

**Approaches to Improve Production and Performance of Channel Catfish
(*Ictalurus punctatus*) Female x Blue Catfish (*I. furcatus*) Male Hybrid Catfish**

by

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Abstract

Xenogenesis was studied as a method for the production of hybrid catfish fry via mating xenogenic males with normal channel catfish *Ictalurus punctatus* females. Spermatogonial type A cells were isolated from blue catfish *I. furcatus*. Cells were transplanted into confirmed triploid channel catfish. The live cells were introduced to the gonads of the host via catheterization or by surgery followed by injection. A mean of 5.23×10^5 cells ($2 \times 10^4 - 1.43 \times 10^6$) were introduced by catheterization and mean of 7.25×10^5 cells ($5 \times 10^4 - 1.8 \times 10^6$) cells injected via surgical injection. DNA was analyzed from biopsies of the gonads and 80% of the fish were xenogenic, having blue catfish cells in their gonads. Ten months after implantation, a sample of 8 males were tested and 7 were xenogenic. The xenogenic males successfully courted normal channel catfish that had been hormone induced, but none of the eggs hatched, indicating inadequate sperm production or an inability to ejaculate. However, some males testes were well-developed morphologically, and large scale sperm production was detected in 3 of 7 xenogenic males examined two years after transplantation and after hormone therapy with luteinizing hormone releasing hormone analogue. Sperm was removed from a male that had been surgically transplanted with blue catfish cells, and used to fertilize eggs from a hand-stripped channel catfish female. One percent of these eggs hatched. All seven surviving 6-month-old progeny of this male had the external morphology, swim bladder shape, nuclear DNA profile and mitochondrial DNA profile of F1 channel catfish female

X blue catfish male hybrids. This indicates that the sire was indeed a xenogenic channel catfish producing only sperm from blue catfish and these progeny were produced through xenogenesis. This is the first report of successful production of xenogenic catfish, and the first report of producing 100% hybrid progeny through xenogenesis in fish.

The second study investigated genotype- environment (GE) interactions of eight genetic types of channel catfish female x blue catfish male catfish hybrids. These hybrids were reared in four different environments; a low density pond, high density pond, split pond and in-pond raceway. Feed conversion ratio (FCR) by environment was better than what is usually expected for research and on farm for ictalurid catfish.

The FCR was 1.28, 1.99, 1.65 and 1.52 for the low density pond, high density pond, split pond and in-pond raceway, respectively. Genetic type, sex, environment and all possible interactions affected body weight for the different genetic types of channel catfish female x blue catfish male hybrids ($P < 0.05$). MS X RG and KS X RG were the largest two genetic types in each environment, indicating that a single genetic enhancement program could address the improvement of hybrid performance for all culture systems used in the catfish industry. Hybrids produced by MS and KS females selected for increased body weight for 8 generations were larger compared to hybrids from MR and KR random controls in all environments. GE interactions were observed in regards to the sex, and differences were found ($P < 0.05$) between males and females for final body weight. Survival in high density pond, split pond and in-pond raceway from stocker stage to the harvest in the current experiment was as high as 96%.

Harvestability was impacted by environment ($P < 0.05$). Fish from the high density pond and the in-pond raceway, respectively, were the easiest to capture.

Dedication

To my little brother Abdullah Saud Alsaqfi who is no longer with us but deeply missed and will forever be remembered.

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List of Abbreviations

AU	Auburn University
PGC	Primordial Germ Cell
SSC	Spermatogonial Stem Cell
mtDNA	Mitochondrial DNA
LHRHa	Luteinizing Hormone Releasing Hormone analogue
GE	Genotype-Environment Interaction

CHAPTER ONE

Introduction

The demand for aquatic food and protein is increasing and the supply from global wild caught sources is not expected to increase since it has reached its maximum sustainable yield (FAO 2012; 2014; Griffiths et al. 2015). The only other source that can meet the increasing demand for fish is aquaculture. Since 1970, aquaculture production has been increasing rapidly and it is expected to pass capture fisheries by 2015 (Lowther 2007; Tidwell 2012; FAO 2010; 2013; 2014). However, the human population will continue to grow and the demand for affordable and high quality protein will also be increasing. As a result more efficient production systems are required, and this has encouraged increased research and experimentation in the field of aquaculture (Naylor, et al. 2000). Many essential improvements in aquaculture have been achieved through the enhancement of husbandry procedures, disease diagnosis and treatments and genetics.

Application of genetics has improved production of several aquaculture species (Dunham 2014). Channel catfish (*Ictalurus punctatus*) has been the primary species used in the aquaculture industry in the United States. Over 62 % of the total value of aquaculture production is catfish (National Agricultural Statistic Service 2012). According to the USDA's National Agricultural Statistics Service (2012), the major producing states, Mississippi, Alabama, Arkansas and Texas accounted for 95% of total sales in 2012. Catfish sales fell 20 % in 2012 to \$ 341million from \$423 million in 2011.

Catfish production in 2003 peaked at more than 300 million kg, but declined to 226 million kilograms in 2007 and during 2013 the total production was 150 million kg (NASS 2012; Hanson and Sites 2014).

There are several factors that contributed to the crisis in catfish industry; increasing fuel and feed cost, competition from foreign imports and the crash of the US economy. Water surface area devoted to catfish culture continued to decline as it decreased 7% to 33,589 ha in 2012 from 36,179 ha in 2011. Water surface area used for production in Mississippi, Alabama and Arkansas during 2014 was 25,778 ha (NASS 2014).

Genetic improvement of catfish is important for this industry to maintain sustainability and profitability. Currently, a larger fish of 0.6 kg to 2 kg is preferred for the fillet market (Green and Engle 2007). The traditional production time for channel catfish to reach 400-600 g is lengthy: up to two years (Wellborn 1988), which is a limitation, for which genetics is one tool to address this impediment.

There are two keys for the US catfish industry to survive and thrive. Firstly, it is critical that aquaculture methods implement more efficient and productive culture systems, such as single cropping, in-pond raceways, split-pond partitioned aquaculture systems and the adoption of the F1 hybrid between the channel catfish female and the blue catfish, *I. furcatus*, male (CxB) which has enhanced production traits. The hybrid

will allow easier adoption of improved culture systems (Brown et al. 2011; Brune et al. 2012).

Hybrids between the seven major species of North American catfish (*Ictalurids*) were first evaluated in 1966 (Giudice 1966, Dupree and Green 1969; Chappell 1979; Dunham and Smitherman 1984, Masser and Dunham 2012), Giudice (1966) indicated that the cross between channel catfish female and blue catfish male showed increased growth and production compared to channel catfish. This hybrid has shown superior characteristics (heterosis) for many traits such as faster growth 20 - 100% (Giudice 1966; Dunham et al. 1987, 1990; Dunham and Brummet 1999, Argue et al. 2003, Brown et al. 2011), especially at high densities, 15-20% better feed conversion (Yant et al. 1976; Li et al. 2004), 50- 100% higher tolerance to low oxygen levels (Dunham and Smitherman 1984), increased resistance to many common diseases, especially bacterial (Ella 1984; Wolters et al. 1996; Dunham et al. 2008; Arias et al. 2012), higher dress-out percentage (Yant 1975, Bosworth et al. 2004, Bosworth 2012) and increased harvestability by seining (Dunham and Argue 1998) and vulnerability to angling (Tave et al. 1981, Dunham et al. 1986).

The production of hybrid catfish embryos has some obstacles; the primary one being the natural reproductive isolation between the channel catfish and the blue catfish, which limits fry production (Goudie, et al.1993; Dunham et al. 1998; Kristanto, et al. 2009). Induced ovulation by hormone injection of the female channel catfish and hand stripping of eggs followed by in-vitro artificial fertilization with blue catfish sperm is

considered the most productive and consistent technique for making the (C×B) hybrid (Masser and Dunham, 2012). Other obstacles are that this technology is labor intensive and the fact that males must be sacrificed, decreasing efficiency of hybrid production.

Although the CxB hybrid exhibits improved performance, not all genetic types of this hybrid have the same level of performance and the potential exists for further genetic enhancement. The strain of the parent species affects the level of heterosis/performance observed in the CB hybrids (Dunham et al. 1987; Ramboux 1990; Dunham et al. 2014ab) and hybrid performance can potentially be improved by selection for combining ability (Bosworth and Waldbieser 2014). Genotype-environment (GE) interactions can be prevalent for hybrid catfish (Dunham et al. 1990). With the advent of new culture systems for hybrid catfish, assessment of GE interactions is critical for design of effective breeding programs to improve hybrid catfish. The complexity introduced by GE interactions suggests that testing may need to involve all possible environments used to rear catfish. Alternatively, just simply developing a single robust genetic type of hybrid may alleviate complexity in the testing paradigm. A critical question in all of this is, then: “Do programs for genetically improving CB hybrids need to be conducted for specific environments since catfish are now cultured in four or more environments (traditional ponds (single or multi-cropping), in-pond raceways and split ponds or will the best fish developed in one environment be the best hybrid for all culture environments?”

The overall objectives of this research were to conduct studies aimed towards improving production of hybrid catfish embryo production, evaluate the differences

among various genetic types of hybrid catfish and determine the extent of genotype-environment interactions among genotypes of hybrid catfish.

This will allow answering of the question of whether a single or multiple genetic improvement programs are needed for enhancing catfish for the current day and future catfish industry in the US.

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CHAPTER TWO

Production of Channel Catfish, *Ictalurus punctatus*, X Blue Catfish, *I. furcatus*, F1 Progeny from the Mating of a Xenogenic Channel Catfish Male and a Normal Channel Catfish Female

Abstract

Putative spermatogonia A from a fresh cell isolate or a density gradient-centrifuged isolate from testes of the blue catfish, *Ictalurus furcatus*, were transplanted into the gonads of triploid channel catfish, *I. punctatus*. The live cells were introduced into the gonads of the host via catheterization or by surgically inserting the cells directly into the gonad. A mean of 5.23×10^5 cells ($2 \times 10^4 - 1.43 \times 10^6$) ($7 \times 10^4 - 1.25 \times 10^5$ cells), respectively were injected. Sixty days post-introduction of the cells into the host, DNA was analyzed from biopsies of the gonads and 80% of the fish were xenogenic, having blue catfish cells in their gonads. Ten months after implantation, a sample of 8 males were tested and 7 were xenogenic. The xenogenic males successfully courted normal channel catfish that had been hormone induced, but none of the eggs hatched, indicating inadequate sperm production or an inability to ejaculate. Male xenogenic treated with luteinizing hormone releasing hormone analogue had well developed testes, and sperm production was detected in 3 of 7 xenogenic males examined two years after transplantation. Sperm were removed from a male that had been surgically-transplanted with blue catfish cells and used to fertilize eggs from a hand-stripped channel catfish

female. One percent of these eggs hatched. All seven surviving 6-month-old progeny of this male had the external morphology, swim bladder shape, nuclear DNA profile and mitochondrial DNA profile of channel catfish female X blue catfish male F1 hybrids. This indicates that the sire was indeed a xenogenic channel catfish producing blue catfish sperm and these progeny were produced through xenogenesis. This is the first report of successful production of xenogenic catfish and the first report of producing 100% hybrid progeny using xenogenesis in catfish.

Introduction

The hybrid resulting from the mating of female channel catfish, *Ictalurus punctatus*, and male blue catfish, *I. furcatus*, (C×B) is the best catfish for pond culture (Dunham et al. 2001; Chatakondi et al. 2005a,b; Dunham et al. 2008). This fish is improved for several important, desirable commercial traits. The inconsistency of hybrid seed production when attempting to mate female channel catfish and male blue catfish has been an impediment to commercial application of the hybrid. However, advancement in induced spawning followed by artificial fertilization has resulted in improved hybrid seed production (Masser and Dunham 1998). However, this technology remains labor intensive and development of new technologies, such as germ cell transplantation (Brinster and Zimmermann 1994) has opened up new avenues of research in reproductive biotechnology and aquaculture (Okutsu et al. 2006; Lacerda et al. 2012).

In the past decade, advancements in stem cell technology have opened the possibility of unique approaches for manipulating reproduction in fish involving germ cell transfer (Yoshizaki et al. 2002; Yoshizaki et al. 2013). The advancements have allowed the development of xenogenesis, a method of reproduction in which successive generations differ from each other. A xenogenic organism is comprised of elements typically foreign to its species. Xenogenesis can also be accomplished intraspecifically and the resulting individuals would be allogenic.

Two types of cells can be isolated and introduced to the host fish: primordial germ cells (PGCs) from developing embryos and type A spermatogonial stem cells (SSC). Three approaches for germ cell transplantations can be used to produce xenogens. First, transplantation of primordial germ cells (PGCs) into blastula-stage fish embryos via micromanipulation. This technique has been demonstrated in zebrafish (*Danio rerio*) (Ciruna et al. 2002; Giraldez 2005; Saito et al. 2008; Saito et al. 2010) and was validated by the observation of F1 offspring showing donor-derived mutant phenotypes. Saito et al. (2008, 2010) reported that a single PGC from pearl danio, loach or goldfish could be microinjected into the blastodisc of zebrafish, thereby producing xenogeneic males that in turn produced gametes 100% of which were derived from the donor. This result revealed that this technique works even when using phylogenetically distant host and donor species (Saito et al. 2010).

The second approach is the isolation of donor germ cells and transplantation into newly hatched triploid fry. An isolated germ cell could be a PGC or a type A spermatogonium; which possesses stem cell potential (Schulz and Miura 2002). These cells divide during spermatogenesis to produce isogenic germ cells committed to meiosis. Okutsu et al. (2006) demonstrated that type A spermatogonia contained a cell population showing stem cell activity in fish when transplanted into developing rainbow trout (*Oncorhynchus mykiss*) embryos. In those experiments, such embryos, produced sperm or eggs derived from the transplanted cells upon becoming adults.

Isolated donor germ cells have been transplanted into the peritoneal cavities of newly hatched triploid masu salmon (*Oncorhynchus masou*) larvae using a microinjector (Yoshizaki et al. 2011a). Newly hatched larvae were chosen as recipients because they did not possess a functional immune system, indicated by the lack of differentiation in both their thymus and T cells. Yoshizaki and colleagues (2005) thereby avoided immune-rejection of exogenous (donor-derived) germ cells. In the first reported study of intra-peritoneal injections, green fluorescent protein (GFP) transgenic rainbow trout were used as donors and wild type rainbow trout were used as the host (Yoshizaki 2005). Primordial germ cells (PGCs) injected into the triploid larvae migrated to and colonized the host gonadal ridge. The donor-derived PGCs proliferated and matured into eggs and sperm in the allogeneic gonads, which resulted in normal offspring showing the donor-derived phenotype (Takeuchi et al. 2003).

Okutsu (2006) investigated the use of spermatogonial stem cells (SSC) in place of PGCs. SSCs isolated from transgenic adult male rainbow trout expressing GFP driven by the *vasa* promoter were injected into the peritoneal cavities of newly hatched rainbow trout larvae. After recipients reached maturation, donor spermatogonia differentiated into spermatozoa in males and fully functional eggs in females. Furthermore, donor-derived spermatozoa and eggs obtained from recipient, 2-year-old, xenogenic masu salmon were able to produce normal rainbow trout offspring. These findings indicated that the testicular germ cells of fish, more specifically the SSCs, possess a high level of sexual plasticity, even after animals reach maturity (Okutsu et al. 2006).

Additionally, spermatogonia were isolated from giant gourami (*Osphronemus goramy*) testis held at 4°C and transplanted into tilapia. The result from this study showed no difference in efficiency of colonization among SSCs from donor testicular tissue preserved for either 24 or 48 hours. Fresh testicular tissue also supported SSC colonization (Andriani 2012). This approach was also applied to marine species such as nibe croaker (*Nibea mitsukurii*) (Higuchi et al. 2011), yellow tail mackerel (*Seriola quinqueradiata*) (Morita et al. 2012) and chub mackerel, (*Scomber japonicus*) (Yazawa et al. 2010).

The plasticity of oogonia was studied by transplanting donor female germ cells into the newly hatched embryos of rainbow trout. Oogonia differentiated into functional eggs in the female recipient and sperm in male recipients; therefore, oogonia share the germ line stem cell function seen in spermatogonia (Yoshizaki et al.2010).

Thirdly, xenogenesis can also be accomplished in juvenile and adult fish. Lacerda et al. (2012) developed techniques for Nile tilapia (*Oreochromis niloticus*) in which spermatogonia were transplanted through the urogenital papilla of adult fish. The recipient fish had their endogenous spermatogenesis suppressed using the cytostatic drug busulfan (1,4-butanediol dimethanesulfonate) in association with high temperature (35 °C). An enriched type A spermatogonial cell suspension was then obtained from donor testes and labeled with the fluorescent lipophilic dye PKH26-GL before being injected into the adult testes of the recipient through the common spermatic duct, which opens into the urogenital papilla. The recipient testes, which were analyzed soon after

transplantation, showed PKH26- labeled germ cells in the lumen of the seminiferous tubules. Based on the appearance and histology of the testis parenchyma, exogenous germ cells did not appear to trigger severe immune-rejection in adult allogenic recipients (Lacerda et al. 2006). A subsequent study also demonstrated the production of donor spermatozoa in recipient testes of tilapia 8 and 9 weeks post-transplantation (Lacerda et al. 2010). After natural fertilization, a generation of progeny demonstrating the genotype of donor animals, which belonged to a different strain of Nile tilapia, was observed through microsatellite DNA analysis.

Majhi et al. (2009) transplanted spermatogonial stem cells (SSCs) from the pejerrey, (*Odontesthes bonariensis*), into the gonads of sexually mature Patagonian pejerrey (*O. hatcheri*), which had been partially depleted of endogenous SSCs with a combination of busulfan treatment coupled with high water temperatures. This results in temporary suppression of native SSCs production, giving the donor stem cells an opportunity to colonize. Host stem cell production and spermatogenesis reinitiates within 6 months after the transplantation. Crosses between *O. hatcheri* males, which had been transplanted with *O. bonariensis* SSCs, and normal *O. hatcheri* females produced both pure *O. hatcheri* fry (88%) and hybrid fry (12%) proving the functionality of the transplanted SSCs, although they were outcompeted by the host SSCs. This system might be improved by using juveniles or adults that had been permanently sterilized by triploidy. Triploid fish are sterile due to the lack of endogenous germ cells (Lacerda et al. 2010). Thus, the current research evaluated using triploid channel catfish (*I. punctatus*) males as hosts to improve upon the system of Majhi et al. (2009).

There are many potential applications of xenogenesis. These include the study of cell and tissue communication, potential resurrection of extinct species and genetic lines if cryopreserved cells exist, rapid multiplication of threatened and endangered species, reproduction of species with limited spawning capacity or those that do not respond well in captivity, genetic cloning of reproductive tissue, accelerated genetic research and development of novel hybridization technologies.

Our objective was to develop interspecific transfer of stem cells into ictalurid catfish resulting in xenogenic host individuals by transferring donor stem cells to juvenile gonads, a life stage for which cell transfer may be technically easier. This is the first step to accomplish the long-term goal to develop technologies that utilizes channel catfish males as xenogenic hosts. The production of the first xenogenic warm water fish in a species native to North America and in ictalurid catfish is reported here.

Materials and Methods

Triploid induction

Channel catfish females were induced to ovulate in 2010 using the general procedures of Lambert et al. (1999), Dunham et al. (2000), Hutson (2006), and Kristanto et al. (2009). Luteinizing hormone releasing hormone analog, des-Gly10,[D-Ala6] LHRH ethyl amide (LHRHa) (85% active ingredient) was administered intramuscularly as a

single 90 μ g/kg body weight dose with EVAC implants (Center of Marine Biotechnology, University of Maryland, Baltimore, MD).

When females began to ovulate, thirty-gram aliquots of eggs were hand-stripped, placed into small pie pans and dry fertilized. Water was added to the pie pan to activate sperm and eggs for fertilization. At three minutes post-fertilization, the eggs were placed in an egg basket and the basket placed in a hydrostatic pressure chamber (Lilyestrom et al. 1999). At five minutes post-fertilization, the fertilized eggs were pressure shocked using a hydrostatic pressure of 6000psi (408.27 atm) for 5 minutes. After pressure shocking, the eggs were removed from the chamber and placed in a flow-through hatching trough fitted with a paddlewheel. .

Hatched fry were grown to 8-10 cm fingerlings within indoor tanks, then were stocked into a 0.04-ha pond and allowed to grow for three years. In 2012, the fish produced in 2010 were harvested and transported to American Sport Fish, Montgomery, Alabama, for ploidy analysis. Each fish was analyzed for ploidy individually using blood from a tail prick. Each sample was analyzed for erythrocyte nuclei volume with a Coulter-counter, as described by Beck and Biggers (1983). Triploid and diploid individuals were separated and transported back to Auburn University. The triploid individuals from 2012 were given unique individualized brands and stocked back into an earthen pond. In 2013, similar procedures were performed with triploids produced in 2010. These triploid individuals were marked with visible implants elastomer (VIE).

Donor cell isolation

Two-year-old, sexually immature donor male blue catfish (mean length 31.8 cm; mean testes weight 0.177g) were harvested and anesthetized with tricaine methanesulfonate (Finquel: MS-222). The ventral surface of the fish surface was cleaned with 70% isopropanol. An incision was made on the ventral side and the testes were removed using a scalpel and forceps. Testes from individual fish were processed separately. The testis tissue was placed into 1.5 mL anti-agent medium (Hank's Balanced Salt Solution, HBSS) with 1.0 $\mu\text{g}/\text{mL}$ NaHCO_3 , 100unit/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (Hyclone). The testicular tissue was then placed on a clean petri dish and any connective tissue or blood was removed using a pair of forceps. The tissue was washed twice with 0.5 mL anti-agent medium. The testes were then shredded using a pair of sterilized scalpel blades, transferred into 12 mL HBSS with 0.25% trypsin (Thermo Scientific), and incubated on ice for 30 min followed by 30 min at 21°C with a magnetic stirrer to form a cell suspension. The cell suspension was then filtered using a 40- μm mesh and centrifuged at 500 g for 10 minutes. The supernatant was discarded and the pellet re-suspended in 1 mL HBSS. The concentration of cells was calculated using a hemocytometer, and the fresh cell isolate was stored on ice until use, which was after 15 minutes to 1.5 hours. Three major size classes of cells were recognized. The size classes had diameters of 8-15 μM , 5-7 μM and < 2.0 μM . The largest were presumed to be SSCs.

Gradient-centrifuged cells were prepared by using a Percoll gradient. A Percoll gradient was made using three concentrations (2 mL of 90%, 70% and 45% each in HBSS) in a 15 mL centrifuge tube. The fresh cell isolate was placed on top of the Percoll

gradient and centrifuged at 800 g for 40 minutes. After centrifugation, the top layer was carefully removed using a micropipette and 2 mL of HBSS added and centrifuged at 500 g for five minutes. The supernatant was discarded and the pellet re-suspended in 1mL culture medium (L-15 with 25 mM HEPES, 50 unit/mL penicillin, 50 µg/mL streptomycin, 1.0 µg/mL NaHCO₃, 0.3 µg/mL L-glutamine, 10% FBS, 5% catfish serum and 1 ng/mL bovine serum fibroblast growth factor bFGF). This solution had a high percentage of spermatogonial cells, which consisted of type A spermatogonia (diameter 12-15 µM), Type B spermatogonia (diameter 10-11 µM) and primary spermatocytes (diameter 8-9 µM), which are the product of mitosis of type B spermatogonia. The concentration of cells was calculated using a hemocytometer, and the cell solution was stored on ice until ready for use.

Cell implantation into host fish

In July 2012, the triploids were harvested. The fish (mean weight = 1.72kg) were anesthetized using 100 mg/L tricaine methanesulfonate (MS-222) and 100 mg/L sodium bicarbonate. Two fish were surgically implanted with a fresh cell (1 female) isolate or gradient centrifuged cells (1 male), and four fish (2 males, 2 females) were catheterized and the fresh cell isolate or gradient centrifuged cells inserted into the gonads. Each host fish received donor cells from a single individual.

For the surgical procedure, a 5-7 cm incision was made on the ventral side of the fish from the pelvic bone anteriorly. The skin and muscle were parted and the gastrointestinal (GI) tract carefully moved aside. The gonad (atrophied as expected for a

triploid) was located on the dorsal wall ventral to the tail kidney. Using a syringe fitted with a 20 gauge needle, 1 mL of the fresh cell isolate or gradient-centrifuged solution ($7 \times 10^4 - 1.25 \times 10^5$ cells) was carefully injected into the anterior part of each gonad in 3 locations in each testis or ovary (0.5 mL in each ovary or testis). Post injection, the GI tract was placed back in the body cavity and the incision closed using biodegradable sutures. The fish were then placed into a recovery tank and allowed to heal for 10 days before stocking into an earthen pond.

For the catheterization procedure, an 8-cm polyethylene tube (0.8 mm i.d., 1.2 mm o.d.) was carefully inserted into the vent and gently fed into the genital tract until 4-5 cm of the tube was inserted into the fish. The gradient centrifuged solution or fresh cell isolate was drawn up into a 1mL syringe, the end of the syringe attached to the exposed end of the polyethylene tube, and the 1 mL of the cell isolate ($2 \times 10^4 - 1.43 \times 10^6$ cells) was injected into the genital tract and the gonads of the fish. The tubing was then drawn out of the vent and the fish were allowed to recuperate in indoor tanks for 10 days before moving into an earthen pond. Sixty days after stocking in the ponds, the fish transplanted in 2012 were seined from the pond and transported to indoor holding facilities. They were anesthetized with MS-222 and a 5-7 cm incision made on the ventral side of the fish from the pelvic bone anteriorly. Tissue samples were excised from the gonads for DNA analysis. Ten months after implantation, seven confirmed xenogenic males were biopsied and examined for sperm production.

In 2013, similar procedures were executed except the cell number was slightly different. For the two surgically-transplanted individuals, 1 mL of the fresh cell isolate or

gradient-centrifuged solution ($1.7 \times 10^5 - 4.5 \times 10^5$ cells) was carefully injected into the anterior part of each gonad in 2 locations. For the eight catheterized individuals, 1 mL of the cell isolate ($5 \times 10^4 - 1.8 \times 10^6$ cells) was injected into the genital tract and the gonads of the fish.

Genomic DNA

The excised gonad samples were placed into 1.5-mL microfuge tubes on ice then held at -80°C until DNA extraction. DNA was extracted using proteinase K digestion followed by protein precipitation and ethanol precipitation with the protocol described by Liu et al. (1998) and Waldbieser (2008) with slight modification. DNA from control samples of diploid channel catfish, blue catfish and hybrid catfish were also extracted.

PCR

Primers used for differential PCR-amplification of channel and blue catfish genes are listed in Table 1 (Waldbieser and Bosworth, 2008). Genes evaluated were follistatin (*Fst*), and hepcidin antimicrobial protein (*Hamp*) for channel catfish and blue catfish. PCR reactions were prepared in 10.0 μL volume containing 20-250 ng genomic DNA in 1 \times buffer (1.0 μl 10 mM Tris-HCl [pH: 9.0], 0.4 μL 50 mM MgCl_2 , 0.8 μL 2.5 mM of each dNTP, 0.6 μL 10 μM each *Fst* primer, 0.3 μL 10 μM each *Hamp*, 0.1 μl 5U/ μL platinum Taq polymerase, 3.9 μL water. The *Fst* and *Hamp* amplification products were analyzed on an ethidium bromide stained 2.5% agarose gel. Amplicon size was measured by TrackIt™ 100 bp™ 100bp DNA Ladder (Invitrogen, Carlsbad, CA). All the reactions for each sample were repeated three times.

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

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

Mating of triploid host with normal channel catfish

The eight transplanted triploid host channel catfish males (putative xenogens) from 2012 that were 3 years old were harvested in May 2013 and 2014 (4 years old), and individually placed in 91cm x 32 cm x 61cm glass aquaria. Normal channel catfish females were seined and induced to ovulate using the general procedures of Lambert et al. (1999), Dunham et al. (2000), Hutson (2006), and Kristanto et al. (2009). LHRHa (85% active ingredient) was administered intramuscularly as a single 90 µg/kg body weight dose with EVAC implants. The females were paired with the xenogenic males in the aquaria. The water flow rate averaging was maintained at 4 L/minute, water temperature 26 – 28°C and dissolved oxygen 4.2 – 5.3 mg/L with the use of air diffusers. Fish transplanted in 2013 were mated in the same manner in 2014. Additionally, xenogenic males produced in 2012 were implanted with 90 µg/kg of LHRHa upon spawning.

After each mating attempt with normal females, transplanted fish were anesthetized with MS-222, and a 5-7cm incision made on the ventral side of the fish,

from the pelvic bone anteriorly. Tissue samples were excised from the gonads of 2012 transplanted fish for DNA analysis to confirm xenogenesis and to examine testicular development. Similar protocols and procedures were repeated in 2014 to only examine gonadal development of the fish transplanted in 2012 (7 males) and 2013 (10 males). Gonadal development was recorded and the fish were transferred to recovery tanks.

After a mating attempt in 2014, the testes were surgically excised from one xenogenic channel catfish male weighing 2.01 kg. Blood and excess tissue were removed using 0.9% saline solution and then the testes macerated to release sperm. The sperm suspension was added to 20 g of eggs (approximately 1,000 eggs) from a channel catfish female in a small pie pan and mixed to dry fertilize. Water was added to activate the eggs and fertilize them. Then the pan was placed in a trough with flowing freshwater for 10 - 15 minutes to harden the eggs. Once hardened, they were transferred into a basket and suspended in a hatching trough with flowing water, paddle wheels and aeration. Treatments of 100 mg/L formalin and 32 mg/L copper sulphate (Cu_2SO_4) were administered to prevent fungus and bacteria every 8 hours. These treatments were given statically for 15 minutes, 3 times per day. Hatched fry were transferred to a 51 L fry rearing tank. The fry were transferred to a recirculating system for further growth. The fry were fed a 40% protein fry feed three times per week (AquaMax™).

Morphological examination

Seven putative hybrid fingerlings, 6-7 cm total length and 2-3 g body weight, produced via xenogenesis were compared to known channel catfish fingerlings of the same size for body shape and color and. Swim bladders were also compared for these two groups of fish.

mtDNA

Genomic DNA was extracted using the previous method followed by cytochrome oxidase I (COI) sequencing. A partial sequence of COI gene was amplified using cocktail primers (Table 2). Seven test samples as well as three control samples were used. Controls included channel catfish, blue catfish and channel catfish female x blue catfish male hybrids. PCR was carried out in a 10- μ L reaction volume in a 0.5-mL micro-centrifuge tube with the following components: up to 10 μ l PCR grade water; 1X PCR buffer; 200 μ M of dATP, dCTP, dGTP and dTTP each; 2 mM MgCl₂; 1 μ M of C_FishF1t1 and C_FishR1t1 each; 0.5 unit of Platinum® Taq DNA polymerase (Invitrogen) and 20 ng DNA. PCR cycling conditions were as follows: initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 40 sec, extension at 72°C for 1 min; and final extension at 72°C for 10 min. PCR products were purified using the Exo / SAP method (Dugan et al.2002) with some modifications (Wong et al. 2011). Purified PCR products were sequenced from both ends using M13F and M13R primers. COI sequences of the 7 test samples were aligned against the control sequences using t-coffee (Genome Campus Hinxton, Cambridge,

United Kingdom) then the multiple sequence alignment was shaded with Boxshade software (SIB and the Vital-IT group).

Table 2. The cocktail name, primers name, ratio, sequence, position and reference for sequencing the cytochrome oxidase I gene of channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatus* and channel catfish female X blue catfish male hybrid controls and 7 putative channel catfish female X blue catfish male hybrids produced via xenogenesis.

Cocktail name	Name	Ratio	Primer sequence 5'-3'	Primer position	Reference
C_FishF1t1	VF2_t1	1	TGTA AACGACGGCCAGTCAACCAACCACA AAGACATTGGCAC	6448–6474	(Ward et al. 2005)
	FishF2_t1	1	TGTA AACGACGGCCAGTCGACTAATCATA AAGATATCGGCAC	6448–6474	(Ward et al. 2005)
C_FishR1t1	FishR2_t1	1	CAGGAAACAGCTATGACACTTCAGGGTGAC CGAAGAATCAGAA	7152–7127	(Ward et al. 2005)
	FR1d_t1	1	CAGGAAACAGCTATGACACCTCAGGGTGTC CGAARAATCARAA	7152–7127	(Ivanova et al. 2007)
-	M13F	-	TGTA AACGACGGCCAGT		(Messing 1983)
-	M13R	-	CAGGAAACAGCTATGAC		(Messing 1983)

Results

Both triploid channel catfish, one of each sex, that were injected with density gradient sorted blue catfish germ line stem cells, putative spermatogonia A, contained blue catfish cells in their testes and ovaries 60 days after injection, based upon DNA evidence (Fig. 1). PCR products from a mixture of 25 cells from channel catfish and 25 cells from blue catfish amplified with both *follistatin* Fst and *hepcidin*, Hamp primers. CC and BL are channel catfish and blue catfish controls, All fish were injected with 1mL of cell solution. A3, A26 and A29 are triploid channel catfish males injected in the testes with $7 \times 10^4 - 1.25 \times 10^5$ cells blue catfish stem cells. A1, A2, A5, A25 and A30 are triploid

channel catfish males inserted with $2 \times 10^4 - 1.43 \times 10^6$ cells from blue catfish, by catheterization through the oviduct.

In all instances, some gonadal growth was evident (size doubled in most cases). One female triploid channel catfish and two males had a mixture of fresh testicular cells from blue catfish introduced by catheterization via the genital and urogenital opening, respectively. After 60 days, blue catfish cells were undetectable in the female, but were found in both males. Ten months after implantation, eight putative xenogenic males were sampled. Three had small paired testes, one a single small testis, and four had significant growth in a single or both testes. Seven catfish, or 87.5% were xenogenic (Fig 1). One of these seven males had normal sperm production at this time. This male had been surgically injected. The males transplanted in 2013 at 3 years of age were surgically checked in 2014, and the testicular tissue was minimally developed at this time. Sperm were not evident.

All xenogenic catfish males and the putative xenogenic males displayed aggressive courtship behavior, Five of seven, (71%) of the xenogenic males transplanted in 2012 were able to induce females to lay eggs in 2013 (Table 2)

Table 3. Number of xenogenic (triploid channel catfish, *Ictalurus punctatus* containing blue catfish, *I. furcatus*, blue catfish cells in their gonads) males, age at transplantation, age at spawning, percent of females induced by xenogenic males to lay eggs and percent of hatching of eggs.

Year	Matings N	Age of males (yr)	Age (yr) of males at transplantation	% females induced by xenogenic males to lay eggs	% hatch
2013	7	3	2	71	0.0
2014	7	4	2	100	0.0
	10		3	70	0.0
2014	1	4	2	<i>in vitro</i> fertilization	1.0*

*Fry were 100% hybrids from *in vitro* fertilization using sperm from xenogenic male crossed with a channel catfish female.

In 2014, 100% of the xenogenic males transplanted in 2012 and 70% of the putative xenogenic males transplanted in 2013 induced females to lay eggs, of which 0% of the eggs hatched. Despite the lack of hatching, 3 of 7 xenogenic males (injected in 2012) had well developed testes when examined in 2014. Fig. 2 illustrates the testicular development of 1 of the 3 xenogenic males from the 2012 group examined in 2014 post-spawning.

Xenogenically produced progeny

Eleven of 1,000 eggs hatched from the spawn produced from the artificially fertilized eggs obtained from a normal channel catfish female that were fertilized with sperm from a xenogenic male. In 2014, tissue samples were collected from the seven

surviving 6-month old fingerlings. These fingerlings had the external morphology of a channel catfish female X blue catfish male hybrid (Fig 4). The channel catfish female X blue catfish male hybrid had characteristics of the blue catfish (paternal predominance) (Dunham et al. 1982), a larger acute angle from the snout to the insertion of the dorsal spine, a dorsal hump, bluish color, very few spots and a relatively straight anal fin. In contrast, channel catfish has a flatter head, no pronounced hump, a greyish color, more spots and a rounded anal fin. The reciprocal hybrid, blue catfish female X channel catfish male has almost an identical appearance to channel catfish (Dunham et al. 1982). The swim bladder (Fig. 5) of the xenogenically-produced progeny was as expected for a channel catfish female X blue catfish male hybrid (bilobate; i.e., heart shaped like a channel catfish with a reduced nipple-like posterior second lobe) (Dunham et al. 1982). In contrast, channel catfish and the blue catfish female X channel catfish male hybrid have a single lobed, heart shaped, swim bladder. Blue catfish have a distinct bilobate swim bladder, and the lobes are similar in size.

Nuclear and mtDNA

The nuclear DNA genotype for follistatin and hepcidin for the progeny from the xenogenic male was that expected for a channel-blue hybrid catfish (Fig. 3).

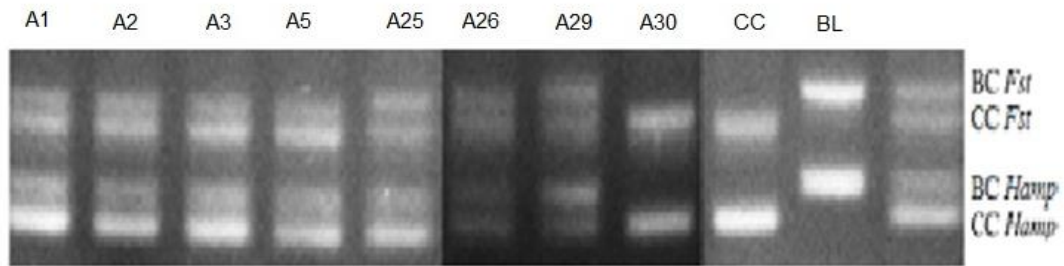


Fig. 1 PCR results for detecting blue catfish (*Ictalurus furcatus*) cells in the testes of triploid channel catfish (*I. punctatus*). Blue catfish and channel catfish cells were differentiated with PCR using follistatin, *Fst* and hepcidin antimicrobial protein, *Hamp* genes as markers. CC was channel catfish control; BC was blue catfish control; H was channel catfish female × blue catfish male hybrid controls. Three recipient fish - A3, A26 and A29 were transplanted by the surgical method and blue catfish cells were detected in the testes of all three fish. Five recipient fish - A1, A2, A5, A25 and A30 were transplanted by the catheterization method and blue catfish cells were detected in the testes of four fish, A1, A2, A5 and A25; blue catfish cells were not detected in fish A30.



Fig. 2. Testicular development of a 4-year-old channel catfish (*Ictalurus punctatus*) (left) xenogenic male transplanted with blue catfish SCC cells at 2 years of age, compared to a

triploid channel catfish male (right). The xenogenic male exhibited normal courtship behavior and induced the female to lay eggs, attempted to fertilize the eggs, but was not able to naturally fertilize the eggs. Well-developed testes with milt were detected and sperm from this male were used to artificially fertilize channel catfish eggs to produce channel catfish (*Ictalurus punctatus*) female X blue catfish (*Ictalurus furcatus*) male hybrid fry.

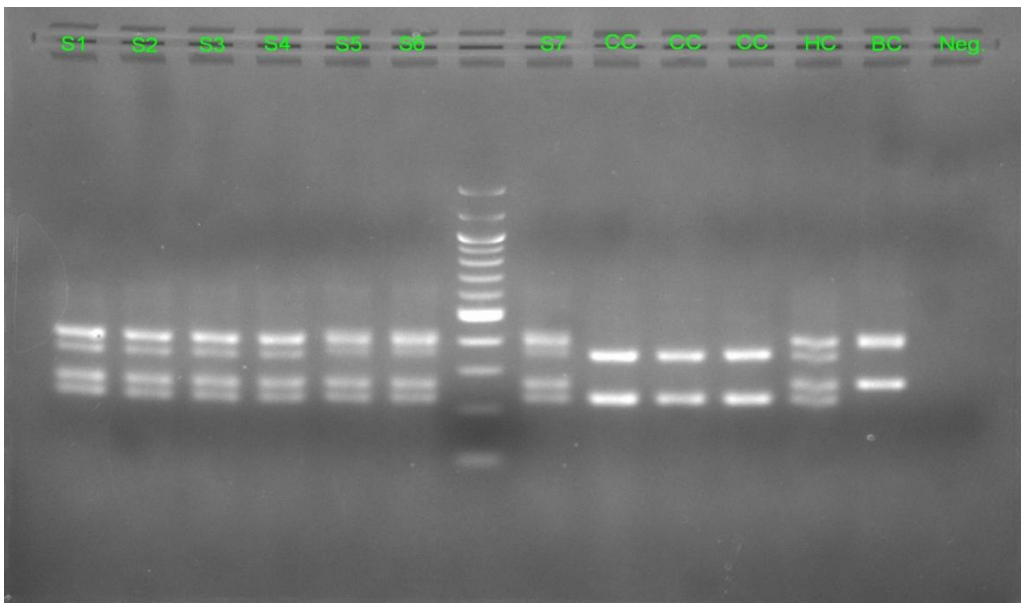


Fig. 3. PCR results for progeny of a triploid channel catfish (*Ictalurus punctatus*) male possessing, which contained blue catfish (*I. furcatus*) cells in its testes and a normal channel catfish female. Blue catfish and channel catfish cells were differentiated using follistatin, *Fst* and hepcidin antimicrobial protein, *Hamp* genes as markers. CC = channel catfish control; BC = blue catfish control; HC= channel catfish female × blue catfish male hybrid control. Seven putative channel catfish female X blue catfish male fingerlings (S1 – S7) produced from an artificial spawn between the channel catfish male

and the xenogenic male. All seven showed the genotype of channel catfish X blue catfish hybrids.



Fig. 4. Channel catfish (*Ictalurus punctatus*) female X blue catfish (*I. furcatus*) male hybrid fingerlings produced from mating a xenogenic male and a channel catfish female (left) compared morphologically to control channel catfish fingerlings (right). The channel catfish female X blue catfish male hybrid has characteristics of the blue catfish (paternal predominance), a steep slope from the snout to the insertion of the dorsal spine, dorsal hump, a bluish color, very few spots and a relatively straight anal fin. In contrast, channel catfish has flatter head, no pronounced hump, a greyish color, more spots and a rounded anal fin. The reciprocal hybrid, blue catfish female X channel catfish male has an almost identical appearance to channel catfish.

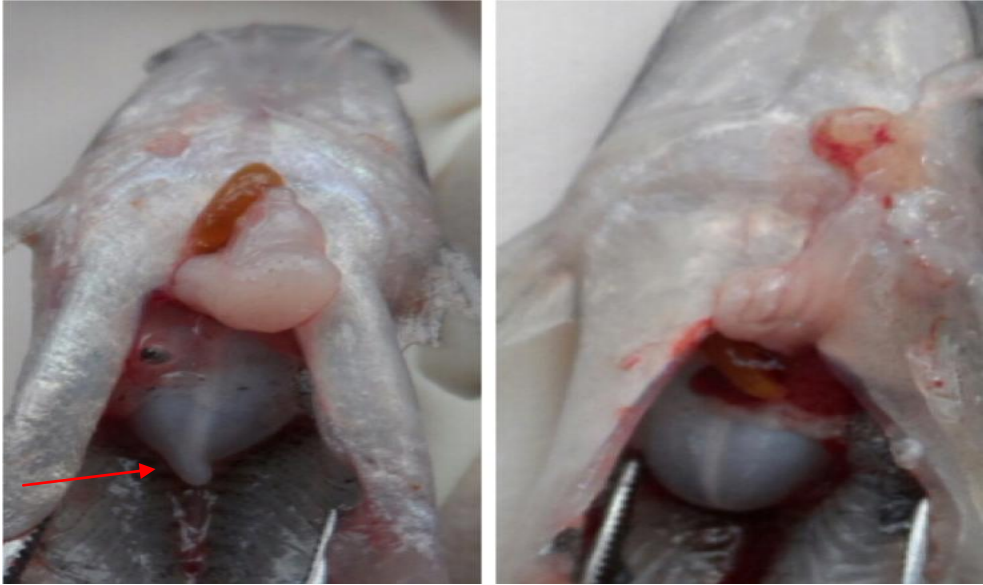


Fig. 5. The swim bladder of a channel catfish, *Ictalurus punctatus*, female X blue catfish, *I. furcatus*, male hybrid catfish fingerling produced from mating a xenogenic male (triploid channel catfish male with blue catfish cells in the testes) and channel catfish female (left) compared with that of a similar-sized channel catfish fingerling (right). Channel catfish and the blue catfish female X channel catfish male hybrid have a single lobed, heart shaped, swim bladder. Blue catfish have a distinct bilobate swim bladder, and the lobes are similar in size. The swim bladder of the xenogenically produced progeny was that expected for a channel catfish female X blue catfish male hybrid (smaller than channel catfish with small nipple like second lobe (Dunham et al. 1982).

High quality mtDNA sequences (Fig. 6) were obtained for the putative hybrids produced by xenogenesis and aligned against the control sequences. Figure 7 represents the multiple sequence alignment of a partial sequence of cytochrome oxidase I gene from sample 1 against channel, hybrid and blue catfish control sequences. The original

sequences were trimmed so that only sequences with good quality were aligned. Except for the single point mutation in samples 1 and 2 at the bases 291 and 294 of the aligned sequence and excluding the first and last part of the alignment, which might be attributed to sequencing errors, the 7 test samples were identical to the channel catfish and hybrid catfish control sequences and varied from the blue catfish control sequence confirming that the dam of these 7 test samples was a channel catfish and there was no mtDNA contribution from the sire.

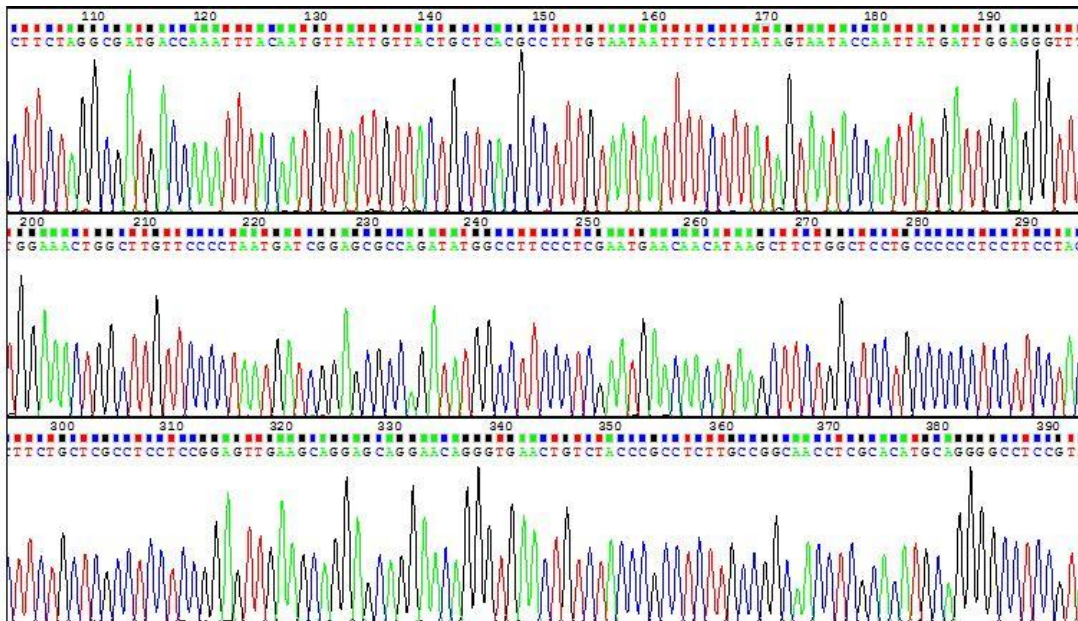


Fig. 6. A four-color mtDNA sequencing chromatogram of a partial COI sequence amplified by C_FishF1t1 and C_FishR1t1 cocktail primers and sequenced by M13F primer from sample 7, a putative channel catfish, *Ictalurus punctatus*, female X blue catfish, *I. furcatus*, male hybrid produced via xenogenesis. Bases 103-392 from sample 7 are shown. Peaks are evenly spaced with minimal baseline noise.

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Blue_Control      1  -----TTATCCGGGCAAG
Channel_Control  1  -----CCCTTAGCCTGCTTATCCGGGCGG
Hybrid_Control   1  -----CGGCCCTTAGCCTGCTTATCCGGGCGG
Sample_1         1  -----TTATCCGGGCGG

Blue_Control      13  AGCTAGCCCAAGCCCCTTGCCTTCTAGGCGATGACCAAATTTAATGTTATTGTCACTG
Channel_Control  25  AATTAGCCCAACCCGGCGCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG
Hybrid_Control   28  AATTAGCCCAACCCGGCGCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG
Sample_1         13  AATTAGCCCAACCCGGCGCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG

Blue_Control      73  CACAGCCTTTGTAATAATTTCTTTATAGTAATGCAATTATGATTGGAGGGTTTGGAA
Channel_Control  85  CTCACGCCTTTGTAATAATTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGGAA
Hybrid_Control   88  CTCACGCCTTTGTAATAATTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGGAA
Sample_1         73  CTCACGCCTTTGTAATAATTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGGAA

Blue_Control      133  ATTGGCTTGTTCCCTAATGATGGGCGCACAGACATGGCCTTCCTCGAATAAACAATA
Channel_Control  145  ACTGGCTTGTTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCTCGAATGAACAACA
Hybrid_Control   148  ACTGGCTTGTTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCTCGAATGAACAACA
Sample_1         133  ACTGGCTTGTTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCTCGAATGAACAACA

Blue_Control      193  TAAGCTTCTGGCTCCTGCCCCCTTCTCTACTTCTGCTCGCCTCCTCGGAGTTGAAG
Channel_Control  205  TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCGGAGTTGAAG
Hybrid_Control   208  TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCGGAGTTGAAG
Sample_1         193  TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCGGAGTTGAAG

Blue_Control      253  GGGAGCGGGACAGGATGAACGTATACCCGCCTCTTGGCGCAACCTCGCACATGCAG
Channel_Control  265  CAGGAGCAGGAACAGGGTGAACGTATACCCGCCTCTTGGCGCAACCTCGCACATGCAG
Hybrid_Control   268  CAGGAGCAGGAACAGGGTGAACGTATACCCGCCTCTTGGCGCAACCTCGCACATGCAG
Sample_1         253  CAGGAGCAGGAACAGGGTGAACGTATACCCGCCTCTTGGCGCAACCTCGCACATGCAG

Blue_Control      313  GGGCCTCGTAGACTTAACCATCTTTTCCCTACACCTTGCAGGAGTTTCATCTATTCTAG
Channel_Control  325  GGGCCTCGTAGATTTAACTATCTTTTCCCTTATCTTGCAGGAGTTTCATCTATTCTTG
Hybrid_Control   328  GGGCCTCGTAGATTTAACTATCTTTTCCCTTATCTTGCAGGAGTTTCATCTATTCTTG
Sample_1         313  GGGCCTCGTAGATTTAACTATCTTTTCCCTTATCTTGCAGGAGTTTCATCTATTCTTG

Blue_Control      373  GGGCCATTAATTTTATTACAACAATTATTAACATAAAACCCCCCGCAATCTACAATATC
Channel_Control  385  GGGCCATTAACTTTATTACAACAATTATTAATATGAAGCCCCCGCAATCTACAATATC
Hybrid_Control   388  GGGCCATTAACTTTATTACAACAATTATTAATATGAAGCCCCCGCAATCTACAATATC
Sample_1         373  GGGCCATTAACTTTATTACAACAATTATTAATATGAAGCCCCCGCAATCTACAATATC

Blue_Control      433  AGACTCCCTATTTGTCTGAGCTGTCCCTAATTACAGCGTCTACTACTACTACCTCC
Channel_Control  445  AAACCCCTATTTGTCTGAGCGTCTAATTACAGCGTCTCTACTACTACTACCTCC
Hybrid_Control   448  AAACCCCTATTTGTCTGAGCGTCTAATTACAGCGTCTCTACTACTACTACCTCC
Sample_1         433  AAACCCCTATTTGTCTGAGCGTCTAATTACAGCGTCTCTACTACTACTACCTCC

Blue_Control      493  CAGTCTAGCTGCTGGCATCACAATCTTCTAACAGACCGAAACTTAAACACACCTTCT
Channel_Control  505  CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAATACTACCTTCT
Hybrid_Control   508  CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAATACTACCTTCT
Sample_1         493  CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAATACTACCTTCT

Blue_Control      553  TTGACCCCGCAGGGGGAGGGGACCCCATCTTTACCAACACCTTTTCTGATTCTTCGGTC
Channel_Control  565  TTGACCTGCAGGGGGAGGCGACCCCATCTTTACCAACACCTTTTCTGATTCTTCGGTC
Hybrid_Control   568  TTGACCTGCAGGGGGAGGCGACCCCATCTTTACCAACACCTTTTCTGATTCTTCGGTC
Sample_1         553  TTGACCTGCAGGGGGAGGCGACCCCATCTTTACCAACACCTTTTCTGATTCTTCGGTC

Blue_Control      613  ACCCTGAAGTGTCA-----A
Channel_Control  625  ACCCTGAAGTGTCAATAGCTTGTTC
Hybrid_Control   628  ACCCTGAAGT-----T
Sample_1         613  ACCCTGAAGT-----G

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Fig. 7. Multiple sequence alignment of a partial sequence of cytochrome oxidase I gene for 1 test sample, which is a putative channel catfish, *Ictalurus punctatus*, female X blue

catfish, *I. furcatus*, male hybrid produce via xenogenesis against channel catfish, hybrid catfish and blue catfish control sequences. Original sequences were trimmed so that only sequences with good quality were aligned. Except for the single point mutation in sample 1 at base 291 of the aligned sequence and excluding the first and last part of the alignment which might be attributed to sequencing errors. The test sample was identical to the channel catfish and hybrid catfish control sequences and varied from the blue catfish control sequence confirming that the dam of this fish was a channel catfish and there was no mtDNA contribution from the sire.

Discussion

Two transplantation techniques, gonadal injection and gonadal catheterization, using blue catfish cells that were either a fresh mixture of all testicular cells or putative germ line stem cells from the testes that were density gradient purified resulted in xenogenic catfish, triploid channel catfish harboring blue catfish cells in their gonads were evaluated. One xenogenic female and 3 xenogenic males were produced. Another sample of 8 males had 7 individuals that were xenogenic. Theoretically, the blue catfish DNA that was detected is from germ line stem cells that were able to colonize and survive in the gonads. This technique was very effective as 80-90% of the manipulated host fish accepted the foreign cells, which colonized and were present 60 days and 10 months later, and may be an improvement compared to transfer of germ line stem cells into blastulae and late stage embryos. In the case of the salmonid xenogens, a 30-70%

success rate was achieved among injected host embryos (Okutsu et al. 2007), and approximately 60% of injected zebrafish became xenogens. Manipulating the juvenile or adult fish is also technically easier than microinjecting the blastulae and hatchings (Lacerda, et al. 2012; Perera 2012).

Based on subjective observation, the gonads of the manipulated fish doubled in size, which is further evidence that germ line stem cells colonized and began to proliferate. Alternatively, this could be natural gonadal growth during the 60-day period or inflammation from an immune response to the donor cells, although such inflammation should have subsided in a two-month period. In fact, it is highly unlikely that the blue catfish contribution to these xenogens is of somatic origin, as successful transplantation of somatic cells usually requires immune suppression of the host to prevent rejection of the foreign cells (Zawada et al. 1998). Additionally, the blue catfish cells utilized were enriched so that the majority of the cells transplanted were spermatogonia. When evaluated at 10 months, one xenogenic male (of 7 examined) was producing sperm, which is further proof that stem cells, not somatic cells, had colonized. This also demonstrates that the system of introducing stem cells to triploid sub-adults has potential and might be used for specific applications even if it is found that it cannot be used for large-scale fry production.

Allogenic and xenogeneic transplantation has been achieved in fish before by manipulating adult Nile tilapia (Lacerda, et al. 2012) and Patagonian pejerrey (Majhi et al. 2009). In the case of Nile tilapia, spermatogonia were transplanted through the

urogenital papilla of adult fish and in the case of the Patagonian pejerrey, the spermatogonia were transplanted by surgery followed by injection of the gonads. In both examples, the recipient fish had their endogenous spermatogenesis suppressed using the cytostatic drug busulfan (1, 4-butanediol dimethanesulfonate) in association with high temperature. Xenogens (pejerrey) and allogens (tilapia) were produced with no apparent immune response against the donor cells and both xenogens and allogens were able to produce progeny with genotypes of the donor cells.

One disadvantage of suppressing spermatogenesis with busulfan is that the native stem cells are not annihilated and after treatment is terminated, natural spermatogenesis returns so these individuals produce both host and donor germ cells and, thus, produce both host and donor progeny. Apparently, there was also no immune response against donor germ line stem cells in the triploid channel catfish as blue catfish cells were present after 60 days at high temperature when the catfish immune system should be operating at maximum efficiency and were still present after 10 months and at least one individual was able to produce progeny originating from the donor cells. The triploid xenogens, in contrast to the busulfan xenogens, should not be able to produce host gametes (Dunham, 2011). Thus, if introduced cells undergo gametogenesis, they alone will contribute to the germ line of resulting offspring. Obviously, this is critical for commercial-scale application, and the experimental protocols for the tilapia and pejerrey could not be scaled industrially because of the mixture of host and donor progeny.

The xenogenic channel catfish males exhibited normal courtship, attempted to fertilize the eggs and were able to induce about 80% of the females to lay egg masses. The xenogenic males showed aggressive mating behavior and guarded the eggs, including severely biting the scientist removing the egg masses. The ability of the triploid xenogenic males to induce females to spawn was an expected result as sterile triploid males in other fish species were able to induce females to lay eggs. Triploid Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) have shown courting and aggressive behavior while spawning (Fjelldal et al. 2010; 2014). Triploid brook trout migrate in response to sexual maturity (Warrillow et al. 1997) and attempt to spawn. Triploid males of salmon and grass carp (*Ctenopharyngodon idella*) have a sex hormone cycle similar to those of diploid males and can mate diploid females though the eggs are not fertilized (Dunham 2011).

A total of 19 egg masses were produced by channel catfish females that were paired with xenogenic males. None of these eggs hatched. In some cases, the testicular development of xenogenic males was inadequate and the testes appeared devoid of sperm. In a few cases, the males had sperm, but the quantity was insufficient to naturally fertilize eggs in an aquarium or they were unable to ejaculate. For some of these fish, the stem cells were introduced via catheterization so the sperm duct had sufficient diameter for ejaculation. However, the testes proper has a series of valves that have not been defined (Dunham and Dunham in preparation) and delivery of ictalurid sperm appears complicated. One possibility is that by the time the stem cells were introduced that these

ducts were irreversibly malformed preventing ejaculation and testes structure and function needs further study.

If the donor cells dictate the temporal development of sperm, this could be another explanation for the low levels of sperm production. These xenogenic males were mated at 3 and 4 years of age, which is the normal age of sexual maturity for the host's (channel catfish) body. However, blue catfish usually do not become sexually mature until 5 years of age. Thus, if the sperm are programmed to mature at 5 years, the xenogenic males would not be able to produce large amounts of sperm until they are one year older.

The xenogenic males appeared to show increasing sexual maturity with time. One year after stem cell introduction only 1 of 7 males from the 2012 group and 0 of 10 males from the 2013 group showed signs of sperm production. Both groups were able to induce 70-71% of the females to deposit eggs one year after the males had been transplanted. For the 2012 group, 43% of the males had obvious sperm production two years after transplantation versus 14% after one year. Additionally, their ability to convince females to deposit eggs increased from 71% to 100%. Perhaps, the level of sexual maturity will increase the third year of transplantation when these males are 5-years-old. Alternatively, the 2012 group received LHRHa therapy in 2014 and this may have been the cause of the increased sperm production. If this is the explanation, more extensive hormone therapy could increase sperm production.

This slow rate of sexual maturity and apparent expansion and maturation of the donor stem cells impedes commercial-scale application. Several possible solutions need evaluation including introduction a larger number of donor cells. The donor cells were injected at 3 locations in each testis. This may have limited the colonization, thus diminishing sperm production, and might be corrected by increasing the number of injections per testes. The xenogenic males and their gonads may need increased hormonal stimulation to increase sperm proliferation and maturation, and this could be introduced exogenously. The xenogenic males in the current experiment were not transplanted until they were 2 and 3 years old. For increased colonization and sperm production, these xenogens may need transplantation at younger ages and smaller sizes to promote colonization and provide increased time for spermatogonia A maturation and transformation into mature sperm.

Possibly, none of these alterations to the protocol will result in improved sperm production or ejaculation obviating large-scale application. Allowing full development of gonads prior to stem cell introduction may limit sperm production, proper testicular function and ejaculation. Introduction of stem cells to triploid blastula or fry may be necessary to influence and guide proper gonadal development. Research would also be needed to increase the efficiency of transformation and survival of injected blastula and fry.

By using sperm from a xenogenic male to fertilize channel catfish eggs, a channel catfish female X blue catfish male hybrid was produced via xenogenesis for the first time.

Even though only 1% of the embryos hatched, this illustrates that SSCs from a blue catfish can be transplanted to a triploid channel catfish host, colonize, become mature sperm and fertilize eggs. This is first report of xenogenesis in catfish and in fish in North America.

A different procedure was utilized to produce pejerrey interspecific hybrids (Majhi et al. 2009). This protocol was more efficient as only 4 out of 20 (20%) transplanted pejerrey produced the donor *O. bonariensis* spermatozoa. Similar to the current experiment, testes from these four were dissected and used to inseminate eggs from normal *O. hatcheri* host species. The resulting progeny were 1.2 -13.3 % *hybrid progeny between the two species* and the remaining progeny were pure *O. bonariensis*. *Thus, the current experiment, which produced 100% channel –blue hybrid catfish, is the first to produce 100% hybrids with xenogenesis. To date, to the best of our knowledge, there are no experiments that have successfully produced xenogenically-derived hybrids through natural or semi-natural spawning.*

The results of the current experiment demonstrate that it is possible to produce catfish hybrids with xenogenesis. If the protocols can be altered to increase the fry production to realistic commercial levels, there are multiple ways to apply the technology in the catfish farming industry. One would be to mate xenogenic males with normal channel catfish females in traditional open ponds. This would greatly reduce the labor and skill required for the current artificial fertilization techniques used to produce hybrid catfish embryos (Su et al. 2013; Chatakondi et al. 2005ab; Masser and Dunham 1998).

The current open pond spawning technique can produce sporadic results and is highly affected by weather patterns (Pawiroredjo et al. 2008).

Alternatively, the mating of the xenogenic males and the channel catfish females could be conducted with aquaria spawning following hormone induction of the females. This technique is highly efficient (Dunham unpublished), reliable and with temperature-controlled water, not dependant on weather patterns. This technique is not labor intensive, does not require much skill compared to artificial fertilization technology and is an intermediate technology that could easily be adopted by most hatcheries.

Even if commercial-scale application of xenogenesis for hybrid catfish production does not materialize, many potential applications exist. These include technology to rapidly expand and conserve endangered catfish species or strains. This would also provide a mechanism to study cellular and tissue communication and physiology and pseudo-cloning of specific genotypes by cloning of gonads. Furthermore, techniques to avoid inbreeding, technology to produce difficult-to-spawn-species, such as blue catfish, possible enhanced gene transfer, and a method to reduce generation intervals can be developed. If cryopreserved testes exist for extinct species, it would also be possible to develop technologies to resurrect extinct species or lines of catfish.

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APPENDIX

Multiple sequence alignment of a partial sequence of cytochrome oxidase I gene from 7 test samples against channel, hybrid and blue catfish control sequences. Original sequences were trimmed so that only sequences with good quality were aligned. Except for the single point mutation in samples 1 and 2 at the bases 291 and 294 of the aligned sequence and excluding the first and last part of the alignment which might be attributed to sequencing errors, the 7 test samples were identical to the channel and hybrid control sequences. On the other hand, the test samples were not identical to the blue catfish control sequence because of point mutation in many locations confirming that the 7 test samples are channel catfish female X blue catfish male hybrids.

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Blue_Control      1  -----TTATCCGGGCGAG
Channel_Control   1  -----CCCTTAGCCTGCTTATCCGGGCGG
Hybrid_Control    1  -----CGGCCCTTAGCCTGCTTATCCGGGCGG
Sample_1          1  -----TTATCCGGGCGG
Sample_2          1  -----GGTGCCTGAGCCGGAATAGTGGGTACCGCCCTTAGCCTGCTTATCCGGGCGG
Sample_3          1  TTGTATTTGGTGCCTGAGCCGG--ATAGTGGGTACGGCCCTTAGCCTGCTTATCCGGGCGG
Sample_4          1  -----CCGGAATAGTGGGTACCGCCCTTAGCCTGCTTATCCGGGCGG
Sample_5          1  -----GGTGCCTGAGCCGGAATAGTGGGTACCGCCCTTAGCCTGCTTATCCGGGCGG
Sample_6          1  -----GTGGGTACGGCGTTAGCCTGCTTATCCGGGCGG
Sample_7          1  -----GGTGCCTGA-CCGGAATAGTGGGTACCGCCCTTAGCCTGCTTATCCGGGCGG
  
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Blue_Control      13  AGCTAGCCCAACCCGGTGGCCCTTCTAGGCGATGACCAAATTTAATGTTATTGTTACTG
Channel_Control   25  AATTAGCCCAACCCGGCGCCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG
Hybrid_Control    28  AATTAGCCCAACCCGGCGCCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG
Sample_1          13  AATTAGCCCAACCCGGCGCCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG
Sample_2          53  AATTAGCCCAACCCGGCGCCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG
Sample_3          60  AATTAGCCCAACCCGGCGCCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG
Sample_4          43  AATTAGCCCAACCCGGCGCCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG
Sample_5          53  AATTAGCCCAACCCGGCGCCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG
Sample_6          35  AATTAGCCCAACCCGGCGCCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG
Sample_7          52  AATTAGCCCAACCCGGCGCCCTTCTAGGCGATGACCAAATTTACAATGTTATTGTTACTG
  
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Blue_Control      73  CCACGCCTTTGTAATAATTTCTTTATAGTAATGCCAATTATGATTGGAGGGTTTGAA
Channel_Control   85  CTCACGCCTTTGTAATAATTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGAA
Hybrid_Control    88  CTCACGCCTTTGTAATAATTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGAA
  
```

Sample_1	73	CTCACGCCTTTGTAATAATTTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGAA
Sample_2	113	CTCACGCCTTTGTAATAATTTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGAA
Sample_3	120	CTCACGCCTTTGTAATAATTTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGAA
Sample_4	103	CTCACGCCTTTGTAATAATTTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGAA
Sample_5	113	CTCACGCCTTTGTAATAATTTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGAA
Sample_6	95	CTCACGCCTTTGTAATAATTTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGAA
Sample_7	112	CTCACGCCTTTGTAATAATTTTCTTTATAGTAATACCAATTATGATTGGAGGGTTTGAA

Blue_Control	133	ATTGGCTTGTCCCTAATGATGGGGCCAGACATGGCCTTCCCTCGAATAAACAATA
Channel_Control	145	ACTGGCTTGTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCCTCGAATGAACAACA
Hybrid_Control	148	ACTGGCTTGTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCCTCGAATGAACAACA
Sample_1	133	ACTGGCTTGTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCCTCGAATGAACAACA
Sample_2	173	ACTGGCTTGTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCCTCGAATGAACAACA
Sample_3	180	ACTGGCTTGTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCCTCGAATGAACAACA
Sample_4	163	ACTGGCTTGTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCCTCGAATGAACAACA
Sample_5	173	ACTGGCTTGTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCCTCGAATGAACAACA
Sample_6	155	ACTGGCTTGTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCCTCGAATGAACAACA
Sample_7	172	ACTGGCTTGTCCCTAATGATCGGAGCGCCAGATATGGCCTTCCCTCGAATGAACAACA

Blue_Control	193	TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCCGGAGTTGAAG
Channel_Control	205	TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCCGGAGTTGAAG
Hybrid_Control	208	TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCCGGAGTTGAAG
Sample_1	193	TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCCGGAGTTGAAG
Sample_2	233	TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCCGGAGTTGAAG
Sample_3	240	TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCCGGAGTTGAAG
Sample_4	223	TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCCGGAGTTGAAG
Sample_5	233	TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCCGGAGTTGAAG
Sample_6	215	TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCCGGAGTTGAAG
Sample_7	232	TAAGCTTCTGGCTCCTGCCCCCTCCTTCTACTTCTGCTCGCCTCCTCCGGAGTTGAAG

Blue_Control	253	CGGGAGCGGGACAGGATGAAGTGTATACCCGCCTCTTGGGGCAACCTCGCACATGCAG
Channel_Control	265	CAGGAGCAGGAACAGGGTGAAGTGTATACCCGCCTCTTGCCGGCAACCTCGCACATGCAG
Hybrid_Control	268	CAGGAGCAGGAACAGGGTGAAGTGTATACCCGCCTCTTGCCGGCAACCTCGCACATGCAG
Sample_1	253	CAGGAGCAGGAACAGGGTGAAGTGTCTACCCGCCTCTTGCCGGCAACCTCGCACATGCAG
Sample_2	293	CAGGAGCAGGAACAGGGTGAAGTGTCTACCCGCCTCTTGCCGGCAACCTCGCACATGCAG
Sample_3	300	CAGGAGCAGGAACAGGGTGAAGTGTCTACCCGCCTCTTGCCGGCAACCTCGCACATGCAG
Sample_4	283	CAGGAGCAGGAACAGGGTGAAGTGTCTACCCGCCTCTTGCCGGCAACCTCGCACATGCAG
Sample_5	293	CAGGAGCAGGAACAGGGTGAAGTGTCTACCCGCCTCTTGCCGGCAACCTCGCACATGCAG
Sample_6	275	CAGGAGCAGGAACAGGGTGAAGTGTCTACCCGCCTCTTGCCGGCAACCTCGCACATGCAG
Sample_7	292	CAGGAGCAGGAACAGGGTGAAGTGTCTACCCGCCTCTTGCCGGCAACCTCGCACATGCAG

Blue_Control	313	GGGCCTCCGTAGATTTAACTATCTTTTCCCTTACACTTGCAGGAGTTTCATCTATTCTTG
Channel_Control	325	GGGCCTCCGTAGATTTAACTATCTTTTCCCTTACACTTGCAGGAGTTTCATCTATTCTTG
Hybrid_Control	328	GGGCCTCCGTAGATTTAACTATCTTTTCCCTTACACTTGCAGGAGTTTCATCTATTCTTG
Sample_1	313	GGGCCTCCGTAGATTTAACTATCTTTTCCCTTACACTTGCAGGAGTTTCATCTATTCTTG
Sample_2	353	GGGCCTCCGTAGATTTAACTATCTTTTCCCTTACACTTGCAGGAGTTTCATCTATTCTTG
Sample_3	360	GGGCCTCCGTAGATTTAACTATCTTTTCCCTTACACTTGCAGGAGTTTCATCTATTCTTG
Sample_4	343	GGGCCTCCGTAGATTTAACTATCTTTTCCCTTACACTTGCAGGAGTTTCATCTATTCTTG
Sample_5	353	GGGCCTCCGTAGATTTAACTATCTTTTCCCTTACACTTGCAGGAGTTTCATCTATTCTTG
Sample_6	335	GGGCCTCCGTAGATTTAACTATCTTTTCCCTTACACTTGCAGGAGTTTCATCTATTCTTG
Sample_7	352	GGGCCTCCGTAGATTTAACTATCTTTTCCCTTACACTTGCAGGAGTTTCATCTATTCTTG

Blue_Control	373	GGGCCATTAATTTTATTACAACAATTATTAACATAAAACCCCCCGCAATTCACAATATC
Channel_Control	385	GGGCCATTAACTTTATTACAACAATTATTAATATGAAGCCCCCGCAATTCACAATATC
Hybrid_Control	388	GGGCCATTAACTTTATTACAACAATTATTAATATGAAGCCCCCGCAATTCACAATATC
Sample_1	373	GGGCCATTAACTTTATTACAACAATTATTAATATGAAGCCCCCGCAATTCACAATATC

Sample_2 413 GGGCCATTAAC TTTATTACAACAATTATTAATATGAAGCCCCCGCAATCTCACAATATC
 Sample_3 420 GGGCCATTAAC TTTATTACAACAATTATTAATATGAAGCCCCCGCAATCTCACAATATC
 Sample_4 403 GGGCCATTAAC TTTATTACAACAATTATTAATATGAAGCCCCCGCAATCTCACAATATC
 Sample_5 413 GGGCCATTAAC TTTATTACAACAATTATTAATATGAAGCCCCCGCAATCTCACAATATC
 Sample_6 395 GGGCCATTAAC TTTATTACAACAATTATTAATATGAAGCCCCCGCAATCTCACAATATC
 Sample_7 412 GGGCCATTAAC TTTATTACAACAATTATTAATATGAAGCCCCCGCAATCTCACAATATC

Blue_Control 433 AGACTCCCCTATTTGCTGAGCGTGTCCCTAATTACAGCCGTCCTACTACTACTATCCCTCC
 Channel_Control 445 AAACCCCCCTATTTGCTGAGCCGTCC TAATTACAGCCGTCCTCTACTACTATCCCTCC
 Hybrid_Control 448 AAACCCCCCTATTTGCTGAGCCGTCC TAATTACAGCCGTCCTCTACTACTATCCCTCC
 Sample_1 433 AAACCCCCCTATTTGCTGAGCCGTCC TAATTACAGCCGTCCTCTACTACTATCCCTCC
 Sample_2 473 AAACCCCCCTATTTGCTGAGCCGTCC TAATTACAGCCGTCCTCTACTACTATCCCTCC
 Sample_3 480 AAACCCCCCTATTTGCTGAGCCGTCC TAATTACAGCCGTCCTCTACTACTATCCCTCC
 Sample_4 463 AAACCCCCCTATTTGCTGAGCCGTCC TAATTACAGCCGTCCTCTACTACTATCCCTCC
 Sample_5 473 AAACCCCCCTATTTGCTGAGCCGTCC TAATTACAGCCGTCCTCTACTACTATCCCTCC
 Sample_6 455 AAACCCCCCTATTTGCTGAGCCGTCC TAATTACAGCCGTCCTCTACTACTATCCCTCC
 Sample_7 472 AAACCCCCCTATTTGCTGAGCCGTCC TAATTACAGCCGTCCTCTACTACTATCCCTCC

Blue_Control 493 CAGTCTAGCTGCTGGCATCACAATACTTCTAACAGACCGAAACTTAAACACCCACTTCT
 Channel_Control 505 CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAACTACTACCTTCT
 Hybrid_Control 508 CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAACTACTACCTTCT
 Sample_1 493 CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAACTACTACCTTCT
 Sample_2 533 CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAACTACTACCTTCT
 Sample_3 540 CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAACTACTACCTTCT
 Sample_4 523 CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAACTACTACCTTCT
 Sample_5 533 CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAACTACTACCTTCT
 Sample_6 515 CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAACTACTACCTTCT
 Sample_7 532 CAGTTTTAGCCGCTGGTATCACAATGCTTCTAACAGACCGAAACTTAAACTACTACCTTCT

Blue_Control 553 TTGACCCG CAGGGGGAGGGGACCCCA TTTCTTTACCAACACCTTTTCTGATTCTTCGGTC
 Channel_Control 565 TTGACCCTGCAGGGGGAGGGGACCCCA TTTCTTTACCAACACCTTTTCTGATTCTTCGGTC
 Hybrid_Control 568 TTGACCCTGCAGGGGGAGGGGACCCCA TTTCTTTACCAACACCTTTTCTGATTCTTCGGTC
 Sample_1 553 TTGACCCTGCAGGGGGAGGGGACCCCA TTTCTTTACCAACACCTTTTCTGATTCTTCGGTC
 Sample_2 593 TTGACCCTGCAGGGGGAGGGGACCCCA TTTCTTTACCAACACCTTTTCTGATTCTTCGGTC
 Sample_3 600 TTGACCCTGCAGGGGGAGGGGACCCCA TTTCTTTACCAACACCTTTTCTGA-----
 Sample_4 583 TTGACCCTGCAGGGGGAGGGGACCCCA TTTCTTTACCAACACCTTTTCTGATTCTTCGGTC
 Sample_5 593 TTGACCCTGCAGGGGGAGGGGACCCCA TTTCTTTACCAACACCTTTTCTGATTCTTCGGTC
 Sample_6 575 TTGACCCTGCAGGGGGAGGGGACCCCA TTTCTTTACCAACACCTTTTCTGATTCTTCGGTC
 Sample_7 592 TTGACCCTGCAGGGGGAGGGGACCCCA TTTCTTTACCAACACCTTTTCTGATTCTTCGGTC

Blue_Control 613 ACCCTGAAGTGTCA-----A
 Channel_Control 625 ACCCTGAAGTGTCA TAGCTTGT TTC
 Hybrid_Control 628 ACCCTGAAGTG-----T
 Sample_1 613 ACCCTGAAGT-----G
 Sample_2 653 ACCCTGAAGTG-----T
 Sample_3 650 -----T
 Sample_4 643 ACCCTGAAGTGTCA-----T
 Sample_5 653 ACCCTGAAGTGTCA-----AG
 Sample_6 635 ACCCTGAAGTG-----T
 Sample_7 652 ACCCTGAAGTGTCA-AGC-----TT

CHAPTER THREE

Genotype-Environment Interactions of Different Genetic Types of Hybrid Catfish

Abstract

Genotype environment (GE) interactions were studied for eight genetic types of channel catfish (*Ictalurus punctatus*) female x blue catfish (*Ictalurus furcatus*) male catfish hybrids. These hybrids were reared in four different environments; a low density pond, high density pond, split pond and in-pond raceway. Feed conversion ratio (FCR) was better than what is usually expected on farm for ictalurid catfish. The FCR was 1.28, 1.99, 1.65 and 1.52 for the low density pond, high density pond, split pond and in-pond raceway, respectively. Genetic type, sex, environment and all possible interactions affected body weight for the different genetic types of channel catfish female x blue catfish male hybrids ($P < 0.05$). MS X RG and KS X RG were the largest two genetic types in each environment, indicating that a single genetic enhancement program could address the improvement of hybrid performance for all culture systems used in the catfish industry. Hybrids produced by MS and KS females selected for increased body weight for 8 generations were larger compared to hybrids from MR and KR random controls in all environments. GE interactions were observed in regards to the sex, and differences were found ($P < 0.05$) between males and females for final body weight. Survival in high density pond, split pond and in-pond raceway from stocker stage to the harvest in the current experiment was as high as 96%. Harvestability was impacted by environment

($P < 0.05$). Fish from the high density pond and the in-pond raceway, respectively, were the easiest to capture.

Introduction

Interspecific hybridization is the mating of two different species. Hundreds of different interspecific fish hybrids have been produced by artificial insemination, including hybrids between white bass (*Morone chrysops*) female x striped bass (*M. saxatilis*) male (Smith 1988), tilapia Mossambique (*Oreochromis mossambicus*) female x Nile tilapia (*O. niloticus*) male (Lim et al. 1993), bluegill (*Lepomis macrochirus*) female x green sunfish (*L. cyanellus*) x male (Wang et al. 1998), walleye (*Stizotiedion vitreum*) female x sauger (*S. canadense*) male (Hearn 1986) and between channel catfish (*Ictalurus punctatus*) female x blue catfish (*I. furcatus*) male (Giudice 1966; Dunham et al. 1987; Dunham et al. 1990). In general, interspecific hybridization programs have a low probability of success (Dunham 2011) and heterosis, especially for growth. Approximately 50 North American catfish have been evaluated, (Dunham and Smitherman 1984) but only one, the channel catfish female *I. punctatus* crossed with blue catfish, *I. furcatus*, males showed heterosis (Giudice 1966) .

The channel-blue hybrid is considered to be the best catfish genotype for pond culture (Dunham et al. 2007). This particular hybrid has shown better performance when compared to the commonly grown channel catfish. Various economically important traits were improved. This included: (15-20%) better feed conversion (Yant 1975), growth (especially at high densities, 20-100%) (Giudice 1966; Yant et al. 1976; Dunham et al. 1987, 1990; Dunham and Brummett 1999, Argue et al. 2003, Li et al. 2004,

Dunham et al. 2007, Brown et al. 2011), bacterial disease resistance (Ella 1984, Wolters et al. 1996, Dunham et al. 2007, Arias et al. 2012) but not viral disease resistance (Plumb and Chappell 1978), and overall survival (10-100%) (Ella 1984, Dunham and Brummett 1999). Additionally, the channel/blue catfish hybrid exhibits heterotic tolerance of low oxygen (50-100%) (Dunham et al. 1983), harvest by seining (50-100%) (Yant et al. 1975; Dunham and Argue 1998), angling vulnerability (100%) (Dunham et al. 1986), carcass yield (10%) (Yant 1975; Huang et al. 1994, Argue et al. 2003, Bosworth et al. 2004, Bosworth et al. 2012) and has more uniform growth and body shape (Yant et al. 1975; Brooks et al. 1982a,b; Dunham et al. 1982).

The strain of the parent species affects the level of heterosis observed in hybrids (Dunham et al. 2014a; Dunham et al. 2014b). Differences in body weight were found among channel-blue catfish hybrids produced from different parental strains or families (Dunham et al. 1987).

Genotype-environment (GE) interactions have been evaluated in many fish species. Growth rate was measured for three genetic groups of common carp, *Cyprinus carpio*, (Chinese and the European race and an inter-racial Chinese X European crossbreed) in five different environments, which differed in stocking rate and feed type (Wohlfarth et al. 1983). The Chinese carp was the fastest growth in all environments followed by Chinese x European. Response curves were constructed by plotting growth in each of the tested environments for each genotype. The differences in slopes and intercepts of the response curves of each genotype demonstrated the genotype-

environment interaction. Saillant et al. (2006) studied genetic parameters for somatic growth rate in sea bass (*Dicentrarchus labrax L.*) in two experiments where 27 (year-class 1997) to 30 (year-class 1998) families of sea bass were raised in four different environments (high temperature and low temperature) and two density regimes. The results from both experiments indicated an important additive genetic component in growth rate of the sea bass in all conditions. Correlations of the estimated breeding values of the sires between treatments and years were moderate, suggesting the occurrence of genotype x environment effects. Genotype-environment interactions were also studied in paradise fish (*Macropodus opercularis*). Three inbred strains and all of their possible F1 crosses were monitored in four different environmental conditions and genotype-environment interactions were found for behavior (Gerlai and Csanyi 1990).

Several other species were investigated in different environments: Strains of rainbow trout, *Oncorhynchus mykiss*, tested for growth (Fishback et al. 2002), five families of Pacific oysters, *Crassostrea gigas*, were studied in five different environments for body weight (Swan et al. 2007) and 30 families of European sea bass *Dicentrarchus labrax L.* were also examined for body weight in four different environments (low temperature, high temperature, low density and high density) (Dupont et al. 2008). GE interactions were observed in all of these comparisons.

Dunham et al. (1990) estimated genotype-environment interactions for growth of blue, channel and hybrid catfish in ponds and cages at varying densities. When fry were communally evaluated, channel catfish grew faster and larger than the CB hybrid at low

density, but at high density a GE interaction occurred and hybrids grew larger than channel catfish. When fry or fingerlings were stocked communally or separately in ponds, hybrid catfish grew faster 30-121% than channel catfish at all densities, but when grown in cages, GE interactions were evident and channel catfish grew faster than hybrid catfish.

Makhubu (2014) estimated GE interactions for eight genetic types of channel catfish (*I. punctatus*) female x blue catfish (*I. furcatus*) male hybrids in three environments, a low density pond, a high density pond and an in-pond raceway grown to stocker size. Genotype x environment, sex x environment and genotype x sex x environment interactions were observed. Fish in the low density pond and the in-pond raceway were of similar size, yet the sexual dimorphism was different in these two environments. Genotype- environment interactions were observed for survival. Significant genotype-environment interactions existed, and multiple breeding programs for the multiple culture systems were recommended.

Genotype-environment (GE) interactions are relevant to farm application of genetically improved fish. Genotype-environment interactions among strains and selected lines are minimal. The GE interaction is more common when comparing intraspecific crossbreeds and parents (Bentsen et al. 1998; Gjerde 2006). However, this interaction is much more prevalent when comparing polyploids, transgenics, species and interspecific hybrids (Dunham et al. 1990; Xie et al. 2007; Dunham 2011). In aquaculture, there are many different culture environments. Catfish are now cultured in upwards to 4 or more

environments: traditional ponds (single or multi-cropping), in-pond raceways and split ponds. Since a variety of farm conditions exist, genotype-environment interactions must be evaluated before improved stocks can be recommended (Dunham et al. 1990).

The current study continues the work of Makhubu (2014) and evaluates different genetic types of CB hybrids when grown to larger sizes and in an additional environment. The objectives were to measure genotype-environment interactions for growth, survival, sexual dimorphism and seinability for different genetic types of channel catfish female x blue catfish male hybrids grown in a low density pond, a high density pond, a split pond and an in-pond raceway, and to identify the best performing genetic type of hybrid under these culture conditions when grown from stocker size to market (food) size.

Materials and Methods

Experimental Fish

Five lines of channel catfish females were hybridized in 2012 with 2 lines of blue catfish males D&B (DB) and Rio Grande (RG) (Makhubu 2014). Channel catfish lines were Kansas Random (KR), Marion Random (MR), Marion Select (MS, selected for 8 generations for increased body weight), Kansas Select (KS, selected for 8 generations for increased body weight) and 103 x KS (an F2 generation cross between NWAC-103 and KS). Ancestry of these fish can be found in Dunham and Smitherman (1983). NWAC-103 was selected for growth for 2 generations and originated from a fast growing strain. These fish were maintained at the Fish Genetics Unit, E.W. Shell Fisheries Research Center, Auburn University, Alabama. Spawning, incubation, fry culture, stocking into different environments and growing the fish to stocker size are found in Makhubu (2014).

Fingerling stocking and harvest of stocker fish

To begin the stocker phase of this experiment, 13-month-old fingerlings were seined in July 2013 from ponds, weighed, heat branded to differentiate the genetic groups at harvest, and stocked communally so that each genetic group was represented in each experimental unit to minimize the component of environmental variation and accentuate the effects of genotype and GE interaction (Brummett 1986; Dunham and Brummett 1999; Wohlfarth and Moav 2014). Each fish was heat branded using the technique of

Moav et al. (1960). In this design, the individual fish are replicates within the environment. Not only does this minimize the component of environmental variation, but is necessary for experiments where unrealistically large numbers of animals would be required to replicate environments. This type of design with individuals as replicates within the individual environments or with individuals nested within a treatment within a single experimental unit is commonly utilized to determine the extent of genotype-environment interactions in animals (Brown and Gacula, Carter et al. 1971, Koger et al. 1975, Sandelin et al. 2002, Case et al. 2014, Durunna et al. 2014).

These hybrids were stocked into three different environments (low density pond, high density pond and in-pond raceway) and were harvested after 5 months to estimate GE interactions for growth, survival, sexual dimorphism and seinability of stocker size hybrids. To initiate the current experiment, all fish were stocked back into the low density pond, high density pond and in-pond raceway on November 12, 2013; January 12, 2014, and January 22, 2014, respectively, to study the growth from stocker size to food fish (Table 1). The initial body weight and numbers (final body weight and numbers at stocker size) for the current study of each genetic type for each environment are presented in Table 2.

Experimental units

Three different environments were used: in-pond raceway, a high density pond and a low density pond at the beginning of this experiment. In August 13, 2014, fish from low density pond and in-pond raceway were randomly sampled, and on September

3, 2014, the hybrids from high density pond were harvested, data collected and fish stocked into a split pond on September 5, 2014. Additional hybrid catfish were stocked to increase the density and to reach 5000 fish (125,000 fish/ha, Table 1). Each individual was heat branded to differentiate them from the study groups. Initial body weight and number for the split pond are also found in Table 1.

The earthen split pond consisted of two areas. The fish or culture pond had a surface area of 0.04 ha (405 m²) and was approximately 1.8 m deep. The waste treatment pond had a surface area of 0.12 ha (1,213.8 m²) and had a similar depth as the culture pond. During the night, two aerators, 1.0 and 0.5 hp, were used (the first from - 1800-0800 h, the second from - 0100 - 0800 h, respectively) in the fish pond to maintain a minimum dissolved oxygen level of 4.0 mg/L and during the day time two 0.5-hp water pumps were used to exchange the water (280 m³/hr) between the two ponds.

The low and high density earthen ponds had a surface area of 0.04 ha (404.6 m²) with an average depth of 1.6 m and 1.0 m, respectively. Water levels were maintained by periodic addition of water compensating for loss due to seepage and evaporation, and a 0.5-hp power aerator was used every night to elevate dissolved oxygen to a minimum value of 3.5 mg/L (2200 - 0600 h).

The raceway was rectangular, built from treated lumber and suspended between walkways of a floating pier, having the dimensions of 1.2 m deep, 1.2 m wide, 5.9 m long, for a total volume of 8.5 m³. It was constructed of a semi-rigid, high density

polyethylene plastic. A set of air lift pumps located at the head end of the raceway circulated pond water into the raceway and through a screened end. It had approximately a 4% slope along its length to assist the movement of fish waste. The raceway was located in a 10-ha pond containing 11 additional in-pond raceways. The stocking densities for the four environments at stocker size and the stocking numbers and weights (kg) for each of the genetic groups in each environment at stocker size are found in Table 1.

Table 1. The stocking densities of the different genetic types of channel catfish (*Ictalurus punctatus*) female x blue catfish (*I. furcatus*) male hybrids catfish cultured in a low density pond, high density pond, split pond and an in-pond raceway

Environments	Density
Low density pond	10,925 fish/ha (0.06 fish/m ³)
High density pond	59,550 fish/ha (3.3 fish/m ³)
Split pond	31,250 fish/ha (1.72 fish/m ³)
In-pond raceway	226 fish/m ³

Table 2. The stocking numbers and mean initial body weights (BW, kg) of the different genetic types of channel catfish (*Ictalurus punctatus*) female x blue catfish (*I. furcatus*) male catfish hybrids cultured in a low density pond, high density pond, split pond and in-pond raceway environments

Genetic type ¹	Environment and initial BW (kg)							
	Low density		High density		Split pond		Raceway	
	No.	BW	No.	BW	No.	BW	No.	BW
103KS X DB	62	0.110	501	0.101	477	0.217	349	0.131
MR X DB	43	0.184	109	0.145	102	0.277	159	0.209
KS X DB	54	0.186	593	0.139	575	0.260	237	0.179
KR X DB	40	0.177	262	0.103	260	0.205	229	0.145
103KS X RG	63	0.136	167	0.109	160	0.241	395	0.146
MS X RG	37	0.253	120	0.176	119	0.370	91	0.217
KR X RG	58	0.202	353	0.104	344	0.217	239	0.130
KS X RG	60	0.235	277	0.155	261	0.312	221	0.224
Grand mean		0.181		0.124		0.249		0.136

¹Blue catfish sires were D&B (DB) and Rio Grande (RG). Channel catfish lines were Kansas random (KR), Marion random (MR), Marion select (MS, selected for 8 generations for increased body weight), Kansas select (KS, selected for 8 generations for increased body weight) and 103KS (an F2 generation cross between NWAC-103 and KS).

Fish culture

The fish in all environments were fed to satiation one time per day with 32% crude protein floating catfish pellets. Dissolved oxygen (DO) and water temperature were measured daily in the early morning using a YSI® Pro20 Dissolved Oxygen Meter (Pentair Aquatic Eco-Systems, Inc. Apopka, FL), aeration was provided during day time only if the dissolved oxygen was critically low (<3 mg/L) during summer. Other water quality parameters such as pH, total ammonia (NH₃), nitrite (NO₂), hardness, alkalinity and chloride (Cl⁻) were measured once a week during the culture period using Fish Farm 9 Test Kit (Pentair Aquatic Eco-Systems, Inc. Apopka, FL). DO was maintained between 4 and 6.5 mg/L, pH was maintained between 6 and 7 mg/L, ammonia was at <1 mg/L, nitrite was maintained at 0.05 mg/L, hardness was maintained between 46 and 68 mg/L, alkalinity maintained between 40 and 84 mg/L, chloride was maintained above 60 mg/L. Low levels of Cl⁻ were observed in the split pond (October 18, 2014) and sodium chloride was applied to increase the Cl⁻ level. Approximately, 910 kg of sodium chloride was added to increase the chloride level from 20 mg/L to 68 mg/L.

Harvesting and data collection

After a grow-out period of 8 months, the high density pond was harvested on September 3, 2014 with a 30-m seine. Seining was done in the morning when the temperature was the lowest to avoid fish stress. The ponds were prepared for seining by reducing the water level approximately 50% the previous day. After 2-3 seine hauls, the remaining fish were caught by completely draining the pond and capturing them with dip nets.

Similar harvesting procedures were repeated for the split-pond harvested on January 14, 2015 (5 months of rearing) and the low density pond harvested on 12/13/15 (8 months of rearing).

A rectangular net with a metal frame was used for harvesting the in-pond raceway. The net was carefully placed at the far end of the raceway, ensuring it touched the raceway bottom, and the raceway was seined four times.

The day following harvest from each environment, individual body weight and sex were determined for each fish in each genetic group and recorded. Data were taken by seine haul to determine seinability for each genetic group on each environment.

Statistical Analysis

Experimental data were analyzed for statistical significance using ANOVA and LSM (Least Square mean) for differences in treatment means.

Body weight

Final individual body weight was analyzed using three-way analysis of variance (ANOVA) at the significance level ($P \leq 0.05$). A three-way ANOVA model was used as below.

$$y_{ijk} = \mu + G_i + S_j + E_k + (GE)_{ik} + (SE)_{jk} + (GS)_{ij} + (GSE)_{ijk} + \epsilon_{ijkl}$$

Where, μ : overall mean body weight; y_{ijk} : final body weight;

G: main effect of genetic type of hybrids; $i=1, 2, 3, \dots, 8$;

S: main effect of gender; $j=F$ or M ;

E: environment; k=1, 2, 3 and 4;

ϵ : random error.

Weight gain

Weight gain (kg) and percent change in body weight from stocker size to harvest for each genetic type within each environment were calculated.

Multiple comparisons on the means of final body weight, net gain and adjusted body weight were performed using the Tukey-Kramer procedure with two/three-way analysis of variance (ANOVA) at the significant level $P \leq 0.05$ (SAS 9.3, SAS Institute Inc. NC, USA). Equal variance, independent and normal distribution of residuals were checked using Bartlett's Test, QQ-plot and residuals plots. When assumption of homogeneity of variance was violated, data were transformed using Box-Cox transformation and the ANOVA was performed on the transformed data.

Survival

Different genetic types of hybrids had different survival in the three environments. To determine influence of the main factors, such as genetic type of hybrids, sex, initial mean body weight, final body weight, net gain, adjusted body weight and environments on the survival of the genetic type of hybrids, a logistic model were used given as below.

$$U = \mu + G_i + S_j + E_k + y_i + I + (GE)_{ik} + (SE)_{jk} + (GS)_{i,j} + (GSE)_{i,j,k} + \epsilon_{ijkl}$$

Where U: either 1 (survival) or 0 (death); μ : overall survival percentage; y_i : either the final body weight, net gain or adjusted final body weight. I: average initial body weight
Goodness of fit was checked using a Hosmer-Lemeshow test. INFLUENCE and IPLOTS were used for regression diagnostics.

Seinability

Seinability in regards to percentage of fish caught in the first haul was analyzed using Tukey's one df F test for additivity (Tukey 1949). The data structure was one observation per cell with two main factors. The model was given below:

$$Y_{ij} = \mu.. + G_i + E_j + D G_i E_j + \epsilon_{ij}.$$

Where, Y_{ij} : First seine haul percentage of a genetic type of hybrid in an environment;

μ : overall mean seinability;

G: main effect of genetic type of hybrid; $i=1, 2, 3, \dots, 8$;

E: main effect of environment; $j=1$ (split- pond), 2 (low density), and 3 (raceway);

D: a real-valued parameter to be estimated.

ϵ : random error

Means of genetic types with common sires (RG or DB) or common dams pooled by ancestry (M, K or 103KS were used as replicates (2-4) to evaluate sire and dam effects on certain traits. Means were compared with a t-test at $P<0.05$. Additionally, hybrids produced by select females were compared to their corresponding controls with an unpaired t-test at $P<0.05$. Males and females of the same genetic type in the same environment were compared with an unpaired t-test at $P<0.05$.

Results

Body weight

Final body weights of the test fish in the 4 environments: the in-pond raceway, low density, high density and split ponds and the relative rank of the genetic types in each environment are found in Table 3. Environment (E), genetic type (G), sex (S), and their interactions; genetic type \times environment (G \times E) and sex \times environment (S \times E) were all significant ($P < 0.05$) (Table 4).

The environment sum of squares was 48.8 times higher than the genetic type \times environment (G \times E) interaction, 128 times higher than the sex \times environment (S \times E) interaction and was 117 times greater than genetic type \times sex \times environment. The genetic type sum of squares was 3.1 times higher than genetic type \times sex \times environment. Thus environment accounted for the majority of the variability followed by genetic type.

There were no significant differences ($P > 0.05$) in final body weight for the low density environment. MS \times RG, 103KS \times RG, KS \times RG had the largest observed a mean body weight of 1.046, 0.790 and 0.780 kg, respectively. MS \times RG had higher ($P < 0.05$) mean body weight in the high density pond environment and the other environments than the other genetic types, but this was confounded by their larger initial body weight. The genetic types KS \times DB, 103 KS \times DB ,KR \times DB and KR \times RG had the lowest body weights ($P < 0.05$) in high density and split pond, except for MR \times DB

which was ($P > 0.05$) from KS X RG in both environments. KS X RG were larger ($P < 0.05$) than other genetic types except for MS X RG in the high density pond, split pond and the in-pond raceway. MS X RG and 103KS X RG began the in-pond raceway experiment at comparable sizes and MS X RG was 38% larger at the end of the experiment.

No sire effect on the final body weight was observed, although the observed mean body weight of hybrids from RG sires was higher than that for hybrids from DB sires in each environment. Dam effects were significant ($P < 0.05$). The pooled mean of M hybrids (0.685 kg) was higher ($P < 0.05$) than that of K (0.561 kg) and 103KS (0.534 kg).

Table 3. Final mean body weights (kg) and the standard deviations (SD) of different genetic types of channel catfish (*Ictalurus punctatus*) female x blue catfish (*I. furcatus*) male hybrid catfish cultured in a low density pond, high density pond, split pond and in-pond raceway.

Genetic type ¹	Mean final body weights ± Standard deviation			
	Low density	High density	Split pond	Raceway
103KS X DB	0.686 ^a ± 0.376	0.217 ^d ± 0.094	0.368 ^e ± 0.163	0.745 ^c ± 0.293
MR X DB	0.684 ^a ± 0.260	0.276 ^{bc} ± 0.131	0.491 ^{bc} ± 0.200	0.909 ^b ± 0.376
KS X DB	0.760 ^a ± 0.354	0.260 ^c ± 0.113	0.433 ^{cd} ± 0.183	0.862 ^b ± 0.316
KR X DB	0.621 ^a ± 0.294	0.204 ^d ± 0.091	0.370 ^e ± 0.162	0.829 ^{bc} ± 0.342
103KS X RG	0.790 ^a ± 0.560	0.241 ^{cd} ± 0.103	0.415 ^{de} ± 0.173	0.812 ^{bc} ± 0.373
MS X RG	1.046 ^a ± 0.295	0.369 ^a ± 0.214	0.580 ^a ± 0.287	1.121 ^a ± 0.484
KR X RG	0.656 ^a ± 0.131	0.216 ^d ± 0.098	0.387 ^e ± 0.179	0.737 ^c ± 0.323
KS X RG	0.780 ^a ± 0.329	0.312 ^b ± 0.155	0.521 ^{ab} ± 0.227	1.029 ^a ± 0.411
C X DB ²	0.688 ± 0.057	0.240 ± 0.034	0.416 ± 0.059	0.836 ± 0.069
C X RG	0.818 ± 0.164	0.285 ± 0.069	0.476 ± 0.090	0.925 ± 0.180
103KS X B ³	0.738 ^y ± 0.074	0.229 ^y ± 0.017	0.392 ^y ± 0.033	0.779 ^y ± 0.047
K X B	0.704 ^y ± 0.078	0.248 ^y ± 0.049	0.425 ^y ± 0.068	0.864 ^y ± 0.122
M X B	0.865 ^x ± 0.256	0.323 ^x ± 0.066	0.536 ^x ± 0.063	1.015 ^x ± 0.150

^{abcde} Means within the same column followed by the same letter are not significantly different (P = 0.05, Tukey multiple range test).

¹Blue catfish sires were D&B (DB) and Rio Grande (RG). Channel catfish lines were Kansas random (KR), Marion random (MR), Marion select (MS, selected for 8 generations for increased body weight), Kansas select (KS, selected for 8 generations for increased body weight) and 103KS (an F2 generation cross between NWAC-103 and KS).

² Sire effects were not significant ($P>0.05$) (t-test)

³Hybrids from pooled M dams were larger ($P<0.05$) than hybrids from pooled 103KS or K dams (t-test)

^{xy} Means within the same column followed by the same letter are not significantly different ($P = 0.05$, Tukey multiple range test).

Table 4. Three-way ANOVA. The interaction of sex (S), genetic type (G) and environment (E) on the final body weight (kg) of the different genetic types of channel catfish (*Ictalurus punctatus*) female x blue catfish (*I. furcatus*) male hybrid catfish cultured in a low density pond, high density pond, split pond and in-pond raceway.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Environment	3	313.878	104.626	1977.64	<.0001
Genotype	7	5.427	0.775	14.65	<.0001
Sex	1	1.578	1.578	29.82	<.0001
Environment*Genotype	21	6.429	0.306	5.79	<.0001
Environment*Sex	3	2.451	0.817	15.44	<.0001
Environ*Genotype*Sex	21	1.768	0.0842	1.59	0.0422

103KS X DB males were larger ($P<0.05$) than their corresponding females in the split pond, but not in the other environments ($P>0.05$) (Table 5). KS X DB males were larger ($P<0.05$) than their females in the split pond and the in-pond raceway, but not ($P>0.05$) in the low and high density ponds. KR X DB, 103KS X RG and KR X RG males were larger ($P<0.05$) than their females in the in-pond raceway, but not in the other environments ($P>0.05$). KS X RG males in the in-pond raceway, high density and split ponds were larger ($P<0.05$) than their females, but not in low density pond ($P>0.05$).

Table 5. Final body weight (kg) and standard deviation (SD) for female and male channel catfish (*Ictalurus punctatus*) female x blue catfish (*I. furcatus*) male hybrids catfish of different genetic types cultured in a low density pond, high density pond, split pond and in-pond raceway.

Genetic type ¹ Sex	Mean adjusted final body weights \pm Standard deviation			
	Low density	High density	Split pond	Raceway
% Sexual Dimorphism				
103KS X DB				
Female	0.710 \pm 0.416	0.213 \pm 0.087	0.352 \pm 0.140 ^a	0.726 \pm 0.275
Male	0.653 \pm 0.402	0.218 \pm 0.099	0.383 \pm 0.180	0.761 \pm 0.307
%	-8	2.3	8.8	4.8
MR X DB				
Female	0.553 \pm 0.167	0.282 \pm 0.148	0.499 \pm 0.199	0.887 \pm 0.352
Male	0.840 \pm 0.280	0.274 \pm 0.123	0.483 \pm 0.203	0.930 \pm 0.399
%	51.9	-2.8	-3.2	4.8
KS X DB				
Female	0.624 \pm 0.312	0.260 \pm 0.099	0.415 \pm 0.162 ^a	0.778 \pm 0.241 ^a
Male	0.998 \pm 0.325	0.260 \pm 0.124	0.452 \pm 0.204	0.969 \pm 0.365
%	59.9	0	2.4	24.5
KR X DB				
Female	0.540 \pm 0.223	0.218 \pm 0.094	0.387 \pm 0.157	0.778 \pm 0.290 ^a
Male	0.743 \pm 0.378	0.199 \pm 0.090	0.355 \pm 0.164	0.873 \pm 0.377
%	37.6	-8.7	-8.3	12.2
103KS X RG				
Female	0.560 \pm 0.111	0.234 \pm 0.093	0.400 \pm 0.171	0.743 \pm 0.310 ^a
Male	1.135 \pm 0.912	0.247 \pm 0.111	0.435 \pm 0.175	0.880 \pm 0.416
%	102.7	5.6	8.8	18.439
MS X RG				
Female	0.910 \pm NA*	0.345 \pm 0.208	0.559 \pm 0.295	1.116 \pm 0.427
Male	1.080 \pm 0.329	0.387 \pm 0.217	0.606 \pm 0.279	1.127 \pm 0.543
%	18.7	12.2	8.4	1
KR X RG				
Female	0.668 \pm 0.125	0.213 \pm 0.095	0.378 \pm 0.169	0.664 \pm 0.269 ^a
Male	0.646 \pm 0.142	0.219 \pm 0.020	0.396 \pm 0.187	0.797 \pm 0.352
%	-3.3	2.8	4.8	20
KS X RG				
Female	0.703 \pm 0.291	0.290 \pm 0.139 ^a	0.482 \pm 0.206 ^a	0.944 \pm 0.355 ^a
Male	0.846 \pm 0.368	0.329 \pm 0.165	0.555 \pm 0.239	1.150 ^a \pm 0.456
%	20.3	13.4	15.1	21.8

¹Blue catfish sires were D&B (DB) and Rio Grande (RG). Channel catfish lines were

Kansas random (KR), Marion random (MR), Marion select (MS, selected for 8

generations for increased body weight), Kansas select (KS, selected for 8 generations for

increased body weight) and 103KS (an F2 generation cross between NWAC-103 and KS).

*only one fish.

^a Means of male and female are different (unpaired t-test, $P < 0.05$).

Percent sexual dimorphism = mean of male - mean of female divided by mean of the female X 100

Weight Gain

Weight gain (kg) and percent change in body weight from stocker size to harvest for each genetic type within each environment are presented in Table 6. Hybrids from both the low density pond and the in-pond raceway more than doubled their body weight. 103KS X DB from the low density pond had the highest observed percentage weight gain among all genetic types in all environments at 524%. Hybrids cultured in the in-pond raceway had the highest grand mean gain compared to the other environments.

Feed conversion ratio (FCR) from stocker size to the current size in each environment was calculated and presented in Table 7. Low density environment has the best FCR followed by the in-pond raceway 1.28 and 1.52, respectively. However, the in-pond raceway has the highest production 1573 kg.

Table 6. Weight gain (kg) and % body weight change from stocker size to the current size of the different genetic types of channel catfish (*Ictalurus punctatus*) female x blue catfish (*I. furcatus*) male hybrids catfish cultured in a low density pond, high density pond and in-pond raceway.

Genetic type ¹	Low density		High density		Split pond		Raceway	
	Gain (kg)	%	Gain (kg)	%	Gain (kg)	%	Gain (kg)	%
103KS X DB	0.576	524	0.116	115	0.151	70	0.614	469
MR X DB	0.500	272	0.131	90	0.214	77	0.700	335
KS X DB	0.574	309	0.121	87	0.173	67	0.683	382
KR X DB	0.444	251	0.101	98	0.165	80	0.684	472
103KS X RG	0.654	481	0.132	121	0.174	72	0.666	456
MS X RG	0.793	313	0.193	110	0.210	57	0.904	417
KR X RG	0.454	225	0.112	108	0.170	78	0.607	467
KS X RG	0.545	232	0.157	101	0.209	67	0.805	359
Grand mean	0.548	303	0.125	101	0.176	71	0.708	521

¹Blue catfish sires were D&B (DB) and Rio Grande (RG). Channel catfish lines were

Kansas random (KR), Marion random (MR), Marion select (MS, selected for 8

generations for increased body weight), Kansas select (KS, selected for 8 generations for

increased body weight) and 103KS (an F2 generation cross between NWAC-103 and

KS).

Table 7. Total production (kg/ha) and feed conversion ratio (FCR) from stocker to food size of the different genetic types of channel catfish (*Ictalurus punctatus*) female x blue catfish (*I. furcatus*) male hybrid catfish cultured in a low density pond, high density pond, split pond and in-pond raceway environments

	Low density	High density	Split pond	Raceway
Kg/ha	968	7,312	8,018	148 (kg/m ³)
FCR	1.28	1.99	1.65	1.52

Survival

The mean survival (%) of the different genetic types of C X B hybrids in the four environments are presented in Table 8. Low density environment had significantly lower survival 7.9 to 25.6 ($P < 0.05$) compared to the other environments. No genetic effect or interactions were found among genetic types for survival ($P > 0.05$). Hybrids from low density had the lowest survival; however, no diseases were observed during the rearing period, but predators might be the reason for low survival rate in this environment.

Table 8. The survival (%) of the different genetic types of channel catfish (*Ictalurus punctatus*) female x blue catfish (*I. furcatus*) male catfish hybrids cultured in low density pond, high density pond, split pond and in-pond raceway environments

Genetic type ¹	Low density*	High density	Split pond	Raceway
103KS X DB	11.3	95.2	97.0	98.3
MR X DB	25.6	93.5	93.6	95.6
KS X DB	20.4	96.9	98.8	93.7
KR X DB	25.0	99.2	88.2	98.7
103KS X RG	7.9	95.8	95.8	98.7
MS X RG	13.5	99.1	92.5	90.1
KR X RG	24.1	97.7	96.9	100.0
KS X RG	21.7	94.2	96.4	95.0

¹Blue catfish sires were D&B (DB) and Rio Grande (RG). Channel catfish lines were

Kansas random (KR), Marion random (MR), Marion select (MS, selected for 8 generations for increased body weight), Kansas select (KS, selected for 8 generations for increased body weight) and 103KS (an F2 generation cross between NWAC-103 and KS).

* Environmental effect was significantly different ($P < 0.05$) from other environments, no genetic effects or interactions were found among genetic types.

Seinability

Environment had a significant effect on seinability ($P < 0.05$, Fig. 1) with fish being more difficult to catch in low density environment and easier to catch in high density pond. No differences were found among genetic types within any of the environments ($P > 0.05$, Table 9). No sire or dam effects were detected on seinability.

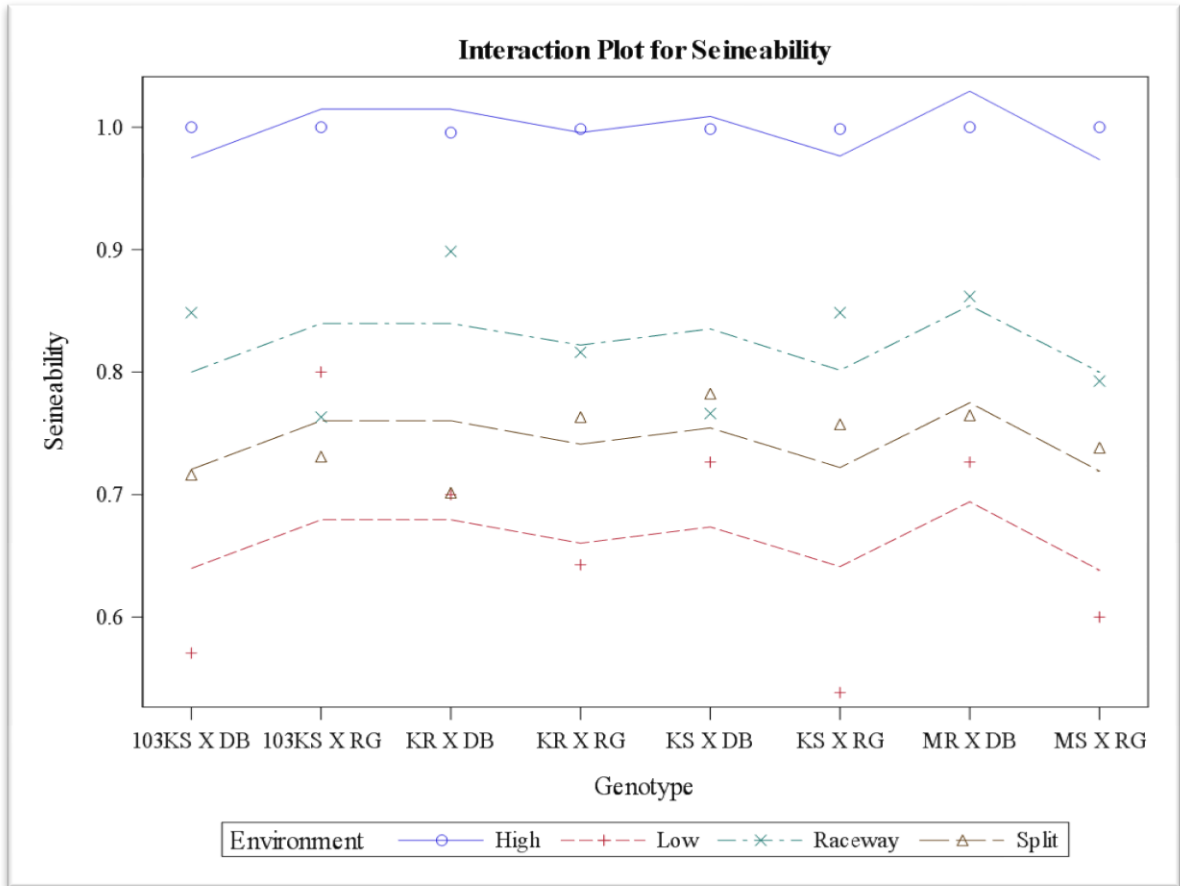
Table 9. The seinability of the different genetic types of channel catfish (*Ictalurus punctatus*) female x blue catfish (*I. furcatus*) male hybrid catfish cultured in a low density pond, high density pond, split pond and in-pond raceway environments.

Genetic type ¹	Low density	High density	Split pond	Raceway
103KS X DB	0.571	1.000	0.716	0.848
MR X DB	0.727	1.000	0.765	0.862
KS X DB	0.727	0.998	0.782	0.766
KR X DB	0.700	0.996	0.701	0.898
103KS X RG	0.80	1.000	0.731	0.764
MS X RG	0.60	1.000	0.739	0.793
KR X RG	0.643	0.998	0.763	0.816
KS X RG	0.538	0.998	0.757	0.848
C X DB	0.681	0.998	0.741	0.844
C X RG	0.645	0.999	0.748	0.805
103KS X B	0.686	1.000	0.724	0.806
K X B	0.652	0.997	0.751	0.832
M X B	0.664	1.000	0.752	0.828

¹Blue catfish sires were D&B (DB) and Rio Grande (RG). Channel catfish lines were

Kansas random (KR), Marion random (MR), Marion select (MS, selected for 8 generations for increased body weight), Kansas select (KS, selected for 8 generations for increased body weight) and 103KS (an F2 generation cross between NWAC-103 and KS).

Figure 1. The seinability of the different genetic types of channel catfish (*Ictalurus punctatus*) female x blue catfish (*I. furcatus*) male hybrids catfish cultured in a low density pond, high density pond, split pond and in-pond raceway environments.



DISCUSSION

The feed conversion ratio (FCR) could not be evaluated by genetic group because of the communal design. However, the FCR by environment was better than what is usually expected for research and on-farm for ictalurid catfish (Pugliese et al. 2012, D'Abramo et al. 2013) with channel catfish FCR ranging from 2.0-3.0. The commercial FCR for channel catfish was estimated at 2.67 (Brown et al. 2011). The FCR achieved during the experiment was superior to industry standards. In the current study, the FCR was 1.28, 1.99, 1.65 and 1.52 for the low density pond, high density pond, split pond and in-pond raceway, respectively. FCR for hybrid catfish under commercial conditions in traditional ponds is approximately 1.7-2.1 (Dunham et al. unpublished), and Brown et al. (2011) were able to achieve a FCR of 1.36 for hybrid catfish in a commercial raceway. The FCR for the low density pond was almost identical to that obtained by Chappell (1979) more than 30 years ago. If the current results can be replicated, it will demonstrate that the high density with aeration was the least efficient system, and despite the high densities, the split pond and raceway can approach the efficiency of low density culture, which has the disadvantage of poor production (kg/ha).

Genetic type, sex and environment and all possible interactions affected final body weight for the different genetic types of channel catfish female x blue catfish male hybrids. MS X RG had the highest observed body weight in all four environments, and statistically MS X RG and KS X RG were the largest two genetic types in each

environment. However, because of its initial size advantage, the MS X RG can only be conclusively shown to be larger (38%) than 103KS X RG in the in-pond raceway. GE interactions were a result of small differences in rank among the remaining genetic groups and the magnitude of the size differences across environments.

Assuming that the initial body weight differences were of genetic origin, the results indicate that a single genetic enhancement program could address the improvement of hybrid performance for all culture systems used in the catfish industry. Differences in body weight were established by the conclusion of the fingerling phase and maintained throughout the remaining culture period in all environments (the correlation of the ranking when pairing environments was 0.76-0.93, $P < 0.05$), indicating that the research to identify the best genetic types of hybrids could be concluded at the end of the fingerling phase and still predict the outcome at market weight. This would simplify genetic evaluation and reduce research costs.

The magnitude of the genetic differences in performance was high among the hybrids with KR X DB were 68.4% and 80.8% smaller than the largest genetic type in the low and high density ponds, respectively. Similarly, KR X RG was 52.1% smaller than the largest genetic type in the in-pond raceway.

An apparent sire effect influenced body weight, although within environments there were no significance differences due to sire. Mean body weight of hybrids from RG sires was 11-19% higher than hybrids produced from DB. Makhubu (2014) reported

a significant sire effect for only one environment during the fingerling phase of this experiment. The difference between RG hybrids and DB hybrids appears to increase during food fish phase. RG hybrids also grew faster than hybrids produced by two Alabama strains of hybrids during the early food fish stage (Dunham et al. 2014a). These series of experiments indicate that RG strain of blue catfish males produce some of the fastest growing genetic types of hybrid catfish.

Within a given strain of blue catfish, sire effects could be less important. Bosworth and Waldbieser (2014) found minimal sire effects among families within the D & B strain of blue catfish for growth. .

In previous experiments, (Jeppsen 1995; Dunham et al. 2014a) dam effects on hybrid growth performance were minimal, but dam effects on hybrid growth were observed in the current study. Hybrids from M strain females had higher mean final body weight in all environments than those from 103KS and K strains. During the fingerling phase of fish in the current experiment, Makhubu (2014) found that hybrids from M and K dams were larger than those from KS103 dams in the low density pond and the in-pond raceway at stocker size.

Bosworth and Waldbieser (2014) reported high dam general combining ability for hybrid catfish growth, which predicts that selection for body weight in channel catfish females will result in channel catfish that produce larger hybrids than females not selected for growth rate. Data from the current experiment substantiate this prediction as

hybrids produced by MS and KS females selected for increased body weight for 8 generations were larger compared to hybrids from MR and KR random controls in all environments. This is in contrast to the findings of Jeppsen (1995), who observed that hybrids from KS selected for increased body weight for four generations had almost identical growth compared to those from KR females. The additional four generations of selection appeared to impact the general combining ability.

The combining ability of RG blue catfish males was more variable than that of DB blue catfish males in all environments based upon the coefficient of variation generated from averaging the means of the genetic types with the common sire. Performance of hybrids sired by DB males may be more predictable regardless of the strain of dam or perhaps based on the results of Bosworth and Waldbieser (2014), Individual DB males may have a more constant combining ability. Alternatively, RG blue catfish males may have a less predictable pattern of combining ability, which could result in combination with certain dams, a much better performing hybrid, but with the risk of a poorer performing hybrid as well.

Genotype- environment interactions were observed in regards to the sex. The interactions: $G \times E$, $S \times E$, and $G \times E \times S$ demonstrated that genetic types and sex responded differently to the variation in environmental conditions. There were significant differences found between males and females for final body weight in high density pond, split pond and the in-pond raceway, but there were no differences in the low density environment, due to small sample size. Makhubu (2014) found the opposite sex

relationship with stocker size channel catfish. Jiang et al. (2008) also found (NWAC103) hybrids males to be significantly larger than females in total weight and length based on sex when channel catfish, blue catfish and their hybrids were cultured in earthen ponds.

Patterns of sexual dimorphism for body weight of hybrid catfish were complex and variable in the current study. Observed sexual dimorphism tended to be the highest with males larger in the low density pond. Perhaps lower density triggers some sexual maturation effects that lead to greater disparity in growth between the two sexes. Sexual dimorphism for body weight in hybrid catfish may also be related to size (Dunham et al. 2014a, Makhubu 2014). Usually, but not always, male hybrid catfish are larger than females (Bosworth and Waldbieser 2014, Dunham et al. 2014a). However, hybrid catfish females at stocker size were larger than males in 5 of the 6 cases when sexual dimorphism for body weight was significant, and in 18 other comparisons males and females were the same size (Makhubu 2014). Makhubu (2014) hypothesized that faster growth of males compared to females may not occur until the hybrids reach larger sizes.

Channel catfish and white catfish, *Ameirus catus*, become sexually dimorphic when much smaller as fingerlings (Brooks et al. 1982) compared to what Makhubu (2014) observed. Fingerling blue catfish lacked sexually dimorphic growth (Brooks et al. 1982), thus paternal predominance (Dunham et al. 1982) may be the explanation for the delayed emergence of the male size advantage in hybrid catfish sired by blue catfish. In the current study, all of the sexual dimorphism with males larger occurred in the low density pond and the in-pond raceway, the two environments with the largest fish. No

significant sexual dimorphism occurred in the high density pond and split pond where the fish were smaller. Considering the large amount of sexual dimorphism in the low density ponds as significant, there were 15 comparisons with males larger and 17 comparisons with males and females not different for body weight. These results support the hypothesis of Makhubu (2014) that the lack of sexual dimorphism and the female size advantage at stocker stage would begin shifting to sexually dimorphic size with the males larger later on as the fish grew.

Size variation of hybrid catfish has become an issue in the US catfish industry. There is a perception that hybrid catfish are more variable in the current culture systems than channel catfish. More or less variability is desirable depending upon the processing or marketing goals. The coefficients of variation for body weight in channel catfish (Konikoff and Lewis 1974) and hybrid catfish (Dunham et al. 1982b, Li et al. 2012) are approximately 30-35%. However, Dunham et al. (2014) observed much higher variability, CV =50-65%) for body weight of hybrid catfish. Most CVs for body weight in the current study were 40-45%, slightly more than expected. The slightly higher variability in the current study might be a result of high stocking density at fry phase or may have been induced during the multiple rearing phase when feed was restricted for some groups.

There were no apparent trends in hybrid variability based upon either environment or genetic type. Dunham et al. (2014) also did not observe any genetic effect

on hybrid size variability. Genetic type of hybrid does not appear to impact variability, thus cannot be used as mechanism to alter size variability in hybrid catfish.

Survival in high density pond, split pond and in-pond raceway from stocker stage to the harvest in the current experiment was higher than what was observed during the fingerling phase (Makhubu 2014), probably because no disease problems occurred and the hybrids of all genetic types were able to tolerate handling stress. No genetic effects were evident and survival for each genetic type was usually 95% or higher. Reported hybrid survival in a raceway environment averaged 90.9% (Brown et al. 2011).

The hybrid catfish in the low density pond had poor survival rate, 7.9-25.6%, compared to those in the other three environments. This is unexpected; if anything, this environment should have equal or higher survival. The poor survival rate in the low density treatment was most likely caused by predators (otters) since no diseases were observed in this pond during the study. The data likely reflect potential differences in predator avoidance.

Seinability or harvestability is another important trait and is undervalued since harvesting can be a significant cost. Dunham and Argue (1998) reported that blue catfish were significantly easier to harvest by seining than channel catfish, and the C x B hybrid was significantly easier to catch than channel catfish. If fish are not captured and still in the culture system, they have a greater chance of mortality from pathogens or predators, and will reach a larger size, increasing their FCR. Additionally, large fish that avoid

capture will compete with smaller fish in the system, which will also severely affect the production and FCR of the next production cycle.

Genetic type did not affect seinability. However, harvestability was impacted by environment. The least harvestable treatment was the low density pond and the in-pond raceway ranked second easiest for capture after the high density pond. This contradicts the results of Makhubu (2014) with the same fish harvested at stocker size as the fish in the in-pond raceways were the most difficult to catch at stocker size. Experience with the in-pond raceway may be improving harvest results in that environment.

CONCLUSION

Genotype, sex, environment and their interactions affected growth among different genetic types of hybrids. Initial size differences at the conclusion of the fingerling growth phase affected the outcome depending upon whether these differences were assumed to be of genetic or environmental origin. If these initial differences are assumed to be of genetic origin, sire and dam effects affect performance of the hybrids and the same genetic types rank the highest in all environments, indicating a need of a single genetic enhancement program to improve hybrids for all culture systems.

Measurement of some traits is affected by age. At stocker size, females were larger than males of equivalent body weight. As the fish grew, this relationship changed and males became larger or equivalent in body weight compared to females after stocker phase. Age and correction methodology will affect conclusions regarding the relative genetic value of channel-blue hybrid catfish as well as genetic management strategies.

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APPENDIX

Environment (E) effect on the final body weight

	Wt_kg_ LSMEAN	Environment	LSMEAN Number
A	0.883	Raceway	3
B	0.763	Low Density	2
C	0.446	Split	4
D	0.262	High Density	1

LS-means with the same letter are not significantly different

Genotype (G) effect on the final body weight

			Wt_kg_ LSMEAN	Genotype	LSMEAN Number
	A		0.766	MS X RG	6
B	A		0.662	KS X RG	8
B			0.595	KS X DB	3
B	C		0.593	MR X DB	2
B	C	D	0.579	103KS X RG	5
	C	D	0.512	KR X DB	4
		D	0.502	103KS X DB	1
		D	0.498	KR X RG	7

Sex (S) comparison effect on the final body weight using Tukey-Kramer Adjustment

S	Wt_kg_ LSMEAN	H0:LSMean1=LSMean2
		Pr > t
F	0.545	<.0001
M	0.632	

Environmental x Sex (ES) interaction on final body weight

		Wt_kg_ LSMEAN	Environment	Sex	LSMEAN Number
	A	0.936	Raceway	M	6
B	A	0.868	Low Density	M	4
B		0.829	Raceway	F	5
	C	0.659	Low Density	F	3
	D	0.458	Split	M	8
	D	0.434	Split	F	7
	E	0.267	High Density	M	2
	E	0.257	High Density	F	1

LS-means with the same letter are not significantly different.

Genotype x Environment (GE) interaction on the final body weight

				Wt_kg_ LSMEAN	Environment	Genotype	LSMEAN Number
F		G		0.369	High Density	MS X RG	6
H		G		0.312	High Density	KS X RG	8
H		G	I	0.276	High Density	MR X DB	2
H			I	0.260	High Density	KS X DB	3
H			I	0.241	High Density	103KS X RG	5
			I	0.216	High Density	KR X RG	7
			I	0.217	High Density	103KS X DB	1
			I	0.204	High Density	KR X DB	4
F	D	E	C	0.580	Split	MS X RG	30
F	D	E	C	0.521	Split	KS X RG	32
F	D	E		0.491	Split	MR X DB	26
F		E		0.433	Split	KS X DB	27
F				0.415	Split	103KS X RG	29
F				0.387	Split	KR X RG	31
F		G		0.370	Split	KR X DB	28
F		G		0.368	Split	103ks X DB	25
		A		1.121	Raceway	MS X RG	22
B		A		1.029	Raceway	KS X RG	24
B		A	C	0.909	Raceway	MR X DB	18
B		A	C	0.862	Raceway	KS X DB	19
B	D		C	0.829	Raceway	KR X DB	20
B	D		C	0.812	Raceway	103KS X RG	21

				Wt_kg_LSMEAN	Environment	Genotype	LSMEAN Number
	D		C	0.745	Raceway	103KS X DB	17
	D		C	0.737	Raceway	KR X RG	23
		A	C	1.046	Low Density	MS X RG	14
B	D	A	C	0.790	Low Density	103KS X RG	13
B	D		C	0.760	Low Density	KS X DB	11
	D		C	0.780	Low Density	KS X RG	16
	D	E	C	0.684	Low Density	MR X DB	10
F	D	E	C	0.686	Low Density	103KS X DB	9
F	D	E	C	0.656	Low Density	KR X RG	15
F	D	E	C	0.621	Low Density	KR X DB	12

Dam effect on final body weight

LS-means with the same letter are not significantly different.			
	weight LSMEAN	Genotype	LSMEAN Number
A	0.685	M	3
B	0.561	K	2
B	0.534	103KS	1

Genotype (G) effect on the final body weight within environment

Low density pond

LS-means with the same letter are not significantly different.			
	Wt_kg_	Genotype	LSMEAN
	LSMEAN		Number
A	1.046	MSRG	6
A	0.790	103KSRG	5
A	0.780	KSRG	8
A	0.760	KSDB	3
A	0.686	103KSDB	1
A	0.684	MRDB	2
A	0.656	KRRG	7
A	0.621	KRDB	4

High density pond

LS-means with the same letter are not significantly different.				
		Wt_kg_	Genotype	LSMEAN
		LSMEAN		Number
	A	0.369	MSRG	6
	B	0.312	KSRG	8
C	B	0.276	MRDB	2
C		0.260	KSDB	3
C	D	0.241	103KSRG	5
	D	0.216	KRRG	7
	D	0.217	103KSDB	1
	D	0.204	KRDB	4

Split pond

LS-means with the same letter are not significantly different.				
		Wt_kg_	Genotype	LSMEAN
		LSMEAN		Number
	A	0.580	MSRG	6
B	A	0.521	KSRG	8
B	C	0.491	MRDB	2
D	C	0.433	KSDB	3
D	E	0.415	103KSRG	5
	E	0.387	KRRG	7
	E	0.370	KRDB	4
	E	0.368	103KSDB	1

Raceway

LS-means with the same letter are not significantly different.				
		Wt_kg_	Genotype	LSMEAN
		LSMEAN		Number
	A	1.121	MSRG	6
	A	1.029	KSRG	8
	B	0.909	MRDB	2
	B	0.862	KSDB	3
C	B	0.829	KRDB	4
C	B	0.812	103KSRG	5
C		0.745	103KSDB	1
C		0.737	KRRG	7