Effect of Host Age and PKH26 Dye Concentration on Stem Cell Viability, Colonization Rate, and Offspring Performance for Production of Xenogenic Catfish

By

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Keywords: Xenogenesis; blue catfish; channel catfish; stem cell; PKH26

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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. Currently, blue catfish donor stem cells are collected and injected into triploid channel catfish embryos without having prior knowledge of the best time for injections. This is problematic, as valuable stem cells may be wasted if a host fish is not injected at the correct point in ontogeny. Thus, this thesis aimed to determine how host age impacts stem cell viability, colonization rate, and offspring performance for production of xenogenic catfish.

To visually confirm colonization and proliferation rates, the concentration of PKH26, a cell tracking dye, was also tested. Here, triploid channel catfish embryos were injected with donor-derived blue catfish stem cells on 0, 1, 2, 3, 4, and 5 days post-hatch (DPH) using PKH26 dye. Injections were found to be successful on each injection day, with 3 DPH having the highest overall survival with and without addition of PKH26 dye after 280 DPH, 46.66% and 58.66% respectively. When visually confirming colonization and proliferation of the PKH26 dyed stem cells there was a significant difference in fluorescence between triploid channel catfish injected on 0 and 5 DPH post-hatch.

Concentrations of PKH26 dye were tested in relation to cell viability and time post-staining. Donor-derived stem cell solutions lost viability post-staining with most concentrations of PKH26. The number of overall cells available for injection declined within five min post exposure to PKH26. Together, these findings, suggest that the injection of stem cells into

triploid channel catfish should take place quickly and specifically within ~5 min post completion of the PKH26 dying process.

Keywords: xenogenesis; blue catfish; channel catfish; hybrid catfish; stem cell; survival; growth

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Chapter One:

Producing xenogenic channel catfish, *Ictalurus punctatus*, with donor-derived stem cells from blue catfish, *I. furcatus*

Abstract

Xenogenesis has been identified as an innovative technology for hybrid catfish (♀ channel catfish, *Ictalurus punctatus* × ♂ blue catfish, *I. furcatus*) embryo production. Currently, donor cells are collected and injected into triploid channel catfish embryos without having prior knowledge of the best time for injections. Here, triploid channel catfish embryos were injected with donor-derived blue catfish stem cells on six different days post-hatch (0, 1, 2, 3, 4, 5) using PKH26 dye to visually confirm colonization and proliferation rates. Injections were found to be successful on each injection day, with 3 DPH having the highest overall survival with and without addition of PKH26 dye after 280 DPH, 46.66% and 58.66% respectively. When visually confirming colonization and proliferation of the PKH26 dyed stem cells there was only a significant difference in fluorescing area between injected triploid channel catfish on days 0 and 5 post-hatch. If the amount of spermatogonia or oogonia present at 90 days reflects the future gamete production, injection at days 0,1 and 5 would be more productive for producing viable xenogenic brood stock.

1. Introduction

1.1. World Aquaculture and the Catfish Industry in the United States

The Global population is increasing and the demand for affordable and sustainable protein sources continues to rise. Overfishing of wild stocks has created a reduction in aquatic biodiversity and shifted the overall ecosystem (Worm et al., 2009). In response, the aquaculture industry is expanding to meet these demands (FAO, 2018), with improved techniques and technologies (Dunham, 2011), as farmers are now being asked to produce more fish in the same amount of space that they have used in the past. In the United States, this has resulted in a shift towards hybrid catfish (female channel catfish, *Ictalurus punctatus* × male blue catfish, *I. furcatus*) production, as these fish can tolerate higher stocking densities (Dunham, 2011). However, there is always the risk of losses when fish are heavily stocked into ponds due to higher stress and potential disease outbreaks. Thus, one of primary keys for meeting future protein demands lies with genetic enhancement techniques, including selection, interspecific hybridization, polyploidy, and transgenic modification (Dunham, 2011).

Siluriformes is one of the most diverse and biogeographically ubiquitous group of teleosts. There are 37 recognized families consisting of over 3,000 species spread across the globe. Catfish represent nearly 11% of all fish and 5.5% of all vertebrates (Armbruster, 2011). Due to this diversity and their ability to tolerate handling, high tolerance to disease, high fecundity, and good feed conversion, catfish are on the global forefront of aquaculture (Jin et al. 2016). Currently, there are multiple catfish that are cultured worldwide, including Tra (*Pangasiuis hypothalamus*), African catfish (*Clarias garienpinus*), walking catfish (*Clarias batrachus*), broadhead catfish (*Clarias macrocephalus*), the hybrid of broadhead catfish female × African

catfish male, blue catfish (*I. furcatus*), channel catfish (*I. punctatus*), and the F1 hybrid of female channel catfish × male blue catfish (Jin et al. 2016).

The U.S. has been a large producer of aquaculture raised channel catfish and the hybrid female channel catfish × male blue catfish. Catfish production accounts for ~68% of all freshwater aquaculture production in the U.S. (NMFS, 2016). The U.S. has recently seen a decline in catfish production, decreasing from 300,278 metric tonnes in 2003 to 136,531 metric tonnes in 2014. The attributed reasons for this decrease in production is due to the growth of imported frozen catfish, primarily *Pangasianodon spp.* (Hanson and Sites, 2015), and the 2007 recession which created high costs for both feed and fuel, as well as contributed to problems with disease and production inefficiencies. Catfish production increased slightly in 2015-2017 and reached 150 million kg in 2017 (Mississippi State University 2017), and continues to grow slowly with 158 million kg catfish produced in 2019 (NASS 2020). Frozen catfish imports have increased from 13,607 metric tonnes to 108,408 metric tonnes in 2005 to 2014, respectively.

The largest competitor to U.S. catfish farmers comes from the Asian export of *Pangasianodon*. Unlike catfish found in the U.S., *Pangasianodon* is able to utilize their swimbladder to absorb oxygen and allows for tolerance of dissolved oxygen levels as low as 0.05 mg/L (FAO, 2019). Because of their respiratory physiology *Pangasianodon* farmers are able to produce their crop at stocking densities higher than that of the U.S. catfish farmer. *Pangasianodon* production ponds are able to reach harvest yields of over 300 metric tonnes/ha (FAO, 2019). In comparison, the average U.S. catfish farmer is able to produce 8,000 kg/ha (Courtwright, 2013) with proper aeration devices in place and some farmers being able to produce upwards of 22,000 kg/ha particularly with intensive pond-based production systems

(Tucker and Hargreaves, 2004; Kumar et al., 2018). Hybrid catfish production has increased to 12,000 kg/ha due to the increased availability of hybrid catfish fry and the adoption of intensive aeration by some commercial producers (Bott et al. 2015; Holland, 2016; Kumar and Engle, 2017). In split-pond systems, production levels can range from 17,000 – 20,000 kg/ha (Brune et al., 2012; Tucker et al., 2014; Kumar et al., 2016; Kumar et al., 2018).

Pangasianodon are reared in non-intensive culture practices due to their respiratory physiology. The lack of need for intensive culture practices allows for high density stocking of *Pangasianodon* and with the decreased need in labor, production costs are lower leading to low exportation prices. *Pangasianodon* is cheaper to import and sell in the U.S. than the channel catfish as fillets are half price (Hanson, 2020). This has created a problematic issue within the U.S. aquaculture industry.

However, foreign imports of catfish are now actually decreasing, likely because of recently implemented USDA inspection programs. As such, a large opportunity exists for increased production, sales, and profitability for the catfish industry. Since the USDA catfish inspection program was implemented, Vietnamese catfish fillet imports have decreased by 55 million kg/yr in 2018 (NOAA 2018), an additional 31 million kg/yr in 2019 (Hanson 2020), and another competing white fish flesh, tilapia is also down 10 million kg/yr (Urner Barry's Foreign Trade Data 2020). This is ~219 million kg/yr round weight. This "empty niche" is equivalent to $908 million in gross income to processors per year ($9.46/kg × 96 million kg/yr), $482 million in gross income to producers per year ($2.2.kg × 219 million kg/yr), and production would more than double if quality product was available to capture this opportunity.

Despite, this tremendous opportunity the void is not being filled. There are three ways to fill this void and to increase competitiveness: 1) increase production efficiency to decrease price, while maintaining or increasing profitability, and 2) increase production/ha as this void cannot be filled overnight (we have less than half the farmers and acreage devoted to catfish farming compared to 2003). Increasing acreage to capture opportunity is a potentially slower process than increasing production and efficiency within the existing footprint. Genetic improvement of channel catfish and hybrid catfish can impact these problems making the U.S. catfish industry more efficient, productive, sustainable, competitive, and profitable. All possibilities of improvement within the U.S. catfish industry, foremost genetic enhancement, must be explored to give catfish farmers a better opportunity.

Over time, the selection of desirable traits has been successful in aquaculture. More than 2000 years ago in the Roman Empire, the first aquaculture practices were recorded (Balon, 1995). The first aquaculturists would breed the largest fish with each other and practiced genetic selection without understanding the genetic basis (Dunham, 2011). Gene frequencies and performance began to become altered due to the domestication of wild-caught fish (Dunham, 2011). The first targets for selection were phenotypic traits such as color, size, and body shape. Organized breeding programs date back to the 1800's when selective breeding of koi (*Cyprinus spp.*) in Japan and goldfish (*Carassius auratus*) in China were performed (Dunham, 2011). The first records of channel catfish being spawned in captivity occurred in 1892 (Dunham and Smitherman, 1983). In 1911 the Kansas Department of Fish and Game started propagating Kansas strain catfish from the Ninnescha River. The early catfish farming operations and federal hatcheries received channel catfish that were propagated from a stock collected from the Red

River in Oklahoma in 1949 (Dunham and Smitherman, 1983). Of that stock, a portion were distributed to the first commercial catfish production farms in Arkansas, Mississippi, and Alabama (Dunham and Smitherman, 1983). However, industry wide, fish came from many different rivers and waterbodies.

There are a large number of desirable traits for commercial aquaculture that the channel catfish already exhibit. Among the major traits include fast growth rate to harvest size, an early age of sexual maturity, ease of spawning, the ability to handle high levels of stress, tolerance of poor water quality, and naturally high resistance to columnaris (*Flavobacterium columnare*) and ich (*Ichthyophthirius multifiliis*). Due to the combination of these traits and the fact that people commonly consumed catfish in the Mississippi River drainage and the South, the channel catfish quickly became the easiest and most widely produced ictalurid for commercial purposes (Dunham et al., 1993; Argue et al., 2003)

A primary focus of genetic enhancement for channel catfish has been to select for increased growth rate to market size (Dunham et al., 1987). Selection for body weight has been extremely successful ranging from 20 to 30% improvement after three generations of selection and 55% improvement after four generations of selection using the Kansas strain of channel catfish (Bondari, 1983; Dunham and Smitherman, 1983; Rezk, 1993; Padi, 1995; Dunham and Brummett, 1999; Rezk et al., 2003; Dunham, 2007). However, there are many desirable traits that exist in other ictalurid species. The white catfish (*Ameiurus catus*) displays fast initial growth. The bullhead catfish (*Ameiurus spp.*) exhibits an increased low dissolved oxygen resistance. Blue catfish possess superior dress out percentage, increased harvestability, better uniformity in growth, and superior resistance to both enteric septicemia of catfish caused by *Edwardsiella*

ictaluri and to channel catfish virus (CCV) (Dunham et al., 1990; Dunham et al., 1993; Argue et al., 2003).

Crossbreeding is a mating system designed to produce improvement through heterosis (Dunham and Elaswad, 2018). Intraspecific crossbreeding of different strains of channel catfish has been shown to improve desirable culture traits such as resistance to enteric septicemia of catfish, dress-out percentage, growth, angling vulnerability, reproduction, lower overall mortality rates, better feed conversion efficiency, and tolerance of low dissolved oxygen (Dunham and Smitherman, 1983; Dunham et al., 1983; Dunham et al., 1986; Dunham et al., 1987; Wolters and Johnson, 1995; Padi, 2003; Padi and Dunham, 2009)

Different species of catfish have distinct culture traits. To attempt to take advantage of all of the specific culture traits interspecific hybridization has been attempted with many different species (Dunham and Elaswad, 2018). The channel female × blue male hybrid is the only interspecific ictalurid hybrid resulting in culture traits superior to the channel catfish (Dunham et al., 1987; Dunham and Brummett, 1999; Argue et al., 2003). The initial research on interspecific hybridization was first attempted in 1966 and used seven ictalurid species, producing 42 different interspecific ictalurid hybrids (Dunham and Masser, 2012). There have been attempts at interspecific hybridization with crosses between channel catfish (*I. punctatus*), blue catfish (*I. furcatus*), white catfish (*A. catus*), black bullhead catfish (*A. melas*), brown bullhead catfish (*A. nebulosus*), yellow bullhead catfish (*A. natalis*), and flathead catfish (*Pylodictis olivaris*) (Dunham et al., 1987; Goudie et al., 1993). All interspecific hybrids produced offspring with observable characteristics of each parents, however they were not necessarily traits that are useful for the industry (Goudie et al., 1993). Channel catfish female and white catfish male hybridization

resulted in overall faster initial growth in aquaria, but slower growth to market size in ponds (Dunham et al., 1987). Blue catfish female and channel catfish male hybridization resulted in a heavy paternal predominance towards the traits of the channel catfish but exhibited few desirable traits of the blue catfish (Dunham et al., 1982).

The reciprocal, channel catfish female and blue catfish male hybrid showed many clear advantages in comparison to the channel catfish. While the first channel × blue hybrid catfish was produced in 1966 (Giudice, 1966), the hybrid did not take hold in the U.S. catfish industry until the early 2000's (Dunham and Masser, 2012; Mischke et al., 2017). Technology was developed from Auburn University that allows for artificial spawning and fertilization based on carp pituitary extract (CPE) injections for induced spawning (Dunham, 1993; Dunham et al., 1998; Lambert et al., 1999; Dunham, 2011; Dunham et al., 2016; Kim et al., 2016).

On a small scale, this technology was adopted by GoldKist (later purchased by Harvest Select) in Inverness, Mississippi starting in 1997. Annual production of hybrid embryos ranged from 1 to 5 million from 1997 to 2004. Auburn University then improved the hybrid embryo production technology with the key being the use of luteinizing hormone-releasing hormone analogue (LHRHa) as the hormone to induce ovulation (Phelps et al., 2011; Perera, 2012; Su et al., 2013). The introduction of LHRHa allowed the doubling and tripling of embryo production efficiency, and this technology was commercialized by Eagle Aquaculture, Auburn, Alabama in 2005. Embryo production rose to 15.2 million in 2005 and was 275 million in 2018 (Fig. 1). Despite this success, there are still obstacles and inefficiencies found in hybrid embryo production. Artificial fertilization for the commercial production of hybrid catfish is labor intensive, and 4 to

8-year-old blue catfish males must be sacrificed for sperm collection to artificially fertilize channel catfish eggs.

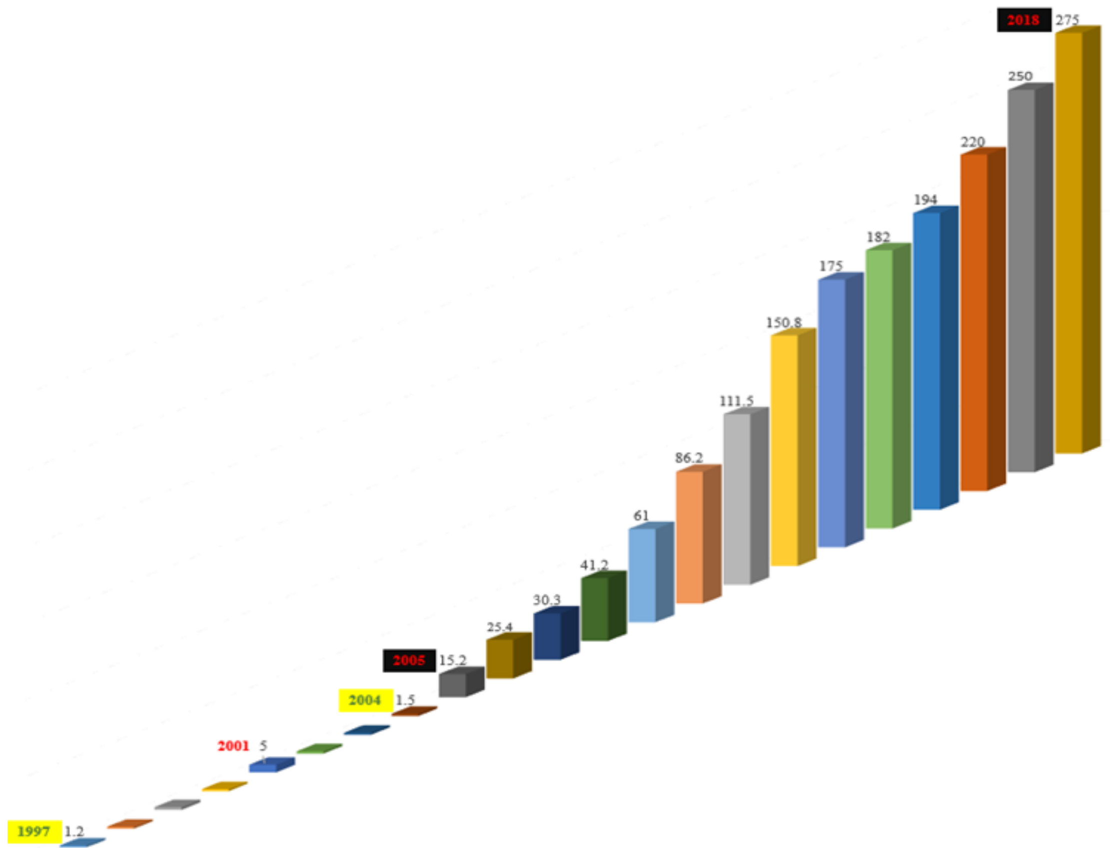

Figure 1: Channel catfish, *Ictalurus punctatus*, female × blue catfish, *I. furcatus*, male hybrid embryos were commercially produced from 1997-2018 in the United States. In 1997, there were 1.2 million hybrid embryos produced but the production of hybrid embryos remarkably increased 230 times more than the production in 1997, reaching 275 million embryos in 2018.

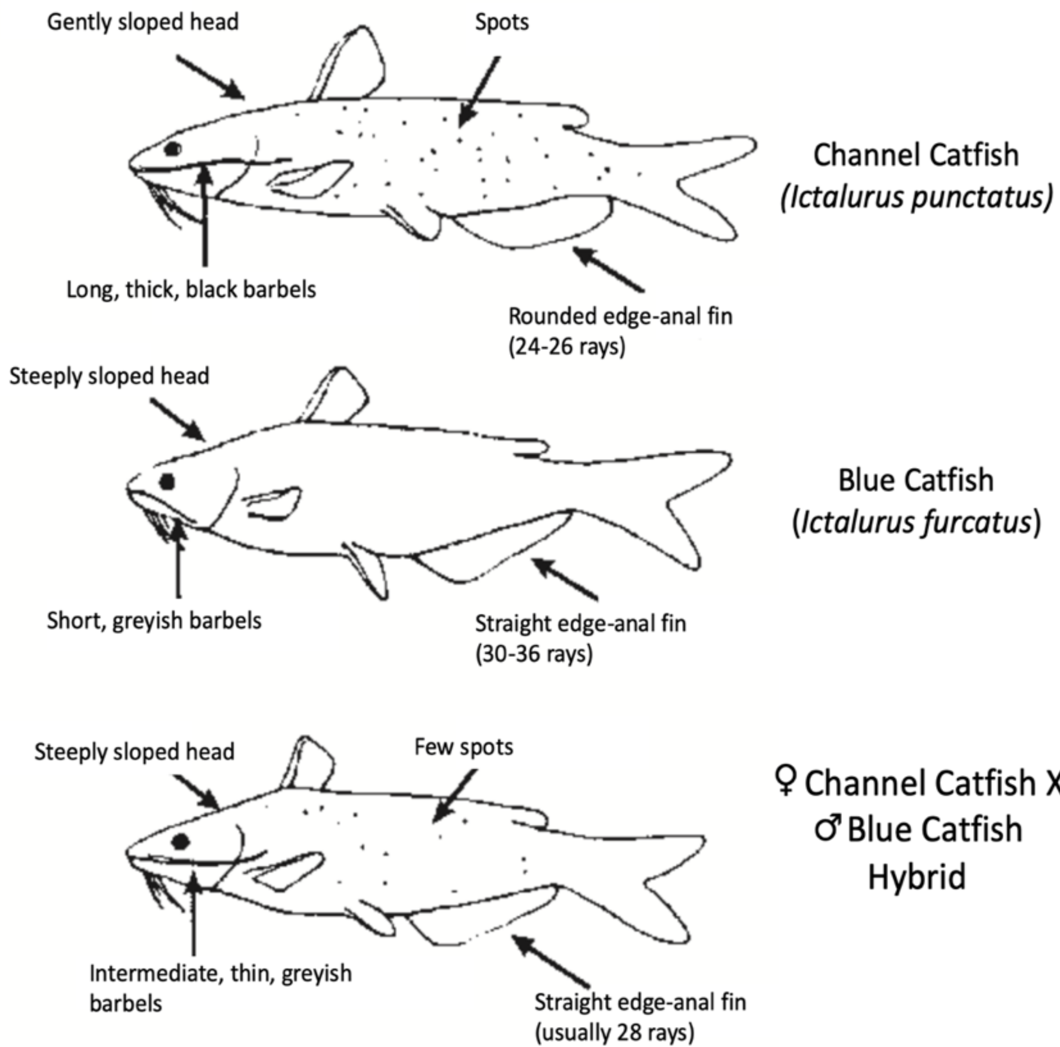

Figure 2: Morphology and external characteristics of the channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatu*s, and hybrid catfish.

The average production cost of hybrid catfish fingerlings is 15 to 22.5% lower than the cost of channel catfish production, even when the fry are bought at a price twice as high for channel catfish fry (Ligeon et al. 2004). Hybrid catfish exhibit superior growth compared to channel catfish and have been found to have a 41% greater body weight than channel catfish (Guidice, 1966) (Fig. 2). When raised in communal pond conditions the hybrids have a more favorable growth rate than channel catfish, growing 35% more rapidly (Dunham and Brummett, 1999). Hybrid catfish have also been observed to grow more rapidly, upwards to twice as fast, than channel catfish when they are stocked at higher densities in ponds (Dunham et al., 1990). Notably, the performance of hybrid catfish in intensive pond-based production systems that have been evaluated or adopted by the US catfish industry such as split ponds, intensively aerated ponds, and in-pond raceway systems is much better than the channel catfish. Hence, most catfish farmers utilizing intensive pond-based production systems in the Southeast choose to grow hybrid catfish (Brown et al., 2011; Brown et al., 2014; Bott et al., 2015; Kumar et al., 2016; Kumar and Engle, 2017; Kumar et al., 2018).

The survival rates of hybrid catfish are also greater than channel catfish with survival rates of hybrid and channel catfish being 93.8% and 85.4%, respectively (Li et al., 2004). Hybrid fry survival was also found to be higher than the survival of channel catfish when they were cultured in separate ponds, 100% and 29.5% respectively, and columnaris was prevalent (Dunham et al., 1990).

Hybrid catfish also have an increased tolerance to diseases as compared to channel catfish. Hybrid catfish and channel catfish had survival rates of 73.8% and 62.0%, respectively when immersed in a bath inoculated with *Edwardsiella ictaluri* (Wolters et al., 1996). Increased

tolerance to low dissolved oxygen has also been exhibited in oxygen-deprived ponds with survival rates of 92.5% for hybrid catfish, and 49.5% for channel catfish (Dunham et al., 1983). In cages, survival rates were 49.0% for hybrid catfish and 12.5% for channel catfish under conditions of critically low dissolved oxygen (Dunham et al., 1983). Additionally, in tanks with dissolved oxygen lowered to 0.6 ppm, the survival rates were 67% for hybrid catfish and 0% for channel catfish (Dunham et al., 1983).

Hybrid catfish consistently have a higher dress-out percentage. Argue et al. (2003) found that hybrid catfish had a dress-out percentage of 61.1% and channel catfish had a dress-out percentage of 57.5%. Hybrids are also known to be easier to seine in contrast to channel catfish. To seine 75.0% of a hybrid catfish population it takes only one seine haul and to capture the same number of channel catfish it takes at least two seine hauls (Yant et al., 1975). Hybrid catfish were also found to be less difficult to seine by Chappell (1979) than channel catfish as 64.65% of the hybrid population was captured while the percentages of channel catfish captured per seine haul during the summer ranged from 18.5% to 24.3%. Dunham et al. (1986) found that hybrids are more easily caught by hook and line when compared to a multitude of genetic lines of channel catfish, leading to a strong benefit for fee fishing enterprises.

Production of hybrid catfish has been accomplished by open-pond spawning, pen spawning, and induced spawning with artificial fertilization (Tave and Smitherman, 1982; Dunham and Smitherman, 1987; Dunham et al., 2000; Dunham and Masser, 2012). Due to general behavioral incompatibility reported by Dunham and Smitherman (1987) open-pond spawning is inconsistent for producing hybrid catfish. Tave and Smitherman (1982) attempted pen spawning between channel catfish females and blue catfish males with a success rate of 40%.

Success rates in later studies have ranged from 0 to 20%, and averaged 15% (Tieman, 1995; Dunham et al. 2000).

Currently, injecting hormones into channel catfish females to stimulate ovulation and hand-stripping of eggs followed by fertilization with blue catfish sperm is considered to be the most efficient method of producing hybrid catfish. Channel catfish females injected with LHRHa at 20 μg/kg body weight followed by a second injection of 100 μg/kg body weight showed high ovulation (52.9 - 95.5%) and fertilization rates (59.1 - 70.0%) when artificially fertilized with blue catfish males (Phelps et al., 2007). Dunham et al. (2016) also reported high hatchery success, where 90.3% of channel catfish females yielded eggs when hand-stripped and 71.5% of their eggs were able to be artificially fertilized with blue catfish sperm to produce hybrid catfish. While the artificial fertilization process is effective it is highly labor intensive and time-consuming (Dunham and Masser, 2012).

Channel catfish are a native species to the U.S. and typically reach sexual maturity two to three years earlier than blue catfish (Tucker and Robinson, 1990). Spawning channel catfish can take place from February to August when water temperature ranges from 21-30°C in the natural environment (Lenz, 1947; Wolters, 1993). For natural spawning, containers are placed in ponds to serve as artificial spawning nests and they are checked every few days for the presence of a fertilized egg mass (Busch, 1983; Steeby, 1987; Tucker and Robinson, 1990). Using the open pond method, channel catfish brooders had a spawning rate between 30 to 50% (Brauhn, 1971; Bondari, 1983; Wolters, 1993).

Using the pen or aquarium spawning method is an alternative but requires more control and effort to obtain fertilized egg masses. The pen method requires selection of individual fish to be placed in pairs in sectioned pens next to a pond bank. The females are allowed to spawn naturally or can be induced with hormones (Graham, 1999). To spawn channel catfish in aquaria, careful selection of individuals and the use of hormones is required (Graham, 1999). Channel catfish had a successful rate (22-58%) for spawning as pairs or groups in rectangular 120 L fiberglass tanks (Bates and Tiersch, 1998). Dunham et al. (Unpublished) routinely obtained 75-90% spawning in aquaria. Unlike channel catfish females, channel catfish males can spawn multiple times a year under hatchery conditions (Legendre et al., 1996). Channel catfish eggs hatch 5-10 days after spawning, depending on water temperature (Wolters, 1993).

1.2. Xenogenesis

Xenogenesis is a method of reproduction in which successive generations differ from each other (Dunham, 2011). This technology could result in an improvement in hybrid catfish embryo production. A key part of that technology is the use of polyploidy. When an individual possesses extra sets of chromosomes it is referred to as polyploidy. Polyploidy has been studied widely in shellfish and fish (Piferrer et al., 2009). Triploidy refers to the 3N state. To induce triploidy, normal fertilization takes place before force retention of the second polar body (Chourraut, 1984; Lou and Purdom, 1984). Force retention is conducted by applying temperature, hydrostatic pressure, or chemical shocks after fertilization (Wolters et al., 1981; Chourrout, 1984; Cassani and Caton, 1986; Johnstone et al., 1989). Temperature shocks, both hot and cold, have large variability in different species when attempting to produce triploids. Reddy et al. (1990) reported the use of

heat shocks at 42 °C in 1 – 2 minutes induced 12% triploidy in rohu (*Labeo rohita*) (Vo, 2019). Triploid rates in Coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha*) shocked at 28 – 30 °C for 10 minutes averaged 58 – 84% (Utter et al., 1983).

Hydrostatic pressure stock to induce triploidy is straightforward and more consistent than other methods such as thermal and chemical shock (Bury, 1989; Dogankaya and Bekcan, 2014). Results from Vo et al. (2019) showed that 82.9 – 100% of channel catfish eggs pressurized at 7,000 psi were triploid. These results show the same general trend found by Lilyestrom (1989) for channel catfish eggs that were put under hydrostatic pressure for five minutes after fertilization with 67 – 100% being triploid, depending on the level of pressure utilized. 7,000 psi is a suitable level for producing triploid catfish with hydrostatic pressure, at this same level 100% of rainbow trout and 98% of grass carp pressurized were triploid (Chourrout, 1984; Aldridge et al., 1990). The hydrostatic pressure method has been utilized to induce triploidy in rainbow trout, *Oncorhynchus mykiss* (Lou and Purdom, 1984), grass carp, *Ctenopharyngodon Idella* (Cassani and Caton, 1986; McCarter, 1988), common carp, *Cyprinus carpio* (Linhart, 1991), bighead carp, *Hypopthalmichthys nobilis* (Aldridge et al., 1990), loach, *Misgurnus fossilis* (Pickett, 2019), zebrafish, *Danio rerio* (Gestl et al., 1997), channel catfish, *I. punctatus* (Rottman et al., 1991), and yellowtail flounder, *Pleuronectes ferrugineus* (Manning and Burton, 2003).

A main motivating factor for producing triploid fish is that they are sterile (Wolters et al., 1981; Chourrout et al., 1984) due to a lack of gonadal development (Casani and Caton, 1986), and production of unbalanced gametes. Since triploid channel catfish are sterile they are incapable of producing their own gametes, which is why they were used as hosts for blue catfish stem cells (Yoshizaki and Lee, 2018). Multiple studies have utilized triploid fish as hosts for germ

cell transplantations (Okutsu et al., 2007; Yoshizaki et al., 2012; Perera et al., 2017; Vo, 2019). Triploidy is extremely useful in controlling the natural spawning in tilapia and preventing the establishment of exotic species. Multiple triploid fishes have shown sterility such as loach (Suzuki et al., 1985), common carp (Cherfas et al., 1994), and Atlantic salmon, *Salmo salar* (Refstie, 1984). Triploid fish can also be beneficial when they show increased growth rate (Chourrout et al., 1986), carcass yield, survival, and flesh quality (Bye and Lincoln, 1986; Hussain et al., 1995; Dunham, 1996).

To accomplish xenogenesis, transplantation of diploid germline stem cells, primordial germ cells (PGCs), type A spermatogonia stem cells (SSCs) or oogonial stem cells (OSCs), derived from donor diploid fish into sterile hosts allows the host to produce the donor-derived gametes (Vo, 2019). PGCs, SSCs, and OSCs are all precursors of gametes that possess the ability to establish or recover gametogenesis (Wylie, 1999; Yoshizaki et al., 2010; Wong et al., 2011). There have been numerous studies on the transplantation of PGCs reported for zebrafish (Lin et al., 1992; Ciruna et al. 2002), medaka, *Oryzias latipes* (Wakamatsu., 1993; Shimada et al., 2008), rainbow trout (Takeuchi et al., 2001; Saito et al., 2010), and the loach (Nakagawa et al., 2002). Studies have been conducted on transplantation of SSCs in species such as masu salmon, *Oncorhynchus masou* (Okutsu et al., 2006, 2007), Nile tilapia, *Oreochromis niloticus* (Lacerda et al., 2006), and channel catfish (Perera et al., 2017; Vo, 2019). OSCs have been successfully transplanted into species such as rainbow trout (Yoshizaki et al., 2010), zebrafish (Wong et al., 2011), and Siberian sturgeon, *Acipenser baerii* (Psenicka et al., 2015).

Xenogenic fish have been produced for multiple species as discussed above, including channel catfish (Perera et al., 2017; Vo, 2019). The main goal for most of these studies was to

demonstrate success in stem cell transplantation. Auburn University worked on the production of xenogenic hybrid catfish progeny. They were specifically focused on using the technology as an improvement for hybridization and were successful in a way that allows for the production of 100% channel catfish female × blue catfish male hybrids, albeit with a low rate of success in regard to producing large numbers of xenogenic brood stock with good fertility (Shang, 2013; Perera et al., 2017; Vo, 2019). For xenogenesis to have practical application in ictalurids, the xenogenesis technology needs to be improved to enable production of large numbers of xenogenic progeny. One of the possible solutions includes determining the ideal stem cell implantation time to then increase the efficiency of xenogenic brood stock rearing.

The long-term goal of this thesis was to improve efficiency of generating xenogenic catfish that display high fertility. The primary objective was to determine the time after hatch that maximizes the colonization of blue catfish stem cells in triploid channel catfish fry. The supporting objective was to determine the effect of injected PKH26 dyed blue catfish on the growth and survival of triploid channel catfish fry.

2. Materials and Methods

2.1. Experimental Fish and Triploid Induction

Sexually mature channel catfish females, channel catfish males and blue catfish males were collected for spawning and stem cell extraction from 0.25 ha aquaculture ponds that were approximately 1.5 meters deep and located at the Fish Genetics Research Unit at the E.W. Shell Fisheries Center in Auburn, Alabama (32.6622° N, 85.4960° W). Fish were fed once daily with commercial floating catfish feed (crude protein: ≥32.00%, crude fat: ≥4.00%, crude fiber: 7.00% and phosphorus: 0.80%; Cargill Animal Nutrition, Franlinton, LA) to satiation. Dissolved oxygen (DO) was monitored twice daily (06:30 and 16:30) and temperature was recorded twice per week using an YSI 55 multi-parameter instrument (605056, YSI Incorporate, Yellow Springs, OH). Total ammonia-N (TAN), nitrite, nitrate, pH, hardness, and alkalinity were also measured three times per week using a water quality test kit (19541-931/19543-91, Easy Strips Tetra, Tetra Holding Blacksburg, VA).

Gravid channel catfish females were intraperitoneally injected with LHRHa at 20 μg/kg body weight followed 12 h later with 100 μg/kg body weight. Fish were placed in spawning bags and submerged in flow-through spawning troughs. Channel catfish males with pronounced secondary sexual characteristics were sacrificed for sperm collection. The channel catfish males that were utilized in this study were euthanized by blunt force trauma to the head and pithing, and their testes were surgically removed with a sterile scalpel and forceps. The testes were then rinsed with the 0.9% saline solution to remove blood (Dunham and Masser, 2012). After removal

of excess blood, the testes were macerated and the sperm strained into 50 mL vials, followed by the addition of 0.9% saline solution to the sperm at a rate of 10 mL per gram of testes.

When the females began to ovulate, several eggs could be seen attached to the spawning bags. The channel catfish females were then anesthetized in 100 mg/L buffered tricaine methanesulfonate (MS-222, Ferndale, WA) until their opercula ceased moving. The females were then hand stripped of their eggs into spawning pans (25 g of eggs/pan) coated in Crisco All-Vegetable Shortening vegetable oil to prevent sticking. Sperm solution was added to the channel catfish eggs at a rate of 2 mL per 25 g of eggs then Fullers' earth solution was added to the sperm-egg solution to activate the fertilization process. Fullers' earth solution was prepared by adding 6 g of Fullers' earth powder (MP Biomedicals, Santa Ana, CA) into 1 L of pond water maintained at 27 °C. At 3 min post-fertilization, the fertilized eggs were transferred into a cylindrical pressure chamber (340 mm height, 70 mm diameter) and placed onto a Carver press (Carver, Inc., Wabash, IN). At 5 min post-fertilization, the eggs were pressure shocked for 5 min. A total of 7,000 psi was applied to induce triploidy. After completion of the pressure shock, the eggs were removed from the chamber and placed in a trough with supplemented calcium chloride at a concentration of 50 ppm for one hour for water hardening before incubating them in a flow-through hatching trough aerated with a paddle wheel. Temperature was recorded twice per day using an YSI 55 multi-parameter instrument (605056, YSI Incorporate, Yellow Springs, OH). The triploid eggs hatched between 133.5 – 145.1 accumulated degree days. Visual inspection of the genital ridge was conducted to confirm triploidy (Fig. 4).

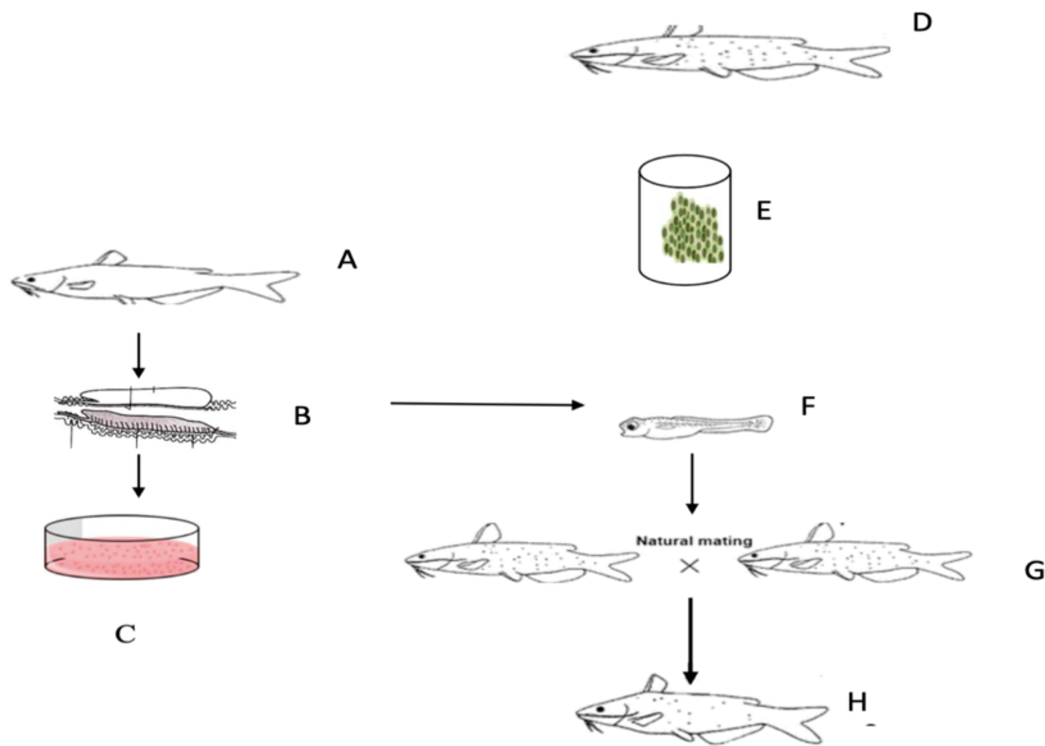

Figure 3: The general procedure for producing channel catfish, *Ictalurus punctatus*, female × blue catfish, *I. furcatus*, male hybrids by mating a xenogenic channel catfish male with a normal channel catfish female. A: Two year old blue catfish were seined and used for isolation of stem cells; B: The gonads were removed from the blue catfish; C: The stem cells were then isolated and concentrated with trypsin ethylenediamine tetraacetic acid (EDTA) 0.25%; D: Diploid channel catfish females were used to produce diploid eggs; E: Induction of triploidy for channel catfish embryos with hydrostatic pressure; F: Triploid channel catfish fry (aged 26.6 to 175.7 accumulated degree days post- hatch) were used as recipients for donor-derived stem cells from blue catfish; G: Mating between normal channel catfish female with transplanted triploid

(xenogenic) channel catfish male; H: Hybrid catfish produced by mating xenogenic channel catfish male with a normal channel catfish female.

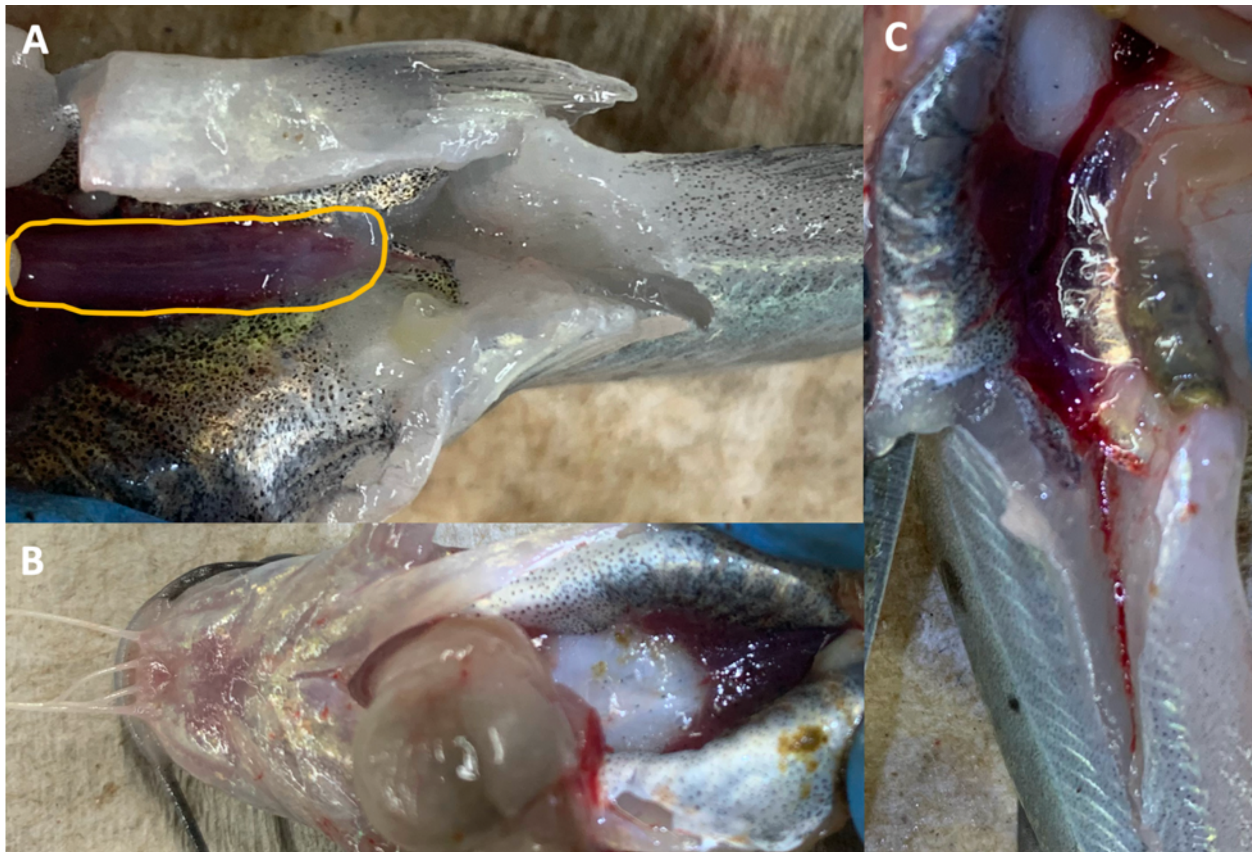

Figure 4: Visual inspection of the genital ridge of both diploid and triploid channel catfish: A: Diploid channel catfish, *Ictalurus punctatus*, female with ovary growth; B & C: Triploid channel catfish showing a lack of gonadal growth confirming triploidy.

2.2. Isolation of donor stem cells from blue catfish

Sexually immature male and female blue catfish (150 – 450 g; 175 – 450 mm) were selected and euthanized via pithing. They were then washed with 0.9% saline and placed on crushed ice before dissection. The blue catfish were then sprayed with a 70% ethyl alcohol solution (Pharmco-Aaper, Shelbyville, KY) to sterilize their exterior surface and were weighed and measured before the abdomen was opened for gonadal extraction. Gonads were removed, weighed, and washed with a 0.5% bleach solution for 1 to 2 min before being placed in a petri dish (100 mm × 15 mm) containing 5 mL of the anti-agent medium Hank's Balanced Salt Solution (HBSS, SH30031.03, GE Healthcare Life Sciences). The gonads were then placed under a biosafety cabinet (The Baker Company, Sanford, ME) to prevent contamination from outside sources, where connective tissues and blood vessels were removed and discarded. The gonads were washed three times with phosphate-buffered saline PBS 10X (PBS; J62692, Alfa Aesar, Haverhill, MA.) and then washed three times with anti-agent medium (HBSS) before being diced with sterile scalpel blades.

The tissue was then transferred to 50 mL flasks containing a magnetic bar that had been autoclaved. In each flask the gonads were added to a 0.25% trypsin - ethylenediamine tetraacetic acid (EDTA; 25200-072, Life Technologies, Carlsbad, CA.) solution that was calculated to 50X gonadal weight. The flasks were then incubated on ice for 30 min and then on a stir plate (Corning, Manassas, VA) for 60 min. The suspension was then filtered using a 70 μm filter and the remaining solution was filtered using a 40 μm filter. The cell solution was then centrifuged at 500 g for 10 min (Sorvall RT6000, ThermoFisher, Waltham, MA.). Thereafter, the supernatant was

discarded, and the pellets were resuspended with 2 mL of Dulbecco's Modified Eagle's Medium/DMEM (10-090-CV, Corning Cellgro, Sigma-Aldrich, St. Louis, MO.) supplemented with 10% fetal bovine serum (FBS; 10438018, Life Technologies), 100 unit/mL Penicillin - Streptomycin (15140-122, Life Technologies), and 200 mM L-glutamine (A2916801, Life Technologies) to provide a favorable environment for the cells. The cell suspension (5 µL) was gently mixed with 45 µL of 0.4% trypan blue (15250061, Life Technologies). Cells (10 µL) were then observed under an Olympus objective microscope (BH2), supplemented with a 20× objective, to determine the total number of cells with the aid of a hemocytometer. The number of cells were counted in four corner quadrants (each 1 mm^2 area) of the hemocytometer. The number of cells in 1 mL was calculated according to Louis and Siegel (2011) with the dilution factor of 2 (cell suspension: trypan blue with 1:1 ratio). Live and dead cells were enumerated for analysis, where the total number of cells per mL = (total number of counted cells × dilution factor × 10^4) / number of squares.

2.3. PKH fluorescent dye addition to stem cells

The isolated cells were centrifuged at 400 g for 5 min to produce a pellet. The supernatant was removed and 1 mL of Diluent C from the PKH26 red fluorescence cell linker kit (Catalog Number CGLDIL, Sigma-Aldrich, St. Louis, MO) was added to resuspend the cells. This was done by gently pipetting to create a homogenous solution. PKH26 was then added to produce a concentration of 0.3% total. The solution was then mixed every 30 s by inversion for 5 min to ensure complete dispersion. Staining was nearly instantaneous, so rapid and homogenous dispersion of cells in the dye solution was essential for bright, uniform, and reproducible labeling.

Because the staining was so rapid, a longer incubation period was not necessary. To end the staining process, 2 mL of 1% BSA was added to the solution and incubated for 1 min to allow binding of the excess dye. The cells were then centrifuged at 400 × g for 10 min at 20-25°C. The supernatant was then removed, the cells were resuspended in 1.5 mL of HBSS and were centrifuged to wash the cells at 400 × g for 5 min and then repeated. Once the second washing was been completed, the supernatant was removed. Thereafter, the cells were resuspended in 1 mL of DMEM and counted using a hemocytometer under an Olympus objective microscope (BH2), supplemented with a 20× objective, to determine the total number of cells.

2.4. Stem cell implantation into the triploid channel catfish host

Triploid channel catfish fry were reared in flow through hatching troughs aerated with a paddlewheel. Temperatures were measured twice daily with the following daily means: 0 (27.5°C), 1 (27.9°C), 2 (28.2°C), 3 (28.3°C), 4 (28.4°C), and 5 DPH (28.4°C). Starting on the first day of hatch until five days post-hatch (DPH) triploid channel catfish fry (7.0 ± 0.4 mm) were anesthetized by placing them in a 10 mg/L MS-222 buffered with 10 mg/L sodium bicarbonate solution. Each day of injection had two groups being injected, one was the control group that was only injected with stem cells and the other group injected with PKH26 dye stained stem cells. The anesthetized fry were then moved to a petri dish and observed microscopically at 1.5X (Amscope, Irvine, CA). Each fry was injected with 1 μL of an unsorted cell suspension containing about 80,000 cells (28,000 stem cells) through a 33-guage needle (outer diameter: 0.20955 mm; inner diameter: 0.108 mm) with a gastight syringe attached to a repeater (Hamilton, Reno, NV). The

fry were laid on their side and the needle was inserted into the cavity between the anal fin and yolk sac where the genital ridge is expected to be formed (Fig. 4). After the injections, the fry were placed in a recovery container that was aerated and then stocked into marked hapas in 360L flow through troughs. Each hapa was stocked at a density of 30 individuals per hapa and were moved into individual 60L aquaria for grow out.

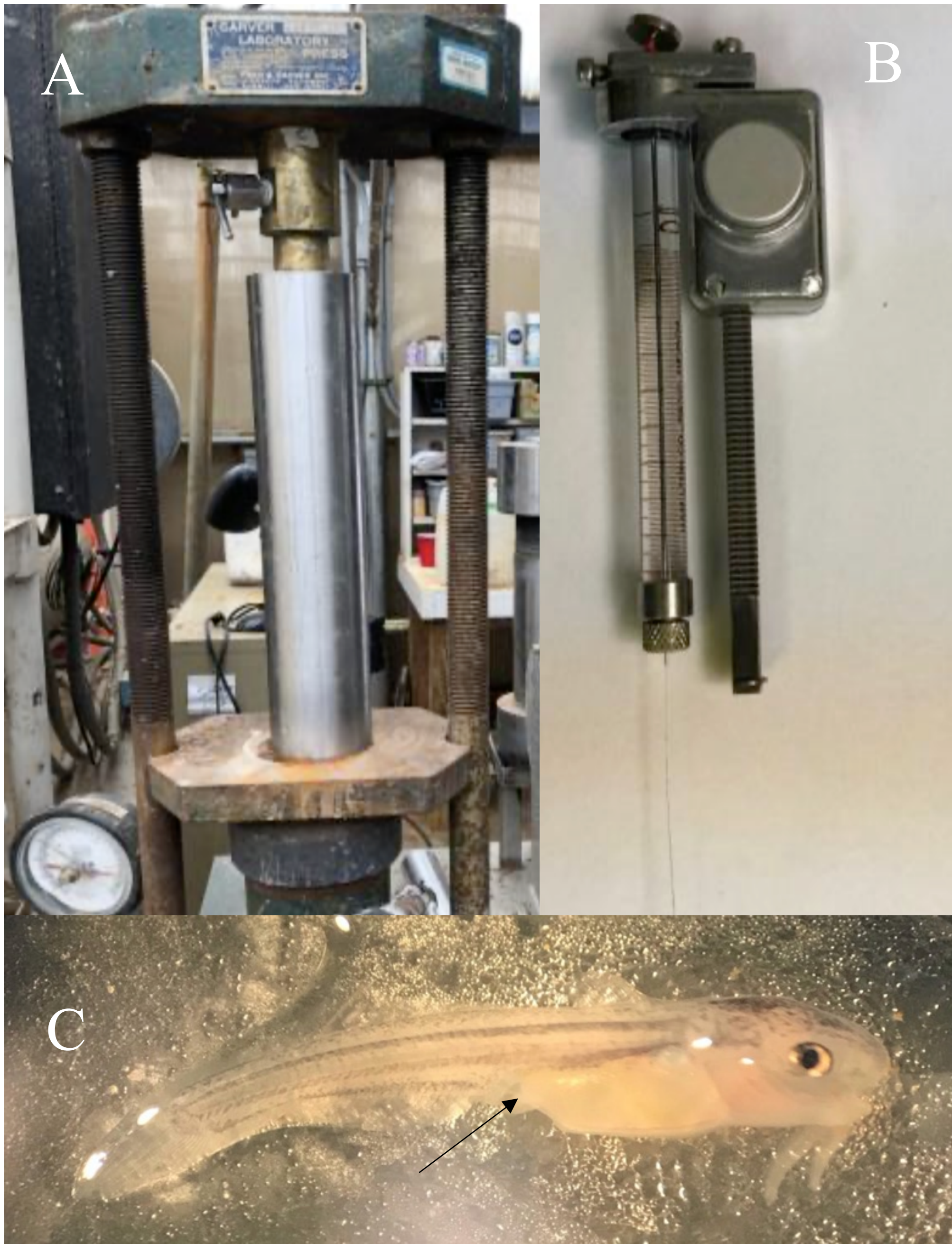

Figure 5: Carver Press with hydrostatic pressure chamber, manual injector, and hatched triploid channel catfish, *Ictalurus punctatus*, fry used for transplantation of blue catfish, *Ictalurus furcatus*, stem cells. A: Carver Press with hydrostatic pressure chamber to induce triploid

embryos. Five min post-fertilization, eggs were loaded into the steel chamber and then pressurized at 7,000 psi for 5 min. B: A gastight syringe attached to a repeater was used to transplant stem cells into the peritoneal cavity of the triploid channel catfish newly hatched fry. C: Channel Catfish fry 36-48 h post-hatch pressurized at 7,000 psi using hydrostatic pressure shock and transplanted with germline stem cells with a gastight syringe attached to a repeater. A pair of genital ridges are located on the wall of the peritoneal cavity. The arrow shows the site where the needle should be inserted into the cavity.

2.5. PKH observation at 90 days

PKH26 dye has a half-life of over 100 days allowing the injected catfish to grow for ~90 days prior to sampling and providing the best opportunity to have gonadal growth prior to sampling. Five fingerlings were collected after at least 90 days from each day post hatch, 0 day – 5 day. Each fingerling was weighed and measured. The fingerlings were sacrificed by pithing, the gonads were surgically removed and applied to a sterile microscope slide. The gonads were then observed using a Zeiss fluorescent microscope (Axio Imager.A2 – Carl Zeiss Microscopy GmbH, Jena, Germany) Once focused on the tissue a grid overlay was added (582,760.376 μm^2) and five random sections of the tissue were photographed. All positive tissue samples had a red glowing mark on the cells. All negative tissue samples had no red fluorescence on the cells. Channel catfish fingerlings from 3-5 DPH that were injected with the stem cell solution without PKH26 dye served as the control.

2.6. Fluorescence Area

Fluorescent sections of fresh samples were used for determination of cell numbers, cell areas, cluster numbers, and cluster areas. A total of 30 samples dyed with PKH26 dye were photographed using a fluorescent microscope and were later analyzed using ImageJ software (http://imagej.nih.gov/ij/). Each fish consisted of five random sections of the gonadal tissue from each PKH26 dyed xenogenic channel catfish from 0- to 5-day post-hatch. ImageJ was calibrated to enumerate the area in μm^2 to determine if there were differences between the number of cells, the number of clusters, the cell area, and the cluster area between each day of injections.

Cells are fluorescing areas that are less than 150μm^2, clusters are fluorescing areas that are greater than 150μm^2.

2.7. Statistics

All data were analyzed using SAS statistical software (v. 9.1; SAS Institute Inc., Cary, NC, USA). Data were arcsine square-root or $\log_{10}$ transformed to meet assumptions of normality and homoscedasticity. The impact of PKH (with or without PKH), day of injection (0 to 5 DPH), month post-injection (1 and 3 months), and corresponding interactions on fry survival was analyzed using a repeated measures factorial ANOVA model. The impact of PKH and day of injection was also analyzed for fry weight at 280 DPH using factorial ANOVA. For the above analyses, if a non-significant interaction was detected the interaction term was removed from the model and re-run. Thereafter, main effects were interpreted. Finally, the effect of day of injection on colonization rate and fluorescence area parameters were analyzed using one-way ANOVA models. Treatment means were contrasted using the Tukey-Kramer test. Alpha was set at 0.05 for main effects and interactions. The coefficient of variance was calculated for each of the means.

3. Results

3.1. Survival and Growth

All higher-order interaction terms were non-significant for fry survival ($P \geq 0.109$). Therefore, the PKH, day of injection, and month post-injection main effects were interpreted. Fish injected with or without PKH showed similar survival ($P = 0.576$). However, day of injection significantly impacted survival, such that fry injected on 0, 1, 2, 4, and 5 DPH had decreased survival compared to those injected on day 3 ($P < 0.001$). Survival also decreased after three months of rearing ($P = 0.005$).

The interaction between injection day and PKH was not significant for fish weight ($P = 0.165$), therefore main effects were interpreted. Injecting fry with PKH had no impact on their growth ($P = 0.331$), while injection day impacted the size of the fish at 280 DPH ($P = 0.005$), such that those injected at 3 DPH were smaller than at 1 and 4 DPH.

Table 1: Mean body weight (g) and mean survival at 90- and 280 days post-hatch of triploid xenogenic channel catfish, *Ictalurus punctatus*, injected with non-dyed or PKH26-dyed blue catfish, *I. furcatus*, stem cells pooled from both juvenile males and females on days 0-, 1-, 2-, 3-, 4- or 5 days post-hatch grown in tanks. Values were analyzed by ANOVA followed by Tukey-Kramer tests and were significantly different for mean body weight and survival for individuals injected 3 DPH ($P < 0.001$).

	Mean Body Weight (g) +/- Standard Deviation				Mean Survival (%) +/- Standard Deviation			
Injection Days Post-hatch	N	90 Days	N	280 Days	N	90 Days	N	280 Days
0	20	6.16 (+/- 1.22)	10	9.15 (+/- 1.61)	20	20 (+/- 14.96)	10	17.33 (+/- 9.33)
0 - PKH26	25	7.15 (+/- 1.59)	11	13.86 (+/- 1.99)	25	28.00 (+/- 12.00)	11	19.66 (+/- 3.67)
1	16	3.61 (+/- 0.67)	10	16.91 (+/- 3.32)	16	19.33 (+/- 3.29)	10	12.44 (+/- 3.32)
1 - PKH26	31	4.00 (+/- 0.99)	20	13.20 (+/- 4.58)	31	27.33 (+/- 17.15)	20	26.22 (+/- 15.29)
2	27	3.72 (+/- 0.64)	23	10.67 (+/- 0.58)	27	30.00 (+/- 8.16)	23	28.66 (+/- 9.84)
2 - PKH26	37	4.21 (+/- 0.98)	1	11.52 (+/- 0.00)	37	15.33 (+/- 7.36)	1	4.00 (+/- 0.00)
3	64	7.09 (+/- 0.37)	59	5.76 (+/- 0.51)	64	61.33 (+/- 11.47)	59	58.66 (+/- 14.72)
3 - PKH26	56	5.64 (+/- 1.38)	45	8.13 (+/- 0.75)	56	53.33 (+/- 10.49)	45	46.66 (+/- 15.43)
4	19	7.26 (+/- 0.15)	14	15.31 (+/- 1.06)	19	18.00 (+/- 2.00)	14	18.72 (+/- 2.00)
4 - PKH26	38	8.01 (+/- 1.72)	11	13.18 (+/- 2.95)	38	16.00 (+/- 5.65)	11	11.33 (+/- 3.39)
5	35	7.33 (+/- 1.76)	35	8.81 (+/- 3.16)	35	34.00 (+/- 12.75)	35	34.32 (+/- 12.75)
5 - PKH26	28	7.54 (+/- 3.19)	17	13.76 (+/- 0.76)	28	20.66 (+/- 3.39)	17	16.54 (+/- 3.26)

3.2. PKH26 Dye Fluorescence

PKH26 binds to lipid regions of cell membranes that were injected into each of the newly hatched triploid fry. From the time of injection, 90 days were allowed to elapse before sampling the gonadal region of the fish. The channel catfish ranged from 70 to 126 mm and from 2.31 to 12.37 g. When the gonadal region was removed from each individual, clear formation of either testes or ovaries were observed, confirming that the stem cell injections were done at the proper placement, proliferated, and formed gonadal organs (Fig. 5). This was seen in both PKH26 dyed individuals and non-PKH26 dyed individuals. Each sample that was removed from an injected channel catfish with PKH26 showed clear florescence (Fig. 6: A – F), while samples that were not injected with PKH26 dye did not fluoresce (Fig. 6: G and H).

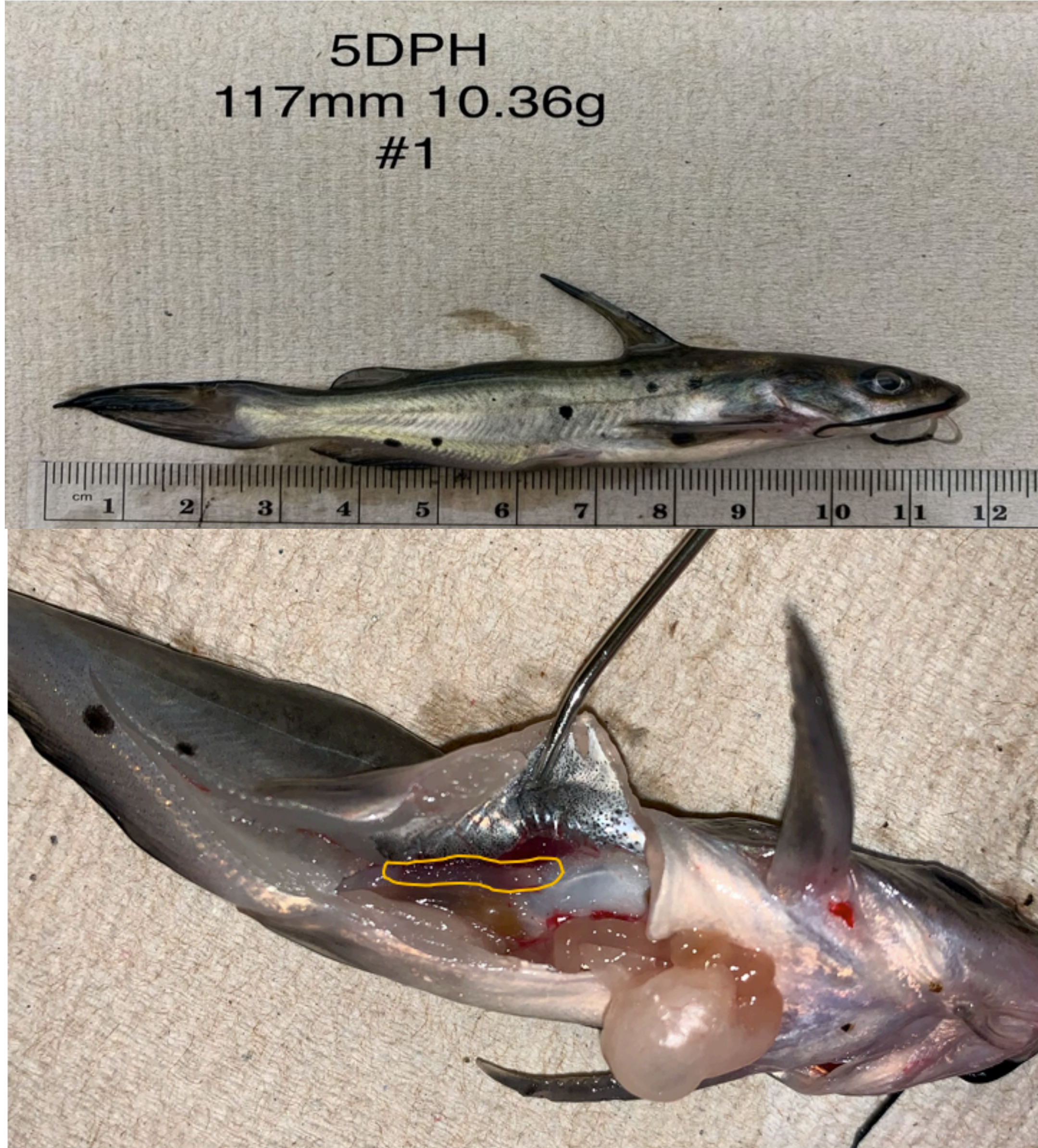

Figure 6: Xenogenic triploid catfish, *Ictalurus punctatus*, 90 days post injection blue catfish, *I. furcatus,* spermatogonia A showing gonadal growth. A: Size and day of injection post hatch before dissection; B: Ovaries of a xenogenic channel catfish being surgically removed.

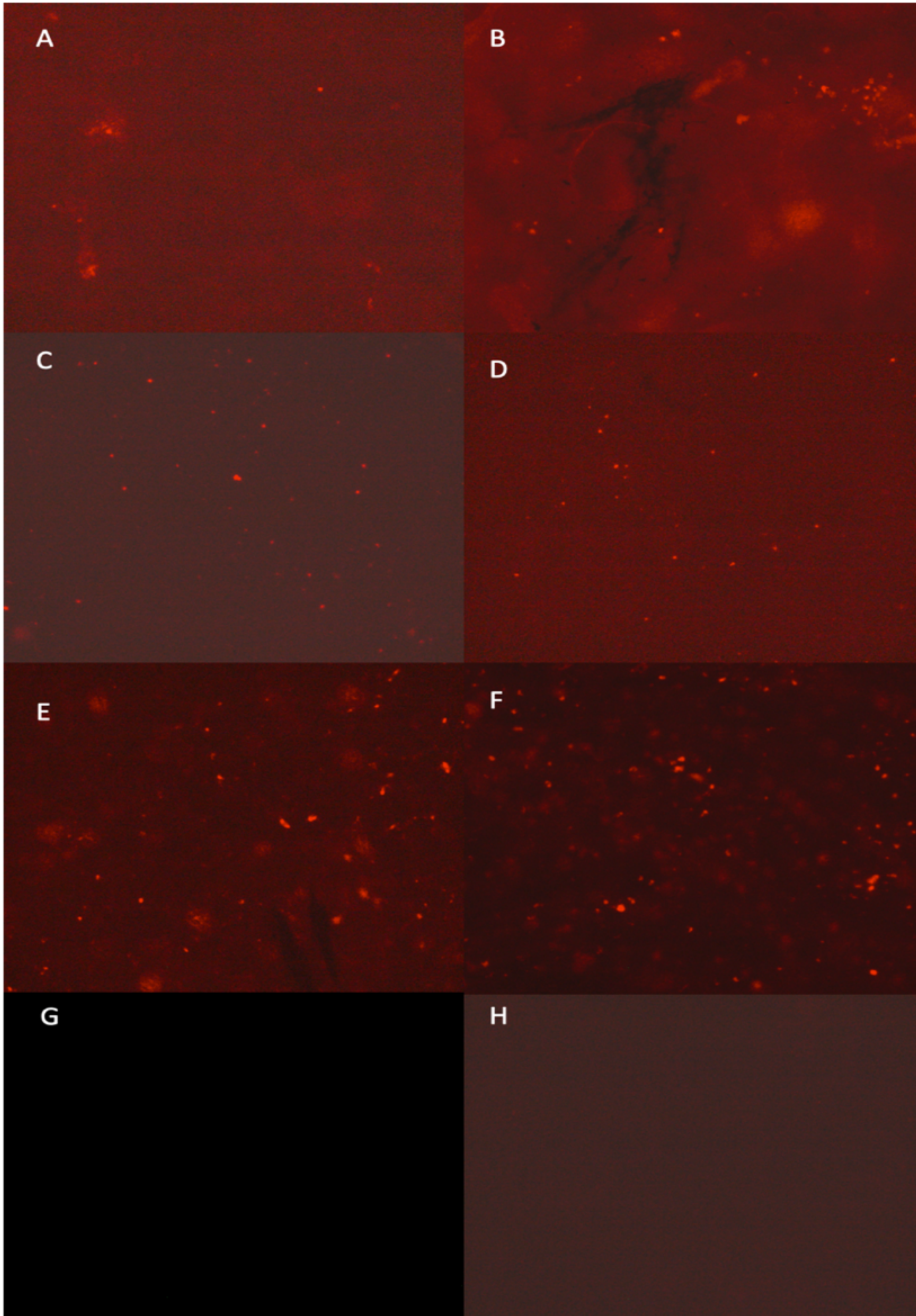

Figure 7: Gonadal tissue expressing fluorescence from PKH26 dyed donor-derived stem cells from blue catfish (*Ictalurus furcatus*) that have proliferated in triploid channel catfish (*I. punctatus*): A: Gonadal tissue from 0-DPH; B: Gonadal tissue from 1-DPH fry; C: Gonadal tissue from 2-DPH fry;

D: Gonadal tissue from 3-DPH fry; E: Gonadal tissue from 4-DPH; F: Gonadal tissue from 5-DPH fry; G: Gonadal tissue from three-day post hatch fry not dyed with PKH26 dye; H: Gonadal tissue from 5-DPH fry not dyed with PKH26 dye.

3.3. Quantifying Fluorescent Labeling

All individuals used in this study were positive for fluorescence from the PKH26 dye that had been injected at least 90 days prior (Table 2). The total area of tissue being viewed was calculated in μm^2, the area for individual cells and clusters were analyzed. Cells were determined to be fluorescing areas less than 150 μm^2 and clusters were determined to be fluorescing areas greater than 150 μm^2 (Fig. 8). Mean square analysis of variance (ANOVA) indicated no effect of interaction between cell means and day of injection, cluster means and day of injection, total area means and day of injection or the coefficient of variation between all of the interactions ($P>0.05$). However, when the data for cell area means, cluster area means, and total area means were transformed using Log10 transformation there was a significant difference found between the cell areas from samples injected on day-0 and 5 ($P = 0.0234$) (Fig. 9). The coefficient of variance was calculated for each of the means, ranging between 0.47 to 0.79.

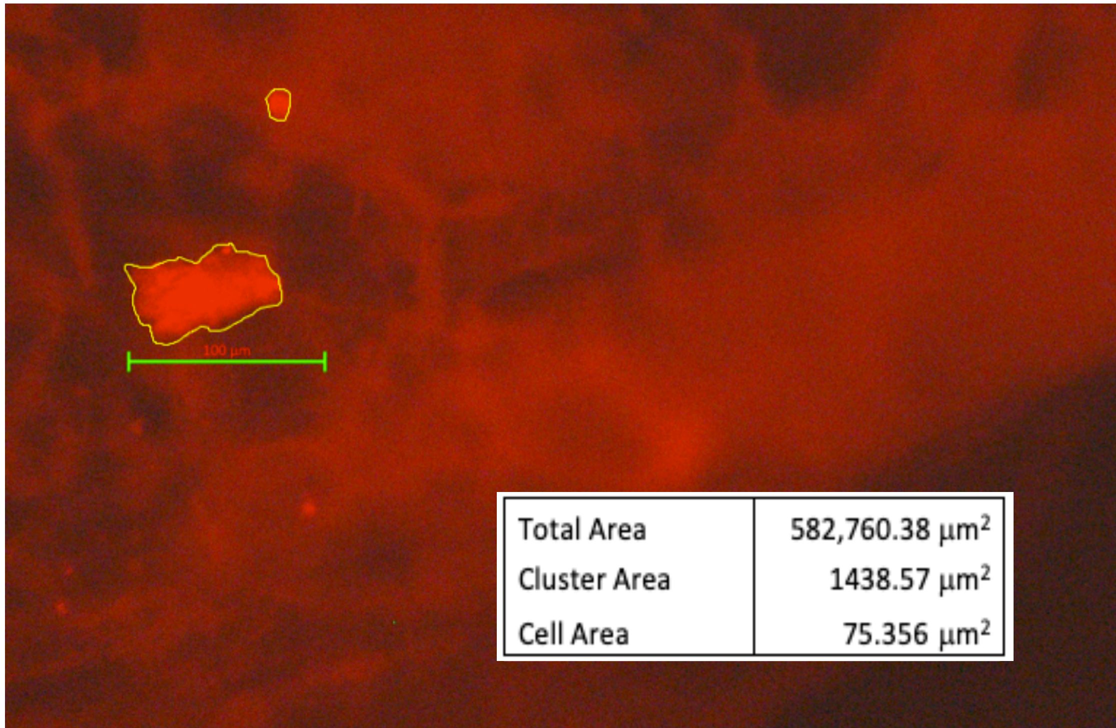

Figure 8: Fluorescence analysis of blue catfish, *Ictalurus furcatus*, gonadal growth from a xenogenic channel catfish, *I. punctatus*, male that was injected with PKH26 ethanolic dyed blue catfish stem cell solution on 3 DPH.

Table 2: Mean weight (g), mean cell area, cluster area, number of cells and clusters in putative triploid xenogenic channel catfish, *Ictalurus punctatus*, injected with PKH26-dyed blue catfish, *I. furcatus,* stem cells pooled from both juvenile males and females. The stem cells were injected into the triploid channel catfish on days 0-, 1-, 2-, 3-, 4-, or 5 days post-hatch and were grown in tanks and five individuals per injection day were sampled after 90 days. The counts are based on fluorescing cells and clusters. Cells are fluorescing areas that are less than 150 μm^2, while clusters are fluorescing areas that are greater than 150 μm^2. clusters are fluorescing areas that are greater than 150μm^2. Values were analyzed using ANOVA followed by Tukey-Kramer tests and were significantly different for cell area on 0- and 5 DPH (P = 0.0234).

Injection Day	N	Mean Cell Area (%) +/- Standard Deviation	Mean Number of Cells +/- Standard Deviation	Mean Cluster Area (%) +/- Standard Deviation	Mean Number of Clusters +/- Standard Deviation
0-Days Post-Hatch	5	0.044 (+/- 0.016)	2.913 (+/- 1.486)	0.157 (+/- 0.101)	4.801 (+/- 2.192)
1-Days Post-Hatch	5	0.137 (+/- 0.068)	6.361 (+/- 3.991)	0.141 (+/- 0.108)	10.683 (+/- 5.544)
2-Days Post-Hatch	5	0.145 (+/- 0.129)	9.911 (+/- 8.273)	0.469 (+/- 0.454)	6.081 (+/- 5.114)
3-Days Post-Hatch	5	0.079 (+/- 0.053)	5.145 (+/- 3.332)	0.553 (+/- 0.561)	3.238 (+/- 1.583)
4-Days Post-Hatch	5	0.127 (+/- 0.088)	8.757 (+/- 5.276)	0.881 (+/- 0.556)	7.000 (+/- 5.932)
5-Days Post-Hatch	5	0.246 (+/- 0.211)	19.825 (+/- 17.416)	0.964 (+/- 0.746)	9.845 (+/- 6.815)

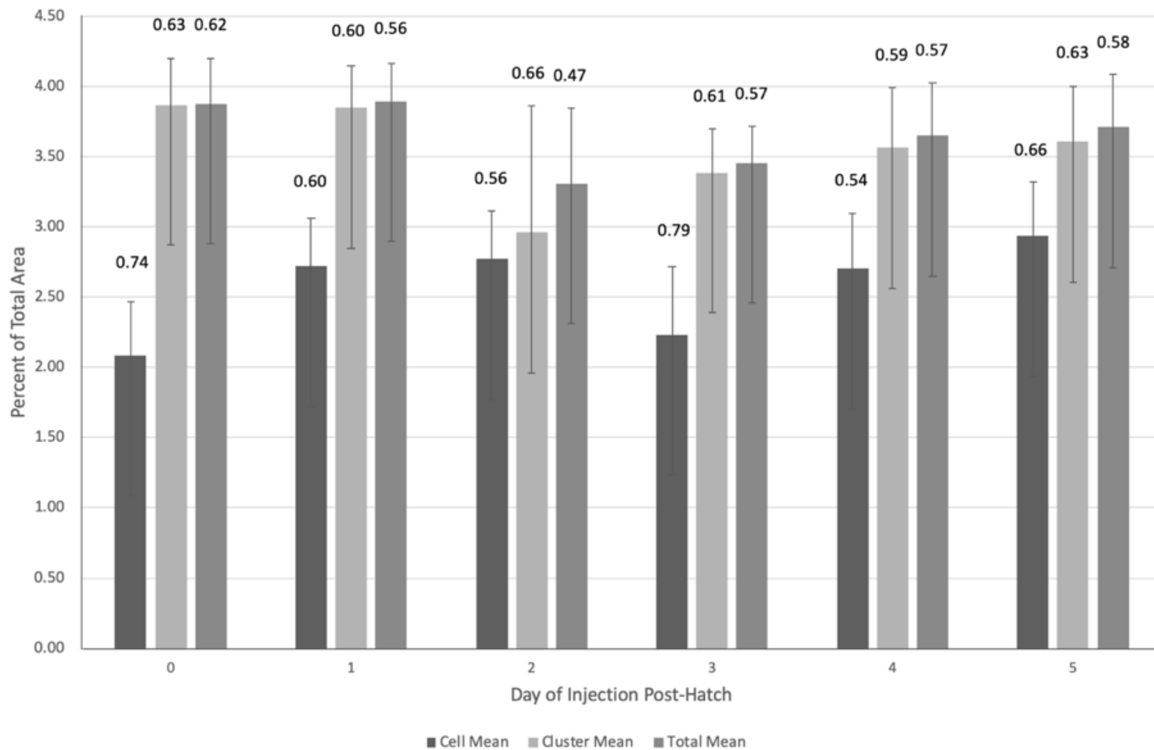

Figure 9: Comparison between day of injection, cell area means, cluster area means, and total means in relation to the total area of tissue sampled (582,760.376 μm^2). Standard deviation bars of the cell, cluster, and total means are represented with the coefficient of variance for each displayed above each bar. Values were analyzed by ANOVA and Tukey-Kramer tests, there was a significant difference between cell area on injection day 0 and 5 (P = 0.0234).

4. Discussion

In an effort to produce a possible replacement for blue catfish (*I. furcatus*) males in the production of channel catfish (*I.* punctatus) female x blue catfish male hybrids, triploid channel catfish were injected at six different days ranging from 0- to 5-DPH. Overall, every day was a success for injection of donor-derived stem cells and for proliferation and production of stem cells in triploid channel catfish as each fish possessed donor stem cells in the gonads 90 days after transplantation. Various observations would suggest that days, 0, 1 and 5 would be the best days for introducing stem cells. Cell area was highest for day 5. In addition, the cell number and combined cell and cluster number was highest for day 5. The observed combined cell and cluster area was highest for days 0, 1, and 5. Variation was quite high among individuals, however, days 2, 3 and 4 consistently had the lowest values and appear the least promising days for colonization and proliferation.

Sterile triploid fish whose germ cells are unable to develop into fertile gametes have been utilized as recipients for stem cell transplantation (Arai, 2001). Fertility of triploid recipients has been found to fully recover by the use of transplantation of diploid donor stem cells (Yoshizaki et al., 2011). Therefore, triploid fish are able to nurse diploid stem cells once they are incorporated into the recipient genital ridge (Vo, 2019). Yoshizaki et al. (2016) stated that stem cell transplantation using triploid or stem cell free recipients resulted in effective colonization when compared to recipients carrying endogenous stem cells.

To determine the effectiveness of transplantation prior to sexual maturation of the xenogenic channel catfish the best available technology is the use of fluorescent labeling of cells.

In the current study, we optimized the day of injection of donor-derived stem cells, 3-DPH, into triploid channel catfish fry in regard to survival. Ninety days after intraperitoneal transplantation of donor-derived blue catfish stem cells, the fluorescently labeled cells were observed inside the recipient gonads following similar observations from Lujic et al. (2018), Psenicka et al. (2015), and Lacerda et al. (2006).

There was pronounced fluorescence in each of the gonads that were tested that had PKH26 dye added to the stem cells. This confirms the proliferation and uptake of the stem cells from the donor blue catfish in all 25 fish evaluated. This is not totally surprising as blue catfish have early sex differentiation similar to that of channel catfish (Patino et al., 1996). The female ovarian activity and ovarian meiosis can be first observed at day 22-post-fertilization, and male lobule structures are typically observed by day 90 post-fertilization (Patino et al., 1996). In catfish, Perera et al. (2017) reported transplanting SSCs from blue catfish into the gonads of sub-adult triploid channel catfish via catherization or surgical insertion (Vo, 2019). Ten-months post-transplantation nearly 90% of the recipients were confirmed as xenogenic (Perera et al., 2017). Two years post-transplantation, a xenogenic channel catfish male was able to fertilize the eggs of a normal channel catfish female (Perera et al., 2017). By injecting the recipient triploid channel catfish directly after hatching and using the PKH26 dye, it is possible to confirm transplantation success and protocols earlier. With earlier confirmation of transplantation success less labor will be involved as protocols can be confirmed as effective earlier, allowing termination of less efficient protocols and the fish from these treatments.

In the current study, the greatest survival rate of triploid fry when injected with donor-derived stem cells was 3-DPH. Unfortunately, this was a day that appeared to have some of the

lowest colonization and proliferation rates, although each fish fluoresced. Hypothetically, these fish would have lower reproductive capacity unless proliferation accelerates. This prediction cannot be confirmed until the fish reach sexual maturity.

Based on the morphological characteristics of the testes and ovaries from the channel catfish that were injected, it is clear that there was success in growth of gonads in triploid catfish using the injected stem cell methodology. A possible negative aspect of utilizing the PKH26 dye is that it kills some cells while dyeing the surviving ones. However, this method predicts that gonadal and gametic growth is possible with the protocols used in this study. The PKH26 technology would be a useful tool to beginning new experimentation with the xenogenesis process with other species.

In summary, xenogenic channel catfish produced by transplanting blue catfish stem cells into the peritoneal cavity of triploid channel catfish on day three post-hatch had the highest survival. However, if the amount of spermatogonia or oogonia present at 90 days reflects the future gamete production, injection at days 0,1 and 5 would be more productive for producing viable xenogenic brood stock. Approximately, twice as many fry would need to be injected on those 3 days to meet production goals, but potentially, less culling would be needed and less wasted culture on non-xenogenic brood stock. The use of PKH26 dye does not impact the survival of injected triploid channel catfish. Further research needs to be focused on 1) the relationship of cell and cluster area and number of the injected stem cells at 90 days to fertility upon sexual maturity, 2) growth of previously injected triploid channel catfish, and (3) mating the channel catfish female with the xenogenic catfish male transplanted with blue catfish stem cells to produce hybrids.

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7. Appendix

Table 3: Xenogenic channel catfish (*Ictalurus punctatus*) survival compared between each day of injection after 90 days post-injection.

Injection Day Survival	Injection Day Survival	Adjusted P-Value
0	1	0.04
0	2	1.00
0	3	0.00
0	4	0.05
0	5	0.97
1	2	0.84
1	3	<.0001
1	4	1.00
1	5	0.07
2	3	0.23
2	4	0.87
2	5	1.00
3	4	<.0001
3	5	<.0001
4	5	0.11

Table 4: Survival between xenogenic channel catfish (*Ictalurus punctatus*) injected with and without PKH ethanolic dye after 90 days.

Injection Survival	Injection Survival	Adjusted P-Value
Survival Without PKH26 Ethanolic Dye	Survival With PKH26 Ethanolic Dye	0.07

Table 5: Xenogenic channel catfish (*Ictalurus punctatus*) survival compared between each day of injection after 280 days post-injection.

Injection Day Survival 280 Days Post-Injection	Injection Day Survival 280 Days Post-Injection	Adjusted P-Value
0	1	1.00
0	2	0.86
0	3	<0.001
0	4	1.00
0	5	0.93
1	2	0.85
1	3	<0.001
1	4	1.00
1	5	0.93
2	3	0.00
2	4	0.94
2	5	1.00
3	4	<0.001
3	5	<0.001
4	5	0.98

Table 6: Survival between xenogenic channel catfish (*Ictalurus punctatus*) injected with and without PKH ethanolic dye after 280 days.

Injection Day Survival 280 Days Post-Injection	Injection Day Survival 280 Days Post-Injection	Adjusted P-Value
Survival Without PKH26 Ethanolic Dye	Survival With PKH26 Ethanolic Dye	0.07

Table 7: Mean weights (g) after 280 days post-injection of xenogenic channel catfish, *Ictalurus punctatus*, injected with and without PKH26 ethanolic dye.

Injection Day	PKH26 Ethanolic Dye	Mean Weight (g) 280 days post-injection
0	No	9.155
0	Yes	13.86
1	No	16.91
1	Yes	13.2
2	No	10.67
2	Yes	11.5
3	No	5.76
3	Yes	8.13
4	No	15.31
4	Yes	13.18
5	No	8.8
5	Yes	13.76

Table 8: Mean length (mm) and weight (g) +/- the standard deviation of PKH26 dyed xenogenic channel catfish, *Ictalurus punctatus*, sampled after 90 days post-injection for visual confirmation of PKH26 dyed stem cells.

Day of Injection	N	Mean Length (mm) +/- Standard Deviation	Mean Weight (g) +/- Standard Deviation
0	5	104.20 (+/- 7.40)	7.15 (+/- 1.79)
1	5	82.00 (+/- 4.85)	4.00 (+/- 1.11)
2	5	84.80 (+/- 9.34)	4.21 (+/- 1.10)
3	5	95.00 (+/- 9.62)	5.65 (+/- 1.55)
4	5	99.20 (+/- 9.78)	8.01 (+/- 1.92)
5	5	103.6 (+/- 20.51)	7.54 (+/- 3.57)

Table 9: Mean length (mm) and weight (g) +/- standard deviation of non-PKH26 dyed xenogenic channel catfish, *Ictalurus punctatus*, sampled after 90 days post-injection.

Day of Injection	N	Mean Length (mm) +/- Standard Deviation	Mean Weight (g) +/- Standard Deviation
3	5	100.25 (+/- 2.98)	7.09 (+/- 0.42)
4	5	101.60 (+/- 2.51)	7.25 (+/- 0.16)
5	5	103.40 (+/- 8.08)	7.33 (+/- 1.77)

Chapter Two

Viability of Blue Catfish, *Ictalurus furcatus*, Donor-Derived Stem Cells Post-Staining with PKH26 Dye

Abstract

Xenogenesis has been identified as an innovative technology for hybrid catfish (♀ channel catfish, *Ictalurus punctatus* × ♂ blue catfish, *I. furcatus*) embryo production. When producing the xenogenic channel catfish the use of PKH26 dye was used to track colonization of donor-derived stem cells into triploid channel catfish, however, possible cytotoxicity had not been tested. Concentrations of PKH26 dye were tested in relation to cell viability and time post-staining. Donor-derived stem cell solutions lost viability post-staining with most concentrations of PKH26. The number of overall cells available for injection declined within five min post exposure to PKH26. These findings suggest that it is crucial to inject the dyed cells immediately to preserve the viability and produce the greatest chance of transplantation success.

1. Introduction

The use of fluorescent dyes dates back almost 150 years (Renz, 2013). Fluorophores are molecules that emit light of a certain wavelength when excited by a light of a different wavelength. When a fluorophore absorbs energy from a photon the energy state becomes excited from the stable low energy ground state to multiple unstable higher energy levels. When this happens, some energy is lost to non-radiative emission and the fluorophores come to a lower energy semi-stable excited state. The fluorophore then returns to ground state by the emission of light at a lower energy, this is referred to as a Stokes shift (Yang et al., 1993).

German chemist Adolf von Baeyer produced the first synthetic fluorophore pigment using phthalic anhydride and resorcinol in 1871 (Lavis and Raines, 2014). At the time Baeyer named this compound resorcinphthalein, however it has since changed and is now known as fluorescein. Fluorescein is a dye with an intense yellow-green fluorescence and serves as the base for several xanthene-based fluorophores (Lavis and Raines, 2014). Albert Coons devised the first method to visualize proteins in animal tissue by adding fluorescein to antibodies (Coons, 1961). Today this method is known as immunofluorescence (Coons, 1961). Since Coons developed his methodology, fluorescent technology has evolved to labeling not just proteins but other biological components including organelles, membranes, lipids and nucleic acids (McKay et al., 1981; Lavis and Raines, 2014).

While fluorescein was an incredible breakthrough, there were vast amounts of autofluorescence that were generated within the short (green) emission wavelengths (McKay et

al., 1981). The result of the autofluorescence was higher background and lower image contrast (McKay et al., 1981). Through time the dyes were refined to develop several other xanthene-based derivatives that are known as rhodamine dyes that include the Texas Red dyes (McKay et al., 1981; Lavis and Raines, 2014). Rhodamine dyes are considered more favorable to use at that time due to their photostability, pH insensitivity, and longer emission wavelengths relative to fluorescein (McKay et al., 1981; Lavis and Raines, 2014). However, there were some drawbacks to the rhodamine dyes, as they suffered from poor solubility and dye to dye quenching during antibody conjugation (McKay et al., 1981; Lavis and Raines, 2014). Nevertheless, this fluorescence technology has potential uses for catfish genetics and reproduction research.

The dye utilized in this study was PKH26. PKH26 is a red fluorescent dye and it offers a half-life greater than 100 days giving time for cell proliferation (Horan and Slezak, 1989; Wallace et al., 1993; Yamamura et al., 1995). Because the PKH26 dye is a red fluorochrome it has excitation (551 nm) and emission (567 nm) characteristics compatible with rhodamine or phycoerythrin detection systems (Wallace et al., 2008). Labeled cells will retain both biological and proliferative activity which is ideal for tracking stem cell division and proliferation (Katajisto et al., 2015). This allows for the visual confirmation of injected cell growth after 90 days post-injection when both male and female channel catfish will have begun gonadal growth. The use of dyes is crucial for visually identifying if transplanted cells colonized and proliferated. Apoptosis is genetically programed and expected. Nevertheless, it is vital to determine if there is any cytotoxicity that occurs from the PKH26 dye (Cummings, 2004), as this can adversely affect the cells.

Hybrid catfish (channel catfish, *Ictalurus punctatus*, female × blue catfish, *I. furcatus,* male), possess a multitude of favorable traits compared to channel catfish, making it very valuable in large scale production aquaculture. However, artificial fertilization for the commercial production of hybrid catfish embryos is labor intensive, as 4 to 8-year-old blue catfish males must be sacrificed for sperm collection to fertilize channel catfish eggs. Xenogenesis is an alternative method for hybrid embryo production that could reduce the labor-intensive aspect and reduce the sacrifice of slow maturing blue catfish. Xenogenesis has been accomplished in catfish, but rates of colonization and proliferation have been low based on rate of xenogenesis and fertility of xenogenic individuals (Shang, 2013; Perera et al., 2017). Tracking stem cells after injection into the host could reveal factors that could improve the generation of xenogenic individuals. However, the dyes used to track cells can have adverse effects on cell viability and survival of the host (Oh et al., 1999). Thus, research is needed regarding the effect of fluorescent dyes on viability of blue catfish stem cells. This knowledge would allow for an optimal volume of dye to be added to the donor-derived stem cells and improve our ability to inject the most triploid channel catfish fry without sacrificing valuable resources.

This study had two major objectives. First, the aim was to isolate donor-derived blue catfish stem cells and then dye them using five different concentrations of PKH26 (0.1, 0.2, 0.3, 0.4, and 0.5%) to determine the lowest required dose to successfully dye donor-derived stem cells. The second aim was to determine how cell viability changes over time [i) before being stained, i) 5 min post-staining, and i) 30 min post-staining)] in an effort to replicate PKH cell exposure between staining and injection into triploid channel catfish fry.

2. Materials and Methods

2.1 Isolation of donor stem cells from blue catfish to test PKH26 concentration

Sexually immature male and female blue catfish (150 – 450 g; 175 – 450 mm) were seined from 0.25 ha aquaculture ponds that were approximately 1.5 meters deep and located at the Fish Genetics Research Unit at the E.W. Shell Fisheries Center in Auburn, Alabama (32.6622° N, 85.4960° W). Fish were fed once daily with commercial floating catfish feed (crude protein: ≥32.00%, crude fat: ≥4.00%, crude fiber: 7.00% and phosphorus: 0.80%; Cargill Animal Nutrition, LA) to satiation. Dissolved oxygen (DO) was monitored twice daily (06:30 and 16:30) and temperature was recorded twice per week using an YSI 55 multi-parameter instrument (605056, YSI Incorporate, OH). Total ammonia-N (TAN), nitrite, nitrate, pH, hardness, and alkalinity were also measured three times per week using a water quality test kit (19541-931/19543-91, Easy Strips Tetra, Tetra Holding, Blacksburg, VA).

Each of the trials was run using pooled stem cells from 9 individual juvenile blue catfish males and females. The selected blue catfish were selected based on length (< 500 mm). Each fish was euthanized via pithing, washed with a 0.9% saline water solution and placed on crushed ice. The blue catfish were then sprayed with a 70% ethyl alcohol solution (Pharmco-Aaper, Shelbyville, KY) to sterilize their exterior surface and weighed and measured before the abdomen was surgically opened for gonadal extraction. Gonads were removed, weighed, and washed with a 0.5% bleach solution for 1 to 2 min before being placed in a petri dish (100 mm × 15 mm) containing 5 mL of the anti-agent medium Hank's Balanced Salt Solution (HBSS, SH30031.03, GE Healthcare Life Sciences, Carlsbad, CA.). The gonads were then placed under a biosafety cabinet

(The Baker Company, Sanford, ME) to prevent contamination from outside sources, where connective tissues and blood vessels were removed and discarded. The gonads were washed three times with phosphate-buffered saline (PBS) at 10X (PBS; J62692, Alfa Aesar, Haverhill, MA.) and then washed three times with anti-agent medium (HBSS) before being diced with sterile scalpel blades. The tissue was then transferred to 50 mL flasks containing a magnetic bar that had been autoclaved. In each flask the gonads were added to a 0.25% trypsin - ethylenediamine tetraacetic acid (EDTA; 25200-072, Life Technologies, Carlsbad, CA.) solution at 50X gonadal weight. The flasks were then incubated on ice for 30 min and then on a stir plate (Corning, Manassas, VA) for 60 min. The suspension was then filtered using a 70 μm filter and the remaining solution was filtered using a 40 μm filter. The cell solution was then centrifuged at 500 g for 10 min (Sorvall RT6000, ThermoFisher, Waltham, MA). Thereafter, the supernatant was discarded, and the pellets were resuspended with 2 mL of Dulbecco's Modified Eagle's Medium/DMEM (10-090-CV, Corning Cellgro, Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; 10438018, Life Technologies), 100 unit/mL Penicillin - Streptomycin (15140-122, Life Technologies), and 200 mM L-glutamine (A2916801, Life Technologies) to provide a favorable environment for the cells. The cell suspension (5 µL) was gently mixed with 45 µL of 0.4% trypan blue (15250061, Life Technologies). Cells (10 uL) were then observed under an Olympus objective microscope (BH2), equipped with a 20× objective, to determine the total number of cells with the aid of a hemocytometer. The number of cells were counted in four corner quadrants (each 1 mm^2 area) of the hemocytometer. The number of cells in 1 mL was calculated according to Louis and Siegel (2011) with the dilution factor of 2 (cell suspension: trypan blue

with 1:1 ratio). Live and dead cells were enumerated for analysis, where the total number of cells per mL = (total number of counted cells × dilution factor × 10^4) / number of squares.

2.2. PKH26 Dye Solution Concentration

The isolated cells from nine blue catfish, 6 males and 3 females, were separated into six equal volumes and were centrifuged at 400 g for 5 min to produce a pellet. The supernatant was removed and 1 mL of Diluent C from the PKH26 red fluorescence cell linker kit (Catalog Number CGLDIL, Sigma-Aldrich, St. Louis, MO) was added to resuspend the cells. This was done by gently pipetting to create a homogenous solution. Each of the six respective centrifuge tubes were labeled from one to five to determine the amount of PKH26 dye solution that was added. Starting with tube one, 0.1% was added and pipetted to mix completely. The next four tubes were each pipetted with 0.2, 0.3, 0.4, 0.5% of PKH26 dye solution respectively. The solutions were then mixed every 30 s by inversion for 5 min to ensure complete dispersion. Staining was nearly instantaneous, as rapid and homogenous dispersion of cells in the dye solution is essential for bright, uniform, and reproducible labeling. Because the staining was so rapid, a longer incubation period was not necessary. To end the staining process, 2 mL of 1% BSA was added to the solution and incubated for 1 min to allow binding of the excess dye. The cells were then centrifuged at 400 × g for 10 min at 20 to 25°C. The supernatant was then removed, the cells were resuspended in 1.5 mL of HBSS, and were centrifuged to wash the cells at 400 ×g for 5 min and then repeated. Once the second washing was completed, the supernatant was removed. Thereafter, the cells were resuspended in 1 mL of DMEM and counted using a hemocytometer under an Olympus objective microscope (BH2), equipped with a 20× objective, to determine the total number of

cells for each treatment (0 – 5 μL of PKH26 dye) before being stained, i) 5 min post-staining, and i) 30 min post-staining to determine if there were differences in cell survival.

2.3. Statistics

Data were analyzed using SAS statistical analysis software (v. 9.1; SAS Institute Inc., Cary, NC, USA). Data were tested for normality (Shapiro–Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). Cell viability data were arcsine square-root transformed to meet assumptions of normality and homoscedasticity. Error bars represent least square means standard error. To examine the effect of PKH26 concentration and time post-staining on cell viability the data were analyzed using a series of repeated measures factorial ANOVA models. Each model contained the PKH26 concentration (0.1, 0.2, 0.3, 0.4, and 0.5%) and time post-staining [0 (control), 5 min, 30 min) main effects, as well as the corresponding PKH26 concentration × time post-staining interaction. If a non-significant interaction was detected the main effects of PKH26 concentration and time post-staining were interpreted. Treatment means were contrasted using the Tukey's test. Alpha was set at 0.05 for main effects and interactions.

3. Results

Viability of cells dyed with and without PKH26 ranged from 43% (0.2% PKH at 30 min post-staining) to 84% (0.4% at 5 min post-staining) (Fig. 10A). There was no significant interaction between PKH26 concentration and time post-staining (P = 0.885). PKH26 concentration had no significant impact on cell viability (P = 0.139). However, time post-staining significantly impacted cell viability (P = 0.026), such that cells which would be stained and not injected after 30 min of PKH exposure would have decreased viability compared to those injected after 5 min (Fig. 10A). Meanwhile, there was no difference detected between the 0 and 5 min PKH exposure times (Fig. 10A).

Live cell production for the different treatments ranged from 2.7×10^7 (0.1% PHK at 30 min post-staining) to 9.7×10^9 (0.4% PKH at 5 min post-staining). The interaction between PKH26 concentration and time post-staining was not significant (P = 0.672). The number of cells available for injection declined within 5 min of exposure to PKH (P < 0.001) (Fig. 10BC). Furthermore, PKH typically impacted the quantity of cells that would be available to inject into fry (P = 0.002), where all treatments expect the 0.4% PHK concentration showed a decline in cells numbers (Fig. 10C).

Table 10: The mean number of live cells contained in 1 mL of the blue catfish, *Ictalurus furcatus,* donor-derived stem cell solution +/- the standard deviation. Each respective 1 mL of solution had a corresponding concentration of PKH26 dye (0.1, 0.2, 0.3, 0.4, and 0.5%) added to determine if there was an increase in cell death over time and in relation to concentration. 30 minutes post-staining had a significant decrease in cell viability when compared to viability at 5 minutes post-staining. Values analyzed with ANOVA and Tukey-Kramer tests. Time post-staining (min) significantly impacted cell viability (P = 0.026), such that cells which would be stained and not injected after 30 min post-PKH26 exposure would have decreased viability compared to those injected after 5 min

	Mean of Live Cell in Stem Cell Solution +/- Standard Deviation		
Concentration of PKH26 Dye	Before Addition of PKH26 Dye	5 Minutes Post-Addition	30 Minutes Post-Addition
0.0%	3.48E+08 (+/- 1.88E+08)	3.51E+08 (+/- 1.28E+08)	3.73E+08 (+/- 1.38E+08)
0.1%	3.59E+08 (+/- 2.07E+08)	1.94E+08 (+/- 1.42E+08)	1.19E+08 (+/- 8.82E+07)
0.2%	4.17E+08 (+/- 2.62E+08)	2.13E+08 (+/- 1.46E+08)	1.32E+08 (+/- 1.11E+07)
0.3%	4.40E+08 (+/- 3.30E+08)	2.37E+08 (+/- 1.60E+08)	1.65E+08 (+/- 9.65E+08)
0.4%	5.01E+08 (+/- 4.02E+08)	2.07E+08 (+/- 5.21E+07)	3.30E+08 (+/- 2.08E+08)
0.5%	4.55E+08 (+/- 3.87E+08)	1.52E+08 (+/- 9.83E+07)	1.52E+08 (+/- 9.35E+07)

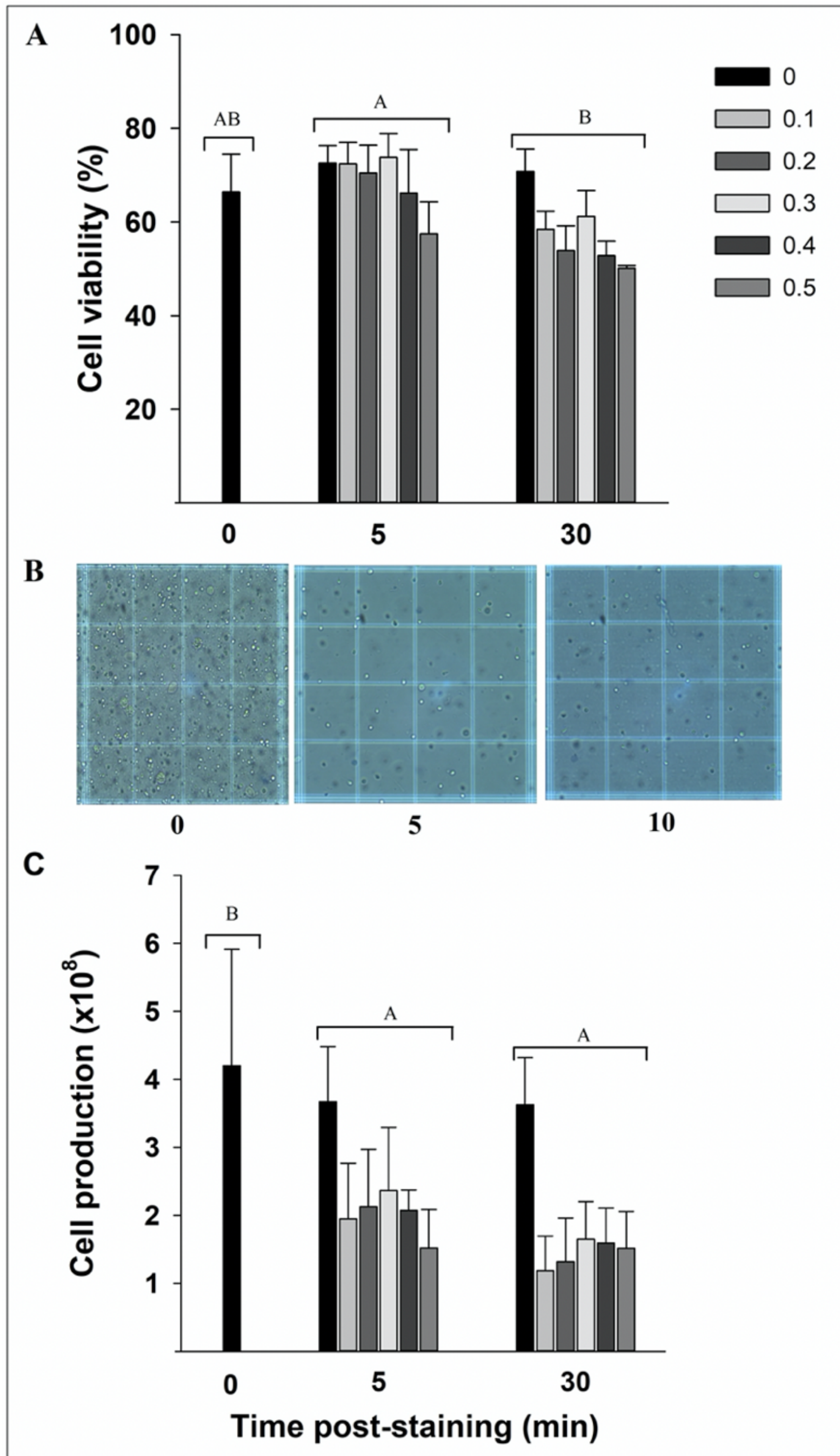
A
100
80
60
40
20
Cell viability (%)
AB
A
B
0
0.1
0.2
0.3
0.4
0.5
0
5
30
B
0
5
10
C
7
6
5
4
3
2
1
Cell production (x10^8)
B
A
A
0
5
30
Time post-staining (min)

Figure 10: Comparison of blue catfish, *Ictalurus furcatus,* donor-derived stem cell solution viability and production. Values were analyzed by ANOVA followed by Tukey-Kramer tests; A: Time post-staining (min) significantly impacted cell viability ($P = 0.026$), such that cells which would be stained and not injected 30 min post-PKH26 exposure would have decreased viability compared to those injected after 5 min; B: Representation of cells available before, 5 min post-, and 30 min post-addition of PKH26 (0.2%); C: Cells available for injection declined within 5 min of exposure to PKH26 ($P < 0.001$). PKH26 typically impacted the quantity was SSCs that would be available to inject into fry ($P = 0.002$), where all treatments expect the 0.4% PHK26 concentration showed a decline in cells numbers.

4. Discussion

In this experiment, PKH26 was utilized to stain donor-derived blue catfish stem cells. PKH26 was used because of its long half-life of over 100 days allowing for the greatest time for growth before visual observation of the labeled cells (Horan and Slezak, 1989; Wallace et al., 1993; Yamamura et al., 1995). The stem cell solutions were stained using 5 different concentrations of PKH26 dye at 0.1, 0.2, 0.3, 0.4, and 0.5%. Time post-staining significantly impacted cell viability such that cells which would be stained and not injected after 30 min of PKH exposure would have decreased viability compared to those injected after 5 min. PKH26 typically impacted the quantity was SSCs that would be available to inject into fry, where all treatments expect the 0.4% PHK26 concentration showed a decline in cells numbers.

There have been studies using PKH26 that have tested the effects of PKH26 dye concentration. Lujic et al. (2018) tested PKH26 dye concentrations utilizing concentrations of 0.0001%, 0.0002%, and 0.0003% to stain stem cells from immature male brown trout, *Salmo trutta*. The PKH26 concentration of 0.0003% was optimal as it emitted the strongest fluorescent signal (Lujic et al., 2018). The effects of phototoxicity on PKH26 labeling of human hematopoietic KG1a progenitor cells line has been examined, where findings showed no effects on cell viability and growth when using PKH26 concentrations up to 0.5% (Oh et al., 1999). However, there was a dramatic decrease in cell viability when the stained cells were exposed to continuous light (Oh et al., 1999). Our results show that low concentrations, 0.1%, of PKH26 can potentially be used to identify if proliferation and colonization has taken place inside the host, while at the same time minimizing cell processing costs. Results showed a difference in cell viability due to time post-staining. The cells which would be stained and not injected after 30 min post-PKH26

exposure would have decreased viability compared to those injected after 5 min post-PKH26 exposure.

Together, these results suggest that host triploid channel catfish should be injected as quickly as possible to maintain high levels of viability. Nevertheless, further studies should test these treatments *in vivo* as it is possible that the cells that died between 5 and 30 min after staining may have been weak and died whether remaining in solution or residing inside the host fish. With an experienced individual injecting host fry, at minimum thirty host fry can be injected with stem cell solutions containing 80,000 cells per injection in 10 min. With multiple experienced injectors and proper equipment, a large amount of host fry can be injected in a short time period. If there are not a surplus of experienced injectors, staggering the time of dye application can be utilized.

In summary, the donor-derived stem cell solutions lost viability post-staining with most concentrations of PKH26. The number of overall cells available for injection declined within five min post exposure to PKH26. The use of the dye is a powerful tool to study transplantation success in the germinal ridge of recipient injected fish. To produce the best results, injection of the solution into the recipient should be accomplished within ~5 min post completion of the PKH26 dying process. In the future less PKH26 dye can be used to stain the stem cell solution and produce the same viability as with no dye added. Future research needs to be focused on 2 main areas: (1) The injection of the stem cell solution dyed using 0.1, 0.2, 0.3, 0.4, and 0.5% of PKH26 dye followed by fluorescent labeling analysis of the gonads from the recipients to visually determine if there is a difference between the concentration and overall cell / cluster numbers, cell / cluster area, and fluorescence intensity; and (2) producing an efficient method of injecting

as many fry in under 30 min to inject the highest number of viable cells. Additionally, short term experiments should be conducted to compare the survival of cells injected at 5 min, 30 min and several hours after injection. Density effects need further analysis, as twice as many dyed cells would need to be used compared to cells not dyed, to evaluate equal numbers of cells between these two treatments within the organism.

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6. Appendix

Table 11: Blue catfish, *Ictalurus furcatus*, donor-derived cell viability compared between concentrations of PKH26.

Amount of PKH26 Dye	Amount of PKH26 Dye	Adjusted P-Value
0	0.1	0.001
0	0.2	0.008
0	0.3	0.046
0	0.4	0.086
0	0.5	0.007
0.1	0.2	0.768
0.1	0.3	0.212
0.1	0.4	0.118
0.1	0.5	0.792
0.2	0.3	0.839
0.2	0.4	0.629
0.2	0.5	1.000
0.3	0.4	0.998
0.3	0.5	0.817
0.4	0.5	0.602

Table 12: Blue catfish, *Ictalurus furcatus*, donor-derived cell viability post-staining with PKH26 compared between three time points.

Time (Minutes)	Time (Minutes)	Adjusted P-Value
0	5	0.002
0	30	0.001
5	30	0.600