu et al., 2019). This positioning underscores *mc4r* significance in metabolic and obesity research. Mutations or errors in the *mc4r* gene can have profound effects on energy balance and metabolism. Such genetic aberrations are often associated with severe obesity, hyperphagia (excessive eating), and metabolic syndrome. Individuals with *mc4r* mutations typically exhibit impaired satiety signaling, leading to increased food intake and reduced energy expenditure. These findings emphasize the critical role of *mc4r* in energy homeostasis and its potential as a therapeutic target for treating metabolic disorders and obesity (Liu et al., 2019). Genetic studies in various species have elucidated the structure and functional importance of *mc4r*. In the channel catfish (*Ictalurus punctatus*), for instance, the *mc4r* gene comprises two exons and a 5,258-base pair (bp) transcript

located on chromosome 20 (Tao et al., 2020). This structural information provides valuable insights into the evolutionary conservation and biological significance of *mc4r* across species, illustrating a highly conserved mechanism for regulating energy balance and metabolism (Liu et al., 2019)

mc4r in Humans, Mice and Rats

Mutations or functional impairments in the *mc4r* gene are closely linked to the development of obesity and related metabolic disorders in humans. Individuals with such mutations often experience excessive eating due to impaired signaling pathways that fail to adequately suppress appetite. This disruption in energy balance leads to increased caloric intake and reduced energy expenditure, resulting in significant weight gain. *mc4r* mutations are one of the most common genetic causes of severe early-onset obesity, highlighting the receptor's essential role in maintaining body weight homeostasis (Farooqi et al., 1999). When *mc4r* is defective, the downstream signaling cascade that normally promotes satiety and increases metabolic rate is disrupted, leading to continuous hunger and decreased energy utilization. This imbalance contributes to the accumulation of adipose tissue and the development of obesity. Furthermore, individuals with *mc4r* mutations often exhibit additional metabolic abnormalities, such as insulin resistance, which further exacerbates weight gain and increases the risk of developing type 2 diabetes and cardiovascular diseases (Vaisse et al., 1998).

Research involving homozygous *mc4r* -deficient mice (*Mus musculus*) has provided significant insights into the receptor's function. These mice exhibit hyperphagic obesity, characterized by excessive food intake, and hyperinsulinemia, a condition marked by elevated levels of insulin in the blood (Huszar et al., 1997). The absence of functional *mc4r* disrupts the normal signaling pathways that regulate satiety and energy balance, leading to uncontrolled eating behavior and

subsequent weight gain. In addition to increased food consumption, *mc4r* -deficient mice display a reduced basal metabolic rate, further contributing to the accumulation of adipose tissue. The combination of hyperphagia and decreased energy expenditure results in a pronounced obese phenotype. Furthermore, these mice often develop metabolic syndrome-like symptoms, including insulin resistance and hyperglycemia, which are indicative of impaired glucose metabolism and increased risk for type 2 diabetes (Chen et al., 2000). Studies in rats have corroborated these findings, demonstrating that mutations or deficiencies in *mc4r* lead to similar metabolic disturbances.

Mc4r is not only crucial for regulating energy homeostasis but also plays a significant role in the human reproductive system. Located primarily in the hypothalamus, *mc4r* is activated by melanocyte-stimulating hormone (MSH), a peptide derived from proopiomelanocortin (POMC), and is antagonized by agouti-related peptide (AGRP) (Mountjoy et al., 1994; Orrù et al., 2017). *Mc4r's* impact on the reproductive system is mediated through its regulation of the hypothalamic-pituitary-gonadal (HPG) axis, which is vital for reproductive hormone release and fertility. Activation of *mc4r* in the hypothalamus modulates the secretion of gonadotropin-releasing hormone (GnRH), which in turn controls the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland (Farah et al., 1991; Caruso et al., 2012; Hawksworth & Burnett, 2019). Figure 15 illustrates the hormonal pathways within the hypothalamic-pituitary-gonadal (HPG) axis, which is essential for the regulation of testosterone and other hormone production. The figure highlights the roles of GnRH analogues in controlling the secretion of LH and FSH from the pituitary gland, which subsequently affects testosterone production in the testes. Activation of *mc4r* by its ligands can affect the release of GnRH, thereby impacting the downstream release of LH and FSH from the pituitary gland and

ultimately regulating testosterone production in the testes. (Hawksworth & Burnett, 2019). These hormones are crucial for the maturation of gametes and the regulation of the menstrual cycle in females, as well as spermatogenesis in males (Hill et al., 2010).

In humans, mutations or functional impairments in the *mc4r* gene have been associated with disruptions in reproductive capabilities. Individuals with *mc4r* deficiencies often exhibit delayed puberty, hypogonadotropic hypogonadism, and reduced fertility. These reproductive anomalies are linked to impaired GnRH release and subsequent downstream effects on LH and FSH secretion (Lubrano-Berthelier et al., 2003). The intricate balance maintained by *mc4r* signaling is essential for normal reproductive function, and disruptions in this pathway can lead to significant reproductive health issues. *Mc4r* plays a role in modulating sexual behavior and libido (Farooqi et al., 2003). This receptor's influence on energy balance and metabolic status indirectly affects reproductive health, as adequate energy reserves are necessary for optimal reproductive function. Thus, *mc4r* mutations that lead to obesity and metabolic dysregulation can also negatively impact reproductive health by altering hormone levels and disrupting the normal function of the HPG axis (Baldini & Phelan, 2019).

Similar hormone regulation regarding *mc4r* has been observed in mice ((Decherf et al., 2010; Israel et al., 2012; Kwon et al., 2016; Dahir et al., 2024). *Mc4r* -deficient mice exhibit notable reproductive deficits. Homozygous *mc4r* knockout mice often show signs of delayed puberty, hypogonadotropic hypogonadism, and reduced fertility same as in humans. Heterozygous *mc4r* knockout mice exhibit milder phenotypes compared to their homozygous counterparts but still show noticeable reproductive abnormalities. These include partial deficiencies in puberty onset, fertility, and hypothalamic-pituitary-gonadal (HPG) axis functionality (Farooqi et al., 2003; Martinelli et al., 2011). Additionally, these mice display

abnormal sexual behaviors, further underscoring the receptor's role in reproductive health (Pritchard et al., 2002). In rats, similar reproductive impairments have been observed with *mc4r* dysfunction (Pritchard et al., 2002; Acevedo-Rodriguez et al., 2018) Disruptions in *mc4r* signaling in rats lead to reduced fertility and altered sexual behavior, consistent with findings in mice. This suggests a conserved mechanism across rodent species whereby *mc4r* is integral to the normal functioning of the reproductive system (Kim et al., 2000).

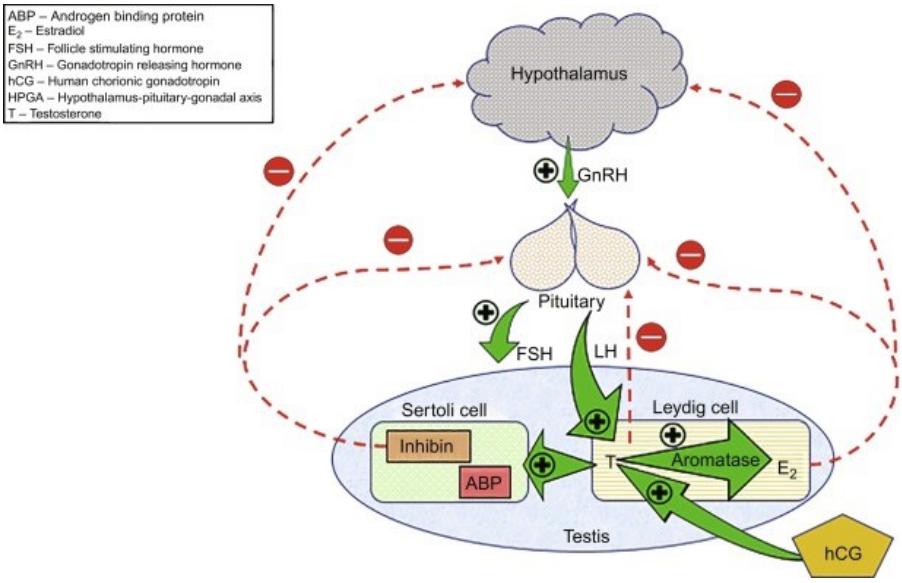

Figure 15. Hormonal Pathways in the Hypothalamic-Pituitary-Gonadal (HPG) Axis. (Dorota J. Hawksworth, Arthur L. BurnettII, in Effects of Lifestyle on Men's Health, 2019)

Depiction of the pathways through which hormonal therapies exert their effects on the hypothalamic-pituitary-gonadal (HPG) axis, which is critical for regulating the production of testosterone and other hormones. Gonadotropin-Releasing Hormone (GnRH) analogues are used to control the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland, influencing testosterone production. Abbreviations in the Figure: ABP: Androgen-binding protein, E_2: Estradiol, FSH: Follicle-stimulating hormone, GnRH:

Gonadotropin-releasing hormone, hCG: Human chorionic gonadotropin, LH: Luteinizing hormone, P: Progesterone, HPG: Hypothalamic-pituitary-gonadal axis, and T: Testosterone

Mc4r in other Animals

In livestock, such as pigs (*Sus scrofa*) and cattle (*Bos taurus*), *mc4r* is integral to regulating body weight (Kim, Larsen, et al., 2000)., feed intake (Houston et al., 2004), fat deposition (Fan et al., 2011), feed efficiency (Silva et al., 2019) and reproductive performance (Klimenko, 2014). . Genetic polymorphisms in the *mc4r* gene are correlated with differences in these traits. For example, specific *mc4r* variants have been associated with increased fat deposition, greater body weight, and higher feed intake in pigs (Kim, Larsen, et al., 2000). In cattle,. *mc4r* polymorphisms can affect carcass traits (Aierqing et al., 2020), including fat thickness (Lotta et al., 2019) and marbling (Prihandini & Maharani, 2019), which are important for meat quality. These genetic variations influence feed intake and energy balance, contributing to differences in growth rates and overall productivity. Furthermore, cattle with certain *mc4r* variants exhibit differences in reproductive traits, such as calving intervals and fertility, underscoring the receptor's broader physiological importance (Madeja et al., 2004). In chickens (*Gallus gallus*) and turkeys (*Meleagris gallopavo*) (Flores et al., 2023; Kubota et al., 2019), *mc4r* is crucial for regulating feed intake and body weight. Mutations in *mc4r* can lead to increased appetite and obesity in chickens, similar to the effects observed in mammalian species. These mutations affect the receptor's ability to regulate hunger signals, resulting in greater food consumption and weight gain (Tao, 2010; Kubota et al., 2019). Additionally, *mc4r* influences reproductive traits in chickens. Variations in the receptor can impact egg production, egg quality, and hatchability, indicating its significant role in avian reproduction. Chickens with *mc4r*

mutations may exhibit altered reproductive cycles and reduced fertility, linking energy balance and reproductive performance (Li & Li, 2006).

Mc4r in Aquatic Species

The melanocortin-4 receptor (*mc4r*) plays a pivotal role in the physiological regulation of aquatic species, significantly influencing growth, feed efficiency, and reproductive success. In Atlantic salmon (*Salmo salar*) (Besnier et al., 2020), polymorphisms within the *mc4r* gene correlate with vital aquacultural traits such as growth rates and body composition (Kleppe et al., 2022). These genetic variants are linked to enhanced feed efficiency and accelerated growth, traits highly valued in commercial breeding programs. The influence of *mc4r* extends to the modulation of appetite and energy expenditure, thereby optimizing growth performance (Kalananthan et al., 2020). Furthermore, *mc4r* contributes to the timing and success of reproductive processes, impacting critical factors like spawning time and egg quality. Similar regulatory mechanisms have been observed in other aquatic species including Barfin flounder (*Verasper moseri*) (Kobayashi et al., 2008), Nile Tilapia (*Oreochromis niloticus*) (Volff et al., 2013), zebrafish (*Danio rerio*) (José Miguel Cerdá-Reverter et al., 2003), goldfish (*Carassius auratus*), and rainbow trout (*Oncorhynchus mykiss*) (Ji et al., 2023).

In zebrafish, disruptions in *mc4r* function lead to increased appetite and obesity, paralleling findings in other vertebrates. These alterations are accompanied by metabolic dysregulations such as impaired glucose metabolism, further emphasizing the receptor's integral role in maintaining energy homeostasis (Sebag et al., 2013). A specific investigation utilizing CRISPR/Cas9 technology to create *mc4r* knockout zebrafish revealed no immediate phenotypic differences from wild-type counterparts during early development (Hruscha et al., 2013). However, as these zebrafish matured, significant variations emerged, including increased food

intake, enhanced growth rates, and elevated body fat percentages (Fei et al., 2017). These observations underscore the importance of *mc4r* during the dynamic early developmental stages, where rapid growth phases are typical. The interruption of *mc4r* function exacerbates these natural physiological processes, leading to notable differences in adult phenotypes and potentially influencing reproductive capabilities.

Mc4r affects Reproductive Activity

The melanocortin-4 receptor (*mc4r*) is increasingly recognized not only for its pivotal role in regulating body weight and adiposity but also for its significant influence on reproductive functions across various species. This receptor's function in reproductive endocrinology offers crucial insights into the interplay between metabolic and reproductive health. In the aquatic realm, the influence of *mc4r* on reproductive functions is both profound and varied. In *Xiphophorus spp*., including swordtails and platyfish, males with polymorphisms in the *mc4r* gene not only nearly double their body weight but also exhibit delayed sexual maturity (Kallman & Borkoski, 1978; Bruger et al., 2020). These physiological changes are accompanied by notable alterations in mating behavior. Larger males with *mc4r* polymorphisms engage in elaborate mating rituals, whereas their smaller counterparts who mature faster and possess normal *mc4r* genes tend to employ sneak mating tactics (Liu et al., 2019). This dichotomy highlights *mc4r*'s role in influencing reproductive strategies, potentially affecting sexual selection processes within populations. In medaka (*Oryzias latipes*), mutations in the *mc4r* gene are linked to enhanced growth rates and slower embryonic development, yet these mutations do not alter the onset of puberty, suggesting a complex interaction between growth and reproductive maturity that may be independent of pubertal timing (Jiang et al., 2017; Liu et al., 2019).

Induced Spawning

Human chorionic gonadotropin (HCG) is a hormone commonly used in aquaculture to induce spawning in fish. It mimics the action of the fish's natural luteinizing hormone (LH), which is essential for triggering ovulation and the release of eggs. HCG binds to LH receptors in the ovaries, promoting the final maturation of oocytes and their subsequent release. This hormone is particularly effective because it can be administered to both males and females to enhance gamete quality and quantity, ensuring successful spawning events (Zohar & Mylonas, 2001). Luteinizing hormone-releasing hormone analog (LHRHa) is another critical hormonal treatment used to induce spawning in fish. LHRHa acts by stimulating the release of endogenous gonadotropins, specifically LH and follicle-stimulating hormone (FSH), from the pituitary gland. These gonadotropins then act on the gonads to promote gamete maturation and ovulation in females and spermiation in males (Figure 15). LHRHa is often used in controlled breeding programs because it can synchronize spawning, increase spawning success rates, and improve the overall reproductive efficiency of cultured fish species (Peter & Yu, 1997).

Objectives

The varied effects of *mc4r* on reproductive activities across species illustrates its integral role in linking metabolic status with reproductive capability (Chen et al., 2000). The receptor's influence extends beyond mere energy regulation, encompassing critical aspects of reproductive health and behavior (Thurston et al., 2022). This multifaceted role makes *mc4r* a compelling target for research, with potential applications in improving reproductive management and understanding metabolic-reproductive interactions (Zohar & Mylonas, 2001). The objectives of this study were to create and evaluate melanocortin-4 receptor (*mc4r*) gene knock-out of F1 channel catfish (*Ictalurus punctatus*) ,2) assess the percentage of spawning pairs, relative fecundity, hatch rate

percentage, and 3) evaluate the effects of hormonal treatments using human chorionic gonadotropin (HCG) and luteinizing hormone-releasing hormone analog (LHRHa) to induce spawning. The primary objective of these experiments was to evaluate the effectiveness of Luteinizing Hormone-Releasing Hormone analog (LHRHa) and Human Chorionic Gonadotropin (HCG) in inducing spawning, and to compare the reproductive success between genetically modified and control groups.

4.2 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.

sgRNA and CRISPR/Cas9 System

For the targeted knockout of the *mc4r* gene in channel catfish, three customized small guide RNAs (sgRNAs) were designed and synthesized using the Maxiscript T7 PCR-based method. The initial step involved designing four gene-specific oligonucleotides (designated *mc4r* -A, *mc4r* -B, *mc4r* -C, and *mc4r* -D) which included the protospacer adjacent motif (PAM) sequences. This design process utilized the CRISPRscan online tool to ensure precise targeting of the *mc4r* gene (GenBank Accession No. LBML01001141.1). Each of these sgRNAs was designed to target exon 1 of the gene to maximize the knockout effect by truncating the protein early in its sequence. The Universal Primer, incorporating the sgRNA scaffold, was procured from Thermo Fisher Scientific. Each oligonucleotide was reconstituted to a concentration of 10mM using DNase/RNase-free water. For sgRNA synthesis, double-stranded DNA (dsDNA) templates were created. This involved mixing 25 µL of 2x EconoTaq Plus Master Mix

(Lucigen), 12.5 µL of the Universal Primer, and 12.5 µL of each gene-specific oligonucleotide. The PCR amplification followed a detailed thermal cycling protocol: an initial denaturation at 95°C for 3 minutes, followed by 5 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds with a ramp speed of -0.2°C/sec, and extension at 72°C for 30 seconds. This was followed by 20 cycles of denaturation at 95°C for 30 seconds and extension at 72°C for 20 seconds with a ramp speed of -0.2°C/sec, concluding with a final extension at 72°C for 10 minutes.

Following the PCR amplification, Taq Polymerase was inactivated by adding 4.8 µL of 0.5M EDTA and incubating the mixture at 75°C for 20 minutes. Confirmation of the PCR product was achieved via electrophoresis on a 1% agarose gel. The subsequent synthesis of sgRNA was carried out using the Maxiscript T7 Kit (Thermo Fisher Scientific), adhering strictly to the manufacturer's protocol. The synthesis reaction consisted of 2 µL of dH2O, 10 µL of the previously generated dsDNA template, 2 µL of 10X buffer, 1 µL each of ATP, CTP, GTP, and UTP, and 2 µL of enzyme mix, incubated at 37°C for 90 minutes.

To eliminate DNA contamination, 1 µL of Turbo DNase I was added to the reaction mix, followed by a brief vortex and an additional incubation at 37°C for 15 minutes. Magnesium ions were chelated by adding 5 µL of 0.5M EDTA, and the Turbo DNase I was subsequently inactivated by heating the mixture at 75°C for 10 minutes. The synthesized sgRNAs were then purified using the Zymo RNA Clean and Concentrator kit (Zymo Research) and stored at -80°C until further use.

The Cas9 protein was sourced from PNA Bio (3541 Old Conejo Rd, Newbury Park, CA 91320) and reconstituted in dH2O to achieve a concentration of 1 mg/mL. Twenty minutes prior to fertilization, four injection solutions were prepared. Each solution consisted of a combination of

Cas9 protein with one of the three individual sgRNAs, and a fourth solution combined all three sgRNAs with Cas9 protein (*mc4r mix*). These mixtures were incubated on ice for 10 minutes. Phenol red was then added to each solution to achieve a final ratio of 1:1:1 for Cas9, sgRNA, and Phenol red, respectively. The final concentrations of Cas9 protein and sgRNA in the injection solutions were maintained at 300-350 ng/µL and 150-200 ng/µL, respectively.

Spawning Brood *Ictalurus punctatus*

Channel catfish brood stock were maintained in 0.04-hectare earthen ponds, with an average depth of 1 meter. These ponds were equipped with a ½ horsepower surface aerator (Air-O-Lator) to ensure that the dissolved oxygen levels remained above 3 mg/L. The brood stock were fed a diet consisting of a 32% protein catfish pellet, administered at a rate of 1-2% of their body weight, five days per week. The Kansas strain of channel catfish was selected for this study due to its demonstrated superior growth rates and high fry output when induced to spawn with luteinizing hormone-releasing hormone analogs (LHRHa).

Selection of individuals for breeding was based on both health status and secondary sexual characteristics indicative of reproductive readiness. Males were identified by their well-developed papillae, large, muscular heads, and bodies that exhibited a pronounced width at the head region. Additionally, males displaying dark coloration and signs of scarring from territorial fighting were considered ideal candidates, as these features are often associated with robust reproductive health. For females, selection criteria included a soft, well-rounded abdomen, which was noticeably wider than the head, along with a swollen urogenital opening, indicating imminent spawning readiness.

To minimize stress and avoid compromising the health of the broodstock, handling was kept to a minimum, and fish were held in tanks only for the shortest duration necessary before spawning. This approach was designed to ensure the maintenance of optimal physiological conditions for successful breeding and high-quality gamete production.

Spawning *mc4r* Knock-out channel catfish

In 2016 and 2017, efforts to spawn *mc4r* mutant broodstock were unsuccessful, despite the broodstock exhibiting robust secondary sexual characteristics. In 2016 two *mc4r* pairs and three control pairs were mated, while in 2017, three *mc4r* pairs and five control pairs were created. During these attempts, male and female *mc4r* mutants were paired in aquaria and administered 100 µg/kg of luteinizing hormone-releasing hormone analog (LHRHa) via implants, following the standard procedure. In 2018, a revised approach involved injecting both male and female P1 *mc4r* mutants intraperitoneally with a combination of 100 µg/kg LHRHa and 1600 international units (IU) of human chorionic gonadotropin (HCG). Complete records of 2018 success is unavailable.

In 2016 and 2017, LHRHa implants administered at 100 µg/kg BW to 5 pairs (both sexes) of *mc4r* KO brood stock failed to produce and spawns while 8 control pairs spawned using the same treatment. When both male and female P1 *mc4r* mutants were injected intraperitoneally with a combination of 100 µg/kg BW LHRHa and 1600 IU/kg BW HCG in 2018,. five wild-type females x P1 *mc4r* mutant males, two P1 *mc4r* mutant females x wild-type males, one P1 *mc4r* mutant female x P1 *mc4r* mutant male, and one pairing of full-sibling wild-type Kansas strain channel catfish all spawned.

The typical sexual characteristics displayed by channel catfish during spawning season were noticeable in the *mc4r* knock-out individuals, Selection was based on sex, size, *mc4r* positive, gravid, and developed individuals. Preliminary experiments demonstrated the necessity of hormone induction to rescue fertility in *mc4r* knockout channel catfish. The hormone-induced spawning treatments compared included:

Table 17 Hormone-induced spawning treatments combinations for *mc4r* knockout gene edited Channel Catfish (*Ictalurus punctatus*) utilizing luteinizing hormone-releasing hormone analog (LHRHa) and/or human chorionic gonadotropin (HCG)

The third injection dose was typically 50-80 hours after the resolving dose of LHRHa implant if *mc4r* positive pairs did not spawn.

Treatment Group	**Priming Dose (20%)**	**Resolving Dose (80%)**	**Time Between Priming Dose and Resolving Does Doses**	**Additional Dose given 50-80 hours later**	**Notes**
1. LHRHa + HCG	20 µg/kg LHRHa	80 µg/kg LHRHa	24 hours	1600 IU/kg HCG	Administered as three separate injections
2. LHRHa + HCG	8 µg/kg LHRHa	32 µg/kg LHRHa	24 hours	1600 IU/kg HCG	Administered as three separate injections
3. LHRHa + HCG	20 µg/kg LHRHa	32 µg/kg LHRHa	24 hours	1600 IU/kg HCG	Administered as three separate injections
4. LHRHa + HCG	20 µg/kg LHRHa + 320 IU/kg HCG	80 µg/kg LHRHa + 1280 IU/kg HCG	24 hours	n/a	Administered as two combined injections
5. LHRHa	20 µg/kg LHRHa	80 µg/kg LHRHa	24 hours	n/a	Administered as two combined injections
6. LHRHa	8 µg/kg LHRHa	32 µg/kg LHRHa	24 hours	n/a	Administered as two combined injections

7. LHRHa + HCG	20 µg/kg LHRHa + 320 IU/kg HCG	80 µg/kg LHRHa + 1280 IU/kg HCG	24 hours	n/a	Administered as two combined injections
8. LHRHa + HCG	8 µg/kg LHRHa + 320 IU/kg HCG	32 µg/kg LHRHa + 1280 IU/kg HCG	24 hours	n/a	Administered as two combined injections
9. Control	20 µg/kg LHRHa	80 µg/kg LHRHa	24 hours	n/a	Administered as two combined injections

In 2019, additional genetic pairings, designated as mc4r x mc4r and mc4r x Control, were generated following the same methodology used in 2018. Broodstock selection involved two- and three-year-old mc4r gene-edited channel catfish with robust secondary sexual characteristics. Both male and female P1 mc4r mutants received intraperitoneal injections of 100 µg/kg LHRHa and 1600 IU of HCG. The goal of the 2019 spawning season was to compare the spawning success of pairs given HCG to those that were not, with HCG administered as a second dose 50-80 hours after the initial LHRHa implant

The goal of the 2020 spawning season was to compare the success of pairs given LHRHa and HCG simultaneously rather than 50-80 hours after the resolving doses.

In 2021, all mc4r pairs received both LHRHa and HCG, with variations in the amount of LHRHa administered and the timing of HCG injections. The goal was to combine and compare the treatments from in 2019 and 2020.

PCR Detection of Genetic Markers

Pelvic fin-clip samples (weighing between 10-20 mg) were collected and stored in sterile 1.5 mL Eppendorf tubes at -80°C until DNA extraction. Genomic DNA extraction was performed using a protocol involving proteinase K digestion and ethanol precipitation. The fin clips were digested in 600 µL of cell lysis buffer (100 mM NaCl, 10 mM Tris, 25 mM EDTA, and 0.5% SDS) along with 2.5 µL of proteinase K in a 55°C water bath for 4-8 hours, with intermittent vortexing. Following digestion, 200 µL of protein precipitation solution (Qiagen, 19300 Germantown Road Germantown, MD 20874) was added, and the mixture was vortexed and stored on ice for 12 minutes. The mixture was then centrifuged at 15,000 rcf for 8 minutes to precipitate the protein. The resulting supernatant, which contained the DNA, was precipitated with isopropanol and centrifuged again at 15,000 rcf for 5 minutes. The DNA pellet was washed twice with 75% ethanol, with gentle inversion five times during each wash, and then dissolved in dH_2O. DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific), and the concentration was adjusted to 500 ng/µL.

To detect genetic markers, a primer set (*mc4r* -F and *mc4r* -R) was designed using Primer3Plus to encompass all potential mutation sites within the *mc4r* gene. PCR amplification was performed using the Expand High FidelityPLUS PCR System (Roche) with 500 ng of genomic DNA. The PCR protocol, executed on a Bio-Rad T100 Thermal Cycler, included an initial denaturation at 95°C for 3 minutes, followed by 34 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 40 seconds with a ramp speed of -0.2°C/second, and extension at 72°C for 40 seconds. A final extension step at 72°C for 10 minutes completed the amplification process.

To detect mutations in the *mc4r* gene, the Surveyor® mutation detection kit (Integrated DNA Technologies) was employed. According to the kit instructions, hybridized DNA products were combined with Nuclease S, Enhancer S, MgCl2, and Reaction Buffer (2). This mixture was incubated at 42°C for one hour to allow for the digestion of mismatched DNA. The resulting digested products were then separated on a 1.5% TBE (Tris borate EDTA) agarose gel and compared against control samples to identify potential mutations.

To confirm and precisely identify these mutations, positive samples identified by the Surveyor® analysis were further analyzed using the TA cloning method. The largest individuals from each treatment, consistently identified as mutants through Surveyor® analysis, were selected for sequencing. Genomic DNA from three mutants per treatment group was amplified using the Expand High FidelityPLUS PCR System (Roche), following the previously described protocol. The PCR products were verified on a 1% TAE agarose gel. These products were then cloned into the TOPO® TA Cloning® Kit (Invitrogen), with 20 clones prepared per sample, and sent to MCLabs for sequencing. The sequences obtained were analyzed and interpreted using the MAFFT sequence alignment tool, providing detailed insights into the specific mutations present in the *mc4r* gene. These broodstock were maintained in 70-liter glass aquaria.

Statistical Analysis

To assess the spawning rates and the impact of various hormone treatments on *mc4r* -positive and control fish pairings, Fisher's Exact Test was utilized by employing the Python programming language. A 2x2 contingency table was constructed to compare spawning success rates between different groups: *mc4r* -positive pairs vs. control pairs, *mc4r* -positive x control pairs vs. control pairs, and pairs with vs. without HCG hormone treatment. Odds ratios and p-values were

calculated to determine the statistical significance of differences in spawning rates between these groups. For continuous variables such as relative fecundity, hatch rate, and fry/kg female body weight, a one-way ANOVA was conducted to compare the means across different groups (*mc4r* Positive x *mc4r* Positive, *mc4r* Positive x Control, Control x Control) to identify statistically significant differences. Additionally, a two-way ANOVA was performed to evaluate the interaction effects of hormone treatments (LHRHa, HCG) and fish pairings on these variables. The combined use of Fisher's Exact Test for categorical spawning data and ANOVA for continuous variables provided a comprehensive analysis of the effects of different treatments and pairings on the reproductive success of *mc4r* -positive and control fish.

4.3 Result

Spawning of *mc4r* Mutant Channel Catfish

The administration of LHRHa followed by HCG significantly enhanced spawning success in *mc4r* gene-edited channel catfish. Specifically, both *mc4r* homozygous pairs (*mc4r* x *mc4r*) and mixed pairs (*mc4r* x control) successfully spawned following treatment with variable doses of LHRHa and a subsequent injection of 1600 IU HCG. In contrast, the control group achieved successful spawning with a single hormone treatment of 100 mg/kg LHRHa alone. Notably, when both hormone treatments (LHRHa and HCG) were administered, spawning parameters—including spawn percentage, relative fecundity, hatch rate, and fry production—were comparable to those observed in the control group for both *mc4r* x *mc4r* and *mc4r* x control pairings. The absence of HCG in the hormone regimen, however, resulted in no spawning activity among the *mc4r* gene-edited catfish, underscoring the essential role of HCG in enabling reproductive success in these genetically modified fish. This finding suggests that HCG is a key component in

the hormonal induction of spawning for *mc4r* gene-edited channel catfish. Analysis revealed significant interactions based on hormone treatments across each individual year with respect to relative fecundity, hatch rate, and fry production per kilogram of female body weight. These interactions indicate that both the timing and the specific hormone treatment influenced reproductive outcomes. However, when examining overall spawning rates, there was no statistically significant difference ($p = 0.133$) between mc4r positive fish receiving HCG and the control group across all three years of study (2019–2021) tables 18-20.

Table 18 Spawning percentage, relative fecundity, hatch rate, and fry/kg female body weight (BW) for melanocortin-4 receptor (MC4R) gene-edited channel catfish (*Ictalurus punctatus*) and normal control channel catfish induced to spawn with combinations of luteinizing hormone releasing hormone analogue (LHRHa) and human chorionic gonadotropin (HCG) in 2019. ANOVA with F-statistics.

LHRHa Dose	**HCG given 50-80 hours later (if given)**	**Female Genotype**	**Male Genotype**	**N**	**Spawn %**	**Relative Fecundity**	**Hatch Rate %**	**Fry/kg Female BW**
20 µg/kg LHRHa + 80 µg/kg LHRHa	1600 IU/kg HCG	MC4R Positive	MC4R Positive	1	100[a]	2397[a]	80.9[a]	1939[a]
20 µg/kg LHRHa + 80 µg/kg LHRHa	n/a	MC4R Positive	MC4R Positive	2	0[b]	0[b]	0[b]	0[b]
20 µg/kg LHRHa + 80 µg/kg LHRHa	1600 IU/kg HCG	MC4R Positive	Control	1	100[a]	1467[a]	69.5[a]	1019[a]
20 µg/kg LHRHa + 80 µg/kg LHRHa	n/a	MC4R Positive	Control	3	0[b]	0[b]	0[b]	0[b]
20 µg/kg LHRHa + 80 µg/kg LHRHa	n/a	Control	Control	5	100[a]	1043.95 (±70.2)[a]	86.24 (±6.1)[a]	900.30 (±110.5)[a]

Mc4r pairs that did not receive HCG did not spawn, indicating the critical role of HCG in inducing spawning in MC4R-positive fish (odds ratio of infinity).

Significant differences between MC4R Positive x MC4R Positive pairs that received HCG versus Control x Control were found for relative fecundity ($F_{(2,4)} = 10.34$, $p = 0.025$), hatch rate ($F_{(2,4)} = 8.52$, $p = 0.032$), and fry/kg female body weight ($F_{(2,4)} = 11.29$, $p = 0.021$) in 2019. Interaction effects between hormone treatments and fish pairings were significant for relative fecundity ($F_{(1,4)} = 8.91$, $p = 0.041$), hatch rate ($F_{(1,4)} = 7.23$, $p = 0.048$), and fry/kg female body weight ($F_{(1,4)} = 9.32$, $p = 0.039$) in 2019.

Letters indicate statistical differences (a, b) in columns where comparisons are applicable.

2020 Spawning Season

In 2020, *mc4r* x *mc4r* and *mc4r* x control successfully spawned. One pair received 100 µg/kg LHRHa along with 1600 IU of HCG, while the second pair received 40 µg/kg LHRHa and the same dose of HCG. Pairs that did not receive HCG failed to spawn. Females untreated with HCG exhibited soft, well-rounded abdomens at the start of the spawning season, which gradually receded without resulting in egg laying.

Table 19 Spawning percentage, relative fecundity, hatch rate, and fry/kg female body weight (BW) for melanocortin-4 receptor (MC4R) gene-edited channel catfish (*Ictalurus punctatus*) and normal control channel catfish induced to spawn with combinations of luteinizing hormone releasing hormone analogue (LHRHa) and human chorionic gonadotropin (HCG) in 2020. ANOVA with F-statistics.

Priming Dose (20%)	**Resolving Dose (80%)**	**Female Genotype**	**Male Genotype**	**N**	**Spawn %**	**Relative Fecundity**	**Hatch Rate %**	**Fry/kg Female BW**
8 µg/kg LHRHa + 320 IU/kg HCG	32 µg/kg LHRHa + 1280 IU/kg HCG	MC4R Positive	MC4R Positive	1	100[a]	2206.47[a]	78.18[a]	1725.02[a]
8 µg/kg LHRHa	32 µg/kg LHRHa	MC4R Positive	MC4R Positive	2	0[b]	0[b]	0[b]	0[b]
20 µg/kg LHRHa + 320 IU/kg HCG	80 µg/kg LHRHa + 1280 IU/kg HCG	MC4R Positive	Control	1	100[a]	23000.00[a]	67.12[a]	15437.60[a]
20 µg/kg LHRHa	80 µg/kg LHRHa	MC4R Positive	Control	3	0[b]	0[b]	0[b]	0[b]
20 µg/kg LHRHa	80 µg/kg LHRHa	Control	Control	5	100[a]	839.13 (±70.2)[a]	84.30 (±6.1)[a]	707.39 (±110.5)[a]

Mc4r pairs that did not receive HCG did not spawn, indicating the essential role of HCG in spawning success for MC4R-positive fish (odds ratio of infinity).

Significant differences were found between MC4R Positive x MC4R Positive pairs that received HCG and Control x Control pairs for relative fecundity ($F_{(2,4)} = 15.67$, $p = 0.018$), hatch rate ($F_{(2,4)} = 10.12$, $p = 0.028$), and fry/kg female body weight ($F_{(2,4)} = 14.23$, $p = 0.022$) in 2020.

Interaction effects between hormone treatments and fish pairings were significant for relative fecundity ($F_{(1,4)} = 12.45$, $p = 0.031$), hatch rate ($F_{(1,4)} = 10.87$, $p = 0.035$), and fry/kg female body weight ($F_{(1,4)} = 13.28$, $p = 0.029$) in 2020.

Letters (a, b) indicate statistical differences in each column where comparisons are relevant

2021 Spawning Season

In 2021, all *mc4r* pairs received both LHRHa and HCG, with variations in the amount of LHRHa administered and the timing of HCG injections successfully spawned. Control successfully spawned with one hormone treatment 100 mg/kg LHRHa. *mc4r* pairs that did not receive HCG did not spawn.

Table 20 Spawning percentage, relative fecundity, hatch rate, and fry/kg female body weight (BW) for melanocortin-4 receptor (MC4R) gene-edited channel catfish (*Ictalurus punctatus*) and normal control channel catfish induced to spawn with combinations of luteinizing hormone releasing hormone analogue (LHRHa) and human chorionic gonadotropin (HCG) in 2021. ANOVA with F-statistics.

Priming Dose (20%)	**Resolving Dose (80%)**	**HCG given 50-80 hours later (if given)**	**Female Genotype**	**Male Genotype**	**N**	**Spawn %**	**Relative Fecundity**	**Hatch Rate %**	**Fry/kg Female BW**
8 µg/kg LHRHa + 320 IU/kg HCG	32 µg/kg LHRHa + 1280 IU/kg HCG		MC4R Positive	MC4R Positive	1	100[a]	2281.25[a]	84.83[a]	1935.18[a]
8 µg/kg LHRHa	32 µg/kg LHRHa	1600 IU/kg HCG	MC4R Positive	MC4R Positive	2	50[b]	1250.74[b]	7.86[b]	98.31[b]
20 µg/kg LHRHa + 320 IU/kg HCG	80 µg/kg LHRHa + 1280 IU/kg HCG		MC4R Positive	MC4R Positive	1	100[a]	1411.85[a]	69.90[a]	986.88[a]
20 µg/kg LHRHa	80 µg/kg LHRHa	1600 IU/kg HCG	MC4R Positive	Control	3	33[b]	766.07[b]	0[b]	0b
20 µg/kg LHRHa	80 µg/kg LHRHa		Control	Control	5	100[a]	6066.67 (±250.45)[a]	75.04 (±5.65)[a]	4552.43 (±300.67)[a]

High spawning success rates were observed in MC4R-positive pairs when both LHRHa and HCG were administered, except in one pair receiving only the initial LHRHa dose, underscoring the importance of HCG for successful spawning in MC4R-positive fish (odds ratio of infinity). Significant differences between MC4R Positive x MC4R Positive pairs that received HCG and Control x Control pairs were found for relative fecundity ($F_{(2,4)} = 11.45$, $p = 0.023$), hatch rate ($F_{(2,4)} = 9.78$, $p = 0.032$), and fry/kg female body weight ($F_{(2,4)} = 12.89$, $p = 0.021$) in 2021. Significant interaction effects between hormone treatments and fish pairings were detected for relative fecundity ($F_{(1,4)} = 9.54$, $p = 0.036$), hatch rate ($F_{(1,4)} = 8.32$, $p = 0.042$), and fry/kg female body weight ($F_{(1,4)} = 10.12$, $p = 0.029$) in 2021.
Letters (a, b) indicate statistical differences in each column where relevant

In 2021 (Table 20), successful MC4R-positive x MC4R-positive spawning was observed in pairs treated with both LHRHa and HCG at varying doses. Notably, a pair receiving 40 µg/kg LHRHa and 1600 IU/kg HCG achieved a high spawning rate (100%), with a relative fecundity of 2281.25 eggs/kg and a hatch rate of 84.83%. Another pair with the same LHRHa dose but only 50% spawning success had significantly lower relative fecundity and hatch rates, emphasizing the importance of complete hormone treatment for optimal biological outcomes. Statistical analysis comparing spawning rates between MC4R-positive and control pairings ($p = 0.133$) indicated no statistically significant difference in spawning rates between these pairings across three years.

A pair treated with a higher initial dose of LHRHa (100 µg/kg) followed by a second dose also achieved successful spawning (100%), though with slightly lower fecundity and hatch rates compared to the first pair, suggesting that timing and dosage of hormone administration can

influence reproductive outcomes. When comparing all three years together, no significant differences were found between the genetic pairings for relative fecundity ($F(2,12) = 0.56$, $p = 0.59$) and no significant interaction effect between hormone treatments and fish pairings ($F(1,12) = 1.11$, $p = 0.31$). Among MC4R-positive x control pairs, only one out of three successfully spawned in 2021 (Table 20), receiving 100 µg/kg LHRHa and 1600 IU/kg HCG. This pair exhibited moderate relative fecundity and hatch rates compared to fully treated MC4R-positive pairs, indicating that while MC4R mutants can reproduce with appropriate hormone treatments, success rates may vary. The spawning success rate showed no significant association with genetic pairings and hormone treatments (odds ratio = 1.67, $p = 1.00$) when analyzing all three years together.

Control pairs consistently demonstrated high spawning success (100%) with high relative fecundity (6066.67 eggs/kg) and hatch rates (75.04%), highlighting the superior reproductive performance of non-mutated control fish under standard hormone treatments compared to the genetically modified MC4R mutants. From 2019 to 2021(Table 18-20), MC4R-positive x MC4R-positive pairs showed high spawning success rates with excellent relative fecundity and hatch rates, indicating the effectiveness of the combined hormone treatment of LHRHa and HCG. MC4R-positive x control pairs demonstrated high relative fecundity and fry yield, suggesting a synergistic effect of the MC4R mutation in females and control males when combined with optimal hormone treatment. Conversely, MC4R-positive x control pairs that did not receive HCG did not spawn, reinforcing the necessity of HCG in the hormone treatment protocol. Comparing MC4R-positive x MC4R-positive and MC4R-positive x control pairs to control x control pairs, the p-value of 0.133 indicates no statistically significant difference in

spawning rates between these pairings. However, the infinite odds ratio suggests that MC4R-positive fish pairings never spawned without HCG, underscoring its crucial role.

When combining data from all three years, no significant differences were found between genetic pairings for relative fecundity ($F(2,12) = 0.56$, $p = 0.59$) and no significant interaction effect between hormone treatments and fish pairings ($F(1,12) = 1.11$, $p = 0.31$). The lack of significance in the combined analysis suggests that while individual years showed notable variations and significant outcomes, these effects were not consistent enough across all years to yield statistically significant results when analyzed together. This could be attributed to year-to-year variations in environmental conditions, sample sizes, or slight differences in hormone administration protocols. Therefore, while significant reproductive enhancements were observed each year, the overall variability diluted the significance in the pooled analysis.

Across all three years of data (Tables 18-20), the inclusion of HCG is shown to be essential for inducing successful spawning in MC4R mutant fish. Without HCG, spawning attempts failed regardless of LHRHa presence

Mutation Analysis

In 2017, microinjection of CRISPR/Cas9 and sgRNA targeting exon 1 of the *mc4r* gene resulted in the survival of 18 fish. Among these, the mutation rate was determined to be 33.3% (6 out of 18 individuals). Notably, the mutation rate varied significantly across different families generated in 2018, with statistical analysis revealing a significant difference ($p<0.05$). Of the six P1 mutants, 33.3% were homozygous or bi-allelic mutants, while the remaining 66.7% were heterozygous. 2018, P1 mutants yielded 398 F1 *mc4r* offspring across eight distinct families. One family was produced by mating an *mc4r* mutant female with an *mc4r* mutant male. Five families resulted from pairing wild-type females with *mc4r* mutant males (*mc4r* a, b, c, d, e x

Control). Additionally, two families were created by crossing *mc4r* mutant females with wild-type males (*mc4r* x control-a,b).

The overall mutation rate among all F1 *mc4r* channel catfish was 42% (170 out of 398 individuals). Within the *mc4r* x *mc4r* family, the mutation rate was particularly high, with 76% (16 out of 21) of the F1 mutants being homozygous or bi-allelic. This data underscores the efficacy of the CRISPR/Cas9 system in generating targeted mutations and highlights the potential for variable mutation rates depending on specific breeding combinations and genetic backgrounds.

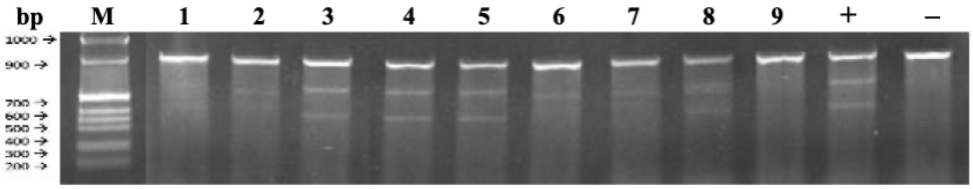

Figure 16. Identification of edited melanocortin-4 receptor (MC4R) gene sequences in channel catfish, *Ictalurus punctatus*, using the surveyor mutation detection assay. All samples were hybridized with an equal volume of non-injected control to detect both homozygotes/bi-allelic and heterozygotes. Wild-type sequences are indicated with a single 932 bp band, while mutations are signified by three bands. Samples 1 and 2 are MC4R * MC4R F1 progeny. Sample 3 is CNTRL x MC4R-1 Fl progeny. Samples 4 and 5 are CNTRL x MC4R-2 F1 progeny. Samples 6 and 7 are CNTRL x MC4R-3 Fl progeny. Samples 8 and 9 are CNTRL x MC4R-4 F1 progeny. Sample + is a previously identified MC4R x MC4R F1 progeny mutant. Sample - came from wild-type control. M indicates 1kb marker.

4.4 Discussion

The knockout of *mc4r* resulted in sterility in channel catfish, demonstrated by the failure of *mc4r* knockout males and females to spawn or produce egg masses. Every mating combination involving *mc4r* mutants, homozygous pairings or reciprocal pairings with control fish,failed to spawn when LHRHa alone was used to induce spawning. In k contrast, control matings of normal channel catfish exhibited high spawning success under LHRHa induction. These results highlight the indispensable role of HCG coupled with LHRHa for enabling spawning in *mc4r*-knockout catfish, which otherwise display impaired natural spawning abilities. This suggests that *mc4r* is essential for reproduction in channel catfish, likely due to its role within the hypothalamus-pituitary-gonad (HPG) axis. The functional role of *mc4r* in fish reproductive systems is further supported by similar findings in other species. Jiang et al. (2017) demonstrated that *mc4r* regulates the secretion of GnRH, FSH, and LH in the spotted scat (*Scatophagus argus*), influencing reproductive function at multiple hormonal levels. In channel catfish, HCG,which possesses both FSH-like and LH-like activity,appears to partially compensate for the disrupted function caused by *mc4r* knockout, likely by supplementing FSH and LH activity essential for initiating ovulation and spermiation. The findings suggest that GnRH and *mc4r* likely work together to regulate LH and FSH levels, a hypothesis that aligns with regulatory patterns observed in other teleost studies (Liu et al., 2019).

When both hormones were applied, reproductive performance metricssuch as spawning rate, relative fecundity, hatch rate, and fry/kg female body weightwere similar between *mc4r* mutants and controls, indicating that optimized hormone regimens can restore reproductive function in gene-edited catfish. This restoration of spawning function in *mc4r*mutants highlights the potential for targeted hormonal interventions to manage

reproduction in genetically modified fish, a strategy that could be applied across various aquaculture species where natural spawning is impaired due to genetic modifications. The reproductive challenges observed in mc4r-knockout channel catfish are consistent with similar findings in xenogenic models and other gene-edited fish.

Studies in xenogenic salmonid broodstock, for instance, have shown that hormonal interventions are often necessary to initiate gamete production in surrogate hosts (Jin et al., 2021),ust as HCG supplementation is required in mc4r-knockout channel catfish to enable spawning, surrogate species also benefit from precise hormonal treatments to facilitate reproduction. Furthermore, the reproductive outcomes in mc4r-knockout channel catfish align with findings in gnrh-deficient zebrafish models, where genetic modifications resulted in sterility without hormonal supplementation (Marvel et al., 2018). Both models demonstrate the critical role of hormonal balance in supporting reproductive function in genetically altered fish.

These results indicate that while *mc4r*-knockout channel catfish can achieve successful spawning under carefully optimized hormonal conditions, their reproductive efficiency (in terms of relative fecundity and hatch rate) were comparable to control fish. This underscores the broader implications of genetic modifications on reproductive performance in aquaculture species. Hormonal regimens tailored to the unique physiological requirements of gene-edited fish, such as *mc4r*-knockouts, can compensate for natural spawning deficiencies and enable effective breeding programs in aquaculture. Future research could refine these hormone protocols to improve spawning outcomes, supporting sustainable and efficient breeding strategies for genetically modified fish.

In conclusion, this study demonstrates that the targeted use of hormonal treatments, specifically the combination of LHRHa and HCG, can successfully restore spawning capabilities

in *mc4r*-knockout channel catfish. These findings contribute to the growing body of knowledge on the role of *mc4r* in fish reproduction and provide a foundation for developing optimized hormonal protocols to manage reproduction in gene-edited aquaculture species.

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