Enhancing the efficiency of hybrid catfish (♀ channel catfish, *Ictalurus punctatus*, × ♂ blue catfish, *I. furcatus*) embryo production through xenogenesis.

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
May 06, 2023

Keywords: Xenogenesis, triploids, stem cell transplantation, cryopreservation, cell colonization, proliferation

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Abstract

Xenogenesis has been identified as an innovative technology for hybrid catfish (♀ channel catfish, *Ictalurus punctatus* × ♂ blue catfish, *I.furcatus*) embryo production. The xenogeneic process can be accomplished by transplanting primordial germ cells (PGCs), spermatogonial stem cells (SSCs), or oogonial stem cells (OSCs), derived from a donor diploid fish into a sterile recipient, which then enables the recipient fish to produce donor-derived gametes. However, still there are some challenges in xenogenesis that need to be optimized in commercial-scale hybrid catfish embryo production. Hence, a series of experiments were conducted to enhance the efficiency of hybrid catfish embryo production through xenogenesis.

Until recently, the timing of transplantation of donor cells into hosts was done with limited knowledge of the best age to inject cells. The age of the host could critically affect the success of germ cell transplantation. Hence, one of the study conducted to identify the best age of the triploid channel catfish to transplant blue catfish stem cells for production of xenogeneic catfish and it was identified that 4 to 6 DPH is a suitable timespan to inject donor-derived stem cells into recipients. However, having a host with a short maturation time and smaller body size than channel catfish would be ideal for commercial application. To address that issue, another study was conducted to assess the effectiveness of triploid white catfish (*Ameiurus catus*) as a host species to transplant blue and channel catfish stem cells during the production process of xenogeneic catfish. It was demonstrated the suitability of white catfish as a host species by transplanting stem cells between 4 - 5 DPH for commercial-scale hybrid catfish production. In all the above experiments, freshly extracted stem cells are used to create xenogens, which is challenging, especially considering germ cell production is donor size and age specific as well as dependent on seasonal cycles. Thus, having

frozen stem cells available in germplasm repositories will help to facilitate xenogenesis technology for hybrid catfish production. Hence another study was conducted to assess the effectiveness of fresh and cryopreserved stem cells for germ cell transplantation to support xenogenesis and it was revealed that cryopreserved donor stem cells can recover in recipient gonads and perform as well as their freshly extracted counterparts. Further, the collection of an adequate number of stem cells for transplantation and cells damaged by proteinases (trypsinization) during the procedure of stem cell extraction were major challenges that required to be addressed. Hence, *in vitro* propagation of stem cells has emerged as a potential solution. Thus, the final study aimed to identify the best culture medium for *in vitro* propagation of blue catfish stem cells for further identification of optimum culture parameters and it was identified that Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) is the most suitable culture medium combination for *in vitro* propagation of SSCs. Overall, these findings will enhance the efficiency of germ cell transplantation for commercial-scale hybrid catfish production.

Keywords: xenogenesis, triploids, stem cell transplantation, cryopreservation, cell colonization, proliferation

Acknowledgment

I would like to dedicate this dissertation to my major supervisors Dr. Rex Dunham and Dr. Ian A. E. Butts, for their enthusiastic guidance, encouragement and continuous support during the research study. With their continuous support, I never lost my eagerness to progress and it was a great honor for me to study under their supervision. I am grateful to Dr. Anthony G. Moss and Dr. Paul Dyce, for evaluating me, being in my PhD committee. In addition, special thank goes to Dr. Xu Wang for accepting the invitation to be the outside reader for my dissertation.

My heartfelt thanks are for all the colleagues and staff of the School of Fisheries, Aquaculture and Aquatic Sciences of Auburn University, for their precious advice and assistance throughout the research. Assistance provided by all of my Genetics Laboratory crew, mainly Veronica Alston, Logan Bern, Jacob Al-Armanazi, Jeremy Gurbatow, Dr. Su, Dr. Shang, Dr. Coogan, Nour El Husseini, Andrew Johnson, Shangjia Li, Dr. Xing, Dr. W. Wang, Jinhai Wang, Dr. Simora, Cuiyu Lu, Dr. Xiaoli Ma, Dr. Hasin, as well as all others is greatly appreciated. Many thanks to my friends from Auburn: Jan Macguire Miles and Carter Miles for their support and inspiration to manage and succeed in all work. Finally, I would like to express my profound gratitude and love to my husband Harsha Galkanda and loving daughters Thini Galkanda and Pini Galkanda, for their support during the study. Real understanding and continued encouragement of my husband was a great inspiration towards my success and he was always beside me sharing the problems and challenges during the study and research. Last but not least, great thanks to my mother, my sister, and brother who always supported me and expected me to finish my research in gold. I know that words are not enough to express my gratitude to them. Thanks again to all the people, who helped me to complete my research.

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Chapter 1

General Introduction

1.1 Catfish Industry in the United States

Over the past several decades, the catfish industry has expanded tremendously in the United States, especially in southern states including Mississippi, Alabama, Arkansas, Louisiana, and Texas (Glahn and Brugger, 1995). Farm-raised catfish production was leading the finfish aquaculture industry, where ~80,000 hectares of land was dedicated to catfish production in 2002 (USDA, 2015). In 2003, ~300 million kg of catfish were produced (USDA, 2015), however because of high production costs, the United States recession and competition with inexpensive imported fish, including Asian catfish, production dropped to 138 million kg by 2011(NASS, 2012). Then catfish production grew slightly to approximately 150 million kg in 2015-2017 and continues to slowly grow with 158 million kg produced in 2019 (NASS, 2020).

1.2 Hybrid Catfish - Channel catfish ♀ × Blue catfish ♂ (C × B)

Until recently, channel catfish, *Ictalurus punctatus* was the prominent species cultured for food fish (Dunham and Elaswad, 2018). However, it was shifted toward the production of hybrid catfish, produced by crossing the channel catfish, ♀ with the blue catfish, *I. furcatus* ♂ due to several improved performances compared with both parent species (Dunham and Brummett, 1999; Brown et al., 2011; Arias et al., 2012; Dunham et al., 2014; Dunham and Elaswad, 2018). The growth rate of the C × B hybrid catfish was 20% to 100% higher than that of channel catfish under high stocking densities (Yant et al., 1976; Dunham et al., 1987; Dunham et al., 1999; Dunham and

Masser, 2012), while feed conversion was 15% - 20% better than their parental species (Yant et al., 1976; Dunham et al., 1987). In addition to the enhanced growth rate, channel-blue hybrid catfish are resistant to low dissolved oxygen levels (Dunham and Masser, 2012), disease-resistant (Dunham et al., 2008; Arias et al., 2012; Dunham and Masser, 2012), have higher survival, dress out percentage, and fillet yield (Bosworth et al., 2004; Dunham and Masser, 2012), higher seinability (Dunham and Argue, 1998), vulnerability to angling (Dunham et al., 1986; Dunham and Masser, 2012), and altered morphometric traits (Bosworth et al., 2004; Dunham et al., 2008) compared to their parents. This led to hybrid catfish becoming a vital component of the United States catfish industry (Dunham and Masser 2012; Perera et al., 2016). Although the hybrid industry has seen sustained growth, there are still challenges due to the lack of natural hybridization between these two species. Artificial fertilization has been considered as an appropriate technology for commercial hybrid catfish production, which still requires additional efforts such as, sacrificing mature blue catfish males for sperm collection, hormonal stimulation of females, extra holding space, special handling techniques, additional feed, and labor cost (Argue et al., 2003; Hu et al., 2011). Hence, new innovative reproduction technologies such as xenogenesis can be used to mitigate some of the challenges in hybrid catfish production.

1.3 Xenogenesis

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). For xenogenesis, there are several types of cells that can be isolated and introduced to a host species, such as primordial germ cells (PGCs) from developing embryos, spermatogonial stem cells

(SSCs), and oogonial stem cells (OSCs) (Yoshizaki et al., 2002; Yoshizaki et al., 2003; Perera et al., 2017). During this procedure, PGCs from developing embryos, SSCs or OSCs from donor fish are typically injected into triploid recipient fish with the objective of producing gametes of the donor fish in the host (Perera et al., 2017; De Siqueira-Silva et al., 2018).

PGCs are the founders of the gametes (De-Felici, 2004), which arise at the earliest stages of gonadal development (Saitou and Yamaji, 2012). They can self-renew and differentiate for both oogonia and spermatogonia after gonadal sex differentiation (Yoshizaki et al., 2003), making them useful as donor cells for xenogenesis (Yoshizaki et al., 2002). However, it is difficult to apply PGC transplantation technology for aquaculture and conservation due to the large number of small-bodied fishes that are needed to obtain enough PGCs. Hence, Okutsu et al (2006) discovered that SSCs and OSCs can be used as alternative donor cell to PGCs.

Both SSCs and OSCs have been used successfully for xenogenesis applications. They have the capability of self-renewal throughout their life. Additionally, it is easier to extract large quantities of these specific donor cells (Yoshizaki and Lee, 2018). Hence, both SSCs (Yoshizaki et al., 2010; Lacerda et al., 2013; Yoshikawa et al., 2016; Perera et al., 2017; Ye et al., 2017; Hettiarachchi et al., 2022) and OSCs (Ye et al., 2017; Yoshizaki and Lee, 2018; Hettiarachchi et al., 2022) transplantation has been successfully applied to improve reproduction for commercially valuable fish species and to conserve endangered populations in several studies.

However, still there are some challenges in xenogenesis that need to be optimized in commercial-scale hybrid catfish embryo production. Hence following experiments were conducted with the major objective of enhancing the efficiency of hybrid catfish embryo production through xenogenesis.

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Chapter 2

Maximizing colonization and proliferation of blue catfish (*Ictalurus furcatus*) donor stem cells for the creation of xenogenic catfish: Identifying the best host age of triploid channel catfish (*I. punctatus*)

Abstract

Xenogenesis is an innovative technology for hybrid catfish (♀ channel catfish, *Ictalurus punctatus*, × ♂ blue catfish, *I. furcatus*) embryo production. The xenogeneic process can be accomplished by transplanting undifferentiated diploid germline stem cells derived from donor fish into sterile recipients. This methodology enables recipients to produce donor-derived gametes. Until recently, the timing of transplantation of donor cells into hosts was done with limited knowledge of the best age to inject cells. The age of the host could critically affect the success of germ cell transplantation. The present study aimed to identify the best age of the triploid channel catfish to transplant blue catfish stem cells for production of xenogeneic catfish. Triploid channel catfish fry were injected with blue catfish stem cells labeled with PKH26 dye from 0 to 18 days post-hatch (DPH). Then at 50 DPH (1st time interval) and 90 DPH (2nd time interval), total length (TL), weight (BW), and survival of recipients were evaluated. Colonization of donor cells was evaluated in recipients using PKH26 dye fluorescence to calculate percent cell (<150 μm^2) and cluster areas (>150 μm^2). PCR determined the percentage of xenogens from gonads. Day of stem cell injection had no impact on TL and BW of recipient fish when evaluated at both sampling intervals. Survival of recipients injected with blue catfish stem cells increased from 0 to 5.4 DPH. After 5.4 DPH, survival remained high (≥82%) for fry injected until 18 DPH. At the 1st time interval, cell and cluster area increased as recipient fish injected from 0 to 5.4 DPH and 0 to 5.6 DPH, respectively.

Thereafter, fluorescent cell and cluster area in the host declined with no further decrease after 11.3 and 10.4 DPH, respectively. At the 2nd time interval, cell and cluster area increased as recipients were injected from 0 to 5.8 DPH and 0 to 5.7 DPH, respectively and significantly declined with no further decrease after 10.2 and 11.3 DPH, respectively. At the 1st time interval, the highest percentage of xenogens were detected when recipients were injected from 3 to 5 DPH (83.3%), while at the 2nd time interval, the highest percentage of xenogens was detected from 4 to 6 DPH (83.3%). Our results show that 4 to 6 DPH is a suitable timespan to inject donor-derived stem cells into recipients. These findings will enhance the efficiency of germ cell transplantation for commercial-scale hybrid catfish production.

Keywords: Xenogenesis, Triploids, Blue catfish, Stem cells

1.0. Introduction

Over the past decade, the United States catfish industry has steadily shifted toward the production of hybrid catfish, produced by crossing the channel catfish, *Ictalurus punctatus* ♀ with the blue catfish, *I. furcatus* ♂ (Torrans and Ott, 2018) due to several superior characteristics, such as fast and uniform growth rate, efficient food conversion, tolerance to low dissolved oxygen, improved disease resistance, higher survival, dress out percentage, fillet yield, and seinability (Dunham et al., 1987; Dunham and Argue, 1998; Bosworth et al., 2004; Arias et al., 2012; Dunham and Masser, 2012; Dunham et al., 2014). However, additional efforts must be taken to produce hybrid catfish due to the lack of natural hybridization between the two species (Argue et al., 2003). Artificial fertilization is the current technology for commercial production of the hybrid catfish, which requires sacrificing mature blue catfish males for sperm collection, hormonal stimulation of females, extra holding space for brood stock, and special handling techniques (Argue et al., 2003; Hu et al., 2011). As a result, new innovative hatchery technologies, such as xenogenesis are attracting considerable attention to produce hybrid catfish minimizing both time and effort in the artificial fertilization process.

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), which can be accomplished by transplanting diploid germline stem cells such as primordial germ cells (PGCs), spermatogonial stem cells (SSCs) or oogonial stem cells (OSCs), derived from donor diploid fish into sterile recipients which then enables recipient fish to produce donor-derived gametes (Yoshizaki et al., 2010; Wong et al., 2011; Perera et al., 2017; De Siqueira-Silva et al., 2018).

PGCs are the founders of the gametes (De-Felici, 2004), which arise at the earliest stages of gonadal development (Saitou and Yamaji, 2012). They can self-renew and differentiate for both oogonia and spermatogonia after gonadal sex differentiation (Yoshizaki et al., 2003), making them useful as donor cells for xenogenesis (Yoshizaki et al., 2002; De-Felici, 2004; Okutsu et al., 2006a). Yoshizaki et al. (2000) performed successful PGC transplantation into newly hatched rainbow trout (*Oncorhynchus mykiss*) larvae, with GFP transgenic rainbow trout as the donor and wild-type rainbow trout as the recipient. Following these findings, Takeuchi et al. (2004) intraperitoneally transplanted rainbow trout PGCs into masu salmon (*O. masou*) gonads and documented the successful production of functional sperm derived from the donor rainbow trout in the host. However, it is difficult to apply PGC transplantation technology for aquaculture and conservation due to the large number of small-bodied fishes that are needed to obtain enough PGCs. In addition, it can be challenging to even collect these PGCs from smaller fish (Yoshizaki and Lee, 2018). Hence, Okutsu et al (2006b) discovered that SSCs and OSCs can be used as alternative donor cell to PGCs.

Both SSCs and OSCs have been used successfully for xenogenesis applications. They have the capability of self-renewal throughout their life and can transmit genes to successive generations. Additionally, it is easier to extract large quantities of these specific donor cells (Brinster and Avarbock, 1994; Yoshizaki and Lee, 2018). SSC transplantation has been successfully introduced to several commercially valuable species, such as masu salmon SSCs into rainbow trout (Kise et al., 2012), jundia catfish, *Rhamdia quelen* SSCs into Nile tilapia, *Oreochromis niloticus* (Silva et al., 2006), GFP transgenic rainbow trout SSCs into wild-type rainbow trout (Okutsu et al., 2006a, 2006b; Okutsu et al., 2007; Lacerda et al., 2018), juvenile pejerrey, *Odontesthes bonariensis* SSCs into patagonian pejerrey, *O.hatchery* (Majhi et al., 2009), Nibe croaker, *Nibea mitsukurii* SSCs

into chub mackerel, *Scomber japonicas* (Yazawa et al., 2010) Japanese char, *Salvelinus leucomaenis* SSCs into wild-type trout (Kise et al., 2012) and blue catfish SSCs into triploid channel catfish (Perera et al., 2017; Hettiarachchi et al., 2022) to produce donor-derived offspring. OSCs have also been tested as donor cells for the xenogenesis process (Yoshizaki et al., 2010; Yoshizaki and Lee, 2018). Specific examples include the injection of GFP transgenic rainbow trout OSCs into wild-type trout (Yoshizaki et al., 2010), transgenic zebrafish, *Danio rerio* OSCs into zebrafish (Wong et al., 2011), and critically endangered Chinese sturgeon, *Acispenser sinens*is OSCs into wild sturgeon, *A. dabryanus* (Ye et al., 2017).

To date, most xenogenic transplantation work (i.e., donor-derived cells into hosts) was done with limited knowledge of the best age to inject cells into the host, and this issue has not been addressed for ictalurid catfish. One potential obstacle could be the rejection of the donor-derived cells by the immune system of the recipient fish (Manning and Nakanishi, 1996). Low levels of colonization of donor cells in channel catfish (Perera et al., 2017) might be due to partial rejection of these cells. To overcome this potential obstacle, embryos and immature fry have been used as recipients because they do not have a mature immune system (Anitha, 2010). The expectation is that embryos and newly hatched fry would not have the ability to reject donor-derived foreign cells. Alternatively, immune response may not be important, but there could still be a critical development stage for maximum receptivity for colonization and proliferation of donor cells, and the overall efficiency and success of germ cell transplantation. However, previous studies have not identified the most suitable host age for cell transplantation.

The present study aimed to identify the best age of the triploid channel catfish (host) to transplant blue catfish stem cells during the production process of xenogeneic ictalurid catfish.

2.0. Materials and methods

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

2.1. Catfish husbandry and triploid induction

Sexually mature channel catfish females and males were obtained from earthen ponds at the Fish Genetics Research Unit at Auburn University, AL, USA. Fish were fed to satiation with 32% protein pellet feed five days per week during summer and three days per week during winter. In addition, the fish were fed 36% protein broodstock feed two months prior to spawning. During the experimental period, dissolved oxygen (DO) was measured twice daily while temperature and salinity were recorded daily. In addition, total ammonia-nitrogen (TAN), nitrite, nitrate, pH, hardness, and alkalinity were measured twice per week. During the broodstock collection period, DO, temperature, pH, TAN, nitrite, nitrate, hardness, and alkalinity levels were 6.8 ± 1.1 mg/L, 27.3 ± 1.6 ^{o}C, 6.8 ± 0.3, <0.5 mg/L, <0.5 mg/L, <20 mg/L, 89.5 ± 15 mg/L, and 35.5 ± 5 mg/L, respectively. Fish were collected by seining ponds with a 3.8 cm mesh seine net.

Three gravid channel catfish females (mean ± SEM weight = 1.9 ± 0.9 kg) were administered intraperitoneal injections of luteinizing hormone-releasing hormone analogue (LHRHa) at 20 μg/kg body weight followed by a 100 μg/kg dose 12 h later. Then, channel catfish females were placed into spawning bags submerged in flow-through spawning tanks (7.5 m × 0.5 m × 0.45 m). Three channel catfish males (mean ± SEM weight = 2.4 ± 0.6 kg) with pronounced secondary

sexual characteristics (i.e., muscular head and pronounced genital pore) were euthanized for sperm collection. Testes were surgically removed with a sterile scalpel and forceps. The testes were rinsed with 0.9% saline solution to remove blood. After the removal of excess blood, the testes were gently dried with paper towels until all blood and moisture were removed. Testes were minced with a scalpel blade. Then the sperm/testes solution was filtered with a 100 μm mesh. Next, 10 mL of 0.9% saline was added for each 1 g of testes (Dunham and Masser, 2012).

When the females began to ovulate, several eggs could be seen attached to the spawning bags. The ovulated females were anesthetized in a 10 L tank with 100 ppm tricaine methanesulfonate (MS-222, Ferndale, WA) with 10 ppm sodium bicarbonate. Eggs were hand stripped into spawning pans coated with Crisco commercial vegetable oil (~25 g of eggs/pan) and dry fertilized with a prepared sperm solution at a rate of 2 mL of sperm solution per 25 g of eggs (Hettiarachchi et al., 2022). At 5 min post-fertilization, the eggs were pressure shocked to induce triploidy, according to standard protocols described by Perera et al. (2017) and Hettiarachchi et al. (2022). After triploid induction, the eggs were placed in a trough for 1 h supplemented with 50 ppm $CaCl_2$ for water hardening. After the hardening process, eggs were incubated in a flow-through hatching trough (7.5 m × 0.4 m × 0.25 m) in hanging baskets (0.15 m × 0.15 m × 0.15 m) which were aerated with a paddle wheel. Ploidy level was verified by measuring erythrocyte nuclei volume with a Coulter-counter at the USFWS Warm Springs Regional Fisheries Center, Warm Springs, GA (Beck and Biggers, 1983).

2.2. Isolation of donor stem cells from immature blue catfish

Sexually immature blue catfish (mean ± SEM length = 30.1 ± 4.8 cm, weight = 419.7 ± 75.6 g) were selected daily (n = 4 fish per day × 19 days = 76 fish) and euthanized. After being euthanized, the external body of the fish was sterilized with 70% ethanol. Then, gonad extraction and stem cell isolation were performed according to the standard protocols described by Shang et al. (2015), Abualreesh et al. (2020, 2021a, 2021b), and Hettiarachchi et al. (2020, 2022). In brief, gonads were removed from the peritoneal cavity and placed on a sterile petri dish (100 mm × 15 mm) containing 5 mL of Hanks' Balanced Salt Solution [(Life Technologies) supplemented with 1.0 μg/mL $NaHCO_3$ (Church & Dwight Co., NG) and 10,000 unit/mL Penicillin - Streptomycin (Life Technologies)]. Then samples were transferred to a biosafety cabinet for cleaning with 0.5% bleach solution prepared with double distilled H_2O. Further cleaning was done with HBSS, and phosphate-buffered saline (Alfa Aesar). Testes were minced with a sterilized scalpel blade. Minced samples were then transferred to a 50 mL autoclaved glass flask for the trypsinization with 0.25% trypsin (Life Technologies). Samples were then incubated on ice followed by a magnetic stirrer. The cell suspension was then filtered with 70 μm and 40 μm cell strainers (VWR International) and centrifuged at 500 g (Eppendorf Centrifuge 5418 R) for 20 min to separate cells. The separated pellet was resuspended in 2 mL of Dulbecco's Modified Eagle's Medium/DMEM (DMEM; Corning cellgro) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 10,000 unit/mL penicillin-streptomycin (Life Technologies), and 200 mM L-glutamine (Life Technologies) to provide a favorable environment for the stem cells.

2.3. Stem cell labelling with PKH26 and implantation into the triploid channel catfish host

The isolated blue catfish stem cells were labeled with PKH26 red fluorescence cell linker (CGLDIL, Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. In brief, cell suspensions were centrifuged at 500 g (Eppendorf Centrifuge 5418 R) for 5 min to obtain a loose pellet. Then, the supernatant was carefully removed and 1 mL of diluent C was gently added to the cell pellet to ensure complete dispersion. Immediately prior to staining, Dye Solution (4 $\times 10^{-6}$ M) was prepared by adding 4 μL of PKH26 dye solution (Catalog Number; P9691) into 1 mL of diluent C and mixed well to disperse. Then, previously prepared cell solution with diluent C was gently added to the prepared PKH26 dye solution and mixed by pipetting. The cell/dye suspension was incubated for 5 min with periodic mixing for the adequate staining. An equal volume of 1% BSA (Bovine Serum Albumin; VWR) was added to prevent further staining and incubated for 1 min to allow binding of excess dye. Finally, the sample was centrifuged at 500 g for 10 min and washed three times with DMEM to remove excess 1% BSA, dilutant C, and PKH26 dye. After the final wash, resuspend the cells in 10 mL of DMEM was used for cell transplantation.

Labelled stem cells were transplanted into triploid channel catfish fry (mean ± SEM) (Fig.1) daily from 0 days post-hatch (DPH) to 18 DPH (i.e., 19 days of injection) with either stem cells labeled with PKH26 or stem cells without PKH26. For each treatment, 90 fry were injected (three replicate for each treatment / 30 fry per treatment) with 1 μL of stem cell suspension containing ~80,000 unsorted cells (The final cell suspension was mainly consisted of undifferentiated stem cells (spermatogonia A and oogonia). However, it contained a minor amount of differentiated stem cells which was hard to totally evacuate. Therefore, during the preset study, stem cell suspension was termed "unsorted cells") with a 33-gauge needle (outer diameter: 0.209 mm; inner diameter: 0.108 mm; Hamilton, Reno, NV). The needle was inserted, and cells were injected into the cavity

between the anal fin and yolk sac where the genital ridge is expected to be formed (Hettiarachchi et al., 2022). Non-injected triploids served as the controls.

2.4. Growth and survival

After stem cell transplantation, fry were transferred to a recirculating aquaculture system (RAS) at a density of 30 fry per cage (0.2 m × 0.2 m × 0.25 m). Fry were fed a standard commercial catfish fry feed (crude protein: 50.00%, crude fat: ≥4.00%, crude fiber: 7.00%, and phosphorus: 0.80%) three times per day to satiation. The feed pellet size was adjusted according to their growth performance. During the experimental period, DO, temperature, salinity, and pH were monitored daily while TAN, nitrite, nitrate, hardness, and alkalinity were measured every two days. DO, temperature, salinity, TAN, nitrite, nitrate, pH, hardness, and alkalinity were maintained within acceptable ranges for channel catfish at 6.8 ± 1.4 mg/L, 26.1 ± 1.2 ^{o}C, 3.6 ± 0.2 ppt, <0.5 mg/L, <0.5 mg/L, <15 mg/L, 6.8 ± 0.2, 89.5 ± 9 ppm, and 35.8 ± 5 ppm, respectively. At 50 and 90 DPH, 30 fingerlings were randomly selected from each treatment to evaluate growth performance using BW (mean ± SEM) and TL (mean ± SEM). In addition, survival (mean ± SEM) was evaluated at 50 DPH (hereafter, 1st time interval) and 90 DPH (hereafter, 2nd time interval).

2.5. PKH26 observations at 50 and 90 DPH

To evaluate stem cell (SSCs and OSCs) proliferation and colonization, 12 fingerlings were randomly sampled from each treatment. The fingerlings were euthanized by pithing. Gonads were surgically removed and placed on a sterile microscope slide (160005, Life Technologies). The

slide was covered with a coverslip and digital images were taken using a Zeiss Imager A2 microscope equipped with a camera (Axio-cam 202) and Zen Pro v.6.1 software (Zeiss, Oberkochen, Germany). Once focused on the tissue, a grid overlay was added to determine the total gonadal area and six random sections of the gonads were photographed (wave length 567 nm; Texas red filter). All tissue samples with donor-derived 'positive' cells glowed red, while 'negative' cells had no fluorescence. Fluorescent images were analyzed using ImageJ software (http://imagej.nih.gov/ij/). ImageJ was calibrated to enumerate the area in μm^2 and cell number, cell area, cluster number, and cluster area were determined using each fluorescent section of the gonad samples. Finally total cell and cluster areas were calculated using average values of six images. Cells were defined as having a fluorescence area <150 μm^2 and clusters as having a fluorescence area >150 μm^2.

2.6. Genomic DNA and PCR analysis

At both time intervals, 6 fingerlings were randomly sampled from each treatment for PCR analysis. The fingerlings were sacrificed by pithing and gonads were surgically removed. The separated gonad samples were placed into 1.5 mL Eppendorf tubes on ice and then held at -80 ^oC until DNA extraction. DNA was extracted using proteinase K digestion followed by protein and ethanol precipitation (Waldbieser and Bosworth, 2008) (Table 1). In brief, a cell lysis solution which contained 600 µL of DNA extraction buffer [100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% sodium dodecyl sulfate] and 3 µL of 20 mg/mL proteinase K (Sigma-Aldrich, St. Louis, MO) were used for control sample digestion. After samples were incubated and digested in the cell lysis solution for 4 to 5 h at 55 °C, proteins were precipitated by protein

precipitation solution (71005-102, VWR) and DNA was precipitated by isopropanol (89108-023, Fisher Scientific). Then the DNA pellet was washed twice with 70% ethanol, air dried for 10 - 20 min, and resuspended in 20 μL RNase/DNasefree water. All DNA samples were quantified with a NanoDrop™ 2000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware) and diluted up to 500 ng/μL. Then the PCR was carried out in a 10 μL reaction volume in microcentrifuge tubes containing 0.6 μL of each primer follistatin (Fst) and hepcidin (Hamp), 5 μL of 2x Eco, 2.2 μL of RNase/DNasefree water, and 1 μL of the DNA sample. The PCR procedures were performed using the following thermal profile: after an initial denaturation at 95 °C for 3 min; PCR amplification was carried out at 95 °C for 1 min, 60 °C for 1 min, and 70 °C for 1 min for 35 cycles. A final extension at 72 °C for 4 min was included. The Fst and Hamp amplification products were resolved on an ethidium-bromide-stained 2.0% agarose gel. All the reactions for each sample were repeated three times.

2.7. Statistical analyses

Breakpoint analysis was run with the Segmented package (Muggeo 2017) in R (R Core Team 2022). The segmented regression or breakpoint analysis was used to determine the optimal age to transplant blue catfish stem cells into triploid channel catfish (host), based on offspring growth, survival, and fluorescent image analyses.

3.0. Results

3.1. Growth and survival

Day of stem cell injection (0 to 18 DPH) had no impact ($P \geq 0.702$) on mean (± SD) TL (Fig. 2A) and BW (Fig. 2B) of recipient fish when evaluated at the 1st (5.0 cm ± 0.2 and 2.5 g ± 0.1, respectively) and 2nd time intervals (14.7 cm ± 0.7 and 15.1 g ± 0.7, respectively). Segmented linear regression showed increasing survival of recipient fish at 1st time interval when injected with blue catfish stem cells from 0 to 5.4 DPH ($P < 0.0001$, $R^2 = 0.77$, $y = 29.1 + 9.14x$; Fig. 2A). Then after 5.4 DPH, survival remained high, where mean (± SD) daily survival was ≥86% ± 3 for fry injected until 18 DPH ($P = 0.934$, Fig. 3A). A similar phenomenon was observed for the survival of recipient fish when evaluated at the 2nd time interval with a breakpoint at 5.3 DPH ($P < 0.0001$, $R^2 = 0.83$, $y = 22.0 + 9.9x$; Fig. 3B). Thereafter, fry survival continued to remain high (≥82% ± 3; $P = 0.181$). Overall, between 5 - 18 DPH the fry injections resulted in higher survival than the rest of the treatments at both time intervals.

3.2. PKH26 observations at 50 and 90 DPH

Fluorescent image analyses of the gonadal region in recipient fish at both time intervals revealed differences in stem cell proliferation and colonization based on the day of injection (Fig 4). For instance, at the 1st time interval, cell area and cluster area of injected recipient fish was increasing from 0 to 5.4 DPH ($P < 0.0001$, $R^2 = 0.68$, $y = 0.91 + 0.33x$; Fig. 5A) and 0 to 5.6 DPH ($P < 0.0001$, $R^2 = 0.65$, $y = 1.24 + 0.37x$; Fig. 5B), respectively. Thereafter, the percentage of

fluorescent cell area ($P < 0.0001$, $R^2 = 0.80$, y = 3.53 - 0.45x; Fig. 5A) and cluster area ($P < 0.0001$, $R^2 = 0.67$, y = 4.52 - 0.51x; Fig. 5B) in the host declined with no further decrease after 11.3 DPH and 10.4 DPH days of injection, respectively. Similarly, at the 2nd time interval, the percentage of fluorescent cell area and cluster area of injected recipient fish were increasing from 0 to 5.8 DPH ($P < 0.0001$, $R^2 = 0.77$, y = 0.95 + 0.34x; Fig. 5C) and 0 to 5.7 DPH ($P < 0.0001$, $R^2 = 0.84$, y = 1.01 + 1.52x; Fig. 5D). Thereafter, percent cell area ($P < 0.0001$, $R^2 = 0.77$, y = 3.69 – 0.46x; Fig. 5C) and cluster area ($P < 0.0001$, $R^2 = 0.85$, y = 10.20 – 1.24x; Fig. 5D) significantly declined with no further decrease after 10.2 DPH and 11.3 DPH. Overall, between 5 - 6 DPH fry injections resulted in the highest cell and cluster areas than the rest of the treatments at both sampling time intervals.

3.3. Genomic DNA and PCR analysis

PCR analysis detected blue catfish donor-derived stem cells in the gonads of triploid channel catfish fry when injected on different days after hatch (Fig. 6). When recipient fish were observed at the first time interval, the highest percentage of xenogens was detected when recipient fish were injected from 3 to 5 DPH (all 83.3%), while the lowest percentage of xenogens was detected on 0, 2, and 11 DPH as well as from 14 to 18 DPH (all 33.3%). At the 2nd time interval, the highest percentage of xenogens was detected from individuals injected from 4 to 6 DPH (all 83.3%), and the lowest percentage of xenogens was detected for those injected from 0 to 2 DPH and from 15 to 18 DPH (all 33.3%).

4.0. Discussion

Most xenogenic transplantation studies were conducted with limited knowledge of the best host age that yields the highest success rates. Filling this knowledge gap, the present study identified 4 to 6 DPH as the most suitable age to transplant donor-derived stem cells into a sterile channel catfish recipient, based on growth performance, survival, proliferation, and colonization.

In the present study, triploid channel catfish fry were used as the recipient fish. Triploids are reproductively sterile with reduced gonadal development and abnormal gametogenesis (Piferrer et al., 2009; Takeuchi et al., 2018). Almost always, triploid induction leads to both male and female sterility, however, there are some rare exceptions (Dunham, 2023). Triploid fry carrying two sets of maternal chromosomes and one set of paternal chromosomes develop normally but become sterile due to abnormal disjunction of chromosomes during meiosis (Yoshizaki and Lee, 2018). Hence, it is believed that blue catfish stem cells have the capability to proliferate and colonize inside the triploid host after transplantation as they would have a competitive advantage. Nevertheless, it is still important to determine the effectiveness of germ cell proliferation and colonization after transplantation. One of the most suitable and cost-effective technologies to determine germ cell proliferation and colonization rates, prior to sexual maturation, is done with the aid of fluorescent membrane dyes, such as PKH26. This dye enables relatively “easy” identification of transplanted cells in recipient gonads (Parish, 1999; Lee and Yoshizaki, 2016; Hettiarachchi et al., 2022). Here, at 50 DPH (1^{st} time interval) and 90 DPH (2^{nd} time interval), intraperitoneal transplantation of donor-derived blue catfish stem cells were observed in recipient gonads using PKH26 dye. Specifically, segmented linear regression showed higher cell and cluster colonization when donor-derived stem cells were injected into recipient fish from 5 to 6 DPH.

According to Manning and Nakanishi (1996) and Yoshizaki and Lee (2018) a potential obstacle for donor cell proliferation and colonization could be the rejection of transplanted cells by the immune system of the recipient fish. Newly hatched fish have delayed maturation of lymphoid organs (Koumans et al., 1994; Zapata et al., 2006). These lymphoid organs are involved in defensive mechanisms by generating immune cells (Bowden, 2008). There are two types of lymphoid organs that can be seen in fish, including the thymus and head kidney which produce and mature the immune system, while the secondary lymphoid organs include the kidney, spleen, and mucosa lymphoid tissue (Zapata et al., 2006; Swain and Nayak, 2009; Zapata, 2022). Thus, due to the delayed maturation of lymphoid organs, they could have delayed immunocompetence (Swain and Nayak, 2009). Hence, newly hatched fish have a limited ability to synthesize specific antibodies after a certain period of hatching, which is species-specific (Ellis. 1998; Swain et al., 2002; Magnadóttir et al., 2004).

Based on immune system maturation in fish, the most suitable host age to transplant donor stem cells should be the first few days after hatching (Manning and Nakanishi, 1996; Yoshizaki and Lee, 2018). However, in our present study, lower cell colonization rates and a lower percentage of xenogens (33.3%) were reported during these initial days of ontogeny, i.e., between 0 to 4 DPH which is contrary to these previous findings of the immune system maturation in fish.

Numerous examples exist of stem cell transplantation using various donor species during different stages of their life cycle. However, most experiments were conducted by transplanting stem cells into embryos or newly hatched host species between 0 to 2 DPH (Takeuchi et al., 2004; Kobayashi et al., 2007; Yoshizaki et al., 2010; Kise et al., 2012). This is primarily because researchers believe that the rejection of the donor-derived cells by the immune system should be lower at these initial stages in ontogeny rather than in later developmental stages (Takeuchi et al.,

2003; Takeuchi et al., 2004; Kutsu et al., 2006; Kobayashi et al., 2007; Kutsu et al., 2007; Yoshizaki et al., 2010; Kise et al., 2012). All these studies showed <50% successful transplantation rates, which is like the present study. Even though immature immunity is an added advantage in newly hatched larvae, there are some technical challenges to inject fry during this early window in ontogeny, as it is often difficult to insert the needle into the small body cavity between the anal fin and yolk-sac (i.e., where the genital ridge is expected to form). Thus, this could be a major reason for the lower transplantation success rates and fry survival we observed between 0 to 4 DPH, although we have preliminary data indicating some of these cells may have strong migratory ability.

Several examples exist for stem cell transplantation using fingerling and adult host stages (Majhi et al., 2009; Lacerda et al., 2010; Nóbrega et al., 2010; Wong et al., 2011; Perera et al., 2017). In line with our observations, these times for injections do not seem ideal, as lower transplantation rates (less than 30%) are typically reported due to the rejection of the donor stem cells via the immune system of the mature host (Yoshizaki and Lee, 2018). However, future studies are also needed to verify the effect of the host immunity on PGC, SSCs and OSCs transplantations, although our circumstantial evidence with fry and preliminary breeding experiments indicate this is likely not problematic in ictalurid catfish.

During the present study, 4 to 6 DPH was the most suitable host age to transplant donor stem cells regarding the percentage of xenogenic individuals with higher donor cell colonization and proliferation. In addition, a significantly higher survival rate was noted during this point in ontogeny, which is an added advantage. Hence, these findings will enhance the efficiency of germ cell transplantation in xenogenesis for commercial-scale hybrid catfish production.

5.0 Declaration of Competing Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

6.0 Acknowledgments

This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2018-67015-27614 from the USDA National Institute of Food and Agriculture. We would like to acknowledge the staff of the Catfish Genetics Research Unit as well as the EW Shell Fisheries Center at Auburn University for fish care, labor, and maintenance. Dr. Matthew K. Litvak was supported by NSERC Discovery Grant (RGPIN-2019-07138).

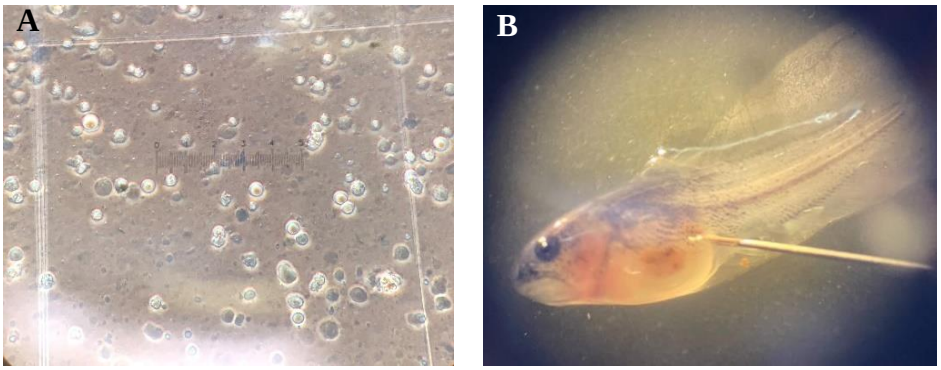

Fig. 1. Stem cells labeled with PKH26 dye (A). Transplantation (intraperitoneally) of donor derived stem cells into triploid channel catfish (*Ictalurus punctatus*) (B). Transplanted stem cells migrate to the genital ridge of the recipient, are incorporated, and initiate oogenesis or spermatogenesis.

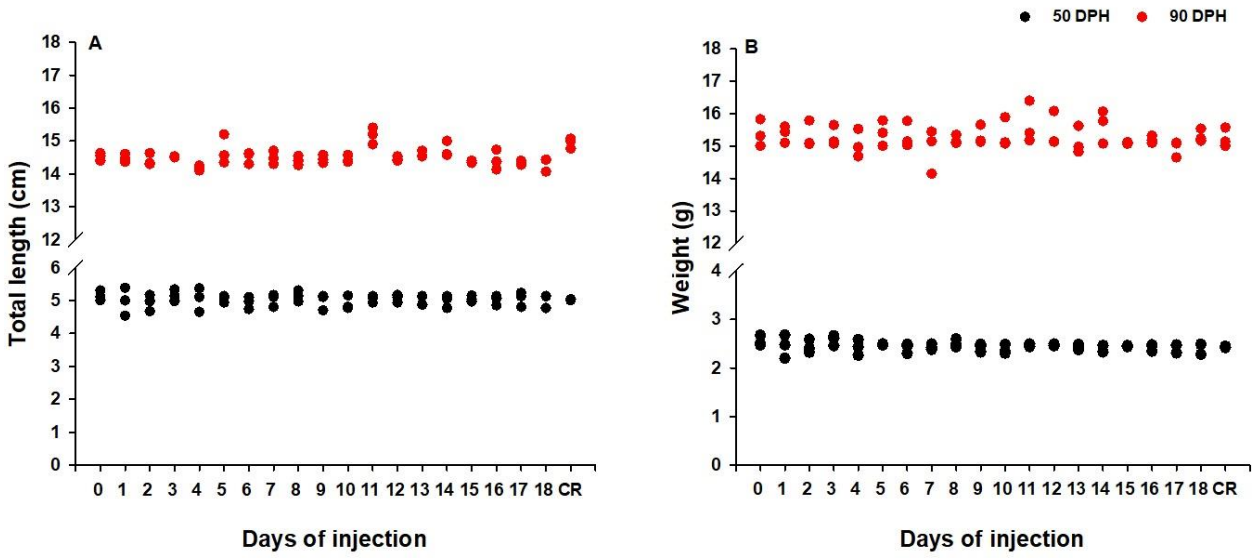

Fig. 2: Total length at 50 days post-hatch (DPH, 1st time interval) and 90 DPH (2nd time interval) (A), body weight at 50 and 90 DPH (B) of triploid xenogenic channel catfish (*Ictalurus punctatus*) injected with blue catfish (*I. furcatus*) stem cells from 0 to 18 DPH or non-injected control (CR) treatment.

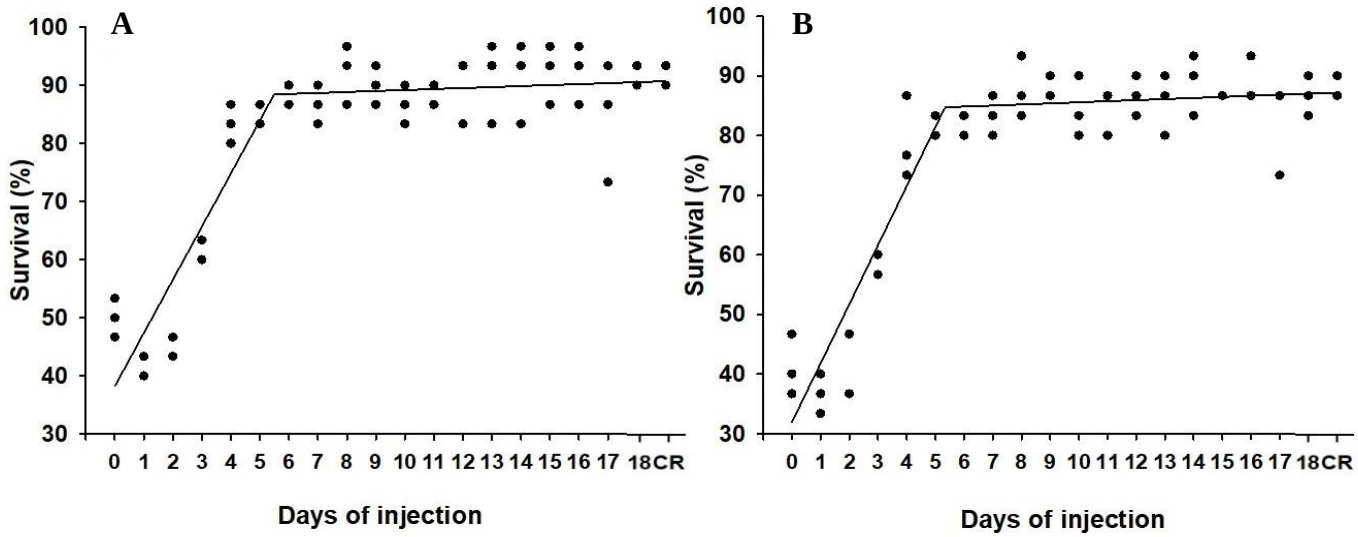

Fig. 3: Percentage survival at 50 days post-hatch (DPH, 1st time interval; A) and 90 DPH (2nd time interval; B) of triploid xenogenic channel catfish (*Ictalurus punctatus*) injected with blue catfish (*I. furcatus*) stem cells from 0 to 18 DPH or non-injected control (CR) treatment. Segmented linear regression or breakpoint analysis was used to determine the optimal age to transplant blue catfish stem cells into triploid channel catfish (host), based on offspring survival.

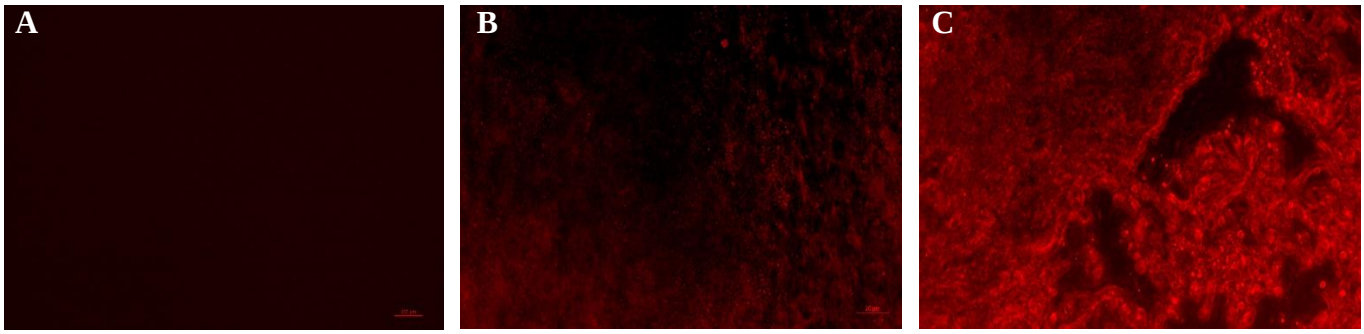

Fig. 4: Gonads expressing fluorescence from PKH26 dyed donor-derived stem cells (spermatogonial stem cells and oogonial stem cells). (A) non-injected control; (B) cells/clusters in triploid channel catfish (*Ictalurus punctatus*) (host) at 45 days post-hatch (DPH) that have been injected with blue catfish (*I. furcatus*) stem cells at 5 DPH; (C), cells/clusters in triploid channel catfish at 90 DPH that have been injected with blue catfish stem cells at 5 DPH.

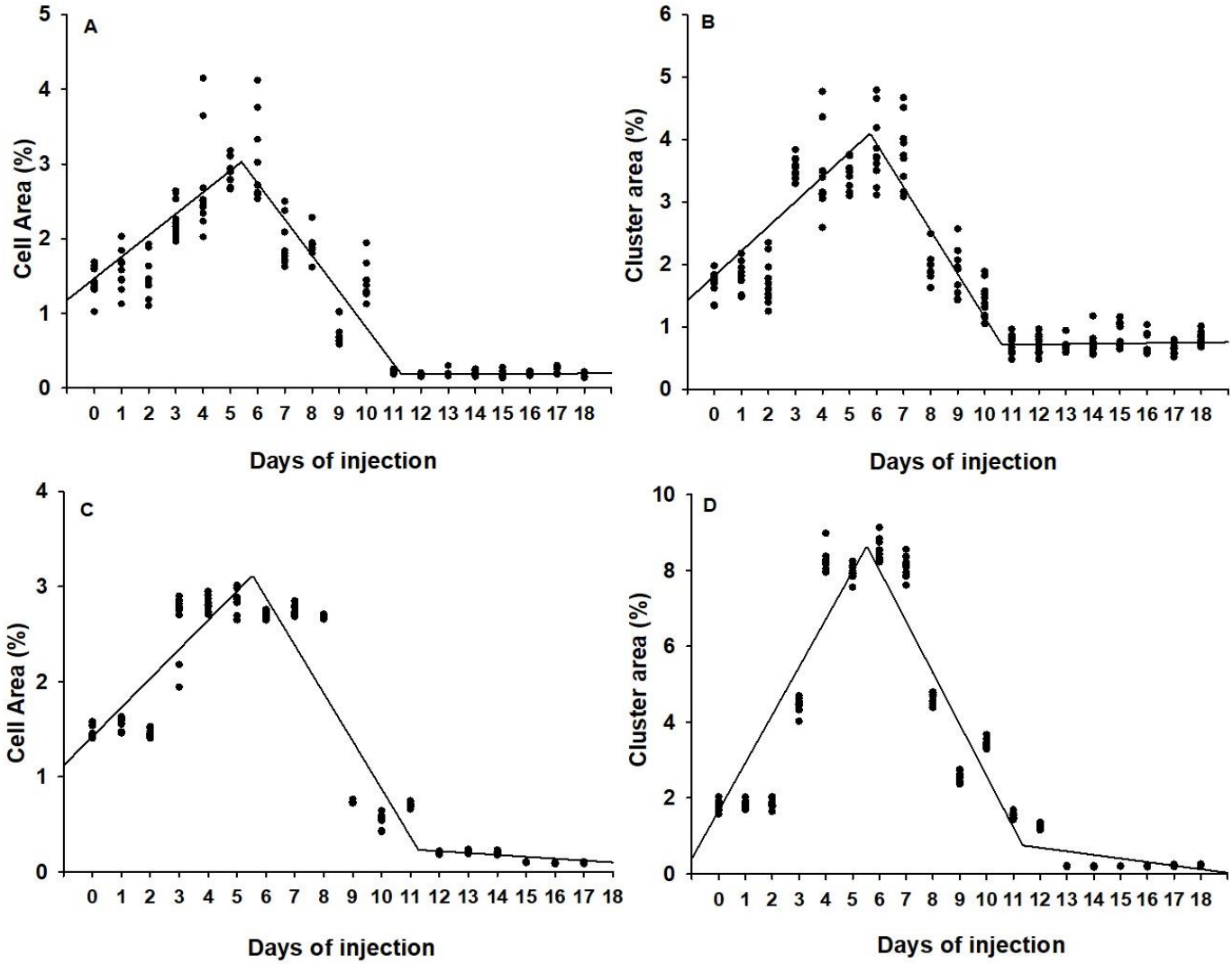

Fig. 5: Percent cell area and cluster area at 50 days post-hatch (DPH; 1st time interval) (A and B) and 90 DPH (C and D; 2nd time interval) of triploid channel catfish (*Ictalurus punctatus*) injected with blue catfish (*I. furcatus*) stem cells from 0 to 18 DPH. Segmented linear regression or breakpoint analysis was used to determine the optimal age to transplant blue catfish stem cells into triploid channel catfish (host), based on fluorescent image analyses. Cells were defined as having a fluorescence area <150 μm^2 and clusters as having a fluorescence area >150 μm^2.

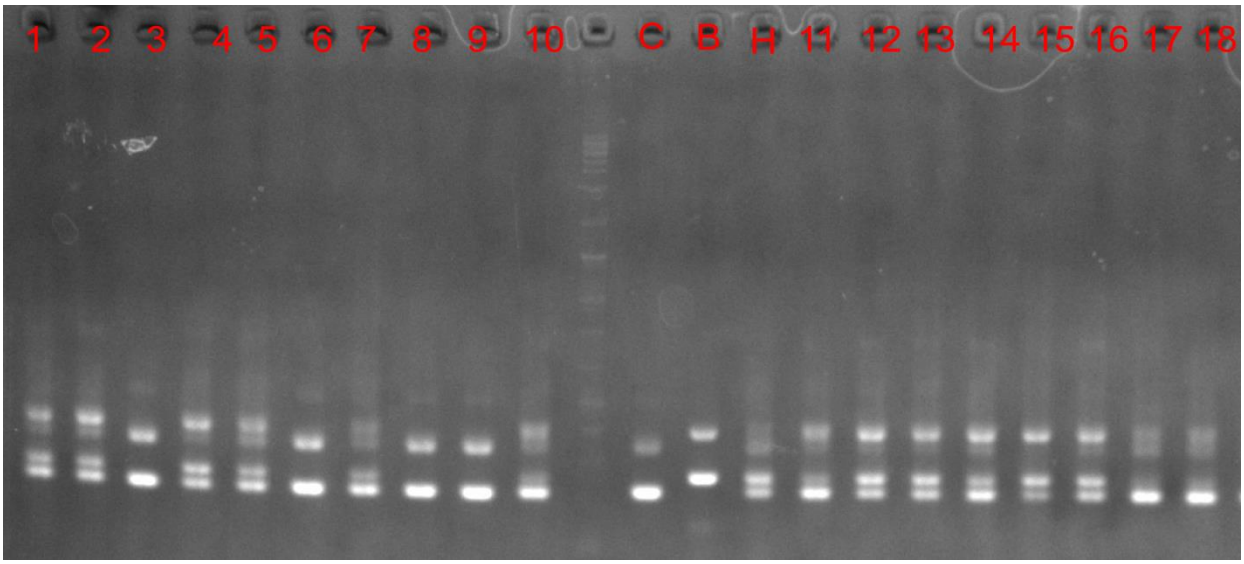

Fig. 6: Sample results from PCR for detecting blue catfish (*Ictalurus furcatus*) donor cells in the testes of triploid channel catfish (*I. punctatus*). Blue catfish and channel catfish cells were differentiated with PCR using follistatin (Fst) and hepcidin antimicrobial protein (Hamp) genes as markers. C = channel catfish control, B = blue catfish control, H = female channel catfish × male blue catfish hybrid controls.

Table 1: Primers used for genes [Fst (follistatin) and Hamp (hepcidin antimicrobial protein)] to differentiate channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*).

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

7.0. Reference

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Chapter 3

Producing xenogenic channel catfish, *Ictalurus punctatus* with cryopreserved testes and ovarian tissues of blue catfish, *I. furcatus*

Abstract

Xenogenesis has been identified as an innovative hatchery technology for hybrid catfish (channel catfish, *Ictalurus punctatus* ♀ × blue catfish, *I. furcatus* ♂) embryo production. The xenogeneic process can be accomplished by transplanting primordial germ cells, spermatogonial stem cells (SSC), or oogonial stem cells (OSC), derived from a donor diploid fish into a sterile recipient, which then enables recipient fish to produce donor-derived gametes. Currently, freshly extracted stem cells are used to create xenogens, which is challenging, especially considering germ cell production is donor size and age specific as well as dependent on seasonal cycles. Thus, having frozen stem cells available in germplasm repositories will help to facilitate xenogenesis technology for hybrid catfish production. This study was conducted to assess the effectiveness of fresh and cryopreserved stem cells for germ cell transplantation to support xenogenesis. At 5 days post hatch (DPH), triploid channel catfish were injected with fresh or cryopreserved SSC or OSC labelled with PKH26 dye. At 45 and 90 DPH, body size (total length and weight) and survival of these recipient fish were evaluated. In addition, colonization of fresh and cryopreserved donor cells was evaluated in recipients using PKH26 dye florescence (for percentage of cell area and cluster area) and PCR from gonadal samples. No significant differences in body size characteristics were detected between the fresh and cryopreserved injection treatments at 45 and 90 DPH. Survival was significantly higher in the control treatment than the SSC and OSC injected treatments at both

sampling days. Fluorescent imaging revealed that percentage of SSC cell area, SSC cluster area, OSC cell area, and OSC cluster area did not significantly differ between the fresh and cryopreserved treatments at 45 and 90 DPH. There was a significant increase in cell area and cluster area from 45 to 90 DPH for all treatments. According to PCR analyses, a high proportion of xenogens (at 45 and 90 DPH, respectively) were detected in recipient fish injected with fresh SSC (72.2 and 83.3%), cryopreserved SSC (61.1 and 66.6%), fresh OSC (66.7 and 61.1%), or cryopreserved OSC (61.1 and 61.1%). Taken together, our results show that cryopreserved donor stem cells can recover in recipient gonads and perform as well as their freshly extracted counterparts. Hence, cryopreserved stem cells can be used for future germ cell transplantation to support xenogenesis. These findings will enhance the efficiency of germ cell transplantation in xenogenesis for commercial scale hybrid catfish production.

Keywords: Xenogenesis, Blue catfish, Cryopreservation, Stem cells

1.0. Introduction

Catfish farming is the largest aquaculture sector in the United States producing nearly 160 million kg in 2019 (NASS, 2020). Like for many other species, the catfish industry faces numerous challenges, such as high production costs, competition with inexpensive imported fish (e.g., Asian catfish), and disease outbreaks, among others, which has major ramifications for total production yields and market value (Oglend, 2020). Until recently, channel catfish, *Ictalurus punctatus* was the primary species propagated for food fish in the United States (Dunham and Elaswad, 2018; Yadav et al., 2020), and ictalurid catfish still dominate total aquaculture production in the United States. Hybrid catfish produced by crossing the channel catfish ♀ with blue catfish, *I. furcatus* ♂ is now a vital component of the catfish industry (Dunham and Masser, 2012; Perera et al., 2017) because of their fast and uniform growth, efficient food conversion, tolerance to low dissolved oxygen, improved disease resistance, higher survival, dress out percentage, fillet yield, and seinability (Yant et al., 1976; Dunham et al., 1983; Dunham et al., 1987; Dunham and Argue, 1998; Bosworth et al., 2004; Arias et al., 2012; Dunham and Masser, 2012; Dunham et al., 2014). The hybrid now accounts for >50% of ictalurid catfish production in the United States (Torrans and Ott, 2018; Griffin et al., 2020).

One impediment to the increased application of hybrid catfish is the additional effort to produce hybrid fry as natural hybridization between channel catfish and blue catfish is rare due to reproductive barriers and timing of spawning (Hu et al., 2011). Therefore, induced ovulation of channel catfish females by administration of hormones, such as luteinizing hormone-releasing hormone analogue (LHRHa), is needed. This is coupled with the need to sacrifice blue catfish

males for sperm collection (Argue et al., 2003; Hu et al., 2011) and in vitro fertilization to produce hybrid embryos at a commercial scale (Su et al., 2013).

Compared to channel catfish, blue catfish have slower growth and maturity rates, reaching sexual maturity after 4-6 years and at larger sizes (Dunham et al., 1994). Thus, blue catfish requires more feed resources, time, space, and expenses for the farmers (Ligeon et al., 2004). The one-time use of the blue catfish males and the labor intense technology make hybrid embryo production challenging though commercially feasible. More efficient hybrid embryo production systems could be established with the aid of new innovative hatchery technologies, such as xenogenesis, to produce channel catfish × blue catfish hybrids for the industry.

Xenogenesis, is a method of reproduction in which successive generations differ from each other (Dunham, 2011). Thus, this innovative hatchery technology can be used to mitigate some of the challenges in hybrid catfish seed production (Dunham and Argue, 2000; Shang et al., 2015). Xenogenesis can be accomplished by transplanting undifferentiated germ cells, such as primordial germ cells (PGC), spermatogonial stem cells (SSC), or oogonial stem cells (OSC) derived from a donor diploid fish into a sterile recipient, which then enables recipient fish to produce donor-derived gametes (Amer et al., 2001; Wong et al., 2011). Overall, this is challenging, especially considering germ cell production is fish size related (Hettiarachchi et al., 2020), age specific (Ryu et al., 2022), and depends on seasonal cycles (Sato et al., 2017). Thus, having frozen stems cells available in germplasm repositories will help facilitate xenogenesis technology for hybrid catfish hatchery production.

Cryopreservation is a long-term storage technique for which biological materials (e.g., cells, gonads) are held at ultra-low temperatures (-196 ^{o}C) to arrest cellular metabolism in a physical state (Routray et al., 2010). Cryopreservation has several benefits and has been recognized for its

importance in maintaining everlasting gene banks and gamete supply for agriculture and aquaculture (Mandawala et al., 2016). If it is possible to use cryopreserved tissues to isolate undifferentiated germ cells, then gonadal tissues of fish can be preserved whenever they are available for xenogenic applications. Cell type, cell size, pH of cryomedia, cell density at freezing, composition of freezing media, type and concentration of cryoprotectants, freezing rate, thawing rate, and post-thaw medium all affect the efficiency of cryopreservation protocols (Garrisi et al., 1992; Tiersch et al., 2007; Lee et al., 2009). Shang (2013) conducted preliminary experiments and demonstrated the capability of cryopreserving blue catfish stem cells. Recently, Abualreesh et al. (2020, 2021ab), successfully cryopreserved blue catfish spermatogonia A and oogonia using 1.0 M dimethyl sulfoxide (DMSO) paired with 0.2 M lactose and egg yolk and froze cells at -0.5^{o}C or -1.0^{o}C/min. A protocol needs to be developed that does not waste these valuable stem cells. Thus, the efficiency of transplantation and colonization between fresh and cryopreserved donor germ cells should be comparable. Although these successful cryopreservation protocols have been developed, their economic application depends upon the ability of the cryopreserved stem cells to colonize and proliferate to accomplish production of xenogenic individuals relative to the effectiveness of using fresh stem cells.

Therefore, in the current study we aim to assess the relative effectiveness of fresh and cryopreserved stem cells for future germ cell transplantation to support xenogenesis. Here, we focus on the ability of cryopreserved blue catfish donor cells to proliferate and colonize inside triploid host fish over a 45 to 90 day period post-transplantation of 5 day old fry.

2.0. Methodology

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

2.1. Catfish husbandry and triploid induction

Sexually mature channel catfish females and males were obtained from 0.04 ha earthen ponds that were located at the Fish Genetics Research Unit at Auburn University, AL. These fish were fed (to satiation) a standard commercial catfish feed (Purina Catfish 32, floating feed; crude protein: ≥32.00%, crude fat: ≥4.00%, crude fiber: 7.00%, and phosphorus: 0.80%) 5 days per week during summer and 3 days per week during winter. In addition, two months prior to spawning, a 36% protein broodstock feed was used. During the broodstock collection period, dissolved oxygen (DO), temperature, pH, total ammonia-nitrogen (TAN), nitrite, nitrate, hardness, and alkalinity levels were 6.9 ± 1.0 mg/L, 27.5 ± 1.4 °C, 7.1 ± 0.2, <0.5 mg/L, <0.5 mg/L, <20 mg/L, 80 ± 15 mg/L, and 30 ± 5 mg/L, respectively.

Four gravid channel catfish females (mean ± SEM weight = 2.5 ± 0.8 kg) were administered intraperitoneal injections of LHRHa at 20 μg/kg body weight followed by a 100 μg/kg dose 12 h later. Then, channel catfish females were placed into spawning bags submerged in flow-through spawning tanks (7.5 m × 0.5 m × 0.45 m). Four channel catfish males (mean ± SEM weight = 1.9 ± 0.5 kg) were euthanized for sperm collection. Testes were surgically removed and then a sperm solution was prepared according to methods described by Dunham and Masser (2012). Briefly,

surgically removed testes were cleaned with 0.9% saline and gently dried with paper towel until all blood and moisture was removed. Testes were minced with a scalpel blade. Then the sperm/testes solution was filtered with a 100 µm mesh. Next, 10 mL of 0.9 % saline was added for each 1 g of testes.

Once eggs were observed on the outside of spawning bags, the ovulated females were anesthetized in a 10 L tank with 100 ppm tricaine methanesulfonate (MS-222, Ferndale, WA). The eggs from ovulating females were hand stripped into spawning pans coated with vegetable oil (~25 g of eggs/pan). The sperm solution was gently added to channel catfish eggs at a rate of 2 mL/25 g of eggs. Next, a Fullers' earth solution was added to the sperm-egg solution to start the fertilization process. This Fullers' earth solution was prepared by mixing 6 g of Fullers' earth powder (Starwest Botanicals, Sacramento, CA) into 1 L of pond water. The fertilized eggs were transferred into a cylindrical pressure chamber (340 mm height, 70 mm diameter), which was then placed onto a Carver press (Carver, Inc., Wabash, IN). At 5 min post-fertilization, the eggs were pressure shocked for 5 min at 7,000 psi (hydrostatic pressure) to induce triploidy. After the pressure shock, the eggs were placed in a trough (for 1 h) supplemented with $CaCl_2$ at 50 ppm for water hardening before incubating in a flow-through hatching trough (7.5 m × 0.4 m × 0.15 m) in hanging baskets which were aerated with a paddle wheel. Ploidy level was verified by measuring erythrocyte nuclei volume with a Coulter-counter at the USFWS Warm Springs Regional Fisheries Center, Warm Springs, GA (Beck and Biggers, 1983).

2.2. Gonad preparation and cryopreservation

Sexually immature blue catfish males (n = 12; mean ± SEM length = 35.6 ± 8.3 cm, weight = 530.7 ± 95.1 g) and females (n = 12; mean ± SEM length = 33.2 ± 6.3 cm, weight = 512.4 ± 84.7 g) were selected and euthanized. After being euthanized, gonad extraction and preparation were performed according to the standard protocols described by Shang et al. (2015), Abualreesh et al. (2020, 2021a,b), and Hettiarachchi et al. (2020).

After gonad preparation, testes or ovaries were minced with a sterilized scalpel blade and pools were made for testes and ovaries separately. Minced gonadal pools were cryopreserved for six months using 1 M DMSO, supplemented with 0.2 M lactose and egg yolk (10%), and frozen at a rate of -1.0°C/min according to the standard protocols described by Abualreesh et al. (2020, 2021a,b).

2.3. Thawing and stem cell isolation

Cryopreserved testes and ovaries of blue catfish were thawed in a 10 ^{o}C water bath for 1 min and then rehydrated using Dulbecco's Modified Eagle's Medium/DMEM [DMEM (10–090-CV, Corning cellgro) supplemented with 10% fetal bovine serum (Life Technologies), 100 unit/mL penicillin - streptomycin (Life Technologies), and 200 mM L-glutamine (Life Technologies). Finally, stem cells were extracted from thawed and freshly separated gonad samples according to standard methods described by Shang et al. (2015), Abualreesh et al. (2020, 2021a,b), and Hettiarachchi et al. (2020). Following extraction, the stem cells were quantified according to standardized protocols (Shang et al., 2015; Abualreesh et al., 2020, 2021a,b; Hettiarachchi et al., 2020).

2.4. Stem cell labelling and implantation into the triploid channel catfish host

The isolated fresh and cryopreserved stem cells were labelled with PKH26 red fluorescence cell linker (CGLDIL, Sigma-Aldrich, St. Louis, MO) following manufacturer's instructions. After stem cells labelling, cells were transplanted into triploid channel catfish fry (mean ± SEM total length = 6.0 ± 0.3 mm) at 5 days post-hatch (DPH). Triploid channel catfish fry were transplanted with either:

(i) Freshly extracted spermatogonia

(ii) Cryopreserved spermatogonia

(iii) Freshly extracted spermatogonia stained with PKH26

(iv) Cryopreserved spermatogonia stained with PKH26

(v) Freshly extracted oogonia

(vi) Cryopreserved oogonia

(vii) Freshly extracted oogonia stained with PKH26

(viii) Cryopreserved oogonia stained with PKH26

(ix) Control (Non injected triploids)

For each treatment, 30 fry × 6 replicates (180 fry) were injected. Each fry was injected with 1 μL of spermatogonia or oogonia cell suspension containing ~80,000 cells through a 33-guage needle (outer diameter: 0.209 mm; inner diameter: 0.108 mm) with a gas tight syringe attached to a repeater (Hamilton, Reno, NV).

2.5. Growth and survival

After stem cell transplantation, fry were placed in a recovery container that was aerated and then transferred to a recirculating aquaculture system (RAS) at a density of 30 fry per 60 L aquaria for further performance assessment. Fry were fed a standard commercial catfish fry feed (Purina Catfish fry feed; crude protein: 40.00%, crude fat: ≥4.00%, crude fiber: 7.00%, and phosphorus: 0.80%) three times per day to satiation and feed pellet size was adjusted based on their growth. DO, temperature, and salinity were monitored daily using a YSI 55 multi-parameter instrument, while TAN, nitrite, nitrate, pH, hardness, and alkalinity were measured daily using a water quality test kit (Easy Strips Tetra). Here, DO, temperature, salinity, TAN, nitrite, nitrate, pH, hardness, and alkalinity were maintained within acceptable ranges for channel catfish at 6.9 ± 1.0 mg/L, 25.4 ± 1.5°C, 3.8 ± 0.3 ppt, <0.5 mg/L, <0.5 mg/L, <15 mg/L, 7.0 ± 0.1, 53.7± 6 ppm, and 35.8 ± 6 ppm, respectively.

To evaluate growth performance, weight (mean ± SEM) and total length (mean ± SEM) of 36 fry per treatment (see *Section 2.5*) were recorded at 45 and 90 DPH as PKH dye can still be observed up to 100 days. Survival (mean ± SEM) was also evaluated at 45 and 90 DPH.

2.6. PKH observations

At 45 and 90 DPH, 18 fingerlings were randomly sampled from each treatment. The fingerlings were sacrificed and then their gonads were surgically removed and applied to a sterile microscope slide. After slide preparation, digital images were taken using a Zeiss Imager A2 microscope equipped with a camera (Axio-cam 202) and Zen Pro v.6.1 software (Zeiss, Oberkochen,

Germany). All tissue samples with donor derived cells were glowing red, while negative tissue samples had no fluorescence. Fluorescent images were analyzed using ImageJ software (http://imagej.nih.gov/ij/). We defined "cells" as having a fluorescence area <150 μm^2 and clusters as having a fluorescence area >150 μm^2. Cell number, cell area, cluster number, and cluster area were determined using fluorescent sections of the gonad samples.

2.7. Genomic DNA and PCR analysis

At 45 and 90 DPH, 18 fingerlings were randomly sampled from each treatment for PCR analysis. The fingerlings were sacrificed by pithing and gonads surgically removed. The separated gonad samples were placed into 1.5 mL eppendorf tubes on ice and then held at −80°C until DNA extraction. DNA was extracted using proteinase K digestion followed by protein and ethanol precipitation (Waldbieser and Bosworth, 2008).

2.8. Statistical analysis

All data were analyzed using SAS statistical analysis software (v.9.4; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro-Wilk test; PROC UNIVARIATE) and homogeneity of variance (plot of residuals vs. predicted values; PROC GPLOT). Post-hoc testing was done with Tukey's test to determine differences among treatments. Values were defined as statistically significant at alpha = 0.05. Growth and survival as well as fluorescent cell and cluster areas were compared among the treatments (see *Section 2.4*) at 45 and 90 DPH using a series of mixed-model and repeated measures factorial ANOVA models.

3.0. Results

3.1. Survival and growth performance

Mean values (± SEM) of total length, weight, and percent survival at 45 and 90 DPH for the eight injection and control treatments are presented in Table 2. No significant differences in total length and weight of fry were detected between treatments at 45 and 90 DPH (all $P > 0.163$) (Table 2). However, survival was significantly higher in the control treatment than the SSC and OSC injected treatments at both sampling days (Table 2).

3.2. PKH26 dye fluorescence and quantifying fluorescent labeling

Based on fluorescent images of the gonadal region of fry at 45 and 90 DPH, >66.7% of individuals in treatments injected with PKH26 had red florescence, indicating donor cell proliferation and colonization (Fig. 1E,F). According to fluorescent image analysis, there were no significant interactions between cell injection treatment and fry age for SSC cell area (Fig. 2A; $P = 0.342$), SSC cluster area (Fig. 2D; $P = 0.404$), OSC cell area (Fig. 3A; $P = 0.970$), and OSC cluster area (Fig. 3D; $P = 0.361$). Additionally, the percentage of SSC cell area (Fig. 2B; $P = 0.893$), SSC cluster area (Fig. 2E; $P = 0.691$), OSC cell area (Fig. 3B; $P = 0.793$), and OSC cluster area (Fig. 3E; $P = 0.697$) did not differ between the fresh and cryopreserved injection treatments. Both SSC (Fig. 2C,F) and OSC (Fig. 3C,F) cell area and cluster area increased from 45 to 90 DPH ($P < 0.0001$).

3.3. Genomic DNA and PCR

PCR analyses confirmed that triploid channel catfish injected with either fresh, cryopreserved SSC or OSC contained blue catfish stem cells in their testes and/or ovaries. At 45 DPH, 72.2%, 61.1%, 66.7%, and 61.1% xenogens were detected in fresh SSC, cryopreserved SSC, fresh OSC, and cryopreserved OSC, respectively (Fig. 4). Finally, at 90 DPH 83.3%, 66.6%, 61.1%, and 61.1% xenogens were detected in fresh SSC, cryopreserved SSC, fresh OSC, and cryopreserved OSC, respectively (Fig. 5).

4.0. Discussion

Transplanting undifferentiated stem cells from a donor species into an interspecific sterile host is an innovative and promising reproductive technology (Lacerda et al., 2010). Currently, freshly extracted stem cells are used for the germ cell transplanting process, which is challenging, especially considering germ cell production is size specific (Hettiarachchi et al., 2020), age specific (Ryu et al., 2022), and depends on seasonal cycles (Sato et al., 2017). Thus, having cryopreserved stem cells available in germplasm repositories will help facilitate stem cell transplantation technologies. If effective, the stem cell transplantation process could facilitate establishment of stem cell cryo-banks with the capability of preserving cells for future transplantation whenever the need arises. In the present study, cryopreserved donor stem cells had an equal ability to recover in recipient channel catfish gonads and perform as well as their freshly extracted counterparts, based on donor cell proliferation and colonization rates inside the host.

One of the best available technologies to determine the effectiveness of germ cell proliferation and colonization prior to sexual maturation is the use of fluorescent labeling of donor derived cells (Parish, 1999; Lee & Yoshizaki, 2016; Ye et al., 2021). In the current study, we observed fluorescently labeled donor-derived fresh and cryopreserved blue catfish stem cells in channel catfish (recipient) gonads at 45 and 90 DPH after intraperitoneal transplantation with equal colonization and proliferation. In addition, there was a significant increase in cell area and cluster area from 45 to 90 DPH in gonads injected with either fresh or cryopreserved SSC/OSC. This indicates that cryopreserved cells can proliferate in triploid channel catfish recipients with comparable success to freshly injected samples.

Cryogenic and xenogeneic techniques have been successfully integrated for conservation of endangered/endemic species and/or to improve reproduction for commercially valuable aquatic species, such as masu salmon (*Oncorhynchus masou*) (Lee et al., 2016), rainbow trout (*O. mykiss*) (Kobayashi et al., 2003; Yoshizaki and Lee, 2018), blue catfish (Abualreesh et al., 2020, 2021 a,b), and tench (*Tinca tinca*) (Linhartova et al., 2014), among others. Based on these findings, it was suggested that stem cells can be stored in liquid nitrogen for several years with no significant reduction in cell survival. More specifically, Ye et al. (2021) demonstrated that American paddlefish (*Polyodon spathula*) SSC cryopreserved in liquid nitrogen for one year could colonize and proliferate in recipient Yangtze sturgeon (*Acipenser dabryanus*), while rainbow trout SSC were transplanted into triploid allogeneic recipients after cryopreservation for two years (Yoshizaki and Lee, 2018). Cryopreserved rainbow trout and Siberian sturgeon (*A. baerii*) SSC were also successfully transplanted into masu salmon and sterlet sturgeon (*A. ruthenus*) recipients respectively, after six months of cryopreservation (Lee at al., 2016; Psenicka et al., 2016). In the present study, blue catfish SSC were cryopreserved for six months following protocols described by Abualreesh et al. (2020, 2021a,b). These cells were then transplanted into triploid channel catfish recipients, showing colonization and proliferation. Together, this suggests the utility of stem cell cryo-banks for future transplantation to restore endangered or valuable populations through xenogenesis.

Despite continuous efforts to work with frozen SSC, limited transplantation experiments have focused on freezing and using female stem cells for xenogeneic applications. Considering the limited successes with egg/embryo cryopreservation (Zhang and Lubzens, 2009; Yoshizaki et al., 2010; Wong et al., 2011), freezing OSC would provide a unique opportunity to conserve maternal variability, as well as individual genomes and cytoplasm. Recently, Franek et al. (2019)

cryopreserved common carp (*Cyprinus carpio*) OSC using slow rate freezing (~1 °C/min) and transplanted these cells into sterile goldfish (*Carassius auratus*) recipients. However, until the current study, no information has been reported on transplantation of cryopreserved OSC in catfish.

In our study, PCR analyses (using specific Fst and Hamp) provided additional evidence that germline stem cells isolated from cryopreserved gonads maintained their viability and characteristics of stem cells. Furthermore, PCR results confirmed high success rates (> 61%) of donor derived gonads in recipient fish at 45 and 90 DPH using SSC and OSC transplantation. Theoretically, the blue catfish DNA that was detected in these triploid channel catfish recipients were able to colonize and proliferation in the gonads. This technique was very effective to prove the host fish accepted the foreign cells, which colonized and were present after 45 and 90 DPH. In the case of salmonid xenogens, a 30 to 50% success rate was achieved among injected host embryos (Okutsu et al. 2007), and approximately 50% of injected zebrafish became xenogens. Additionally, a 40% success rate was achieved when common carp spermatogonia were transplanted into goldfish (Franek et al., 2019). Thus, success rate of xenogen production was high in the current study when compared to previous studies.

In conclusion, the current study demonstrated that blue catfish cryopreserved and fresh SSC and OSC have equal ability to colonize a channel catfish host and proliferate. It is notable that cryopreserved testis and ovarian tissues of blue catfish can provide thousands of viable SSC and OSC respectively, which is sufficient to transplant into many recipients. Hence, this technology could become a viable tool for use in future xenogenesis translation efforts, especially with the availability of frozen stem cells throughout the year. In conclusion, results of the current study

reveal the feasibility of long-term cryogenic storage of SSC and OSC to support catfish aquaculture production and conservation efforts.

5.0. Declaration of Competing Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

6.0. Acknowledgements

This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2018-67015-27614 from the USDA National Institute of Food and Agriculture. We would like to acknowledge the staff of the Catfish Genetics Research Unit as well as the EW Shell Fisheries Center at Auburn University for fish care, labor, and maintenance.

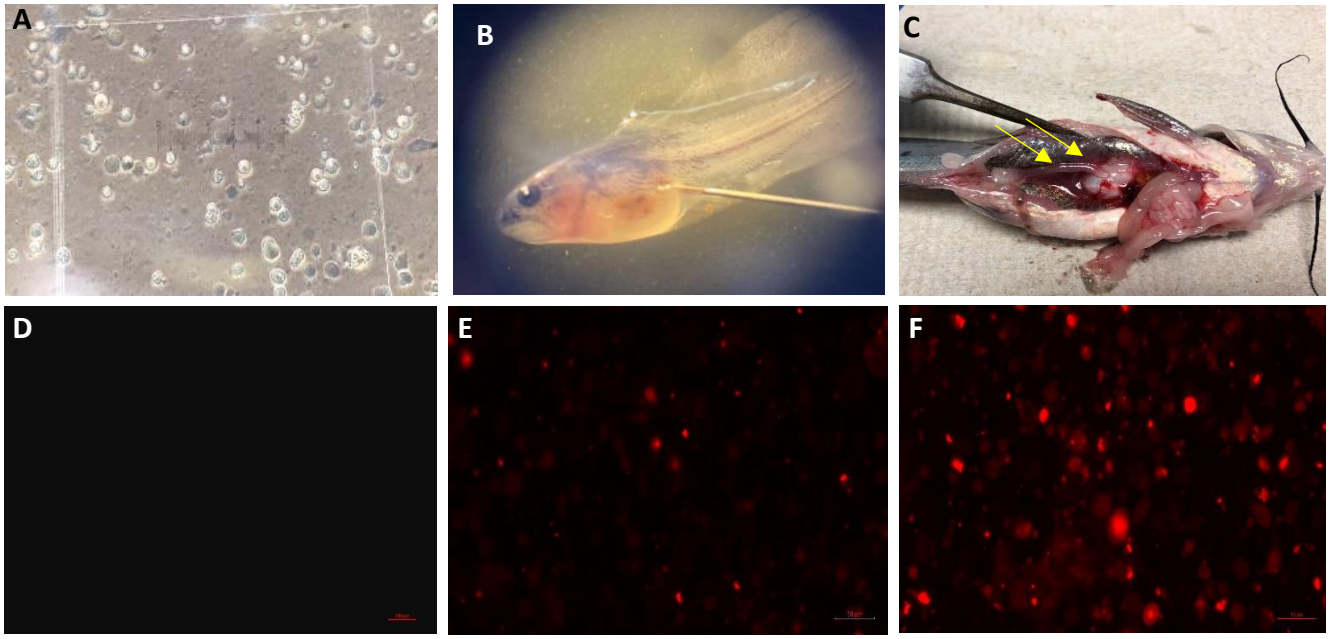

Fig. 1: Stem cells labeled with PKH26 dye (A). Transplantation (intraperitoneally) of donor derived stem cells into triploid channel catfish (*Ictalurus punctatus*). Transplanted stem cells migrate to the genital ridge of the recipient, are incorporated, and initiate oogenesis or spermatogenesis (B). Gonadal growth of the xenogen (C). Gonadal tissues expressing fluorescence from PKH26 dyed donor derived stem cells. The non-injected control treatment (D) showed no cell florescence, while the cryopreserved treatments showed proliferation and increased cell fluorescence from 45 DPH (E) to 90 DPH (F).

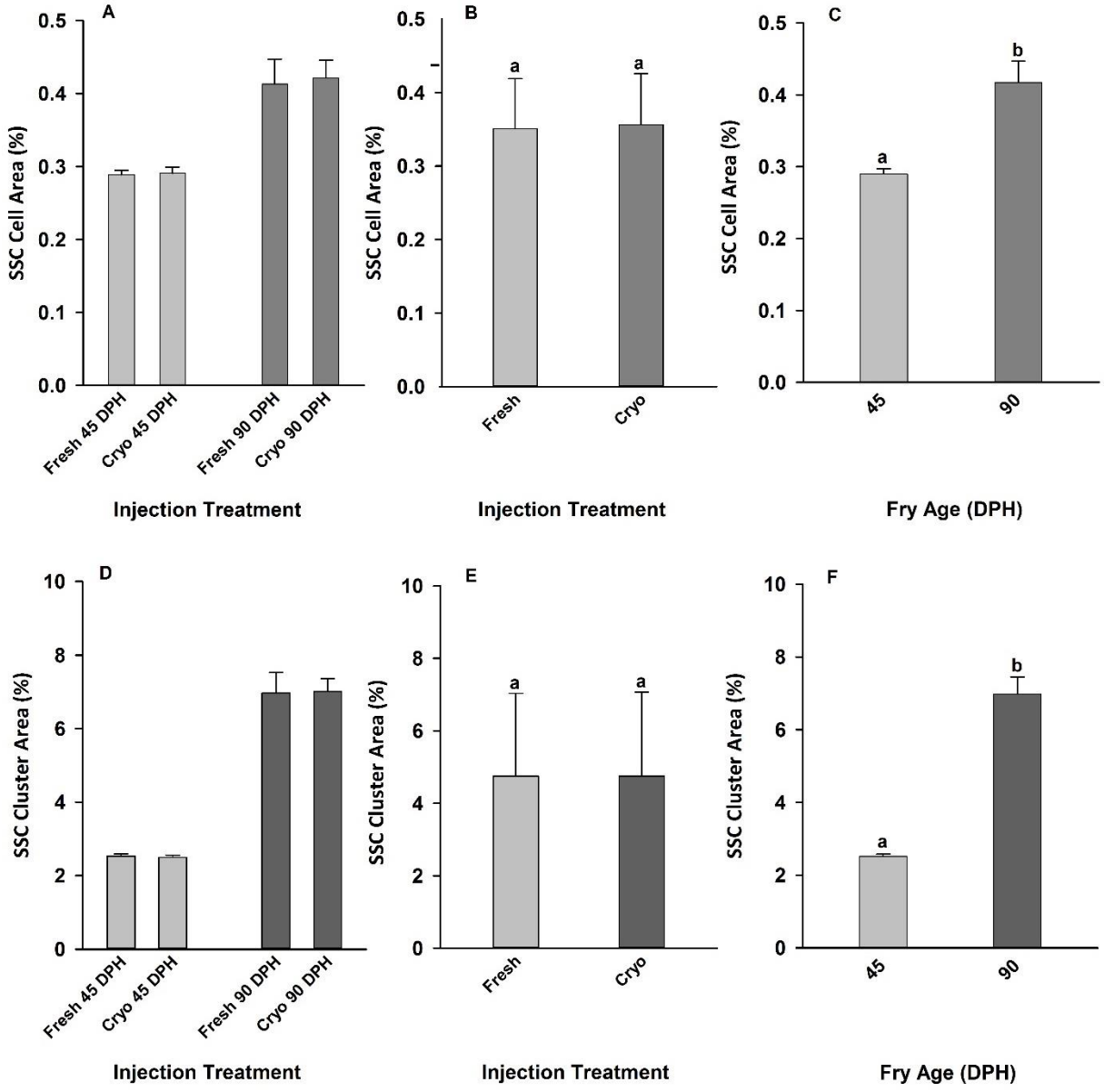

Fig. 2: Percent cell area at 45 and 90 days post hatch (DPH) for triploid channel catfish (*Ictalurus punctatus*) injected with fresh and cryopreserved (Cryo) blue catfish (*I. furcatus*) spermatogonial stem cells (SSC) (A). Comparison of cell area for the fresh and cryopreserved treatments (B). Comparison of cell area at 45 and 90 DPH (C). Percent cluster area at 45 and 90 DPH for triploid channel catfish injected with fresh and cryopreserved blue catfish SSC (D). Comparison of cluster area for the fresh and cryopreserved treatments (E). Comparison of cluster area at 45 and 90 DPH

(F). Bars represent least square means + SEM. Letters represent significant differences among temperature treatments ($p < 0.05$).

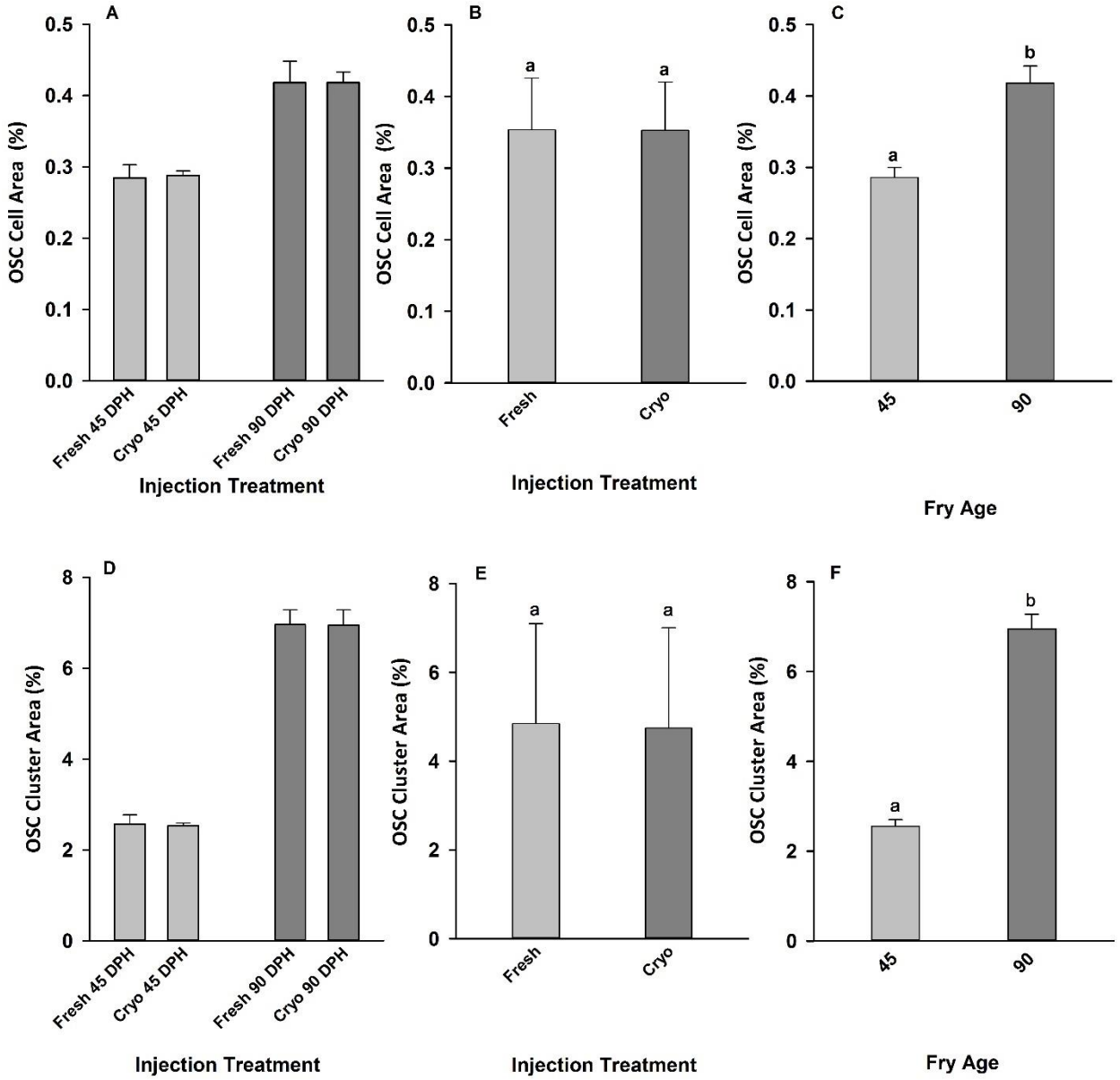

Fig. 3: Percent cell area at 45 and 90 days post hatch (DPH) for triploid channel catfish (*Ictalurus punctatus*) injected with fresh and cryopreserved (Cryo) blue catfish (*I. furcatus*) oogonial stem cells (OSC) (A). Comparison of cell area for the fresh and cryopreserved treatments (B). Comparison of cell area at 45 and 90 DPH (C). Percent cluster area at 45 and 90 DPH for triploid channel catfish injected with fresh and cryopreserved blue catfish OSC (D). Comparison of cluster area for the fresh and cryopreserved treatments (E). Comparison of cluster area at 45 and 90 DPH

(F). Bars represent least square means + SEM. Letters represent significant differences among temperature treatments ($p < 0.05$).

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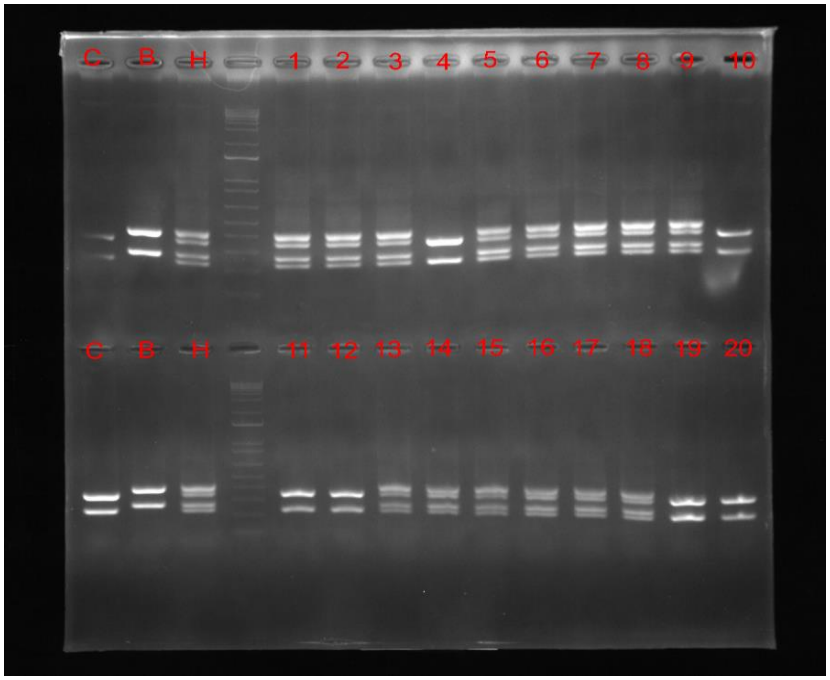

Fig. 4: Results from PCR for detecting blue catfish (*Ictalurus furcatus*) donor cells in the testes of triploid channel catfish (*I. punctatus*) injected with fresh spermatogonial stem cells (SSC), cryopreserved SSC, fresh oogonial stem cells (OSC), and cryopreserved OSC at 45 days post hatch (DPH). Blue catfish and channel catfish cells were differentiated with PCR using follistatin (Fst) and hepcidin antimicrobial protein (Hamp) genes as markers. C = channel catfish control, B = blue catfish control, H = female channel catfish × male blue catfish hybrid controls. Sample #1-5: Gonad samples from fry injected with fresh SSC; Sample #6-10: Gonad samples from fry injected with fresh OSC; Sample #11-15: Gonad samples from fry injected with cryopreserved SSC; Sample #16-20: Gonad samples from fry injected with cryopreserved OSC.

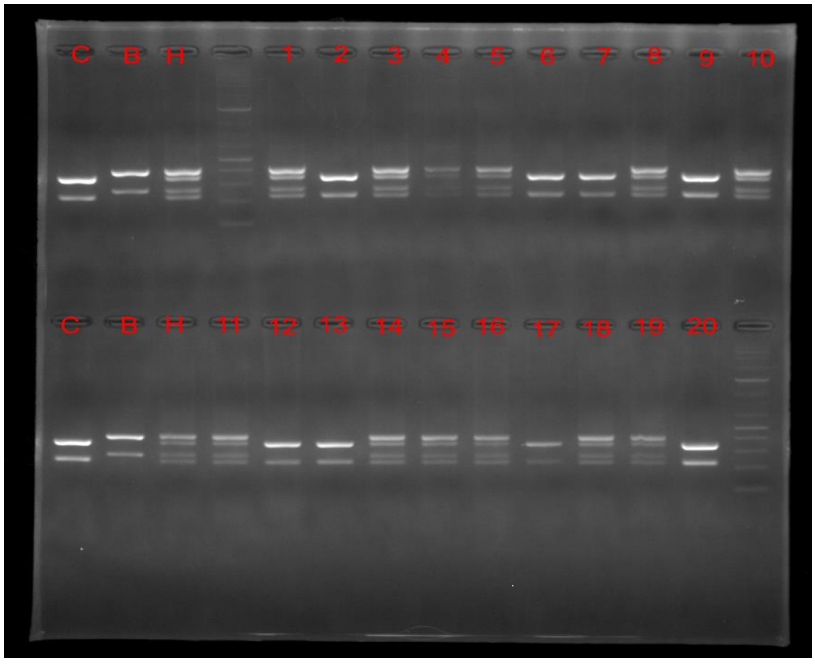

Fig. 5: Results from PCR for detecting blue catfish (*Ictalurus furcatus*) donor cells in the testes of triploid channel catfish (*I. punctatus*) injected with fresh spermatogonial stem cells (SSC), cryopreserved SSC, fresh oogonial stem cells (OSC), and cryopreserved OSC at 90 days post hatch (DPH). Blue catfish and channel catfish cells were differentiated with PCR using follistatin (Fst) and hepcidin antimicrobial protein (Hamp) genes as markers. C = channel catfish control, B = blue catfish control, H = female channel catfish × male blue catfish hybrid controls. Sample #1-5: Gonad samples from fry injected with fresh SSC; Sample #6-10: Gonad samples from fry injected with fresh OSC; Sample # 11-15: Gonad samples from fry injected with cryopreserved SSC; Sample #16-20: Gonad samples from fry injected with cryopreserved OSC.

Table 1: Primers used for genes [Fst (follistatin) and Hamp (hepcidin antimicrobial protein)] to differentiate channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*). Primers were previously described by Waldbieser and Bosworth (2008).

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

Table 2: Total length (mean ± SEM), weight (mean ± SEM), and percent survival (mean ± SEM) at 45 and 90 days post hatch (DPH) of triploid xenogenic channel catfish (*Ictalurus punctatus*) injected with blue catfish (*I. furcatus*) spermatogonial (SSC) and oogonial (OSC) stem cells.

Treatment	Growth				% Survival	
	45 DPH		90 DPH			
	Total length (cm)	Weight (g)	Total length (cm)	Weight (g)	45 Days	90 Days
Fresh SSCs injected fry	4.60 ± 0.12[a]	2.70 ± 0.11[a]	13.49 ± 0.11[a]	13.46 ± 0.20[a]	72.7 ± 5.34[a]	66.1 ± 4.91[a]
Cryopreserved SSCs injected fry	4.63 ± 0.11[a]	2.74 ± 0.03[a]	13.43 ± 0.12[a]	13.47 ± 0.11[a]	73.3 ± 5.96[a]	68.3 ± 5.04[a]
Fresh SSCs with PKH26 injected fry	4.62 ± 0.17[a]	2.69 ± 0.19[a]	13.45 ± 0.16[a]	13.56 ± 0.17[a]	72.2 ± 5.43[a]	67.77 ± 5.83[a]
Cryopreserved SSCs with PKH26 injected fry	4.63 ± 0.17[a]	2.70 ± 0.09[a]	13.44 ± 0.11[a]	13.50 ± 0.06[a]	71.6 ± 8.1[a]	66.67 ± 8.68[a]
Fresh OSCs injected fry	4.61 ± 0.12[a]	2.71 ± 0.17[a]	13.49 ± 0.18[a]	13.49 ± 0.09[a]	72.2 ± 5.02[a]	65.57 ± 4.56[a]
Cryopreserved OSCs injected fry	4.64 ± 0.10[a]	2.68 ± 0.05[a]	13.44 ± 0.18[a]	13.49 ± 0.24[a]	72.7 ± 6.12[a]	67.78 ± 5.00[a]
Cryopreserved OSCs with PKH26 injected fry	4.79 ± 0.12[a]	2.70 ± 0.52[a]	13.46 ± 0.11[a]	13.49 ± 0.23[a]	73.8 ± 4.43[a]	68.33 ± 4.07[a]
Fresh OSCs injected fry	4.65 ± 0.13[a]	2.71 ± 0.06[a]	13.44 ± 0.13[a]	13.49 ± 0.32[a]	71.1 ± 7.20[a]	66.10 ± 6.45[a]
Non-injected control	4.66 ± 0.12[a]	2.73 ± 0.04[a]	13.33 ± 0.17[a]	13.50 ± 0.17[a]	86.6 ± 3.66[b]	81.1 ± 1.70[b]
P-value	0.4450	0.4360	0.1627	0.8690	0.0011	0.0003

Note: Values with different superscripts within the same column are significantly different based on Tukey pairwise comparisons.

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Chapter 4

Production of xenogenic catfish by transplanting blue catfish (*Ictalurus furcatus*) and channel catfish (*I. punctatus*) stem cells into white catfish (*Ameiurus catus*) triploid fry

Abstract

Xenogenesis has been identified as a potential alternative for hybrid catfish (channel catfish, *Ictalurus punctatus* ♀ × blue catfish, *I. furcatus* ♂) embryo production. The xenogeneic process can be accomplished by transplanting primordial germ cells, spermatogonial or oogonial stem cells, derived from a donor diploid fish into a sterile recipient. The capability of hybrid catfish production through xenogenesis has been highlighted in literature by transplanting blue catfish stem cells into a triploid channel catfish host. However, having a host with a short maturation time and smaller body size than channel catfish would be ideal for commercial application. Hence the present study was conducted to assess the effectiveness of triploid white catfish as a host species to transplant blue and channel catfish stem cells during the production process of xenogeneic catfish. Triploid white catfish fry were injected either with blue catfish stem cells (BSCs) or channel catfish stem cells (CSCs) labeled with PKH26 dye from 0 to 12 days post-hatch (DPH). Then at 45 DPH and 90 DPH, growth performances (total length, weight) and survival of recipients were evaluated. Colonization of donor cells was evaluated in recipients using PKH26 dye fluorescence to calculate percent cell and cluster areas. PCR determined the percentage of xenogens from gonads samples.

No significant differences in growth performances were detected among treatments at 45 and 90 DPH. Overall, a significant increase in survival was detected when injected with blue and channel catfish stem cells from 0 to 5.6 DPH and the highest survivals were reported between 4 - 5.6 DPH in all the treatments. After 5.6 DPH, survival remained high (≥81.2%). Overall, at both 45 and 90 DPH sampling periods, significant increases in cell and cluster area in recipients were detected for fish injected from 0 to 5.2 DPH and the highest values were reported between 4 - 5.2 DPH in all the treatments. Thereafter, fluorescent cell and cluster area in the host declined with no further decrease after 10 DPH.

At the 45 DPH sampling, the highest percentage of xenogens were detected in BSCs and CSCs treatments between 4 - 5 DPH and 3 - 5 DPH, respectively. At the 90 DPH sampling, it was detected from 4 - 6 DPH in BSCs and CSCs treatments, respectively. The current study demonstrated the suitability of white catfish as a host species by transplanting stem cells between 4 - 5 DPH, these findings can use to enhance the efficiency of germ cell transplantation in xenogenesis.

Keywords: Xenogenesis, Triploids, Blue catfish stem cells, Channel catfish stem cells

1.0. Introduction

Catfish farming is the largest aquaculture industry in the United States topping out at 347 million pounds produced annually and accounting for approximately 62 % of all United States aquaculture production in 2019 (NOAA, 2022). Until recently, channel catfish, *Ictalurus punctatus* was the prominent species cultured for food fish (Dunham and Elaswad, 2018). However, it was shifted toward the production of hybrid catfish, produced by crossing the channel catfish, ♀ with the blue catfish, *I. furcatus* ♂ due to several improved performances in growth, disease resistance, dissolved oxygen tolerance, feed conversion, fillet yield, dress out percentage, and harvestability compared with both parent species (Dunham and Brummett, 1999; Brown et al., 2011; Arias et al., 2012; Dunham et al., 2014; Dunham and Elaswad, 2018). Although the hybrid industry has seen sustained growth, there are still challenges due to the lack of natural hybridization between these two species. Artificial fertilization has been considered as an appropriate technology for commercial hybrid catfish production, which still requires additional efforts such as sacrificing mature blue catfish males for sperm collection, hormonal stimulation of females, extra holding space, special handling techniques, additional feed, and labor cost (Argue et al., 2003; Hu et al., 2011). Hence, new innovative reproduction technologies such as xenogenesis can be used to mitigate some of the challenges in hybrid catfish production.

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). Xenogenesis can be accomplished by transplanting undifferentiated germ cells, such as primordial germ cells (PGCs), spermatogonial stem cells (SSCs), or oogonial stem cells (OSCs) derived from a donor diploid fish into a sterile recipient, which then enables recipient fish to produce donor-

derived gametes (Amer et al., 2001; Yoshizaki et al., 2010; Wong et al., 2011; Perera et al., 2017; De Siqueira-Silva et al., 2018). PGCs, SSCs, and OSCs possess the self-renewal ability, making them useful as donor cells for xenogenesis transplantation. After transplantation, these cells have the ability to migrate and colonize, giving rise to female or male germ cells, after which the gonad differentiates into an ovary or a testis, depending on the individual's genetic sex. (Yoshizaki and Lee, 2018). Due to these unique characteristics, there are numerous examples exist of the success of blue catfish SSCs and OSCs transplantations in xenogenesis for commercial-scale hybrid catfish production (Perera et al., 2017; Hettiarachchi et al., 2022; Hettiarachchi et al., 2023a).

In all these experiments triploid channel catfish was used as the host species during the stem cell transplantation. Channel catfish usually reach sexual maturity in 2 – 4 years (Dunham and Smintherman,1981; Goudie et al., 1983). However, commercially it is better to use a host species that have a short maturation time which can directly affect to reduce the feed cost, labor cost, holding space, and easiness of handling. Hence, as a solution, white catfish (*Ameiurus catus*) has the advantages of short maturation time and small handling size, making them a potential host model for commercial-scale hybrid catfish production. According to Dunham and Smintherman (1981) and Fobes (2013), white catfish can become mature in 1 - 2 years compared to 2 - 4 years for channel catfish. White catfish are considered the smallest catfish out of all the other North American catfish species, further, they begin to reach sexual maturity as early as 15.2 cm in fork length in northern California populations (Borgeson and Mc Cammon, 1967) and up to 23.0 cm total length in New Jersey populations (Keller, 2010). Spawning of white catfish could be observed in early May when the temperature of the water ranges from 19 - 21^{o}C (Wang, 2010). In addition, White catfish grow rapidly as fingerlings, but their growth is slow when they begin sexually maturing at 1 year old. (Dunham and Smitherman, 1984).

Further, Fobes (2013) reported that using luteinizing hormone-releasing hormone analog (LHRHa) implants at 90µg/kg in domestic white catfish resulted in 100% ovulation of the females after 72 hours. Hence, white catfish has several desirable characteristics over channel catfish as a host during the xenogenesis process. Due to these major advantages of white catfish, the present study aimed to identify the suitability of triploid white catfish as a host species and identify the best age to transplant blue and channel catfish stem cells during the production process of xenogeneic catfish.

2.0. Materials and methods

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

2.1. Broodstock management and triploid induction

Broodstock was cultured in 0.04 ha earthen ponds averaging 1 m in depth at the EW Shell Fisheries Center at Auburn University, AL, USA. Fish were fed to satiation with 32% protein pellet feed five days per week during summer and three days per week during winter. In addition, the fish were fed 36% protein broodstock feed two months prior to spawning. During the sampling, gravid white catfish females (n = 3; mean ± SEM weight = 0.7 ± 0.2 kg) and sexually mature males (n = 3; mean ± SEM weight = 0.6 ± 0.3 kg) were collected by seining the pond with a 3.8 cm mesh seine net.

Gravid females were administered intraperitoneal injections of LHRHa at 90µg/kg body weight. Then, white catfish females and males (1: 1 ratio) were placed in 60 L aquaria in a calm environment. After 48 hrs, each tank was carefully observed. When the females began to ovulate (few eggs could be seen at the bottom of the tank) male and female were separated. Then male was euthanized for sperm collection according to the standard protocol described by Dunham and Masser (2012). In brief, testes were surgically removed with a sterile scalpel and forceps. Then the testes were rinsed with 0.9% saline solution to remove blood. After the removal of excess blood, the testes were gently dried. Testes were minced with a scalpel blade. Then the sperm/testes

solution was filtered with a 100 μm mesh. Next, 10 mL of 0.9% saline was added for each 1 g of testes.

Then the ovulated females were anesthetized with 100 ppm tricaine methanesulfonate (MS-222, Ferndale, WA) and 10 ppm sodium bicarbonate. Eggs were hand stripped into spawning pans coated with Crisco commercial vegetable oil (~25 g of eggs/pan) and fertilized with a prepared sperm solution at a rate of 2 mL of white catfish sperm solution per 25 g of eggs (Hettiarachchi et al., 2022, 2023a). Next, a Fullers' earth (MP Biomedicals, Santa Ana, CA) solution was added to the sperm-egg solution to start the fertilization process. Fuller's Earth solution was prepared by adding 6 g of powdered Fuller's Earth to 1 liter of pond water. After 3 min, triploid induction was carried out according to standard protocols described by Perera et al. (2017) and Hettiarachchi et al. (2022, 2023a). Briefly, the fertilized eggs were transferred into a steel chamber and after an additional 2 minutes hydrostatic pressurize was applied at 7,000 psi for 5 minutes. After pressure shock, the eggs were removed from the chamber and placed in a trough supplemented with $CaCl_2$ at 50 ppm for water hardening before incubating. After 1 hr eggs were incubated in hanging mesh baskets (7 m × 0.4 m × 0.2 m) placed in flow-through hatching troughs which were aerated with paddle wheels. Ploidy level was verified by measuring erythrocyte nuclei volume with a Coulter-counter at the USFWS Warm Springs Regional Fisheries Center, Warm Springs, GA (Beck and Biggers, 1983).

2.2. Isolation of donor stem cells from immature blue and channel catfish

Sexually immature channel catfish (mean ± SEM length = 25.3 ± 5.9 cm, weight = 399.5 ± 100.3 g) and blue catfish (mean ± SEM length = 33.5 ± 5.2 cm, weight = 488.2 ± 84.3 g) were selected daily (n = 4) and euthanized. After being euthanized, gonad extraction, blue catfish stem cells (BSCs), and channel catfish stem cells (CSCs) isolation were performed according to the standard protocols described by Shang et al. (2015), Abualreesh et al. (2020, 2021a, 2021b), and Hettiarachchi et al. (2020, 2022, 2023a, 2023b). In Brief, extracted blue and channel catfish gonads were separately placed on a sterile petri dish (100 mm × 15 mm) containing 5 mL of Hanks' Balanced Salt Solution [(HBSS, SH30048.24, GE Healthcare Life Sciences) supplemented with 1.0 µg/mL $NaHCO_3$ (Church & Dwight Co., NG) and 100 unit/mL Penicillin - Streptomycin (115140–122, Life Technologies)]. Samples were cleaned with freshly prepared 0.5% bleach solution for 2 min, then HBSS and phosphate-buffered saline (PBS; J62701, Alfa Aesar). Then samples were minced with a sterilized scalpel blade and the trypsinization process was conducted by adding 0.25% trypsin - ethylenediamine tetraacetic acid (EDTA; 25243–261, Life Technologies) at 50 times the weight of each sample. After the trypsinization process, each cell suspension was filtered using a 70 µm and 40 µm cell strainers (352364 /382398, VWR International) and centrifuged at 500g (Eppendorf Centrifuge 5418 R) for 10 min. Then, the resulting pellet was resuspended in 2 mL of Dulbecco's Modified Eagle's Medium/DMEM [DMEM (10–173-CV, Corning cellgro,) supplemented with 10% fetal bovine serum (FBS; 10438044, Life Technologies), 100 unit/mL penicillin-streptomycin (15140-164, Life Technologies), and 200 mM L-glutamine (A2916993, Life Technologies).

2.3. Stem cell labeling with PKH26 and transplantation into the triploid white catfish host

The isolated blue catfish and channel stem cells were labeled with PKH26 red fluorescence cell linker (CGLDIL, Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Channel and blue catfish stem cells were transplanted into triploid channel catfish fry daily from 0 days post-hatch (DPH) to 12 DPH with either stem cells (channel/ blue) labeled with PKH26 or without PKH26 according to standard methods described by Hettiarachchi et al. (2022, 2023a). In brief, each day white catfish triploid fry (three replicates for each treatment / 30 fry per treatment) were anesthetized by placing them in a 10 mg/L tricaine methanesulfonate (MS-222) buffered with 10 mg/L sodium bicarbonate solution. Anesthetized fry were placed in the petri dish and observed microscopically at 1.5 × (Amscope, Irvine, CA) and injected with 1 uL unsorted cell suspension (channel / blue) containing 80,000 cells through a 33 gauge needle (outer diameter: 0.209 mm; inner diameter: 0.108 mm; Hamilton, Reno, NV) with a manual microinjector (Hamilton, Reno, NV). Fry were laid on their side and the needle was gently inserted in the cavity between the anal fin and yolk sac where the genital ridge is expected to be formed (Fig. 1). After injection, the fry were placed in a recovery container.

2.4. Growth and survival

Stem cell transplanted fry were transferred back to fry cages (0.2 m × 0.2 m × 0.25 m) in flow-through troughs at a density of 30 fry per cage. In the beginning, fry were fed a standard commercial catfish fry feed (crude protein: 50.00%, crude fat: ≥4.00%, crude fiber: 7.00%, and phosphorus: 0.80%) three times per day to satiation the gradually feed pellet size was increased according to their growth performance. At 45 and 90 DPH, 10 fingerlings were randomly selected

from each treatment and their Total length (TL) and Body weight (BW) were recorded to determine the growth performances. Further, survival was calculated at each sampling period.

2.5. PKH26 observations at 45 and 90 DPH

Nine fingerlings were randomly selected from each treatment to evaluate the proliferation and colonization of transplanted BSCs and CSCs inside the triploid white catfish. The fingerlings were sacrificed by pithing and their gonads were surgically removed. Each gonadal section was placed on a sterile microscope slide (1600221, Life Technologies) and covered with a coverslip. After slide preparation, digital images were taken using a Zeiss Imager A2 microscope equipped with a camera (Axio-cam 202) and Zen Pro v.6.1 software (Zeiss, Oberkochen, Germany). All tissue samples with donor-derived 'positive' cells glowed red, while 'negative' cells had no fluorescence. Six fluorescent images from each gonad were analyzed using ImageJ software and cell number, cell area, cluster number, and cluster area were determined. Cells were defined as having a fluorescence area <150 μm^2 and clusters as having a fluorescence area >150 μm^2.

2.6. Genomic DNA and PCR analysis

At each sampling period (45 DPH and 90 DPH), 5 fingerlings were randomly selected for the DNA extraction and PCR analysis. The separated gonad samples were placed into 1.5 mL Eppendorf tubes on ice and then held at -80 ^oC until DNA extraction. DNA was extracted using

proteinase K digestion followed by protein and ethanol precipitation (Waldbieser and Bosworth, 2008) (Table 1). The PCR was carried out in a 10 μL reaction volume in microcentrifuge tubes containing 0.6 μL of each primer follistatin (Fst) and hepcidin (Hamp), 5 μL of 2x Eco, 2.2 μL of RNase/Dnase free water, and 1 μL of the DNA sample. The Fst and Hamp amplification products were resolved on an ethidium-bromide-stained 2.0% agarose gel.

2.7. Statistical analyses

Breakpoint analysis was run with the Segmented package (Muggeo 2017) in R (R Core Team 2022). The segmented regression or breakpoint analysis was used to determine the optimal age to transplant blue and channel catfish stem cells into triploid white catfish (host), based on offspring growth, survival, and fluorescent image analyses.

3.0. Results

3.1. Growth Performances and Survival

No significant differences in TL and TW of fry have resulted in both BSCs and CSCs treatments at 45 and 90 DPH (all P > 0.097) (Fig. 3). Segmented linear regression showed a significant increase in survival of recipient fish at 45 DPH sampling when injected with blue catfish stem cells from 0 to 4 DPH ($P < 0.0001$, $R^2 = 0.72$, y = 42.7 + 7.0 x; Fig. 4A) and channel catfish stem cells from 0 to 4.5 DPH ($P < 0.0001$, $R^2 = 0.86$, y = 33.2 + 11.0 x; Fig. 4C). After 4 DPH and 4.5 DPH survival were remained constant (≥87.3% ± 2.9; $P = 0.134$) in blue and channel stem cells transplanted treatments, respectively. A similar fluctuation was detected at 90 DPH sampling, survival was gradually increased in the recipient fish when injected with blue catfish stem cells from 0 - 5.5 DPH ($P < 0.0001$, $R^2 = 0.93$, y = 19.76 + 9.41 x; Fig. 4B) and channel catfish stem cells from 0 to 4.5 DPH ($P < 0.0001$, $R^2 = 0.91$, y = 22.7 + 9.56 x; Fig. 4D). Thereafter, the survival remained high (≥81.2% ± 2.1; $P = 0.154$) for fry injected until 12 DPH in both blue and channel stem cells transplanted treatments.

3.2. Quantifying fluorescent labeling

Day of stem cell injection (0 to 12 DPH) had a significant impact on stem cell proliferation and colonization in the host fish (Fig. 2). Briefly, at 45 DPH, a significant increase in cell area and cluster area in recipient BSCs transplanted fish was detected for individuals injected 0 to 5 DPH

($P < 0.0001$, $R^2 = 0.58$, y = 0.18 + 0.054 x; Fig. 5A) and 0 to 4.8 DPH ($P < 0.0001$, $R^2 = 0.89$, y = -0.94 + 1.05 x; Fig. 5B), respectively. After the peak levels, the percentage cell area ($P < 0.0001$, $R^2 = 0.89$, y = 6.56 - 1.11x; Fig. 5A) and cluster area ($P < 0.0001$, $R^2 = 0.88$, y = 6.57 – 1.12x; Fig. 5B) were gradually declined with no further decrease after 8.6 DPH and 9.2 DPH days of injection, respectively. A similar trend was detected at 90 DPH. Briefly, the percentage cell area and cluster area increased when recipient fish were injected from 0 to 4.2 DPH ($P < 0.0001$, $R^2 = 0.81$, y = -1.67 + 1.58x; Fig. 5C) and 0 to 5.2 DPH ($P < 0.0001$, $R^2 = 0.76$, y = -2.20 + 2.30x; Fig. 5D), respectively. After reaching to the peak level cell ($P < 0.0001$, $R^2 = 0.91$, y = 8.34 – 1.37x; Fig. 5C) and cluster ($P < 0.0001$, $R^2 = 0.84$, y = 14.47 – 2.11x; Fig. 5D) areas were gradually decreased, respectively.

Similarly, the Day of stem cell injection (0 to 12 DPH) had a significant impact CSCs proliferation and colonization in the triploid white catfish (host). For instance, at the 45 DPH sampling period, a significant increase in cell area and cluster area in recipient fish was detected for individuals injected 0 to 4.9 DPH ($P < 0.0001$, $R^2 = 0.59$, y = 0.11 + 0.04x; Fig. 6A) and 0 to 4.8 DPH ($P < 0.0001$, $R^2 = 0.87$, y = 0.03 + 0.78x; Fig. 6B), respectively. After reaching the peak level, the percentage of fluorescent cell area ($P < 0.0001$, $R^2 = 0.82$, y = 0.39 - 0.05x; Fig. 6A) and cluster area ($P < 0.0001$, $R^2 = 0.91$, y = 5.65 - 0.81x; Fig. 6B) in the host declined with no further decrease after 7.6 DPH and 10.0 DPH, respectively. Similarly, at 90 DPH sampling period, the percentage cell area and cluster area gradually increased from 0 to 4.4 DPH ($P < 0.0001$, $R^2 = 0.76$, y = -2.89 + 1.76x; Fig. 6C) and 0 to 5.1 DPH ($P < 0.0001$, $R^2 = 0.82$, y = -4.14 + 3.11x; Fig. 6D), respectively. Thereafter, the percentage cell area ($P < 0.0001$, $R^2 = 0.95$, y = 9.12 – 1.53x; Fig. 6C) and cluster area ($P < 0.0001$, $R^2 = 0.83$, y = 19.08 – 3.66x; Fig. 6D) in the host declined with no further decrease after 8.8 DPH and 8.3 DPH, respectively.

3.3. Genomic DNA and PCR analysis

PCR analysis indicated transplanted blue catfish donor-derived stem cells in the gonads of recipient white catfish fry during the 45 and 90 DPH sampling periods (Fig. 6). At the 45 DPH sampling period, in BSCs and CSCs transplanted treatments the highest percentage of xenogens were detected when the recipient fish were injected from 4 to 5 DPH (all 80.0%) and 3 to 5 DPH (80.0%), respectively. At the 90 DPH sampling period, the percentage of xenogens was detected from 4 to 6 DPH in both BSCs and CSCs transplanted treatments.

4.0. Discussion

The potential of hybrid catfish production through xenogenesis has been highlighted in the literature by transplanting blue catfish stem cells into a triploid channel catfish host (Perera et al., 2017; Hettiarachchi et al., 2022; Hettiarachchi et al., 2023a). However, having a host with a short maturation time and a smaller body size than channel catfish would be ideal for commercial applications. Hence, filling this research gap, our current study identified the suitability of white catfish as a host for hybrid catfish production and identified 4 to 5 DPH as the most suitable age to transplant donor-derived stem cells into a sterile white catfish recipient, based on growth performance, survival, proliferation, and colonization.

The fluorescent cell linker dye PKH 26 offers the longest *in vivo* half-life, more than 100 days which makes it ideal for *in vivo* cell tracking, cell proliferation, and colonization studies (Thomas-Mudge et al., 2004; Singh et al., 2022). Hence, it has been successfully utilized in staining germ cells in several cell transplantation studies (Parish, 1999; Lacerda et al., 2006; Pšenička et al., 2015; Lee and Yoshizaki, 2016; Ye et al., 2021). In the present study, we observed fluorescently labeled donor-derived blue and channel catfish stem cells in white catfish (host) gonads at 45 and 90 DPH after intraperitoneal transplantation which indicates the capability of colonization and proliferation of donor-derived gametes. Further, both cell and cluster areas were significantly increased (doubled) when it moved from 45 DPH to 90 DPH by indicating clear proliferation and colonization of the donor-derived cells. Specifically, segmented linear regression showed significantly higher cell and cluster colonization when donor-derived stem cells were injected into recipient fish between 4 - 5 DPH. A similar trend was detected in our previous study

which was conducted using channel catfish as the host where significantly higher cell and cluster colonization was noted when injected into recipient fish between 4 - 6 DPH (Hettiarachchi et al., 2023 a). According to Dunham and Smitherman (1984), white catfish grow more rapidly as fingerlings than channel catfish. The growth performance differences at their fingerling stage could be the reason for slight time differences among white and channel catfish. According to the resulting data, the average TL and TW of channel catfish at 50 DPH was (mean ± SEM) 5.1 cm and 2.46 g, respectively. For the white catfish, it was (mean ± SEM) 7.5 cm and 4.45g, respectively. However, the higher growth performance of white catfish at their fingerling stage could be an added advantage during the stem cell transplantation procedure since there are some practical challenges to injecting fry during this early small life stage, as it is often difficult to insert the needle into the small body cavity between the anal fin and yolk-sac (i.e., where the genital ridge is expected to form). Thus, having a higher growth rate can mitigate this practical issue which is proving the suitability of white catfish as a host species.

Yoshizaki and Lee (2018) stated that a potential obstacle to donor cell proliferation and colonization could be the rejection of transplanted cells by the immune system of the recipient fish. To overcome this potential obstacle, newly hatched larvae were used as recipients in several studies due to their immature immune system immediately after hatching (Manning and Nakanishi, 1996; Takeuchi et al., 2004; Kobayashi et al., 2007; Yoshizaki et al., 2010). This was proved during the present study where cluster and cell area were gradually decreased after 5 DPH with the fish growth. However, lower cluster and cell areas were detected between 0 – 3 DPH, which could be due to technical challenges to transplanting/injecting cells to fry during this early small life stage.

Even though there are several desirable characteristics exist in white catfish as a host species, still there are some drawbacks. Low survival and low seinability of white catfish during the pond culture, are the major challenges that require to overcome in commercial applications. Especially in pond culture, white catfish have a low survival rate and low seinability/harvestability. In addition, like many other *ictalurid* catfish, white catfish are not totally resistant to several bacterial, parasite, and viral diseases such as Columnaris (*Flexibacter columnare*), Enteric septicemia of catfish (*Edwardsiella ictaluri*), Aeromonas (*Aeromonas hydrophila*), Ich (*Ichthyophthirius multifilis*), and catfish virus. Further, white catfish males are aggressive and fight with each other when held communally (Fobes, 2013). This results in frequent wounds in males which is leading to secondary infections and increased disease susceptibility. Even though seinability is considered an undervalued trait, it could significantly increase the production cost in commercial applications (Dunham and Masser, 2012) which could be a major drawback of white catfish pond culture. Culturing white catfish in recirculating aquaculture systems (RAS) could be an alternative option to mitigate these harvestability and disease resistance issues. In RAS, it could control fighting by providing hiding spots, giving more attention to aggressive males, and isolating aggressive male fish while leaving out harvestability issues.

In conclusion, the current study demonstrated the suitability of white catfish as a host species by transplanting stem cells at 4 - 5 DPH, and these findings can use to enhance the efficiency of germ cell transplantation in xenogenesis for commercial-scale hybrid catfish production.

5.0. Declaration of Competing Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

6.0. Acknowledgments

This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2018-67015-27614 from the USDA National Institute of Food and Agriculture. We would like to acknowledge the Catfish Genetics Research Unit staff and the EW Shell Fisheries Center at Auburn University for fish care, labor, and maintenance.

Fig. 1. Stem cells labeled with PKH26 dye (A). Transplantation (intraperitoneally) of donor-derived stem cells into triploid white catfish (*Ameiurus catus*) (B). Gonadal separation from triploid white catfish at 45 days post-hatch (DPH) (C).

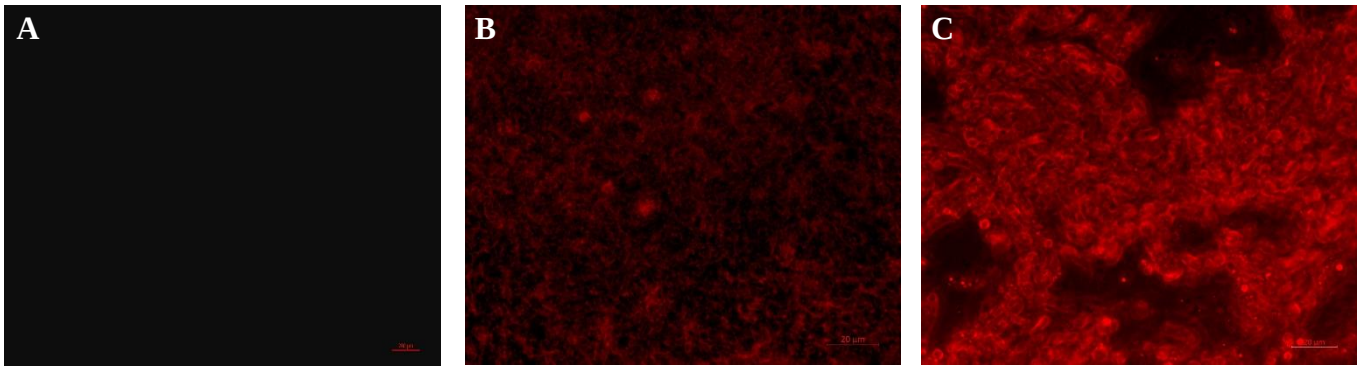

Fig. 2. Gonadal tissues expressing fluorescence from PKH26 dyed donor-derived stem cells. The non-injected control treatment (A) showed no fluorescence, while triploid white catfish (*Ameiurus catus*) (host) at 45 days post-hatch (DPH) (B) and 90 DPH (C) that have been injected with channel catfish (*Ictalurus punctatus*) stem cells at 4 DPH.

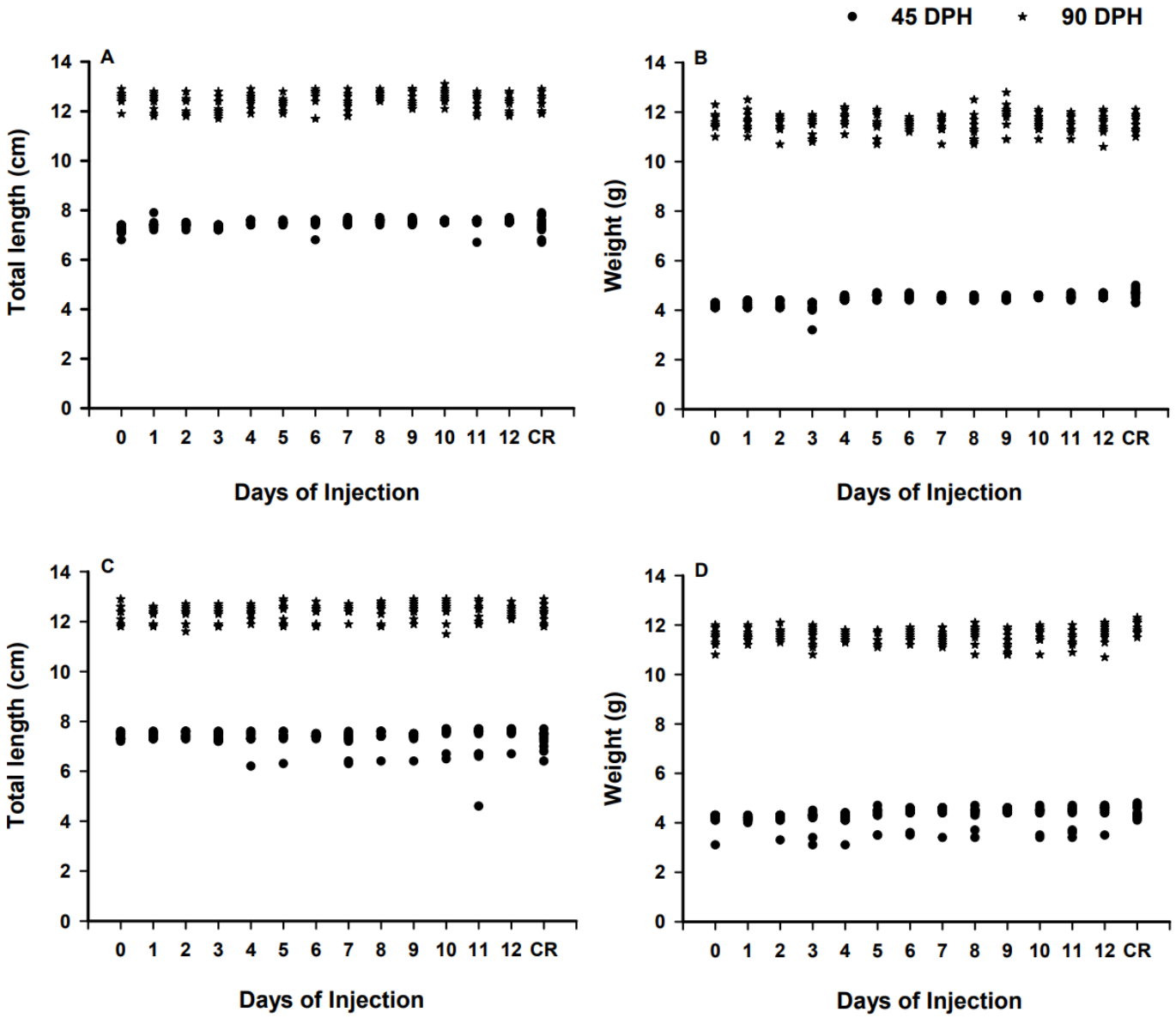

Fig. 3: Total length at 45 days post-hatch (DPH) and 90 DPH (A), body weight at 45 and 90 DPH (B) of triploid xenogenic white catfish (*Ameiurus catus*) injected with blue catfish (*Ictalurus furcatus*) stem cells and channel catfish (*I. punctatus*) stem cells (Total length at 45 and 90 DPH; C), (Body weight at 45 and 90 DPH; D) from 0 to 12 DPH or non-injected control (CR) treatment.

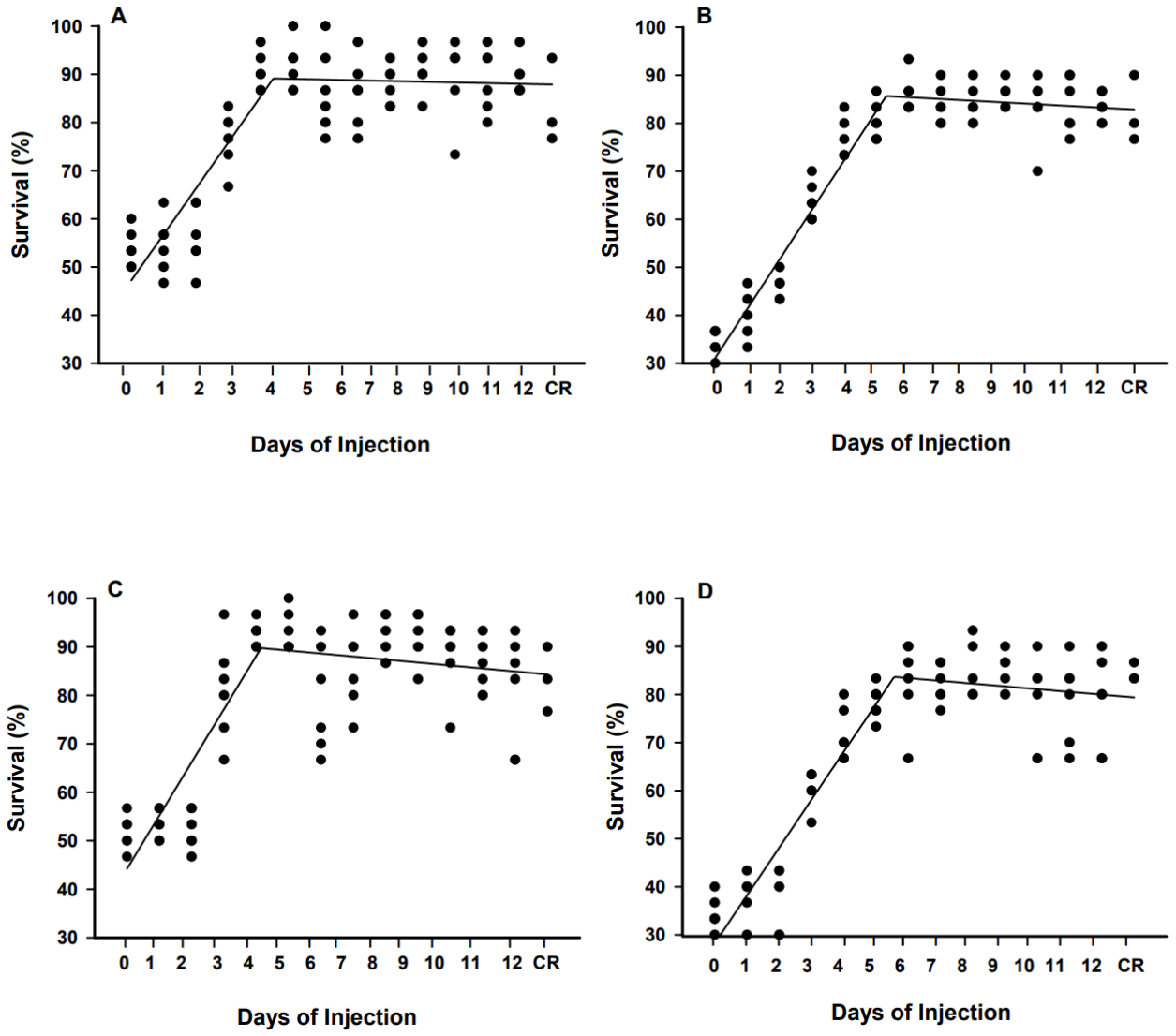

Fig. 4: Percentage survival of triploid xenogenic white catfish (*Ameiurus catus*) injected with blue catfish (*I. furcatus*) stem cells [45 days post-hatch (DPH; A), 90 DPH (B)] and channel catfish (*Ictalurus punctatus*) stem cells [45 DPH (C), 90 DPH (D)] from 0 to 12 DPH or non-injected control (CR) treatment.

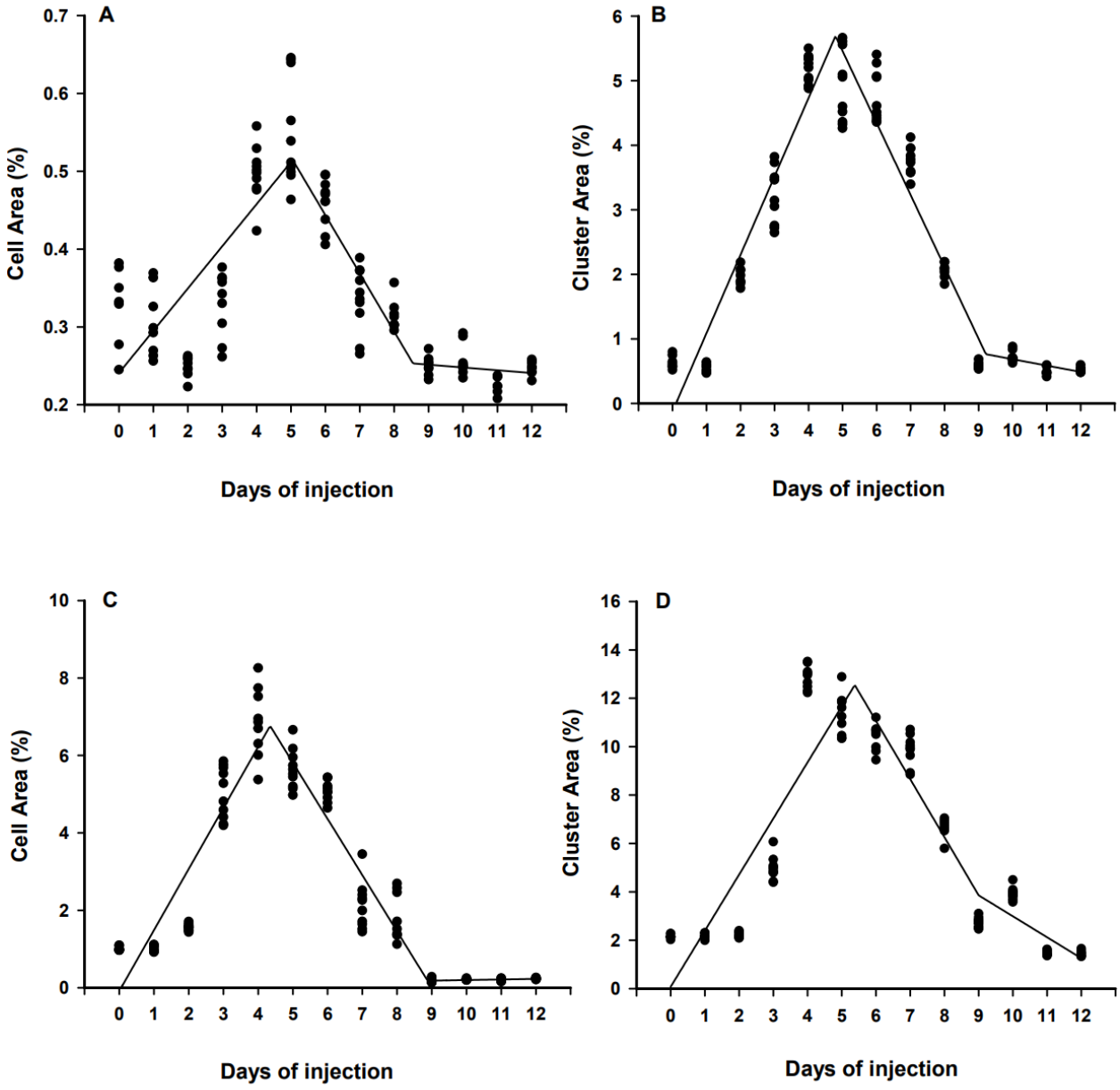

Fig. 5: Percent cell area and cluster area at 45 days post-hatch (DPH) (A and B) and 90 DPH (C and D) of triploid white catfish (*Ameiurus catus*) injected with blue catfish (*Ictalurus furcatus*) stem cells from 0 to 12 DPH.

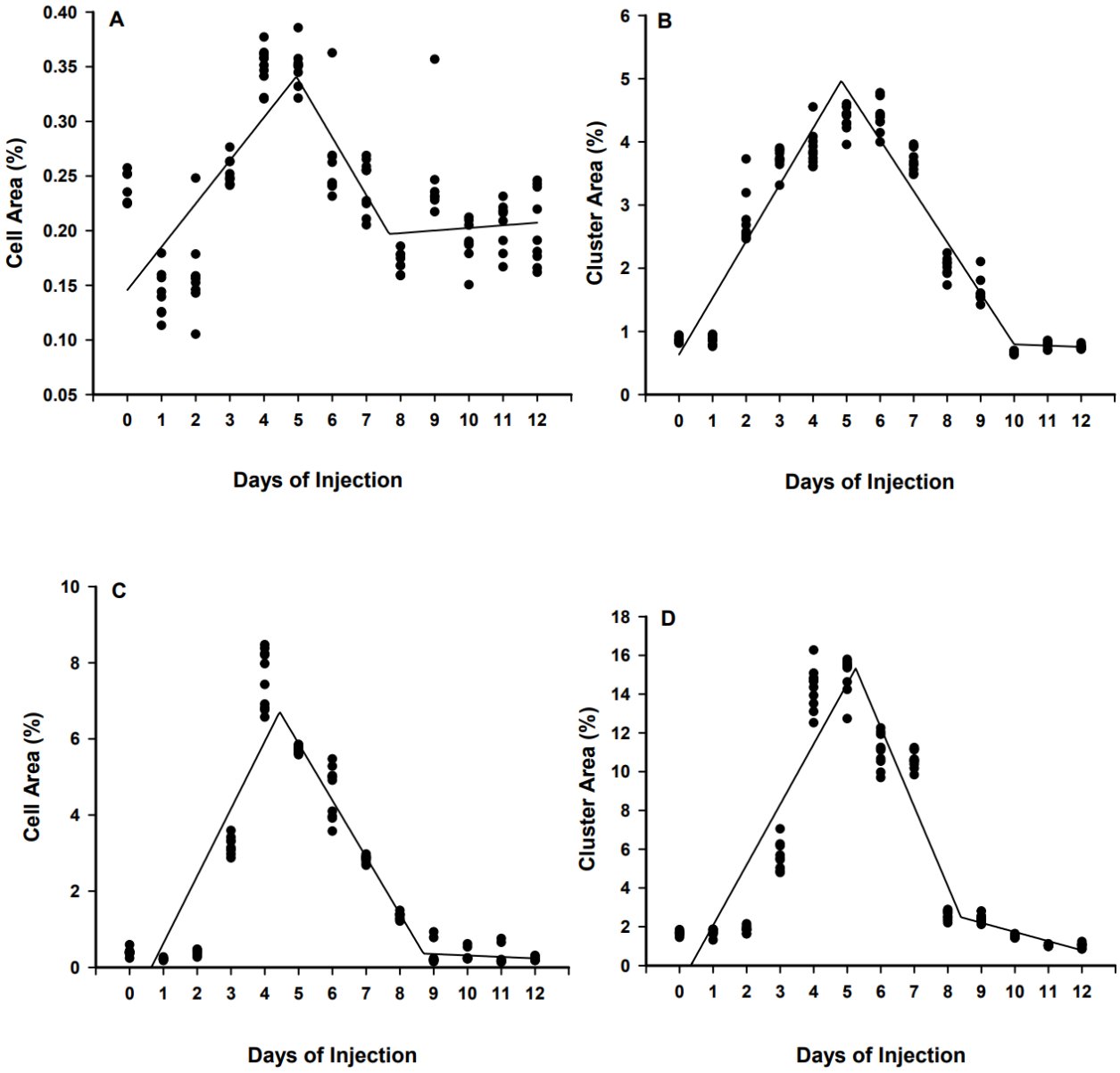

Fig. 6: Percent cell area and cluster area at 45 days post-hatch (DPH) (A and B) and 90 DPH (C and D) of triploid white catfish (*Ameiurus catus*) injected with channel catfish (*Ictalurus punctatus*) stem cells from 0 to 12 DPH.

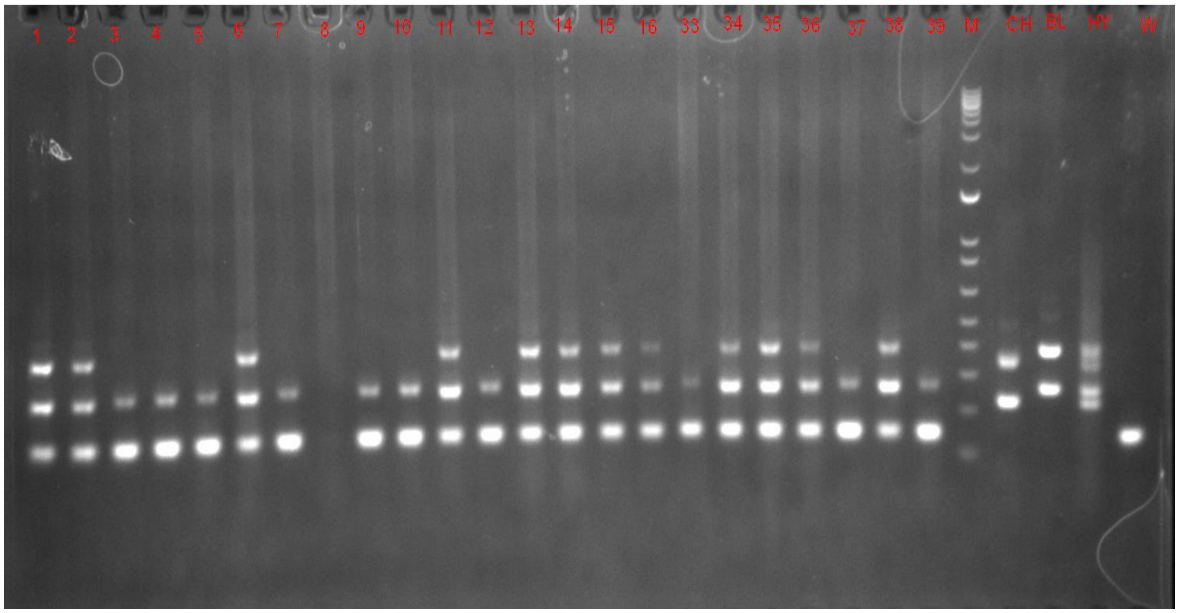

Fig. 7: Sample results from PCR for detecting blue catfish (*Ictalurus furcatus*) donor cells in the testes of triploid white catfish (*I. punctatus*). Blue catfish and white catfish cells were differentiated with PCR using follistatin (Fst) and hepcidin antimicrobial protein (Hamp) genes as markers. W = white catfish control, B = blue catfish control.

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Chapter 5

In vitro culturing of blue catfish (*Ictalurus furcatus*) stem cells for xenogenic channel catfish (*I. punctatus*) production

Abstract

Xenogenesis is emerging as an innovative technology for hybrid catfish (channel catfish, *Ictalurus punctatus* ♀ × blue catfish, *I. furcatus* ♂) hatchery production. The xenogeneic process can be accomplished by transplanting primordial germ cells, spermatogonial stem cells, or oogonial stem cells, derived from a donor diploid fish into a sterile recipient, which then enables the recipient fish to produce donor-derived gametes. Currently, freshly extracted stem cells are used to create xenogens, which is challenging, especially considering the collection of an adequate number of stem cells for transplantation and cells damaged by proteinases (trypsinization) during the procedure of stem cell extraction. Hence, *in vitro* propagation of stem cells has emerged as a potential solution. The present study aimed to identify the best culture medium for *in vitro* propagation of blue catfish stem cells for further identification of optimum culture parameters. Isolated stem cells were seeded at a concentration of 2.6×10^6 cells/mL in four different cell culture media: Dulbecco's Modified Eagle's Medium (DMEM); Minimum Essential Medium (MEM); Leibovitz's Medium (L15); and RPMI. Each cell culture medium was factorially supplemented with three different concentrations (0%, 5%, 10%) of fetal bovine serum (FBS) in cell culture well plates. Spermatogonia A viability and live spermatogonia A counts were then recorded after 1 to 8 days of incubation. Repeated measures ANOVA models indicated significant Cell Culture Media

× FBS Concentration × Incubation Time interactions for spermatogonia A viability ($P < 0.0001$) and live counts ($P < 0.0001$). As such, the models were decomposed into a series of lower-order ANOVA models at each incubation time. DMEM supplemented with 10% FBS resulted in the highest spermatogonia A viability and live spermatogonia A during the incubation period. Overall, our results show that DMEM supplemented with 10% FBS is the most suitable culture medium combination for *in vitro* propagation of spermatogonia A. These findings can be used to enhance the efficiency of germ cell culture and further success of commercial-scale hybrid catfish production via xenogenesis.

Keywords: Xenogenesis, in vitro, cell culture, Spermatogonial stem cell

1.0. Introduction

Hybrid catfish produced by crossing channel catfish (*Ictalurus punctatus*) ♀ with blue catfish (*I. furcatus*) ♂, is the only commercially valuable hybrid among 42 interspecific catfish hybrids in North America. Overall, this hybrid exhibits superior performance compared to the commonly grown channel catfish (Dunham et al., 2008). Although the hybrid industry has seen sustained growth, there are still challenges, largely due to the lack of natural hybridization between the two species and the need to sacrifice blue catfish males for sperm collection (Hu et al., 2011). In addition, blue catfish have slower growth and maturity rates compared to channel catfish, reaching sexual maturity after 4 to 6 years and at larger sizes (Dunham et al., 1994). Hence, blue catfish require more space, feed resources, time, and expenses for farmers (Ligeon et al., 2004). As a result, new hatchery techniques and technologies, such as xenogenesis, are attracting attention to produce this hybrid for commercial production.

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). This can be accomplished by transplanting primordial germ cells (PGCs), spermatogonial stem cells (SSCs), or oogonial stem cells (OSCs), derived from a donor diploid fish into a sterile recipient, then enabling recipient fish to produce donor-derived gametes (Takeuchi et al., 2004; Takeuchi et al., 2009; Dunham, 2011; Higuchi et al., 2011; Perera et al., 2017; De Siqueira-Silva et al., 2018). The PGCs, SSCs, and OSCs can undergo self-renewal throughout their life, making them useful as donor cells for xenogeneic transplantation (Yoshizaki et al., 2010; Yoshizaki and Lee, 2018). In addition, after transplantation, these undifferentiated cells can migrate towards the genital ridge and colonize, giving rise to female or male germ cells, after the gonad has differentiated into an

ovary or a testis, depending on the individual's genetic sex. This innovative hatchery technology has been successfully applied to improve reproduction for commercially valuable fish species and to conserve endangered populations using either PGC (Yoshizaki et al., 2000; Yoshizaki et al., 2002; Takeuchi et al., 2004; Saito et al., 2008; Saito et al., 2011), SSCs (Nagler et al., 2001; Okutsu et al., 2006; Silva et al., 2006; Saito et al., 2008; Majhi et al., 2009; Takeuchi et al., 2009; Yoshizaki et al., 2010; Lacerda et al., 2013; Pacchiarini et al., 2014; Yoshikawa et al., 2016; Perera et al., 2017; Ye et al., 2017; Hettiarachchi et al., 2022), or OSCs for transplantation (Yoshizaki et al., 2010; Wong et al., 2011; Ye et al., 2017; Yoshizaki and Lee, 2018; Hettiarachchi et al., 2022).

All these above experiments used freshly extracted stem cells (PGCs, SSCs, OSCs). However, one of the major disadvantages of using freshly extracted stem cells is that they can be damaged by proteinases (trypsinization), which is an unavoidable step during testes/ovary dissociation procedures (Alberts et al., 2002; Houreld et al., 2018). As such, long-term, or short-term culture prior to transplantation could enable the healing process to counteract this damage. Furthermore, in some species of fish it is challenging to collect an adequate number of donor germ cells for transplantation, therefore in vitro propagation of donor germ cells has emerged as a potential solution (Yoshizaki and Lee, 2018). The development of methods for in vitro culture of stem cells could improve the efficiency of germ cell transplantation, as donor cells could be colonized to get an adequate number of cells for transplantation (Hong et al., 2004; Shikina et al., 2008; Shikina and Yoshizaki, 2010; Lacerda et al., 2013). In addition, in vitro culture would minimize the sacrifice of donor species to extract stem cells, which could be a major advantage for commercialization using xenogenic technology.

Thus, the present study was conducted to identify the best culture medium for in vitro propagation of blue catfish stem cells for further identification of optimum culture parameters, focusing on concentrating on fetal bovine serum.

2.0. Materials and methods

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

2.1. Blue catfish husbandry and stem cell isolation

Immature blue catfish were obtained from earthen ponds (0.04 ha) at the Fish Genetics Research Unit at Auburn University, AL, USA. Fish were fed to satiation with 32% protein pellet feed five days per week during summer and three days per week during winter. During the experimental period, dissolved oxygen (DO) was measured twice daily while temperature and salinity were recorded daily. In addition, total ammonia-nitrogen (TAN), nitrite, nitrate, pH, hardness, and alkalinity were measured twice per week. During the sampling period, DO, temperature, pH, TAN, nitrite, nitrate, hardness, and alkalinity levels were 7.0 ± 1.8 mg/L, 26.5 ± 1.9 ^{o}C, 7.0 ± 0.5, <0.5 mg/L, <0.5 mg/L, <20 mg/L, 89.5 ± 15 mg/L, and 35.5 ± 5 mg/L, respectively. Fish were collected by seining ponds with a 3.8 cm mesh seine.

Immature blue catfish (n = 12; mean ± SEM length = 32.6 ± 7.9 cm, weight = 410.73 ± 87.41 g) were selected and euthanized. After being euthanized, the external body of the fish was sterilized with 70% ethanol. Then, gonad extraction and stem cell isolation were performed according to standard protocols described by Shang et al. (2015), Abualreesh et al. (2020, 2021a, 2021b), and Hettiarachchi et al. (2020, 2022). In brief, testes were removed from the peritoneal cavity and

placed on a sterile petri dish (100 mm × 15 mm) containing 5 mL of Hanks' Balanced Salt Solution (HBSS, SH30031.03, GE Healthcare Life Sciences) supplemented with 1.0 μg/mL $NaHCO_3$ (Church & Dwight Co., NG) and 10,000 unit/mL Penicillin - Streptomycin (I15140–122, Life Technologies). Samples were then transferred to a biosafety cabinet for further cleaning with 0.5% bleach solution prepared with double distilled H_2O, HBSS, and three rinses with phosphate-buffered saline (PBS; J62692, Alfa Aesar). Next, testes were minced with a sterilized scalpel blade and transferred to 50 mL autoclaved glass flasks for the trypsinization process [0.25% trypsin (25210 - 082, Life Technologies)]. Samples were then incubated on ice for 30 min followed by 1 h at 22 °C with a magnetic stirrer. The cell suspensions were then filtered [70 and 40 μm cell strainer with nylon mesh (352340, VWR International)] and centrifuged at 500 g (Eppendorf Centrifuge 5418 R) for 20 min to separate cells. The resulting pellets were resuspended in 2 mL of Dulbecco's Modified Eagle's Medium/DMEM (DMEM, 10–090-CV, Corning cellgro) supplemented with 10% fetal bovine serum (FBS; 10438018, Life Technologies), 10,000 unit/mL penicillin-streptomycin (15140-122, Life Technologies), and 200 mM L-glutamine (A2916801, Life Technologies) to provide a favorable environment for the stem cells.

2.2. Stem cell culturing

Isolated stem cells were seeded at a concentration of 2.6×10^6 cells/mL in four different cell culture media: (1) DMEM; (2) Minimum Essential Medium/MEM (10-010-CMR, Corning cellgro); (3) Leibovitz's Medium/L-15 (10-047CVR, Corning cellgro); and (4) RPIM (SH300027.1, HyClone). Each cell culture medium was then factorially supplemented with three different fetal bovine serum (FBS; 10438076, Life Technologies) concentrations (0%, 5%, 10%)

in cell culture well plates (growth area 9.6 cm^2; 10861-653, VWR). All culture media was supplemented with equal concentrations of 10,000 units/mL penicillin-streptomycin (15140-156, Life Technologies) and 200 mM L-glutamine (A2916871, Life Technologies) to provide a favorable environment for the cells. Isolated cells (2.6×10^6 cells/mL) were incubated in an air-jacketed incubator with 5% CO_2 at 30 ^oC (10810-888; VWR). The cultured cells were observed daily under an Olympus objective microscope (BH2), equipped with a 20× objective, to determine the total number of cells, and the total number of live and dead spermatogonia A, with the aid of a hemocytometer and 0.4% trypan blue (15250061, Life Technologies). The number of cells in 1 mL was calculated according to Louis and Siegel (2011) with a dilution factor of 2 (cell suspension: trypan blue with a 1:1 ratio). The viability index formula was used to obtain the percentage of live cells in each treatment, in which the proportion of the total live spermatogonia A counted from each treatment was divided by the total number of spermatogonia A × 100.

2.3. Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.4; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro-Wilk test; PROC UNIVARIATE) and homogeneity of variance (plot of residuals vs. predicted values; PROC GPLOT). Post-hoc testing was done with Tukey's test to determine differences among treatments. Values were defined as statistically significant at alpha < 0.05. Spermatogonia A viability and live spermatogonia A counts were compared among Cell Culture Media (DMEM; MEM; Leibovitz's Medium/L-15; RPIM), FBS Concentration (hereafter, termed Concentration; 0, 5, 10%) and Incubation Time (0 to 8 days) using a series of repeated measures factorial ANOVA models. If a

significant Cell Culture Media × Concentration × Incubation Time interaction was detected the saturated model was broken down into a series of two-way ANOVAs to determine the effect of Cell Culture Media and FBS Concentration at each Incubation Time. Again, if a significant interaction was detected the 2-way ANOVA model was broken down into a series of 1-way ANOVA models to determine the effect of Cell Culture Media at each Concentration and the effect of Concentration at each Cell Culture Media. If the 2-way interaction was not significant the Cell Culture Media and Media Concentration main effects were interpreted.

3.0. Results

3.1. Spermatogonia A viability

The repeated measures ANOVA model indicated a significant Cell Culture Media × Concentration × Incubation Time interaction for spermatogonia A viability ($P < 0.0001$). Therefore, the model was decomposed into a series of two-way ANOVA models at each incubation time. The Cell Culture Media × Concentration interaction was not significant on Day 1 ($P = 0.296$), whereas the Cell Culture Media ($P < 0.0001$) and Concentration ($P < 0.0001$) main effects were significant, such that DMEM and MEM at 5 and 10% had the highest viability. The Cell Culture Media × Concentration interaction was significant from Day 2 to Day 8 ($P \leq 0.004$); thus, the model was broken down into a series of 1-way ANOVAs at each time (see Section 2.3; Fig. 3). When examining the effect of Cell Culture Media at each Concentration, both DMEM and MEM resulted in the highest spermatogonia A viability when incubated with 5% and 10% FBS. Cell

viability gradually decreased when FBS concentration decreased (Fig. 2). By Day 8, DMEM supplemented with 10% FBS was the only treatment that had viable cells (35.5% viability).

When examining the effect of Concentration at each Cell Culture Media the 5 and 10 % FBS treatments had higher spermatogonia A viability than the 0 % FBS treatments in all cell culture media. However, when the cells reached day 8, DMEM supplemented with 10% FBS had 35.5% viability while the other treatments had no viable cells (Fig. 3).

3.2. Live Spermatogonia A

Similar to spermatogonia A viability, the repeated measures ANOVA model revealed a significant Cell Culture Media × Concentration × Incubation Time interaction for live spermatogonia A counts ($P = 0.001$). Therefore, the model was decomposed into a series of two-way ANOVA models at each incubation time. At Day 1 ($P = 0.371$) the Cell Culture Media × Concentration interaction was not significant. However, significant Cell Culture Media × Concentration interactions were detected between Day 2 to Day 8 ($P \leq 0.0004$). The ANOVAs were therefore decomposed into a series of 1-way ANOVAs at each time to determine the effect of the Cell Culture Media at each Concentration and the effect of Concentration on each Cell Culture Media (Fig. 4).

A significant effect was detected for all one-way ANOVAs when the models were decomposed to determine the effect of the Cell Culture Media at each Concentration ($P < 0.006$). Here, DMEM had the highest spermatogonia A counts when supplemented with 10% FBS. At day 8, DMEM supplemented with 10% FBS was the only treatment with live spermatogonia A. A similar trend was detected when examining the effect of Concentration at each Cell Culture Media.

4.0. Discussion

The success of SSCs transplantation from a donor species into an interspecific sterile host has been identified in the literature using various species (Silva et al., 2006; Saito et al., 2008; Majhi et al., 2009; Takeuchi et al., 2009; Yoshizaki et al., 2010; Lacerda et al., 2013; Yoshikawa et al., 2016; Perera et al., 2017; Ye et al., 2017; Hettiarachchi et al., 2022). However, in all those experiments, freshly extracted SSCs were used, which is challenging especially considering the collection of an adequate number of SSCs for transplantation and cells damaged by proteinases (trypsinization) during the procedure of stem cell extraction. Thus, in vitro propagation of SSCs has emerged as a potential solution that provides time for the healing process and provides an adequate number of cells through cell proliferation and colonization (Yoshizaki and Lee, 2018). Hence, the present study was carried out to identify the best culture medium for in vitro propagation of blue catfish stem cells. In the present study, there was a significant effect of FBS concentrations (0%, 5%, and 10%) and cell culture media (DMEM, MEM, L15, and RPMI) on spermatogonia A viability and live spermatogonia A counts. Overall, it was revealed that DMEM supplemented with 10% FBS resulted in the highest spermatogonia A viability and live spermatogonia A counts throughout the experimental period when enriched with 5% CO_2 at 30 ^{0}C than the MEM, L- 15, and RPMI cell culture media, indicating that specific combinations of these factors can greatly influence the success of *in vitro* blue catfish SSCs propagation.

A typical culture medium is composed of amino acids, vitamins, inorganic salts, glucose, and serum as a source of growth factors and hormones (Yao and Asayama, 2017; Oneill et al., 2021; Okamoto et al., 2022). Overall, the cell culture medium supports cell survival, proliferation,

colonization, as well as other cellular functions. Several examples exist for the use of DMEM as a culture media (Brevini et al., 2007; Wu et al., 2009; Chase et al., 2010; Chen et al., 2010; Mazzetti et al., 2010; Chase et al., 2012; Behnia et al., 2013; Bui et al., 2014). In line with previous studies, our results showed that DMEM provides a favorable environment for blue catfish SSCs when compared to MEM, L15, and RPMI. Even though all these media are synthetic/ artificial, each has its own chemical composition. For instance, DMEM and MEM contain a lower concentration of glucose (1.0 g/L) than RPMI and L15 (2.0 g/L) (Lewinska et al., 2007; Wu et al., 2009). Further, DMEM and MEM have a higher concentration of calcium (1.8 mM) and a lower concentration of phosphate (1.0 mM) than RPMI and L15 which is 0.8 mM of calcium and 5.0 mM of phosphate, respectively (Wu et al., 2009; Krawczyk et al., 2018). These different chemical compositions could directly affect spermatogonia A viability and the counts, resulting in lower spermatogonia A viability and spermatogonia A counts in both L15 and RPMI vs. DMEM and MEM. Even though DMEM and MEM performed better, overall DMEM had higher spermatogonia A viability and live spermatogonia A counts than MEM. The only difference between DMEM and MEM is DMEM contains 4 times higher concentrations of amino acids and vitamins than MEM (Chang et al., 1995; Yang and Xiong, 2012) which may be caused to provide a better environment for SSCs culture.

During the 1st day of incubation in DMEM and MEM, spermatogonia A were dispersed and separated from each other. Cells were either suspended in a culture medium or weakly attached to the culture plate. By Day 3, spermatogonia A incubated in DMEM and MEM supplemented with 10 % FBS started to form clumps (cell-cell adhesion) by attaching to each other and adhering to the culture plate. Cell adhesion and binding is mainly controlled by a subset of cell surface proteins called Cell Adhesion Molecules (CAMs) (Albelda and Buck, 1990; Alberts et al., 2002; Golias et

al., 2011; Farahani et al., 2014). Treating the tissue with low concentrations of a proteolytic enzyme such as 0.25% trypsin disrupts and digests the CAMs interactions that hold cells together (Alberts et al., 2002; Houreld et al., 2018). However, with favorable environmental conditions, CAMs can be repaired and repair CAMs signaling that directly or indirectly modulates stem cell proliferation, self-renewal property, adhesion, and multilineage differentiation (Alberts et al., 2002; Kinney et al., 2014; Lee and Cho, 2017; Abdal et al., 2018). These above observations provide promising results for culturing spermatogonia A and their ability to heal potential damages caused by proteinases with DMEM and MEM culture media. However, during the present study, to obtain accurate data during each sampling day, cells were partially harvested by gently mixing the culture medium. That was the major reason for the decreased cell count and none developed larger clumps over time. Similar clump formations during rainbow trout (*Oncorhynchus mykiss*) stem cell culture was reported by Shikina et al. (2013). Further, it was reported that cultured rainbow trout spermatogonia for a short period with DMEM resulted better transplantation results than freshly prepared rainbow trout stem cells.

Furthermore, Shikina and Yoshizaki (2008) reported that DMEM supplemented with both 5% and 10% FBS provides better environmental conditions than 1% and 20% FBS for rainbow trout spermatogonia A. However, during the present study, it was revealed that DMEM supplemented with 10% FBS provided a better environment for blue catfish spermatogonia A than 0% and 5% FBS. FBS is the most popular and widely applicable serum as it serves as a source of amino acids, proteins, vitamins, carbohydrates, lipids, hormones, growth factors, inorganic salts, trace elements, and other compounds. FBS also improves the pH-buffering capacity of the medium (Chelladurai et al., 2021). According to Yao and Asayama (2017), FBS helps to reduce shear stress (physical damage that is caused by pipette manipulation and stirring) which could be

the major reason for the better results from 10% FBS than the lower concentrations (0% and 5%). Furthermore, FBS has the ability to alter the conditions at a culture substratum, allowing the adherent cells to readily proliferate (Jung et al., 2012).

In n a previous study, Hettiarachchi et al (2022) found that cryopreserved blue catfish (donor) stem cells had an equal ability to recover in recipient channel catfish gonads and perform as well as their freshly extracted counterparts, based on donor cell proliferation and colonization rates inside the host. Further, in both fresh and cryopreserved treatments, cell and cluster areas doubled from 45 DPH to 90 DPH [cluster area increased from (~2.4 % to ~6.2%) and cell area increased from ~0.28% to ~0.4%]. However, there is a high possibility that cell transplantation success could be increased if we can culture cryopreserved stem cells for a short period before transplantation, which could enable the healing of the proteinases damaged.

The culture system of spermatogonia A could be used as a gene transfer system, such as gene targeting. Spermatogonia A would be an attractive alternative material for gene targeting (Capecchi, 1989). A recent report on knockout mouse production using a spermatogonial stem cell line (Oatley and Brinster, 2006) clearly demonstrated that this cell type is well suited to produce transgenic animals. Therefore, further experiments need to be conducted to establish a transplantable cell line derived from spermatogonia A that can colonize recipient gonads and differentiate into functional sperm and eggs through xenogenesis. In addition, future studies are also needed to compare freshly extracted vs. short-term/long-term cultured stem cells and their effect on stem cell proliferation and colonization post-transplantation.

5.0. Declaration of Competing Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

6.0. Acknowledgments

This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2018-67015-27614 from the USDA National Institute of Food and Agriculture. We would like to acknowledge the staff of the Catfish Genetics Research Unit as well as the EW Shell Fisheries Center at Auburn University for fish care, labor, and maintenance.

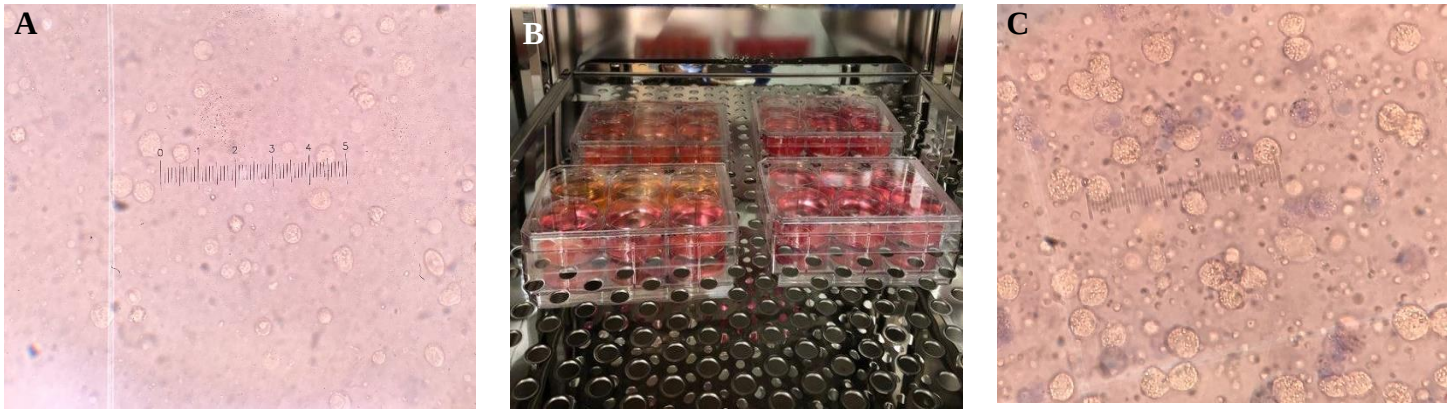

Fig. 1: Cultured spermatogonial stem cells in DMEM cell culture medium supplemented with 10% fetal bovine (FBS) at Day 1(A). Spermatogonial stem cells incubating at 30 ^{0}C and 5.0% CO_2 in different culture plates (B). Cultured spermatogonial stem cells in DMEM cell culture medium supplemented with 10% FBS at Day 3; spermatogonial stem cells started to form clumps and adhered to the culture plate (C).

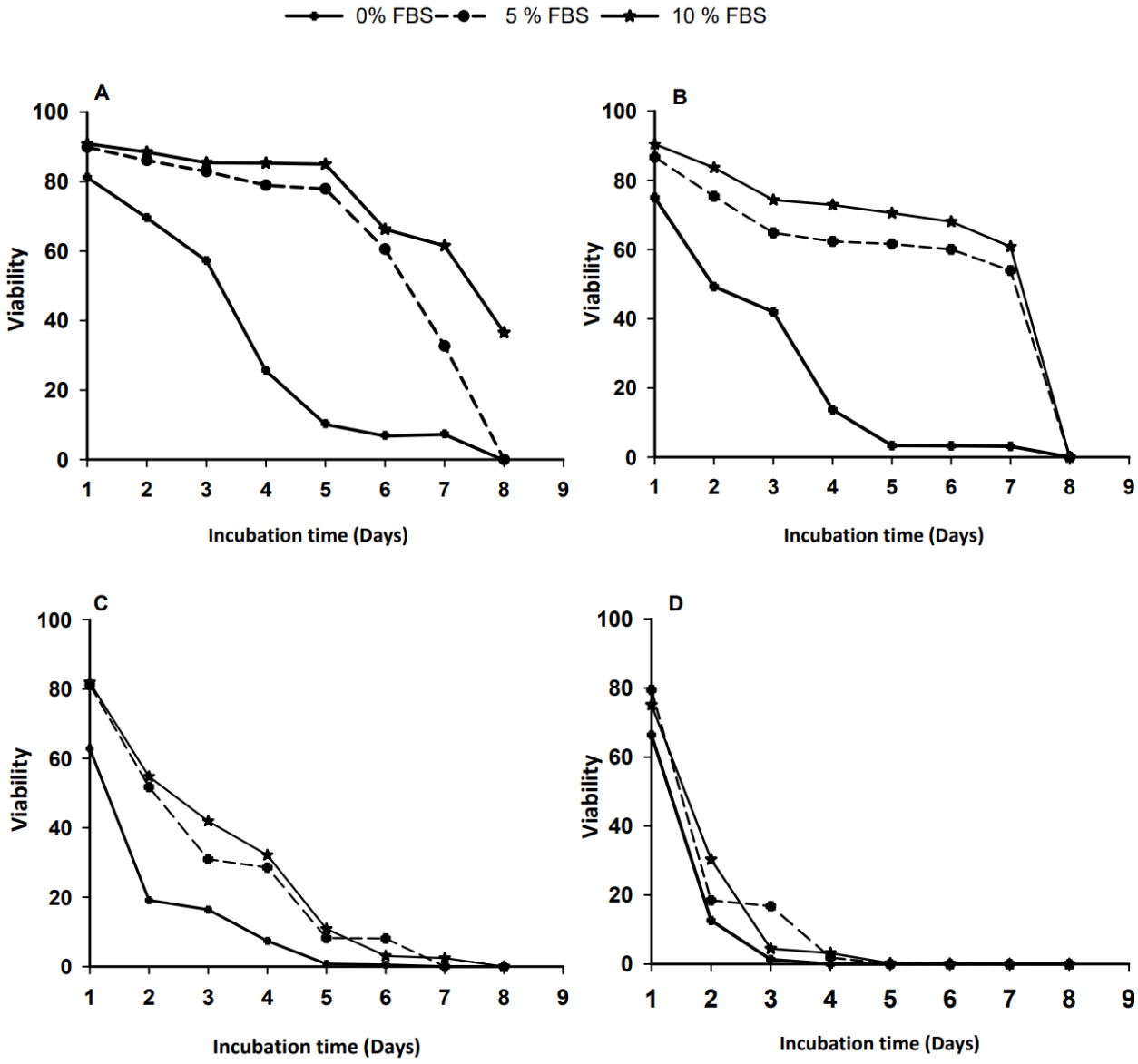

Fig. 2: Spermatogonia A viability of blue catfish (*Ictalurus furcatus*) in different culture media (Dulbecco's Modified Eagle's Medium/DMEM, Minimum Essential Medium/MEM, Leibovitz's Medium/L-15, and RPMI) and Fetal Bovine Serum / FBS concentrations (0%, 5%, and 10%).

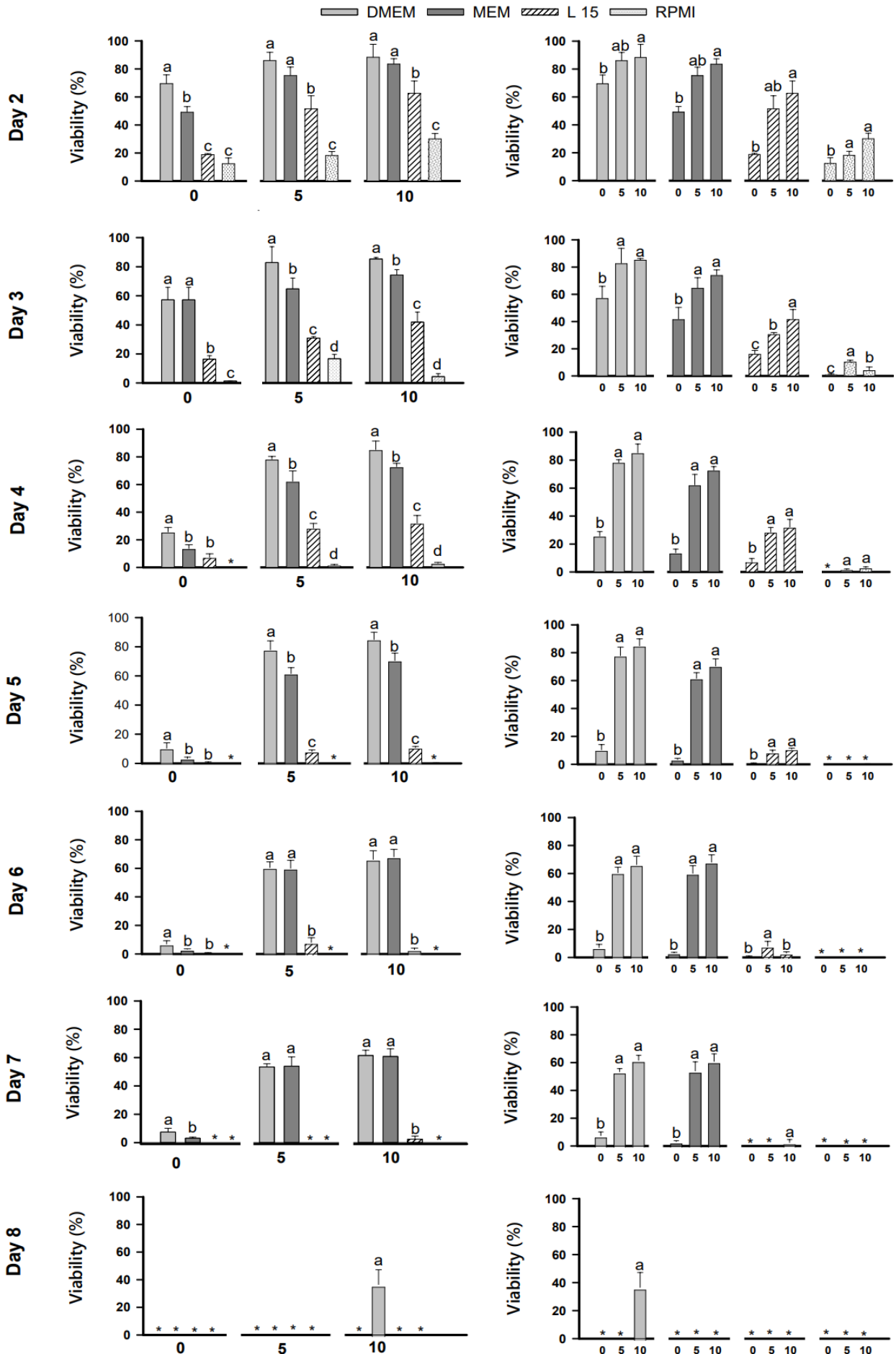
DMEM
MEM
L 15
RPMI
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Viability (%)
0
5
10

Fig. 3: Effect of culture media (Dulbecco's Modified Eagle's Medium/DMEM, Minimum Essential Medium/MEM, Leibovitz's Medium/L-15, and RPMI) and Fetal Bovine Serum / FBS concentrations (0%, 5%, and 10%) on blue catfish (*Ictalurus furcatus*) spermatogonia A viability. A series of one-way ANOVA models were run on sampling days (day 2 - day 8). Letters represent significant differences among treatments ($p < 0.001$). *Note: * zero viability*

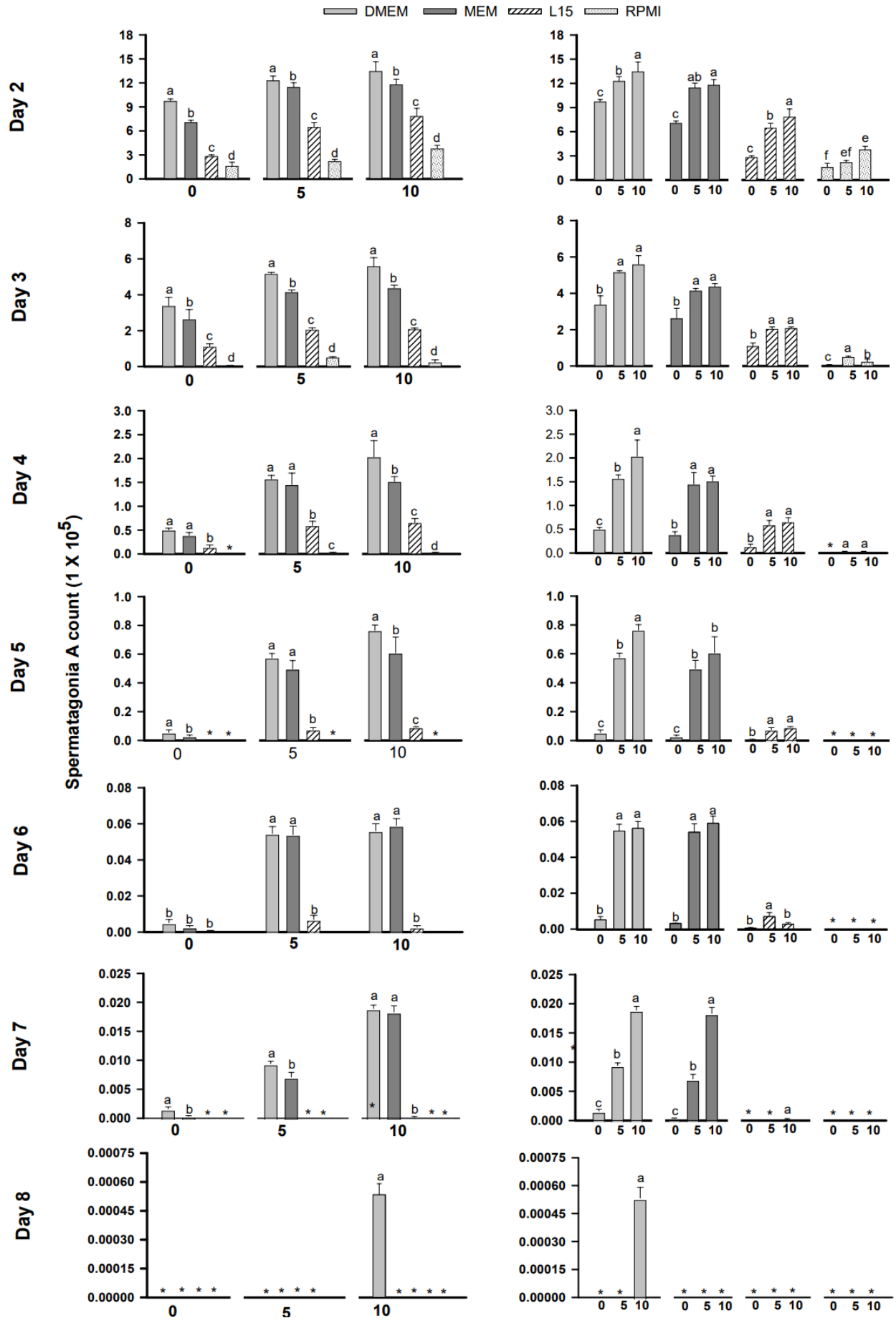
DMEM
MEM
L15
RPMI
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Spermatagonia A count (1 X 10^5)
0
5
10

Fig. 4: Effect of culture media (Dulbecco's Modified Eagle's Medium/DMEM, Minimum Essential Medium/MEM, Leibovitz's Medium/L-15, and RPMI) and Fetal Bovine Serum / FBS concentrations (0%, 5%, and 10%) on blue catfish (*Ictalurus furcatus*) spermatogonia A counts. A series of one-way ANOVA models were run on sampling days (day 2 - day 8). Letters represent significant differences among treatments ($p < 0.001$) *Note: * zero Spermatogonia A*

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