OPTIMIZATION OF STEM CELL EXTRACTION FROM BLUE CATFISH (*Ictalurus furcatus*) TO PRODUCE ALLOXENOGENIC CATFISH: EFFECTS OF BODY SIZE, SEX STEROIDS, AND SEASONALITY

By

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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, donor cells are collected from randomly selected immature fish without having prior knowledge regarding the best donor size. Immature blue catfish males with a total length (T_L) of 38.1 cm and total weight (T_W) of 400.2 g, and immature blue catfish females with a T_L of 31.2 cm and T_W of 410.1 g were the best sizes to yield the maximum number of spermatogonial stem cells (SSCs) and oogonial stem cells (OSCs), respectively. In addition, testosterone (T) and 11-ketotestosterone (11-KT) levels were identified as reliable biomarkers to predict the maximum number of SSCs, which were 50.9 pg/mL and 44.7 pg/mL, respectively. The potential use of mature blue catfish males and females as alternative donors was evaluated. However, stem cell extraction was time consuming and labor intensive, thus the smaller, immature fish were a better source of stem cells.

Key words: xenogenesis; blue catfish; androgen; stem cell; seasonal variation; gonodosomatic index

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CHAPTER I

1.0 GENERAL INTRODUCTION

1.1 World Aquaculture and the Catfish Industry in the United States

The aquaculture industry has been expanding rapidly, exceeding the annual growth rates of poultry, pork, dairy, and beef industries (Smith et al., 2010, Troell et al., 2014). Aquaculture is developing, expanding, and intensifying in almost all regions of the world in parallel to the increase of global population (Subasinghe et al., 2009; Watson et al., 2016). As a result, total global aquaculture production in 2017 was 111.95 Mt, which is 18.32 Mt greater than the production from global capture fisheries production (93.63 Mt) in the same year (FAO, 2019; Tacon, 2020). Therefore, aquaculture is considered to be the opportunity to bridge the supply and demand gap of aquatic food in most regions of the world. Asia has accounted for nearly 89% of world aquaculture production for over two decades while Africa and America have lifted their respective shares up to 2.0% and 3.2%, respectively (FAO, 2019).

The aquaculture industry in the United States has become well established over the last 50 years, but still faces significant challenges to maintain continued growth. The main aquaculture species in the United States are catfish, trout, salmon, tilapia, hybrid striped bass, and shellfish including crawfish and oysters (FAO, 2018). Catfish are the major contributor to United States aquaculture on a volume basis. Catfish belong to the family Ictaluridae, which comprised of about 51 species native to North America including channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus),* brown bullhead (*Ameiurus nebulosus),* and white catfish (*A. catus),* which all play a

vital role in food and sport fish industries (Fine et al., 1997; Sullivan et al., 2006, Boxrucker and Kuklinski, 2006; Green and Rawles, 2010).

Commercial production of catfish in the United States was first established in Kansas in the 1930s. Over the past several decades, the industry has expanded tremendously, especially in southern states including Mississippi, Alabama, Arkansas, Louisiana, and Texas (Glahn and Brugger, 1995). Farm-raised catfish production has rapidly developed and is now leading the finfish aquaculture industry, where it ~80,000 hectares of land was dedicated to catfish production in 2002 (Hanson and Sites, 2015). In 2003, ~300 million kilograms of catfish were processed (Hanson, 2006; Hanson and Sites, 2015), however by 2014 a 65% decline in total farming hectares had occurred, as compared to 2002-2003. There were several factors contributing to the decline in the US catfish industry, such as increased fuel, feed, and labor costs, the recession, competition with imported catfish from Asia, and disease outbreaks (Dunham and Smitherman, 1984; FAO, 2011; Hanson and Sites, 2015).

Total catfish production in United States in 2018 and 2019 was 104.4 million kilogrames and 93.8 million kilogrames, respectively (NASS, 2019). During this time, Mississippi produced 44.31 million kilogrames of catfish, being the top producer of the country (42.2% of total production), while Alabama produced 32.7 million kilogrames (31.3% of total production). Improved hatchery, grow-out culture techniques, disease diagnosis and disease treatments are some of the key processes contributing to the success of the industry. In addition, several genetic and biotechnology applications have been examined, such as strain selection, intraspecific crossbreeding, mass selection, and interspecific hybridization with impactful results (Dunham and Smitherman, 1984; Green and Rawles, 2010; Dunham and Masser, 2012).

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

The hybrid between the female channel catfish and male blue catfish is the only variety (C × B hybrid) of 42 ictalurid hybrids tested in United States, which resulted in commercially desirable characteristics when grown in earthen ponds compared to their parent species (Dunham and Masser 2012; Dunham et al., 2014, Smitherman et al., 1983: Dunham et al., 1982). 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 et al., 1983; 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; Huang et al., 1994), 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 U.S catfish industry (Dunham and Masser 2012; Perera et al., 2016).

Initially, C × B hybrid production was not economically feasible due to numerous challenges, such as costly hormonal stimulation of females, difficulties in mating and preparing spawning environment, manual stripping of channel catfish eggs and artificial fertilization with blue catfish sperm, essentially, reproductive isolation (Argue et al., 2003; Hu et al., 2011). In

addition, blue catfish must be sacrificed to obtain sperm during the hybridization procedure, also blue catfish has slower growth and maturity rates, late sexual maturity (after 4-6 years), and higher consumption of feed due to their larger ultimate size. Meanwhile, xenogenesis has been identified as an innovative hatchery technology to produce C×B hybrids that might be used to address those above-mentioned inefficiencies in production of seed (Dunham and Argue, 2000; Okutsu, et al., 2006).

1.3 Xenogenesis

Xenogenesis is a method of reproduction in which successive generations differ from each other (Dunham, 2011). Xenogenic organisms are comprised of elements typically foreign to its species (Lacerda et al., 2006; Takeuchi et al., 2007; Dunham, 2011; Higuchi et al., 2011). 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., 2016; De Siqueira-Silva et al., 2018). 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., 2016; De Siqueira-Silva et al., 2018). This technique has been successfully applied for conservation of endangered/endemic species and to improve reproduction for commercially valuable fish species, such as zebrafish, *Danio rerio* (Saito et al., 2008; Lacerda et al., 2013), rainbow trout, *Oncorhynchus mykiss* (Nagler et al., 2001; Okutsuet al., 2006), pejerrey, *Odontesthes bonariensis* (Majhi et al., 2009), and nibe

croaker, *Nibea mitsukurii* (Takeuchi et al., 2009; Yoshikawa et al., 2016). SSCs transplantation between two different species or closely related teleost species has also been examined, and specific examples include rainbow trout SSCs injected into cherry salmon, *Oncorhynchus masou* (Yoshizaki et al., 2010), jundia catfish, *Rhamdia quelen* SSCs injected into nile tilapia *Oreochromis niloticus* (Silva et al., 2006), senegalese sole, *Solea senegalensis* SSCs injected into turbot *Scophthalmus maximus* (Pacchiarini et al., 2014), siberian sturgeon, *Acispenser baerii* SSCs injected into starlet sturgeon, *A. ruthenus* (Pšenička et al., 2015), tiger puffer, *Takifugu rubripes* SSCs injected into grass puffer *T. niphobles* (Hamasaki et al., 2017), Chinese sturgeon, *Acipenser sinensis* SSCs injected into Dabry's sturgeon, *A. dabryanus* (Ye et al., 2017) and blue catfish SSCs injected into triploid channel catfish (Perera et al., 2016). Procedures for transplantation of OSCs is similar to SSCs, and was successfully applied in rainbow trout, *O. mykiss* (Yoshizaki et al., 2010), zebrafish *D.rerio* (Wong et al., 2011), and siberian sturgeon, *Acipenser baerii* (Psenicka et al., 2015).

One of the constraints encountered in all previous xenogenic experiments was the collection of donor cells from randomly selected immature donor species, rather than having precise prior knowledge on the relationship between the quantity or quality of extracted donor cells and size of donor fish. This information is critical to select the best donor stage to increase efficiency of germ cell transplantation. Therefore, the relationships between the quantity of stem cells and certain biomarkers such as fish body size (total length, body weight) and sex steroid hormonal levels in the blood [testosterone (T) and 11-ketotestosterone (11-KT)] would be valuable to determine the optimal time for donor cell extraction.

1.4 Testosterone and 11-Ketotestosterone

The sex steroids, testosterone (T) and 11-ketotestosterone (11-KT), are key regulatory hormones that play a vital role in development and function of the male reproductive system (Young et al., 2005). Sex steroid hormones are synthesized in the fish testis (Wei-Xin, 1988; Vizziano et al.,1996; Miura et al.,1999; Higuchi et al., 2011) and can regulate major mechanisms of spermatogenesis, such as spermatogonial cells A and B multiplication, proliferation, spermatocyte formation, and cell differentiation up to spermatozoa (Billard et al., 1982; Miura et al., 1991; Schlinger and Arnold., 1992; Hess et al., 1995; Pudney, 1995; Magee et al., 2006; Golshan et al., 2019). As germ cell differentiation and proliferation is mainly regulated by sex steroids, evaluation of plasma T and 11-KT quantities in blood would provide reliable data on selecting the best stages for donor fish for germ cell transplantation.

1.5 Objectives

Since the success of SSCs transplantation for xenogenesis is mainly dependent upon the quality and quantity of donor stem cells, the aim of this study was to identify relationships between stem cell production and total length (T_L), total weight (T_W), and sex steroid hormones levels (T and 11-KT) of donor fish. In addition, it was planned to evaluate seasonal changes of germ cell multiplication and proliferation in mature fish with the objective of determining the efficacy of mature fish in germ cell transplantation.

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CHAPTER II

DONOR BODY SIZE AND SEX STEROID HORMONES IMPACT GAMETOGONIA PRODUCTION FOR CREATION OF XENOGENIC CATFISH

Abstract

Xenogenesis is emerging as an innovative technology for hybrid catfish (♀ channel catfish, *Ictalurus punctatus* × ♂ blue catfish, *I.furcatus*) production, where primordial germ cells (PGC), spermatogonial stem cells (SSCs) or oogonial stem cells (OSCs) are transplanted to a sterilized host species to produce xenogens. Currently, donor cells are collected from randomly selected immature fish without having prior knowledge regarding the best donor stage, which is critical to increase the efficiency and success of germ cell transplantation in catfish breeding. Therefore, the current study was carried out with the objective of determining the relationships between quantity of stem cells, total length (T_L), total weight (T_W), and the level of sex steroid hormones [testosterone (T) and 11-ketotestosterone (11-KT)] in blue catfish (donor fish). Positive quadratic relationships were observed between T_L and the number of extracted live SSCs ($r^2 = 0.50$; $P < 0.0001$) as well as T_W and the number of live SSCs ($r^2 = 0.37$; $P < 0.0001$), revealing that a T_L of 38.1 cm and T_W of 400.2 g, yield the highest number of extracted live SSCs. Similar to the findings from males, T_L ($r^2 = 0.77$; $P < 0.0001$) and T_W ($r^2 = 0.65$; $P < 0.0001$), also revealed positive quadratic relationships to the number of extracted live OSCs, where 31.2 cm and 410.1g females yielded the greatest number of extracted live cells. Positive quadratic relationships were also detected between levels of T ($r^2 = 0.59$; $P < 0.0001$), 11-KT ($r^2 = 0.69$; $P < 0.0001$) and number of extracted live SSCs in male catfish, where 50.9 pg/mL of T and 44.7 pg/mL of 11-KT yielded the

maximum number of live SSCs. Overall, these relationships between the quantity of stem cells and body size as well as the profile of sex steroid hormones reveal promising results as reliable biomarkers to enhance the efficiency of germ cell transplantation. These results can also be used to make precise predictions on the number of stem cells at a certain body size without sacrificing precious fish housed in living genetic repositories.

Key words: xenogenesi; genetic enhancement; breeding program; blue catfish; androgen; stem cell

1. Introduction

Catfish farming in Alabama, Arkansas, Mississippi, and Texas accounts for nearly 70% of total United States freshwater aquaculture production (Torrans and Ott, 2018). Hybrid catfish produced by crossing the channel catfish, *Ictalurus punctatus* ♀ with the blue catfish, *I. furcatus* ♂, is a vital component of the United States catfish industry (Dunham and Masser, 2012; Perera et al., 2016) because of their 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 (Yant et al., 1976; Dunham et al., 1983; Dunham et al., 1987; Dunham and Argue, 1998; Bosworth et al., 2004; Dunham and Masser, 2012; Arias et al., 2012; Dunham et al., 2014). Although the hybrid industry has seen sustained growth, there are still challenges, largely due to lack of natural hybridization between the two species and the need to sacrifice blue catfish males for sperm collection (Argue et al., 2003; Hu et al., 2011). Recent advances in artificial spawning and assisted reproduction have produced genetically distinct hybrids with even more superior production characteristics (Dunham and Masser, 2012; Bosworth, 2012; Boxrucker and Kuklinski, 2006). Nevertheless, this technology remains labor intensive, leading to the need for new innovative hatchery technologies, such as xenogenesis (Dunham and Argue, 2000).

Xenogenesis is a method of reproduction in which successive generations differ from each other resulting in xenogens, which are organisms comprised of elements typically foreign to its species (Cavaco et al., 1999; Takeuchi et al., 2004; Takeuchi et al., 2009; Dunham, 2011; Higuchi et al., 2011). 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., 2016; De Siqueira-Silva et al., 2018;). 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., 2016; De Siqueira-Silva et al., 2018).

The SSCs have the ability to undergo self-renewal throughout their life and can transmit genes to successive generations (Brinster and Avarbock, 1994). Based on this concept, Brinster and Zimmermann (1994) as well as Brinster and Avarbock (1994) developed a technique to transfer SSCs from donor testes into infertile recipient testes. During their experiments, male germ cells were isolated from donors and concentrated spermatogonial cell suspensions were transplanted to the tubules in testes of recipient males. Consequently, they demonstrated that transplanted SSCs can undergo the normal spermatogenesis process in the recipient male and produce functional spermatozoa, which can fertilize eggs and produce offspring. Following these findings, their technique has been successfully applied for conservation of endangered/endemic species and to improve reproduction for commercially valuable fish species, such as zebrafish, *Danio rerio* (Saito et al., 2008; Lacerda et al., 2013), rainbow trout, *Oncorhynchus mykiss* (Nagler et al., 2001; Okutsuet al., 2006), Pejerrey, *Odontesthes bonariensis* (Majhi et al., 2009), and Nibe Croaker, *Nibea mitsukurii* (Takeuchi et al., 2009; Yoshikawa et al., 2016). SSCs transplantation between two different species or closely related teleost species has also been examined, and specific examples include rainbow trout SSCs injected into cherry salmon, *Oncorhynchus masou* (Yoshizaki et al., 2010), jundia catfish, *Rhamdia quelen* SSCs injected into Nile tilapia *Oreochromis niloticus* (Silva et al., 2006), Senegalese sole, *Solea senegalensis* SSCs injected into turbot *Scophthalmus maximus* (Pacchiarini et al., 2014), Siberian sturgeon, *Acispenser baerii* SSCs injected into starlet sturgeon, *A. ruthenus* (Pšenička et al., 2015), tiger puffer, *Takifugu rubripes*

SSCs injected into grass puffer *T. niphobles* (Hamasaki et al., 2017), Chinese sturgeon, *Acipenser sinensis* SSCs injected into Dabry's sturgeon, *A. dabryanus* (Ye et al., 2017) and blue catfish SSCs injected into triploid channel catfish (Perera et al., 2016).

The OSCs also possess self-renewal ability, making them useful as donor cells for xenogenesis translation. Yoshizaki et al. (2010) were able to produce 50% offspring with donor-derived phenotypes by allogeneically transplanting ovarian cell suspensions prepared from 6 to 9-month-old pvasa-Gfp transgenic trout in sterile triploid rainbow trout. Wong et al. (2011) demonstrated the success of ovarian germ cell colonization in the gonads of the host, following transplantation of ovarian germ cells extracted from zebrafish in sterile hybrid larvae (cross between pearl danio and zebrafish), which was further proved through the findings of Majhi et al. (2014) and Ye et al. (2017), who conducted a similar experiment using critically endangered Chinese sturgeons (*A. sinensis* as the donor and *A. dabryanus* as the host) and Pejerrey (*O. bonariensis* as the donor and *O. hatcheri* as the host).

Once isolated these stem cells become a valuable resource for facilitation of future conservation and aquaculture breeding efforts. Therefore, we need reliable biomarkers that determine the optimal time for donor cell extraction. In this regard, relationships between the quantity of stem cells and fish body size as well as sex steroid hormonal levels would be valuable, as these biomarkers tend to vary based on fish age (Fontoura et al., 2009; Agarwal and Raghuvanshi, 2009) and reproductive status (George and Pandian, 1995; Amer et al., 2001). Thus, the aim of the present study was to link stem cell production and viability to donor body size [total length (T_L) and total weight (T_W)] and the sex steroid hormones, testosterone (T) and 11-ketotestosterone (11-KT).

2. Materials and Methods

2.1. Catfish husbandry

Fish for the current experiment were obtained from 0.25 ha aquaculture ponds that were ~4 feet deep and located at Fish Genetics Research Unit at Auburn University, AL, USA (32.6622° N, 85.4960°W). These fish were fed a standard commercial catfish feed (Purina Catfish 32, floating feeds; crude protein: ≥32.00%, crude fat: ≥4.00%, crude fiber: 7.00% and phosphorus 0.80%) once per day up to satiation. Dissolved oxygen (DO) was monitored twice daily (06:30 and 16:30) while temperature and salinity were recorded twice per week using an YSI 55 multi-parameter instrument (605056, YSI). Total ammonia-N (TAN), nitrite, nitrate, pH, hardness, and alkalinity were also measured twice per week using a water quality test kit (19541-931/19543-91, Easy Strips Tetra). During the sampling period, DO, temperature, salinity, pH, TAN, nitrite, nitrate, hardness and alkalinity levels were 6.9 ± 2.0 mg/L, 28.8 ± 1.8 ^{o}C, 0.1 ± 0.01 g/L, 7.2 ± 0.6, 0 mg/L, 0 mg/L, 0 mg/L, 75 ± 15 mg/L, and 0 mg/L, respectively. Fish were selected to encompass a wide range in size (10 to 75 cm) to ensure that discrete developmental stages were collected during June – August. Fish were collected by seining the pond with a 1¼ inch mesh seine net. Consequently, fish were anesthetized using 300 mg/L solution of Tricaine Methanesulfonate (MS-222) and transferred to the laboratory on ice for the determination of T_L (± 0.1 cm), T_W (± 0.05 g), stem cell isolation, stem cell quantification, and sex steroid analysis, which are mentioned below.

2.2. Stem cell isolation and quantification

The external body area of each catfish was sterilized with 70% ethanol and then stem cells were isolated according to methods described by Shang et al. (2015). In brief, testes or ovaries were carefully removed from the peritoneal cavity to avoid contamination with connective tissues, the peritoneum, and blood vessels. After recording weights, the testes and/or ovaries were placed on a sterile petri dish (100 mm × 15 mm) containing 5 mL of anti-agent medium [Hanks' Balanced Salt solution (HBSS, SH30031.03, GE Healthcare Life Sciences) supplemented with 1.0 μg/mL $NaHCO_3$ (Church & Dwight Co., NG) and 100 unit/mL Penicillin - Streptomycin (I15140-122, Life Technologies)] which were then transferred to a biosafety cabinet for cleaning and sterilization. Within the biosafety cabinet, any connective tissue and blood cells were removed using sterile scalpel blades. Testes and ovaries were rinsed three times with 1 mL of anti-agent medium, and then soaked in a 5 mL 0.5% bleach solution prepared with double-distilled H_2O for 2 min. This was followed by three rinses with HBSS and three rinses with phosphate-buffered saline (PBS; J62692, Alfa Aesar). Each testis or ovary was then minced with a sterilized scalpel blade and transferred to a 50 mL autoclaved glass flask with stir bar. To each sample, 0.25% trypsin - Ethylenediamine tetraacetic acid (EDTA; 25200-072, Life Technologies) was added at 50 times of the weight of the testis or ovary. Samples were then incubated on crushed ice for 30 min followed by 1 h at 22 °C with a magnetic stirrer to achieve higher digestion efficiency. The cell suspension from each replicate was then filtered using a 40 μm cell strainer with nylon mesh (352340, VWR International) and centrifuged at 2900 RPM (Eppendorf Centrifuge 5418 R) for 20 min to separate cells from trypsin. The resulting supernatant was discarded, and the pellet was resuspended in 2 mL of Dulbecco's Modified Eagle's Medium/DMEM [DMEM (10-090-CV, Corning cellgro,) supplement with 10% fetal bovine serum (FBS; 10438018, Life Technologies), 100 unit/mL Penicillin - Streptomycin (15140-122, Life Technologies), and 200 mM L-glutamine

(A2916801, Life Technologies) to provide a favorable environment for the cells. Following this, 5 µL of cell suspension 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), supplemented with a 20× objective, to determine the total number of cells, total number of live and dead cells, total number of live and dead SSCs, and total number of live and dead OSCs with the aid of 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), as mentioned below. Three counts were done for each sample and mean used for further analysis, where the total number of cells per mL = (total number of counted cells × dilution factor × 10^4) / number of squares.

2.3. Sex steroid analyses

2.3.1. Blood Sample collection

Blood (~1 mL) was collected from the caudal vein of male catfish (n =30) in order to quantify T and 11-KT levels using 19 G 1.5 needles (305187, Cayman chemicals). Immediately after collection, the samples were held in 1.5 mL eppendorf tubes (without anticoagulant) for 24 h at 4 °C. Thereafter, they were centrifuged (3000 RPM for 15 min at 4 °C with Eppendorf Centrifuge 5418 R) for serum separation. Serum was stored at -40 °C for further analysis.

2.3.2. Enzyme-linked immunosorbent assays (ELISA)

Serum T and 11-KT were quantified using Cayman ELISA kits (11-KT ELISA, 582751, Cayman Chemical and T ELISA, 582701, Cayman Chemical) following manufacturer's instructions. Standards (8 × 50 µL) or serum samples (50 µL) were added to 50 µL of T-acetylcholinesterase (AChE) conjugate (T tracer) and 50 µL of T-specific rabbit antiserum in a 96-well plate and incubated for 2 h at room temperature (~21 °C). The plate was then washed five times with wash buffer to remove any unbound reagents and 200 µL of Ellman's reagent (contains substrate to AChE) was added to each wall. The plate was covered with plastic film and placed on an orbital shaker (Standard Analog 1000 Orbital Shaker, Troemner™ Talboys™) and kept in the dark for 60 min. The yellow intensity in each well was measured using a microplate reader (Multiskan™ FC Microplate Photometer) at 405 nm. Assay sensitivity (80% B/B_0) was 6 pg/mL, in the range of 3.9 to 500 pg/mL. The procedure for 11-KT was identical to those specified above, except that 11-KT-acetylcholinesterase (AChE) conjugate was used and the rabbit antiserum binding sites were 11-KT specific. In addition, the incubation was performed at 4 °C for 18 h, and the assay developed on an orbital shaker in the dark for 90 min before measuring using a microplate reader at 405 nm. Sensitivity (80% B/B_0) was 1.3 pg/mL, in the range of 0.78 to 1000 pg/mL.

2.4. Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA). Quadratic regressions were used to relate (i) T_L and T_W to the number of live

SSCs and OSCs and (ii) T_L and T_W to sex steroid levels in males (PROC REG; SAS Institute 2003). Quadratic equations were chosen a-priori to fit the data based on the available literature (Siemsen et al., 2010). The T_L, T_W, T and 11-KT yielding the greatest quantity of stem cells was determined using the first derivative of the quadratic equation set to 0 (Odibat and Momani, 2008). Additionally, Alpha was set at 0.05.

3. Results

Blue catfish males ranged from 10.2 to 74.8 cm (Fig. 1A) and weighed from 60.4 to 1100.5 g (Fig. 1B). Up to 9.321×10^5 live SSCs (Type A) were extracted from these males. There was a positive quadratic relationship between T_L and the number of extracted live SSCs ($y = -0.0066x^2 +0.4947x - 3.4616$; $r^2 = 0.50$; $P < 0.0001$; Fig. 1A) as well as T_W and live SSCs ($y = 1.8845E-005x^2 + 0.0137x+ 3.0045$; $r^2 = 0.37$; $P < 0.0001$; Fig. 1B). Setting the derivative to zero provided the T_L and T_W at which the maximum number of live SSCs could be extracted. Based on this regression, stem cells can be extracted from 38.1 cm fish or those that weighed 400.2 g to obtain the most SSCs for future germplasm banking and xenogeneic transplantation. Similar positive quadratic relationships were also detected between the T_L ($y = -0.0292x^2+ 1.7843x-19.4856$; $r^2 = 0.77$; $P < 0.0001$; Fig. 1C) and T_W ($y = -5.1063E-005x^2+0.0423x-1.3481$; $r^2 = 0.65$; $P < 0.0001$; Fig. 1D) of females and the number of extracted live OSCs. According to this regression, maximum OSCs can be isolated from 31.2 cm or 410.1g blue catfish females.

The sex steroids, T and 11-KT were measured in male blue catfish and ranged from 48.9 to 53.6 pg/mL and 35.1 to 54.9 pg/mL, respectively (Fig. 2). Positive quadratic relationships were also detected between T ($y = -1.0212 x^2+ 103.9297 x- 2638.4$; $r^2 = 0.59$; $P < 0.0001$; Fig. 2A) and

11-KT ($y = -0.0821 x^2+7.1804 x- 149.594$; $r^2 = 0.69$; $P < 0.0001$; Fig. 2B) of males and the number of extracted live SSCs. Based on this regression, the maximum number of live SSCs can be isolated when T and 11-KT profiles, are 50.9 pg/mL or 44.7 pg/ml 11-KT, respectively.

4. Discussion

Over the past 15 years, the United States catfish industry has steadily shifted towards the production of hybrid catfish, which now represent 70% of the total annual catfish production (Torrans and Ott, 2018). Induced spawning followed by artificial fertilization is the main technology used to produce hybrid catfish fry (Dunham et al., 1986; Bart and Dunham, 1990; Dunham and Brummett, 1999; Dunham et al., 2008; Green and Rawles, 2010). Unfortunately, this is a labor-intensive procedure that requires sacrificing males for testes and sperm production (Hu et al., 2011). Thus, new and innovative techniques and technologies for producing large quantities of fry are urgently needed (Bosworth et al., 2005). Consequently, xenogenesis has been identified as a method to address those inefficiencies in hybrid catfish breeding. Ultimately, this procedure works by transplanting PGCs from developing embryos, SSCs, or OSCs into a host species. These cells then have self-renewal ability and the capability of transmitting genes between the two successive generations for facilitating artificial spawning between species that may be difficult to breed in captivity (Perera et al., 2016; de Siqueira-Silva et al., 2018).

The success of SSCs transplantation through xenogenesis has been highlighted in the literature using various species (Silva et al., 2006; Okutsu et al., 2006; Okutsu et al., 2007; Takeuchi et al., 2009; Morita et al., 2010; Lacerda et al., 2013; Farlora et al., 2014; Pacchiarini et

al., 2014; Perera et al., 2016; Ye et al., 2017). In all experiments, donor cells were collected from randomly selected immature donor species, rather than having precise prior knowledge on the relationship between the quantity or quality of extracted donor cells and size characteristics of the donor fish, which is critical to select the best donor stage to increase efficiency of germ cell transplantation. Filling this research gap, our current study identified the relationship between live SSCs and T_L of blue catfish males, which resulted in a nonlinear polynomial dome shaped function. Based on this regression, the optimal length of blue catfish males for production of SSCs was determined to be 38.1 cm. A gradual reduction of SSCs following a specific stage of maturation was noted by Dadziea and Wangila (1980), which was assumed to be due to active transformation of those cells into successive stages. Perera et al. (2016) demonstrated the success of blue catfish SSCs transplantation to mature triploid channel catfish, where two-year-old sexually immature male blue catfish with a mean length of 31.8 cm were used to collect donor cells up to 4.5×10^5. Thus, they have used a sub-optimal size of blue catfish for stem cell extraction then would be predicted, based on our regression function.

In line with this relationship between live SSCs and T_L, a significant positive quadratic relationship was also detected between SSCs and T_W of blue catfish males, where 400.2 g was regarded as the optimum weight to yield the maximum number of live SSCs. Despite these results, no work has revealed such relationships between quantity of donor cells and size characters of donor fish, as studies have mostly focused on evaluating relationships between size and reproductive indices, such as gonadosomatic index (GSI) and sperm density (Stockley et al., 1997; Poole and Dillane, 1998; Nash et al., 1998; Minier et al., 2000; Fontoura et al., 2009; Agarwal et al., 2009; Islam et al., 2012). Therefore, using this new information will increase the availability of SSCs for transplantation and creation of catfish xenogens.

Numerous examples exist for successful OSC transplantation using numerous donor species, such as 6- to 9-month old rainbow trout with mean a T_L of 25.2 cm (Yoshizaki et al., 2010), pejerrey with a mean of T_W of 310 g and T_L 34.2 cm (Majhi et al., 2014), as well as three-month old zebrafish (Wong et al., 2011) and Chinese sturgeon (Ye et al., 2017). However, in all these studies, no prior information or special techniques were used during the selection process; i.e. immature female were collected. Therefore, the resulting regressions from our study can also be used as a biomarker to predict the number of OSC for harvest from different sized female blue catfish.

Cell differentiation during spermatogenesis is regulated by sex steroid hormones, mainly T and 11-KT (Plant and Marshall, 2001; Koulish et al., 2002; McLachlan et al., 2002; Schulz and Miura, 2002). Sex steroid hormones synthesized in the fish testis (Vizziano et al.,1996; Wei-Xin, 1988; Miura et al., 1999) can regulate major mechanisms of spermatogenesis, such as spermatogonial cells A and B multiplication, proliferation, spermatocyte formation, and cell differentiation up to spermatozoa (Billard et al., 1982; Fostier, 1983; Miura et al., 1991b; Pudney, 1995; Hess et al.,1995; Cavaco et al., 2001; Magee et al., 2006). Since the success of SSCs transplantation for xenogenesis is mainly dependent upon the quality and quantity of donor stem cells, the relationship between levels of sex hormones and germ cell development is expected to give reliable data for selecting the best stages for donor fish, rather than sacrificing individuals to reveal similar information. Based on Campbell et al. (2003) and Fostier et al. (1982), elevation of plasma T and 11-KT indicated the start of spermatogonial proliferation during the annual reproductive cycle. Furthermore, they showed a positive association between particular sex steroids and the spermatogenesis cycle. Observations of our study coincide with findings by Campbell et al. (2003) and Fostier et al. (1982), where a gradual decline of SSCs type A (after the

peak) could be due to germ cell differentiation from spermatids into spermatozoa. Similar observations during spermatogenesis corresponding to different levels of T and 11-KT are also available for numerous species including tilapia, *Oreochromis niloticus* (Tokalov and Gutzeit,2015), common carp (Koldras et al., 1990), Japanese eel, *Anguilla japonica* (Miura et al., 1991a), goldfish (Kobayashi et al., 1991), African catfish (Vermeulen et al., 1994), medaka, *Oryzias latipes* (Saiki et al., 1997), Japanese huchen, *Hucho perryi* (Amer et al., 2001), and zebrafish (Sakai, 2002). Though the functional characteristics of T and 11-KT are similar across studies, the ranges and peak levels of particular hormones differ due to differences in species, age of individuals, and analytical methods. Nevertheless, our results reveal a direct relationship between T and 11-KT in catfish which could be used a biomarker to determine the optimal time for donor cell extraction.

In conclusion, the relationships between quantity of stem cells and body size and the level of sex steroid hormones revealed promising results as reliable biomarkers to enhance the efficiency of germ cell transplantation. However, when considering the coefficient of determination (r^2) values of each model as an estimation of goodness of fit, T_L has a higher accuracy in predicting number of stem cells (r^2 values of 0.50 and 0.77 respectively in male and female fish) compared to the T_W of fish (r^2 values of 0.37 and 0.65 respectively in male and female fish). Even though T and 11-KT can reliably predict the number of stem cells in blue catfish (i.e. $r^2 > 0.69$) these parameters seems to have less practical, since their quantification is more time consuming and costly. Ultimately, these data could be used to make precise predictions on the number of stem cell at a specific body size, without sacrificing fish to enhance the efficiency of germ cell transplantation in xenogenesis.

5. Reference

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6. Figures

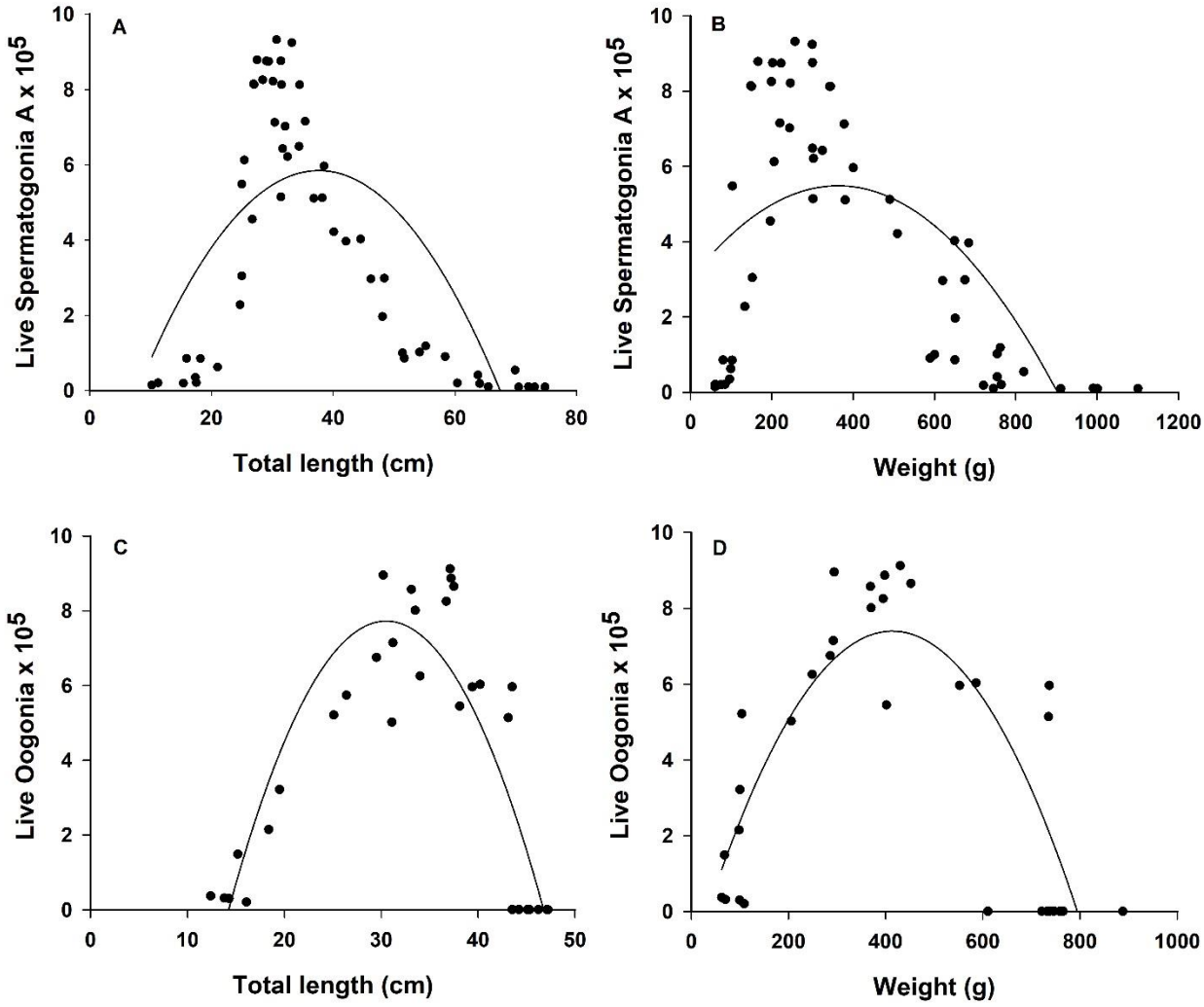

Fig. 2.1. Polynomial regressions ($y = -0.0066x^2 + 0.4947x - 3.4616$; $r^2 = 0.50$; $P < 0.0001$) between live spermatogonial stem cells (SSCs) and total length (T_L) of blue catfish (*Ictalurus furcatus*) males (Fig 1-A), live SSCs and total weight (T_w) ($y = 1.8845E\text{-}005x^2 + 0.0137x + 3.0045$; $r^2 = 0.37$; $P < 0.0001$) of blue catfish (*I.furcatus*) males (Fig 1-B), live oogonial stem cells (OSCs) and T_L ($y = -0.0292x^2 + 1.7843x - 19.4856$; $r^2 = 0.77$; $P < 0.0001$) of blue catfish (*I.furcatus*) females (Fig 1-

C) and live OSCs and T_w (y = -5.1063E-005x^2+0.0423x-1.3481; r^2 = 0.65; P < 0.0001) of blue catfish (*I.furcatus*) females (Fig 1-D) raised in earthen ponds.

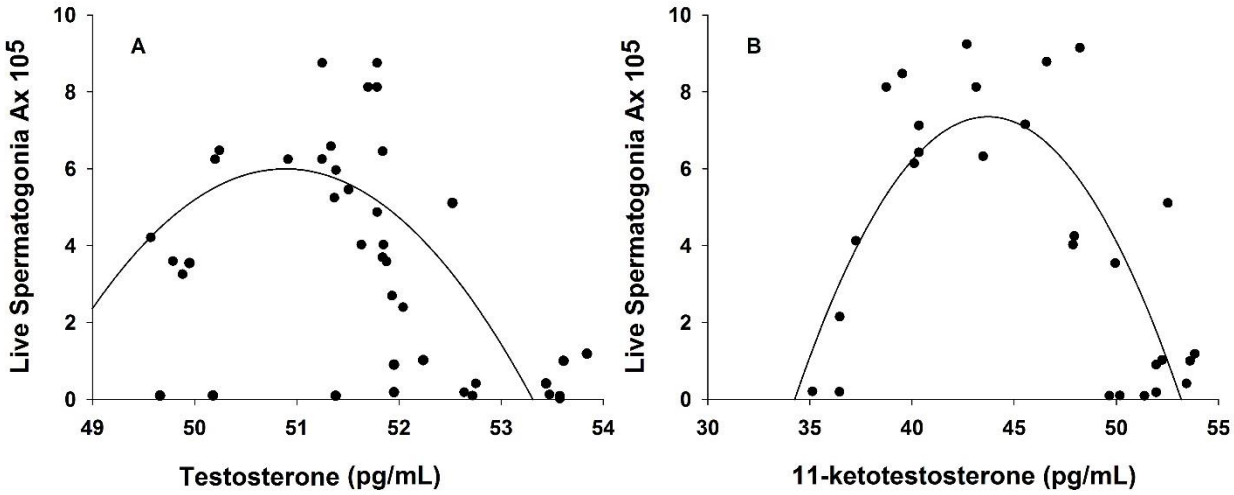

Fig. 2. Polynomial regressions between live spermatogonial stem cells (SSCs) and testosterone (T) (y = -1.0212 x^2+ 103.9297 x- 2638.4; r^2 = 0.59; P < 0.0001) of blue catfish (*I.furcatus*) male (Fig 2 -A), Live SSCs and 11-ketotestosterone (11-KT) (y = -0.0821 x^2+7.1804 x- 149.594; r^2 = 0.69; P < 0.0001) of blue catfish (*I.furcatus*) male (Fig 2- B).

CHAPTER III

EFFECTS OF SEASONALITY AND SEX STEROID HORMONES FOR OPTIMIZATION OF STEM CELL EXTRACTION FROM MATURE BLUE CATFISH (*ICTALURUS FURCATUS*) TO PRODUCE ALLOXENOGENIC CATFISH.

Abstract

Xenogenesis can be used an innovative tool for hybrid catfish (♀ channel catfish, *Ictalurus punctatus* × ♂ blue catfish, *I.furcatus*) seed production. This xenogeneic process can be accomplished by transplanting diploid 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. Usually, stem cells are collected from immature fish, however, there is potential to collect donor-derived cells from mature fish during certain seasons of the year. The current study evaluates seasonal variations in germ cell count in mature fish (> 50 cm total length). Live SSCs type A production ranged from 0.19×10^4 to 7.053×10^4 per fish in blue catfish males, where the highest production was recorded in April (7.053×10^4). SSCs counts gradually decreased from April to November, where the lowest production ($P < 0.0001$) was reported in November (0.19×10^4) and thereafter gradually increased from December onwards. Mean GSI of blue catfish males ranged from 0.067% to 0.318%, which was significantly higher in November, December, and January compared to levels reported in July, August, and September. The highest quantity of live OSCs ($P < 0.0001$) in female blue catfish were observed in April (9.6×10^2) which gradually decreased to zero over the months of May, June and July. No OSCs were extracted during the months of August to February because ovaries

with full of immature oocytes. GSI levels of females remained consistent in the range of 11.3% - 6.7% from April to February, which showed no significant differences between the months. Results reflect a clear temporal variation in live SSCs type A and GSI in mature males, while the count of live OSCs were found to be extremely low (9.6×10^2 to 0) in mature female blue catfish throughout the year. This reveals the potential of using mature blue catfish males as donor species during the months of April, May and July, but at the cost of time and labor intensiveness in cell isolation procedures (specifically in trypsinization and filtering) compared to the process in immature fish.

Key words: xenogenesis; blue catfish; seasonal variation; stem cell; gonado somatic index

1.0 Introduction

The hybrid between channel catfish, *Ictalurus puncatatus* ♀ and blue catfish, *I. furcatus* ♂ is extensively cultured in the Southern U.S. due to their improved performances in growth, disease resistance, dissolved oxygen tolerance, feed conversion, fillet yield, dress out percentage, and harvestability compared to both parent species (Yant et al., 1976; Dunham et al., 1987; Dunham et al., 1999; Brown et al., 2011; Arias et al., 2012; Dunham and Masser, 2012; Perera et al., 2016). Although hybrids have clearly demonstrated performance benefits, challenges still remain for efficiently producing embryos, as manual stripping of gametes for artificial fertilization is labor-intensive and requires the sacrifice of blue catfish males for testes preparation (Argue et al., 2003; Hu et al., 2011). Additionally, blue catfish mature at larger sizes and older ages than channel catfish, resulting in a large financial investment in the male broodstock as well as costs for pond space while waiting for sexual maturity (Argue et al., 2003; Hu et al., 2011; Dunham and Masser, 2012).

Xenogenesis, which is a method of reproduction in which successive generations differ from each other (Dunham, 2011), is a potentially innovative hatchery technology to mitigate some of the challenges in hybrid seed production (Dunham and Argue, 2000; Shang et al., 2015). This process can be accomplished by transplanting diploid primordial germ cells (PGCs), spermatogonial stem cells (SSCs) or oogonial stem cells (OSCs), derived from a donor diploid fish into sterile recipients, which enable recipient fish to produce donor-derived gametes (Yoshizaki et al., 2010; Wong et al., 2011). Since the process of xenogenesis is dependent upon the availability of germ cells from donors, it is crucial to have precise prior knowledge regarding the best donor stage to yield the maximum quantity of high-quality cells. However, the majority of previous

studies (Okutsu et al., 2006; Silva et al., 2006; Okutsu et al., 2007; Takeuchi et al., 2009; Morita et al., 2010; Yoshizaki et al., 2010; Wong et al., 2011; Lacerda et al., 2013; Farlora et al., 2014; Majhi et al., 2014; Pacchiarini et al., 2014; Perera et al., 2016; Ye et al., 2017) on germ cell transplantation were conducted with no prior knowledge on the best donor stage.

Hettiarachchi et al. (unpublished) revealed that immature blue catfish with a total length (T_L) of 38.1 cm and total weight (T_W) of 400.2 g were the best donor body sizes to yield the maximum number of SSCs in male fish, while the highest number of OSCs could be extracted from female fish that were 31.2 cm and 410.1g. Results from this study also revealed that fewer SSCs and OSCs were found in mature fish than in immature fish, which is likely due to the conversion of stem cells to gametes following maturation.

Immature testes contain SSCs together with somatic cells, leydig cells, and sertoli cells. Together, they function in unison to complete spermatogenesis and sperm maturation (Nagahama et al., 1994 and Nishimura and Tanaka, 2014). Spermatogenesis is a highly organized and coordinated process to form mature spermatozoa through proliferation and differentiation of diploid spermatogonia (De Rooij and Russell, 2000). The process of spermatogenesis can be divided in to three different phases based on its function; the mitotic or spermatogonial phase consists of different generations of spermatogonia (undifferentiated spermatogonia A and B or differentiating spermatogonia), the meiotic phase consists of primary and secondary spermatocyte and the final phase is the spermiogenic phase, which produces haploid spermatids with motile, flagellate spermatozoa (Guan et al., 2006; Conrad et al., 2008). The mitotic or spermatogonial phases seems to be the best stage to extract donor cells for germ cell transplantation, since the testis consists of undifferentiated spermatogonia A and B (Ohmura et al., 2004). However, even during the mitotic phase, the variation in number of spermatogonial cells seems to be high

depending on the species, maturity stage, environmental or seasonal variations (Nóbrega et al., 2009).

Though some of the tropical species maintain their spermatogenic activities independent of the season, many species living at higher latitudes tend to show seasonal variations in spermatogenic activity ((Billard, 1986; Kubota and Brinster, 2006). Rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus sp*., *Cirrhinus sp*.), and pike (*Esox lucius*) show active spermatogenesis during summer corresponding to the rise of water temperature while the active spermatogenesis of tench (*Tinca tinca),* common bream (*Abramis brama*), whiting (*Merlangius merlangus*) and sea bream starts during late spring or at the beginning of autumn. Some fish species like killifish mummichog (*Fundulus heteroclitus*), stickleback (*Gasterosteidae*), and common roach (*Rutilus rutilus*) show active spermatogenesis from the beginning of autumn until the following spring (Billard and Breton, 1978; Billard, 1986). Those species exhibiting clear cut seasonal variation in spermatogenesis have proliferating spermatogonia only at the beginning of the spermatogenesis process (Kubota and Brinster, 2006), which fills testicular tubules with only two types of germ cells; large numbers of spermatozoa and some scattered, quiescent type spermatogonia A after the completion of spermatogenesis (Billard, 1986). In contrast, species that do not show clear cut seasonal variation in reproduction have all different types of germ cells, such as spermatogonia and spermatocytes throughout the year such as in common goldfish (*Carassius auratus*) and guppy (*Poecilia reticulata*) (Billard,1986).

In females, follicle stimulating hormone (FSH), luteinizing hormone (LH), progestin and estrogen seem to induce the process of oogenesis in ovaries (Miura et al., 2007) which initially contains oogonia and inactive somatic cells. The process of oogenesis can be divided into four different phases based on morphological and physiological features; the proliferation phase,

primary growth phase, secondary growth phase, and maturation phase (Le Menn et al. 2007). Formation of PGCs, transformation of PGCs into oogonia and subsequently into primary oocytes happens during the proliferation phase (Le Menn et al., 2007; Selman et al., 1993; Wallace and Selman, 1981), which is the best stage to collect OSCs for germ cell transplantation (Lubzens et al., 2010). However, similar to the observations in males, seasonal changes such as atmospheric and water temperature have direct effects on hormonal mechanisms of fish, which ultimately controls oogonial proliferation in mature fish (Wildner et al., 2013). Therefore, in the process of collecting germ cells from mature fish, it is critical to have precise information on seasonal variations of the germ cell count, which was not explored previously in blue catfish.

The present study evaluates the effect of seasonal changes on quantity of stem cells in mature fish, with the objective of determining the potential to use mature fish as a donor species in germ cell transplantation.

2.0 Materials and Methods

2.1. Catfish husbandry

Blue catfish for this experiment were collected from 0.25 ha aquaculture ponds that were ~1.2m deep and located at the Fish Genetics Research Unit at Auburn University, AL, USA (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 twice per week using a water quality test kit (19541-931/19543-91, Easy Strips Tetra, Tetra Holding (US), VA). Mature fish, > 50 cm total length (T_L) were collected monthly over the full annual cycle by seining ponds with a $3\times3cm^2$ mesh sized seine net. Consequently, fish were anesthetized using a buffered 300 mg/L solution of tricaine methanesulfonate (MS-222) and transferred to the laboratory on ice for the determination of T_L (± 0.1 cm), total weight (T_W) (± 0.05 g), gonadal weight (± 0.05 g), gonado-somatic index (GSI), and stem cell counts.

$$\text{GSI} = \frac{Gonad\ Weight\ (g)}{Total\ tissue\ weight\ (g)} \times 100$$

2.2. Stem cell isolation and quantification

The external body area of each catfish was sterilized with 70% ethanol and then stem cells were isolated according to methods described by Shang et al. (2015). In brief, after recording T_L, T_W, and gonadal weight of fish, the testes or ovaries were carefully removed from the peritoneal cavity to avoid contamination with connective tissues, the peritoneum, and blood vessels. Testes or ovaries were placed on a sterile petri dish (100 mm × 15 mm) containing 5 mL of anti-agent medium [Hanks' Balanced Salt solution (HBSS, SH30031.03, GE Healthcare Life Sciences, MA) supplemented with 1.0 μg/mL $NaHCO_3$ (Church & Dwight Co., NG) and 100 unit/mL Penicillin - Streptomycin (I15140-122, Life Technologies,CA)] which were then transferred to a biosafety cabinet for cleaning and sterilization. Within the biosafety cabinet, any connective tissue and blood cells were removed using sterile scalpel blades. Testes and ovaries were rinsed three times with 1 mL of anti-agent medium, and then soaked in a 5 mL 0.5% bleach solution prepared with double-distilled H_2O for 2 min. This was followed by three rinses with HBSS and three rinses with phosphate-buffered saline (PBS; J62692, Alfa Aesar, MA). Each testis or ovary was then minced with a sterilized scalpel blade and transferred to a 50 mL autoclaved glass flask with a stir bar. For each sample, 0.25% trypsin ethylenediamine tetraacetic acid (EDTA; 25200-072, Life Technologies) was added at 50 times of the weight of the testis or ovary. Samples were then incubated on crushed ice for 30 min followed by 1 h at 22 °C with a magnetic stirrer to achieve higher digestion efficiency. The cell suspension from each replicate was then filtered using a 70 μm (352350, VWR International) and 40 μm (352340, VWR International) cell strainer with nylon mesh and centrifuged at 2900 RPM (Eppendorf Centrifuge 5418 R) for 20 min to separate cells

from trypsin. The resulting supernatant was discarded, and the pellet was resuspended in 2 mL of Dulbecco's Modified Eagle's Medium [DMEM (10-090-CV, Corning cellgro)] supplement with 10% fetal bovine serum (FBS; 10438018, Life Technologies), 100 unit/mL penicillin-streptomycin (15140-122, Life Technologies), and 200 mM L-glutamine (A2916801, Life Technologies) to provide a favorable environment for the cells. Thereafter, 5 µL of the cell suspension was gently mixed with 45 µL of 0.4% trypan blue (15250061, Life Technologies). Cells (10 µL) were then observed under a BH2 Olympus objective microscope, supplemented with a 20× objective, to determine the total number of cells, total number of live and dead cells, total number of live and dead SSCs, and total number of live and dead OSCs with the aid of a hemocytometer. Number of cells were counted in four corner quadrants (each 1 mm^2 area) of the hemocytometer in triplicate and cell density (cells/mL) was calculated according to Louis and Siegel (2011) with the dilution factor of 10 (cell suspension: trypan blue with 1:9 ratio).

$$\text{Total number of cells per mL} = \frac{\text{total number of counted cells } \times \text{ dilution factor } \times \text{ 10\^4}}{\text{number of squares}}$$

2.4. Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro-Wilk test; PROC UNIVARIATE; SAS Institute, 2003) and homogeneity of variance (plot of residuals vs. predicted values; PROC GPLOT; SAS Institute, 2003). Stem cell data was log transformed (Zar, 1996). Alpha was set at

0.05 for main effects and interactions. Temporal changes in stem cell production and GSI were analyzed using repeated measures mixed model ANOVA (PROC MIXED).

3.0 Results

Live SSCs type A production ranged from 0.19×10^4 to 7.053×10^4 in blue catfish males, and the highest valves were recorded in April (7.053×10^4). SSCs type A counts gradually decreased from April to November with the lowest density, 0.19×10^4, reported in November followed by a gradual increase from December onwards (Figure 1). No significant differences in live SSCs type A was revealed between the months of April, May and June, while the SSCs type A count in male blue catfish during these months were significantly higher ($P < 0.0001$) than that the cell count in July- January. No significant differences in the cell count were revealed between the months of October and November, which showed the lowest numbers compared to the SSCs type A in rest of the months studied. Mean GSI of blue catfish males ranged from 0.067% to 0.318%, which was higher ($P < 0.0001$) in the months of November, December and January compared to the GSI levels in July, August and September (Figure 2). Lowest GSI was observed in July (0.068%), while the highest was noted in November (0.318%) with a similar pattern observed in SSCs type A abundance (Figure 1).

The highest number of live oogonia cells in female blue catfish were observed in April (9.6×10^2) which was gradually decreased to zero over the months of May, June and July. No oogonial cells were extracted during the months of August to January (Figure 2). Unlike in males, GSI levels of females remained consistent with no differences (P) among months, and ranging

from 11.3% - 6.7% from April to January in the range of 11.3% - 6.7% from April to January which showed no significant differences between the months (Figure 2).

4.0 Discussion

Results from the current study revealed the importance of April to June to collect maximum number of SSCs type A from mature blue catfish males compared to rest of the year (from July to February). However, all these values during April to June were significantly lower than that in immature blue catfish males (T_L of 38.1 cm and T_W of 400.2g), which had mean SSCs count of 9.321×10^5 during the months of April- August (Hettiarachchi *et al.*, unpublished). The least number of SSCs type A were observed in the months of September and October indicating that, this is an inefficient time for sampling mature male blue catfish during particular months to use as a donor fish in xenogenesis. Water temperature and SSCs number fluctuation has a same pattern during this period. Higher number of live SSCs type A obtained while having high water temperature and when water temperature gradually decreasing harvested cell number also decreased. Similar patterns of variations in SSCs type A count during and following the spawning season were noted by Billard (1986) in common roach (*Rutilus rutilus*), Scott and Sumpter (1988) in rainbow trout (*Salmo gairdneri*) and Mohamed et al (2001) in tench (*Tinca tinca*) which was assumed to be due to the active transformation of those cells into successive stages to produce spermatozoa. In addition, residual spermatozoa left in the testis or spermatic duct system were found to be removed by Sertoli cells via phagocytosis at the end of the spawning season (Billard

et al., 1972; Van den Hurk et al., 1978) indicating the onset of a new cycle with increasing the number of SSCs type A (Van den Hurk et al., 1978; Brandão et al, 2013).

In blue catfish males, consistent GSI values were recorded in October to June except for a decline in July, August and September, reaching to its lowest value at the end of the spawning period (in July). Similar decline at the end of the spermiogenesis phase in GSI was noted in rainbow trout (*Oncorhynchus mykiss*) (Scott and Sumpter,1988), in Japanese huchen (*Parahucho perryi*) (Amer et al.,2001), in blue spotted tilapia (*Oreochromis leucostictus*) (Siddiqui ,1977) and in long whiskers catfish (*Mystus gulio*) (Khan et al .,2002) due to the elimination and phagocytosis of the spermatid cytoplasm by Sertoli cells (Billard et al., 1972; Van den Hurk et al., 1978).

Live OSCs count of mature blue catfish females in April was significantly higher than that of May, June and July. However, all these values were significantly lower than that in immature blue catfish (total length of 31.2 cm and total weight of 410.1g), with observed mean OSCs count of 9.124×10^5 during the months of April- August (Hettiarachchi et al., unpublished). These results reveal the efficacy of collecting stem cells from immature female fish compared to mature females for the purpose of germ cell transplantation in xenogenesis. Extremely low levels of OSCs in April-July or non-availability of OSCs during the rest of the months in mature fish could be due to the interference of reproductive mechanisms, which resulted in ovaries full of immature oocytes. In the early maturing phase, ovaries are full of eggs that are a deep yellow color, which occupy 2/3 to 3/4 of the body cavity (West and Grant, 1990; Brandão et al, 2013). This could be the reason for the significantly higher GSI values of mature female blue catfish (GSI in the range of 6.71-11.31 in 1.5kg female fish) observed during the current study compared to the immature females (GSI of 0.109 in 400g fish) (Hettiarachchi et al., unpublished).

In conclusion, the present study revealed the probability of using mature male and female blue catfish to extract SSCs type A and oogonia, during certain periods of the year. Clear temporal variations were observed in live SSCs type A and GSI in males, while the count of live oogonia cells was found to be extremely low or zero in mature female blue catfish compared to immature females. The result indicates the potential to use mature blue catfish males as donor species during April, May and July, but less success due to time and labor intensity in cell isolation procedures (specifically in trypsinization and filtering) and yield of fewer stem cell numbers add further challenges to use of mature fish compared to immature fish.

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6. Figure

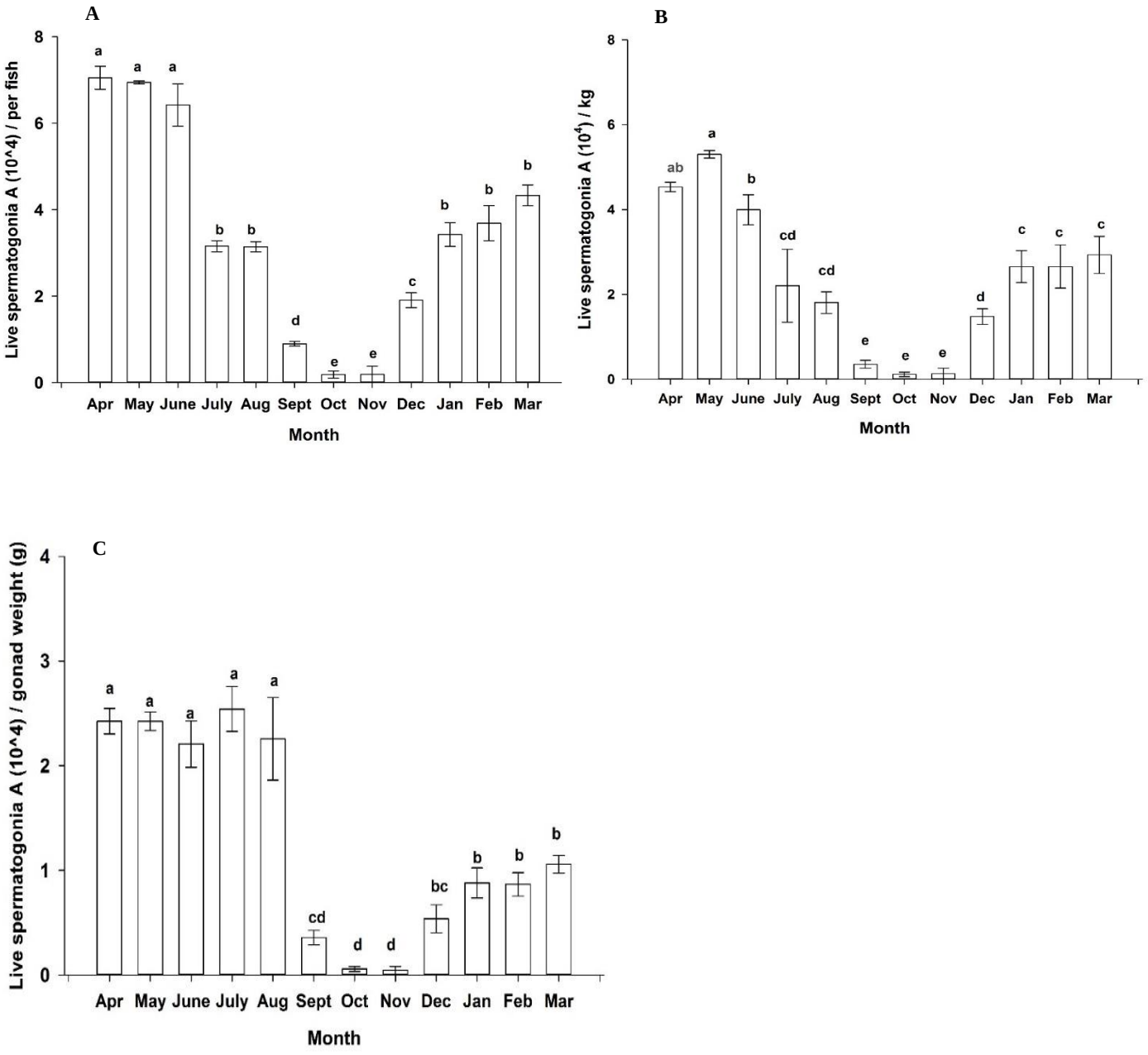

Fig.3.1. Change in the number of live spermatogonial stem cells (SSCs) type A in adult blue catfish males (> 50cm) (*Ictalurus furcatus*) per fish (Fig 3-A), per kg of fish (Fig 3-B) and per gonad weight (Fig 3-C).

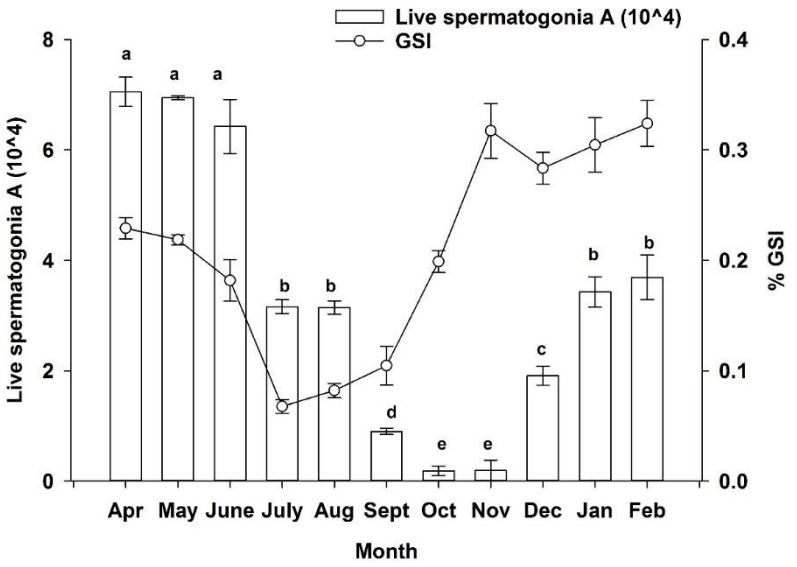

Fig.3.2. Change in the number of live spermatogonial stem cells (SSCs) type A and gonadosomatic index (GSI) during the April to February period in adult blue catfish males (> 50cm) (*Ictalurus furcatus*) (mean ± SEM).

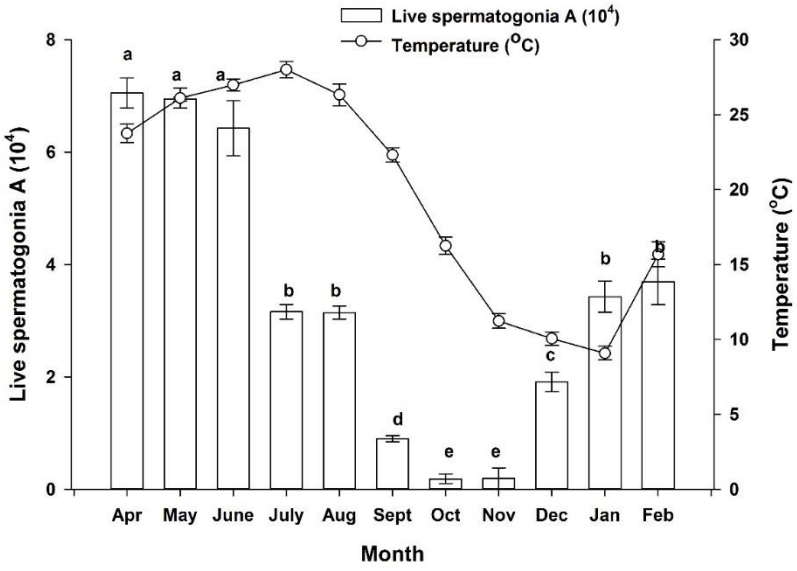

Fig.3.3. Change in the number of live spermatogonial stem cells (SSCs) type A and temperature during the April to February period in adult blue catfish males (> 50cm) (*Ictalurus furcatus*) (mean ± SEM).

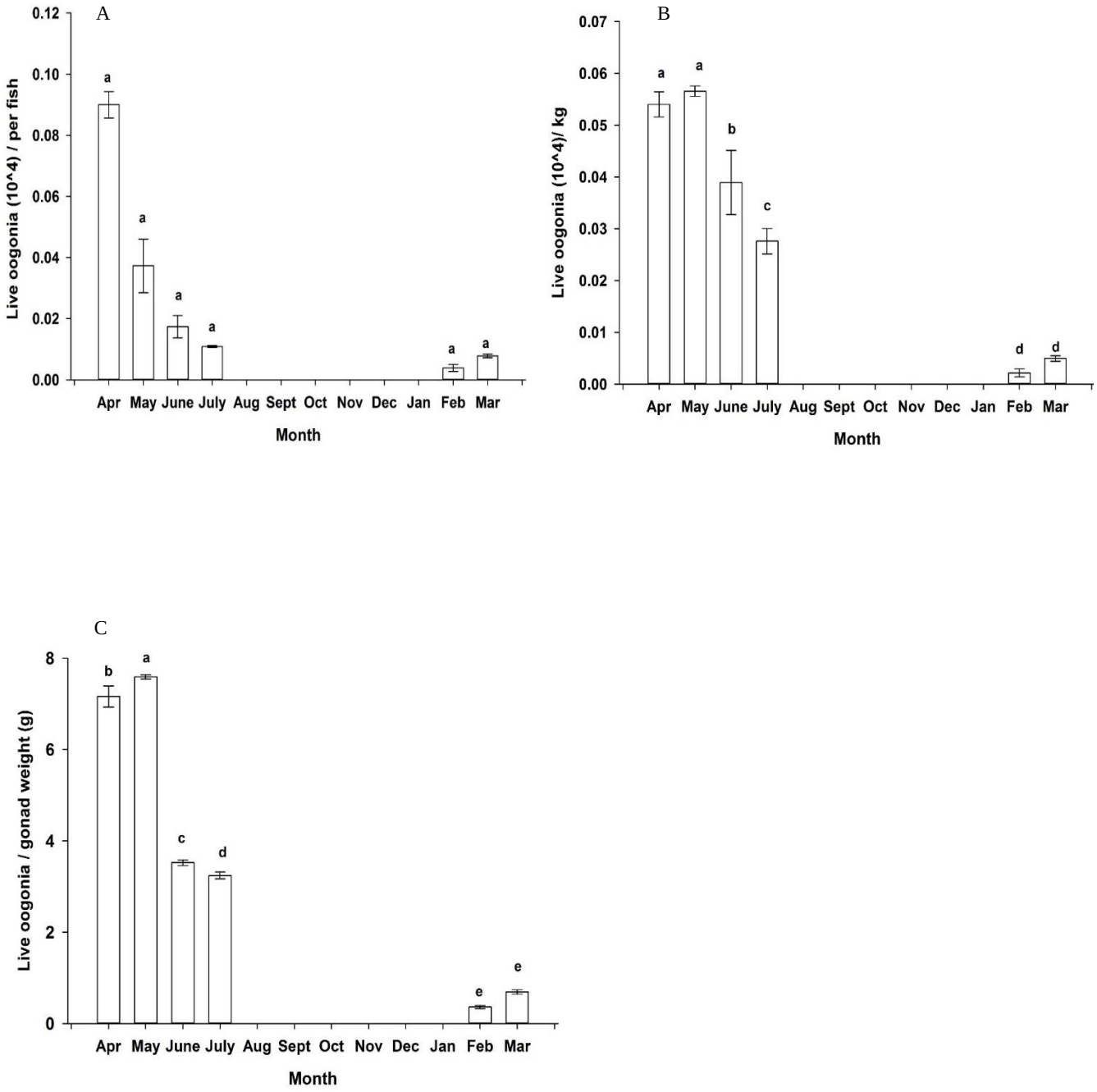

Fig.3.4. Change in the number of live oogonial stem cells (OSCs) in adult blue catfish females (> 50cm) (*Ictalurus furcatus*) per fish (Fig 3.4-A), per kg of fish (Fig 3.4-B) and per gonad weight (Fig 3.5-C).

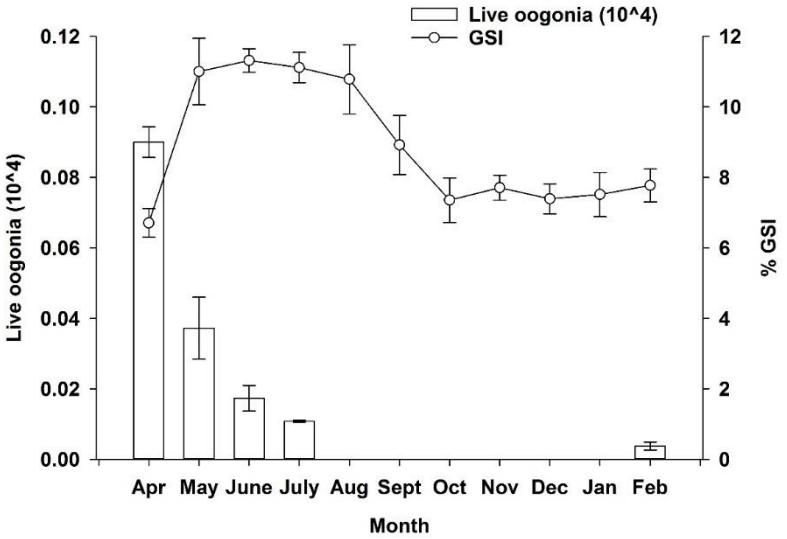

Fig.3.5. Change in the number of live Oogonial stem cells (OSCs) and gonadosomatic index (GSI) during the April to February period in adult blue catfish females (> 50cm) (*Ictalurus furcatus*) (mean ± SEM).

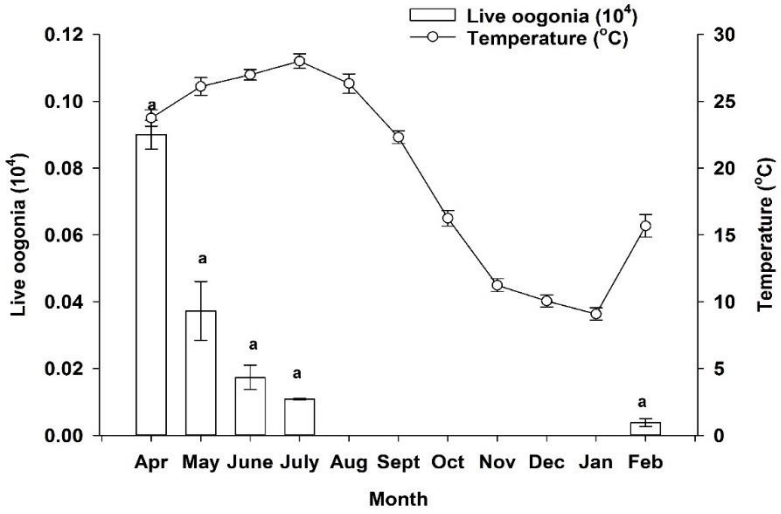

Fig.3.6. Change in the number of live Oogonial stem cells (OSCs) and water temperature during the April to February period in adult blue catfish females (> 50cm) (*Ictalurus furcatus*) (mean ± SEM).