Studies for Improvement of Reproductive Biotechnology for Production of Channel Catfish (*Ictalurus punctatus*) Female X Blue Catfish (*Ictalurus furcatus*) Male Hybrid Embryos

by

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Abstract

Investigative studies were conducted on the relative effectiveness between carp pituitary extract (CPE), luteinizing hormone releasing hormone analog (LHRHa) injections and LHRHa implants for producing hybrid catfish embryos. Data from the past 15 years, which included, 25 on CPE, 20 on LHRHa injections, and 20 for LHRHa implants, respectively, were evaluated. LHRHa administered as an injection or implant produced more ($P<0.001$) fry/kg than CPE. Mean fry/kg female body weight (all females) produced was 948 for, CPE 2,483 LHRHa injections and 2,765 for LHRHa. There was not a significant difference in fry/kg between the two LHRHa treatments. The coefficient of variance indicated more consistent results for CPE (CV=37.5), and LHRHa implants (CV= 35.9) than LHRHA injections (CV= 49.9).

The second study investigated the effectiveness of OVA-EAZE a Luteinizing Hormone Releasing Hormone analog (LHRHa). This study investigated des-Gly^{10},[D-Ala^{6}] LHRH Ethyl amide (LHRHa) administered in two doses of 20 μg per kilogram of female channel catfish body weight as a priming dose, followed 12 hours later by a resolving injection of 100 μg per kilogram of body weight, to determine its effectiveness in inducing ovulation in channel catfish *(Ictalurus punctatus)*. A double blind study was conducted at two sites concurrently with 50 treatment, and 25 control fish at each site using similar protocols. All eggs were fertilized using blue catfish *(Ictalurus furcatus)* sperm in order to produce hybrid fry. At both sites treated females had a higher ovulation rate (92%, $P<0.001$), and (84%, $P<0.001$), compared to sham injected controls (4%) and (4.2%). When secondary variables such as; eggs/kg, egg quality

score, latency, hatch percentage, and fry/kg were also evaluated at both locations for the treated fish, it showed the secondary variables for females ovulated at both locations easily exceeded minimum industry standards for channel catfish females. At Baxter Land Company (AR), eggs/kg, egg quality score, latency, hatch percentage, and fry/kg were 8,665, 3.5, 44.4 hours, 31.3% and 2,401, while at Auburn University, eggs/kg, egg quality score, latency, hatch percentage and fry/kg were 10,385, 3.8, 46.2, 41.3% and 3,700 for females injected with LHRHa.

The third study focused on the toxicity and target animal safety of OVA-EAZE, luteinizing hormone releasing hormone analogue (LHRHa). The experimental animals (channel catfish females) were administered 0, 180, 540 and 900-µg/kg female body weight of LHRHa and observed for seven days. Seven-day survival, as well as the histopathology of the spleen, ovary, liver, kidney, gill, muscle, heart, stomach and intestine of fish was assessed to determine the possible toxic effects of LHRHa. The seven-day survival was 62.5, 87.5, 75.0 and 75.0% between the four treatments and was not significantly different ($P = 0.05$). Of those fish that died, the time to death was not different among treatments. The mean severity of the mouth kidney and eye abnormalities was higher ($P = 0.05$) for the sham-injected controls. The observed liver discoloration tended to increase with dosage, however, there were no significant ($P=0.05$) differences among treatments. No consistent or significant ($P = 0.05$) trends in histopathological abnormalities existed among treatments. Observed intestinal inflammation appeared to be more prevalent with increasing dosage. Bacterial and parasitic load also appeared random and there were no significant differences.

The final study focuses on xenogenics as a novel method for the production of hybrid catfish fry. Putative spermatogonia A, and primordial germ cells from a fresh cell isolate or a

density gradient-centrifuged isolate from blue catfish, testes were inserted into the, gonads of confirmed triploid channel catfish. The live cells were introduced to the gonads of the host via catheterization or by surgically accessing the gonads and inserting the cells directly into the ovaries or testes with an injection of (mean: $5.23x10^5$) cells. Sixty days post introduction of the cells into the host, DNA was analyzed from biopsies of the gonads. Two triploid channel catfish, inoculated with density gradient sorted blue catfish stem cells introduced by injection contained viable blue catfish cells in their ovaries and testes 60 days later. Three triploid channel catfish that were inoculated with testicular cells from blue catfish introduced by catheterization via the genital and urogenital opening respectively, contained viable blue catfish cells in their ovaries and testes 60 days later. This is the first report of successful production of xenogenic catfish in the United States.

Dedication

To my loving grandfather Mr. Harry David, grandmother Catherine W. Perera
and to my dearest sister Anushka Perera

Thank you for watching over me.

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List of Abbreviations

AU	Auburn University
BLC	Baxter Land Company
FDA	Food and Drug Administration
LHRHa	Luteinizing Hormone Releasing Hormone analogue
CPE	Carp Pituitary Extract
HCG	Human Chorionic Gonadotropin.

CHAPTER ONE

Introduction

The beginnings of aquaculture can be traced back to Asia, specifically to ancient China (Boyd and Tucker, 1998; Costa-Pierce, 2008; Nash, 2011; Dunham, 2011). The concept of growing fish in ponds most likely arose when inhabitants of coastal areas noticed fish and other aquatic organisms being washed into ponds by monsoon floods and then being trapped in these inland pools when the water level receded. Over time these trapped aquatic organisms would provide these coastal inhabitants and their families with a convenient supply of easily obtainable food. This was especially essential at times of the year when other sources were limited. Eventually, this concept, through natural progression, evolved into the idea of stocking ponds with food fish, and the concept of aquaculture (Nash 2011).

With the current increase in population and the ongoing worldwide food crisis, the demand for cheap, high yield, quality proteins has increased (Naylor, et al. 2000). This has encouraged increased research and experimentation in the field of agriculture (Naylor, et al. 2000). Within agriculture, a discipline that has been highly invested in is fish culture. This increased investment has resulted in what we see as modern aquaculture. Aquaculture is currently a multi-million dollar business and are present in almost every country in the world (Holts 1985; Peggy Hsieh, Leong, and Rudloe 2001; Standley et al. 2009). Channel catfish *(Ictalurus punctatus)* is the primary species used in the aquaculture industry in the United States. Commercial catfish

production currently generates over 62 % of the total value of aquaculture production (National Agricultural Statistic Service 2012). From the first commercial production in ponds in the 1930s in Kansas, 1940s in Arkansas, 1950s in Mississippi and 1960s in Alabama, catfish production rapidly grew to reach annual sales of 317,515 metric tons in 2008. The value of the catfish harvest in the United States reached $ 980 million in 2008 (Mississippi State University Extension Services; USDA, 2008). However due to the economic downturn and competition from foreign imports sales dropped to $ 403 million in 2010. In 2011 the industry has shown signs of recovery with a 5% increase in sales to $ 423 million (National Agricultural Statistic Service 2012).

Rapid growth of the catfish industry in the 1980s and the 1990s led to it becoming one of the most important agricultural activities in states such as Mississippi, Arkansas, Alabama, and Louisiana. The combined production acreage of these four states makes up 92% of all catfish production acreage (National Agricultural Statistic Service 2012). As of July 1st 2012 Catfish production totaled 40,307 hectares, with 348 commercial operations. Of this the Delta region in Mississippi accounted for 51% (20,477 hectares) of the total land area devoted to catfish farming (NASS, USDA 2012). However this is significantly lower compared to the peak of catfish farming in 2002 when 79,626 hectares was used for catfish production (National Agricultural Statistic Service 2012).

Modern research has enabled commercial aquaculture operations to adopt new technologies. Aquaculturists realize that by controlling environmental conditions, such as water quality,

oxygen, temperature, feed rate, and stocking densities, they have been able to lower costs and to obtain higher yields (Lee, 2000).

In the commercial catfish industry, an important factor that affects the success of fish farms is the time needed to produce a marketable fish. As time between hatch and reaching a marketable product increase the overall production cost also increase due to feed, aeration, labor, pond management. Furthermore, with increased production time the farmer faces the risk of diseases, unexpected catastrophes, a drop in market demand, competition from imported aquaculture products, thus increasing the rick to his financial investment (Hargreaves, 2002). In the case of the channel catfish, (*Ictalurus punctatus*) the traditional production time was about two years for a 400-600 g fish (Giudice, 1966; Wellborn, 1988). However as of lately catfish processsors have prefered a larger fish ranging from 0.6 kg – 2 kg for the fillet market (Green and Engle 2007). Since hybridization of other animals has yielded more rapidly growing individuals, this technique could potentially be useful in producing faster growing crops (Giudice, 1966). Hybrids between the seven major species of North American catfish (*Ictalurids*) were first researched and evaluated in 1966 (Masser and Dunham 2012).

Initial experiments conducted by Giudice in 1966 indicated that the cross between channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*) (CxB) showed significantly faster growth and increased production compared to channel catfish. Continued research on hybrid catfish has shown that the CxB hybrid exhibits superior characteristics for the following traits: faster growth, better feed conversion, higher tolerance to low oxygen, increased resistance to many common diseases, higher tolerance to crowded growth conditions in ponds, more

uniformity in size and shape and higher dressout percentage (Masser and Dunham 2012). Additionally, the CxB hybrid has an increased harvestability by seining and increased vulnerability to angling (Goudie, et al. 1993; Masser and Dunham, 2012).

The primary obstacle in hybrid catfish production is the natural reproductive isolation between the channel catfish and the blue catfish, which limits adequate, fry production (Goudie, et al. 1993; Dunham, Liu, and Argue, 1998; Hutson, 2006; Kristanto, et al. 2009). Unfortunately, many fish species show reproductive dysfunction when reared in a captive environment. Reproductive barriers between channel catfish and blue catfish further compound this problem. In many cultured species of fishes, females fail to undergo final oocyte maturation, thus impacting ovulation and spawning (Zohar and Mylonas 2001). In the past 50 years, manipulations of environmental factors such as temperature, photoperiod, salinity, vegetation, and substrate have been researched and have shown to improve the reliability of spawning in some species (Zohar and Mylonas 2001). Hormone induced ovulation and hand stripping of channel catfish eggs followed by in-vitro artificial fertilization with blue catfish sperm is considered the most productive and consistent technique for making (C×B) hybrid catfish (Masser and Dunham, 2012).

Since the 1930's a variety of hormonal approaches have been tried and tested successfully on many cultured species of fishes (Zohar and Mylonas 2001). The objective was to affect the brain-pituitary-gonadal axis to induce follicular development, vitellogenesis, and ovulation. These hormonal treatments originated as crude ground extracts from the endocrine systems of mature fishes, primarily a crude form of ground pituitary extract (Zohar and Mylonas, 2001). In

the past 30 years with the advancement of research and a better understanding of the reproductive physiology of fishes, significant developments were made in refining these crude extracts to their functional components as well as identifying specific target locations in the brain-pituitary-gonadal axis. With advancements in the fields of chemistry, biochemistry and engineering, we are now capable of designing and producing synthetic hormones. Currently, various synthetic, highly potent analogs of the gonadotropin-releasing hormone (GnRHa) are available (Peter, et al. 1993; Crim and Bettles, 1997; Zohar and Mylonas, 2001), as well as sustained-release delivery systems for their controlled administration (Cheng-Sheng and Chiu-Liao 1985; Breton et al. 1990). The use of gonadotropins to induce fish to spawn has many advantages in the aquaculture industry. In many species of fish, gravid females respond to gonadotropins or gonadotropin releasing hormone injections such as human chorionic gonadotropins (HCG) by spawning (Sneed and Clemens 1960). The latency period ranges between 12-72 hours depending on temperature and the species of fish. A major advantage for the fish culturist is the ability to predict and control spawns based on a planned schedule. This makes resource allocation efficient, which in turn reduces cost in commercial operations. Another major advantage in using hormones to control spawning is the ability to stock ponds with fry of the same age and uniform size (Sneed and Clemens 1960). This reduces cannibalism as well as intraspecific competition for resources. Another major advantage of hormone induced spawning is to reduce vertical transmission of diseases, since the brood stock, eggs and fry are not housed together (Sneed and Clemens 1960). Hormonal induction is essential for production of hybrid fish. Therefore, the development of hormonal induction methods and techniques has contributed significantly to the development of more reliable, less species-specific methods for the control of reproduction of captive brood stocks.

Acetone-dried carp pituitary extract (CPE) has been used widely around the world to induce spawning of several commercially cultured fishes such as northern pike (*Esox lucius*) (Szabo, 2001), Asian catfish (*Clarius batrachus*) (Sahoo, Giri, and Chandra, 2008), pangasius *(Pangasius sutchi*) (Chand, Singh, and Mandal, 2011), sturgeon (*Acipenser oxyrhynchus Desoto*) (Parauka, et al. 1991), spotted seatrout *(Cynoscion nebulosus)* (Colura, Maclorowski and Henderson-Arzapalo 1990) rainbow *(Labeo erythrurus)* and red tail black sharks *(Labeo bicolor)* (Shireman and Gildea 1989). In the hybrid catfish industry in the United States, the use of CPE has been widespread because it was the first spawning aid to allow commercial scale application and the simplicity of its use (Dunham, Liu, and Argue, 1998; Dunham, Bart, and Kucuktas, 1999; Dunham and Argue, 2000).

In addition to CPE, human chorionic gonadotropin, HCG, has also been used to induce spawning in several species of commercially grown fishes (Sneed and Clemens, 1959), such as silver carp *(Hypophthalmichthys molitrix)* (El-Hawarry, Nemaatallah, and Shinaway 2012), Eurasian perch, (*Perca fluviatilis*) (Żarski et al. 2012), pikeperch (*Sander lucioperca* L.) (Křišt'an et al. 2012), spotted seatrout *(Cynoscion nebulosus)* (Colura, Maclorowski, and Henderson-Arzapalo, 1990),), grey mullet (Kuo, Shehadeh, and Nash, 1973) and striped bass (Yeager, 2006). HCG was not effective for producing hybrid catfish (Kristanto 2004).

Luteinizing hormone releasing hormone analogs, LHRHa, has been studied as a spawning aid for the past several years with significant success. It is currently used in several commercially cultured species internationally, such as Persian sturgeon *(Acipenser persicus)* (Amini et al. 2012), bullseye puffer (*Sphoeroides annulatus*) (Garcia-Ortega, 2008), asp (*Aspius aspius*)

(Targońska et al. 2010), fat snook, (*Centropomus parallelus*) (Cerqueira and Tsuzuki 2008), yellow catfish (*Pelteobagrus fulvidraco*) (Wang et al. 2009), and Asian catfish (*Heteropneustes fossilis*) (Nayak et al. 2011).

Synthetic LHRHa has also been used in the United States with channel catfish to produce hybrid catfish. The research goals have been primarily directed towards dose responses, efficient spawning, increased fecundity and increased survival. Furthermore, with the development of in vitro fertilization techniques and the increased knowledge on strain selection, hybridization and transgenics in catfish, hormone induced hand stripping has become essential. Therefore LHRHa along with CPE, and HCG are invaluable tools in improving the catfish industry as a whole in the United States as well as research.

In the past decade, several studies demonstrated that LHRHa can successfully ovulate channel catfish (Silverstein, Bosworth, and Wolters 1999; Barrero et al. 2008; Chatakondi et al. 2011; Chatakondi and Torrans 2012). Several studies focused on the production of the CxB hybrids using LHRHa showing great promise (Hutson 2006; Gima 2008; Quintero et al. 2009).

The overall objective of this dissertation was to conduct studies aimed towards improving technology to produce hybrid catfish embryos. The initial chapters focus on studies that demonstrate the effectiveness and safety of des Gly^{10}, [D-Ala^{6}] ethylamide, a synthetic LHRH analog. The research presented addresses the effectiveness of this drug in a research setting as well as a commercial setting, and its efficacy in ovulating channel catfish. Secondary variables such as latency, fecundity, egg quality, hybrid hatch rate and hybrid fry/kg were also

investigated. The drug LHRHa is also compared to CPE in a comparative study between CPE and two forms of LHRHa (liquid injection versus implant) to determine the relative effectiveness between the three treatments in the production of CxB hybrid fry. To fully understand the properties and consequences of any drug used in agriculture, it is essential to conduct studies to determine the potential toxicological effects on the target animal. Currently there are no studies addressing the toxicological effects of LHRHa on channel catfish. The fourth chapter of this dissertation addresses the issue of toxicity of LHRHa on channel catfish. Preliminary investigations and analysis were conducted using the external pathology of the fish as well as the histopathology of the internal organs, of experimental fish that were administered different doses of LHRHa. The final chapter focuses on novel methods to produce CxB hybrid fry using natural spawning techniques. Xenogenesis and stem cell transfer techniques are used in order to transplant primordial germ cells from blue catfish into sterile channel catfish hosts with the expectation the introduced blue catfish cells proliferate and mature into sperm and ova. This would provide an alternative technology for the production of channel female x blue male hybrid catfish or for propagating difficult to spawn blue catfish.

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CHAPTER TWO

Relative effectiveness of carp pituitary extract, luteinizing hormone releasing hormone analog (LHRAa) injections and LHRHa implants for producing hybrid catfish fry

Abstract

Adoption of the hybrid catfish (channel catfish, *Ictalurus punctatus,* female x blue catfish *I. furcatus,* male) is increasing in the catfish industry. The most effective way to produce embryos is hormone induced spawning of females coupled with hand stripping and *in vitro* fertilization. The success of common carp pituitary extract (CPE), luteinizing hormone releasing hormone analog (LHRHa) injections and LHRHa implants for producing hybrid catfish embryos was investigated over the past 15 years. Data from 65 studies, 25, 20 and 20 for CPE, LHRHa injections, and LHRHa implants, respectively, were evaluated. Both LHRHa treatments were significantly better in producing hybrid catfish fry than the CPE treatments, while there was not a significant difference between the two LHRHa treatments. Mean fry/kg female body weight (all females) produced was 948, 2,483 and 2,765 for CPE, LHRHa injections, and LHRHa implants, respectively. LHRHa administered as an injection or implant produced more ($P<0.001$) fry/kg than CPE. LHRHa implants (CV=37.5), and CPE (CV= 35.9) gave a more consistent result than LHRHA injections (CV=49.9). LHRHa is a more economical ovulating hormone for producing hybrid catfish fry than CPE. This advantage is even more important if brood fish, space or time are limiting.

Introduction

Catfish are one of the most important aquaculture species in the United States, consecutively ranked from 2006-2010 by the National Fisheries Institute (NFI) as the sixth most consumed seafood in the United States in the top 10 most consumed seafoods category (NFI, 2010). Annual catfish production in the United States has been relatively unstable in recent years peaking at 662 million pounds of processed catfish in 2003. Total productivity declined to 472 million pounds in 2011 (Hanson and Sites, 2010) with the four top catfish producing states: Mississippi, Alabama, Arkansas, and Texas accounting for 95 percent of catfish sales (USDA, 2012).

The channel catfish, *Ictalurus punctatus*, is the primary aquaculture species produced in the United States (NFI, 2010). The hybrid between the channel catfish female × blue catfish (*I. furcatus*) male exhibits strong heterosis and superior performance traits for disease resistance, growth rate, oxygen tolerance, tolerance to crowding, feed conversion, uniformity in body shape and growth, processing yields and seinability (Masser and Dunham, 1998; Dunham et al., 2008). Furthermore, the channel female/blue male hybrid catfish grows faster than the reciprocal hybrid (blue catfish female × channel catfish male) (Chappell, 1979; Dunham et al., 1982). The culture of hybrid catfish may help reverse the trend of declining production in the catfish industry and increase profits, and make a positive impact on local economies in the fore mentioned states.

Hybrid catfish fry production is relatively difficult compared to intraspecific catfish fry production due to natural reproductive barriers. To counter this reproduction problem, ovulation is induced in female channel catfish using hormones, eggs hand-stripped, and then the eggs are

artificially fertilized with blue catfish sperm (Dunham, et al., 2000). The egg masses can be incubated, and hatched using traditional procedures used to hatch channel catfish eggs after fertilization.

The efficiency of different hormones including luteinizing hormone releasing hormone analog (LHRHa), common carp pituitary extract (CPE), catfish pituitary extract and human chorionic gonadotropin (HCG) have been investigated for induced spawning of channel catfish brood stock using varied concentrations of these hormones. LHRHa has been given to induce spawning of channel catfish held in aquaria by intraperitoneal or intracranial injection (Busch and Steeby, 1990). Daily doses of 1, 10 and 50 μg/kg LHRHa intracranial injections yielded from 50% - 70% spawning success, whereas 100 μg/kg LHRHa delivered either by intraperitoneal or intracranial injection had 75% - 100% success (Busch and Steeby, 1990). The same investigators reported that single daily doses of 1 μg/kg and 10 μg/kg LHRHa induced spawning in channel catfish, and these dosages were reported to be as economical as standard dosages of human chorionic HCG or CPE. LHRHa injection was reported to increase the pond-spawning rate for randomly injected 3-year-old female channel catfish to 18.8% compared to 4.8% for the control, indicating that young fish that have little possibility to spawn could be hormonally induced to spawn (Barrero, et al., 2008).

The uses of CPE and LHRHa have been demonstrated to be efficient and effective for producing hybrid catfish (Kim, 1996; Kristanto, 2004; Hutson, 2006; Kristanto, et al., 2009). LHRHa or salmon gonadotropin hormone releasing hormone GnRH applications were more effective compared to CPE and Ovaprim in producing hybrid catfish (Kristanto, 2004).

The two most common and effective-hormones used for spawning catfish are CPE and LHRHa although both hormones have advantages and disadvantages, the debate continues regarding which is more effective. The objectives of the present study were to determine the relative effectiveness of CPE, LHRHa Injections and LHRHa implants for producing hybrid catfish fry.

Materials and Methods

Experimental fish

Experiments were conducted over a 15-year period (1996 - 2010) at several locations with most of the work conducted at the Fish Genetics Research Unit, E. W. Shell Fisheries Research Center, Auburn University, AL. Experiments were also conducted at commercial fish farms in Alabama and Mississippi. General procedures follow: channel catfish and blue catfish brood stock with good, secondary sexual characteristics were seined from ponds and brought to the hatchery. Fish were randomly placed in spawning tanks sometimes as a group and other times segregated in spawning bags, and in all cases with flowing water in the hatchery. Channel catfish females weighed from 1.0 - 6.0 kg and the male blue catfish weighed from 2.0 - 20.0 kg. The water temperatures ranged from 22 - 31°C in these experiments.

Experimental procedures

Sixty-five observations for the hormone treatments were made during 15 years of studies, 25, 20 and 20 for CPE, LHRHa injections, and LHRHa implants, respectively. Hybrid catfish embryos (channel catfish female x blue catfish male) were produced using common carp pituitary extract (CPE, Stoller Fisheries, Spirit Lake, Iowa, USA.) (n = 25 studies) studies, luteinizing hormone

releasing hormone analog (LHRHa, Sigma Chemical Company, St. Louis, MO; American Peptide Company, Sunnyvale, CA, CS Bio, Menlo Park, CA, Syndel Laboratories, Qualicum Beach, BC, Canada.) (n=20 studies) injections and LHRHa (EVAC) implants (Center of Marine Biotechnology, University of Maryland, Baltimore, MD) (n=20studies).

In general, artificial spawning procedures were those of Lambert et al. (1999), Dunham et al. (2000), Hutson (2006), and Kristanto et al. (2009). Channel catfish females were injected or implanted with the spawning hormones. LHRHa was diluted with a commercial 0.85% physiological saline solution and injected intraperitoneally with a priming dose of 20-30 µg (85% active ingredient) /kg that was followed twelve hours later by a 100-150 µg/kg resolving dosage. LHRHa implants were administered intramuscularly as a single dose, 75-100 µg/kg body weight, posterior and ventral to the dorsal fin. CPE was dissolved for 30-45 min in 0.85% saline before injection with a priming dose of 2 mg/kg, followed 12 hours later with a resolving dose at 8 mg/kg, respectively.

Ovulation occurred 24 to 48 hours after the resolving dose or 36-72 hours after implantation. Ovulating females that were releasing eggs were anesthetized with 100 mg/L tricaine methanesulfonate (MS-222) and 100 ppm sodium bicarbonate. Females that were not ovulating remained in the trough and were checked periodically. The anesthetized females were dried with a towel, and eggs were hand-stripped into pans or bowl lubricated with vegetable shortening until eggs could no longer be stripped.

Testes were excised from euthanized blue catfish males. Blood and excess tissue were removed using Hanks' balanced salt solution (HBSS, 160.0 g NaCl, 8.0 g KCl, 2.8 g $CaCl_2$, 4.0 g $MgSO_4 \cdot 7H_2O$, 2.4 g $NaHPO_4 \cdot 7H_2O$, 1.2 g KH_2PO_4, 7.0g $NaHCO_3$, and 20.0 g glucose in 20 L reverse osmosis-distilled H_20, pH=6) or saline. Testes were then macerated in either HBSS or 0.9 % saline. The sperm was added to the eggs and mixed to dry fertilize. Water was added to cover and activate the egg and sperm mixture. After 2 minutes, more water was added into the pan or bowl to prepare the fertilized eggs for water hardening. The egg masses were placed in a flow-through trough for 15 to 45 min for further water hardening. Egg masses were then moved into egg baskets suspended in hatching troughs fitted with paddle wheels for gentle agitation and incubation. Fry were removed upon hatching and counted.

Partial budget

Hybrid fry production budgets were calculated for production of 10 million hybrid fry. For CxB hybrid embryo production budgets it was assumed that the cost of brood stock was $6.6/kg for females and $11/kg for males. The hormone costs were $225/g, and $45/mg for CPE and LHRHa, respectively. One kg of males was assumed to be needed to fertilize eggs from 6kg of females. The recommended stock density in spawning ponds is usually 900 to 1,300 kg/ha (Kelly, 2004), but farmers often exceed these values. In this study, the density of 1,400 kg body weight of brood stock per ha was used for the budget.

Statistical analysis

Fry per kg was calculated by dividing the total number of fry produced by the weight of the total body weight of the females in the treatment. This value is the ultimate measurement of hybrid

egg production and production efficiency. Mean number of fry/kg was calculated for each study. Seven to 2,000 females per hormone treatment were used in each study. Grand means were calculated by averaging the means of each study for each hormone treatment.

The fry data for the three treatments, LHRH implant, LHRH injection and CPE, were analyzed using Graph Pad Prism statistical package (GraphPad Prism version 5.02 for Mac OS X, GraphPad Software, San Diego California USA). Kruskal–Wallis one-way analysis of variance (ANOVA) was used to compare all these three treatments for producing hybrid catfish fry with an alpha value of 0.05. Differences between treatments were tested further using Dunn's Multiple Comparison Test.

Results

Fry/kg

Mean fry/kg female body weight produced was 948 ± SD, 2,483 ± SD and 2,765 ± SD for CPE, LHRHa injections, and LHRHa implants, respectively. The Kruskal-Wallis statistic was 37.61 ($P<0.0001$), indicating that a significant difference existed between at least two of the treatments. The following post post-hoc analysis indicted a significant difference between the CPE treatment and the two LHRH treatments (Table 1, Figure 1). LHRHa injections and implants produced more ($P<0.001$) fry/kg than CPE. The observed results for LHRHa implants and CPE were less variable (CV= 35.9 and 37.5, respectively) than for LHRHa injections (CV=49.9) (Table 2, Figure 1).

Table 1. Comparison of three treatments CPE injection, LHRH implant and injection for producing channel catfish (*Ictalurus punctatus*) female x blue catfish (*Ictalurus furcatus*) male hybrid fry using Dunn's Multiple Comparison Test

Dunn's Multiple Comparison Test	Difference in rank sum
LHRH Implant vs LHRH Injection	5.85
LHRH Implant vs CPE	32.11***
LHRH Injection vs CPE	26.26***

No difference was found between LHRHa implant and injection. However, significant differences were observed between LHRHa implant and CPE and also LHRHa injection and CPE injection ($P<0.05$).

Table 2. Comparison of three different hormone CPE, LHRHa implant and LHRHa injection during 15-year period for producing channel catfish (*Ictalurus punctatus*) female x blue catfish (*Ictalurus furcatus*) fry

Treatment	N	Mean(Fry/kg)	SD	CV
CPE	25	948 ± SD	356	37.5
LHRHa injection	20	2483 ± SD	1239	49.9
LHRHa implant	20	2765 ± SD	992	35.9

N = number of experiments in a 15-year period; LHRHa implants and injections gave more fry/kg than CPE ($P< 0.001$); SD = standard deviation; CV = coefficient of variation.

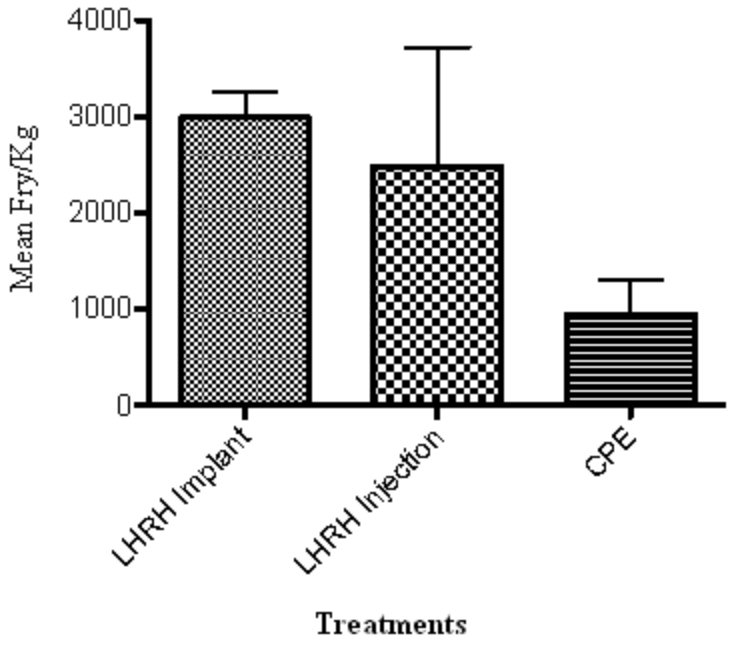

Figure 1. Mean fry survival (mean + SD) of three different treatments for producing CB hybrid fry. Mean fry survival of LHRHa implant and injection are significantly different ($P<0.05$) from the CPE injection. SD=standard deviation.

Partial budget for production 10 million fry

To produce 10 million CB hybrid fry, the weight of females needed when using CPE and LHRHa were 10,548 kg and 4,027 kg, respectively. The cost for the purchase of the females would be $69,620 for CPE and $26,578 for LHRHa injection. A 105 g of CPE hormone at a cost of $23,734 would be needed (Table 3). For LHRHa injections the amount would range from 80.3 to 120.8 mg for the priming dose and 402.7 to 604.1 mg for the resolving dose. The corresponding price for the production of 10 X 10^6 hybrid fry would range from $21,748 to $32,621.

Table 3. Partial budget for producing 10 million channel catfish (*Ictalurus punctatus*) female X blue catfish (*Ictalurus furcatus*) male hybrid fry

Treatment	Fry/kg	Hormone cost ($)	kg of ♀ needed	Cost of ♀($)	Ha of ♀	kg of ♂ needed	Cost of ♂($)	Ha of ♂
CPE	948	23,734	10,548	69,620	7.5	1758	19,338	0.5
LHRHa injection	2483	27,184	4027	26,578	2.9	671	7381	0.2

Estimated area required to hold brood stock capable of producing 10 million fry under CPE and LHRHa treatments is projected to be 8.8 and 3.4ha, respectively. The kg body weight of the corresponding blue catfish males to produce those 10 million fry would be 1,758 kg for CPE and 671 kg for LHRHa. The cost of males would be $19,338 and $7,381 for CPE and LHRHa.

Based on the data presented above, that the number of male blue catfish and female channel catfish needed to produce 10 million hybrid catfish fry was much less if LHRHa is used to stimulate ovulation. This value is almost 1/3 of that compared to CPE. Brood stock costs were about $51,000 more for CPE per 10 million fry, and the acreage needed for brood stock almost triple that of LHRHa.

Discussion

Both LHRHa injection and implant protocols can be effectively used to induce female channel catfish to ovulate and produce channel-blue hybrid catfish embryos. An advantage of the implant was that only a single handling and implantation of the fish is required versus two for the injections, thus reducing handling stress and labor. LHRHa was more economical than CPE for producing hybrid fry. Because of the lesser amount of brooders needed to produce 10 million hybrid fry LHRHa was more effective, the cost of producing 10 million hybrid fry could be reduced by more than $40,000 by reducing the number of brood stock required and the acreage devoted to brood stock production and preparation. Use of LHRHa to induce female channel catfish to ovulate could greatly expand the commercial production of hybrid catfish.

Exogenously administered hormone is an effective way to mimic environmental and hormonal factors that trigger egg maturation and ovulation in fish for efficient hatchery seed (egg, embryo and fry) production. Hormonal manipulations may also facilitate interspecific hybridization and artificial fertilization for genetic selection and enhancement programs (Mylonas et al., 2010; Mehdi and Mousavi, 2011). A major advantage of hormone induction is to synchronize ovulation while accelerating gametogenesis. Both must act together in order to have efficient production of hybrid catfish fry.

LHRH induces the secretion of gonadotropins or luteinizing hormones (LH), stimulating final oocyte maturation, ovulation and spermiation (Zohar and Mylonas, 2001; Mehdi and Mousavi, 2011). There are some advantages of LHRHa application such as repeatable application without reduction in efficiency, coupled with action at a higher level on the hypothalamus- pituitary-gonadal (HPG) axis, and LHRHa can be synthesized in pure form (Chatakondi et al., 2011) for enhanced efficiency. Although CPE is the most widely used hormone in the world to induce spawning in fish, the drawbacks for CPE were summarized as variability in its quality, limited supplies, action on a lower level in the HPG axis, and potential to transmit disease from donor to recipient fish (Dunham et al., 2000; Chatakondi et al., 2011).

Because of other disadvantages of using CPE, such as unstable source of production and source vendors, researchers in recent years have also developed channel catfish pituitary to induce spawning in aquaculture. Catfish pituitary has also been proven to be effective, and was administered with the same dosage regimes as CPE (Green and Yant, 2011). Green and Yant (2011) report that catfish pituitary gave similar or better ovulation rates than LHRHa injections,

and a greater number of viable embryos at 36 h. The number of viable embryos at 36h is not a good indicator of eventual hatch (Lambert, 1998), and Green and Yant (2011) do not report any hatch data.

The data in the current study indicates that use of LHRHa injections and implants can double or triple hybrid catfish fry production compared to CPE (Tables 1, 2; Fig. 1), which agrees with the results of Kristanto (2004). Kristanto et al. (2009) also reported that LHRHa injections could yield more than 2,000 fry/kg. Chatakondi et al. (2011) reported much lower mean fry/kg when inducing channel catfish females with LHRHa injections than found in the current study, and those of Kristanto et al. (2009) and Hutson (2006). Chatakondi et al. (2011) utilized LHRHa doses 20-100 % less than used in the current study. Green and Yant (2011) also used lower doses of LHRHa, and did not obtain the same effectiveness as reported in the current study. Total doses of 100 μg/kg (85% active ingredient) or less are inadequate for maximum effectiveness of LHRHa for hand stripping and artificial fertilization. There was no difference between the LHRHa injections and LHRHa implants over a 15-year period, which contradicts a previous study in which LHRHa implants were more effective than the injections (Hutson, 2006). However, the smaller coefficient of variation (CV) of LHRHa implants is an advantage, suggesting a more consistent result.

Conclusion

In conclusion, in these comparative studies conducted on the relative effectiveness of CPE, LHRHa Injections and LHRHa implants for producing hybrid catfish fry. We see that the LHRH treatments were significantly better than the CPE treatments. When the LHRH treatments were

compared between implant and injection, no significant difference was seen between the two treatments. When the coefficient of variance was analyzed between the two LHRHa treatments it was observed that the implant gave less variable results. Cost analyses between LHRH and CPE indicate an advantage in using LHRH. Though the cost of producing the implant has not still been analyzed, we believe the benefits of improved and consistent ovulation and handling ease of the implants, would offset any production cost. Based on these findings we recommend LHRH implants as an efficient method for the production of CxB hybrid fry.

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CHAPTER THREE

Effectiveness of luteinizing hormone-releasing hormone analog des Gly^{10},[D-Ala^{6}] ethylamide (lhrha) injections on ovulation of the female channel catfish, (*Ictalurus punctatus)*

Abstract

A double blind study was conducted to study and evaluate the effectiveness of a 20 µg/kg female body weight, priming dose (85% active ingredient) of luteinizing hormone releasing hormone analog (LHRHa) followed 12 hours later by a 100 µg/kg of female body weight of LHRHa resolving dose for ovulating female channel catfish, *Ictalurus punctatus*. The study was concurrently conducted at two sites; a research facility Auburn University, AL and a commercial hatchery; Baxter Land Company, AR. Fifty fish were treated with LHRHa while 24-25 fish were treated with a sham saline injection and used as controls fish at each site. Ovulated eggs were fertilized with the sperm of blue catfish, *I. furcatus*, incubated and hatched. The primary variable evaluated was rate of ovulation. At Baxter Land Company, treated females had a higher ovulation rate (92%, P<0.00) compared to sham injected controls (4.0%). At Auburn University, treated females had a higher ovulation rate (84%, P<0.00) than that of sham injected controls (4.2%). Secondary variables such as eggs/kg, egg quality score, latency, hatch percentage, and fry/kg were also evaluated at both locations At Baxter Land Company (AR), eggs/kg, egg quality score, latency, hatch percentage, and fry/kg were 8,665, 3.5, 44.4 hrs, 31.3% and 2,401 respectively for females injected with LHRHa. At Auburn University, eggs/kg, egg quality

score, latency, hatch percentage and fry/kg were 10,385, 3.8, 46.2, 41.3% and 3,700 respectively for females injected with LHRHa.

Introduction

The sexual maturation, ovulation and spawning of teleosts is controlled by the hypothalamic-pituitary-gonadal (HPG) axis. The signal pathway consists of the hypothalamus secreting hormones, which act on the pituitary causing it to release gonadotropins. The gonadotropins act on the gonads, which secrete steroid hormones that are essential for many functions including gamete production, maturation ovulation and release (Zohar 1988; Dunham and Argue 2000; Chatakondi et al. 2011). For the past 80 years, a variety of hormonal methodologies and tactics have been researched and tested successfully on many cultured species of fishes (Zohar and Mylonas, 2001). The objective has been to affect the brain-pituitary-gonadal axis to induce follicular development, vitellogenesis, and ovulation (Zohar and Mylonas, 2001).

Carp pituitary extract (CPE) is used worldwide to induce the spawning of fishes (Dunham, et al. 2000). It is easily available to commercial farmers as there is limited regulation. There is also over 50 years of research conducted on CPE as a spawning aid, which has been tested on many aquaculture species (Peter, et al. 1993).

A disadvantage to CPE is the large irregularity in luteinizing hormone (LH) concentrations in the ground crude pituitary extract (Chatakondi, et al. 2011). Furthermore, as CPE is a crude protein product. It contains additional pituitary hormones in addition to LH (Chatakondi, et al. 2011). These additional hormones could present adverse physiological affects to the fish. CPE acts on a

lower level of the HPG axis giving mixed or variable results when used for induced spawning (Chatakondi, et al. 2011).

In the past 20 years there has been an advancement and development in the design, production, and use of synthetic hormones in aquaculture. Various synthetic, highly potent analogs of the gonadotropin-releasing hormone (GnRHa) are available (Peter, Lin, and Van Der Kraak 1988), as well as sustained-release delivery systems for their controlled administration (Tamaru et al. 1990). This has made it possible for a more precise targeting of the action of the hormone on specific parts of the HPG axis. Synthetic LHRHa has been studied since the mid 80's and have shown considerable potential as an ovulatory agent (Crim and Bettles, 1997; Kristanto, 2004; Kristanto, et al. 2009; Chatakondi, et al. 2011). The advantages of LHRHa include; its smaller decapeptides which do not trigger the immune system. This allows its repeated use without a decrease in its efficiency (Chatakondi, et al. 2011). The mode of its action consists of inducing the natural release of endogenous LH from the anterior pituitary to overcome the endocrine disruption observed in the lower levels of the HPG axis in maturing fish to promote final oocyte maturation. It targets at a higher level in the HPG axis, therefore the stimulation of reproductive events are more balanced and coordinated. Since it is a synthetic hormone, it is produced under sterile conditions, and reduces the possibility of contamination (Chatakondi, et al. 2011). LHRHa has successfully applied to a wide variety of fishes (Crim and Bettles, 1997; Yeager, 2006; Chatakondi, et al. 2011).

There are very few drugs approved by the US Food and Drug Administration (FDA) for use in fish (Reimschuessel et al. 2005). As of 2012 there were only 20 drugs approved by the FDA for

aquaculture (FDA, Approved Drugs for Use in Aquaculture). Of these 20 drugs, only Chorulon® - NADA 140-927, an injectable chorionic gonadotropin, is approved as an aid in improving spawning function in male and female brood finfish. Although there are several reasons for this lack of approved drugs in the fields of aquaculture, the primary factor is a lack of pharmaceutical sponsors willing to invest in the research needed to generate the data to support a drug approval (Reimschuessel et al. 2005). Such data include demonstration of drug efficacy and safety in the target species, human food safety, environmental impact assessments, and demonstrating appropriate chemical manufacturing controls (FDA). The overall cost of obtaining the experimental data required for a New Animal Drug Application (NADA) could be in excess of $40 million (Reimschuessel et al. 2005; Squibb 2005).

For a new drug to be approved by FDA, first the primary sponsor of the drug is required to submit a NADA along with supporting data, including all adverse effects associated with the drug's use. The NADA must also include information on the drug's chemistry; composition and component ingredients; manufacturing methods, facilities, and controls; proposed labeling; analytical methods for residue detection and analysis if applicable; an environmental assessment; and other information. Once the NADA has been submitted along with the appropriate information and data, an extensive review process is performed by FDA scientist to determine if the data was developed in accordance with Good Laboratory Practice Regulations (GLP), or clinical trial guidance. If the studies were conducted properly, the data is evaluated with respect to drug safety and effectiveness. The animal safety data for a drug product must relate to the dosage levels and routes of administration proposed in the labeling. The primary objective is to determine the safety of the product relative to labeled usage. At the conclusion of the animal

safety review, a summary is prepared which explains why the product is safe or not shown to be safe. If the product has been shown to be safe but some restrictions or constraints on use are needed, all warning and precaution statements are to be placed on the label, must be enumerated and included in the summary, as well as any expected side effects. All effectiveness data submitted must relate either directly or indirectly to the specific label and labeling claims made for the product. The sponsor must demonstrate that the product produces the claimed effect (FDA).

The objective of this study was to generate the required data for the submission of an NADA to the FDA for the approval of LHRHa as a spawning aid. This study focuses on the efficacy of the drug to obtain dose confirmation. Non-pivotal supporting experiments have been conducted from 2000-2010 under a variety of commercial and research conditions in Alabama, Mississippi and Texas. In these studies, dose responses were evaluated and the efficacy of the drug investigated. These studies indicated doses of 20/100 and 30/150 (priming/resolving dose in µg/kg female body weight), resulted in ovulation rates, which ranged from 24.0-100.0%, with a mean of 65% (Kristanto, 2004; Hutson, 2006; Ballenger, 2007; Phelps, et al. 2007; Durland, et al. 2009; Kristanto, et al. 2009; Phelps, et al., 2011; Dunham, 2012). These studies also indicate the secondary variables of, hatch ranging from 2.3-57.1% with a mean of 30.3%, and fry/kg ranged from 78-3,556 with a mean of 1,894 when hybrid catfish fry were produced.

A dose range of 120-180 µg per kg female body weight will be proposed for the label, administered as a priming dose of 20-30 µg per kg fish body weight, followed 12 hours later with a resolving dose of 100-150 µg per kg fish body weight. The objective of the effectiveness

study was to confirm that a priming injection of 20 µg of des-Gly^{10},[D-Ala^{6}] LHRH Ethyl amide (LHRHa) per kilogram of female channel catfish body weight, followed 12 hours later by a resolving injection of 100 µg per kilogram of body weight, is effective for ovulation. The secondary objective was to evaluate variables such as; eggs/kg, egg quality score, latency, hybrid hatch percentage, and hybrid fry/kg of the treated fish and the sham treatments.

Materials and Methods

Experimental design

The study was conducted at two locations, Auburn University, Alabama (AU) and Baxter Land Company, Arkansas (BLC). The experiments were conducted at these two sites between May 23rd and June 1st 2011. Fifty females were used at each site in the treatment group, while 24 and 25 individuals were respectively used at AU and BLC as controls. Each female channel catfish was placed in an individual spawning bag, which was placed in a tank until ovulation and tracked individually. At BLC a single large common ovulation tank was used, while at AU several smaller tanks were used with multiple individuals in each. Each female channel catfish constituted a single experimental unit representing a single replicate at both sites.

The treatment group received an LHRHa treatment at 120 µg/kg female body weight of OVA-EASE (85% active ingredient) in two injections of 20 µg/kg priming dose followed 12 hours later by a resolving dose of 100 µg/kg LHRHa. Solutions were made at 20 µg/mL, and 100 µg/mL concentrations and used for the priming and resolving doses respectively. The control group received a sham commercially purchased 0.9% saline (Hospira) injection at 1 mL/kg body weight. At both sites, fish from the different treatment groups were handled in an identical

manner to maintain consistency. The study was double blind with only the primary investigator having access to the random method by which fish numbers were designated as treatment or control and which fish numbers ultimately corresponded to individuals that received the LHRHa treatment versus the control for the duration of the whole study. The primary investigator prepared all injections behind a screen and not in view of fish handlers. The treatment and control injection solutions were the same in appearance. All injection data was recorded and retained by the primary investigator. All other personnel involved in the study were blinded. The primary investigator was not allowed to collect ovulation, egg fertilization or hatch data.

Diets

At BLC the fish were fed a floating catfish pellet feed containing 35% protein in accordance with the industry's generally accepted feeding schedule of five times a week from March-October and once per week between November-February. At AU the fish were fed a commercially purchased floating catfish pellet containing 36% protein. The fish were fed five times a week from March-October and once a week between November-February. During the eight weeks prior to the study, female channel catfish at AU were given a 36% protein floating brood stock feed containing 6% fish oil, 500 ppm vitamin C, 65 ppm vitamin E, 0.5% docosahexaenoic acid/omega 3(DHA), 0.5% arachidionic acid/omega 6 with standard vitamin and mineral packs. Study fish at both sites were not fed the day prior to harvest nor at any time throughout the duration of the study.

Harvest

At BLC, consistent with the commercial operator's normal operating procedure, study fish were harvested the day prior to initiation of the study. The fish were captured in a seine with the fish

crowded together as a single unit. The fish were then netted in a random fashion and evaluated at pond-side for gravidness and evaluated for health. Any fish that showed signs of diseases, lesions, or poor physical health were excluded from the study. Selected fish were then placed loose in a hauling tank for transport to the hatchery. Upon arrival at the hatchery, the fish were transferred, and held together in a 13,450 L containment tank were they were allowed to acclimate for approximately 24 hours.

Seventy-five fish were needed for the study; however, an additional 56 fish were selected and transported to the hatchery, as potential replacements should the primary investigator reject a fish in his initial health/gravidness evaluation. On the day of injection, the fish were netted in a random manner, health and gravidness was evaluated by the primary investigator, each selected fish was weighed (to the closest 0.01 kg), assigned an identification number and placed in a 60 x 90 cm, 32 mm spawning mesh bag. The identification number assigned to the fish along with its weight was recorded on a tag, which was attached to the mesh bag. Thereafter, the bag with the fish in it was suspended in a holding tank. At the completion of the random procedures of netting and identification number assignment in the hatchery a health evaluation of the unused 56 fish was conducted and documented by the investigator.

At AU, the study fish were harvested in a similar manner early on the day of injection. The fish were captured in a seine where segregation and escape was not possible and the fish were crowded together as a single unit. The fish were then netted in a random manner and evaluated for gravidness, and given a initial health evaluation as done at BLC. Selected fish were weighed, and placed in individual 60 x 90 cm, 32 mm mesh spawning bags pond-side. The bags were

sequentially numbered and tagged with the fish number and fish weight. The bags were then suspended in the hauling tank for transport to the hatchery. Upon arrival at the hatchery, the bagged fish were unloaded and placed sequentially in 836 L tanks, at 8-9 fish per tank, according to the number on the bag, and allowed to acclimate for 12 hours. A flow of 24 L/min, along with aeration was maintained in each tank

An additional 14 fish were selected at AU and transported to the hatchery as potential replacements should the investigator reject a fish in his initial health/gravidness evaluation. After the 75 fish were selected for the study, a health evaluation of the unused 14 fish was conducted and documented, and returned to a 0.1 ha pond.

At both sites, experimental fish were not fed the day prior to harvest/capture nor at any time during the conduct of the experiment. The fish resumed the normal feeding regime upon return to the ponds. No medications or vaccines were administered during the acclimation period or experiment.

Selection criterion

At both experimental locations, fish were selected in a random manner by seining crew personnel with multiple years of experience in assessing brood quality. The primary investigator performed a second evaluation of gravidness and health when the fish arrived at the hatchery. Inclusion criteria for selection consisted of: no evidence of disease, lesions or lethargy, minimum age of three years, gravidness, swollen genital openings with reddish color was preferable but not essential. Fish not meeting the inclusion criteria were excluded from the study.

Treatments

The LHRHa treatment consisted of two doses; a priming dose of 20 μg/kg and a resolving dose of 100 μg/kg. The LHRHa treatments were made by dissolving the appropriate amounts of LHRHa (C.S. Bio) in 0.9% sodium chloride (Hospira). For the ease of calculating the appropriate volume needed for administration to the females, two stock solutions were prepared at 20 μg/ml and 100 μg/ml concentrations respectively. Each stock solution was prepared immediately prior to the start of the administration of the injections. For the control treatment, a volume of 1 ml/kg of the 0.9% saline was administered twice, to correspond with the priming and resolving doses of the LHRHa. The allocation of channel catfish females to treatment groups was done by using the fish identification numbers and a random number generator in Microsoft Excel 2010.

Treatment administration

The LHRHa treatments and sham controls were administered in a similar manner at both experimental sites. In both locations, the treated female channel catfish were administered two injections consisting of a priming dose of 20 μg/kg fish body weight followed 12 hours later with a resolving dose of 100 μg/kg fish body weight. Control fish were administered two injections of 0.9% saline (without LHRHa) at the same times and in the same manner as treated fish.

At both experimental sites, the primary investigator worked behind a screen and not in view of the fish handlers, recorded the weight of each numbered fish, determined and recorded the appropriate priming LHRHa solution or sham dose (1 ml/kg body weight) and loaded a 3 or 5 ml

syringe with a 21.5-gauge needle. The investigator then stepped from behind the screen and administered the priming dose to the fish, which at BLC was removed from the holding tank and positioned on a table. At the AU site the treatment was administered without removing the fish from the containment tank. Each bagged and numbered fish was gently raised to the surface, turned to expose the belly and injected. At both sites treatments, and control doses were administered by intraperitoneal injection.

The resolving dose was determined, prepared and documented and administered 12 hours later intraperitoneally in the same manner (screened and not in view of the fish handlers) as the priming dose. At both sites the treatment or sham resolving dose was administered without removing the fish from the containment tank.

Male blue catfish sperm preparation

Blue catfish males were seined and selected based upon several external characteristics such as skin color, muscular development, leanness and size of the papilla. Individuals that were not selected were returned back to holding ponds. Fish with any signs of disease were also excluded from the study. The blue catfish males were weighed, sacrificed, and their testes removed. The testes were then carefully cleaned using a 300-mOsmol/kg solution of salt (this solution was made by taking 36 g of salt and adding distilled water up to 4 L), and trimmed with scissors to remove excess tissue and blood. The testes were then weighed and macerated with 10 ml (± 0.1 ml) of the salt solution per gram of testes. Thereafter, the solution was filtered using a 100-micron mesh. The sperm concentration was determined by using actual counts on a hemocytometer with a microscope and a spectrophotometer with a wavelength of 440 nm. A

standard curve with the best fit for the sperm concentration was calculated. This curve was used to determine the concentration of sperm/ml throughout this study. The sperm that was prepped was used within 24 hours of processing and was stored at 4^0C in a refrigerator.

Ovulation

At 38 hours (at BLC) and 36 hours (at AU) after the priming injection the experimental fish were carefully checked for ovulation and the release of eggs. Signs of egg release were checked by gently bringing the fish to the surface and checking the spawning bag for any eggs. The time of ovulation and water temperature was recorded. Ovulation checks were conducted on fish that had not ovulated at four-hour intervals for the next 36 hours for a total of 10 potential egg-stripping sessions. Total ovulation time allowed for the experiment from administration of the priming dose injection was 73.5 hours at BLC and 72 hours at AU. At this point, all non-ovulated fish were anesthetized and checked for ovulation by attempting manual stripping of the eggs.

Egg stripping

At the conclusion of each ovulation check, if egg release was evident the female was removed from the ovulation tank and anesthetized in a 100 ppm solution of MS-222 that had equal parts of $NaHCO_3$. The $NaHCO_3$ was added to maintain pH at neutral. The ovulated fish was anesthetized while still contained in the 32 mm mesh bag. The fish were anesthetized for approximately 60 seconds or until the fish lost equilibrium, the gills were beating at about 6 beats per minute and the fish could be handled without struggle. Once the fish was adequately anesthetized, the top of the mesh bag was rolled down to the level of the fish and the fish removed from the bag and anesthesia solution while placing a finger on the vent to prevent egg

release. Each anesthetized fish was dipped in a 9 ppt salt solution to rinse the anesthesia solution from the body. The fish was then dried completely with a towel to prevent water from dripping from the body of the fish and pre-maturely activating the eggs prior to fertilization. The towel was then wrapped around the head of the fish (to prevent water dripping from the gills) and taken to the egg stripping station

All eggs from a single ovulating female were stripped directly into a 2 L plastic tub (BLC) or an 20 cm diameter pie pan (AU) lightly coated with vegetable shortening. The anesthetized female was cradled with its head up and tail down, with the genital opening over the greased container. Pressure was applied on the right and left side of the body cavity and the ovary repeatedly stroked from the anterior region to the genital opening.

At BLC if the eggs stripped cleanly (little or no blood/excessive fluid and no egg clumps), they were taken to the fertilization station. If the eggs did not strip cleanly, the eggs were run through a screen to remove clots and clumps. Notes were made about egg quality and the presence of any blood. The screening process entailed pouring the eggs from the tub over a stainless steel screen with 0.65 cm diameter holes. A large container was positioned below the screen to collect the good eggs as they filtered through the screen. Clots and clumps remained on top of the screen and were discarded. The container containing the good eggs was then transferred to the fertilization station.

At AU, if the eggs stripped cleanly (little or no blood/excessive fluid and no egg clumps), the egg mass of a single female was separated into smaller masses by transferring the desired amount into separate, tared, 20 cm diameter pie pans. The egg mass was then weighed and

delivered to the fertilization station. If the eggs did not strip cleanly, the eggs were covered with 0.9% saline until the saline was 2 cm above the eggs. The saline-blood solution was then decanted and the clots and clumps removed by hand before dividing the egg mass into tared pans, weighing and transfer to the fertilization station.

At both study locations egg quality was evaluated at the stripping table and prior to screening or cleaning of the eggs. An egg quality score (EQS) (Table. 1) was documented for each egg mass. Poor quality eggs (White small under developed eggs, with excessive blood and clumps) from which a low hatch was expected were discarded. Eggs were kept if a 10% or greater hatch was expected.

Table. 1 Egg Quality Score (EQS) Guide

1—White, excessive clumps/blood/fluid (discarded)
2—Free flowing, white, containing clumps, blood and extra fluid
3—Free flowing, mostly white with some yellow, with some clumps/blood
4—Free flowing, pale yellow, sticky
5—Free flowing, golden yellow, without blood and clumps

Upon completion of the egg stripping process, each fish was fin-clipped as a method of identifying the fish if needed during the recovery period. Each fish was then transferred to the recovery containment tanks, observed for 6 hours prior to being transferred to a hauling tank and returned to a pond. At BLC four fish died during this recovery period and necropsies were performed on them. At AU no mortalities occurred. However, on one occasion the recovery period was shortened due to ammonia accumulation in the recovery tank.

Fertilization

When the eggs arrived at the fertilization station at BLC, 100 ml aliquots of the eggs were placed in lightly greased 20 cm diameter pie pans. If the final aliquot contained less than 60 ml of eggs, these eggs were evenly distributed among the previous aliquots. If the final aliquot was 60 ml or greater is was poured into a pie pan of its own. The amount of sperm solution required for fertilization was determined by egg volume. At AU, egg mass size and amount of sperm needed was determined gravimetrically. The stripped eggs were aliquoted into greased pie pans with about 100 g of eggs per pie pan. At both sites the total egg volume from each individual fish along with the fish tag number and the male number were recorded as well as marked on the pie pan prior to fertilization. At BLC based on the egg volume in each pie pan, either 2 ml of 3.25 x 10^7 or 3 ml of a 2.13 x 10^7 blue catfish sperm solution per 100 ml of egg volume was drawn into a syringe and evenly distributed over the eggs, while at AU the blue catfish sperm was added to each pie pan at the rate of 2 ml of 3.25 X10^7/ml sperm.

Water taken from the egg-hardening trough was added to the fertilized eggs to activate the eggs and sperm. Water was added to raise the water level so that 0.5 cm covered the eggs. The eggs and sperm were gently swirled in the water for 30 seconds to distribute the sperm and facilitate the fertilization of the eggs (egg fertilization occurs within a 1-2 minute period). The numbered pans were then allowed to sit for 10 minutes before transfer to the water-hardening trough.
At both experimental sites the egg masses along with their pie pans and tags were placed gently in a hardening trough, with flowing water. The water level was maintained at about six inches above the top of the pie pan. The eggs were allowed to harden for approximately 30 minutes.

Egg sampling

The total number of eggs per kilogram of fish body weight was determined at both experimental locations by sampling every 10th fish in each egg stripping/fertilization session. At BLC a volumetric method was used; by taking a 5 ml egg sample and counting the number of eggs in the sample and dividing the number by the volume to obtain the number of eggs per unit volume. From this data a mean for all the females that ovulated was calculated and applied to each female's total egg volume to determine the number of eggs per female, except for females from which the data was generated.

At AU a similar method was used, with the only difference being the number of eggs were determined gravimetrically. Five-gram egg samples were collected from every 10th fish and counted. As before, number of eggs per unit mass was determined, and this data applied to each female's total egg volume to determine the number of eggs per female, except for the females from which the counts were taken and their actual mean egg size was utilized. When the eggs were stripped, any fish that appeared to be a potential outlier was sampled.

Egg incubation and hatch

At both sites the eggs were incubated in 380 L hatching troughs with flow through, aeration and paddle wheels. Water was exchanged in the hatching troughs 2.5 times per hour with a minimum flow of 16 L per minute. At BLC the hatching baskets were divided in half with each half sufficient in size to accommodate two egg masses (4 egg masses per basket). Initially, only one egg mass was placed in each section of the divided basket (2 egg masses per basket) in order to

further facilitate the forming and hardening of the egg mass. After 8-12 hours after the initial introduction the single egg masses were consolidated to four egg masses to a hatching basket depending on the needs of the commercial operation. In the consolidation process, egg masses from a numbered fish were segregated in 1-2 baskets depending on the number of egg masses given by the fish. At AU Each egg mass was placed in a single basket that was suspended in the trough between the paddles. Each trough contained several baskets with egg masses. Eggs from an individual spawning time block was assigned to a single trough. Once the eggs were placed in a trough they remained at the same location till hatch. Egg masses at both sites were checked and flipped twice daily to examine for fungus and dead material.

Egg treatments

At both locations, the eggs were treated every 8 hours with a 100 ppm of formalin (Paraside-F). During the treatment the flow was stopped and the appropriate amount of formalin diluted in a 2 L container of hatching trough water. The formalin treatment was evenly distributed between the egg baskets. The eggs were treated for 15 minutes and the water flow turned back on. Each trough contained eggs from a single spawning session. Therefore, eggs in a single trough were similar in age. Four of egg masses developed fungus at AU. These were moved to a separate hatching trough and treated with two treatments of 32 ppm copper sulfate followed by a formalin treatment daily for 5 days.

Hatch estimate and fry counts

At BLC and AU an actual sac fry count for each study fish was not possible in that the eggs from several females were incubated in the same hatching trough. Actual counts would be possible

only for a mixture of fry for multiple treatments or females in a single trough, but not for individual egg masses/spawns. Estimation of the hatch rate for an individual fish was done by visually estimating the percentage of live embryos 24 hours prior to hatching, weighing the entire egg mass and weighing and counting a one gram sub-sample from the eggs mass.

After the hatching was complete the fry were captured by siphoning them into a large graduated cylinder and the total volume measured, to determine the total number of fry hatched in each trough. A 5 ml sample from each trough was enumerated. The fry volume for each trough was documented, and the total number of fry estimated.

Due to excessive temperature, the embryos developed more rapidly than normal at AU. Individuals began hatching between normal checking periods, thus the opportunity to estimate individual hatch rate was missed. Post hatch, the hatch rate and fry/kg were calculated by capturing the fry by siphoning the fry out of the trough and onto a large net. The total weight of the fry was recorded. A sub sample of 5 g was weighed and the number of fry counted. The number of fry per unit weight, and the total weight gave the number of fry in each hatching trough.

At both study locations, water quality was measured and recorded (using a LaMotte Model AQ-2 water quality kit) for the brood stock as well as the hatching troughs (Table 2).

Table 2. Water quality parameters for the brood stock held in ovulation tanks and eggs in the hatching troughs.

Temperature:	24-30C^0
Oxygen:	5-10 ppm
Ammonia:	<0.25 ppm
Water Exchange/Flow Rate:	brood stock 2times/hour; eggs 3times/hour
Hardness:	>30 ppm

Data Calculations, Statistical Methodologies and Analysis

Primary variables

Individual female channel catfish in individual spawning bags constituted replications. One tank and a completely random design were used at Baxter Land Company. Nine tanks and a randomized block design were used at Auburn University. The experiment was initiated with 25 control females and 50 treated females at each of the two locations.

Ovulation rate was the primary effectiveness variable. The data were analyzed by site. Fisher's exact test was used to compare ovulation rate between treated and control groups at both locations. At Auburn University, ovulation was first compared for each tank. If a significant difference was demonstrated at $\alpha = 0.25$ for 6 of 9 tanks, all data were pooled for analysis. The hypothesis of a difference between group ovulation rate for the pooled data was tested at $\alpha = 0.05$ for all data at the Arkansas commercial site and for the data pooled across tanks at the Auburn University site. Additionally, exact 95% confidence intervals were estimated for ovulation rate for all groups. No data were transformed.

Secondary variables

Our expectation was that the control females would not ovulate, thus they would have no observation for latency, fecundity, egg quality, hatch rate and fry/kg, and no statistical comparison could be made to the treated fish. One control fish ovulated at each location. A sample of one was insufficient to allow meaningful statistical analysis between the treated and control females. We took the approach that success for the treated fish for the secondary variables would be defined by their means having met a minimum value expected in the catfish industry for a profitable hybrid hatchery operation (Ligeon et al. 2004; Umali 2007). The minimum standards were 65 hours for latency, 3,500 eggs/kg for fecundity, 2.8 for egg quality, 15.0% for hatch rate and 1,000 fry/kg.

Number of observations, mean, standard deviation and range were calculated for each trait. No data were transformed. Means and standard deviations were calculated using the Microsoft Excel 2009 program.

Results

At both study locations, the first fish started to ovulate at approximately 36 hours after the priming dose (Table 3). The majority of the females, 72% at Baxter Land Company (AR) and 74% at Auburn University, ovulated 40-48 hours after the priming dose.

Table 3 Number (N) and percentage (%) of channel catfish, *Ictalurus punctatus*, females that ovulated and were stripped of eggs at certain time points (approximate hours after the initial injection (hrs.) after injection with luteinizing hormone releasing hormone analog at Baxter Land Company (Baxter) and Auburn University.

Stripping Session	Hours To Ovulation	Baxter		Auburn University	
		N	% Ovulation	N	% Ovulation
1	36	5	10.0	1	2.0
2	40	13	26.0	8	16.0
3	44	13	26.0	23	46.0
4	48	10	20.0	6	12.0
5	52	2	4.0	0	0.0
6	56	2	4.0	0	0.0
7	60	0	0.0	3	6.0
8	64	1	2.0	0	0.0
9	68	0	0.0	0	0.0
10	72	0	0.0	1	2.0

At both locations the females given the treatment of 20 µg/kg of LHRHa followed 12 hours later with a resolving dose of 100 µg/kg had a higher percentage ovulation when compared to the sham injected controls ($P<0.001$). At Baxter 92% of the LHRHa treated fish ovulated (92%, $P<0.001$). At Auburn University 84% of the LHRHa induced fish ovulated (84%, $P<0.001$) (Table 4). Of the fish that received the sham injection 4% at BLC and 4.2% at AU spawned (Table 4).

Table 4. Mean, standard deviation (SD) and range of body weight, % ovulation and 95% confidence interval (CI) for ovulation for channel catfish, *Ictalurus punctatus,* given a priming injection of 20 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, followed by 100 µg/kg 12 hours later or given a sham injection of 0.9% saline at Baxter Land Company, Arkansas and Auburn University, Alabama.

Location	Treatment	Body Weight				% Ovulation	
		N	Mean	SD	Range	% Ovulation**	95% CI
Baxter (AR)	Injection*	50	2.70	0.59	1.61-3.93	92.0	80.7-97.8
	Sham Injection	25	2.73	0.66	1.62-4.46	4.0	0.00-20.4
Auburn University	Injection	50	1.41	0.34	0.56-2.08	84.0	70.9-92.8
	Sham Injection	24	1.61	0.38	1.00-2.84	4.2	0.00-21.1

* One control and one treated fish released bad eggs
** Injection significantly different from sham injection at both study locations (P <0.00, Fisher's Exact Test)

At Auburn ovulation rates for treated female channel catfish were calculated by individual tank. In four of the nine tanks used, 100% of the LHRHa treated fish ovulated, while the other five tanks had 71.4%, 75.0%, 75.0%, 83.3% and 71.4% ovulation. The ovulation percentages for control females in the same 9 tanks were 0.0, 0.0, 0.0, 0.0, 0.0, 33.3, 0.0, 0.0 and 0.0% (Table 5).

Table 5. Mean ovulation rate (%) for female channel catfish, *Ictalurus punctatus*, given a priming injection of 20 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, followed by 100 µg/kg 12 hours later (treated) or given a sham injection of 0.9% saline (control) at Auburn University, Alabama. Fisher's exact test was used to test the differences in mean ovulation of treated and control females by tank.

Tank #	Treated		Control		Probability*
	Number	% Ovulation	Number	% Ovulation	
1	6	100.0	3	0.0	0.012
2	4	75.0	4	0.0	0.071
3	5	100.0	3	0.0	0.017
4	7	71.4	2	0.0	0.167
5	4	75.0	4	0.0	0.143
6	6	100.0	3	33.3	0.107
7	5	100.0	3	0.0	0.017
8	6	83.3	2	0.0	0.101
9	7	71.4	1	0.0	0.375

* Probability that the treated and control means are different (Fisher's Exact Test).

Eight of the nine tanks met the criteria of having a difference in the means at $\alpha = 0.05$. Since six or more of the tanks met this criterion, the data from all the tanks were pooled and re-analyzed. Analysis of the pooled data showed that female channel catfish injected with LHRHa had an ovulation rate of 84%, which was higher than that of sham injected controls, 4.2% (Table 4).

Means for secondary variables easily exceeded minimum industry standards for females ovulated at both locations. At Baxter Land Company (AR), eggs/kg, egg quality score, latency, hatch percentage and fry/kg were 8,665, 3.5, 44.4 hours, 31.3% and 2,401 for females injected with LHRHa. At Auburn University, eggs/kg, egg quality score, latency, hatch percentage and fry/kg were 10,385, 3.8, 46.2, 41.3% and 3,700 for females injected with LHRHa (Table 6).

Table 6 Mean, standard deviation (SD) and range of eggs/kg, egg quality score, latency, hatch % and fry/kg female body weight for channel catfish, *Ictalurus punctatus,* given a priming injection of 20 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, followed by 100 µg/kg 12 hours later or given a sham injection of 0.9% saline at Baxter Land Company, Arkansas and Auburn University, Alabama.

Location	Treatment	Eggs/kg			Egg Quality Score			Latency (Hrs.)		
		Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Baxter	Treated	8,665	1,869	3,796-12,728	3.5	0.8	2.0-5.0	44.4	5.8	36.1-63.9
(AR)	Control	6,456(1)	-	-	3.0	-	-	56.4	-	-
Auburn	Treated	10,385	2,170	6,080-15,732	3.8	0.9	1.0-5.0	46.2	6.9	36.5-71.6
University	Control	12,696(1)	-	-	4.0	-	-	61.1	-	-

Table 6 (cont.)

Location	Treatment	Hatch %			Fry/kg		
		Mean	SD	Range	Mean	SD	Range
Baxter	Treated	31.3	17.4	0.0-75.0	2401	1,674	0 - 6,232
	Control	29.8	-	-	93	1,674	-
Auburn	Treated	41.3	2.9	34.5-41.9**	3700	580**	2,807-,130**
University	Control	*	-	-	< 250***	-	-

* Mixed with injection and identify lost due to rapid hatching
**Standard deviation and range based on tank means
***Maximum possible value 29.8 0-6,232

No experimental fish at Auburn University and four experimental fish at Baxter Land Company died during the experiment (treated-8%, sham-0%). The observed mortality of the injected fish at Baxter Land Company was not significantly different ($P>0.05$) from that of sham injected females. One of the control females used at AU was misidentified and was actually a blue catfish female. Thus, the actual number of controls was 24 at this site. At AU there was also a temporary spike in the water temperature during the egg incubation period due to a heat wave

Discussion

The effects of synthetic LHRH decapeptides on the release and of both LH and follicle stimulation hormone has been well documented in a variety of species of animals such as mammals, avians, amphibians, and fish (Lam, Pandey, and Nagahama 1976). Several studies conducted on the efficacy of GnRH and LHRHa on the ovulation of fish species over the past 40 years such as spotted sea trout (*Cynoscion nebulosus*) (Thomas and Boyd 1989), asp (*Aspius aspius*) (Targońska et al. 2010), sea bass (*Lates calcarifer*) (Harvey et al. 1985), gold fish (*Carassius auratus*) (Lam, Pandey, and Nagahama 1976), milk fish (*Chanos chanos*) (Lee et al. 1986), silver carp (*Hypophthalmichthys molitrix*) (Naeem et al. 2005), channel catfish (Ictalurus punctatus) (Chatakondi et al. 2011), show that there are multiple factors that that may influence the effectiveness of LHRHa in stimulating the ovulation in fish. Even though the endocrine action of GnRH on the HPG axis and the down stream signal pathway is similar in teleost, there are several environmental or external factors that could effect the signal pathway, resulting in unique differences in different species of fishes to stimulation by externally administered LHRH. This fact holds true not only to synthetic hormones, but also to any administered drug.

When determining the efficacy of a drug the primary factor that need to taken into account is the target species. In this instance the target species is a teleost, however the reaction of LHRHa to different species is different. For instance even thought in many species of fish LHRHa induces ovulation and the release of eggs, there are occasions where LHRH does not stimulate an ovulatory response (Shireman and Gildea 1989). Furthermore the method and frequency of administering the drug also determines the efficacy of the drug. The efficacy of LHRHa in teleost, has been evaluated when administered via intramuscular, intraperitoneal, implants and oral routes have been investigated (Donaldson, 1996). The results show that the optimal method for administering this drug is dependent and unique to the species. The same can be said about the dose response of synthetic LHRHa. Several factors such as temperature, nutrition, spawning condition, stress, age and season (late/early) within the spawning period affect dose response (Phelps et al. 2011).

The analog used in this study, LHRH analog Gly^{10}, [$D\text{-}Ala^{6}$] LH-RH Ethylamide has been used and researched extensively and is currently the most commonly used form in fisheries sciences and aquaculture (Donaldson 1996; Kahkesh et al. 2010). Dose response studies conducted by Kristanto (2004) established a dose of 120-180 µg/kg would induce final maturation ovulation and egg release in channel catfish. In consequent years these recommended dose ranges have been used successfully to spawn channel catfish in commercial as well as laboratory settings (Dunham 2012).

This study was conducted at two locations concurrently, Auburn University; AL and Baxter Land Company; AR. The results were highly similar at both locations, being geographically separated, with different types of water resources, sizes of tanks, strains of fish and other subtle differences. This shows that the results obtained for LHRHa induced spawning and ovulation at research facilities are highly relevant to what can be expected at commercial locations if the protocol is the same.

The data from the trials were consistent with results obtained in non-pivotal studies done in the past. The ovulation rates of the LHRHa treated fish were 84% and 92% at AU and BLC respectively. These ovulation rates are higher than the mean ovulation rate of 66.5% calculated from previous studies done, however they do fall within the range (24%-100%) (Kristanto, 2004; Hutson, 2006; Ballenger, 2007; Phelps, et al. 2007; Durland, et al. 2009; Kristanto, et al. 2009; Phelps, et al., 2011, Dunham 2012). The relatively high ovulation rate could be attributed to the fact that this trial was conducted at the peak of the spawning season, and also to the fact that the females were in good condition.

The mean relative fecundity (ovulated eggs per kg of female body weight) ranged from 8,665 at BLC to 10,385 at AU. This is slightly higher than normal mean industry fecundity (Dunham personal communication). This might be explained by the experiment being done at the peak of the spawning season, good brood stock preparation, use of experienced egg stripping personnel and adherence to protocol.

Previous studies on CxB hybrid fry production had hatching rates ranging from 2.3-57.1% with a mean of 30.3% and fry/kg ranging from 78-3,556 with a mean of 1,894 (Kristanto, 2004; Hutson, 2006; Ballenger, 2007; Phelps, et al. 2007; Durland, et al. 2009; Kristanto, et al. 2009; Phelps, et al., 2011). The current study exceeded those mean results with a hatch percentage, and fry/kg of 31.3% and 2,401, respectively, at BLC, and 41.3% and 3,700, respectively at AU. Adherence to the protocol used in the current study, could result in greater hybrid fry production in the catfish industry.

Currently, the primary obstacles for the expansion of the hybrid catfish industry, is the general acceptance of the hybrid, and lack of blue catfish brood stock. Future research avenues could include developing hatchery techniques to improve survival rates of the hybrid fry. Future research should also include improved spawning techniques for blue catfish. Currently as the catfish industry is primarily based on channel catfish production, a limited amount of work has been done on spawning techniques for blue catfish. Future studies would include investigating the efficacy of LHRHa, and dose response for blue catfish females. As blue catfish males are a critical part of hybrid catfish production a improvement in propagation techniques, and strain selection would benefit the hybrid industry.

Conclusion

Channel catfish ovulation rate is the primary indication of LHRHa efficacy. In the study conducted in 2011 at Auburn, and Baxter land farms, a 20 µg/kg priming dose of LHRHa followed 12 hours later by a resolving dose of 100 µg/kg was shown to be effective for inducing

ovulation in female channel catfish, confirming dose response. There was not a significant difference in ovulation rates between the two experimental sites. This would indicate that the efficacy of LHRHa should be similar in either a research or commercial setting. Application of this LHRHa treatment on a commercial scale throughout the catfish industry would yield in improved hybrid catfish fry production, which in turn would have a positive economic impact in not only in aquaculture, but in the in the US agriculture industry as a whole.

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CHAPTER FOUR

Toxicity, target animal safety and health of female channel catfish injected with luteinizing hormone releasing hormone analog des-gly^{10}, [d-ala^{6}] LH-RH ethylamide (LHRHa)

Abstract

Double blind studies were conducted to determine the toxicity, target animal safety and health of channel catfish, *Ictalurus punctatus,* females injected with OVA-EASE (85% LH-RH) luteinizing hormone releasing hormone analogue (LHRHa) (OVA-EAZE, Eagle Aquaculture, Auburn, AL). The primary variable evaluated was survival. Secondary variables included mean time to death for fish that died in the study, external health and the histopathology of the spleen, ovary, liver, kidney, gill, muscle, heart, stomach and intestine. Seven-day survival of 0X, 1X, 3X and 5X treatments (0,180,540 and 900 µg/kg female body weight) were 62.5, 87.5, 75.0 and 75.0% respectively and were not significantly different among treatments (P = 0.05). Of those fish that died, the time to death was not different among treatments. 1) Sham injected controls generally had higher observed frequencies of abnormalities when necropsied, however, there were no significant differences among treatments. 2) The mean severity of the mouth, kidney and eye abnormalities was higher (P = 0.05) for the sham-injected controls. The observed liver discoloration tended to increase with dosage, however, there were no significant (P=0.05) differences among treatments.

Histopathology was conducted on the spleen, ovary, liver, kidney, gill, muscle, heart, stomach and intestine. 3) No consistent or significant (P = 0.05) trends in histopathological abnormalities existed among treatments. Observed intestinal inflammation appeared to be more prevalent with increasing dosage. 4) Bacterial and parasitic load also appeared random as revealed by histopathology. Bacteria appeared to be more prevalent in the controls and parasites more prevalent in the fish receiving LHRHa, although there were no significant differences. 5) Females that were given an injection of 20 μg/kg female body weight followed by a resolving dose of 100 μg/kg and that ovulated tended to have better health after 2-3 days than females that did not ovulate or that were sham injected with saline.

Introduction

Growth of the aquaculture industry and the growing concern over bio-ethics

The aquaculture industry has grown rapidly over the past 30 years. This growth has been driven by consumer demand for high quality, low cost protein and the diminishing population stocks of wild fish due to over fishing (Naylor et al. 2000). Currently cultured species of aquatic animals span more than five different phyla (Conte, 2004). The welfare of cultured organisms has become a concern and a priority for some segments of society and bioethics an increasing aspect of animal production (Kaiser, 2005). Animal welfare practices are primarily directed towards vertebrates, finfish, and not emphasized as much for aquatic invertebrates (Conte, 2004).

Fish, in addition to being important as a commercially, cultured species in fisheries and aquaculture is widely used in research. There are four primary stages of concern for animal welfare in aquaculture: breeding, grow-out, capture and handling, and slaughter (Ashley, 2007).

Animal welfare protocols in the aquaculture industry primarily focus on good animal husbandry, water quality, stocking density, feed and nutrition, capture, slaughter, transport, and euthanizing techniques and protocols (Ashley, 2007). The effect of spawning aid treatments on the welfare of the animal is a topic that has not been addressed.

Impact and regulation of LHRHa

LHRHa acts upon the pituitary gland, which releases polypeptide and glycoprotein hormones and does not release androgen hormones (Pickford and Atz 1957). The purpose of LHRHa is to stimulate the release of luteinizing hormone, LH.

Luteinizing hormone is involved in final oocyte maturation, ovulation, and spermiation. Catfish have a single gene for luteinizing hormone and produce a single LH (Liu, Kim, and Karsi 2001). The pars distalis is stimulated to or naturally releases luteinizing hormone. The hormones released from the pituitary are highly susceptible to degradation and are characterized by very short biological ½-lives. Although there is a lack of information regarding the ½-lives of many teleost pituitary hormones, the ½-lives of some avian, amphibian, and mammalian pituitary hormones have been characterized and range from 3 minutes to 12.5 hours (Roos and Jørgensen 1974; Leng and Luckman 1998; Phillips et al. 1998). Peptide hormones spilled into water would have a very short ½-life (seconds to minutes).

LHRHa has been used to stimulate channel catfish females to spawn for the production of channel x blue hybrid fry. Chorulon (human chorionic gonadotropin) (Merck Animal Health) is the only approved spawning aid for finfish. Kristanto (2004) demonstrated that Chorulon does not appear to be effective for producing hybrid catfish and it is not used in the catfish industry. Alternatives to LHRHa include common carp pituitary, catfish pituitary and luteinizing hormone-releasing hormone analog (LHRHa) implants, none of which are approved compounds. Hybrid catfish are produced and legally consumed under an FDA Investigational New Animal Drug (INAD), Eventually, LHRHa or other spawning aids must be approved for use as spawning aids for food fish or the INADs will be withdrawn and no legal means for the artificial fertilization of channel catfish eggs to produce hybrids would be available.

For a drug such as spawning aid to be approved for use by FDA, not only does it need to be demonstrated as effective, it also needs to be evaluated for its potential toxicity and health effects of the target animal (FDA). No information is available on the toxicity or effects of LHRHa on the health of channel catfish. Therefore it is essential that a pivotal study be conducted to determine the toxicity of LHRHa on channel catfish

Degradation of hormones

Studies conducted by Goren et al. (1990) and Zohar et al. (1990) were the first to describe in detail the pathways and kinetics of degradation of native and modified forms of hormones, specifically gonadotropin releasing hormone (=LHRH) to induce ovulation in mature fish (Goren et al. 1990; Zohar et al. 1990). The central (pituitary) and peripheral (kidney and liver) degradation patterns were studied *in vivo*. The half-life of LHRHa was 7.6, 5.0 and 12.9 hours

for in the pituitary, kidney and liver, respectively in gilthead sea bream (Zohar et al. 1990). In general, peptidase activity is much more intense in the pituitaries than kidney and liver of the fish. Their studies proved that peptidases degrade LHRH in all the three sites suggesting that the degrading enzymes were not specific, but were general proteolytic enzymes. Their studies concluded that fractions of the LHRH were rapidly degraded by two endopeptidases (Tyr^5-Gly^6 and Pro^9-$Gly^{10}NH_2$) to minimize their accumulation to reflect low bioactivity in liver and kidney tissues reducing their potential to inactivate the hormonal action. Regardless of the sources of hormones evaluated, 33 to 51% of the peptide is cleaved within the first 45 minutes of incubation and more than 78 to 87% in 180 minutes. The enzymatic degradation of exogenous hormones at both its neurosecretory fibers and its target cells, the gonadotropes, are suggestive of regulatory mechanism in final oocyte maturation and ovulatory activity in fish

The primary objective of this study was to determine the toxicity of des-Gly^{10}, [D-Ala^6] LH-RH Ethylamide (LHRHa) on channel catfish females. Additional objectives were to determine the effects of LHRHa on the health of channel catfish females induced to ovulate with LHRHa.

Materials and Methods

Experimental design

These procedures were conducted at Auburn University, Alabama at the EW Shell Research Center. The experiments were conducted between June 13th and June 23rd 2011. Thirty-two channel catfish (*Ictalurus punctatus)* females were used. The design utilized was 0X, 1X, 3X, and 5X of a recommended high dosage of 180 µg OVA-EASE (85% active ingredient)/kg

female body weight. The four treatments utilized were 0, 180, 540, and 900 µg OVA- EASE/kg of female body weight. The 0 µg/kg control was sham injected with 0.9% physiological saline. Eight replicate females were used per treatment. A completely random design was implemented with all fish represented in a single, communal tank. Females were assigned to treatment groups using a random number generator on Microsoft Excel 2010. Females were individually marked by clipping barbels. Necropsies were conducted if any fish died throughout the course of the experiment. Furthermore, tissue samples from several organs were taken and preserved for further histopathological analysis. At the end of seven days, all fish were sacrificed, necropsies conducted and tissues collected for histopathological analysis.

Studies were conducted to assess the efficacy of LHRHa injections for ovulating channel catfish females in 2011 at two locations. Health observations and survival data were obtained for the fish in this study. The locations were, Auburn University, Alabama (AU) and Baxter Land Company, Arkansas (BLC). The experiments were conducted between May 23rd and June 1st 2011. Fifty females were used at each site in the treatment group, while 24 and 25 individuals were respectively used at AU and BLC as controls. Each female channel catfish was placed in an individual spawning bag, which was placed in a tank until ovulation and tracked individually. At BLC a single large common ovulation tank was used, while at AU several smaller tanks were used with multiple individuals in each.

The treatment group received an LHRHa treatment at 120 µg/kg female body weight (85% active ingredient) in two injections of 20 µg/kg priming dose followed 12 hours later by a resolving dose of 100 µg/kg. LHRHa. Solutions were made at 20 µg/mL, and 100 µg/mL

concentrations and used for the priming and resolving doses respectively. 0.9% saline (Hospira) was used as the solvent. The control group received a sham injection of commercially purchased 0.9% saline (Hospira) injection at 1 ml/kg body weight.

All studies were conducted double-blinded. Only the primary investigator had access to the random method by which fish numbers were designated as treatment or control and which fish numbers ultimately corresponded to which treatments (treatment or control). The primary investigator prepared all injections behind a screen out of view of fish handlers. All injection data was recorded and retained by the primary investigator. All other personnel involved in the study were not informed or aware of the treatments. The primary investigator did not collect survival, necropsy or histopathological data.

Diets

Fish were fed a 36% protein floating catfish feed in accordance with the industry's generally accepted feeding schedule of five times a week from March-October and once a week between November-February. During the eight weeks prior to the study, female channel catfish were prepared with a 36% protein floating brood stock feed containing 6% fish oil, 500 ppm vitamin C, 65 ppm vitamin E, 0.5% docosahexaenoic acid/omega 3(DHA), 0.5% arachidonic acid/omega 6 (ARA) with standard vitamin and mineral packs. Study fish were not fed the day prior to harvest nor during the evaluation period.

Capture

Study fish were harvested on the morning of the day of injection. The fish were captured in a seine where segregation and escape was not possible and the fish were crowded together as a single unit. The fish were then netted in an indiscriminate fashion and evaluated for health and inclusion in the study. The experimental fish were selected in a random fashion by seine crew personnel with multiple years of experience in assessing fish health. Inclusion criteria for selection consisted of no evidence of diseases, lesions or lethargy. Selected fish were weighed and sequentially numbered with a coding system of clipped fins and barbels to identify individuals and placed in the hauling tank for transport to the experimental tank for the toxicity test. For the ovulation studies, fin clipping was done after ovulation or after the final attempt to collect eggs. These fish were observed for six to eight hours prior to being returned to the ponds. These fish were acclimated in the hauling tank for a period of 15-60 minutes before being returned to a pond.

For the toxicity study, upon arrival at the experimental tank, the fish were unloaded individually, their identity code read, and a second evaluation of the health of the fish completed by the primary investigator prior to the administration of the injection. The injected fish were then transferred into an outdoor 14,527 L experimental tank for the toxicity study. Handling of the fish for the ovulation study and spawning details were described by Perera (elsewhere in this thesis, 2012).

Application of Treatments

The treatments were administered as a single, intraperitoneal injection loaded a 5 ml syringe/21.5-gauge needle. The OVA-EASE LHRHa treatments, 0, 180, 540, and 900 µg/kg (0X, 1X, 3X and 5X) treatments were made by dissolving the appropriate amounts of OVA-EASE in 0.9% commercially produced saline. All injections were administered at 1.0 ml of solution per kg female body weight.

For the ovulation studies, the LHRHa treatment consisted of two doses; a priming dose of 20 µg/kg and a resolving dose of 100 µg/kg. The LHRHa treatments were made by dissolving the appropriate amounts of LHRHa (C.S Bio) in 0.9% sodium chloride (Hospira). For the control treatment, a volume of 1 ml/kg of the 0.9% saline was administered twice, to correspond with the priming and resolving doses of the LHRHa.

Environmental conditions

The fish were placed in a single 5.8 m X 2.75 m X 0.91 m flow-through, outdoor experimental tank post injection for the toxicity study. The mean flow rate was 6,960 L/hr (116 L/min X 60 min) resulting in a complete water exchange every 2.09 hours. Oxygen, hardness, pH and flow rate were always maintained in the desired range for the study (Table. 1).

Table 1.Water quality for the toxicity study

Temperature:	24-30C^0
Oxygen:	5-10ppm
Ammonia:	<0.25ppm
Water Exchange/Flow Rate:	6,960L/hr
Hardness:	>30ppm

Health observations

Study fish were observed 3-6 times daily for any changes in health. Daily observations were made regarding normal/regular ventilation rate (gills are pumping at a regular, steady rate), color of the fish (not pale), no development of lesions, lethargy and fish are stable and balanced in the water column. Any adverse reactions, including death, water quality, or other reactions were recorded. The first three days of the study the fish were checked, and observed six times daily (every four hours) as that is when the majority of the lethargy and death occurred, probably because of the stress of harvesting and handling. After that point, lethargic fish were not observed, mortality slowed, and the fish were observed three times daily (every 6-10 hours). The health assessment for the efficacy study in 2011was primarily done visually and the assessment was done purely on the fish's outward appearance. The internal organs were not examined, except any fish that died were necropsied.

Necropsy procedures

Necropsy was performed on all expired fish, and all remaining fish were sacrificed and necropsied at the end of the study. The fish were examined externally for lesions, hemorrhages, edema, cuts, swelling, discoloration and any other abnormality. The injection site was examined for abnormalities. An incision was made from the anus through the pelvic girdle and up to the abdominal musculature just above the heart. Internal organs were examined for lesions, hemorrhages, edema, swelling, discoloration and any other abnormality. Tissue samples from the spleen, ovary, liver, kidney, gill, muscle, heart, stomach and intestine was removed and placed in Bouin's fixative until histopathological studies could be performed.

Histopathology

Tissues were processed for histopathology immediately after tissues/organs were excised with all samples routinely preserved in Bouin's fixative (minimum period of fixation is 24 hours at ambient temperature). For each fish, tissues from the nine organs (spleen, ovary, liver, kidney, gill, muscle, heart, stomach and intestine) were placed in a 200 ml jar containing Bouin's fixative. Prior to histopathology the tissue samples were rinsed and placed in containers with a 50% isopropanol solution (Fisher Scientific). Fixed tissues were processed routinely by using a dehydration gradient with graded isopropyl alcohol, clearing in xylene, and embedding in paraffin blocks using automated processing and embedding equipment (Tissue Tek VIP; Sakura Finetek, Torrance, CA). The gill tissues were decalcified in RDO solution (Apex Inc., Plainfield, IL, USA) for one hour. Sections of each tissue sample were cut and placed in cassettes labeled with the corresponding identification number. Cassettes were placed in a Sakura Tissue-Tek VIP model E150 for tissue processing. When the process was complete, the cassettes were removed, and the tissue blocked in paraffin wax using the Tissue-Tek Tissue Embedding Console System. Using a razor blade, excess paraffin was trimmed from blocks to the make tissue sample fit easily on a slide. The tissue blocks were then cut into 6 μm thick ribbons with an American Optical "820" Spencer Microtome. The ribbons were transferred to an adjacent Fisher Tissue Prep Flotation Bath model 135 set at 45˚C. Selected tissue sections were then cut using a metal wire coated with histosol and placed on silane coated slides. The slides were labeled with corresponding identification numbers using a solvent resistant marker. Slides were set vertical to air dry before being placed on a Fisher Slide Warmer set at 45˚C. Before staining, slides were heated in a 600^{o} C oven for 15 to 30 minutes. The slides were then stained using Hematoxylin

and Eosin stain after Shehand and Hrapchak (1980) prior to microscopic examination. Stained slides were mounted with a Surgipath Micromount and a cover glass applied.

The histologic sections of both treatment and control groups were examined and scored for cellular hypertrophy, hyperplasia, necrosis, neoplasia and inflammation (as evident from infiltration of macrophages and other leukocytes). Scoring was carried out by board certified anatomic pathologist (Leah Kuhnt, DVM, MS, ACVP, Alabama Department of Agriculture and Industries), who was unaware of the treatment applied. Other lesions or variations from normal, including the presence of bacteria and parasites, were noted. Severity of changes were scored as follows: "0" for tissues considered normal, "1" for mild changes, "2" for moderate changes, and "3" for severe changes. Photomicrographs of histopathology sections from the channel catfish females injected with 0, 180, 540 or 900 µl/kg body weight, were taken and recorded.

Primary variables

Fisher's multi-treatment exact test was used to compare survival rate among treatments. The procedures and programs followed are found at udel.edu/~mcdonald/statfishershtml and www.physics.csbsju.edu/cgi-bin/stats/exact. The hypothesis of a difference between survival rate was tested at $\alpha = 0.05$. Additionally, exact 95% confidence intervals were estimated for survival rate for all groups (statpages.org/confint.html). No data were transformed.

Secondary variables

A Fisher's multi-treatment exact test was used to compare the frequency of abnormalities and the frequency of severe abnormalities of all organs and tissues among treatments. Procedures and

programs followed are found at udel.edu/~mcdonald/statfishershtml and www.physics.csbsju.edu/cgi-bin/stats/exact). Additionally, these frequencies were weighted by averaging the severity of the abnormalities and reanalyzed using Fisher's multi-treatment exact test. The hypothesis of a difference among groups was tested at $\alpha = 0.05$. No data were transformed.

Supporting non-pivotal studies

Supporting non-pivotal studies were conducted at Auburn University to determine the possible toxicity of LHRHa. In 2004, a mixture of channel catfish females in excellent spawning condition from three randomly selected strains were given a single injection of LHRHa to determine toxicity. The design utilized five treatments. They were 0, 180, 540, 900 and 1,800 µg LHRHa/kg of female body weight. The 0 µg/kg control was sham injected with physiological saline. A blinded study was conducted, as the observers did not know which treatment was in which tank. Five to six-replicate females were used per treatment. Treated fish were held in 1000 L tanks with flow through reservoir water, and compressed air for 6 days, observed for any abnormal behavior, mortality or lesions and then transferred to a 0.04 ha pond to determine longer term effects on survival. Temperature in the tanks was approximately 28.7^0 C. The fish were held in the pond for 112 days, and then were harvested to determine total mortality.

In 2008, toxicity of LHRHa delivered as an implant was examined at Auburn University. Channel catfish females were administered a single implant of 0, 100, 300 or 500 µg/kg female body weight of LHRHa. Controls were sham implanted. The fish were held in tanks for eight

days. Mortality through out the eight days was recorded and percentage mortality per treatment was determined

Results

Survival, 2011 toxicity study, Auburn University

Seven-day survival of 0X, 1X, 3X and 5X treatments were 62.5, 87.5, 75.0 and 75.0% respectively and were not significantly different among treatments (P = 0.05) (Table 2).

Table 2. Mean, standard deviation (SD) and range of body weight, % survival, 95% confidence interval (CI) for survival and time to death for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n = 8 for each treatment) and examined in 2011 for 7 days at Auburn University, Alabama. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test) for survival.

	Body Weight			
Treatment	Kg	Range	Survival % (95% CI)	Time to Death (d)
0	2.46 ± 0.81	1.39-3.39	62.5 (24.5-91.5)	4.3 ± 0.5
180	2.50 ± 0.86	0.95-3.35	87.5 (47.4-99.7)	2.0
540	2.65 ± 0.81	1.81-3.77	75.0 (34.9-96.8)	2.5 ± 0.5
900	2.40 ± 0.96	1.37-3.84	75.0 (34.9-96.8)	3.0 ± 2.0

Of the fish that died, the time to death was not significantly different among treatments. The first three days of the study the fish were checked, and observed six times daily (every 4 hours). After that point, lethargic fish were not observed, mortality slowed, and the fish were observed three times daily (every 6-10 hours).

Survival, 2004 toxicity study, Auburn University

In the study done in 2004, during the first day, all fish appeared healthy, except one fish in the 540 µg treatment group lost equilibrium and some fish in the 1800 µg treatment group appeared lethargic during part of the day. The sham injected control had 100% survival and exhibited no unusual behavior during the entire 6 days in tanks. The control did not deposit any eggs. For the 180 µg treatment, one fish was discolored by day three. This fish was sick on day four and eggs were observed in the tank on day four. The same fish was found dead on day five. It had been attacked and chewed by the other fish probably because of its weakened state. On day six, one fish had a mildly chewed tail and, upon moving the fish to ponds, one fish appeared completely spawned out. In the treatment 540 µg group, one fish had developed a patch of fungus on its tail by day five. Egg deposition was also observed in this treatment. On day six, one fish had a chewed tail, one had a bad eye and three fish were dropping eggs. No mortality or unusual behavior was observed for the 900 µg treatment and eggs were also observed for these fish during the first five days. On day six, one fish had hemorrhaged fins and one was dropping eggs. During the second day a sick fish and a lethargic fish were observed for the 1800 µg treatment. On day three, one fish was dead and on day four, eggs were observed, as was the case for all treatments except the control. On day five, the fish in this treatment group appeared somewhat lethargic. On day six, one fish had abrasions on the lip, one had lesions on the head and protruding genitals and a third fish was dropping eggs.

Sham injected controls had 100% survival during the entire study period (Table 3). Seventeen and 20 percent of the 1X and 10X fish died during the first seven days in tanks, respectively, but none of the 3X and 5X fish died during this period. No dosage effect was evident. Fifty to 80

percent of LHRHa injected fish had died by the end of the 112-day holding period in the ponds. Again, there was no dosage effect during this phase. There may have been genetic effects on toxicity. Excluding controls, three lines randomly utilized in this experiment had 55, 100 and 0 % survival.

Table 3. Cumulative mortality of channel catfish females administered a single injection of 0, 180, 540, 900 or 1,800 µg LHRHa/kg of female body weight in 2004 at Auburn University. Controls were injected with physiological saline.

		Cumulative Mortality (%)	
Dose	N	Tanks (6 days)	Pond (112 days)
0	5	0	0
180	6	17	50
540	5	0	80
900	5	0	80
1800	5	20	60

Survival, 2008 toxicity study, Auburn University

In the study done in 2008 using LHRHa implants, mortality was 25.0, 25.0, 25.0 and 12.5% for 0, 100, 300 or 500 µg LHRHa/kg of female body weight, respectively (Table 4). There was no relationship between dosage rate and mortality.

Table 4. Cumulative mortality of channel catfish females administered a single implant of 0, 100, 300 or 500 µg/kg of female body weight of LHRHa in 2008 at Auburn University. Controls were sham implanted.

		Mortality (%)
Dose	N	Tanks (8 days)
0	8	25.0[1]
100	8	25.0
300	8	25.0[2]
500	8	12.5[2]

[1] One fish died due to aggression by the other females. Deleting this fish gives a mortality of 12.5%. All other mortalities exhibited no signs of aggression or infection.
[2] Two days after implantation eggs were observed in the tank from 1 or more females.

Histopathology and health assessment

Sham injected controls in the toxicity study generally had higher observed frequencies of abnormalities when necropsied, however, there were no significant differences among treatments. The mean severity of the kidney and eye abnormalities was higher (P=0.05) for the sham-injected controls. The observed liver discoloration tended to increase with dosage, however, there were no significant (P=0.05) differences among treatments. Not surprisingly, the fish that died had the most severe observed abnormalities as revealed by necropsy (Table 5).

Table 5. Mean % of fish demonstrating abnormalities, the severity of the abnormality and % of fish demonstrating severe abnormalities for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline and examined in 2011 for 7 days at Auburn University, Alabama.

Treatment	Frequency of Fish Demonstrating Abnormality										
	Inject Site	Body	Head	Eye	Mouth	Ovary	Intestine	Kidney	Heart	Liver	Gill
0	0.0	0.4	0.3	0.3	0.1	0.1	0.0	0.3	0.0	0.5	0.3
180	0.0	0.3	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.6	0.0
540	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0
900	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.9	0.1

Table 5 (continued)

Treatment	Mean Severity of Abnormality[1]										
	Inject Site	Body	Head	Eye	Mouth	Ovary	Intestine	Kidney	Heart	Liver	Gill
0	0.0	1.1	0.3	0.5[2]	0.1	0.3	0.0	0.8[2]	0.0	0.6	0.5
180	0.0	0.4	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.8	0.0
540	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0
900	0.0	0.3	0.0	0.0	0.0	0.1	0.0	0.0	0.0	1.0	0.3

[1] Scored on a value scale of 0-3: 0 = Normal, 1 = Mild, 2 = Moderate, 3 = Severe

[2] Significant differences were found among treatments at P=0.05 (Fisher's Multi-treatment Exact Test.)

Table 5 (continued)

Treatment	Frequency of Fish Demonstrating Severe Abnormality[3]										
	Inject Site	Body	Head	Eye	Mouth	Ovary	Intestine	Kidney	Heart	Liver	Gill
0	0.0	0.4	0.0	0.3	0.0	0.1	0.0	0.3	0.0	0.1	0.1
180	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0
540	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
900	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1

[3]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

Health observations from the efficacy study

In the 2011 study that was done on the efficacy of LHRHa as a spawning aid, the health assessment done post spawning showed no adverse reactions attributed to the administrati the LHRHa. No experimental fish at Auburn University and four experimental fish (treate sham-0%) at Baxter Land Company died during the experiment. The mortality of the inje fish at Baxter Land Company was not significantly different (P=0.05) from that of sham i females (Table 6).

At Baxter Land Company, the appearance and health of the injected (treated) fish was slig worse than the sham injected fish; however, this was due to the four injected fish that died stripping of the eggs and only the incidence of body lesions of injected fish was more freq < 0.0) than that of sham injected controls. Appearance and health of the remaining 92% of injected (treated) fish was identical to that of sham injected controls. No abnormality was at the site of injection for injected or sham injected females.

At Auburn University, the fish that were treated and ovulated appeared to have better heal physical appearance than fish that did not ovulate. Sham injected controls had the worst appearance and health. Abrasions and assorted adverse health observations were much mo common in sham injected controls. The incidence of mouth abrasions and mouth discolora was more frequent ($P < 0.05$) in sham-injected females than LHRHa injected females. The incidence of mouth, eye and tail abrasions was more frequent ($P < 0.05$) in non-ovulating ovulating LHRHa injected females. No abnormality was found at the site of injection for i or sham injected females.

Table 6 Mean health observations for female channel catfish, *Ictalurus punctatus*, given a priming injection of 20µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, followed by 100µg//kg 12 hours later or given a sham injection of 0.9% saline at Baxter Land Company, Arkansas and Auburn University, Alabama.

Location/ Treatment	Mortality** (%)	Mean health observation score[1]								
		I* site	Body Lesion	Discoloration Body	Discoloration M*	Discoloration Gill	Head	O* Rup*	Int* Hem*	Kid*
Baxter (AR)										
Injection	8.0	0.00	0.14[a]	0.06	0.08	0.04	0.00	0.00	0.04	0.00
Sham Injection	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Auburn Uni.(AL)										
Injection	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ovulated	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Non-ovulated	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sham Injection	0.0	0.00	0.00	0.00	0.32[a]	0.00	0.00	0.00	0.00	0.00

(Cont'd)

Location/ Treatment	Mean health observation score[1]									
	Stomach				Abrasion			Eye Edema	Ovary Prot*	Gen* Tear
	Ht*	Liver	Ed*	Prot*	Mouth	Eye	Tail			
Baxter (AR)										
Injection	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sham Injection	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Auburn Uni. (AL)										
Injection	0.00	0.00	0.00	0.00	0.34	0.12	0.16	0.02	0.04	0.02
Ovulated	0.00	0.00	0.00	0.00	0.12	0.07	0.00	0.02	0.05	0.02
Non-ovulated	0.00	0.00	0.00	0.00	1.50[b]	0.38[b]	1.00[b]	0.00	0.00	0.00
Sham Injection	0.00	0.00	0.00	0.00	1.38[a]	0.20	0.16	0.00	0.00	0.00

[1] 0 = normal, 1 = slight, 2 = moderate, 3 = severe
* I = Injection, M = mouth, O = Ovary, Rup = rupture, Int = Intestine, Hem = hemorrhage, Kid = kidney, Ht = heart, Ed = edema, Prot = protrusion (stomach in throat, ovary in genital opening), Gen = genital opening
** During recovery
[a] Means between LHRHa and sham injected females are different ($P < 0.05$, Fisher's Exact Test)
[b] Means between LHRHa injected ovulating and non-ovulating females are different ($P < 0.05$, Fisher's Exact Test)

At Auburn University in 2004 and 2008 with LHRHa injection and LHRHa. In the early experiments, implanting or injecting channel catfish females with LHRHa followed by ha stripping of the eggs resulted in high mortality rates long-term (112 days). However, there no trends of dosage with survival rate. Mortality was 25.0, 25.0, 25.0 and 12.5% for 0, 10 or 500 µg LHRHa/kg of female body weight, respectively. There was no relationship betw dosage rate and mortality.

Histopathology (Toxicity study)

Histopathology was conducted on the spleen, ovary, liver, kidney, gill, muscle, heart, stomach and intestine. No consistent or significant (P= 0.05) trends in histopathological abnormalities existed among treatments (Tables 7-15). Observed intestinal inflammation appeared to be more prevalent with increasing dosage (Table 15). Inflammation of the spl was only detected in the 5X treatment. The 5X treatment was the only treatment for which inflammation of the ovary was not detected. Hypertrophy, necrosis and inflammation were detected in the liver for the control, necrosis in the 5X treatment and inflammation of the liver in all treatments. All treatments had some individuals with hyperplasia of the kidney, but necrosis was only detected in the control and 1X. All treatments had some individuals exhibiting hyperplasia and inflammation of the gills. Only the control and the 5X treatmen had individuals exhibiting inflammation in the muscle. Only the control and the 1X treatm had individuals with inflammation of some heart cells (Tables 7-15).

Table 7. Histopathology of the spleen for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n=8 per treatment) at Auburn University, Alabama. Frequency, mean and frequency of severe cellular hypertrophy, hyperplasia; necrosis, neoplasia and inflammation were determined on a scale of 0-3: 0 = normal; 1= mild; 2 = moderate; 3 = severe. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test).

	Frequency of Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.0
180	0.0	0.0	0.0	0.0	0.0
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.1

Table 7 (continued)

	Mean Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.0
180	0.0	0.0	0.0	0.0	0.0
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.3

Table 7 (continued)

	Frequency of Severe Cellular[1]				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.0
180	0.0	0.0	0.0	0.0	0.0
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.1

[1]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

Table 8. Histopathology of the ovary for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n=8 per treatment) at Auburn University, Alabama. Frequency, mean and frequency of severe cellular hypertrophy, hyperplasia; necrosis, neoplasia and inflammation were determined on a scale of 0-3: 0 = normal; 1= mild; 2 = moderate; 3 = severe. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test).

	Frequency of Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.1
180	0.0	0.0	0.0	0.0	0.3
540	0.0	0.0	0.0	0.0	0.1
900	0.0	0.0	0.0	0.0	0.0

Table 8 (continued)

	Mean Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.1
180	0.0	0.0	0.0	0.0	0.4
540	0.0	0.0	0.0	0.0	0.1
900	0.0	0.0	0.0	0.0	0.0

Table 8 (continued)

	Frequency of Severe Cellular[1]				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.0
180	0.0	0.0	0.0	0.0	0.1
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.1

[1]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

Table 9. Histopathology of the liver for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n=8 per treatment) at Auburn University, Alabama. Frequency, mean and frequency of severe cellular hypertrophy, hyperplasia; necrosis, neoplasia and inflammation were determined on a scale of 0-3: 0 = normal; 1= mild; 2 = moderate; 3 = severe. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test).

	Frequency of Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.3
180	0.0	0.0	0.3	0.0	0.4
540	0.0	0.0	0.0	0.0	0.1
900	0.0	0.0	0.1	0.0	0.3

Table 9 (continued)

	Mean Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.3
180	0.0	0.0	0.3	0.0	0.5
540	0.0	0.0	0.0	0.0	0.1
900	0.0	0.0	0.1	0.0	0.3

Table 9 (continued)

	Frequency of Severe Cellular[1]				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.0
180	0.0	0.0	0.0	0.0	0.1
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.0

[1]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

Table 10. Histopathology of the kidney for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n=8 per treatment) at Auburn University, Alabama. Frequency, mean and frequency of severe cellular hypertrophy, hyperplasia; necrosis, neoplasia and inflammation were determined on a scale of 0-3: 0 = normal; 1= mild; 2 = moderate; 3 = severe. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test).

	Frequency of Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.3	0.0	0.0	0.0
180	0.0	0.5	0.1	0.0	0.0
540	0.0	0.3	0.0	0.0	0.0
900	0.0	0.3	0.0	0.0	0.0

Table 10 (continued)

	Mean Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.3	0.0	0.0	0.0
180	0.0	0.6	0.1	0.0	0.0
540	0.0	0.4	0.0	0.0	0.0
900	0.0	0.3	0.0	0.0	0.0

Table 10 (continued)

	Frequency of Severe Cellular[1]				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.0
180	0.0	0.1	0.0	0.0	0.0
540	0.0	0.1	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.0

[1]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

Table 11. Histopathology of the gill for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n=8 per treatment) at Auburn University, Alabama. Frequency and mean cellular hypertrophy, hyperplasia; necrosis, neoplasia and inflammation were determined on a scale of 0-3: 0 = normal; 1= mild; 2 = moderate; 3 = severe. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test). No moderate or severe abnormalities were observed.

	Frequency of Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.1	0.0	0.0	0.1
180	0.0	0.3	0.0	0.0	0.5
540	0.0	0.1	0.0	0.0	0.1
900	0.0	0.3	0.0	0.0	0.3

Table 11 (continued)

	Mean Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.1	0.0	0.0	0.1
180	0.0	0.3	0.0	0.0	0.5
540	0.0	0.1	0.0	0.0	0.1
900	0.0	0.2	0.0	0.0	0.3

Table 11 (continued)

	Frequency of Severe Cellular[1]				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.0
180	0.0	0.0	0.0	0.0	0.0
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.0

[1]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

Table 12. Histopathology of the muscle for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n=8 per treatment) at Auburn University, Alabama. Frequency and mean cellular hypertrophy, hyperplasia; necrosis, neoplasia and inflammation were determined on a scale of 0-3: 0 = normal; 1= mild; 2 = moderate; 3 = severe. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test). No moderate or severe abnormalities were observed.

	Frequency of Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.1
180	0.0	0.0	0.0	0.0	0.0
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.1

Table 12 (continued)

	Mean Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.1
180	0.0	0.0	0.0	0.0	0.0
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.1

Table 12 (continued)

	Frequency of Severe Cellular[1]				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.0
180	0.0	0.0	0.0	0.0	0.0
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.0

[1]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

Table 13. Histopathology of the heart for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n=8 per treatment) at Auburn University, Alabama. Frequency, mean and frequency of severe cellular hypertrophy, hyperplasia; necrosis, neoplasia and inflammation were determined on a scale of 0-3: 0 = normal; 1= mild; 2 = moderate; 3 = severe. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test).

	Frequency of Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.1
180	0.0	0.0	0.0	0.0	0.1
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.0

Table 13 (continued)

	Mean Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.3
180	0.0	0.0	0.0	0.0	0.1
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.0

Table 13 (continued)

	Frequency of Severe Cellular[1]				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.1
180	0.0	0.0	0.0	0.0	0.0
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.0

[1]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

Table 14. Histopathology of the stomach for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n=8 per treatment) at Auburn University, Alabama. Frequency, mean cellular and frequency of severe hypertrophy, hyperplasia; necrosis, neoplasia and inflammation were determined on a scale of 0-3: 0 = normal; 1= mild; 2 = moderate; 3 = severe. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test).

	Frequency of Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.3
180	0.0	0.0	0.0	0.0	0.4
540	0.0	0.0	0.0	0.0	0.1
900	0.0	0.0	0.0	0.0	0.3

Table 14 (continued)

	Mean Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.3
180	0.0	0.0	0.0	0.0	0.5
540	0.0	0.0	0.0	0.0	0.1
900	0.0	0.0	0.0	0.0	0.3

Table 14 (continued)

	Frequency of Severe Cellular[1]				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.0
180	0.0	0.0	0.0	0.0	0.1
540	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.1

[1]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

Table 15. Histopathology of the intestine for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n=8 per treatment) at Auburn University, Alabama. Frequency, mean and frequency of severe cellular hypertrophy, hyperplasia; necrosis, neoplasia and inflammation were determined on a scale of 0-3: 0 = normal; 1= mild; 2 = moderate; 3 = severe. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test).

	Frequency of Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.4
180	0.0	0.0	0.0	0.0	0.4
540	0.0	0.0	0.0	0.0	0.5
900	0.0	0.0	0.0	0.0	0.5

Table 15 (continued)

	Mean Cellular				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.4
180	0.0	0.0	0.0	0.0	0.8
540	0.0	0.0	0.0	0.0	0.6
900	0.0	0.0	0.0	0.0	0.6

Table 15 (continued)

	Frequency of Severe Cellular[1]				
Treatment	Hypertrophy	Hyperplasia	Necrosis	Neoplasia	Inflammation
0	0.0	0.0	0.0	0.0	0.0
180	0.0	0.0	0.0	0.0	0.3
540	0.0	0.0	0.0	0.0	0.1
900	0.0	0.0	0.0	0.0	0.1

[1]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

Bacterial and parasitic load also appeared random as revealed by histopathology. Bacteria appeared to be more prevalent in the controls and parasites more prevalent in the fish receiving LHRHa, although there were no significant differences (Table 16). All treatments exhibited inflammation of some stomach and intestinal cells (Table14-15). Stomach and intestinal bacteria were only detected in a single fish (Table 17).

Table 16. Bacterial and parasitic frequency, mean severity and frequency of severe infection found during histopathological examination of the spleen, ovary, liver, kidney and gill for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n=8 per treatment) at Auburn University, Alabama. Bacterial and parasitic load were subjectively estimated on a scale of 0-3: 0 = normal; 1= mild; 2 = moderate; 3 = severe. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test).

	Frequency of Bacteria (B) and Parasites (P)									
	Spleen		Ovary		Liver		Kidney		Gill	
Treatment	B	P	B	P	B	P	B	P	B	P
0	0.3	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.3
180	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.6
540	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.6
900	0.2	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.5

Table 16 (continued)

	Mean Bacteria (B) and Parasites (P)									
	Spleen		Ovary		Liver		Kidney		Gill	
Treatment	B	P	B	P	B	P	B	P	B	P
0	0.5	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.3
180	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.9
540	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.9
900	0.2	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.9

Table 16 (continued)

Frequency of Severe[1] Numbers of Bacteria (B) and Parasites (P)

	Spleen		Ovary		Liver		Kidney		Gill	
Treatment	B	P	B	P	B	P	B	P	B	P
0	0.3	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
180	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3
540	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
900	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4

[1]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

Table 17. Bacterial and parasitic frequency, mean severity and frequency of severe infection found during histopathological examination of the muscle, heart, stomach and intestine for channel catfish, *Ictalurus punctatus,* females given an injection of 180, 540 or 900 µg/kg female body weight of luteinizing hormone releasing hormone analog, LHRHa, or a sham injection of 0.9% saline (n=8 per treatment) at Auburn University, Alabama. Bacterial and parasitic load were subjectively estimated on a scale of 0-3: 0 = normal; 1= mild; 2 = moderate; 3 = severe. No significant differences were found at P= 0.05 (Fisher's Multi-treatment Exact Test).

	Frequency of Bacteria (B) and Parasites (P)							
	Muscle		Heart		Stomach		Intestine	
Treatment	B	P	B	P	B	P	B	P
0	0.0	0.0	0.1	0.0	0.1	0.0	0.3	0.0
180	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.4
540	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1

Table 17 (continued)

	Mean Bacteria (B) and Parasites (P)							
	Muscle		Heart		Stomach		Intestine	
Treatment	B	P	B	P	B	P	B	P
0	0.0	0.0	0.1	0.0	0.3	0.0	0.3	0.0
180	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.4
540	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1

Table 17 (continued)

	Frequency of Severe[1] Numbers of Bacteria (B) and Parasites (P)							
	Muscle		Heart		Stomach		Intestine	
Treatment	B	P	B	P	B	P	B	P
0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
180	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
540	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
900	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[1]Severe; for this analysis 0 and 1 are not considered severe and scores of 2 and 3 are considered severe.

All treatments had some fish with gill flukes with the observed higher prevalence in the LHRHa treated fish. The 1X and 5X treatments had some individuals that had parasites in the stomach and intestine.

Discussion

Survival rates

Survival was 62.5, 87.5, 75.0 and 75.0% survival for 0, 180, 540, and 900 µg/kg treatments respectively. These means were not statistically significant, and do not appear to be dose related, but to be random and were likely due to handling stress rather than the effects of LHRHa dosages. The survival data from the two non-pivotal studies support this conclusion as the mean survival is very consistent among treatments 81.3, 85.3, 87.5, 87.5 and 80.0% for 0X, 1X, 3X, 5X and 10X, respectively. Many of the problems and much of the mortality in the tank phase of the experiment appeared to be a result of fighting. Furthermore, the adverse effects of being stimulated to ovulate without removal of the eggs could have caused mortality.

Data from field observations also support the hypothesis that injections of LHRHa are safe, and do not have a large impact on survival of female brood stock. Typical long-term (one-year) survival in industry and at research institutions is approximately 75% (Renee Beam, personal communication; Nagaraj Chatakondi, personal communication) after injection, ovulation and hand stripping. One-year survival of channel catfish females implanted with LHRHa accompanied with hand stripping of eggs was approximately 78, 91, 95 and 64% for an average of 82% from 4 ponds at Eagle Aquaculture, Pike Road, Alabama. This matches up well with the

84.3% average survival that we observed when all the treatments and studies were combined even though half the treatments are overdoses. The stress of anesthesia, hand stripping, handling and water quality in recovery tanks are the variables most likely responsible for mortality. Some level of mortality is also expected from normal aging and attrition.

Sources of stress

It is a well-known fact that induced suppression or stimulation of the reproductive function in fish can lead to heightened stress. This is further magnified when spawning is done outside the normal seasons or natural environment (Barton and Iwama 1991). The elevation of plasma corticosteroids, mainly cortisol, in teleost fish in response to various types of stressful stimuli has been well documented (Patiño, Redding, and Schreck 1987). As a response to elevated cortisol levels stress responses can vary among species, strains and between wild and hatchery reared fish (Barton and Iwama 1991). Examples of these variations are changes in plasma corticosteroids in fish after handling or confinement or resistance to disease (Barton and Iwama 1991). Such differences appear, at least in part, to have a genetic basis (Williamson and Carmichael 1986).

Similarly, the magnitude of stress responses may be influenced by the fish's developmental or maturational state (Barton et al. 1985). Quantitative differences in responses to stress show the importance of being familiar with the genetic background and life history when carrying out stress investigations with fish and interpreting the findings. Future studies regarding the survival of LHRHa injected channel catfish after hand stripping should address variables such as strain, age, and maturational state,

LHRHa-injected individuals at BLC showed a higher incidence of body lesions relative to its controls, while at Auburn University all the fish (treated and untreated) showed lesser health abnormalities. The lesions could possibly have been caused due to handling of the fish at BLC during strip spawning. This could have also caused the four mortalities at BLC. The necropsies conducted on the dead fish did not show any significant changes to the internal anatomy.

At Auburn University, abrasions to the eyes, mouth and tail were observed in all the treatments. This can be attributed to the fish rubbing up against the mesh bag. The abrasions to the eye, mouth and tail was significantly higher in the LHRHa non-ovulated females relative to the LHRHa injected ovulated females. The non-ovulated females remained in the mesh bags till the end of the study, while the ovulated females were taken out of the bags when they started to drop eggs, and post spawning were placed in a common tank. Therefore this result is expected as the non-ovulating females spent more time in the mesh bags (till the end of the study), while the ovulation females were in the bags for a relatively shorter time. This would also explain as to why the sham injected females had a significantly higher incidence of mouth abrasions relative to the LHRHa injected females. This was not observed at BLC, so this reaction could be a result of water quality and pathogen load differences. An alternative explanation could be that the ovulating females experience physiological changes that help them tolerate the stress of the hatchery environment and post-stripping environment. When channel catfish females naturally spawn, they experience rough courtship with males, and appear extremely emaciated after ovulating all of their eggs, which can result in 25% or more weight loss. These females appear to be extra slimy after spawning (R. Dunham, personal communication). Females in a ovulation ready state that ovulate eggs may go through some physiological and immune responses to

increase their chances of survival, and this could be another future area of research.

The results of the health evaluation indicated that the sham injected controls had higher frequencies of abnormalities relative to the LHRHa treatments. When the severity of the abnormalities was evaluated there was a significantly higher degree of severity of the abnormality in the eye and kidney of the control group. Survival was relatively lower in the control group. Currently there are no studies that clearly indicate therapeutic effects of LHRH on fish in reducing stress. However this is a possibility if ovulating females go through physiological changes to assist them in surviving reproductive stress, these controls would not have the same benefit.

In the case of the control, although the fish that died had the most severe observed abnormalities as revealed by necropsy, they had no tissue irregularities as indicated by histopathological examination. However, two of three of these controls had large numbers of bacteria in the spleen, and all three to some extent had bacteria in one or more of the following tissues, heart, stomach and intestine, which could be a cause of death. Alternatively, this could be the onset of decomposition. Only a single fish died in treatment 1X. This fish had normal tissue characteristics, but a low number of kidney bacteria, and was the only fish in this treatment with detectable bacteria. The same result was observed for a fish that died in treatment 3X. Again in treatment 5X, the tissue irregularities of the two fish that died and the six fish that lived were observed to be similar, but one of two fish that died had low numbers of bacteria in the spleen and liver. Across all treatments, bacteria were only detected in fish that died. Parasitic infestation was low. All but one dead fish across

treatments had no gill parasites, and gill parasites were observed to be much more prevalent in live fish.

The lack of parasites on the deceased fish is an expected result as parasites start vacating the fish in less than an hour after death. The greater prevalence of bacteria in the dead fish has two explanations. At the temperatures observed in this study, bacteria can start populating in the body in one hour or less after death. Alternatively, these fish may have died because they had low, undetectable levels of infection at the beginning of the study (although externally they appeared completely healthy), and the stress of harvest and handling allowed bacteria to populate in the tissues of these fish compared to the fish that survived. The tissue distribution of the bacteria (primarily internal organs) is suggestive, but not conclusive evidence of the former explanation (initial decomposition), although stomach and intestinal bacteria were only detected in a single fish.

In this study the primary focus was channel catfish females. In future studies, health evaluation of LHRHa injected blue catfish males should be conducted as LHRHa shows promise to increase reproductive efficiency of males (Kristanto 2004; Hutson 2006).

Conclusion

Survival data from the pivotal study, non-pivotal studies and large scale industry observations; indicates that when LHRHa is administered to channel catfish females that 75-80% of the fish should survive, and that the mortality is no different from unperturbed fish. In fact the data make it clear that most of the mortality is related to stresses other than the LHRHa itself. No consistent significant differences in health were observed among 0X and the 1X, 3X and 5X treatments in this study. The random nature among treatments of the adverse health observations ranging from survival, and necropsies of injected fish along with the histopathology of tissues from injected fish suggests that these problems are related to handling stress rather than the effects of LHRHa dosages. The data from this study as well as data previously published from our lab indicate that LHRHa is safe for use as a spawning aid for channel catfish females.

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CHAPTER FIVE

Production of xenogenic catfish by delivery of putative blue catfish stem cells into the gonads of triploid channel catfish

Abstract

Putative spermatogonia A, and primordial germ cells from a fresh cell isolate or a density gradient-centrifuged isolate from blue catfish, *Ictalurus furcatus,* testes were inserted into the, gonads of confirmed triploid channel catfish, *I. punctatus*. The live cells were introduced to the gonads of the host via catheterization or by surgically accessing the gonads and inserting the cells directly into the ovaries or testes with an injection of $2x10^4 – 1.43x10^6$ (mean: $5.23x10^5$) cells. Sixty days post introduction of the cells into the host, DNA was analyzed from biopsies of the gonads. Both triploid channel catfish, one of each sex, that were inoculated with density gradient sorted blue catfish stem cells introduced by injection contained viable blue catfish cells in their ovaries and testes 60 days later. Two female triploid channel catfish and two male channel catfish were inoculated with testicular cells from blue catfish introduced by catheterization via the genital and urogenital opening, respectively. After 60 days, blue catfish cells were undetectable in one female, but were found in both males and the second female. This is the first report of successful production of xenogenic catfish. If the introduced blue catfish cells proliferate and mature into sperm and ova, an alternative technology will be available for

the production of channel female X blue male hybrid catfish or for propagating difficult to spawn blue catfish.

Introduction

Forty percent of all aquaculture production is lost to disease. Thus, disease constitutes the greatest production problem in catfish farming and all of global aquaculture (Owens, 2012). Bacterial diseases cause more economic damage within the catfish industry than eukaryotic parasites and viruses (Tucker, 2012). The bacterial diseases, columnaris and enteric septicemia, cause 80% of the losses from infectious diseases. With the new rise of epidemics from virulent atypical *Aeromonas hydrophila* and *Edwardsiella tarda* the impact of bacterial disease continues to grow disproportionately when compared with all other disease.

Genetic enhancement of catfish is a key component to combat disease and for improving the sustainability, profitability and competitiveness of the catfish industry. Using multiple breeding programs will make maximum genetic improvement of catfish and fish in the future. This is indicated by positive results from initial research examining simultaneous application of multiple genetic enhancement programs (Dunham, 2011). Interspecific hybridization of catfish is one genetic strategy to increase disease resistance.

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. 2005ab; Dunham et al. 2008). This fish is improved (15-20%) for feed conversion (Yant et al. 1975), growth (especially at high densities, 20- 100%, Dunham et al.

1987, 1990; Jeppsen 1995; Dunham and Brummett 1999), disease resistance and survival (10-100%) (Ella 1984; Dunham et al. 1990; Jeppsen 1995; Wolters et al. 1996, Dunham and Brummett 1999), tolerance to low dissolved oxygen (Dunham et al. 1983b), harvest by seining (50-100%) (Yant et al.1975; Chappell 1979; Dunham and Argue 1998), hook-and line vulnerability (Tave et al. 1981), dress out percentage and fillet percentage (5-10%) (Yant 1975, Chatakondi et al. 2000) and has more uniform growth and body shape (Yant et al. 1975, Brooks et al. 1982a,b, Dunham et al. 1982) compared to the commonly grown channel catfish. Body composition and flavor of the hybrid and the channel catfish are similar (Huang et al. 1994). This is the most powerful example of genetic improvement in farmed fish in the world in regards to number of traits improved simultaneously and potential economic impact.

Large-scale field trials have confirmed the outstanding performance of the hybrid in commercial settings and in processing plants, resulting in high demand and premium prices for fingerling hybrids. The typical commercial production rates of 15,000 kg/ha of hybrid catfish are, almost double that of channel catfish (Chatakondi et al. 2005ab; Whitis unpublished), and well over 100 farmers are using hybrid catfish in commercial farms in the United States. Hybrid embryo production has steadily improved from 1-2 million in 2004 to 65 million in 2009, and in 2012 is approximately 150 million (B. Bosworth, USDA-ARS, MS, personal communication).

The primary hurdle in hybrid catfish production is the adequate production of fry. Hormone induced ovulation and hand stripping of channel catfish eggs followed by in-vitro artificial fertilization with blue catfish sperm is considered the most productive and consistent technique for making C×B hybrid catfish (Dunham and Masser, 2012), but it is labor intensive. Reproductive technology needs to be improved to make hybrid embryo production

In the past decade, advancements in biotechnology have allowed several labs around the world to take a unique approach towards manipulating teleost fish reproduction. This unique approach involves germ cell transfer (Yoshizaki et al. 2002; Yoshizaki et al. 2003). Although the use of germ cell transplantation has been relatively well established in mammals (Lacerda et al. 2012), the technique has only been adapted for use in fish after 2000 (Lacerda et al. 2012).

During the last decade, several different approaches have been developed for germ cell transplantation in fish using recipients of various ages and life stages, such as blastula-stage embryos, newly hatched larvae and sexually mature specimens (Lacerda et al. 2012). Because germ cells can develop into live organisms through maturation and fertilization processes, germ cell transplantation in fish has opened up new avenues of research in reproductive biotechnology and aquaculture. For instance, the use of xenotransplantation (the transplantation of living cells, tissues or organs from one species to another) in fish has lead to advances in the conservation of endangered species and the production of commercially valuable fish using surrogate recipients (Okutsu et al. 2006; Lacerda et al. 2012). Further, xenogenesis could also facilitate the engineering of transgenic fish. Furthering our understanding of germ-line stem cells would contribute significantly to advances regarding germ cell transplantation in fish (Lacerda, Costa, et al. 2012).

There are three primary approaches that have been explored for germ-cell transplantation in teleost to produce xenogens. Intraspecific or interspecific transplantation of primordial germ cells (PGCs) into blastula-stage fish embryos through micromanipulation can produce xenogens. The PGCs are harvested from donor embryos and transplanted into blastula stage recipients that

have had their endogenous PGC development blocked by the injection of a dead end antisense morpholino oligonucleotide (MO). This technique, which resulted in the complete replacement of the recipient germline with donor PGCs, has been successfully demonstrated in zebrafish (*Danio rerio*) (Ciruna et al. 2002; Giraldez 2005) and was validated by the observation of F1 offspring generation showing donor-derived mutant phenotypes. Saito (2007) demonstrated that a single PGC from a pearl danio, loach or goldfish could be microinjected onto the blastodisc of a germ cell deficient zebrafish blastula producing xenogenic males and females the produced donor gametes 100% of the time (Giraldez 2005; Saito et al. 2007). In each instance the host produced sperm from the respective donor showing that the technique is viable even when using physiologically distant host and donor species (Saito et al. 2007). Only males were produced as knockdown of dead end results in all male progeny.

The second approach has been the transplantation of PGCs into newly hatched fish embryos. Isolated donor germ cells were transplanted into the peritoneal cavities of newly hatched larvae using a microinjector (Yoshizaki et al. 2011). Newly hatched larvae were chosen to be used as recipients as they did not possess a functional immune system, as indicated by the lack of differentiation in both their thymus and their T cells. Lack of a functional immune system allowed the avoidance of 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). PGCs were able to migrate to the gonadal ridge of the host and colonize. 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, 2003). Okutsu (2006) investigated the use of spermatogonial stem cells (SSC) in place of PGCs. SSCs isolated from

adult male vasa - GFP rainbow trout were injected into the peritoneal cavities of male and female newly hatched rainbow trout larvae. After recipients reached maturation, donor spermatogonia differentiated into spermatozoa in males and were also able to differentiate into fully functional eggs in females. Furthermore, donor-derived spermatozoa and eggs obtained from recipient fish were able to produce normal 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).

In 2007, the intra-peritoneal SSC transplantation involving two different species was investigated. Spermatogonial xenotransplantation was performed using vasa - GFP rainbow trout donors into sterile triploid masu salmon hatchling recipients with transplanted trout spermatogonia undergoing spermatogenesis and oogenesis in male and female recipients, respectively. Approximately two years after transplantation, triploid salmon recipients produced only trout sperm and eggs (Okutsu et al. 2007). Protocols have been established for other species such as nibe croaker (*Nibea mitsukurii*), yellow tail mackerel (*Trachurus novaezelandiae*) and chub mackerel (*Scomber japonicas*) (Yazawa et al. 2010).

As shown in the above studies, SSCs possess a great degree of plasticity and can differentiate in to functional eggs and sperm in the host. In order to determine the potential plasticity of oogonia, Yoshizaki (2010) transplanted female germ cells from donors to newly hatched embryos. Two years after transplantation, donor-derived oogonia had differentiated into functional eggs in the female recipient and sperm in male recipients. Oogonia isolated from sexually differentiated ovaries contain a cell population that can differentiate into fully functional

sperm (Yoshizaki et al. 2010). The results of these studies clearly indicate that both the spermatogonia and oogonia of fish, even those isolated from the gonads after sex differentiation, contain cell populations with a high level of sexual plasticity, and that the sex of a fish germ cell is determined solely by the somatic micro-environment.

The third approach has been germ cell transplantation in adult fish (Lacerda et al. 2012). Techniques have been developed in Nile tilapia (*Oreochromis niloticus*), in which spermatogonia were transplanted through the urogenital papilla of adult fish. In the initial stages of this method, the recipient fish had their endogenous spermatogenesis suppressed using the cytostatic drug, busulfan (1,4-butanediol dimethanesulfonate), in association with high temperature (35^0C). An enriched type A spermatogonial cell suspension was then obtained from donor testis 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. This method demonstrated the feasibility of using sexually mature fish as recipients in germ cell transplantation. 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 has also demonstrated complete spermatogenesis in a tilapia recipient after observations taken between 8 and 9 weeks post transplantation revealed the production of donor spermatozoa in recipient testes (Lacerda et al. 2010). Furthermore, 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. Additionally, cryopreserved tilapia

spermatogonia were also able to efficiently proliferate and differentiate into spermatozoa in recipient testes after transplantation (Lacerda, Batlouni, et al. 2010).

An effective intra-gonadal surgical model for xenogeneic germ cell transplantation in sexually competent fish has also been developed (Majhi et al. 2009). In the development of this model, germ cells were isolated from juvenile pejerrey (*Odontesthes bonariensis*), while sexually competent Patagonian pejerrey (*Odontesthes hatcheri*) were prepared as recipients for donor germ cell transplantation. Endogenous spermatogenesis was also depleted using busulfan treatment coupled with high temperatures (Lacerda et al. 2012). Donor germ cells were subsequently labeled with CFDA-SE and injected into the recipient gonad. Injection of the germ cell suspension was made possible in this procedure by a long midline incision made in the abdomen of the recipient followed by the exposition of the gonads from the coelomic cavity. Transplanted spermatogonial cells were able to colonize the gonads of individual Patagonian pejerrey and generate donor-derived sperm within 6 months post transplantation. The resulting sperm was used for artificial insemination, and subsequent DNA analysis confirmed that some of the resulting offspring possessed donor-derived DNA (Majhi et al. 2009).

Our goal is to develop a protocol for the interspecific transfer of embryonic germ cells between different species of catfish to increase the efficiency of reproduction for catfish hybridization. Efficiency for CxB hybrid embryo production might be improved by transplanting blue catfish spermatogonial stem cells (SSC) into triploid channel catfish males and mating them with normal channel catfish females. If successful, this could result in the production of CxB hybrids through natural open pond spawning. Our primary research objective was to isolate primordial germ cells (PGCs) and/or spermatogonia A (spermatogonial stem cells, SSCs), and successfully

transplant them from a diploid donor catfish into a triploid host catfish, which will later produce diploid offspring from the donor. This would represent the first application of this breakthrough technology to a major, warm-water, aquaculture species native to the United States such as catfish.

Materials and Methods

Capture and harvest of brood stock

The channel catfish brood stock was maintained in 0.04 ha ponds throughout the growing season. The brood stock was fed a commercial floating catfish pellet containing 36% protein. The fish were fed five times a week from March-October and once a week between November-February. Prior to the start of the experiment in 2009, fish were captured by seining the pond. The captured fish were evaluated for health and separated by sex. Selected females were placed in individual 60 x 90 cm, 32 mm mesh spawning bags pond-side, and transported to the green house and placed in 836 L fiberglass tanks with a flow through water (24 L/min), and aeration. Nine to twelve females in spawning bags were housed in each tank. The spawning bags along with the fish were suspended in the tank using clothes pegs. The males were transported to the green house and housed in another fiberglass tank with flow through and aeration communally. The fish were allowed to acclimate for six to eight hours prior to hormonal induction and artificial spawning.

Artificial spawning procedures were those of Lambert et al. (1999), Dunham et al. (2000), Hutson (2006), and Kristanto et al. (2009). Channel catfish females were implanted with the spawning hormone LHRHa. LHRHa (EVAC) implants (Center of Marine Biotechnology, University of Maryland, Baltimore, MD) were administered intramuscularly as a single dose, 90 µg/kg body weight, posterior and ventral to the dorsal fin.

Ovulation occurred 36-72 hours after implantation. Ovulating females that were releasing eggs were anesthetized with 100 mg/L tricaine methanesulfonate **(**Finquel® MS-222®**)** and 100 ppm sodium bicarbonate. Females that were not ovulating remained in the trough and were checked periodically. The anesthetized females were dried with a towel, and eggs were hand-stripped into pans lubricated with vegetable shortening until eggs could no longer be stripped.

Testes were excised from euthanized channel catfish males. Blood and excess tissue were removed using Hanks' balanced salt solution (HBSS: 160.0 g NaCl, 8.0 g KCl, 2.8 g $CaCl_2$, 4.0 g $MgSO_4 \cdot 7H_2O$, 2.4 g $NaHPO_4 \cdot 7H_2O$, 1.2 g KH_2PO_4, 7.0 g $NaHCO_3$, and 20.0 g glucose in 20 L reverse osmosis-distilled H_20, pH=6) or saline. Testes were then macerated in either HBSS or 0.9 % saline. The sperm was added to the eggs and mixed to dry fertilize.

The pans with the egg/sperm mixture were moved to the pressure shock station. Thirty-gram aliquots of the dry fertilized eggs were placed in small pie pans. Water was added to a single pie pan to activate and commence fertilization of the eggs. At three minutes post fertilization the eggs were placed in an egg basket, and the basket placed in the pressure chamber. At five minutes post fertilization, the fertilized eggs were pressure-shocked using a hydrostatic pressure

of 6000 psi (408.27 atm) for 5 minutes. After pressure shocking, the eggs were removed from the chamber and placed in a hatching trough fitted with a paddle wheel and flow through.

Seven days post hatch, fry were removed and placed in fry tanks. The fry were fed 40% protein fry feed (AquaMax™) three times a day and grown to 8-10 cm fingerlings. The fingerlings were stocked in a 0.04 ha pond and allowed to grow for three years. The fish were fed five times a week from March-October and once a week between November-February with a 36% protein-floating pellet.

In April 2012, all fish 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. The blood was placed in a cuvette, along with containing 5ml of Isoton® diluent and 2 drops of Zap-Oglobin II© (a digestive enzyme) (Beckman Coulter). Each sample was analyzed for erythrocyte nuclei volume with a Coulter-counter (Beckman Coulter), using the protocol described by Beck (Beck and Biggers 1983). Triploid and diploid individuals were separated, and transported back to Auburn University. The diploid individuals were discarded, and the triploid individuals were given unique individualized brands and placed back in a pond.

Donor cell isolation

Donor male blue catfish (25-30 cm, two year old) were harvested and anesthetized with tricaine methanesulfonate (MS-222). The ventral surface of the fish surface was wiped down with by 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 placed separately in 1.5 ml anti-agent medium (HBSS with 1.0 µg/ml $NaHCO_3$, 100 unit/ml penicillin and 100 µg/ml streptomycin). Thereafter the testicular tissue was placed on a clean petri dish and any connective tissue or blood was removed using a pair or forceps. Next the tissue was washed twice with 0.5 ml anti-agent medium. The testes were then shredded using a pair of sterilized scalpel blade and transferred into 12 ml HBSS with 0.25% trypsin, 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* (gravitational acceleration) for 10 minutes. The supernatant was discarded and the pellet resuspended in 1 ml HBSS. The concentration of cells was calculated using a hemocytometer, and the cell solution stored on ice till ready for use. This solution was considered to be a fresh cell isolate.

Gradient-centrifuged cells were prepared by using a Percoll gradient (GE Healthcare). 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 25mM HEPES, 50 uint/ml penicillin, 50µg/ml streptomycin, 1.0µg/ml $NaHCO_3$, 0.3µg/ml L-glutamine, 10% FBS, 5% catfish serum and 1ng/ml bFGF). The concentration of cells was calculated using a hemocytometer, and the cell solution stored on ice till ready for use.

Cell implantation of the host

In July 2012, the triploids were harvested. The fish (mean weight = 1.72Kg) were anesthetized using 100mg/L tricaine methanesulfonate (MS-222) and 100ppm 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.

For the surgical procedure a 5-7cm incision was made on the ventral side of the fish, from the pelvic bone cranially. The skin and muscle was parted and the gastrointestinal tract carefully moved aside. The gonad (gonad was small or atrophied) was located on the dorsal wall ventral to the tail kidney. Using a syringe fitted with a 30 gauge needle 1ml of the fresh cell isolate or gradient centrifuged solution ($7x10^4 – 1.25x10^5$ cells) was carefully injected into the cranial part of each gonad (0.5ml in each ovary or testis). Post injection, the GI tract was placed back in the body cavity and the incision closed using biodegradable sutures. Next the fish were placed in a recovery tank and observed and allowed to heal for 10 days, and then moved to a 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 by depressing the plunger 1ml of the cell isolate ($2x10^4 – 1.43x10^6$ cells) was driven into the genital tract and the gonads of the fish. The tubing was then drawn out of the vent, and the fish were observed and allowed to recuperate in the green house for 10 days, and moved into a pond.

Sixty days later the implanted fish 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 cranially. The skin and muscle was parted and the gastrointestinal tract carefully moved aside. Tissue samples were excised from the gonads for DNA analysis. The incision was closed with biodegradable sutures. The fish were observed and allowed to heal for 5 days and returned in to a pond.

Genomic DNA

The excised gonad samples were placed in a 1.5 ml microfuge tube on ice during tissue collection. Thereafter the samples were transferred to a freezer and held at ˉ80C 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 little modification. Cell lysis solution contained 600 ul of DNA extraction cocktail buffer (100mM/L NaCl, 10mM/L Tris-HCl, pH 8, 25mM/L EDTA, 0.5%/L sodium dodecyl sulfate) and 3 ul 20mg/ml freshly made proteinase K. Briefly, the gonad samples were incubated and digested with proteinase K (Sigma cat# P6556-500mg) at 55°C overnight, proteins were precipitated using protein precipitation solution (Qiagen cat# 158912) and DNA was extracted with isopropanol. DNA was washed twice with 70% ethanol, air-dried, resuspended in Rnase/Dnase free water, and quantified with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). DNA from control samples of diploid channel catfish, blue catfish and hybrid catfish were extracted the same way.

PCR

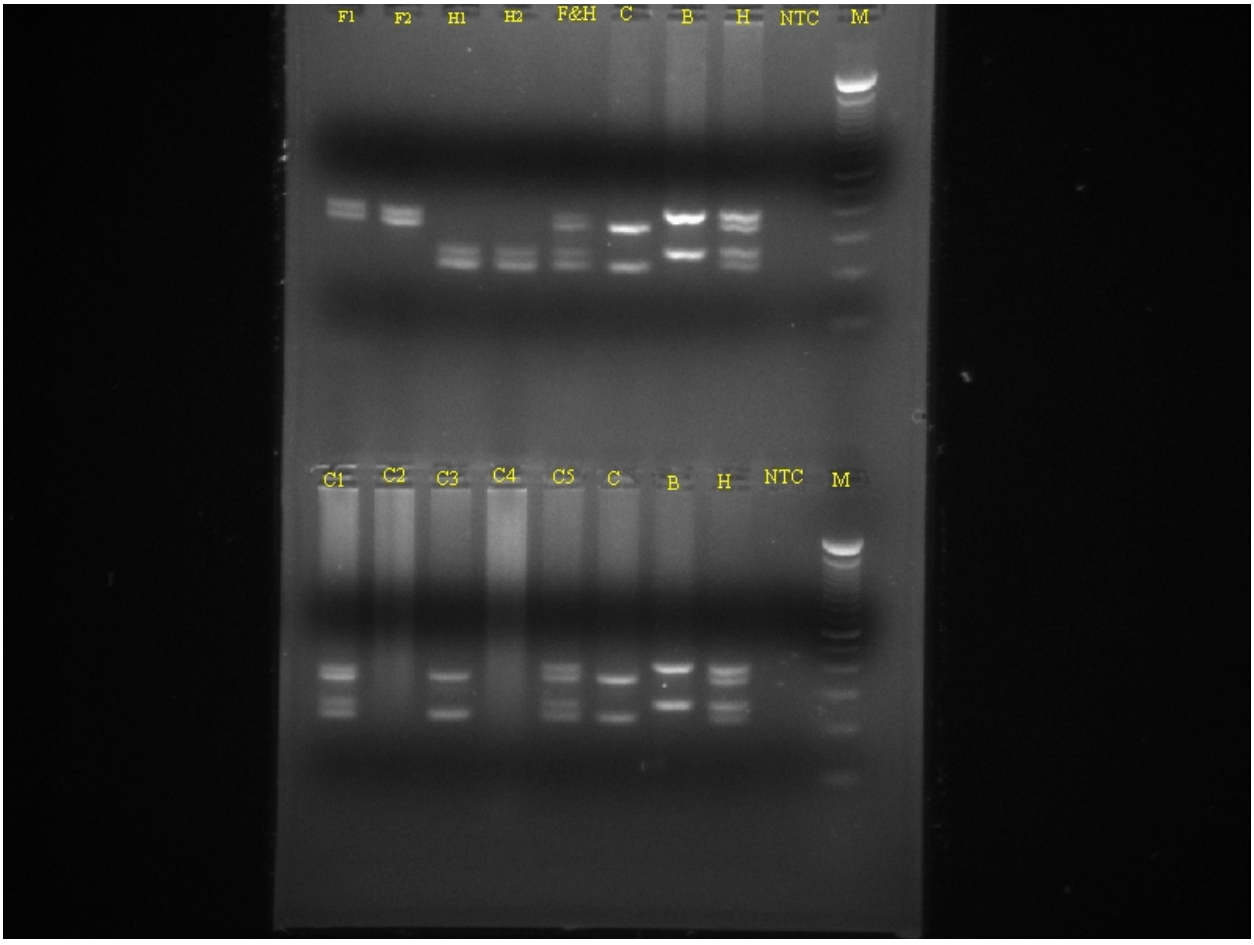
PCR primers were previously described by Waldbieser and Bosworth (2008). Primers used for differential PCR-amplification of channel and blue catfish genes are listed in Table 1. Genes evaluated were follistatin, *Fst,*and hepcidin, *Hamp* antimicrobial protein for channel catfish and blue catfish. The PCR reactions were prepared in 10.0ul volume containing 20-250ng genomic DNA in 1× buffer (1.0 ul 10 mM Tris-HCl[pH:9.0], 0.4 ul 50 mM $MgCl_2$, 0.8 ul 2.5 mM of each dNTP, 0.6 ul 10 pmM each *Fst* primer, 0.3 ul 10 pmM each *Hamp*,0.1ul 5 U/ul platinum Taq polymerase, 3.9 ul water. The PCR procedures were performed using the following thermo profile: after an initial denaturation at 95^{o}C for 3 min; PCR amplification were carried out at 95°C for 1min, 65°C for 1 min, and 70°C for 1 min for 3 cycles as the first step; and at 95°C for 30s, 63°C for 30s, and 72°C for 1 min for 35 cycles as the second step. A final extension at 72°C for 10 min was included. The *Fst* and *Hamp* amplification products were analyzed on an ethidium-bromide-stained 2.5% agarose gel. Tracklt™ 100bp DNA Ladder (cat no: 10488-058, Invitrogen, USA) was used to check the band size after electrophoresis. All the reactions for each sample were repeated three times.

Table 1. Primers were used to differentiated channel catfish and blue catfish. *Fst* stands for *follistatin*, *Hamp* stands for *hepcidin* antimicrobial protein for channel catfish and blue catfish, respectively.

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

Results

Both triploid channel catfish, one of each sex, that were injected with density gradient sorted blue catfish stem cells, putative spermatogonia A and primordial germ cells, contained blue catfish cells in their testes and ovaries, 60 days after injection. In all instances, some gonadal growth was evident (doubles 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 a single female, that was catheterized (Figure 1.).



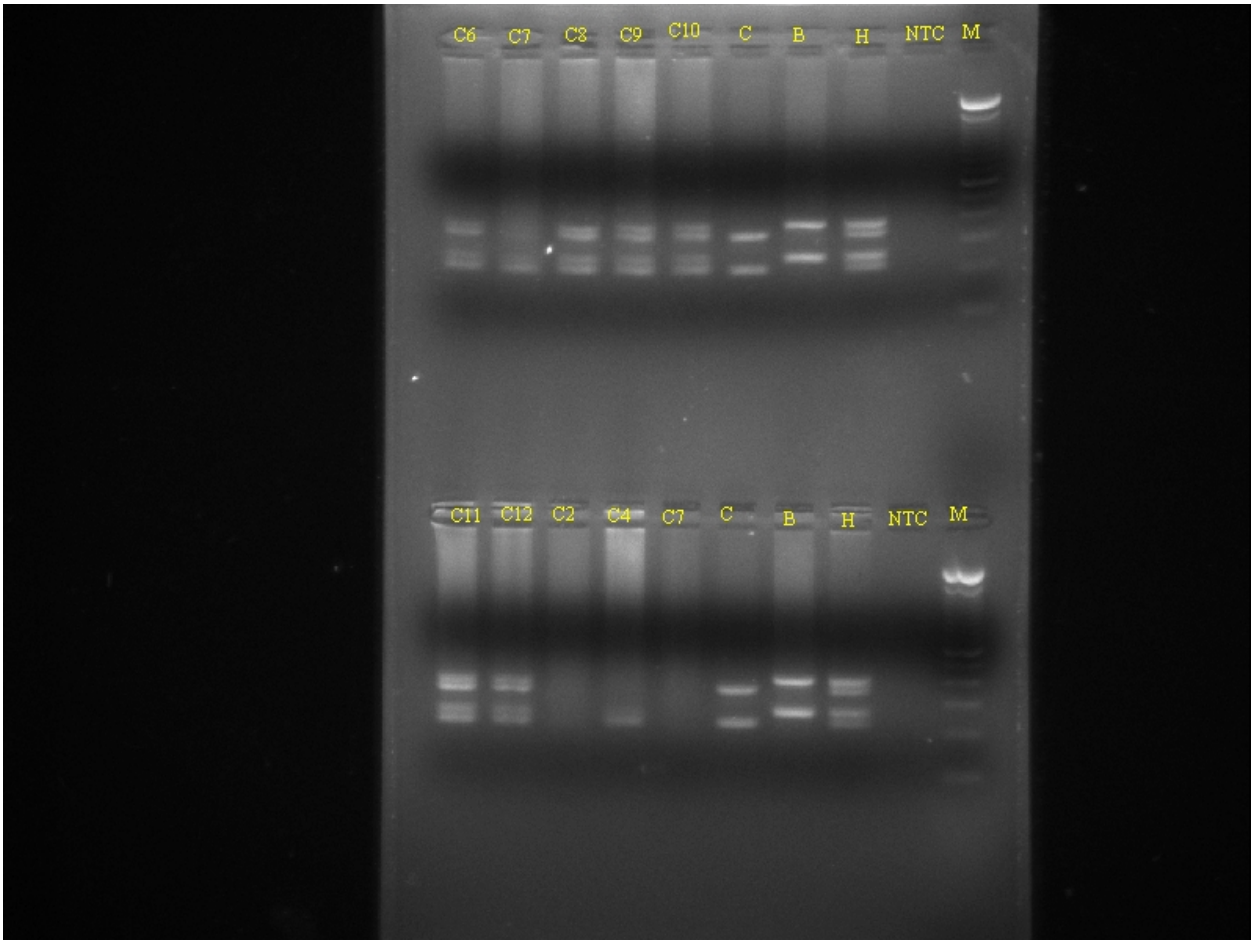


Fig. 1 (A,B) PCR results for xenogenic catfish (triploid channel catfish, *Ictalurus punctatus,* with blue catfish, *I. furcatus,* cells in their gonads). F1 and F2 are PCR products from a mixture of 25 cells from channel catfish and 25cells from blue catfish amplified with primers for the *follistatin*, Fst, gene. H1 and H2 are PCR products from a mixture of 25 cells from channel catfish and 25cells from blue catfish amplified with primers for the *hepcidin, Hamp,* gene. F&H are PCR products from a mixture of 25 cells from channel catfish and 25cells from blue catfish amplified with both Fst and Hamp primers. C and B are channel catfish and blue catfish controls. H, C4-7, 10-11 are channel catfish female X blue catfish male hybrid controls. NTC is a no DNA template control (negative control). M is a 100bp marker. All fish were injected with one ml of cell solution. C1 is a male triploid channel catfish injected in the testes with 7x 10^4 /ml putative blue catfish stem cells. C12 a female triploid channel catfish injected in the ovaries with 1.25x10^5/ml putative blue catfish stem cells. C2 and C3 are triploid channel catfish females inserted with a mixture of testicular cells from blue catfish, 1.43x 10^6/ml and 1.3x 10^5/ml, respectively, by catheterization through the oviduct. C8 and C9 are triploid channel catfish males inserted with a mixture of testicular cells from blue catfish, 1.43x 10^6/ml and 2x 10^4/ml, respectively, by catheterization through the urogenital opening.

Discussion

Two insertion techniques, gonadal injection and catheterization, using blue catfish cells that were either a fresh mixture of all testicular cells or putative stem cells from the testes that were density gradient purified resulted in xenogenic catfish, triploid channel catfish harboring blue catfish in their gonads. One xenogenic female and 3 xenogenic males were produced. Theoretically, the blue catfish DNA that was detected is from stem cells that were able to colonize and survive in the gonads. This technique was very effective as 80% of the manipulated fish accepted the foreign cells, which colonized and were present 60 days later, and may be an improvement compared to transfer of stems cells into blastulas 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 blastulas and hatchlings.

Xenogens have been produced twice before by manipulating adult fish, Nile tilapia (Lacerda, Costa, 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 were produced, there apparently was no immune response against the donor cells, and the xenogens 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. They will produce both host and donor progeny. Apparently, there is also no immune response against donor stem cells in the triploid channel catfish as blue catfish cells were present after 60 days at temperatures of 27-32^{o}C when the catfish immune system should be operating at maximum efficiency. The triploid xenogens, in contrast to the busulfan xenogens, should not be able to produce host gametes (Dunham 2011), thus, if these introduced cells become sperm or ova, they will only have the genotype of the donor, blue catfish, making our technique with triploid host of much greater utility.

Assuming that these transferred cells will mature into fully functional gametes, many potential applications exist. These include technology to rapidly expand and conserve endangered species or strains, if cryopreserved testes exist, a technology to resurrect extinct species or lines, a mechanism to study cellular and tissue communication and physiology, cloning of specific genotypes, a mechanism to avoid inbreeding, technology to produce difficult to spawn species, alternative hybridization techniques, possible enhanced gene transfer and a method to reduce generation intervals.

The functionality of the stem cell transfer technique in the catfish yet needs to be demonstrated. The fish produced in this study need to be grown and maintained to demonstrate the longer term persistence of the donor cells in the host, the reproductive behavior of these xenogens and the production of fertilizable mature gametes.

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